

Evidence of a landlocked reproducing population of the marine pejerrey *Odontesthes argentinensis* (Actinopterygii; Atherinopsidae)

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Abstract

In South America, the order Atheriniformes includes the monophyletic genus *Odontesthes* with 20 species that inhabit freshwater, estuarine and coastal environments. Pejerrey *Odontesthes argentinensis* is widely distributed in coastal and estuarine areas of the Atlantic Ocean and is known to foray into estuaries of river systems, particularly in conditions of elevated salinity. However, to our knowledge, a landlocked self-sustaining population has never been recorded. In this study, we examined the pejerrey population of Salada de Pedro Luro Lake (south-east of Buenos Aires Province, Argentina) to clarify its taxonomic identity. An integrative taxonomic analysis based on traditional meristic, landmark-based morphometrics and genetic techniques suggests that the Salada de Pedro Luro pejerrey population represents a novel case of physiological and morphological adaptation of a marine pejerrey species

to a landlocked environment and emphasises the environmental plasticity of this group of fishes.

KEYWORDS

Atherinopsidae, biogeography, environmental plasticity, integrative taxonomy, landlocked population

1 | INTRODUCTION

Neotropical silverside, pejerrey, species of the order Atheriniformes are found in lakes, rivers, estuaries and marine coastal areas in tropical and temperate regions (Dyer & Chernoff, 1996; Eschmeyer & Fong, 2014). In South America, all known species belong to the family Atherinopsidae. Within this family, the genus *Odontesthes* Evermann & Kendall 1906 is monophyletic, encompassing 20 species found in freshwater, estuaries and coastal areas (Campanella *et al.*, 2015; Wingert *et al.*, 2017). Whereas some species inhabit either marine or freshwater environments, others can undertake movements between marine, brackish and freshwater environments (Avigliano *et al.*, 2014; Dyer & Chernoff, 1996; González- Castro *et al.*, 2016). Not surprisingly, several studies have pointed out the capacity of pejerrey for developing populations in coastal lagoons and estuaries, highlighting the potential for local populations with different genetic and phenotypic characteristics (Beheregaray *et al.*, 2002; Beheregaray & Sunnucks, 2001; González- Castro *et al.*, 2009, 2016; Mancini *et al.*, 2016). Although all of them have a marine ancestor group (Beheregaray & Sunnucks, 2001; Campanella *et al.*, 2015), the landlocking of marine Atheriniformes represents a major factor for speciation, as has been observed in Australia (Potter *et al.*, 1986), Mexico (Echelle & Echelle, 1984), USA (Johnson, 1975) and Europe (Henderson & Bamber, 1987).

In southern South America *Odontesthes argentinensis* (Valenciennes 1835) is a widely distributed species in coastal areas of the Atlantic Ocean (Cousseau & Perrotta, 2004; Dyer, 1993; Llopart *et al.*, 2013) whereas the congeneric *Odontesthes bonariensis* (Valenciennes 1835) inhabits inland waters and estuaries (Ringuelet, 1975). A remarkable characteristic of *O. argentinensis* is its ability to colonise and establish resident populations in open coastal lagoons as has been observed in Los Patos Lagoon (Brazil; Chao *et al.*, 1986) and Mar Chiquita Lagoon, Argentina (González- Castro *et al.*, 2009; 2016), where phenotypic and physiological divergences compared with adjacent marine conspecifics have been observed. Differences in egg morphology, habitat use and reproductive patterns have been reported for *O. argentinensis* entering these environments (Bemvenuti, 1987; Moresco & Bemvenuti, 2006). Moreover, allozyme variability has been observed in the Los Patos lagoon population, supporting the idea that the estuarine population is under a process of speciation (Beheregaray & Levy, 2000). On the other hand, *O. bonariensis* occupies estuarine and temperate shallow and eutrophic lakes and reservoirs (Baigún & Anderson, 1994) characterised by high limnological variability (Diovisalvi *et al.*, 2015; Quirós *et al.*, 2002a, 2002b). Although *O. bonariensis* is usually found in oligohaline to mesohaline waterbodies (Baigún & Anderson, 1994; Colautti *et al.*, 2006,

2015), it can also inhabit lakes with intermediate to high salinity such as Chasicó Lake, Argentina (salinity of 28; Berasain *et al.*, 2015; Kopprio *et al.*, 2010) and other inland lakes, with salinity levels up to 50 (Bucher & Etchegoin, 2006; Mancini *et al.*, 2016). Both species can coexist as it has been observed in the low salinity, inner zone of Mar Chiquita coastal Lagoon (Bruno, 2014; Cousseau *et al.*, 2001; Gonzalez- Castro *et al.*, 2009; 2016). These examples demonstrate that marine pejerrey populations can colonise estuarine areas and coastal lagoons, but there are no previous records of an inland-water landlocked population.

The Salada de Pedro Luro Lake, located at the limit between Pampean and Patagonian regions, Argentina and 55 km from the Atlantic Ocean, is a brackish to hypersaline shallow lake placed over old marine grounds and represents a relic of the marine coastal environment that covered the region 6000 years ago (Melo *et al.*, 2013). Historical records indicate that the Salada de Pedro Luro Lake developed a well-established pejerrey fishery from the end of the 19th century to 1936 (MacDonagh & Thormahlen, 1945), but did not provide taxonomic information. The same authors indicated that the lake was stocked several times with *O. bonariensis* from Chascomús and Chasicó Pampean lakes. The first classification was made by Pianta de Risso & Risso (1953), who identified the pejerrey from Salada de Pedro Luro Lake (hereafter SPL pejerrey) as *Austromeniidia bonariensis* (junior synonym of *O. bonariensis*) but noticed some morphological differences with the same species found in other Pampean lakes. More recently, Bemvenuti (2002) included specimens from Salada de Pedro Luro Lake deposited in the Museo de La Plata collection (MLP I. V.52. 1 6) to make the morphological re-description of *O. argentinensis*. In addition, pejerrey specimens collected in the lake in 2012 by us, did not conform entirely to any of the available descriptions of pejerrey species (Bemvenuti, 2002, 2005; Dyer, 2006; González- Castro *et al.*, 2016; Malabarba & Dyer, 2002; Piacentino & Torno, 1987). These facts suggest a confused taxonomic identification of SPL pejerrey and a possible shift in the original population owing the introduction of *O. bonariensis* in the middle of the past century.

Since, to our knowledge, *O. argentinensis* has never been documented to inhabit inland lakes, the presence of a landlocked ecotype represents a major challenge in terms of understanding how morphological and physiological adaptations have evolved to colonise this new environment. Nevertheless, the conflicting reports on the identity of the pejerrey from Salada de Pedro Luro Lake also suggest that the study of this population and perhaps of other pejerrey species at the α -taxonomic level (Schlick-Steiner *et al.*, 2010), cannot be tackled by using traditional taxonomic criteria and would require an integrative taxonomic perspective (Dayrat, 2005; Padial *et al.*, 2010). Although

such an approach has not been applied previously for Atheriniformes, examples are available for other fish species (Gomes *et al.*, 2015; Rosso *et al.*, 2018). In this context, the goal of this study was to clarify the taxonomic identity of SPL pejerrey by using integrative taxonomy based on meristic, landmark-based morphometrics and genetic techniques and to hypothesize about its possible origins.

2 | MATERIALS AND METHODS

Care during collection and handling of fish for this study complied with the Buenos Aires Province (Argentina) Wildlife and Fisheries Authority guidelines and policies (Law 11,477). The collections for this study were not a part of faunal surveys and they did not employ any type of experimental procedure, surgery or chemical agents that would induce neuromuscular blockage or injury on the collected organisms. All fish collected were euthanised as humanely as possible by anaesthetic overdose to prevent unnecessary suffering.

2.1 | Study area

Salada de Pedro Luro Lake (39° 27' S, 62° 42' W) is a sub-circular polymictic lake with a surface area of c. 400 ha and mean and maximum depths of c. 2.5 and 3 m, respectively (Figure 1). According to Alfonso *et al.* (2015), limnological and climatological variables show a wide range of values. This system is alkaline (pH range 9.3–10.5), mesotrophic-eutrophic (total phosphorous range 36–298 $\mu\text{g l}^{-1}$), relatively transparent (Secchi depth 0.6–2.9 m) and saline (20–52; Alfonso *et al.*, 2015). According to the Köppen & Geiger system (Kottek *et al.*, 2006), the climate is classified as BSk (cold semi-arid) with an annual mean precipitation of 405 mm year^{-1} . The Salada de Pedro Luro Lake receives excess water originally diverted for irrigation of agricultural areas from the Colorado River through a complex network of irrigation channels that are managed according to agriculture requirements. The Colorado River water conductivity ranges between 1 and 4.5 mS cm^{-1} and irrigation channel gates are closed when water conductivity reaches 1.8 mS cm^{-1} to prevent soil salinisation (CORFO,). There is no outflow from Salada Pedro Luro Lake and it behaves as an endorheic system where water is lost due to evaporation and possible groundwater infiltration (Alfonso *et al.*, 2015). Drainage water of the system flows to the sea through saline soils causing progressive increase in water conductivity before reaching the marine environment. It is also important to note that sea tides in this area have an average amplitude of 2.8 m and, due to the scarce slope of the territory, their influence can be detected in the continent up to 60 km from the mouth of Colorado River (Perillo *et al.*, 1999).

2.2 | Fish collection

Fish samples were obtained in September, November of 2012, August, October, December 2013, 2014 and 2015 using 1.9 m height floating gillnets of different bar mesh sizes (14, 19, 21, 25,

28, 32, 36 and 40 mm), fyke nets (Colautti, 1998) and a beach seine net (30 × 1.5 m and 1.5 cm bar mesh size). Gillnets and fyke nets (2) were set in central and coastal areas of the lake, respectively, for 12 h overnight. For the beach seine nets, two hauls perpendicular to the shore were made during daytime. For all individuals, standard length (L_S , mm), total mass (M , g) and gonadal mass (M_G , g) were measured. A maximum of 30 fish from every sampling date were euthanised by an overdose of benzocaine solution, dissected and classified according to sex and identified as juvenile or adult based on visual inspection of gonad development following Vazzoler (1996). Sagitta otoliths were removed and preserved in paper envelopes. Fin clip tissue was collected from several individuals, preserved in 95% ethanol and stored in the Laboratorio de Biotecnología Acuática-FCByF-UNR (Rosario, Argentina) collection and in the Laboratory of Population Biology of the Tokyo University of Marine Science and Technology collection (Tokyo, Japan). The entire body of 12 individuals ranging from 165 to 258 mm L_S and the gill arches from 30 SPL pejerrey ranging from 160 to 340 mm L_S were preserved in formalin. In addition, 20 fish were brought to the laboratory in ice for meristic and morphological studies. In the laboratory, four complete skeletons of fish >180 mm L_S and eighteen post-cranial axial skeletons of fish between 210 and 370 mm L_S were prepared according to Bemvenuti (2005) and Rojo (1988), by removing their muscles and soft tissues using water at 70°C. Bones were dried and preserved in paper envelopes. Voucher specimens of SPL pejerrey (10) were deposited in the ichthyology collection of the Fundación de Historia Natural Félix de Azara, Buenos Aires, Argentina (CFA-IC-9183).

Samples of other *Odontesthes* spp. collected in this and other studies between October 2012 and November 2015 were used in the comparative morphological and genetic analyses described below. Fish were captured using similar procedures and fishing gears as described for Salada de Pedro Luro Lake and were taxonomically identified following the morphological criteria provided by Bemvenuti (2002), Dyer (2006) and Piacentino & Torno (1987). Thus, specimens collected in coastal marine environments were identified as *O. argentinensis* from Mar del Plata and San Blás, *Odontesthes incisa* (Jenyns 1841) from Santa Teresita and *Odontesthes smitti* (Lahille 1929) from Necochea and those from inland waters (Río de la Plata, Salada de Monasterio Lake, Cochicó Lake, Chascomús Lake) as *O. bonariensis* (Figure 1).

2.3 | Analysis of population biological parameters

The population structure was analysed by means of length-frequency distribution using 2012 fish samples. The potential for reproduction was assessed by estimation of gonado-somatic index (I_{GS}) during the spring (August, October and December), in 2013, 2014 and 2015. Only fish >230 mm in L_S were used for I_{GS} analysis considering the minimum maturation sizes reported for *O. bonariensis* and *O. argentinensis* by Calvo & Dadone (1972) and Llopart *et al.* (2013), respectively.

2.4 | Meristic, morphological and morphometrical analyses

In order to taxonomically classify the SPL pejerrey, different meristic and morphological features of taxonomic relevance to the *Odontesthes* genus (Bemvenuti, 2002; Malabarba & Dyer, 2002; Mancini *et al.*, 2016; Tombari *et al.*, 2010) were analysed in 12 SPL pejerrey individuals. These included observation of the presence-

absence of predorsal crenulate scales, the presence of scales over the cleithrum, the number of precaudal and caudal vertebrae, the number of fin rays in the pectoral, pelvic, anal, first dorsal (D1) and second dorsal (D2) fins, the number of scales on the lateral line above the lateral line, the number of rows of scales between the lateral line and D1, the number of rows of scales counted on a vertical line at half distance of D2, the number of rows of subocular and dorsal scales between left and right lateral lines (Bemvenuti, 2002; Dyer, 2000,

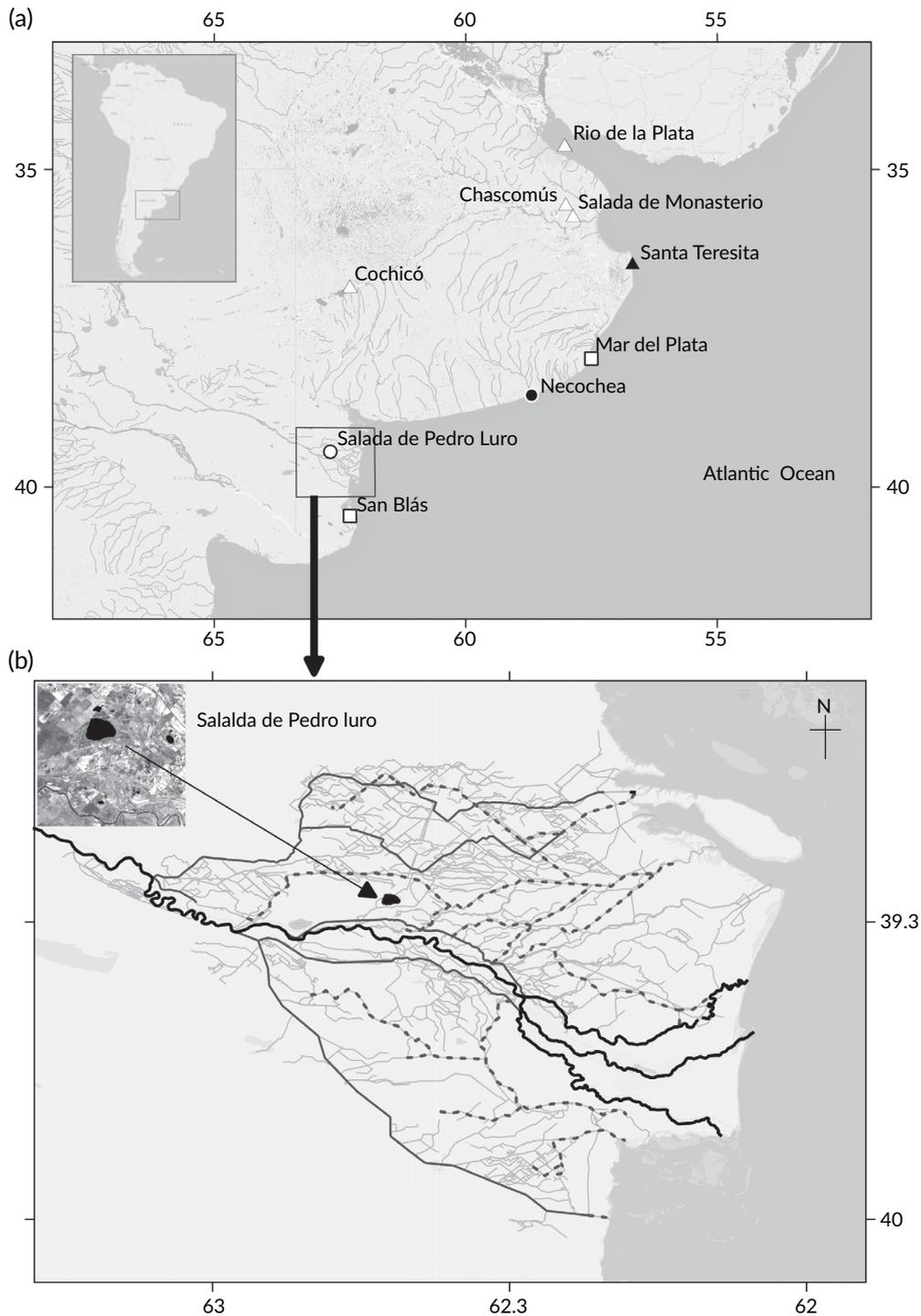


FIGURE 1 (a) Geographical location of Salada de Pedro Luro Lake and other sampling sites for *Odontesthes* spp. (○) SPL pejerrey, (□) *O. argentinensis*, (△) *O. banariensis*, (▲) *O. incisa*, and (●) *O. smitti*. (b) Colorado River and their branches, main irrigation channels, main drainage channels and minor channels. (black line—) Colorado river, (grey line—) main irrigation channels, (dotted grey line—) main drainage channels, and (light grey line—) minor channels

2006; Malabarba & Dyer, 2002). In bilateral structures, counts were made on the left side of the fish.

Description of the skeletal characteristics of SPL pejerrey included the basipterygium and haemal arches from the caudal vertebra in side view, the haemal funnel form in frontal view starting from the second caudal vertebra, as well as the frontal bone separation, teeth along the premaxilla, back subopercle apophysis, top interopercle notch, the suture between scapula and caracoid and the presence of teeth on dentary, vomer and endopterygoid bones. These structures were assessed under a binocular microscope Motic SFC-11C with incidental and transmitted light according to Bemvenuti (2002, 2005), Malabarba & Dyer (2002); Dyer (2006), González-Castro *et al.* (2016) and Piacentino & Torno (1987).

The numbers of gill rakers on the lower limb of the first arch (ceratobranchial) was independently counted by two readers under a binocular microscope (Olympus SZ61; www.olympus-lifescience.com). If the number of gill rakers counted differed between readers, both readers viewed the structure together until a consensus was reached. According to Dyer (2006), this is an important taxonomic feature in the *Odontesthes* genus. Therefore, the range of number of gill rakers found in SPL pejerrey was compared with values reported for other *Odontesthes* species by Bemvenuti (2002) and Piacentino (1999).

A comparative geometric morphometric analysis of the haemal funnels was conducted on 19 SPL pejerrey, 14 specimens classified as *O. bonariensis* from the Río de la Plata ($n = 6$), Salada de Monasterio Lake ($n = 6$) and Cochicó Lake ($n = 2$) and 15 specimens classified as *O. argentinensis* from San Blás. The Cartesian coordinates of 16 anatomical landmarks located on the second, third, fourth and fifth haemal funnels were determined. The first haemal funnel was not included in the analysis due to its great variability and extreme thinness. Landmark coordinates for each specimen were scaled, translated and rotated using the generalised Procrustes superimposition (GLS, also called GPA; Rohlf, 1999). The thin-plate spline (TPS) procedure was employed to compare shape differences among the three groups, using both the uniform and non-uniform shape components (Bookstein, 1991; Rohlf & Marcus, 1993). This method is not only an effective visualisation 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 (Rohlf, 1999). A principal component analysis (PCA) of the partial warps matrix was performed (usually named as relative warp analysis, RWA), to describe the major trends in shape variation. To examine the potential for differences in shape when classifying SPL pejerrey specimens, the relative warp scores (RWS) were subjected to discriminant analysis (DA; Nie *et al.*, 2004) using cross-validation.

Principal Components Analysis (PCA) was used to describe otolith shape variation among SPL pejerrey, *O. argentinensis* (San Blás) and *O. bonariensis* (Chascomús and Salada de Monasterio lakes). Fifty right sagitta otoliths per group were digitised (sulcus side upwards) with a camera (Moticam 1000, Motic; www.motic.com) mounted on a stereomicroscope. The digital images were used to describe the

overall shape by elliptical Fourier analysis (EFA) using the contour coordinates (Kuhl & Giardina, 1982). For each individual, chain codes were registered on the contour to calculate the elliptical Fourier descriptors (EFD) of the first five harmonics using the software Shape 1.3 (Iwata & Ukai, 2002). Each harmonic comprises four coefficients resulting in 17 coefficients per individual. Each otolith was normalised by the program for size and orientation, which caused the degeneration of the first three coefficients to fixed values: $a_1 = 1$, $b_1 = c_1 = 0$ (Kuhl & Giardina, 1982). The average shape of the otolith for each group was reconstructed from normalised coefficient mean values of the EFDs using inverse Fourier transformations (Iwata & Ukai, 2002). A cross-classification analysis was performed with the Past 1.99 program (Hammer & Harper, 2010) to assess the correct assignment of individuals to each fish group based on the otolith shape.

2.5 | Salinity tolerance of embryos

Fertilised eggs were obtained by artificial insemination using gametes from a single female and male captured in the Salada de Pedro Luro Lake or from captive-reared *O. bonariensis* broodstock from the IIB-INTECH (Chascomús). Eggs from Salada de Pedro Luro Lake were transported in a refrigerated box to the IIB-INTECH aquaculture facilities. For the salinity tolerance test, triplicate groups of 100 embryos from both groups were incubated in 1 l plastic jars at salinities of 5, 15, 30 and 45 by addition of NaCl to dechlorinated tap water, at a temperature of 19°C. The jars were provided with constant aeration and the water in each jar was completely replaced every 2 days until hatching. The effects of salinity on hatched eggs by group were tested using one-way ANOVA followed by the Tukey's test ($P < 0.05$).

2.6 | Genetic analyses

Analyses of microsatellites and *amhy* (Y-linked anti-Müllerian hormone) gene promoter polymorphism were performed in order to provide insight on the genetic relation of SPL pejerrey to other *Odontesthes* species. The *amhy* gene promoter region was included because previous studies revealed that this gene shows size polymorphism among species of *Odontesthes* (Hattori *et al.* and R. S. Hattori pers. comm., October 2017).

The microsatellite analysis included SPL pejerrey ($n = 36$), *O. bonariensis* from Chascomús Lake ($n = 20$), *O. argentinensis* from Mar del Plata ($n = 47$), *O. incisa* (Jenyns, 1841) from Santa Teresita ($n = 13$) and *O. smitti* (Lahille, 1929) from Necochea ($n = 12$). *O. incisa* and *O. smitti* samples were used as outgroups. Genomic DNA was isolated according to Villanova *et al.* (2015). Nine polymorphic microsatellite loci developed for *O. bonariensis* were amplified and used for genetic analysis (Villanova *et al.*, 2013, 2018). Amplified fragments were genotyped using an ABI 3730 DNA sequencer (Applied Biosystems; www.appliedbiosystems.com) and results obtained using

Peak scanner 1 (Applied Biosystems). Alleles were scored using a GeneScan 500 LIZ Size Standard (Applied Biosystems). To detect the presence of scoring errors or the possible presence of null alleles, we analysed the genotypic matrices obtained with the Micro-Checker software (van Oosterhout *et al.*, 2004). Genetic distance estimation and comparison among species were performed using an unweighted pair group method with arithmetic mean (UPGMA) tree based on DA distance (Nei *et al.*, 1983) by Populations, 1.2.30 software package (Langella, 1999). The resulting tree was displayed using Newick format tree file in Treedisplay employing POPTREEW. Node confidence was inferred with 1000 bootstrap replicates (Takezaki *et al.*, 2014). A factorial correspondence analysis (FCA) was performed using the GENETIX software program (Belkhir *et al.*, 2002) to graphically represent the distribution of genetic variability between pejerrey from each sampling location in multidimensional space. Assignment tests were carried out to assign SPL pejerrey individual samples to a given reference population using GeneClass2 (Piry *et al.*, 2004). Bayesian and genetic distance methods were tested for populations and individuals. Reference groups were *O. bonariensis*, *O. argentinensis*, *O. incisa* and *O. smitti*. Considering the hypothesis that SPL pejerrey was an *O. argentinensis* or *O. bonariensis* population or a mix of both species living at the Salada de Pedro Luro Lake, SPL pejerrey genetic differentiation with *O. bonariensis* and *O. argentinensis* was estimated through Pairwise F_{ST} values and their significance level ($P \leq 0.05$) were calculated using Arlequin 3.1 (Schneider *et al.*, 2000) to test the hypotheses that SPL pejerrey was an *O. argentinensis* population, an *O. bonariensis* population, or a mix of both species. In addition, Bayesian analysis of population subdivision and clustering of individual genotypes were implemented with STRUCTURE 2.2 (Pritchard *et al.*, 2000) by the Markov chain Monte Carlo (MCMC) method. One to eight genetic groups (clusters) were considered ($K = 1-8$). Ten independent runs employing an admixture model were implemented with a burn-in period length of 100,000 iterations, followed by 500,000 MCMC replicates. Evanno's correction (Evanno *et al.*, 2005) was implemented and visualised using the STRUCTURE Harvester software (Earl, 2012). Thus, δK values were used to infer the most likely K value. In addition, the presence of hybrids between *O. bonariensis* and *O. argentinensis* was explored among SPL pejerrey individual samples using NewHybrids 1.1 (Anderson & Thompson, 2002).

A primer set that amplifies the 5' flanking region of *amhy*, designed based on sequences MG707233.1 and HM153804.1 from GenBank (www.ncbi.nlm.nih.gov), was used for genotyping male specimens of SPL pejerrey ($n = 46$), *O. bonariensis* from Chascomús ($n = 105$) and Cochicó ($n = 34$) and *O. argentinensis* from San Blás ($n = 5$) in the *amhy* gene promoter region analysis. The primers were *Infyprofw-2* (taa act agc ttc agc cct gag tgg cca ctt) and *Ynfyporrv* (aag ggc aat tcc acc ggt acc cga gcg ctc gag ata tca cc) and the amplification conditions were initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 2 min and a final elongation step at 72°C for 5 min. Samples were electrophoresed in 1% agarose gel and stained with ethidium bromide according to a previous study (Yamamoto *et al.*, 2014).

3 | RESULTS

3.1 | Population biological variables

The length-frequency distribution of fish collected in 2012 was multimodal and included fish of all developmental stages from larvae to adults (10–360 mm L_S ; Figure 2).

The trends in gonad maturation during spring were similar in years 2013–2015, with maximum I_{GS} in October compared with August and December for both males and females (Table 1). The I_{GS} values for October, in particular for females, were in the range considered as active oogenesis and pre-spawning (Calvo & Dadone, 1972).

3.2 | Meristic, morphological and morphometric analysis

The meristic features of SPL pejerrey ($n = 12$; 185–268 mm L_S) compared with *O. argentinensis* and *O. bonariensis* are presented in Table 2 where there is a clear overlapping in most of characters analysed. The presence of scales in the cleithrum was observed in 91.6% of the fish. Crenulated scales were present in the pre-dorsal region of every fish. In all specimens the upper lobe of the caudal fin was larger than the lower one. The osteological analysis revealed that the haemal expansions of the caudal vertebrae had a round form and dorso-ventral striations on their surface. It is important to note that these four features are shared exclusively with *O. argentinensis* and represent taxonomic characters to differentiate from *O. bonariensis*. The first haemal spine covered $1/5$ th to $1/3$ rd of the length of the first caudal vertebra. The first haemal arch expansion was broader than the other ones, being such difference more visible after the fourth caudal vertebra. After the fourth vertebra, the expansion was mainly anterior and the length of the haemal arch was similar to the haemal expansion. Haemal expansions were visible up to the 13–14 caudal vertebra (Figure 3a).

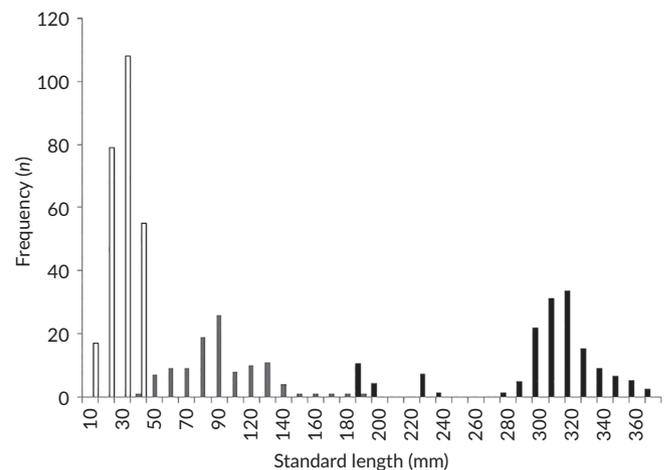


FIGURE 2 Length-frequency distribution of pejerrey collected with different sampling gears in the Salada de Pedro Luro Lake during 2012. ■, Seine-net samples; ■, gillnet samples; □, fyke net samples

TABLE 1 Mean (\pm SE) gonadosomatic Index of male and female pejerrey *Odontesthes* spp. from Salada de Pedro Luro Lake, between August and December of 2013–2015. The number of fish analysed in each sampling is indicated within parenthesis

Sex	Month	Year		
		2013	2014	2015
Female	August	3.71 \pm 0.21 (11)	2.73 \pm 0.27 (6)	2.65 \pm 0.47 (11)
	October	7.58 \pm 0.74 (14)	5.94 \pm 0.85 (12)	6.82 \pm 1.83 (9)
	December	1.15 \pm 0.31 (3)	3.12 \pm 0.40 (14)	3.99 \pm 2.78 (9)
Male	August	1.94 \pm 0.21 (9)	2.01 \pm 0.36 (3)	0.90 \pm 0.30 (6)
	October	4.62 \pm 0.28 (3)	2.57 \pm 0.25 (8)	3.20 \pm 0.22 (11)
	December	1.34 \pm 0.65 (4)	1.60 (1)	0.73 \pm 0.10 (11)

In front view, the urosome showed a circular shape (Figure 3b). The central borders of the frontal bones were partially separated by a fontanelle avoiding contact in the medium region. Premaxilla teeth were inserted in great part of the bone, but they did not cover its entire length. The dentary showed up to three rows of teeth being the external the largest ones. Presence of a medium patch, two lateral rows of teeth in the vomer, a posterior sub-opercle apophysis, a notch in the upper border of the interopercle, a serrated suture between the caracoid and the scapula and the presence of small and numerous teeth in the endopterygoid were also observed. In the basipterygium, the lateral process was acute in its extreme, the internal plate overpassed half of the bone and the frontal and posterior spines were long. The SPL pejerrey had 20 to 23 (mode of 22) gill rakers in the first ceratobranchial and this range overlapped completely with *O. argentinensis* and partially with *O. incisa* (Table 3).

In the geometric morphometric analysis of the haemal funnel, the first two RWs among the 28 obtained explained 64% (36% and 28%) of the total variance for the GLS-RWA relative to the shape of the haemal funnels in the three groups of pejerrey. The pattern of morphological variation described by the first two RWs is shown in Figure 4a. A complete segregation between *O. argentinensis* and *O. bonariensis* was observed along the second relative warp axis, while specimens from Salada de Pedro Luro Lake were clustered in an intermediate morphospace of both nominal species. Shape changes were

expressed in the angle variation between haemal funnels and their respective vertebrae, as well as in the haemal funnel width and length. The data corresponding to the 28 RWs of the RWA were used to perform the DA with specimens of SPL pejerrey, *O. argentinensis* and *O. bonariensis*. The first canonical discrimination function explained 84% of the total data variance (Wilks' $\lambda = 0.012$, $P < 0.001$). The DA correctly classified 100% of the fish according to the original groups whereas the cross-validation analysis correctly classified 75% of the fish according to their haemal funnel shape (Table 4). Misclassifications (cross-validation analysis) ranged between 5.3% and 26.7% and SPL pejerrey was the only group with misclassified individuals. On the other hand, both *O. argentinensis* and *O. bonariensis* showed only misclassifications with SPL pejerrey, the three groups were defined and their centroids and individuals were separated both on the first and second discriminant functions with no overlap (Figure 4b).

The geometrical interpretation of the PCA 1 in the otolith shape analysis reveals a change in regularity for the dorsal border explaining 36.6% of variation (Figure 5). The PCA 2 was associated, in turn, with a gradient of dorsoventral expansion for the otolith accounting for 25.4% of variation. Therefore, the first two axes in the PCA explained 62% of the accumulated variation in otolith shape. The otoliths of fish from each group presented a typical shape with significant statistical differences ($P < 0.05$). Assignment of individuals to the *Odontesthes* groups based on the outline method correctly classified 92% of the

TABLE 2 Meristic characters of pejerrey from Salada de Pedro Luro Lake (SPL pejerrey), and values range of such characters in *Odontesthes argentinensis* and *Odontesthes bonariensis*

Character	SPL pejerrey		<i>O. argentinensis</i>	<i>O. bonariensis</i>
	Mode	Range	Range	Range
Pre-caudal vertebrae	23	22–23.0	22–25	26–27
Caudal vertebrae	24	23–24	23–24	22–23
Pectoral-fin rays	14	12–14	13–16	13–15
Pelvic-fin rays	5	5–6		6
Anal-fin rays	18	17–19	16–19	15–19
Dorsal 1 (D1) fin rays	4	3–5	3–6	4–6
Dorsal 2 (D2) fin rays	10	8–11	8–11	8–11
Scales above lateral line	54	53–56	48–56	50–60
Scales rows between D2 and anal fins	10	8–10		9–11
Scales rows between D1 and lateral line	4.5	4.5	4.5	
Dorsal scale rows	10	10–11		9–10
Sub-ocular scales rows	3	3–4	3–4	2–3

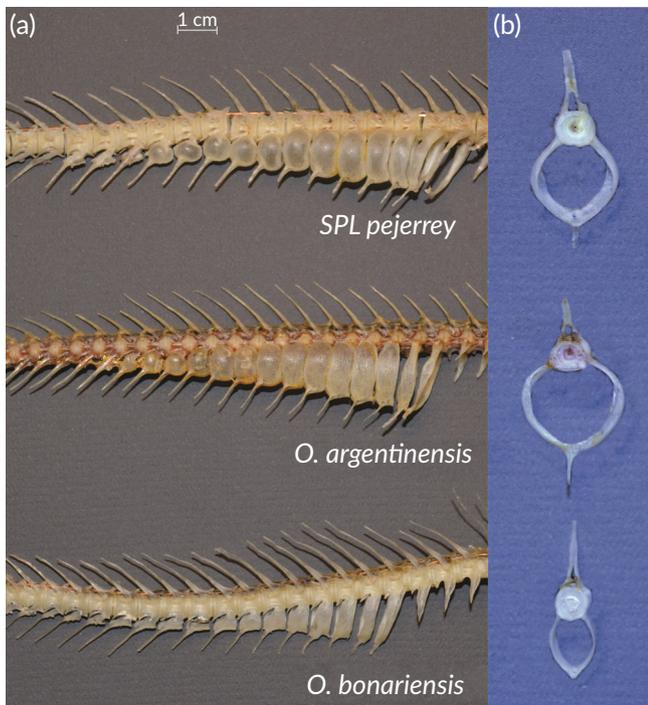


FIGURE 3 (a) Side and (b) front view of haemal funnels of pejerrey from Salada de Pedro Luro lake (SPL pejerrey), *Odontesthes argentinensis* and *Odontesthes bonariensis*

TABLE 3 Number of gill rakers on ceratobranchial of the first branchial arch in pejerrey from Salada de Pedro Luro Lake (SPL pejerrey) and other species of *Odontesthes*

Species	Number of gill rakers (range)
SPL pejerrey	20 to 23
<i>O. argentinensis</i>	20 to 24 ^b (full overlapping)
<i>O. bonariensis</i>	32 to 37 ^b
<i>O. humensis</i>	15 to 19 ^a
<i>O. incisa</i>	22 to 30 ^a , ^a (partial overlapping)
<i>O. miriniensis</i>	24 to 29 ^b
<i>O. nigricans</i>	11 to 16 ^a
<i>O. perugiae</i>	24 to 30 ^b
<i>O. retropinis</i>	46 to 50 ^b
<i>O. smitti</i>	27 to 31 ^a

^aBemvenuti, 2002.

^bPiacentino, 1999.

samples (Table 5). The few individuals that were misclassified belonged to the SPL pejerrey group.

3.3 | Salinity tolerance of embryos

Larvae from SPL pejerrey and *O. bonariensis* hatched after 14 days of incubation at 19°C. The two groups showed similar survival (range 73.5%–88.1%) at low and moderate salinities but SPL pejerrey had a

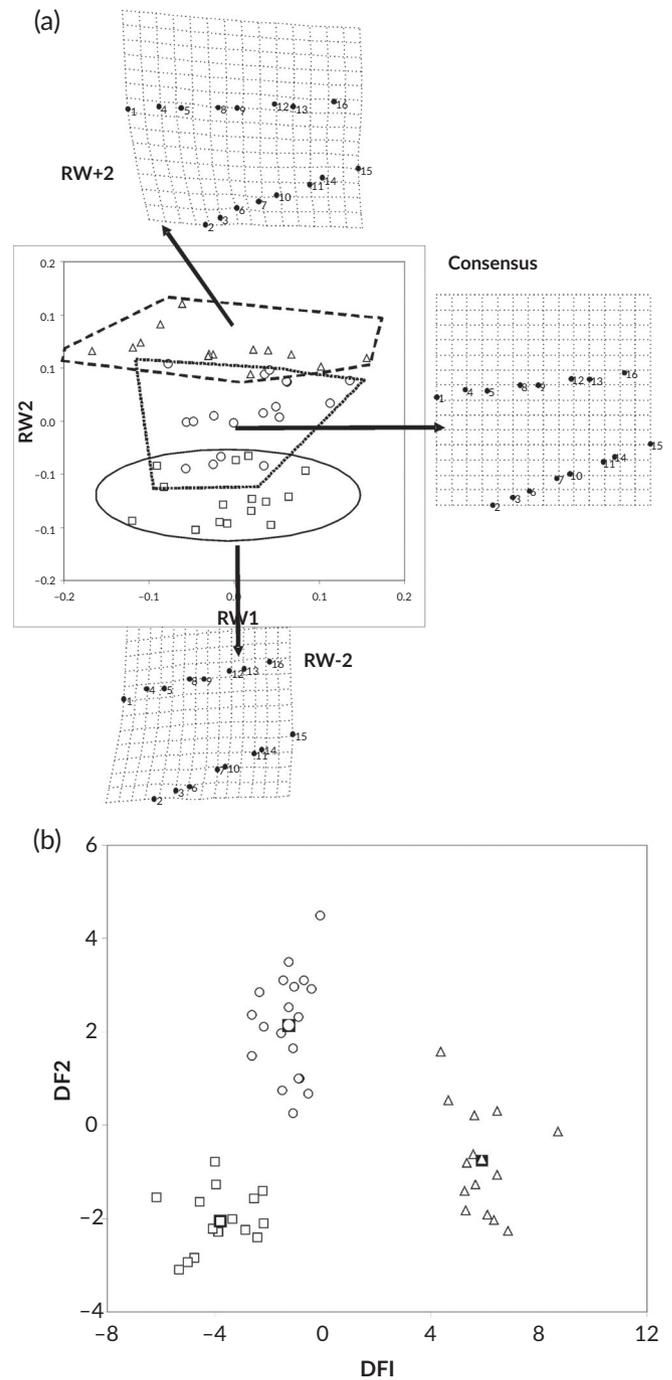


FIGURE 4 (a) Relative warp analysis (RW1 v. RW2) based on landmark coordinates obtained from pejerrey from Salada de Pedro Luro Lake (SPL pejerrey; O), *Odontesthes argentinensis* (□), and *Odontesthes bonariensis* (△). Thin-plate spline transformation grids for the extreme points of RW2 are shown; they 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. (b) Discriminant analysis of haemal funnels based on landmark coordinates for the same pejerrey groups shown in (a). Symbols as in (a)

significantly higher hatching rate than *O. bonariensis* at a salinity of 30 (Table 6). Embryos from the two sources did not hatch at a salinity of 45.

TABLE 4 Predicted Group Membership according to cross-validated discriminant analysis, based on the relative warps scores of landmarks coordinates for the haemal funnel of pejerrey from Salada Pedro Luro Lake (SPL pejerrey), *Odontesthes argentinensis* and *Odontesthes bonariensis*

Groups	SPL pejerrey	<i>Odontesthes argentinensis</i>	<i>Odontesthes bonariensis</i>
SPL pejerrey	13 (68.4%)	5 (26.3%)	1 (5.3%)
<i>Odontesthes argentinensis</i>	4 (26.7%)	11 (73.3%)	0
<i>Odontesthes bonariensis</i>	2 (14.3%)	0	12 (85.7%)

3.4 | Genetic analyses

The Micro-Checker analysis suggested consistent genotyping at all loci. Stuttering and large allele dropout were not suggested in any case. The UPGMA tree based on Nei's genetic distance matrix detected four main clades corresponding to, *O. argentinensis*, *O. bonariensis*, *O. smitti* and *O. incisa*. Pejerrey from Salada de Pedro Luro Lake were included in the *O. argentinensis* clade (Figure 6a). The FCA analysis supported these results (Figure 6b). In addition, genetic assignment tests based on Bayesian and genetic distance methods implemented in GeneClass2, showed that SPL pejerrey individuals were assigned to the *O. argentinensis* group and rejected from *O. bonariensis*, *O. smitti* and *O. incisa* groups (Supporting Information Table S1). The STRUCTURE analysis of SPL pejerrey, *O. argentinensis* and *O. bonariensis*, indicated that the most probable number of clusters in the total sample was $K = 2$ (Supporting Information

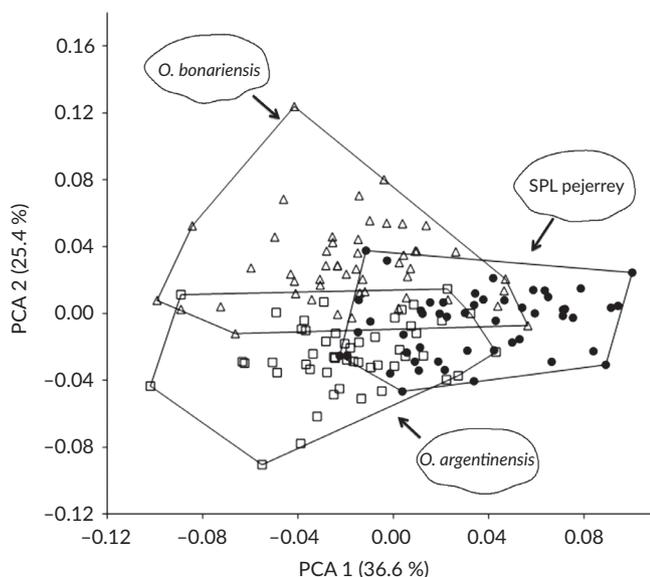


FIGURE 5 Average shape and principal component analysis of five harmonics derived from the contours of sagittal otoliths of pejerrey from Salada de Pedro Luro Lake (SPL pejerrey; $n = 50$; \square), *Odontesthes argentinensis* ($n = 50$; \circ) and *Odontesthes bonariensis* ($n = 50$; \triangle)

TABLE 5 Cross-classification analysis of each fish group of pejerrey from Salada Pedro Luro Lake (SPL pejerrey) *Odontesthes argentinensis* and *Odontesthes bonariensis* according to otolith analysis

	SPL pejerrey	<i>Odontesthes argentinensis</i>	<i>Odontesthes bonariensis</i>
SPL pejerrey	46	3 (6.4%)	1 (2.2%)
<i>Odontesthes argentinensis</i>	3 (6.5%)	47	0
<i>Odontesthes bonariensis</i>	4 (8.7%)	0	46

Figure S1a). The most likely population structure according to this analysis is shown in Supporting Information Figure 1b. Thus, individuals from Salada de Pedro Luro Lake were mainly assigned to group 2, which is defined by *O. argentinensis*. Moreover, SPL pejerrey had a lower pairwise F_{ST} value ($F_{ST} = 0.02$; $P < 0.001$) with *O. argentinensis* from the Atlantic (Mar del Plata) than with *O. bonariensis* from Chascomús Lake ($F_{ST} = 0.19$; $P < 0.001$). SPL pejerrey hybrid individuals between *O. argentinensis* and *O. bonariensis* species were not found using NewHybrids (Supporting Information Table S2).

Genotyping based on the *amhy* promoter sequence revealed the presence of two fragments of different sizes among the samples of *O. bonariensis* from Chascomús Lake and Cochicó Lake that had a shorter fragment compared with those from SPL pejerrey and *O. argentinensis* from the Atlantic coast (San Blás; Figure 7).

4 | DISCUSSION

The SPL pejerrey exhibits demographic characteristics that indicate a well established population characterised by the presence of all developmental stages from larvae and early juveniles to adults and a multimodal length-frequency distribution that suggests the presence of multiple year-classes. There were also clear signs of gonadal maturation and imminent spawning as evidenced by the maturation and pre-spawning-stage I_{GS} values (Calvo & Dadone, 1972) recorded during the presumed reproductive season.

Comparison of the meristic and osteological characteristics of SPL pejerrey with those of other species of *Odontesthes* reveals that SPL pejerrey differs from *Odontesthes perugiae* Evermann & Kendall 1906 in the partial dentition of the dentary bone and from *O. incisa* in the uniformity in size of such teeth. Likewise, the presence of teeth over the endopterygoid bone allows to separate SPL pejerrey from *Odontesthes humensis* de Buen 1953 (Bemvenuti, 2005). In addition, the shape of the haemal expansions and the number of scales above the lateral line clearly differentiate SPL pejerrey from *Odontesthes hatcheri* (Eigenmann 1909), *O. incisa*, *Odontesthes nigricans* (Richardson 1848) and *Odontesthes platensis* (Berg 1895) (Mancini et al., 2016; Piacentino & Torno, 1987; Tombari et al., 2010). Some SPL pejerrey characters, such as the number of fin rays, small teeth in the endopterygoids and the absence of teeth in the premaxilla, are shared by *O. argentinensis* and *O. bonariensis*. On the other hand, the presence of scales in the cleithrum, the larger upper lobe in the caudal

TABLE 6 Mean (\pm SE; $n = 3$) hatching rates of pejerrey from Salada Pedro Luro Lake (SPL pejerrey) and *Odontesthes bonariensis* eggs incubated at different salinities

Fish source	Salinity			
	5	15	30	45
SPL pejerrey	88.1 \pm 0.6	82.3 \pm 0.3	40 \pm 0.7*	0
<i>Odontesthes bonariensis</i>	73.5 \pm 2.4	76.5 \pm 1.4	15 \pm 0.5*	0

* $P < 0.05$.

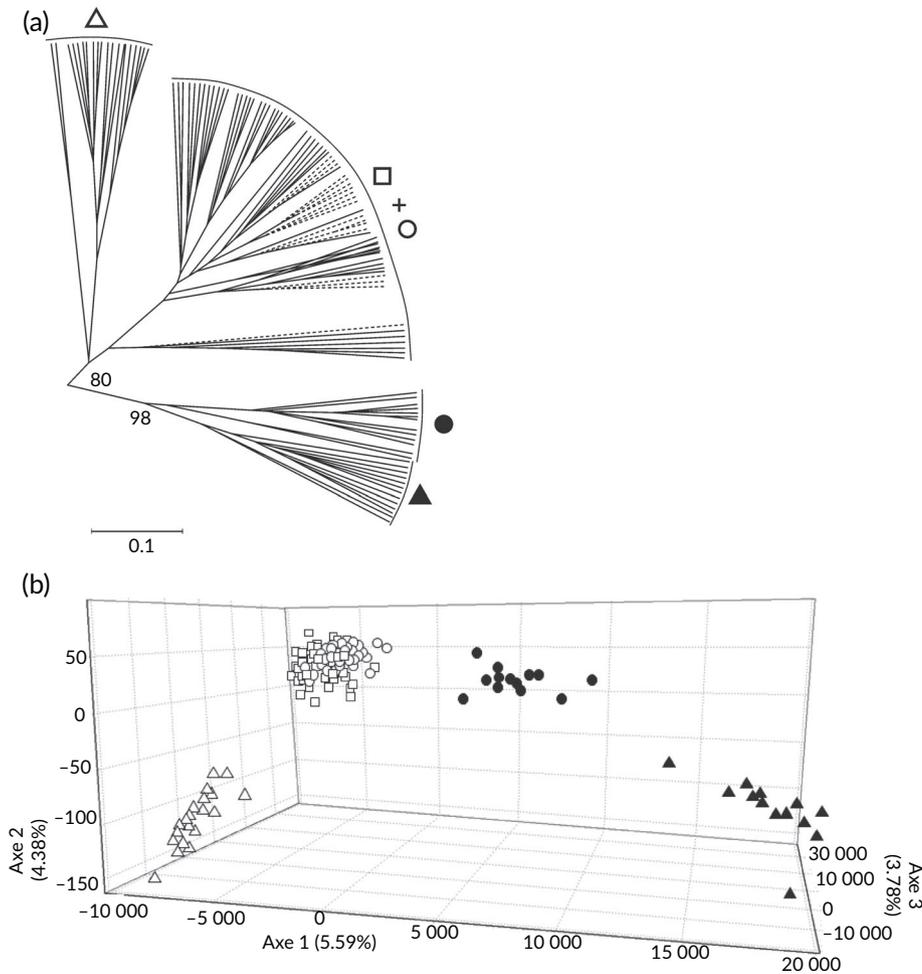


FIGURE 6 (a) Unweighted pair group method with arithmetic mean (UPGMA) tree based on Nei's distance (D_a) showing the relationships among populations of pejerrey from Salada de Pedro Luro Lake (SPL pejerrey) with *Odontesthes argentinensis*, *Odontesthes bonariensis*, *Odontesthes smitti*, and *Odontesthes incisa*. Bootstrap support was generated from 1000 replicates. Only values $> 50\%$ are shown (\circ) SPL pejerrey, (\square) *O. argentinensis*, (\triangle) *O. bonariensis*, (\bullet) *O. smitti*, and (\blacktriangle) *O. incisa*. (b) Factorial correspondence analysis (FCA) plot of the genetic relationships between the groups shown in (a)

fin, the crenulate pre-dorsal scales, the circular frontal appearance of the urosome vertebrae and the number of gills rakers of SPL pejerrey are typical characteristics of *O. argentinensis* (Bemvenuti, 2002; Dyer, 2006; González-Castro et al., 2016; Mancini et al., 2016; Tombari et al., 2010).

The integrative taxonomic approach based on the geometric morphometric analysis of the haemal funnel, otoliths and the genetic analysis corroborated the findings above that place SPL pejerrey as intermediate morphotype between *O. argentinensis* and *O. bonariensis*, but more closely related to the former. For example, the cross-validated discriminant analysis shows both the intermediate position of this group, also the dissimilarity in shape between the nominal species *O. argentinensis* and *O. bonariensis*. Furthermore, it highlights the

existence of morphological features of these populations that are sufficient to discriminate the Salada de Pedro Luro Lake population from other closer species of the *Odontesthes* genus. Interestingly, although hybrid individuals between *O. bonariensis* and *O. argentinensis* were found at Salada Grande Lake (Buenos Aires province, General Madariaga district; Tejedor, 2001), no hybrid individuals were found in Salada de Pedro Luro Lake. Thus, the STRUCTURE analysis, the NewHybrids analysis and the microsatellite analysis all suggested that the current pejerrey population of Salada de Pedro Luro Lake is derived solely from *O. argentinensis*. Essentially the same conclusion can be derived from genotyping based on *amhy* promoter amplification.

When populations occupy different environments, divergent selection pressures can result in phenotypic differentiation in traits

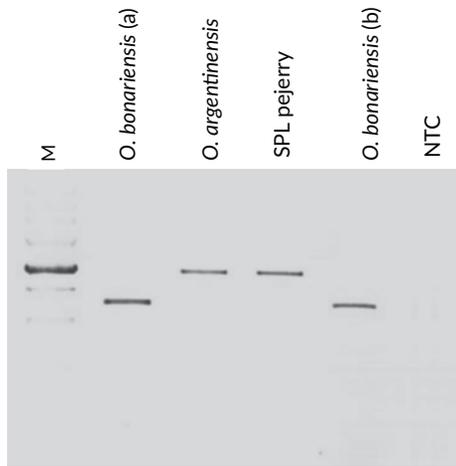


FIGURE 7 Polymorphism analysis of the *amhy* gene promoter in pejerrey from Salada de Pedro Luro Lake (SPL pejerrey), *Odontesthes argentinensis* from the Atlantic coast (San Blas) and *Odontesthes bonariensis* from Chascomús Lake (a) and Cochicó Lake (b). M, marker; NTC, non-template control

that confer a local fitness advantage (Kawecki & Ebert, 2004). Indeed, it is known that morphological characters of otoliths and other skeletal structures, such as those that constitute the distinctive characters of SPL pejerrey, can be related to the ecological niche occupied by the pejerrey population in Salada de Pedro Luro Lake and reflect local adaptations (Lombarte & Popper, 2004; Schulz-Mirbach *et al.*, 2008) and reflect specific adaptations (Tuset *et al.*, 2012). In the absence of any homogenising effects of gene flow, divergent selection can lead to the evolution of locally adapted ecotypes, where resident genotypes produce phenotypes with higher relative fitness than genotypes originating from other habitats (Kawecki & Ebert, 2004). Alternatively, adaptive phenotypic plasticity can move population phenotypes toward a local optimum without any genetic differentiation in response to environmental cues (Price *et al.*, 2003), and this may be the case with the SPL pejerrey. Evidence of local adaptation in morphology due to differences in selection pressure has been documented in a number of fish species (Snorrason *et al.*, 1994; Taylor & McPhail, 1985; Toline & Baker, 1993). In *Percichthys trucha* (Valenciennes 1833) for example, a common Patagonian freshwater species, within-species differentiation in phenotypic characters were related to the use of environmental resources (Ruzzante *et al.*, 2012). Future studies must address whether SPL pejerrey resident genotypes have been selected to produce adaptive phenotypes or if adaptation has been produced without genetic differentiation.

Differentiation due to isolation has been previously documented in other Atheriniformes (*e.g.*, subfamily Menidiinae; Bloom *et al.*, 2013). A high plasticity that enables animals to move from marine to brackish environments has been reported in the family Atherinidae (Bamber & Henderson, 1988). Likewise, in the Mar Chiquita Lagoon, Argentina, *O. argentinensis* showed reproductive isolation and significant meristic and body shape differences compared with individuals from the sea despite both environments remained connected (Gonzalez- Castro *et al.*, 2016). It has been suggested that the same

species can behave as well-differentiated populations, or even as incipient ecological species (González- Castro *et al.*, 2009; 2016). This is in agreement with previous studies showing that the successful colonisation and ecological shift of estuarine environments could promote speciation of marine pejerrey (Beheregaray & Sunnucks, 2001; Bemvenuti, 2002; 2005; González- Castro *et al.*, 2016). In southern South America, such features could have been favoured by the decrease of sea level during late Holocene (Angulo *et al.*, 2006; Islas, 2012), allowing the development of coastal lagoons with suitable saline conditions for colonisation by eurihaline species such as the pejerrey. When marine species colonise estuarine or freshwater environments, new selective pressures can account for divergent natural selection and adaptation, in the long term promoting new lineages through speciation (Beheregaray & Sunnucks, 2001; Betancur *et al.*, 2012; Lescaik *et al.*, 2015; Schluter, 2000). In the case of Salada de Pedro Luro Lake, it should be noted that the naturally elevated salinity may have precluded the establishment of other species and the presence of hybrids. Therefore, the impoverished fish fauna conditions of the lake may have offered ecological opportunities for other tolerant species such as *O. argentinensis* to colonise this lake entering through oligohaline or mesohaline corridors. As noted by Losos (2010) and Yoder *et al.* (2010), ecological opportunity in isolated lakes is a direct function of resource abundance and an inverse function of competition, when occasional connections occur.

During extensive faunal surveys carried out in the study area (Almirón *et al.*, 1997), the presence of *O. argentinensis* was only registered in the mouth of the Colorado River. Thus, the possibility of this marine species reaching the endorheic Salada de Pedro Luro Lake seem to be very limited. However, considering that drainage water flows to the sea through saline soils causing progressive increase in water conductivity before reaching the coast and that marine tides can be detected in the continent up to 60 km from the mouth of Colorado River (Perillo *et al.*, 1999), it is possible that under very exceptional occasions, such as the event which supposedly originated this population, the *O. argentinensis* might reach the Salada de Pedro Luro Lake through drainage channels. On the other hand, the absence of *O. bonariensis* and their hybrids at Salada de Pedro Luro Lake is somewhat puzzling. It is known that *O. bonariensis* was introduced into the lake several times (G. E. Berasain pers. comm., November 2012) and that the species is the most abundant pejerrey inhabiting this section of the Colorado River (Almirón *et al.*, 1997) from where it could reach this lake. This species can also be found in brackish environments (Campanella *et al.*, 2015; Dyer, 2006) and in several saline lakes (Berasain *et al.*, 2015; Bucher & Etchegoin, 2006; Kopprio *et al.*, 2010; Mancini *et al.*, 2016) as well. However, high saline conditions may represent a physiological barrier for embryo development, as demonstrated in the salinity tolerance test conducted in this study. Thus, reproduction may be only successful in water bodies with moderate salinity (Berasain *et al.*, 2015). The lack of permanent natural freshwater inlets and the very high salinity reached in Salada de Pedro Luro Lake during dry years may explain why this species did not establish a stable population and therefore did not produced hybrid individuals.

We conclude that the SPL pejerrey population represents a group harbouring unique characteristics that shows a case of phenotypic differentiation due to local adaptation, phenotypic plasticity, or both, from *O. argentinensis* through partial or total isolation. More efforts should be focused however, in defining the SPL pejerrey population structure at the genetic and body shape-morphogeometric levels using finer-scale analysis. This approach would provide additional clues to determine if the Salada de Pedro Luro Lake population derives from an estuarine ancestral and currently represents a case of incipient speciation. Further studies should also be directed toward understanding the role of the Colorado River and irrigation–drainage channels as potential corridors connecting the Salada de Pedro Luro Lake to the Atlantic Ocean. This study offers novel evidence and documents for the first time that marine pejerrey can maintain landlocked populations. Studies on unique environments such as Salada de Pedro Luro Lake will foster our understanding of marine pejerrey colonisation mechanisms and speciation processes and help devise management and conservation strategies for these species and environments.

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CONTRIBUTIONS

C.D., M.L. and B.C. substantial contributions to conception and design, acquisition of data, analysis and interpretation of data and intellectual contribution to manuscript drafting. S.C.A. acquisition of data, reviewing critically the manuscript adding a relevant intellectual contribution and improvements. G.-C.M., V.G.V., H.R.S., L.I., L.I.-V.S., M.P. specific analysis and interpretation of data and intellectual contribution to manuscript drafting. M.M., M.T., B.G., G.F., S.P., S.V., D.-P. O. dF.-P. and Y.Y. acquisition of data, analysis and interpretation of data and intellectual contribution to manuscript drafting.

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SUPPORTING INFORMATION

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