Neotropical Ichthyology, 13(1): 137-150, 2015

DOI: 10.1590/1982-0224-20130094

Populations of *Odontesthes* (Teleostei: Atheriniformes) in the Andean region of Southern South America: body shape and hybrid individuals

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The original distribution area of the Patagonian 'pejerrey' *Odontesthes hatcheri* has been subjected to the introduction of a related species; the Bonaerensean 'pejerrey' *Odontesthes bonariensis.* This species currently coexists with *O. hatcheri* in lakes and reservoirs, and can interbreed and produce fertile hybrid offspring. The purposes of this study were; a) the extensive sampling of Patagonian and Andean-Cuyan populations of pejerrey, b) the species identification according to taxonomic key, c) validation of taxonomic results on the basis of mitochondrial DNA composition, and d) applying morphometric analysis to explore the effects of hybridization and environmental conditions on body shape. Cytochrome *b* sequence analysis showed a high degree of genetic divergence between species and low intraspecific variation in *O. hatcheri*. Geometric Morphometric Analyses detected shape differences in agreement with diagnostic characteristics of each species. Putative hybrids exhibiting intermediate diagnostic characteristics were identified by Geometric Morphometric Analysis. Significant regressions between body shape and total phosphorus and altitude were found, suggesting a dependence on trophic web structure. This multi-level approach suggests the introgression of *O. bonariensis* into several *O. hatcheri* populations throughout Patagonia. Managers should take this into account when considering further exotic introductions into regions where non-native fishes have not yet become established.

La distribución original del 'pejerrey' patagónico *Odontesthes hatcheri* ha sido sometida en las últimas décadas a la introducción de una especie relacionada; el 'pejerrey' Bonaerense *Odontesthes bonariensis*. Ambas especies coexisten actualmente en algunos lagos y embalses debido a prácticas de siembra y pueden cruzarse y producir progenie híbrida y fértil. Los propósitos de este estudio fueron a) un amplio muestreo de las poblaciones patagónicas y andino-cuyanas del pejerrey, b) la identificación de las especies de acuerdo con la clave taxonómica, c) la validación de los resultados taxonómicos sobre la base de la composición del ADN mitocondrial y d) aplicar el análisis morfométrico para explorar los efectos de la hibridización y las condiciones ambientales sobre la forma corporal. El análisis de la secuencia del Citocromo *b* mostró un alto grado de divergencia genética entre ambas especies y una muy baja variación intraespecífica en *O. hatcheri.* El análisis de la Morfometría Geométrica detectó diferencias de forma coincidentes con las características diagnósticas de cada especie. Presuntos híbridos exhibiendo características diagnósticas intermedias fueron identificados por el análisis de la Morfometría Geométrica. Regresiones significativas entre la forma corporal y la concentración total de fósforo y la altitud fueron halladas, sugiriendo una dependencia con la estructura de la trama trófica. Este enfoque múltiple sugiere la introgresión de genes de *O. bonariensis* dentro de varias poblaciones de *O. hatcheri* a lo largo de la Patagonia. Las autoridades de aplicación deberían tomar en cuenta estos riesgos al momento de considerar nuevas introducciones de especies exóticas en regiones donde estas especies no se encuentren previamente establecidas.

Keywords: Geometric morphometrics, Patagonia, Pejerrey, Stocking.

Introduction

The Patagonian pejerrey, *Odontesthes hatcheri* (Eigenmann, 1909) is a native freshwater species from the Andean Region of southern South America (Dyer, 2000; López *et al.,* 2008), encompassing a vast latitudinal range, from 27ºS to 54ºS. This species is commonly found in rivers, lakes, and reservoirs of both Atlantic and Pacific Patagonian drainages (Aigo *et al.,* 2008).

The native distribution area of the Patagonian pejerrey (Fig. 1) has been subject to stocking practices, with *Odontesthes bonariensis* (Valenciennes, 1835) stocking

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carried out by three hatcheries (Estación Hidrobiológica de Chascomús 35º36'S, 58º01'W, Estación de Piscicultura de Embalse 32º13'S, 64º29'W, and Estación de Piscicultura Río Limay 38º59'S, 68º14'W) since 1930 (Dyer, 2006; Crichigno *et al.,* 2013). Crichigno *et al.* (2013) differentiated *O. hatcheri, O. bonariensis* and intermediate individuals on the basis of head shape. The taxonomic categories included a non-negligible number of individuals considered misclassified cases. The closer the hatcheries participating in stocking programs (Estación de Piscicultura de Embalse and Estación de Piscicultura Río Limay) were to the sampling site, the higher the percentage of misclassified individuals.

Odontesthes bonariensis, a commercially important species that has been used extensively in aquaculture since the early 1900s (Somoza *et al.,* 2008), is a dominant species in the limnetic zone of the 'lagunas' (Gómez *et al*., 2007) of Buenos Aires Province in Argentina and in diverse environments within the Plata-Paraná basin (Fig. 1). This ubiquitous species is highly valued as food and as a sport fish. In consequence, it has been introduced in Argentina and Chile and also exported overseas to Japan and Italy (López *et al*., 1991; Somoza *et al*., 2008). This species is able to establish populations in environments where mean summer air temperatures are higher than 20ºC (Cussac *et al*., 2009). The southernmost locality known for *O. bonariensis* in Patagonia is the Reservoir Ezequiel Ramos Mexía on the Limay river (39º30´S, 69º00´W, Aigo *et al.,* 2008).

Both species have disjoint natural distributions (Fig. 1); *O. hatcheri* in the southwest and *O. bonariensis* in the north east (Baigún & Ferriz, 2003; Dyer, 2006; Aigo *et al*., 2008), and both are remarkably tolerant of high salinity levels but not of sea water (Tsuzuki *et al*., 2000a, b; Gómez & Ferriz, 2001). Genetic data from Sommer *et al.* (2010) indicate that *O. hatcheri* has a closer relationship with marine species than with *O. bonariensis*. Both species hybridize in captivity (Strüssmann *et al*., 1997a) and probably also in the wild (Dyer, 2000). This suggests that the extent of hybridization in natural habitats where both species coexist may be significant (Strüssmann *et al*., 1997a).

The successful interbreeding of hybrids with one or both parental forms leads to introgression of varying degrees (Turner, 1999). To characterize individuals along such a continuum, and to account for various types of hybrids, many authors have resorted to multivariate analysis (Crespin & Berrebi, 1999). Multidimensional morphology (Crespin & Berrebi, 1999) and geometric morphometrics (Valentin et al., 2002; Bart Jr et al., 2010; Tobler & Carson, 2010) have proven useful tools in the study of the morphological transition across hybrid zones between species.

The aim of this study is to review the taxonomic status of native populations of *O. hatcheri* after the introduction of *O. bonariensis*. The study involves; a) extensive sampling of Patagonian and Andean-Cuyan populations of the Patagonian pejerrey, b) species identification according to taxonomic key, c) genetic analyses used to validate taxonomic results based on the mitochondrial DNA composition, and d) morphometric analysis used to explore the effects of hybridization and environmental conditions on body shape.

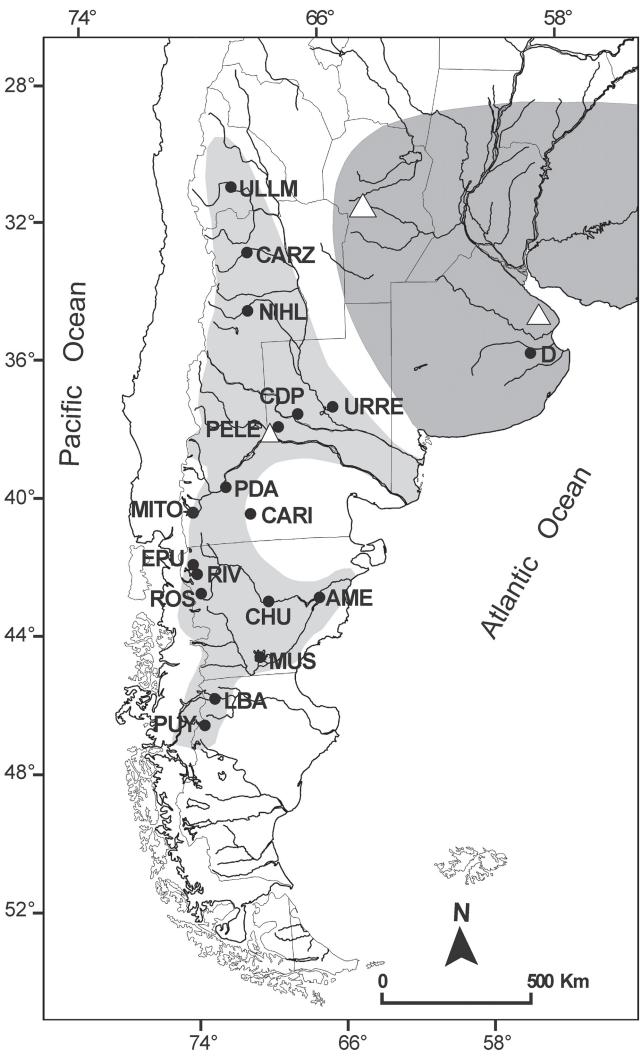

Fig. 1. Distribution of *O. hatcheri* (light gray) and *O. bonariensis* (dark gray) described by Dyer (2006) and sampling localities: ULLM, Ullum Reservoir; CARZ, Carrizal Reservoir; NIHL, Nihuil Reservoir; D, Lake San Lorenzo; URRE, Lake Urre Lauquen; CDP, Casa de Piedra Reservoir; PELE, Lake Pellegrini; PDA, Piedra del Aguila Reservoir; MITO, Lake Morenito; CARI, Lake Carilafquen; EPU, Lake Epuyén; RIV, Lake Rivadavia; ROS, Lake Rosario; AME, Florentino Ameghino Reservoir; CHU, Chubut River at Los Altares; MUS, Lake Musters; LBA, Lake Buenos Aires; PUY, Lake Pueyrredón. White triangles show the location of the three hatcheries (Estación Hidrobiológica de Chascomús 35º36'S, 58º01'W, Estación de Piscicultura de Embalse 32º13'S, 64º29'W, and Estación de Piscicultura Río Limay 38º59'S, 68º14'W), sources of stocking practices.

Materials and Methods

Collection sites and sampling procedures. Fish samples were obtained from 18 localities throughout the Andean Region, covering most of the native distribution of *O. hatcheri*. Individuals were caught using seine or gill nets in streams or littoral areas of lakes and reservoirs (Table 1, Fig. 1). A sample from a natural population of *O. bonariensis* was obtained from the native range of this species (Lake San Lorenzo, locality D in Fig. 1, Table 1) as a reference. To study interactions between the two species and the formation of hybrids, four localities were targeted where both species were present (NIHL, URRE, CDP, PELE, in Fig. 1). Captured fish were immediately killed by an overdose of anesthetic (benzocaine 1:10000). Digital images of the left side of the body were taken for each fresh specimen, taking care to minimize parallax error. Fish were measured (Standard Length= SL), weighed, and Condition Factor (body mass . SL^{-3}) was calculated. Tissue samples (muscle) were preserved in 96% ethanol for DNA analysis. Species identities were determined following the dichotomous key proposed by Dyer (2006). Specimens corresponding to Lake Pueyrredón, the type locality of *O. hatcheri* (Ringuelet *et al.,* 1967), were deposited in *Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata,* Argentina (Voucher Museum ID: MLP 9858 to MLP 9877). The same Sample ID was used to include these individuals in The Barcode of Life Data System (Ratnasingham & Hebert, 2007).

Table 1. Sampling localities, basins, latitude, longitude, mean summer air temperature 1961-1990 (Temp, data from Servicio Meteorológico Nacional, Argentina, www.smn.gov.ar), species identification, and SL of *Odontesthes* specimens used in this study. All river drainages flow to the Atlantic, unless otherwise noted (Pacific). *OB*: *O. bonariensis* , *OH*: *O. hatcheri,* Hy: presumptive hybrids, showing key characters of both species; CA: Cluster Analysis; GMA: Geometric Morphometrics Analysis; N: number of specimens. In bold, differences between key, genetic, and morphological criteria. Presumptive hybrids were highlighted as * in Fig. 3 and bold characters in Table 2.

Sampling locality and river basin	Latitude (S) Longitude (W)	Temp (ºC)	Species based on key characters (N)	Species based on mtDNA (N)	Species based on CA applied to GMA data (N)	SL (cm) (mean and range)
ULLM, Ullum Reservoir, Colorado basin	31º28' 68º40'	22	*OB* (25)	*OB* (1)	*OB* (25)	8.83 (6.06-14.14)
CARZ, El Carrizal Reservoir, Colorado basin	33°20' 68°43'	22	*OB* (30)	*OB* (1)	*OB* (30)	17.00 (13.62-30. 90)
NIHL, El Nihuil Reservoir, Colorado basin	35°04' 68°45'	22	***OH* (12)**	***OB* (2)** *OH* (7)	***OH* (12)**	7.44 (4.85-9.71)
D, Lake San Lorenzo; Salado del Sur basin	36°5' 58° 1'	20-22	*OB* (34)	*OB* (2)	*OB* (34)	19.66 (15.54-23.98)
URRE, Lake Urre Lauquen; Colorado basin	38°5' 65°50'	22-24	OB (6)	OB (2) OH (4)	OB (4) **OH (1)**	12.48 (7.05-16.35)
CDP, Casa de Piedra Reservoir, Colorado basin	38°15' 67°30'	22-24	*OB* (1) *OH* (8)	*OB* (1) *OH* (7)	*OB* (1) *OH* (8)	7.75 (3.92-26.58)
PELE, Lake Pellegrini; Negro basin	38°41' 67°59'	22	**Hy (26)** *OH* (19)	***OB* (5)** *OH* (18)	***OB* (6)** ***OH* (36)**	27.51 (22.32-39.40)
PDA, Piedra del Aguila Reservoir; Negro basin	40° 24' 70° 6'	16-18	*OH* (30)		*OH* (30)	9.46 (12.20-7.80)
MITO, Lake Morenito; Negro basin	41°03' 71°31'	12-14	*OH* (27)	*OH* (6)	*OH* (27)	31.37 (21.77-38.00)
CARI, Lake Carilafquen Chica; endorheic basin	41°12' 69° 25'	16-18	*OH* (24)	*OH* (2)	*OH* (24)	20.93 (26.70-14.03)
EPU, Lake Epuyen; Puelo basin (Pacific)	42°10' 71° 31'	12-14	*OH* (6)	*OH* (11)	*OH* (6)	5.02 (4.14-5.63)
RIV, Lake Rivadavia; Futaleufú basin (Pacific)	42°30' 71°45'	12-14	*OH* (28)	*OH* (11)	*OH* (28)	7.21 (6.49-7.80)
ROS, Lake Rosario; Futaleufú basin (Pacific)	43°15' 71°20'	12-14	*OH* (27)	*OH* (3)	*OH* (27)	28.49 (15.91-33.09)
AME, Florentino Ameghino Reservoir, Chubut basin	43°42' 66°29'	18-20	***OH* (15)**	***OB* (2)** *OH* (7)	***OH* (15)**	17.75 (13.17-32.90)
CHU, Chubut River (Los Altares); Chubut basin	43°51' 68°48'	18	*OH* (29)	*OH* (11)	*OH* (28)	8.04 (5.15-11.26)
MUS, Lake Musters; Chubut basin	45°28' 69°10'	16-18	*OH* (25)	*OH* (11)	*OH* (25)	11.68 (5.70-22.94)
LBA, Lake Buenos Aires; Aysen basin (Pacific)	46°29' 71°28'	14	*OH* (36)	*OH* (23)	*OH* (36)	17.59 (8.91-27.17)
PUY, Lake Pueyrredón; Baker basin (Pacific)	47°23' 71°55'	12-14	*OH* (29)	*OH* (12)	*OH* (29)	14.15 (9.24-39.05)

Genetic analysis. To assess maternal genetic identity, mitochondrial DNA (mtDNA) sequences of the cytochrome *b* gene (cyt*b*) were collected from a subsample of individuals (Table 1) and two outgroup specimens (*O. smitti* (Lahille, 1929), a strictly marine species obtained from a coastal South Atlantic locality near Puerto Madryn, Argentina). DNA was extracted from ethanol-preserved tissues using a Promega Wizard kit (Promega Corp.). The primer (forward) GLU31 (Unmack *et al*., 2009) and a species-specific primer designed for this study (Pej15929 5'-CGGCGTTCGGTTTACAAGAC-3') were used to amplify cyt*b* by PCR. The target DNA fragment was amplified in 12 µl reactions using 25 ng of template DNA, 0.25 mM of each primer, 0.625 units of Taq DNA polymerase, 0.1 mM of each dNTP, 1.25 µL of 10X reaction buffer and 1.25 µl of 25 mM MgCl2. Amplification thermocycler parameters were: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 50.7°C for 45 s, 72°C for 60 s, and, finally, 72°C for 10 min. PCR products were purified using 96-well Excelapure filter plates (Edge Biosystems). Templates were sequenced from both directions using the same primers used for PCR amplification and resolved on an Applied Biosystems 3730 XL automated sequencer (Brigham Young University DNA Sequencing Center). Raw chromatograms were assembled into contigs and edited using Sequencher version 4.8 (Gene Codes Corp.). Sequences were then aligned using MAFFT version 6 (Katoh & Toh, 2008). Identical sequences obtained from multiple individuals were identified and collapsed to unique haplotypes using FaBOX (Villesen, 2007) before phylogenetic analysis.

The cyt*b* alignment was partitioned by codon position and an independent model of sequence evolution was determined for each partition using the Treefinder software version 13.0.0 (Jobb, 2008). Phylogenetic relationships between unique haplotypes were estimated by Maximum Likelihood (ML) analyses using said software and 100 bootstrap pseudoreplicates to estimate support for the resulting phylogeny. In order to obtain information regarding zygotic genetic identity in *Odontesthes*, nuclear microsatellite markers are being developed (G. Ortí pers. obs.).

Geometric morphometric analysis. Morphometric characters were scored and analyzed to quantify anatomical differences between species.

Fifteen landmarks (Fig. 2) were digitized from 436 individuals with the TpsDig v2.10 software (Rohlf, 2006). Landmarks used were: 1, anterior tip of the premaxilla; 2, eye center; 3, posterior process of symplectic bone; 4, dorsal insertion of the pectoral fin; 5, anterior insertion of the first dorsal fin; 6, anterior insertion of the second dorsal fin; 7, dorsal insertion of the caudal fin; 8, posterior end of the lateral line; 9, ventral end of the caudal fin; 10, anterior insertion of the anal fin; 11, anus; 12, distal tip of the pelvic fin onto fish body; 13, anterior insertion of the pelvic fin; 14, vertical tie of landmark 12 onto the lateral line; and 15, vertical tie of landmark 10 onto the lateral line. These were chosen to include the diagnostic anatomical features for the Dyer (2006) identification key and other standard points commonly used for fish morphometrics. Landmark configurations for each specimen were aligned, rotated, translated and scaled by a Generalized Procrustes Analysis (GPA), (Rohlf & Slice, 1990) using a consensus configuration as a reference (Rohlf and Slice, 1990; Rohlf and Marcus, 1993). The unbending command, landmarks 4, 8, and two landmarks on the lateral line (14 and 15) were used to correct body position. After the unbending process landmarks 14 and 15 were eliminated from the analysis. Partial (PW) and Relative Warps (RW) were calculated using TpsRelw v1.35 (Rohlf, 2003a). RWs allowed the visualization of mean body shape of each population by means of deformation grids relative to the consensus shape. Cluster Analysis (CA, centroid clustering and average linkage between groups) and Discriminant Analysis (DA) were performed with SPSS software employing the PW and uniform coordinates (weight matrix, Rohlf, 1996) in order to group fish on the basis of overall body shape, test differences between species and presumptive hybrids, and within species between capture sites, respectively. The percentage of individuals correctly grouped and the probability of each individual being correctly grouped were obtained from the analysis.

In order to assess the effects of size on body shape within each species, TPSRegr 1.28 (Rohlf, 2003b) and polynomial regressions between Discriminant Functions (DFs) and independent variables such as sampling sites and SL, were assayed. The degree of the polynomial was assessed on the basis of R^2 and the significance of the polynomial coefficients. In order to visualize deformation grids for sampling sites where shape differences were not explained by size effect, a new set of RWs was obtained.

The effects of environmental factors on body shape were studied. Discriminant functions based on geometric morphometric analysis were used to correlate body shape with the following environmental parameters of sampling sites: mean summer air temperature (ºC, as an estimation of summer surface water temperature, Livingstone & Lotter, 1998), latitude (°S), longitude (°W), area of the lake or reservoir (km^2), perimeter (km), perimeter/area ratio (P_{AR}), coastline development (L_{CD}, Wetzel, 1981), mean depth (m), altitude (m a.s.l.), conductivity ($\mu S.cm^{-1}$), chlorophyll a ($mg.m^{-3}$), Total Phosphorous (TP, $mg.m^{-3}$), Total Nitrogen (TN, $mg.m^{-3}$), and TN:TP ratio. Most of these data came from the ARLARE database (Quirós *et al.,* 1988; Quirós, 1997) and other publications (IARH-INCYTH, 1995; Colautti *et al.,* 1998; Díaz *et al*., 2000; Modenutti *et al.,* 2000). Altitude, area, and perimeter were obtained from Google Earth (www.earth.google.com) and processed with Image Pro Plus 6.0 software. Correlation and stepwise regression analyses were used to select independent variables (using a Bonferroni correction for multiple comparisons).

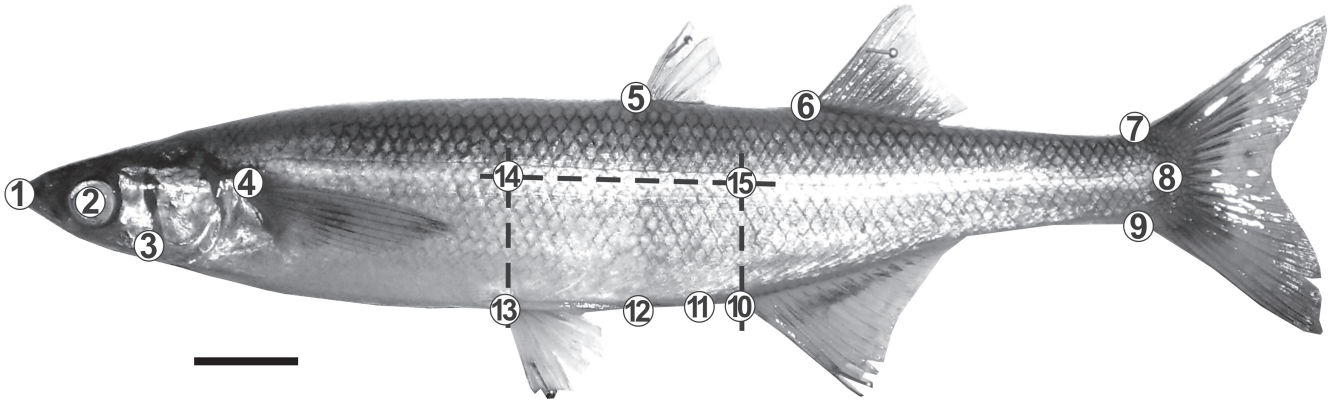

Fig. 2. Landmarks used in geometrics morphometrics analysis: 1, anterior tip of the premaxilla; 2, eye center; 3, posterior process of symplectic bone; 4, dorsal insertion of the pectoral fin; 5, anterior insertion of the first dorsal fin; 6, anterior insertion of the second dorsal fin; 7, dorsal insertion of the caudal fin; 8, posterior end of the lateral line; 9, ventral end of the caudal fin; 10, anterior insertion of the anal fin; 11, anus; 12, distal tip of the pelvic fin onto fish body; 13, anterior insertion of the pelvic fin; 14, vertical tie of landmark 12 onto the lateral line; 15, vertical tie of landmark 10 onto the lateral line. Black line scale: 2 cm.

Results

Species identification. The identification key based on adult morphological and meristic characters (most importantly; size of dorsal scales and position of the first dorsal fin in relation to the position of the pelvic fin and anus) provided unambiguous diagnosis for most samples (Table 1). However, some fish from PELE exhibited inconsistencies when checked against key characters and identification for a group of individuals with mixed specific characters (Table 2) was problematic. These fish exhibited dorsal fin position as expected for *O. bonariensis,* dorsal scale size (number of rows) typical of *O. hatcheri,* and mitochondrial DNA corresponding to *O. bonariensis* (N= 5) or to *O. hatcheri* (N= 10, Table 2, Fig. 3).

Cytochrome *b* sequences (1,125 bp) were obtained for a total of 151 specimens, including the two outgroup individuals (*O. smitti*, collected from a coastal marine location near Puerto Madryn). Among the 151 sequences, 72 unique haplotypes were identified and used for phylogenetic analysis (GenBank Accessions KJ499111-KJ499182). The most frequent haplotype (haplotype 5) was shared by a total of 50 individuals collected from several localities (see Fig. 1), ranging from the Salado/Colorado basin (localities NIHL, URRE), to the Chubut basin (AME, CHU, MUS), and the Pacific-flowing Baker basin (PUY, LBA). This common haplotype, widely distributed throughout the native range of *O. hatcheri,* suggests that either range expansion or an extensive gene flow between all these currently separated basins must have occurred in the recent past. Other examples of such widespread distribution of shared haplotypes include fish collected from URRE and AME (Salado/Colorado and Chubut basins, respectively). Other unique haplotypes were shared by individuals obtained from the same locality (e.g., by two individuals from CDP, by nine individuals from EPU, by two individuals from MUS, and by several individuals from PELE and RIV). Two haplotypes were shared by individuals obtained from different localities within the Rio Negro basin (by individuals from MITO and PELE). It is important to note that all these common alleles were shared by individuals assigned to the same species according to morphological criteria; common alleles were never shared between species.

Phylogenetic analysis of the 72 unique haplotypes was conducted to test for reciprocal monophyly of alleles for the three nominal species included in the study. A partitioned maximum likelihood (ML) approach was implemented using three partitions, optimizing an independent model for each codon position in the alignment. The "propose model" routine in Treefinder based on the AICc recommended TN+G models for first and second codon position sites and the HKY+G model for third codon positions of the cyt*b* gene. Parameters for these models were optimized independently for each data partition and three replicate searches were conducted. The resulting phylogeny is shown in Fig. 3. The *O. hatcheri* and *O. bonariensis* haplotypes formed strongly supported (100 percent bootstrap values) reciprocally monophyletic groups, with 7% average sequence difference between groups (uncorrected distance). In contrast, sequence differences within each group were smaller than 1% in every case. Average differences from the outgroup sequences were 3% for *O. hatcheri* and 6% for *O. bonariensis*. Genetic identity based on the cyt*b* sequences is robust to intraspecific variation and provides an unambiguous signature for comparison with morphological data.

A total of 436 fish were digitized and used for morphometric analyses (Table 1, sample size for each locality ranged from 5 to 36). Considering all individuals, the RW1 and RW2 explained 68.2% of total variation (Table 3). Main differences involved relative antero-posterior position of dorsal and pelvic fins, body height and head length (Fig. 4). Cluster analysis applied to all the individuals showed three main groups; *O. bonariensis, O.*

hatcheri, and presumptive hybrids. In effect, Discriminant Analysis of the three groups showed two significant functions: pairwise significant differences ($P < 0.0001$) and 89% of cases grouped correctly. Separation of the two species was clear for DF1. The difference between *O. hatcheri* and presumptive hybrids was observed using the residual of DF2 versus SL (Fig. 5, Table 4). Discriminant Analysis also provided the probability of membership to the original group. In several sites, an appreciable number of taxonomically identified *O. hatcheri* individuals had low probabilities of being *O. hatcheri*. This was not observed for *O. bonariensis* (Fig. 6).

Table 2. Meristic and morphological characters applied to the identification (following Dyer, 2006) of 45 individuals from Lake Pellegrini. Results from mtDNA analysis are compared with identification based on taxonomic key: origin of first dorsal fin over posterior half of pelvic fin (*O. hatcheri*) or anterior to anus and posterior to pelvic fin (*O. bonariensis),* number of dorsal scale rows between lateral lines >12 (*O. hatcheri)* or <11(*O. bonariensis);* number of gill rakes on the lower arm of first branchial arch, 21-27 (*O. hatcheri)* or >30 (32-38) (*O. bonariensis)*; upper jaw prognathous (*O. hatcheri)* or lower jaw prognathous in large specimens (*O. bonariensis).* N: number of specimens. In bold, the presumptive hybrids individuals identified on meristic and morphological characters, those are highlighted with * in Fig. 3).

Origin of first dorsal fin		Number of dorsal scale rows between lateral lines	Number of gill rakes on the lower arm of first branchial arch	Jaw prognathism	Species based on key (N)	Species based on mtDNA (N)
Over posterior half of pelvic fin	Anterior to anus and posterior to pelvic fin					
	X	13	30	upper	hybrid?	***O. bonariensis***
	X	12	29	lower	hybrid?	-
	X	12	31	no	hybrid?	***O. bonariensis***
	X	13	31	upper	hybrid?	***O. bonariensis***
	X	12	28	lower	hybrid?	***O. bonariensis***
	X	13	30	lower	hybrid?	***O. bonariensis***
X		15	22	upper	*O. hatcheri*	-
X		15	23	no	*O. hatcheri*	*O. hatcheri*
X		15	23	upper	*O. hatcheri* (2)	*O. hatcheri*
X		15	24	no	*O. hatcheri*	*O. hatcheri*
X		15	24	upper	*O. hatcheri* (4)	-
X		15	25	upper	*O. hatcheri* (2)	*O. hatcheri* (2)
X		16	24	upper	*O. hatcheri* (2)	*O. hatcheri*
X		16	25	upper	*O. hatcheri* (4)	*O. hatcheri* (2)
X		16	26	upper	*O. hatcheri* (2)	*O. hatcheri*
	X	13	27	no	hybrid?	***O. hatcheri***
	X	14	24	upper	hybrid?	-
	X	15	23	upper	hybrid? (3)	***O. hatcheri* (2)**
	X	15	25	no	hybrid?	***O. hatcheri***
	X	16	24	upper	hybrid?	***O. hatcheri***
	X	16	25	upper	hybrid?	***O. hatcheri***
	X	15	22	upper	hybrid?	***O. hatcheri***
	X	15	23	upper	hybrid? (2)	-
	X	15	24	upper	hybrid?	***O. hatcheri***
	X	15	25	upper	hybrid? (2)	-
	X	16	22	upper	hybrid?	***O. hatcheri***
	X	16	24	upper	hybrid? (2)	-
	X	16	25	upper	hybrid?	-
	X	17	24	upper	hybrid? (2)	O. hatcheri

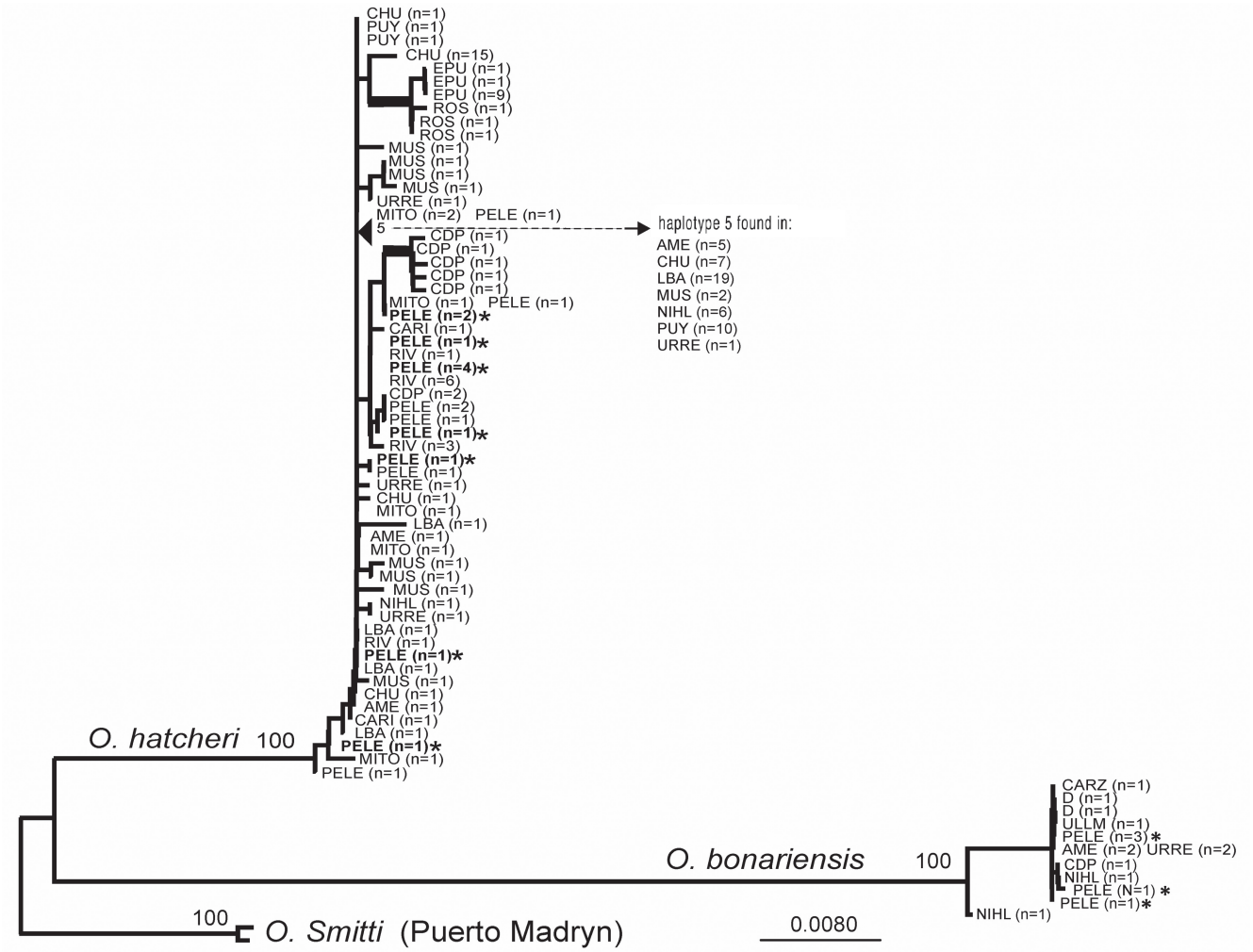

Fig. 3. Maximum likelihood phylogeny for 72 unique haplotypes obtained from *O. hatcheri*, *O. bonariensis*, and *O. smitti*. Haplotype labels indicate their sampling locality (as explained in Fig. 1 and Table 1) and the number of individuals (in parentheses). Several common haplotypes were recovered from several individuals from the same or different localities (most significantly haplotype 5). Monophyly of haplotypes from each species is supported by 100% bootstrap values, and two groups of *O. hatcheri* haplotypes (from EPU and CDP, indicated by heavier lines) also received strong bootstrap support. All other nodes were weakly supported by bootstrap analyses. *: presumptive hybrids individuals identified on meristic and morphological characters (Table 2).

Table 3. Interspecific and intraspecific morphological variation of *Odontesthes* species. Variance explained (%) by first and second RW is indicated. N: number of specimens.

Data subsets	N	Variance explained (%)	
		RW1	RW2
all individuals	436	48.30	19.92
O. bonariensis	100	46.68	19.69
O. bonariensis from CARZ and D	64	45.69	13.88
O. hatcheri	315	44.81	16.21
O. hatcheri from PUY, NIHL, CDP, and PDA	79	42.34	19.35

Variation within species. RW1 and RW2 explain a great proportion of the total variation of *O. hatcheri*. Similar results were obtained for *O. bonariensis* (Table 3). There were significant relationships between body shape and fish size for each sampling locality. These relationships were significantly different between sites for both *O. bonariensis* and *O. hatcheri* (Tpsregr, n= 100, $P < 0.001$; Tpsregr, n=315, $P < 0.001$, respectively).

Discriminant Analysis for *O. bonariensis* populations produced 4 significant DFs, identifying 5 groups. DF1 and DF2 depended significantly on size (Table 4). Notwithstanding this dependence, DF1 revealed body shape differences between CARZ and D (Fig. 7). In effect, it can be seen that 'pejerrey' of CARZ had slightly longer caudal peduncles than D individuals (Fig. 7). Another noticeable trend is that DF1 (r= 0.346, n= 100, $P = 0.005$) and DF2 (r= -0.431, n= 100, $P< 0.001$) residuals (from regression with SL) showed a significant correlation with the Condition Factor.

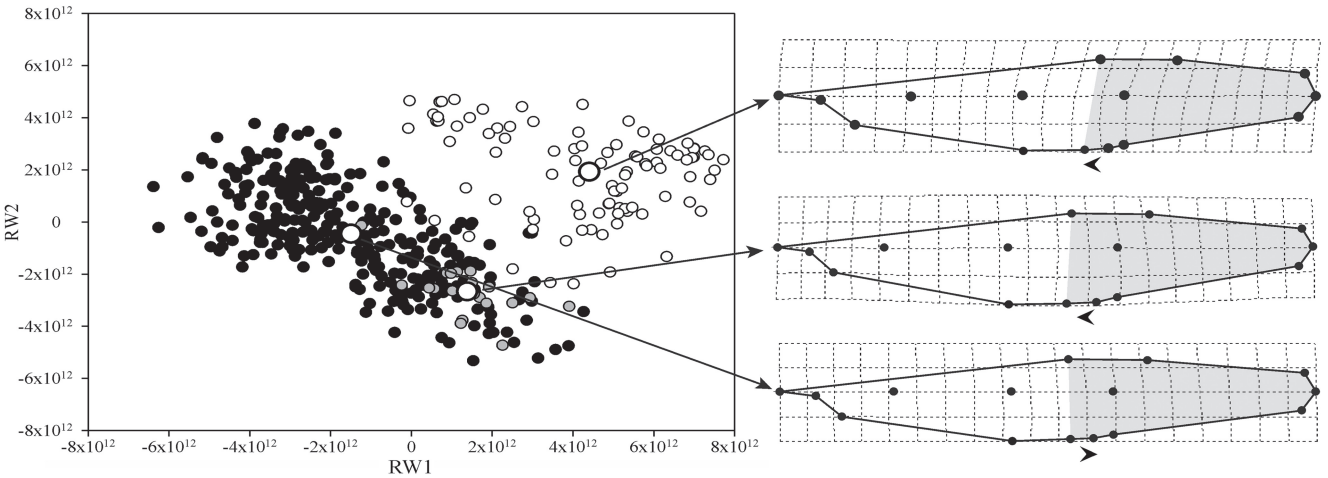

Fig. 4. Geometric Morphometric Analysis applied to *Odontesthes* individuals. RW2 versus RW1 and deformation grids (tied to group means) for *Odontesthes bonariensis* (white circle), *O. hatcheri* (black circle) and presumptive hybrids (gray circle). Arrowheads indicate displacement of landmarks relative to consensus. Shaded area shows relative position of landmarks 5 (anterior insertion of the first dorsal fin) and 12 (distal tip of the pelvic fin onto fish body).

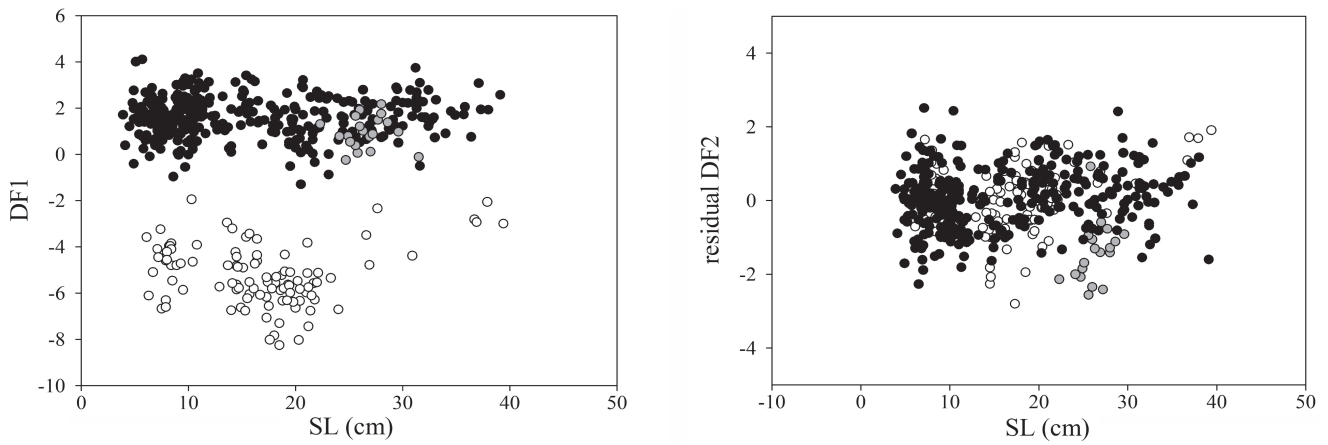

Fig. 5. Morphometric differences between species. DF1 and residual DF2 (of the regression of DF2 versus Standard length) vs. Standard length (SL). *Odontesthes bonariensis* (white circle), *O. hatcheri* (black circle), and presumptive hybrids (gray circle).

Table 4. Discriminant Analyses results for two species of *Odontesthes*.

Groups	N	Cases correctly grouped (%)	Discriminant Function	Variance explained (%)	Canonical correlation	Eigenvalues	Wilks' Lambda signification	Regression with size signification
O. bonariensis, O. hatcheri, and presumptive hybrids (3 groups)	436	89.0						
			1	97.5	0.943	8.019	0.000	>0.05
			2	2.5	0.411	0.203	0.000	0.000
O. bonariensis sampling sites (5 groups)	100	100	1	56.2	0.953	9,894	0.000	0.001
			2	33.8	0.925	5.954	0.000	0.002
			3	6.9	0.741	1.216	0.000	>0.05
			4	3.1	0.595	0.549	0.007	>0.05
O. hatcheri sampling sites (12 groups)	315	90.8	1	47.6	0.944	8.113	0.000	0.000
			2	14.6	0.845	2.491	0.000	>0.05
			3	1.1	0.809	1.899	0.000	>0.05
			4	6.7	0.731	1.146	0.000	>0.05
			5	5.7	0.701	0.966	0.000	>0.05
			6	4.4	0.655	0.752	0.000	>0.05
			7	3.3	0.602	0.569	0.000	>0.05
			8	2.7	0.565	0.469	0.000	>0.05
			9	1.6	0.460	0.269	0.000	>0.05
			10	1.2	0.406	0.197	0.000	>0.05
			11	0.6	0.314	0.110	0.006	>0.05

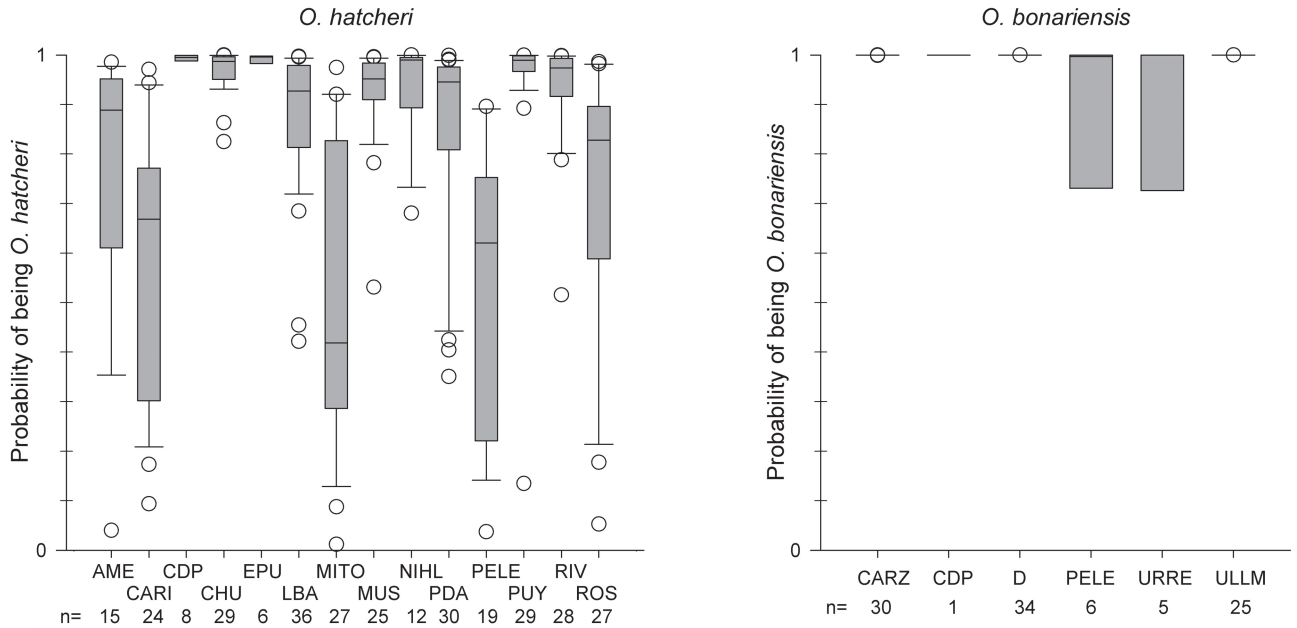

Fig. 6. Probability for taxonomically identified *Odontesthes hatcheri* individuals of being *O. hatcheri* (left) and probability of taxonomically identified *O. bonariensis* individuals of being *O. bonariensis* (right). Number of fish, median, quartiles, and data outside 10 and 90th percentile are indicated. Water bodies are named as in Fig. 1.

Discriminant Analysis for *O. hatcheri* revealed 11 significant DFs for the 13 sampling sites (Table 4). DF1 showed significant dependence on size (Fig. 7), but this was not observed for the other DFs. The DF3 versus DF2 graph (Fig. 8) showed major differences between body shapes corresponding to PUY (the type locality of *O. hatcheri*, Ringuelet *et al*., 1967) and the PDA, CDP and NIHL reservoirs. The deformation grids showed that CDP individuals had lower body, and the landmarks related to anus, pelvic and anal fins (10, 11 and 12) in an anterior position, in contrast to that observed in individuals from PUY (Fig. 8, Table 3). DF1 (residuals of the regression of DF1 versus SL), DF2, and DF4 showed a significant correlation (r= -0.265, n= 315, P< 0.001; r= 0.197, n= 315, P< 0.001 and r= 0.192, n= 315, P= 0.002, respectively) with Condition Factor. Although the number of significant DFs allows discrimination of all the sampling sites, the differences that could be visualized were extremely low (Fig. 8).

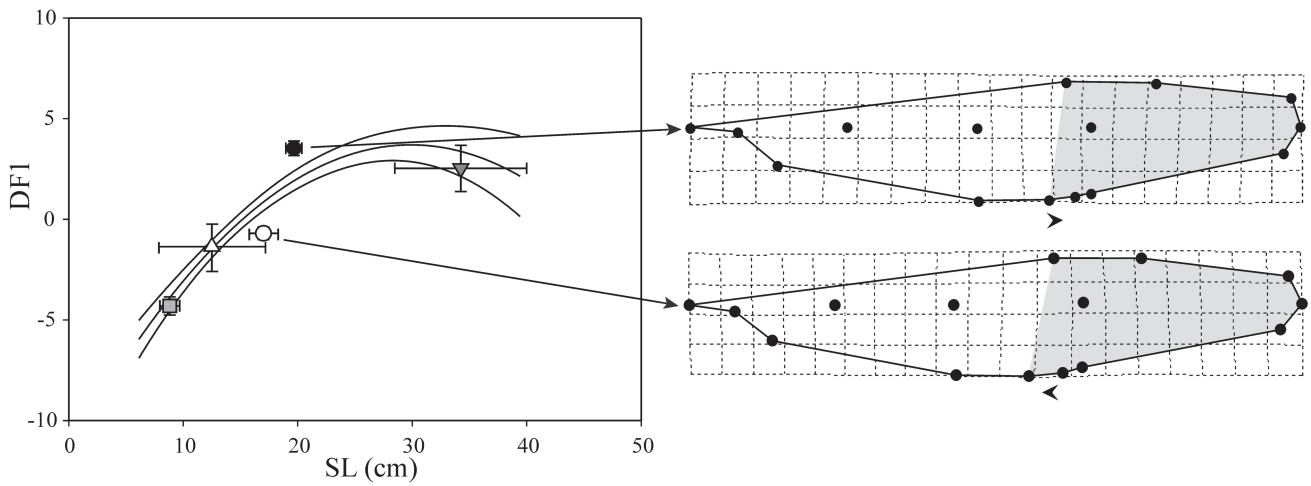

Fig. 7. Geometric Morphometric Analysis applied to *Odontesthes bonariensis* individuals. Left: plot of DF1 vs. Standard length (SL), showing quadratic fit with 95% confidence interval (N= 100) and means and 95% confidence intervals by sampling sites (locality labels as in Fig. 1) ULLM (gray square), CARZ (white circle), D (black square), URRE (white triangle), PELE (gray triangle). Right: Deformation grids correspond to a relative warps analysis (see Table 3) involving only CARZ and D. Arrows indicate displacement of landmarks relative to consensus. Shaded area remarks relative position of landmarks 5 (anterior insertion of the first dorsal fin) and 12 (distal tip of the pelvic fin onto fish body).

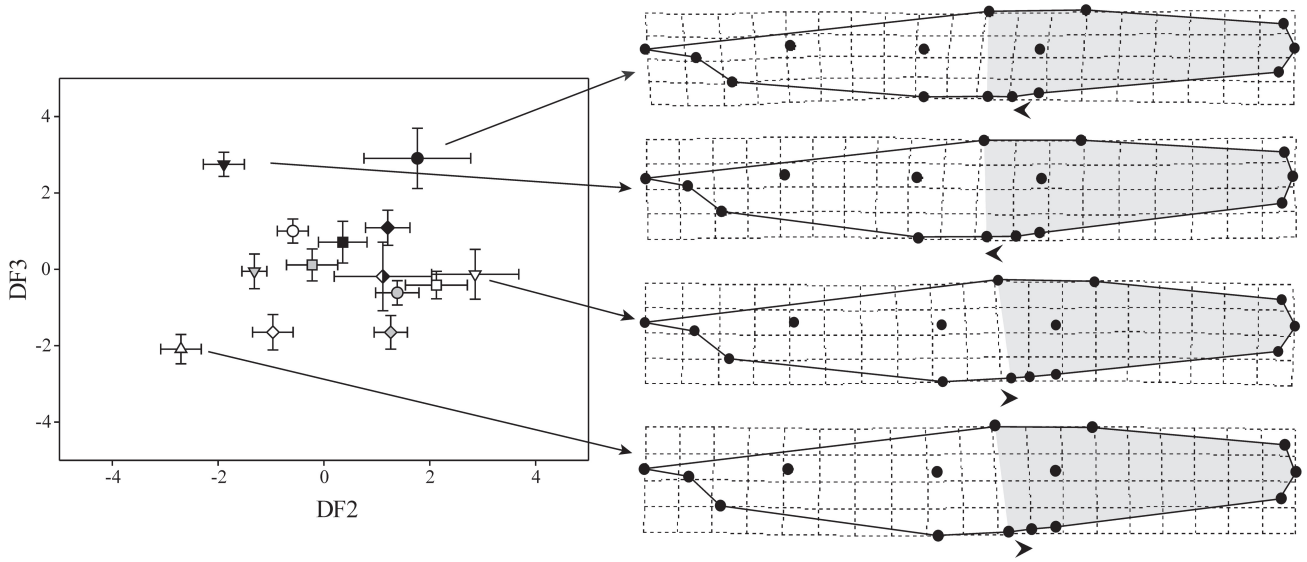

Fig. 8. Geometric Morphometric Analysis applied to *O. hatcheri* individuals. Left: plot of DF3 vs. DF2 showing means and 95% confidence intervals by sampling sites (locality labels as in Fig. 1) NIHL (white triangle), CDP (black circle), 7: PELE (gray square), PDA (black triangle), MITO (black diamond), CARI (white square), EPU (black and white diamond), RIV (gray circle), ROS (white diamond), AME (black square), CHU (gray diamond), MUS (gray triangle), LBA (white circle), and PUY (white triangle). Right: deformation grids correspond to a relative warps analysis involving only CDP, PDA, and NIHL and PUY. Arrowheads indicate displacement of landmarks relative to consensus. Shaded area remarks relative position of landmarks 5 (anterior insertion of the first dorsal fin) and 12 (distal tip of the pelvic fin onto fish body).

Body shape and environmental parameters. Regarding geographic, physical and chemical parameters of lakes and reservoirs, significant regressions were found between DF1 residuals of *O. hatcheri* and phosphorous concentration (TP, regression, $n = 14$, $P < 0.005$), and altitude (regression, $n = 14$, $P < 0.001$).

Discussion

Species boundaries between *O. hatcheri* and *O. bonariensis* are clearly revealed by morphological and genetic traits, in agreement with current taxonomy. Although morphological differences between these two species were small and mainly related to the position of dorsal fins, the degree of genetic variation in the cyt*b* gene (7%) is significant. Genetic distances estimated from mtDNA have been used in many instances to validate vertebrate species (Johns & Avise, 1998), indicating that more than 90% of vertebrate sister species exhibit a cyt*b* divergence of at least 2%. At intra-specific level, variation between *O. hatcheri* populations observed in this study was low (less than 1%), with no clear distinction of haplogroups or geographic orientation. A common cyt*b* haplotype (haplotype 5, Fig. 3) was widely distributed, encompassing most of the species' range.

In just one sampling locality (PELE) a group of individuals displayed intermediate morphology, as already reported (Dyer, 2006). In agreement with Crichigno *et al*. (2013), our current analyses showed that intermediate individuals fall closer to the morphospace of *O. hatcheri* (Fig. 5) but DA was able to differentiate between them and *O. hatcheri*. In addition to PELE, four reservoirs and one lake in the distribution area of *O. hatcheri* have been reported to contain *O. bonariensis* (Liotta, 2006). The Ezequiel Ramos Mexía reservoir, three reservoirs used for this study (NIHL, CDP, and AME), and one lake (URRE) included both species, but did not yield intermediate forms as in PELE. Only the last-mentioned has an artisanal fishery.

The distribution of O. *bonariensis* in the colder lakes and reservoirs of the Andean Region could be limited by temperature-dependent factors (Strüssmann *et al*., 2010). These species show a very different degree of temperature-dependent sex determination (TSD; Strüssmann *et al*., 1997b). *Odontesthes bonariensis* individuals exposed to a temperature of approximately 17ºC from hatching to juvenile stage all became female (Strüssmann *et al*., 1997b). In temperate regions, mean air and water temperatures are likely to correspond most closely in midsummer (Livingstone & Lotter, 1998). *Odontesthes hatcheri* breed in late spring to early summer (Cussac *et al*., 1992) and *O. bonariensis* in spring and autumn (Ringuelet, 1943). As Cussac *et al*. (2009) have already suggested, the results of this survey (Ameghino reservoir being the southernmost locality) confirm that *O. bonariensis* is absent when mean summer air temperatures are lower than 20ºC (www.smn.gov.ar; Liotta, 2006, Table 1). According to the above discussion, *O. bonariensis* populations in colder lakes towards the south of the distribution area (higher latitude) are expected to have a higher proportion of females than

males. Therefore, if hybridization occurs in these lakes, hybrids are likely to carry *O. bonariensis* mtDNA. As Ringuelet (1943) noted, however, artificial fertilization is a common practice when fishermen capture ripe 'pejerrey', releasing fertilized eggs back to the lake. This practice, the probable natural segregation of reproductive habitats, and artificial bias in the capturing carried out by the artisan fishery could be explanations as to why Lake Pellegrini is the only site where conspicuous intermediate individuals were reported and why their cyt*b* haplotype identification is unexpected.

Several methods have been proposed in the literature for the identification of fish hybrids. External morphology is unreliable when used as the sole means of identification in such analyses, particularly for hybrid individuals beyond the F1 generation (Hashimoto *et al.,* 2012). Simple PCR-based techniques that can differentiate F1 and post-F1 hybrids from wild types using different microsatellite markers have been developed for several species (Hashimoto *et al.,* 2012; Allu *et al.,* 2014; Hasselman *et al.,* 2014). At present, nuclear microsatellite markers are being developed in order to estimate the probability of individuals belonging to distinct hybrids or purebred *Odontesthes* categories (G. Ortí pers. obs.).

Both *O. hatcheri* and *O. bonariensis* are extremely susceptible to stress, frequently dying due to handling (Tsuzuki *et al.*, 2000a,b). In consequence, brood stocks were never established in Argentina before 2001, when Somoza *et al.* (2008) implemented spontaneous spawning of *O. bonariensis* in captivity. Thus, the southernmost hatchery (Piscicultura Río Limay) participating since 1930 in 'pejerrey' stocking programs throughout the Andean Cuyan and Patagonian Provinces (Crichigno *et al.,* 2013) always stocked fish obtained from adults caught in neighboring sites, e.g. Lake Pellegrini. In consequence, and in agreement with the results of Crichigno *et al.* (2013), even lakes and reservoirs with water temperature too low for *O. bonariensis* could have received low-temperature-resistant hybrid and / or introgressed individuals, resulting in *O. hatcheri* populations with high morphological variation. There, taxonomically assigned *O. hatcheri* individuals could have a low probability of being classified as *O. hatcheri* in geometric morphometric analysis (Fig. 6).

Intraspecific variation in morphology within each species suggests, though in a slightly different way for each one, that morphological variation mainly affects body height, caudal peduncle size, and the relationship between body height and the Condition Factor. In particular, morphological variation within *O. hatcheri* involves body height and the relative positions of the anus, pelvic and anal fins. In agreement with Aigo *et al.* (2008), who positively relate abundance of *O. hatcheri* to the area of the lake, and the dependence of cephalic shape of *O. hatcheri* on coastline development (Crichigno *et al.,* 2013), significant relationships between DFs and independent variables affecting quality and quantity of planktonic food availability (TP and altitude) were observed. Part of this variation could be ascribed to phenotypic plasticity (Crichigno *et al.,* 2012).

In conclusion, present results increase biological knowledge of these species and provide new insights into the conservation of *O. hatcheri* populations. The poor diversity of Patagonian fish fauna successfully overcame a long history of environmental changes in Patagonia (Cussac *et al.,* 2009) and *O. hatcheri* was naturally distributed across more than 18 latitudinal degrees. However, the human practice of stocking fish provoked a dispersal of *O. bonariensis* in the distributional range of *O. hatcheri.* The native species is now absent and replaced by *O. bonariensis* in the north of their original distributional range (Table 1) and the ability of both species to hybridize could have created a hybrid zone.

Current taxonomy was clearly confirmed by morphological and genetic traits. Morphometric analyses can effectively discriminate between the species and their putative hybrids, in spite of the very small morphological differences, even when morphological variation includes the possible effects of genetic introgression, environmental dependence, and phenotypic plasticity (Crichigno *et al.,* 2012).

Previous (Crichigno *et al.,* 2013) and present results suggest the introgression of *O. bonariensis* into several *O. hatcheri* populations. Introgression in tilapia (Deines *et al.,* 2014) and cyprinid species (Deinhardt, 2013; Meraner *et al.,* 2013) have threatened biodiversity, signalizing that biological invasions, non-native introductions, and introgressive hybridization may have a negative impact on the genetic resources available for aquaculture and fisheries, being major drivers for the decline of native freshwater fishes. Managers should take these risks into account when considering further exotic introductions into regions where non-native fishes have not yet become established.

Acknowledgments

We thank Sonia Crichigno for her contribution to the analysis and laboratory work, Gustavo Somoza for samples from Lake San Lorenzo, and Feliciano Gómez for samples from Lake Pellegrini. We would like to acknowledge the following institutions for grants supporting the present project: Universidad Nacional del Comahue (04B147), CONICET (PIP 112-200801-00282), and FONCYT (PICT 2005-35241) of Argentina, and the NSF-PIRE award (OISE 0530267, USA) for support of collaborative research on Patagonian Biodiversity given to the following institutions (listed alphabetically): Brigham Young University, Centro Nacional Patagónico, Dalhousie University, Darwinion Botanical Institute, Universidad Austral de Chile, Universidad Nacional del Comahue, Universidad de Concepción, and University of Nebraska.

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Submitted June 10, 2013
Accepted October 15, 2014 by Cláudio de Oliveira
Published March 31, 2015