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ORIGINAL PAPER

Displacement of native Patagonian freshwater silverside populations (*Odontesthes hatcheri*, Atherinopsidae) by introgressive hybridization with introduced *O. bonariensis*

Eva C. Rueda · Kerry A. Mullaney · Cecilia Conte-Grand · Evelyn M. Habit · Víctor Cussac · Guillermo Ortí

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Abstract The Patagonian silverside *Odontesthes hatcheri* is a native fish restricted to streams and lakes of Patagonia (Argentina and Chile). Stocking programs to enhance recreational fisheries in man-made reservoirs have introduced a nonnative, closely-related species (the pejerrey *O. bonariensis*) in Patagonia almost a century ago, and yet little is known about the invasiveness of this species. To evaluate the impact of these introductions we analyze genetic data (microsatellite markers and mitochondrial DNA) to quantify the incidence of hybridization between these two species and assess potential effects on native population structure. Phylogeographic analyses reveal weak geographic differentiation among populations of *O. hatcheri*, in agreement with previous studies for other freshwater fishes in Patagonia strongly influenced by Quaternary glaciations and hydrographic basin changes since the last glaciation. However, many populations have unique genetic pools. In several areas, introductions resulted in extensive hybridization, with high frequencies of F2 and backcrossed hybrids in natural populations, and in some cases *O. bonariensis* has completely displaced the

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E. C. Rueda
Departamento de Ciencias Naturales, FHUC-UNL, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Litoral, Ciudad Universitaria, 3000 Santa Fe, Argentina
e-mail: evarueda@fbcb.unl.edu.ar

K. A. Mullaney · G. Ortí (✉)
Department of Biological Sciences, The George Washington University, 2023 G Street, NW, Washington, DC 20052, USA
e-mail: gorti@gwu.edu

C. Conte-Grand · V. Cussac
Instituto de Investigaciones en Biodiversidad y Medioambiente (INIBIOMA), Universidad Nacional del Comahue – Consejo Nacionalde Investigaciones Científicas y Técnicas (CONICET), 8400 Bariloche, Río Negro, Argentina
e-mail: cussacve@comahue-conicet.gob.ar

E. M. Habit
Departamento de Sistemas Acuáticos, Facultad de Ciencias Ambientales y Centro EULA, Universidad de Concepción and Centro de Investigaciones en Ecosistemas Patagónicos, Concepción, Chile
e-mail: ehabit@udec.cl

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native populations. The negative impact of these introductions on native populations is correlated to temperature, a critical parameter in the face of global warming, suggesting that invasiveness of *O. bonariensis* may increase in the future. Our results advise against continuing stocking programs to preserve the integrity of natural populations of the Patagonian silverside.

Keywords Invasive species · Microsatellite markers · Mitochondrial DNA · Stocking · Global warming · Pejerrey

Introduction

The genus *Odontesthes* Evermann & Kendall 1906 includes 19 nominal species of silverside fishes distributed in freshwater, brackish, and marine habitats in southern South America (Eschmeyer and Fricke 2015). The largest and most emblematic species of this genus is *Odontesthes bonariensis* (Valenciennes 1835), that lives in rivers and shallow lakes of the Pampean (Fig. 1 "Pampas") and Great Rivers Provinces (sensu López et al. 2008), encompassing the Paraná and Uruguay river basins and extending geographically to coastal areas of Uruguay and southern Brazil (Dyer 2006). Commonly known as "pejerrey," *O. bonariensis* is commercially important, highly valued in food and sport fisheries, and has been used extensively in aquaculture since the early 1900s, stocked throughout Argentina, Chile, Bolivia, and Perú, and also exported overseas to Japan, Israel, and Italy (Somoza et al. 2008). This species also has been established as an experimental model to study sex-determination and endocrinology in fishes since it exhibits a unique temperature-sex-determination mechanism (Fernandino et al. 2015).

The Patagonian pejerrey, *Odontesthes hatcheri* (Eigenmann 1909), is readily differentiated from *O. bonariensis* based on its non-overlapping native geographic range, morphology, and molecular characters (Conte-Grand et al. 2015). It is commonly found in rivers, freshwater lakes, and reservoirs of Patagonia flowing to the Atlantic and Pacific Oceans, extending to the north along the eastern slope of the Andes into the Cuyo region (Fig. 1) (Dyer 2006; Gómez et al. 2006; Menni 2004; Ringuelet et al. 1967). Biogeographically, the native distribution of *O. hatcheri* occupies the Patagonian Province (sensu Dyer 2000) and Cuyo, the Andean-Cuyan Region in Argentina (sensu López et al. 2008); therefore it is parapatric with respect to *O. bonariensis*. Several diagnostic morphometric and meristic characters as well as deep mitochondrial DNA (mtDNA) sequence divergence (7 % for the cytochrome *b* gene) separate *O. bonariensis* and *O. hatcheri* (Conte-Grand et al. 2015; Dyer 2006). Molecular phylogenetic studies show that these two species are placed in separate clades within the tribe Sorgentinini, each having close affinities with different marine silverside species (Campanella et al. 2015; Heras and Roldan 2011). In spite of these differences, both species hybridize in captivity and reciprocal hybrids have been successfully bred to give rise to viable F2 individuals (Strüssmann et al. 1997a).

The native area of the Patagonian pejerrey has been stocked repeatedly with *O. bonariensis* starting around 1940 (Crichigno et al. 2013; Dyer 2006; Somoza et al. 2008). Introduced larvae originated from three hatcheries (Fig. 1): Estación Hidrobiológica de Chascomús (EHC: 35°36′S, 58°01′W), Estación de Piscicultura de Embalse (EPE: 32°13′S, 64°29′W), and Estación de Piscicultura Río Limay (EPL: 38°59′S, 68°14′W). *Odontesthes bonariensis* has been able to establish populations in environments where the mean summer air temperature is higher than 20°C (Cussac et al. 2009), most successfully in the northern areas (Cuyo), extending southward into Patagonia up to the Reservoir Ezequiel Ramos Mexía on the Limay river (39°30′S, 69°00′W), the southernmost locality reported for *O. bonariensis* (Aigo et al. 2008). In some localities, introduced *O. bonariensis* seem to have displaced the native Patagonian pejerrey, and several natural populations of *O. hatcheri* are suspected to contain putative hybrid individuals. Crichigno et al. (2013) characterized *O. hatcheri*, *O. bonariensis*, and morphologically intermediate individuals from several Patagonian sites on the basis of head shape. Their study identified a non-negligible number of putative hybrids that increased in frequency in study sites geographically closer to hatcheries participating in stocking programs (EPE and EPL, Fig. 1). A follow-up study comparing body shape among O. *bonariensis* and *O. hatcheri* from wild populations, farmed stocks, and artificially hybridized individuals, showed that purebred individuals are

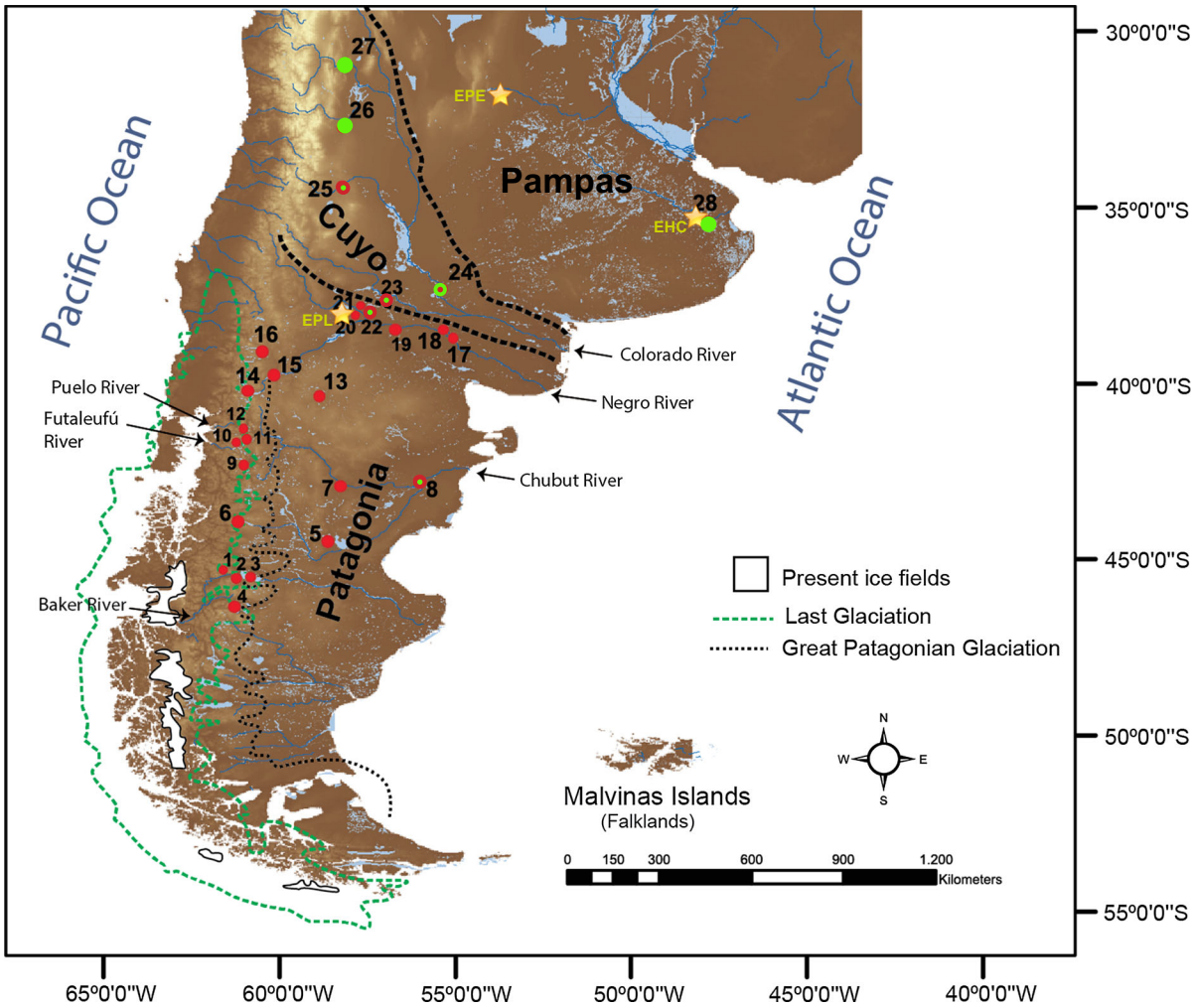


Fig. 1 Sampling localities for *O. hatcheri* and *O. bonariensis* in the Patagonian and Andean-Cuyan (Cuyo) ichthyological provinces (sensu López et al. 2008; see Table 1 for additional information). Major river basins flowing to the Altlantic (Colorado, Chubut and Negro rivers) and to the Pacific (Puelo, Futaleufú and Baker rivers) Oceans are labeled and the ranges of past and present ice caps are indicated with *dotted lines*. Hatcheries where *O. bonariensis* are raised for stocking are indicated by *yellow stars* (*EHC* Estación Hidrobiológica de Chascomús, *EPE* Estación de Piscicultura de Embalse, *EPL* Estación de Piscicultura Rio Limay). Results of genetic identity of species and hybrids are color-coded as follows: *Solid red dots* indicate populations harboring gene pools that are 100 % *O. hatcheri* (locality 4, Lake Pueyrredon, is the type locality for *O. hatcheri*). *Solid green dots* are populations 100 % *O. bonariensis* (locality 28 in the Pampas, is a typical locality for *O. bonariensis*). *Red dots* containing a smaller green dot represent populations with *O. hatcheri* gene pool that also contain *O. hatcheri* × *O. bonariensis* hybrids. *Green dots* containing smaller red dot are *O. bonariensis* populations that also contain hybrids

more slender than both farmed hybrid fish and putatively introgressed wild fish (Crichigno et al. 2014). Conte-Grand et al. (2015) found individuals with intermediate morphologies sampled from several lakes and reservoirs in Patagonia and Cuyo that harbored mitochondrial cytochrome *b* sequences typical of *O. bonariensis*, suggesting that hybrid fish may indeed be common in natural environments. A recent study analyzing genetic divergence at mtDNA and microsatellite loci among the pejerrey (*O. bonariensis*) and its close relatives (e.g., *O. argentiniensis*, *O. perugia*, and *O. humensis*) suggests that promiscuous gene flow and hybridization among freshwater species of *Odontesthes* is common, blurring species boundaries and complicating delimitation of management units (García et al. 2014).

Hybridization between native fish and introduced hatchery stocks is a growing problem for aquatic environments and may endanger native species (Neville and Dunham 2011). The extent of hybridization between *O. bonariensis* and *O. hatcheri* in natural habitats remains unknown, hampering management and conservation efforts that rely on accurate surveys of natural populations. External morphology used to document intermediate forms usually provides the first line of evidence to suspect hybridization, but turns unreliable when used as the sole means of hybrid identification, particularly for individuals beyond the F1 generation (Hashimoto et al. 2010, 2013). Therefore, genetic information has become essential to survey natural populations (Ferguson et al. 1995). Analyses of mtDNA can help in many cases, but also lack the power to discriminate the extent of introgression in natural populations without additional evidence (Conte-Grand et al. 2015). Distinction of F1 and post-F1 hybrids from wild types has been efficiently achieved using nuclear microsatellite data (e.g., Hashimoto et al. 2013; Sanz et al. 2008). In this study we apply microsatellite markers developed for *Odontesthes* to document the extent of hybridization and introgression in natural populations of *O. hatcheri* that have been subject to stocking with *O. bonarienesis*.

Man-made disturbance on natural populations through stocking practices is but one of the most recent factors affecting aquatic ecosystems in Patagonia. Many localities where *O. hatcheri* currently lives were covered by glacial ice during the Plesitocene (Fig. 1), and paleoecological conditions may have been quite different in historical times. Catastrophic rearrangements of hydrological basins during the retreat of continental glaciers in Patagonia caused drainage reversals in several rivers anciently flowing to the Atlantic (Turner et al. 2005), capturing headwaters east of the Andes that now flow to the Pacific (e.g. Baker, Puelo, Futaleufú rivers, Fig. 1). Large and powerful rivers crossing the Patagonian steppe during glacial and interglacial periods (Martínez and Kutschker 2011) and extended paleolakes at the fringes of melting glaciers could have facilitated north–south dispersal of fishes across basins (Ponce et al. 2011), as demonstrated for catfishes, galaxiid and percichthyid fishes with broad distributions in Patagonia (Muñoz-Ramirez et al. 2014; Ruzzante et al. 2008). A similar pattern has been reported for *O. hatcheri*, with low intraspecific variation among populations of (<1 % sequence divergence at the cytochrome *b* gene) and many common haplotypes shared across currently isolated river drainages (Conte-Grand et al. 2015), but the phylogeographic structure of this species remains unknown.

In this study, we present analyses for both, mtDNA and microsatellite genetic variation among populations of *O. hatcheri* sampled throughout its native range to characterize its phylogeographic structure and assess the effect of stocking on natural populations of a putative invasive species such as *O. bonariensis*. A deeper understanding of natural variation and the patterns shaped by historical processes is critical for gauging the effect of introductions and current management practices.

Methods

Sample collection and DNA extraction

We analyzed a total of 482 Individuals collected from a wide range of freshwater habitats spanning the native distribution of *Odontesthes hatcheri* in Argentina and Chile (Fig. 1; Table 1). The locations collected include the northern Cuyo region in Argentina where many man-made reservoirs have been stocked with *O. bonariensis*, and Patagonian basins towards the southern range of the distribution that flow to both Pacific and Atlantic Oceans, some of which also have been stocked. Individuals were caught between 2006 and 2008 using seines or gill nets in streams and littoral areas of lakes and reservoirs and immediately euthanized by an overdose of anesthetic (MS 222, >300 mg/L). We also analyzed 38 specimens of *Odontesthes bonariensis* from Laguna San Lorenzo (Río Salado basin, Buenos Aires Province, locality 28 in Fig. 1). Specimens corresponding to Lake Pueyrredón, the type locality of *O. hatcheri* (Ringuelet et al. 1967), were deposited at the Fish Collection of Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Argentina (Voucher Museum ID: MLP 9858 to MLP 9877). Seven specimens from diverse localities have been donated to The Barcode of Life Data System (http://www.boldsystems.org) and have COI sequences available online (Table 1). Voucher specimens from additional localities also are kept at the author's institutions. Many specimens analyzed in this study have been used previously for

morphometric analysis to explore the effects of hybridization and environmental conditions on body shape and to sequence cytochrome *b* for species identification (Conte-Grand et al. 2015). Tissue samples (fin clips or whole specimens) were preserved in 95 % ethanol for DNA extraction. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and stored at −20°C.

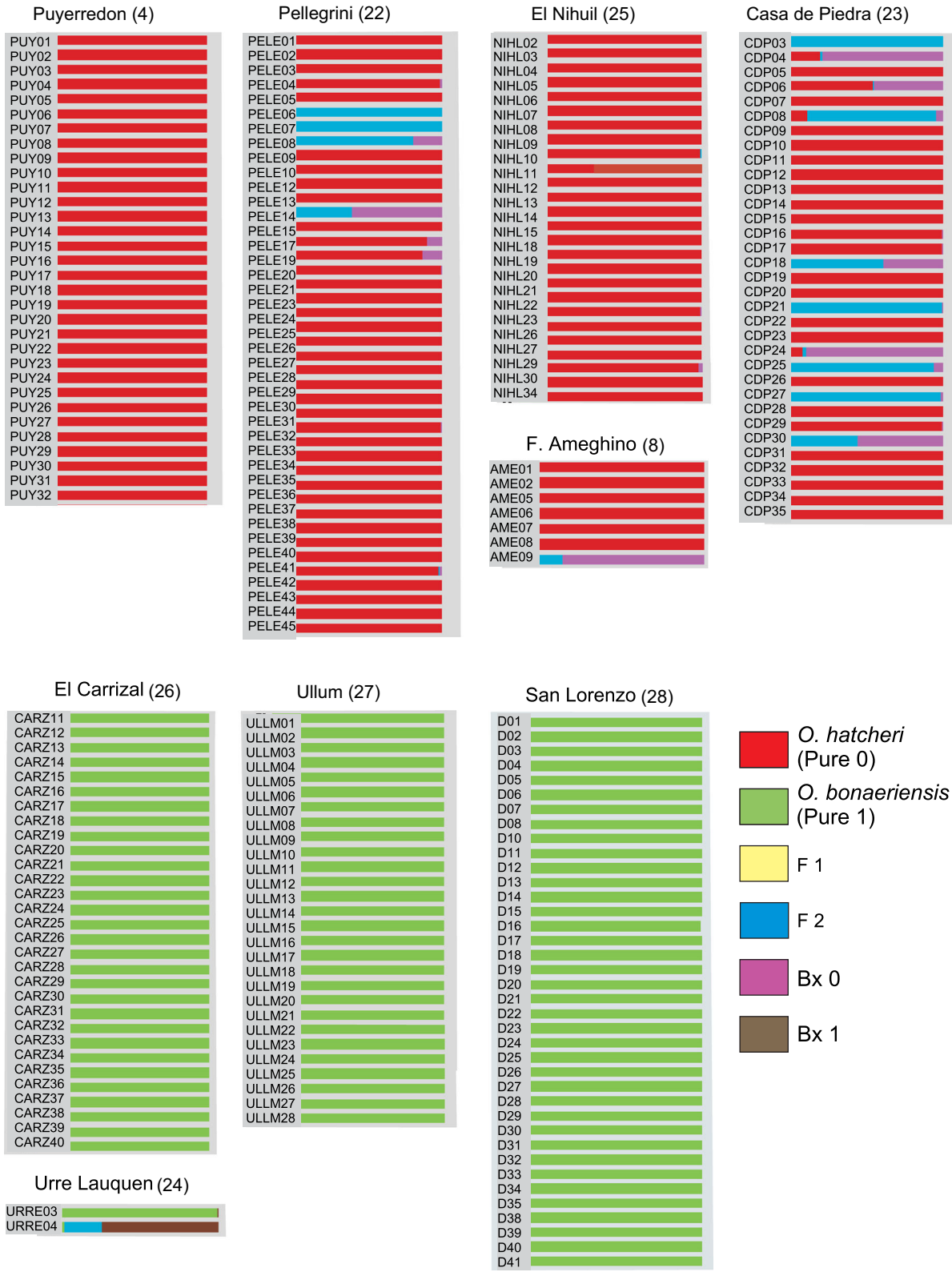
Mitochondrial DNA analysis

Mitochondrial cytochrome *b* (cytb) sequences were obtained from a subsample of 183 individuals (Table 1), counting 149 that had been sequenced before by Conte-Grand et al. (2015). PCR to amplify cytb used primers GLU31 (Unmack et al. 2009) and the species-specific primer Pej15929 5′-CGGC GTTCGGTTTACAAGAC-3′ (Conte-Grand et al. 2015). The target DNA fragment was amplified in 12 ul 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 10× reaction buffer and 1.25 µL of 25 mM $MgCl_2$. Amplification thermo-cycler parameters were: 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 51 °C for 45 s, and 72 °C for 60 s, with a final extension step at 72 °C for 5 min. The PCR products were checked on agarose gels and resulting amplicons were sent for purification and sequencing from both directions to High Throughput Sequencing Solutions (HTSeq.org), University of Washington, Seattle, Washington. Sequences were edited using Geneious v6 (created by Biomatters, http://www.geneious.com/) and aligned with MAFFT version 6 (Katoh and Toh 2008) using default settings and visually inspected to verify the expected open reading frame. Identical sequences obtained from multiple individuals were identified and collapsed to unique haplotypes with Geneious before phylogenetic analysis. Genealogy and phylogeographic structure of haplotypes was assessed by constructing a haplotype network with the program NETWORK v. 4.6.0.0 (http://www.fluxus-engineering.com/sharenet.htm), which implements the median-joining method. The number of haplotypes (n), haplotype diversity (h), and nucleotide diversity (π), were estimated using DnaSP 5 (Librado and Rozas 2009). Genetic differentiation between populations was estimated by φ_{ST} with the Tamura-Nei substitution model implemented in ARLEQUIN version 3.5 (Excoffier and Lischer 2010). Hierarchical genetic structure was assessed using analysis of molecular variance (AMOVA) based on 10,000 random permutations in ARLEQUIN with populations grouped according to river basin (Table 1).

Microsatellite analysis

A total of 469 individuals were assayed for 13 microsatellite loci developed for *Odontesthes bonariensis*. These markers have been reported to be selectively neutral, unliked, free of technical artifacts in allele scoring, and useful for broodstock management and assessment of variation in natural populations and contributed to the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu) by Takashi Sakamoto and collaborators (Almany et al. 2009). Between 20 and 41 individuals per population were assayed for a total of 15 populations (Table 1), but fewer individuals were available for two populations (URRE and AME). Microsatellite loci were amplified by PCR (20 µl reaction volume), following the conditions reported by T. Sakamoto, containing 1× buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM each primer (one with fluorescent marker) and 0.25 U of T*aq* (Promega). Amplified microsatellite fragments were scored for length with an ABI 3730*xl* analyzer® (Applied Biosystems) using LIZ500 as the internal lane size standard. Fragment lengths were assigned to allelic classes with GENEMAPPER 3.1® (Applied Biosystems) and all assayed individuals were genotyped. CONVERT (Glaubitz 2004) was used to reformat the data files for downstream analysis. The presence of null alleles and deviation from Hardy–Weinberg equilibrium was tested in MICRO-CHECKER (van Oosterhout et al. 2004). Linkage disequilibrium (LD) and Hardy–Weingberg equilibrium (HWE) for each locus was checked using the log-likelihood ratio statistic in GENEPOP version 4.2 with 10,000 dememorization steps, 100 batches, and 10,000 iterations per batch (Raymond and Rousset 1995). Standard diversity indexes including observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles averaged across loci (A), and mean total heterozygosity across sites were calculated with ARLEQUIN version 3.5 (Excoffier and Lischer 2010).

To identify hybrid individuals and estimate population-level hybridization, we carried out admixture analyses using a Bayesian clustering approach


Puyerredon (4)
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PUY02
PUY03
PUY04
PUY05
PUY06
PUY07
PUY08
PUY09
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PUY11
PUY12
PUY13
PUY14
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PUY22
PUY23
PUY24
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PUY26
PUY27
PUY28
PUY29
PUY30
PUY31
PUY32
Pellegrini (22)
PELE01
PELE02
PELE03
PELE04
PELE05
PELE06
PELE07
PELE08
PELE09
PELE10
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El Nihuil (25)
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NIHL21
NIHL22
NIHL23
NIHL26
NIHL27
NIHL29
NIHL30
NIHL34
Casa de Piedra (23)
CDP03
CDP04
CDP05
CDP06
CDP07
CDP08
CDP09
CDP10
CDP11
CDP12
CDP13
CDP14
CDP15
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CDP35
F. Ameghino (8)
AME01
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AME08
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El Carrizal (26)
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Ullum (27)
ULLM01
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ULLM18
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ULLM24
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ULLM27
ULLM28
San Lorenzo (28)
D01
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D32
D33
D34
D35
D38
D39
D40
D41
Urre Lauquen (24)
URRE03
URRE04
O. hatcheri (Pure 0)
O. bonaeriensis (Pure 1)
F 1
F 2
Bx 0
Bx 1

◀**Fig. 2** Graphic representation of hybrid detection obtained with NEWHYBRIDS based on microsatellite data. Each panel displays the probability assigned to the multilocus genotype for each individual (sample code number of individual shown on the *left*). Only representative localities are shown (locality numbers correspond to sites shown in Fig. 1): Pueyrredón and San Lorenzo are used as references for pure *O. hatcheri* (*solid red*) and pure O. *bonariensis* (*solid green*) populations, respectively; Pellegrini, El Nihuil, Casa de Piedra, and F. Ameghino have native *O. hatcheri* populations with a significant proportion of introgressed genes from *O. bonariensis* (F2 or backcross hybrid individuals are indicated with different colors); El Carrizal and Ullum reservoirs harbor 100 % *O. bonariensis* populations (but are in the native distributional range of *O. hatcheri*); the Urre Lauquen population seems to harbor *O. bonariensis* gene pool with some hybrids (small sample size available). All other populations surveyed but not shown were 100 % *O. hatcheri* (*red dots* in Fig. 1). MtDNA haplotype identity for all populations is shown in Table 1 and Suppl. Table 1)

implemented in the program NEWHYBRIDS version 1.1 beta (Anderson 2008). This method assumes that the sample is drawn from a mixture of pure individuals and hybrids and fits a model where q is the probability that an individual belongs to several genotype frequency classes; in this study: pure-bred parentals, F1 hybrids, or two backcross categories. Population samples from Lake Pueyrredón and Laguna San Lorenzo were used as references for pure *O. hatcheri* and pure *O. bonariensis*, respectively. Lake Pueyrredón is the type locality for *O. hatcheri* and has no history of stocking with *O. bonariensis*. Analyses were carried out for all samples jointly and also separately for each of population paired with the two pure-species references. In all cases, no prior species information was assumed for tested populations.

Additional population genetic analysis of microsatellite loci were carried out by assuming the infinite allele model (IAM) (Kimura and Crow 1964). To assess population structure, an Analysis of Molecular Variance (AMOVA) among sampled populations was performed with ARLEQUIN. Differentiation between pairs of populations was estimated across loci using the fixation index F_{ST} (Weir and Cockerham 1984) and evaluated with 1000 random permutations. To test for isolation by distance, correlation between F_{ST} and geographic distance was estimated with the Mantel test using 10.000 permutations.

To further assess genetic relationships among sampled individuals and populations, their multilocus genotypes were clustered using the Bayesian approach implemented in STRUCTURE version 2.1 (Falush et al. 2003, 2007; Pritchard et al. 2000). Calculations were carried out under the non-admixture model assuming independent allele frequencies, given the high interspecific differentiation, and no prior population information. The parameter settings applied were: 10 independent replicates each for a number of populations (K) ranging from 1 to 20, a burn-in of 100,000 steps followed by 300,000 MCMC iterations. The most likely number of clusters was estimated with the K statistic (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl 2012). Multimodality in individual and population coefficient across different runs were accounted for using the permutation procedure in CLUMPP (Jakobsson and Rosenberg 2007). The resulting matrix of Q values was graphically displayed through DISTRUCT (Rosenberg 2004).

Results

Molecular identification of hybrids using microsatellites

We first report results of hybrid detection obtained with NEWHYBRIDS to estimate the frequency of *O. hatcheri* × *O. bonariesis* hybrids in natural habitats. This software assigns probabilities to each individual for carrying pure *O. hatcheri* or *O. bonariensis* multilocus genotypes (Fig. 2, shown in red and green, respectively), as well as genotypes of different hybrid classes (F1s or backcrosses). Population samples used as references for pure *O. hatcheri* (Pueyrredon lake) and pure *O. bonariensis* (San Lorenzo) were included in all analyses. No differences were observed between analyses that included all populations simultaneously or a single population with the two references. Among all sites, a total of 19 specimens were identified as hybrids with different probabilities of being F2 or backcrosses to the parental genotype predominant in the population. Our results detected hybrid individuals with different frequencies among the following populations tested (Fig. 2): Pellegrini Lake (Fig. 1, locality 22) with 7 hybrids out of 41 individuals assayed (17 % hybrid population); F. Ameghino Reservoir (Fig. 1, locality 8) with 1 hybrid out of 7 individuals (14 %); Casa de Piedra Reservoir (Fig. 1, locality 23) with 10 out of 33 (30 % hybrids); and Urre Lauquen Lake (Fig. 1, locality 24) with 1 hybrid out of two individuals (50 % hybrids). Evidence from cytb

haplotype affiliation (Table 1 and Suppl. Table 1) confirms the presence of hybrid individuals detected with NEWHYBRIDS in Reservoir F. Ameghino (2 out of 9, or 22 %), Pellegrini Lake (5 out of 18, or 22 %), Casa de Piedra Reservoir (1 out of 8, or 12 %), and Urre Lauquen (2 out of 6, or 33 %). We did not detect hybrids in other populations with the microsatellite assay, but two individuals (NIHL025 and NIHL028) from El Nihuil Reservoir (Figure, 1 locality 25), for which we could not obtain microsatellite genotypes for this analysis, carried typical *O. bonariensis* cytb haplotypes (Table 1 and Suppl. Table 1), indicating that this locality may harbor a significant proportion of hybrids that were undetected by analysis with NEWHYBRIDS. All populations sampled from the Cuyo region (Rio Colorado basin), where *O. hatcheri* used to be the only native pejerrey species, contain individuals with *O. bonariensis* multilocus genotype (and/or mtDNA haplotypes), most notably El Carrizal (locality 26) and Ullum Reservoirs (locality 27), where 100 % of the individuals sampled have *O. bonariensis* multilocus genotype (Fig. 2) and cytb haplotype. Only two localities surveyed in Cuyo (locality 23: Casa de Piedra and locality 25: El Nihuil) still contain *O. hatcheri* populations with a fraction of F2 and backcross hybrids. In Patagonia, the southernmost population with introduced *O. bonariensis* genotypes is F. Ameghino Reservoir in the Chubut basin (Fig. 1, locality 8). In conclusion, these results confirm a measurable impact on natural populations of the Patagonian silverside as a result of introductions of *O. bonariensis*, particularly towards the northern reaches of its distribution. Hybrid individuals detected by these analyses were excluded for subsequent analyses of microsatellite variation among *O. hatcheri* populations.

MtDNA phylogeography and population structure

Cytochrome *b* sequences (1125 bp) were analyzed for a total of 183 specimens (Table 1). Only two specimens were used as a reference for *O. bonariensis* from their native area in Laguna San Lorenzo (Fig. 1, locality 28). Among all sequences, 35 unique haplotypes were identified, 31 corresponding to *O. hatcheri* and 4 haplotypes to *O. bonariensis* (for GenBank Accession numbers and the complete list of individuals carrying each haplotype see Suppl. Table 1). Most of these sequences were presented in an earlier phylogenetic comparison (Conte-Grand et al. 2015) that unambiguously resolved reciprocal monophyly and deep interspecific divergence (7 % sequence divergence for cytb), therefore haplotypes of the two species were treated separately for population structure analyses. As noted above, several localities in the native area of *O. hatcheri* were found to contain individuals carrying *O. bonariensis* haplotypes (Table 1): F. Ameghino (locality 8), Pellegrini (locality 22), Casa de Piedra (locality 23), Urre Lauquen (locality 24), El Nihuil (locality 25), El Carrizal (locality 26), and Ullum (locality 27). These results confirm significant levels of *O. bonariensis* mtDNA introgression into these populations.

Haplotype networks are presented in Fig. 3. The most frequent haplotype for *O. hatcheri* (H1, Fig. 3) was shared by a total of 97 individuals collected from all river basins in Patagonia and Cuyo, except the Puelo river basin (localities 11 and 12). The second most frequent haplotype (H9, Fig. 3) differs from H1 by one mutation and is represented by 10 individuals widely distributed in Patagonia in the Puelo, Negro, Colorado, and Chubut river basins. The haplotype network displays a typical star-shaped pattern, with H1 in the center and no apparent segregation of haplotypes according to geography, river basin, or Oceanic drainage. Results from AMOVA (Table 2), in fact, show that most cytb variation is distributed within populations (about 82 %), with less than 0.5 % explained by comparisons among river basins. In spite of relatively small sample sizes for each locality, several estimates of genetic differentiation (measured as pairwise ϕ_{ST}) among river basins (Table 3) or populations (not shown) were significant, suggesting that local populations differ in their haplotype frequencies, given that they may have been isolated for a considerable amount of time.

Microsatellite variation and population structure

After removing 19 putative hybrid detected by NEWHYBRIDS, a sample of 441 individuals (345 pure *O. hatcheri* and 96 pure *O. bonariensis*) was used to assess population structure based on microsatellite genotypes. Urre Lauquen (locality 24) and F. Ameghino (locality 8) samples were not used because sample sizes were too small (2 and 7 individuals, respectively). Diversity parameters estimated using ARLEQUIN, such as the mean number of alleles (A) and

Table 1 List of sampling localities depicted in Fig. 1 and genetic diversity indices for *Odontesthes hatcheri* based on mitochondrial and microsatellite markers

Species	Country	Basin (drainage)	Site latitude/longitude (S/W)	(Map #) site code	Mitochondrial DNA		Microsatellite analysis			
					N	π	N	H_E (±SD)	H_O (±SD)	A (±SD)
Odontesthes hatcheri	Chile	Baker River (Pac)	Laguna Esmeralda 46°33′/71°58′	(1) ESM	6[a]	0.0000	–	–	–	–
			General Carrera Lake (Chile) 46°30′/72°0′	(2) LGC	10[a]	0.0000	30	0.5315 ± 0.2792	0.4630 ± 0.2962	5.07 ±3.05
	Argentina		Buenos Aires Lake (Argentina) 46°29′/71°28′	(3) LBA	12	0.0000	33	0.5594 ± 0.2986	0.4728 ± 0.2621	6.69 ±4.42
			Pueyrredón Lake 47°23′/71°55′	(4) PUY	12[a]	0.0000	32	0.4288 ± 0.2405	0.4129 ± 0. 2791	3.69 ±2.23
		Chubut River (Atl)	Musters Lake 45°28′/69°10′	(5) MUS	11	0.0023	30	0.5603 ± 0.3599	0.5241 ± 0.3356	7.69 ±6.45
			La Plata Lake 44°52′/71°49′	(6) PLAT	1	0.0000	–	–	–	–
			Chubut River 43°51′/68°48′	(7) CHU	11	0.0008	32	0.6274 ± 0.2829	0.5517 ± 0.2794	8.92 ±5.71
			Florentino Ameghino Reservoir 43°42′/66°29′	(8) AME	7 (2)	0.0000	7	–	–	–
		Futaleufú River (Pac)	Rosario Lake 43°15′/71°20′56″	(9) ROS	3	0.0000	–	–	–	–
			Rivadavia Lake 42°36′/71°39′	(10) RIV	11	0.0005	30	0.3299 ± 0.3279	0.3183 ± 0.3290	2.84 ±2.89
		Puelo River (Pac)	Epuyén Lake 42° 27′/71°40′	(11) EPU	11	0.0002	20	0.4094 ± 0.2934	0.2963 ± 0.1982	3.69 ±2.43
			Puelo Lake 42°9′/71°38′	(12) PUEL	4	0.0000	–	–	–	–
		Endorheic	Carilafquen Lake 41°12′/69°25′	(13) CARI	2	0.0000	29	0.5884 ± 0.2874	0.5444 ± 0.2773	8.76 ±4.93

Table 1 continued

Species	Country	Basin (drainage)	Site latitude/longitude (S/W)	(Map #) site code	Mitochondrial DNA		Microsatellite analysis			
					N	π	N	H_E (±SD)	H_O (±SD)	A (±SD)
		Negro River (Atl)	Morenito Lake 41°03′/71°31′	(14) MITO	6	0.0015	29	0.4870 ± 0.3442	0.4360 ±0.2847	6.76 ±5.71
			Alicura Reservoir 40°35′/70°45′	(15) ALIC	5	0.0026	–	–	–	–
			Centro de Ecología Aplicada del Neuquén 39°54′/71°06′	(16) CEAN	4	0.0026	–	–	–	–
			Pomona 39°28′/65°30′	(17) POMO	1	0.0000	–	–	–	–
			Choele Choel 39°17′/65°40′	(18) CHOEL	4[a]	0.0024	–	–	–	–
			Villa Regina 39°16′/67°11′	(19) VREG	2	0.0011	–	–	–	–
			Isla Jordan 38°59′/67°58′	(20) RNIJ	6[a]	0.0004	–	–	–	–
			Piscicultura Rio Limay 38°59′/68°14′	(21) PLOT	2	0.0000	–	–	–	–
			Pellegrini Lake 38°41′/67°59′	(22) PELE	18 (5)	0.0012	41	0.6837 ± 0.2067	0.6173 ± 0.2459	10.69 ±4.96
		Colorado River (Atl)	Casa de Piedra Reservoir 38°15′/67°30′	(23) CDP	7 (1)[a]	0.0033	33	0.5852 ± 0.3225	0.5411 ±0.3065	7.84 ±4.89
			Urre Lauquen Lake 38°5′/65°50′	(24) URRE	4 (2)[a]	0.0016	2	–	–	–
			El Nihuil Reservoir 35°04′/68°45′	(25) NIHL	7 (2)	0.0003	25	0.3162 ± 0.2700	0.2217 ±0.2474	3.69 ±2.67
			El Carrizal Reservoir 33°20′/68°43′	(26) CARZ	(1)[a]	–	30	0.5459 ± 0.1977	0.4710 ± 0.1817	4.23 ± 2.25
			Ullum Reservoir 31°28′/68°40′	(27) ULLM	(1)[a]	–	28	0.5728 ± 0.1956	0.5577 ± 0.2075	4.30 ± 2.46

Table 1 continued

Species	Country	Basin (drainage)	Site latitude/longitude (S/W)	(Map #) site code	Mitochondrial DNA		Microsatellite analysis			
					N	π	N	H_E (±SD)	H_O (±SD)	A (±SD)
O. bona-riensis		Salado River (Atl)	San Lorenzo Lake 36°5′/58°1′	(28) D	(2)	–	38	0.5939 ± 0.2374	0.5297 ± 0.2428	6.15 ± 2.71

Odontesthes bonaeriensis individuals from their native Salado basin (D) and *O. hatcheri* individuals collected from stocked areas that carry *O. bonariensis* haplotypes also are listed

Pac Pacific Ocean, *Atl* Atlantic Ocean, *N* sample size for *O. hatcheri* and (*O. bonariensis*) mitochondrial haplotypes or sample size for microsatellite analysis; π nucleotide diversity (*O. hatcheri* only), *A* mean number of alleles, H_O mean observed heterozygosity, H_E mean expected heterozygosity

[a] COI sequence available at www.boldsystems.org. LGC and LBA is the same lake that spans the national border between Argentina and Chile (and receive different names in each country)

mean expected and observed heterozygosity (H_E and H_O), for population samples with sample sizes $N > 20$ are shown in Table 1. The total observed number of alleles per locus varied between 2 and 39. According to Microchecker results, only one locus (OBO21) presented evidence for a null allele. Thus, allele frequencies for OBO21 were adjusted using the Chakraborty correction. Total H_E (13 loci) averaged across samples was $H_E = 0.6972$ (±0.2448, range 0.31622–0.68371) and H_O ranged from 0.2217 to 0.5571. HWE tests with Microchecker and GENEPOP detected an excess of homocygotes, rejecting conditions of equilibrium ($p \leq 0.05$) for the total sample. Reductions in heterozygosity may be explained by Wahlund effects due to population structure, as shown below. The exact test for linkage disequilibrium showed negligible levels of linkage among loci, always under 5 % of the loci with significant values at any particular locality. These results suggest that variation at microsatellite loci is suitable for population genetic analysis.

In agreement with cytb results, genetic divergence between *O. bonariensis* and *O. hatcheri* at microsatellite loci is significant (Fig. 4a). STRUCTURE analyses also separate introduced *O. bonariensis* individuals from Ullum (locality 27) and El Carrizal (locality 26) from the native population (San Lorenzo, locality 28), but overall variation among populations was low (average $F_{ST} = 0.06$) with 94 % of the variance explained within populations. Hierarchical AMOVA for *O. hatcheri* (Table 2) also shows high levels of variation within populations (80 % of the variance), only 19 % among populations within river basins, and 1.4 % among river basins. F_{ST} values among localities and river basins (Table 3), however, are relatively high, reaching ca. 0.5 (e.g., Futaleufú vs. Puelo basins). The Mantel test used to infer correlation between genetic and geographic distances showed a positive ($r = 0.16$) but not significant ($p > 0.05$) relationship between these two variables, suggesting that genetic differentiation is not directly related to geographic distance. STRUCTURE analyses for pure *O. hatcheri* populations suggested a most likely value of $K = 11$ (Fig. 4b). Therefore, each population sampled can be differentiated from the others by their specific genetic signature. Populations from rivers flowing to the Pacific slope (Baker, Puelo, and Futaleufu basins) are not grouped together but all populations are different.

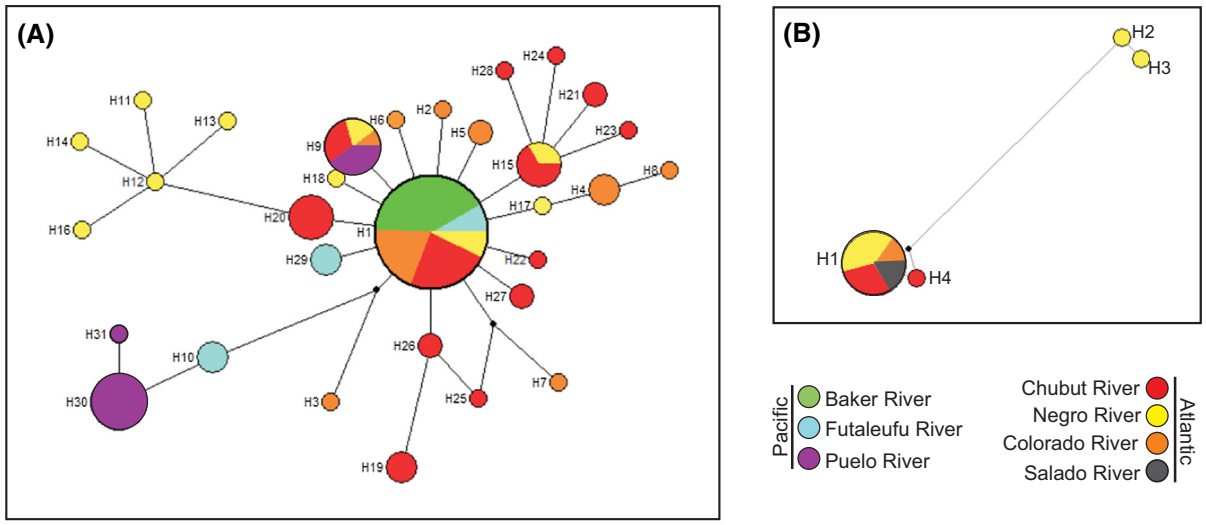

Fig. 3 Cytochrome *b* haplotype networks inferred separately for **a** *O. hatcheri* haplotypes H1–H31 and **b** *O. bonariensis* haplotypes H1–H4. Size of the circles is proportional to number of individuals carrying each haplotype and *colors* indicate river basin of origin, as shown. The complete list (identity and locality) of individuals carrying each haplotype is in Suppl. Table 1

Table 2 Analysis of molecular variance (AMOVA) for *O. hatcheri*. Population samples were grouped according to river basin (N = 6 groups: Baker, Chubut, Futaleufú, Puelo, Negro, and Colorado, see Table 1)

Components of variation	SS	Variance component	% Variation	*p* value
mtDNA (cytb)				
Among groups (basins)	7.352	0.00493	0.44	<0.005
Among populations within groups	24.314	0.19941	17.75	<0.005
Within populations	147.936	0.91886	81.81	<0.005
Microsatellites (13 loci)				
Among groups (basins)	21,940.765	4.12499	1.43	<0.005
Among populations within groups	17,357.576	54.17836	18.76	<0.005
Within populations	157,193.472	230.48896	79.81	0.40078

SS sum of squares

Table 3 Estimates of population genetic differentiation among river basins based on cytb sequences (pairwise ϕ_{ST}, below diagonal) and microsatellite markers (pairwise F_{ST}, above diagonal) for *Odontesthes hatcherix*

Basin	Baker	Chubut	Futaleufu	Puelo	Negro	Colorado
Baker	❖	0.06942	0.13730	0.29063	0.10122	0.12214
Chubut	0.04422	❖	0.19371	0.25596	0.05036	0.03259
Futaleufu	0.36199	0.06613	❖	0.41063	0.19126	0.07489
Puelo	0.36478	0.17918	0.27120	❖	0.31628	0.07563
Negro	0.10482	0.09854	0.03187	0.29945	❖	0.29291
Colorado	0.25152	0.14229	0.08833	0.28335	0.05458	❖

Underlined numbers indicate non significant values ($p > 0.05$)

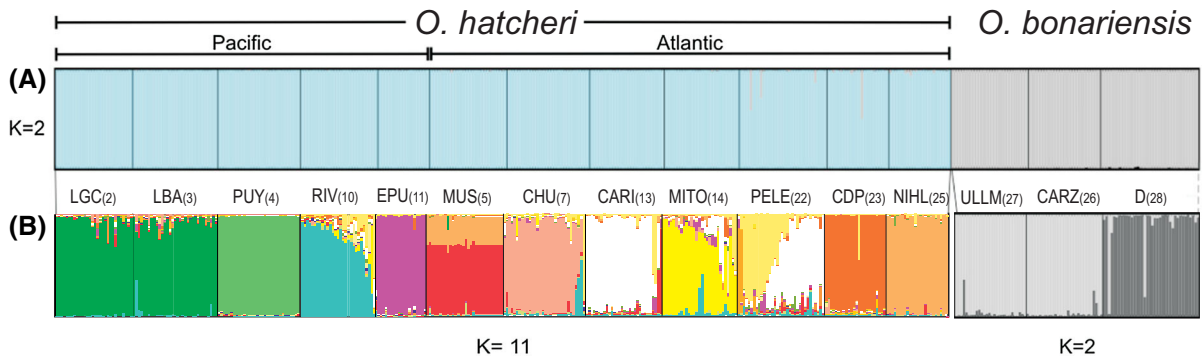

Fig. 4 Population structure based on microsatellite data obtained with the program STRUCTURE. **a** All population samples analyzed together (*O. hatcheri* plus *O. bonariensis*) results in clear separation of the two species. **b** Each species analyzed separately results in 11 clusters for *O. hatcheri*, each from a different locality, and only two clusters for *O. bonariensis*, one for the native population in Buenos Aires and the other from the introduced populations in Cuyo. Locality code and number (in parenthesis) follow Fig. 1 (see Table 1 for more information)

Discusion

We here addressed two main issues: (1) the effects of introductions of *O. bonariensis* by molecular identification of hybrids using microsatellites and (2) the phylogeography of *O. hatcheri* as a backdrop to better understand the consequences of man-made alterations of habitats and stocking practices. Both are complementary and informative for managing conservation efforts directed to this endemic species of Patagonia.

Extensive hybridization: new evidence from microsatellites

Hybridization is a natural phenomenon long recognized to affect many species of fishes (Hubbs 1955; Schwartz 1981). Like in many agricultural activities, inter-specific hybrids have been produced in aquaculture and stocking programs to transfer desirable traits, such as increased growth rate, between species and for production of sterile fish or mono-sex offspring to control unwanted reproduction, to facilitate harvesting, and to increase overall hardiness in culture conditions (Bartley et al. 2000). Such genetic manipulations have been applied to many commercially important freshwater fishes such as *Oncorhynchus* (Bartley and Gall 1991; Young et al. 2001), Serrasalmids (Hashimoto et al. 2011, 2014), *Pseudoplatystom*a (Hashimoto et al. 2013; Prado et al. 2012), *Leporinu*s (Hashimoto et al. 2010), and many other species but not to pejerrey. Recurrent translocation of *O. bonariensis* into man-made reservoirs and lakes that altered the native habitat of *O. hatcheri* in Cuyo and Patagonia started in the early twentieth century (Dyer 2006; Somoza et al. 2008), mainly in the Negro and Colorado river basins and most intensively in Mendoza province. A perceived positive impact of these introductions on sport fisheries and tourism helped launch a national plan to "repopulate" freshwater habitats in Argentina with *O. bonariensis* fry produced first in hatcheries in Buenos Aires province, and later in other parts of the country (Somoza et al. 2008). Maintenance of captive broodstock for egg and sperm production in hatcheries, however, was not part of the routine procedures used for pejerrey propagation until after the early 2000s. Poor survival after handling or during long-term rearing conditions used at the time precluded establishment of breeding programs to manipulate or engineer genetic composition of brood stocks used for pejerrey aquaculture. All introductions performed during the twentieth century (and some that continue nowadays), relied on methods well established by the 1940s that depended exclusively on capture of wild broodstock during the breeding season, collection of egg and sperm for artificial fertilization in situ (in the field), with little or no rearing of larvae in hatcheries (Ringuelet 1943). Hence, exotic pejerrey larvae recurrently introduced to populate man-made reservoirs in Patagonia and Cuyo were unlikely to be homogeneous or selected genetic stocks. In fact, comparisons of observed heterozygosity between populations of introduced *O. bonariensis* from Ullum, (locality 27) and El Carrizal (locality 26) reservoirs and a natural population from San Lorenzo

lagoon (locality 28) show no difference in this parameter (0.47 and 0.56 vs. 0.53, respectively). The introduced populations, however, carry a lower average number of alleles per locus (4.2 and 4.3 versus 6.2, respectively; Table 1).

Data on the invasiveness of *O. bonariensis* following introductions throughout Argentina as well as in other countries (Chile, Bolivia, and Peru) are somewhat conflicting. Some studies report negative effects on native populations in Bolivia and Peru, for example by damaging native *Orestias* species in lake Titicaca where introduced *O. bonariensis* currently support an important commercial fishery (FAO 1997), or by outcompeting native *Trichomycterus* catfishes in some Chilean streams (Dyer, 1998). Our results show that some introductions in Patagonia also had drastic effects, replacing native *O. hatcheri* populations or at least reducing them to a minumum in three reservoirs on the Colorado river basin (Ullum, El Carrizal, and Urre Lauquen, localities 24, 26, and 27), and establishing significant presence of F2 hybrids and backcrossed individuals in other two reservoirs, Casa de Piedra (locality 23) and El Nihuil (locality 25, Fig. 2). The predominant gene pool in the latter populations, however, continues to correspond to the native *O. hatcheri* (Fig. 2; Table 1). Further south, introductions into the Negro river basin resulted in hybridization with *O. hatcheri* in Pellegrini Lake (locality 22). Most noteworthy, our results show for the first time establishment of *O. bonariensis* in the Chubut basin (F. Ameghino reservoir, locality 8), extending its presence south of the Rio Negro basin. In these populations, however, the native *O. hatcheri* gene pool still prevails, possibly because this species is better adapted to lower temperatures than *O. bonariensis* (Cussac et al. 2009). A narrow temperature range for optimal growth of pejerrey adults (25–27 °C) and post-larval stages (17–24 °C) (Strüssmann and Yasuda 2005) may curb any competitive advantage of *O. bonariensis* over *O. hatcheri* in cold Patagonian habitats. But the opposite may be true in the Cuyo area with higher water temperatures. A recent report comparing phenotypes of wild and farmed *O. bonariensis* and *O. hatcheri* specimens, farmed hybrid specimens, and presumptive introgressed specimens from wild populations (Crichigno et al. 2014) showed that under laboratory conditions, hybrids lacked body deformations when reared at high temperature during early life, a common outcome in purebred *O. hatcheri* (at 25 °C or higher) and *O. bonariensis* (at 29 °C or higher). This result suggests that hybrids may have increased tolerance to extreme temperatures during development (Inazawa et al. 2011), consistent with invasive hybridization observed in the warmer Cuyo region. No other factors are known to have caused negative impacts on the native *O. hatcheri* populations in this area. Temperature, in fact, is directly related to another important trait that may limit introgression of *O. bonariensis* into southern Patagonia: temperature-dependent sex determination (Yamamoto et al. 2013, 2014) *O. bonariensis* hatchlings reared at water temperatures of 17 or 29 °C during post-hatching weeks 1–5 develop population sex ratios of 100 % females or males, respectively (Strüssmann et al. 1997b), suggesting that cold water temperatures in southern Patagonian environments may bias sex ratios dramatically in this species. The sex determination mechanism in F2 or backcrossed hybrids seems to depends on complex interactions between temperature and genotype, but is strongly affected by parental genotype (Inazawa et al. 2011), suggesting that this potential barrier for introgression may become weaker and the invasiveness of *O. bonariensis* will increase as global warming trends advance in Patagonia.

Phylogeography of *Odontesthes hatcheri*

Intraspecific mtDNA differentiation among *O. hatcheri* populations observed in this study was low, less than 1 % among cytb haplotypes. The haplotype network (Fig. 3) displayed a star-like pattern with no separation or clustering of haplotypes into groups, consistent with a scenario of recent demographic expansion most likely influenced by recent founder-flush events towards the end of Pleistocene glaciations. Widespread sharing of mtDNA haplotypes among localities, river basins, oceanic drainages, and an overall lack of geographic orientation in the distribution of cytb variation is similar to patterns observed in *Percichthys trucha and Galaxias platei*, *two* co-distributed freshwater fishes native of Patagonia (Ruzzante et al. 2006; Zemlak et al. 2008). Collectively, these studies strongly suggest relatively recent mixing of freshwater populations east of the Andes. A recent symposium focused on understanding the effect of paleogeography and paleoclimatology on biodiversity of Patagonia (Ruzzante and Rabassa

2011) summarized research on the major natural forces shaping Patagonian landscapes since the Jurassic. Relevant to the species involved in our study, two major mechanisms were proposed that might explain the observed lack of population structure across currently isolated river drainages and large geographic expanses (Ruzzante et al. 2011). First, what are now disjunct lakes and different river basins may have been part of larger proglacial lakes formed during the retreat of the most recent Plesitocene glaciers (see extent of glaciations in Fig. 1). Geological evidence indicates the existence of several large palaeolakes east of the Andes during the last deglaciation (Turner et al. 2005) and the presence of large and powerful rivers crossing the Patagonian steppe (up to ten times larger than the current rivers) during the glaciations and glacial termination periods in the Pleistocene (Martínez and Kutschker 2011). The latter also may explain a second mechanism: the formation of many braided and deltaic connections among these large rivers on the exposed continental shelf (Martínez and Kutschker 2011; Ponce et al. 2011). During glacial periods, large flat areas exposed by lower sea level may have been dotted with shallow and relatively warm and productive freshwater bodies, providing preferred habitats for *O. hatcheri* and connections among river basins. Therefore, geological processes operating since the Quaternary glaciations, starting hundreds of thousands of years ago, may have provided repeated connections among the large, currently separated, Patagonian river systems. An alternative explanation for admixture of populations among river systems could involve marine dispersal between the river mouths along the extended South Atlantic coast of Patagonia. Although *O. bonariensis* has eurihaline habits, tolerating salinities close to sea water (Gómez et al. 2006), and some studies have suggested current or recent gene flow between this species, its marine relative *O. argentiniensis* (García et al. 2014), and other sympatric freshwater congeners, none of these characteristics pertain to *O. hatcheri.* Preliminary surveys of cytb haplotypes among marine coastal species of *Odontesthes* in Patagonia (*O. smitti*, *O. incisa*, and *O. nigricans*), in addition to those already mentioned for *O. argentiniensis*, show no evidence of mtDNA introgression with *O. hatcheri* and haplotypes of *O. hatcheri* have never been obtained from marine environments (G. Ortí, unpublished results). The only evidence for introgression, presented in this and our previous study, is a consequence of man-made introductions of *O. bonariensis* into Patagonia. In contrast, marine dispersal has been shown to be a common mechanism of colonization and range expansion for *Galaxias maculatus*, another native freshwater fish species of Patagonia (Zemlak et al. 2010). *G. maculatus*, on the other hand, showed a relatively pronounced genetic structure which appears to be a consequence of variations in its anadromous life history, a consequence of landlocking after isolation from ancestral marine populations, or to other factors limiting dispersal of non-migratory populations (Zemlak et al. 2010).

Microsatellite data analyzed in the present study, in contrast to mtDNA data, were able to discriminate local populations of *O. hatcheri* (Fig. 4; Table 3) such that each lake or river population sampled contains a unique combination of genotypes. This pattern is not inconsistent with the previous discussion based on mtDNA data; it merely adds power to discriminate local populations that have been isolated long enough to accumulate differences in microsatellite genotypes since they became isolated under the current hydrological configuration.

Another feature that receives much attention in discussions of Patagonian phylogeography is the rise of the Andes (Ruzzante and Rabassa 2011), a formidable barrier to gene flow for some freshwater species (Ruzzante et al. 2006, 2008, 2011; Zemlak et al. 2008, 2010, 2011). In contrast to other species with geographic distributions on both sides of the Andes (*P. trucha*, *G. maculatus*, *G. platei*), *O. hatcheri* populations from drainages flowing to the Pacific are not differentiated from populations in Atlantic basins. Samples from the Baker, Puelo and Futaleufú drainages share common cytb haplotypes with most other populations, and microsatellite distances fail to cluster them together. It is interesting to note that our sampling efforts failed to obtain *O. hatcheri* individuals from the lower stretches of the Puelo and Futaleufú rivers in Chile (rivers that have at their headwaters the Epuyen and Rivadavia lakes, respectively), in spite of intensively seining suitable natural habitat where these fish could have existed. Possible reason for extirpation from these lower stretches may be intensified predatory pressure from salmonids in Chile, a pattern documented for other native species (Habit and Cussac 2016; Habit et al. 2012). The origin of these three river systems (Baker, Puelo, Futaleufú)

from headwater lakes in Argentina may be explained by recent drainage reversals, as huge paleolakes caused the collapse of western barriers to water flow at the end of the last glacial cycle (Tatur et al. 2002; Turner et al. 2005; Zemlak et al. 2010). It is surprising that *O. hatcheri* is currently absent from these rivers. Furthermore, the absence of other *Odontesthes* species is noteworthy, such as the commonly found *O. mauleanum* in Chilean rivers flowing to the Pacific in areas further north.

Conclusions

Our study clearly documents a significant frequency of hybrids in natural populations of *O. hatcheri* that have been stocked with *O. bonariensis* for commercial or recreational reasons. Introduction of *O. bonarienes* to Patagonia may have produced positive social impacts and minor commercial benefits (U.S. Fish and Wildlife Service 2014) but the adverse impact on natural populations may be more widespread than previously thought. This effect is clearly dependent on temperature, as evidenced by the significantly greater frequency of *O. bonariensis* genotypes in populations from the warmer Cuyo region at the northern end of Patagonia. In some cases, *O. bonariensis* seems to have completely displaced the local *O. hatcheri* populations. Global warming trends could extend this threat towards the southern range of the distribution in the future (Cussac et al. 2009); therefore it is recommended to stop further introductions of *O. bonariensis* into Patagonia. Population structure revealed by microsatellite markers suggest that some local populations of *O. hatcheri* contain unique combination of genotypes that may reflect local adaptations and could be lost by introduction of exotic species. It seems somewhat paradoxical that *O. hatcheri* has been able to survive the repeated introduction of exotic salmonids that are formidable predators, but may be outcompeted by introduction of a congener under conditions predicted to be increasingly common in Patagonia in the near future.

Acknowledgments This study is a new contribution to a more comprehensive project for conservation of biodiversity based on reconstructing phylogeographic histories of several distinct species (or species complexes) native to the Patagonian region of southern Argentina and Chile (http://patagonia.byu.edu). We thank Gustavo Somoza for samples from Lake San Lorenzo and Feliciano Gómez for samples from Lake Pellegrini, and D. Ruzzante for insightful comments on the manuscript. We would like to acknowledge the following grants for supporting this study: Universidad Nacional del Comahue (04B147), CONICET (PIP 112-200801-00282), and FONCYT (PICT 2005-35241) of Argentina to VC; FONDECYT 108002 to EH; George Washington University (startup funds to GO); and the NSF-PIRE award (OISE-0530267, USA) 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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Supplementary Table 1 – List of cytochrome b unique haplotypes, GenBank Accession numbers, and individuals found to carry them (N=number of individuals). Individuals are labeled by locality code followed by specimen number. Locality codes are listed in Table 1. Haplotype networks for *O. hatcheri* (H1 to H31) and *O. bonariensis* (H1 to H4) are shown in Figure 3.

Species	GenBank Accession	Haplotype (N)	Specimens with this haplotype
Odontesthes hatcheri	KJ499113	H1 (97)	AME01, AME05, AME06, AME07, AME08, AME09, AME13, CARI02, CARI03, CHOEL04, CHU002, CHU01, CHU06, CHU07, CHU09, CHU10, CHU12, CHU13, CHU14, ESM31, ESM32, ESM33, ESM34, ESM36, ESM37, LBA001, LBA002, LBA06, LBA07, LBA08, LBA09, LBA10, LBA11, LBA12, LBA13, LBA14, LBA15, LGCBJ01, LGCBJ02, LGCBJ04, LGCBU31, LGCBU32, LGCBU33, LGCBU34, LGCBU35, LGCBU36, LGCBU37, MITO03, MITOA71, MITOF72, MITOF73, MUS08, MUS12, MUS14, NIHL14, NIHL15, NIHL16, NIHL17, NIHL18, NIHL19, PELE03, PELE04, PELE12, PELE14, PELE19, PELE20, PELE23, PELE25, PLOTT03, PLOTT04, POMO01, PUY001, PUY002, PUY06, PUY07, PUY08, PUY09, PUY10, PUY11, PUY12, PUY13, PUY14, PUY15, RNIJ01, RNIJ02, RNIJ04, RNIJ05, RNIJ11, RIV001, RIV002, RIV07, RIV10, RIV11, RIV12, RIV14, RIV15, URRE05
	KJ499127	H2 (1)	CHU08
	KJ499128	H3 (1)	CHU15
	KJ499148	H4 (3)	MUS01, MUS07, MUS13
	KJ499152	H5 (2)	MUS02, MUS15
	KJ499147	H6 (1)	MUS06
	KJ499149	H7 (1)	MUS09
	KJ499151	H8 (1)	MUS11
	KJ499180	H9 (10)	ALIC01, ALIC03, ALIC05, NIHL20, PLAT01, PUEL02, PUEL03, PUEL05, PUEL06, URRE03

	KY001660	H10 (3)	ROS04, ROS05, ROS06
	KJ499119	H11 (1)	CDP01
	KJ499121	H12 (1)	CDP13
	KJ499122	H13 (1)	CDP16
	KJ499123	H14 (1)	CDP17
	KJ499124	H15 (6)	CDP18, CDP20, PELE01, PELE08, PELE24, VREG01
	KJ499125	H16 (1)	CDDP35
	KJ499181	H17 (1)	URRE04
	KJ499182	H18 (1)	URRE06
	KJ499144	H19 (3)	ALIC02, ALIC04, MITOM71
	KJ499142	H20 (6)	CEAN01, CHOEL03, MITOF71, PELE15, PELE17, PELE21
	KY001661	H21 (2)	CEAN02, CEAN06
	KY001662	H22 (1)	CEAN03
	KY001663	H23 (1)	CHOEL01
	KY001664	H24 (1)	CHOEL05
	KJ499158	H25 (1)	PELE05
	KJ499160	H26 (2)	PELE09, RNIJ03
	KJ499161	H27 (2)	PELE10, PELE26
	KY001665	H28 (1)	VREG04
	KJ499174	H29 (3)	RIV06, RIV08, RIV13
	KJ499132	H30 (10)	EPU01, EPU02, EPU07, EPU08, EPU09, EPU10, EPU12, EPU13, EPU14, EPU15
	KJ499133	H31 (1)	EPU06

Species	GenBank Accession	Haplotype (N)	Specimens with this haplotype
O. bonariensis	KJ499130	H1 (13)	AME03, AME04, D4, D6, CARZ01, NIHL25, PELE11, PELE16, PELE18, PELE22, ULLM01, URRE01, URRE02
	KJ499120	H2 (1)	CDP02
	KJ499154	H3 (1)	NIHL28
	KJ499159	H4 (1)	PELE07