


# Biogeography, habitat transitions and hybridization in a radiation of South American silverside fishes revealed by mitochondrial and genomic RAD data

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## Abstract

Rivers and lake systems in the southern cone of South America have been widely influenced by historical glaciations, carrying important implications for the evolution of aquatic organisms, including prompting transitions between marine and freshwater habitats and by triggering hybridization among incipient species via waterway connectivity and stream capture events. Silverside fishes (*Odontesthes*) in the region comprise a radiation of 19 marine and freshwater species that have been hypothesized on the basis of morphological or mitochondrial DNA data to have either transitioned repeatedly into continental waters from the sea or colonized marine habitats following freshwater diversification. New double digest restriction-site associated DNA data presented here provide a robust framework to investigate the biogeographical history of and habitat transitions in *Odontesthes*. We show that *Odontesthes* silversides originally diversified in the Pacific but independently colonized the Atlantic three times, producing three independent marine-to-freshwater transitions. Our results also indicate recent introgression of marine mitochondrial haplotypes into two freshwater clades, with more recurring instances of hybridization among

Atlantic- versus Pacific-slope species. In Pacific freshwater drainages, hybridization with a marine species appears to be geographically isolated and may be related to glaciation events. Substantial structural differences of estuarine gradients between these two geographical areas may have influenced the frequency, intensity and evolutionary effects of hybridization events.

#### KEYWORDS

cytochrome *b*, glaciation, introgression, *Odontesthes*, Patagonia, single nucleotide polymorphism

## 1 | INTRODUCTION

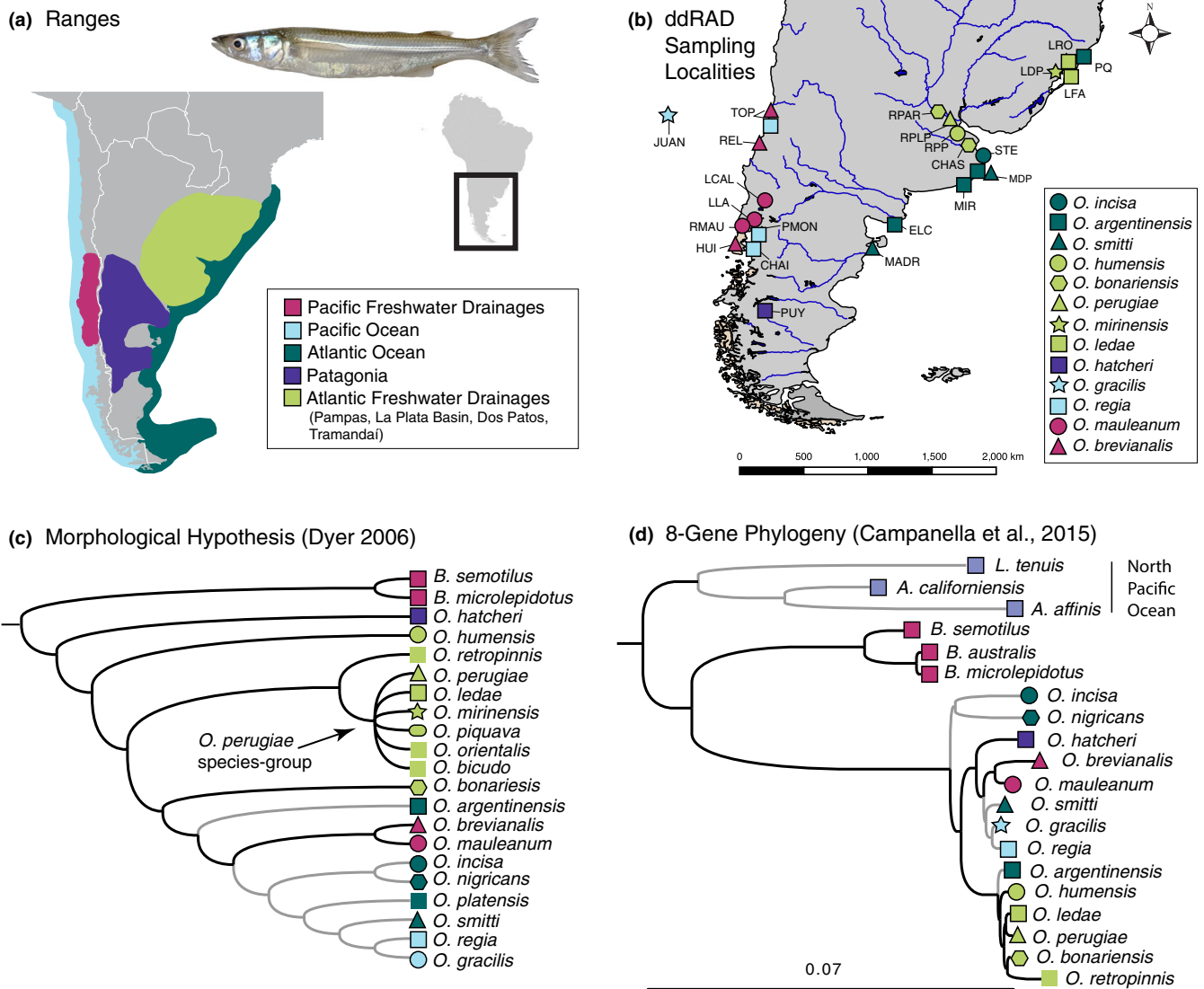
Compared to the hyperdiverse tropics, the temperate freshwater lakes and rivers of southern South America harbour a modest fish fauna, following a latitudinal trend towards lowest species diversity in southern Patagonia. Many fish species in Patagonia are endemic (Abell et al., 2008), of local economic and ecological importance (Cussac et al., 2004), and both the rapidly changing climate and introductions of invasive species are threats to these unique fishes (Becker, Crichigno, & Cussac, 2018; Rueda et al., 2017; Strüssmann, Conover, Somoza, & Miranda, 2010). Species inhabiting this region have evolutionary histories heavily influenced by temperature, the rise of the Andes, historical connectivity of rivers and lakes, and glaciation events (Cussac et al., 2016; Habit, Gonzalez, Ruzzante, & Walde, 2012; Ortubay, Gómez, & Cussac, 1997; Unmack, Barriga, Battini, Habit, & Johnson, 2012; Unmack, Bennin, Habit, Victoriano, & Johnson, 2009).

Freshwater colonization of the Amazon and Paraná River Basins by marine lineages such as drums, pufferfishes and anchovies are well documented (Bloom & Lovejoy, 2017). However, less is known about the origin, relationships, and biogeographical and phylogeographical history of marine-derived lineages in southernmost South America, where repeated glaciation cycles extirpated freshwater fish populations or relegated them to refugia (Cussac, Fernandez, Gomez, & Lopez, 2009; Cussac et al., 2004; Ruzzante et al., 2006, 2008; Ruzzante, Walde, Macchi, Alonso, & Barriga, 2011; Zemlak, Habit, & Walde, 2008; Zemlak, Habit, Walde, Carrea, & Ruzzante, 2010; Zemlak, Walde, Habit, & Ruzzante, 2011), and recent changes in sea level resulted in rapid recolonization (Cussac et al., 2004) or speciation (Beheregaray & Sunnucks, 2001; Beheregaray, Sunnucks, & Briscoe, 2002). A majority of these studies have relied on mitochondrial DNA (mtDNA) or microsatellites to make inferences, but applying modern high-throughput sequencing methods to these organisms should aid in elucidating the processes and histories underlying speciation among the unique fish lineages inhabiting this region.

Restriction-enzyme associated DNA (RAD) markers provide genome-wide data that have demonstrated utility for resolving phylogenetic relationships among rapidly diverged species (Wagner et al., 2013) where a handful of polymerase chain reaction (PCR)-based markers lack resolution. They also are useful for detecting gene flow and introgression among populations and species, processes that can

confound phylogenetic inferences and species delimitation (Eaton & Ree, 2013). Although genomic data have revolutionized phylogenetics, mtDNA markers, traditionally popular in the field of phylogeography (Avice, Arnold, & Ball, 1987), remain useful for understanding evolutionary patterns. Unlike nuclear markers, mtDNA does not readily recombine, and in cases of introgression it may be retained intact over generations. There are numerous reports of mtDNA haplotypes crossing species boundaries that unambiguously reveal ancient introgression events that, otherwise, may have left a weak or undetectable nuclear signature (Bryson, Smith, Nieto-Montes de Oca, García-Vázquez, & Riddle, 2014; Good, Vanderpool, Keeble, & Bi, 2015; Willis, Farias, & Orti, 2014). Discordance between mtDNA genealogies and nuclear DNA phylogenies has been extensively debated, but it is generally accepted that incomplete lineage sorting of ancestral polymorphism, introgressive hybridization and sex-biased dispersal rates are the most common causes of mito-nuclear discordance in various organisms (Toews & Brelsford, 2012).

New World silversides (family Atherinopsidae) have repeatedly transitioned between marine and freshwater habitats, but precise knowledge of their phylogenetic history, and therefore the chronology and frequency of inferred habitat transitions, remains contentious (Bloom, Weir, Piller, & Lovejoy, 2013; Campanella et al., 2015; Dyer, 2006). Some freshwater silverside species can osmoregulate in brackish water (Hughes, Somoza, & Nguyen, 2017; Silveira, Martins, & Domingues, 2018; Tsuzuki, Aikawá, Strüssmann, & Takashima, 2000), suggesting that reversals from freshwater to marine habitats may be possible, despite this being a relatively rare phenomenon among ray-finned fishes (Betancur-R, Ortí, & Pyron, 2015). With seven marine and 12 freshwater recognized species (Fricke, Eschmeyer, & van der Laan, 2019) distributed across coastal marine habitats and in rivers and lakes exclusively in temperate South America (Figure 1a,b), the atherinopsid genus *Odontesthes* (locally known as pejerrey in Spanish or peixe-rei in Portuguese) provides a unique system to study the history and evolutionary consequences of habitat transitions. A phylogenetic hypothesis based on morphological evidence (Figure 1c) implies that freshwater habitats comprised the ancestral range for this genus (Dyer, 2006). Conversely, a recent molecular phylogeny (Campanella et al., 2015) reached the opposite conclusion (Figure 1d), instead requiring several instances of marine dispersal from the Pacific to the Atlantic and subsequent freshwater



**FIGURE 1** (a) Marine and freshwater areas currently occupied by species of *Odontesthes* in southern South America, and photograph of *Odontesthes perugiae* by Y. P. Cardoso. (b) Sampling localities for ddRAD-sequenced samples (see Appendix S1: Table S2 for description of population codes). (c) 2006 Morphological phylogenetic hypothesis for all *Odontesthes* species (Dyer, 2006), although lacking the newly described *O. yacuman* (Wingert, Ferrer, & Malabarba, 2017). Marine lineages are shown with grey branches. Shapes with black outlines indicate that *cytb* or ddRAD data were collected for this study for that species. (d) Maximum likelihood molecular hypothesis based on seven nuclear genes and *cytb* (Campanella et al., 2015), including three outgroups that inhabit the northern Pacific Ocean that are not otherwise included in this study. Branch lengths for molecular hypotheses are shown in substitutions per site, and grey branches indicate marine lineages. Species included in this study have black outlines around their shapes [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

colonization by silversides. Studies based only on mtDNA sequences either included very few taxa or failed to resolve species boundaries and their relationships (García et al., 2014; González-Castro, Rosso, Delpiani, Mabrugaña, & Díaz de Astarloa, 2019; González-Castro, Rosso, Mabrugaña, & Díaz de Astarloa, 2016; Heras & Roldán, 2011). A series of studies on species of *Odontesthes* along the southwestern Atlantic coastline have shown significant genetic and phenotypic differentiation between marine and estuarine or freshwater populations, as populations adapt to different salinities without physical barriers separating them to restrict gene flow (Beheregaray, Levy, & Gold, 2000; Beheregaray & Sunnucks, 2001; Beheregaray et al., 2002; Endler, 1977; González-Castro et al., 2019, 2016).

An influential study on the mode of diversification of silversides proposed a hypothesis based on repeated colonization of brackish lagoon systems and estuaries by marine populations, facilitated by adaptive genetic and phenotypic plasticity (Bamber & Henderson, 1988), making them a ripe system to study the competing processes of gene flow and selection as populations invade new environments. The replicated nature of these ecological transitions in *Odontesthes* makes them particularly appealing, but we do not currently have a clear understanding of how many times they have made marine-to-freshwater transitions. Several studies have suggested that a marine lineage currently represented by *Odontesthes argentinensis* has invaded estuaries multiple times to establish freshwater populations resulting in speciation,

spawning both the phenotypically diverse “*O. perugiae* species-group” (Figure 1c) in the coastal lagoons of southern Brazil (Beheregaray et al., 2000, 2002; Beheregaray & Sunnucks, 2001) and a unique population in the Mar Chiquita Lagoon in Argentina (González-Castro et al., 2019, 2016). However, other work contradicts the hypothesis that the *O. perugiae* species-group is marine-derived. Phylogenetic relationships proposed using morphology fail to place *O. argentinensis* as the sister-group to the *O. perugiae* complex (Dyer, 1998, 2006; Figure 1c), contradicting the hypothesis of a marine-derived origin for this group. Furthermore, published mtDNA genealogies (García et al., 2014; González-Castro et al., 2016, 2019; Heras & Roldán, 2011) support a clade that includes the marine species *O. argentinensis* and all freshwater species endemic to the La Plata River Basin and the coastal lagoons of Uruguay and southern Brazil (e.g., the “*O. perugiae* group” plus *O. bonariensis*, *O. retropinnis* and *O. humensis*), but mtDNA consistently fails to establish species boundaries among these taxa or their phylogenetic relationships, potentially due to a history of gene flow among species (García et al., 2014) and limited information content of mitochondrial markers.

Hybridization between marine and incipient or established freshwater species has been proposed to be widespread among *Odontesthes* species in the southwestern Atlantic Basin (García et al., 2014; González-Castro et al., 2016, 2019). The coastline from northern Argentina to southern Brazil has many large estuaries and floodplains, such as the La Plata estuary, Mar Chiquita Lagoon and Dos Patos Lagoon, creating potential secondary contact zones between marine and freshwater species. In contrast, the steep southeastern Pacific coastline is punctuated with rivers draining snowmelt from the Andes mountain range, creating a sharper boundary between marine and freshwater habitats (Griffiths, 2018), and making hybridization less likely. Given the relative ease with which mtDNA may cross species boundaries in the event of hybridization, nuclear data also are necessary to elucidate the evolutionary history of *Odontesthes*.

Here we analyse a large data set of mtDNA sequences for species in *Odontesthes* to generate hypotheses about species boundaries and introgression that we further test with genome-wide RAD markers. We propose a new phylogenetic framework for this group and use it to formulate biogeographical hypotheses and to infer the history of habitat transitions. Finally, we explore a unique pattern of marine introgression detected by mtDNA analysis in a lake in southern Chile with nuclear data. The combination of mtDNA and nuclear RAD markers provides a powerful tool to disentangle species limits and to resolve phylogenetic relationships among species in the face of past or ongoing gene flow across species boundaries.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling and DNA extraction

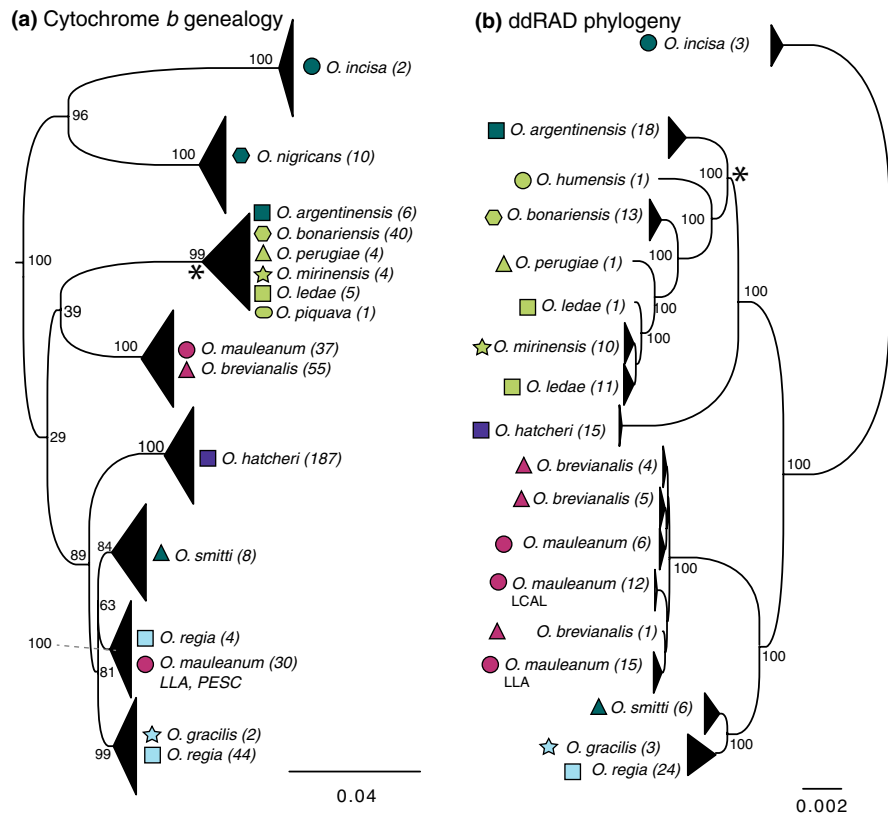
Silversides were collected via seine or gill nets between 2006 and 2014, and immediately killed via an overdose of MS-222, with fin clips preserved in 95% ethanol for DNA extraction. Sampling localities in

Argentina, Uruguay, Brazil and Chile span the range of *Odontesthes* and are listed in Table S1 for cytochrome *b* (*cytb*) sequences, and Appendix S1: Table S2 and Figure 1b for double digest RAD (ddRAD) sequences. *Odontesthes brevianalis* and *O. mauleanum* were difficult to distinguish in the field, and are named here based on typical habitat, which is estuarine/riverine for *O. brevianalis*, and primarily lacustrine for *O. mauleanum*. These two species are separated by a single morphological character, the presence of “noticeably crenate scales” along the side of *O. mauleanum*, or restricted only to the caudal peduncle for *O. brevianalis* (Dyer, 2006). Genomic DNA for *cytb* sequencing was extracted with a DNeasy Blood & Tissue Kit (Qiagen). Samples for RAD sequencing were extracted in 96-plate format via Autogen automated DNA extraction at the Laboratory of Analytical Biology at the Smithsonian National Museum of Natural History.

### 2.2 | mtDNA analysis

This data set expands upon a published set of sequences collected to investigate putative hybridization between *O. hatcheri* and introduced *O. bonariensis* (Conte-Grand, Sommer, Ortí, & Cussac, 2015; Rueda et al., 2017). We used the forward primer GLU31 (Unmack et al., 2009) and the *Odontesthes*-specific reverse primer Pej15929 (Conte-Grand et al., 2015) to amplify the target fragment of the *cytb* mitochondrial gene, following the same protocol as Rueda et al. (2017). PCR products were purified on 96-well Excerpture plates (Edge Biosystems), and cycle-sequenced at the Brigham Young University DNA sequencing centre. Contigs were assembled from raw chromatograms in SEQUENCHER version 4.8 (Gene Codes Corp.), and then aligned in MAFFT version 1.30b (Katoh & Standley, 2013). Our data set for *cytb* sequences included 450 individuals representing 14 nominal species of *Odontesthes* (of 19 described), and four sequences from its sister genus *Basilichthys*, collapsed into 171 unique haplotypes. The number of individuals per species is shown in parentheses in Figure 2(a). New sequences are accessioned on GenBank (MK983245–MK983379; Table S1).

Sequences were partitioned by codon position and the best substitution model for each partition was evaluated with MODELFINDER (Kalyaanamoorthy, Minh, Wong, Haeseler, & Jermin, 2017), followed by maximum-likelihood inference in IQ-TREE 1.6.0 (Nguyen, Schmidt, Haeseler, & Minh, 2015), and 1,000 bootstrap replicates using the ultrafast bootstrap method UFBOOT2 (Hoang, Chernomor, Haeseler, Minh, & Vinh, 2018). Haplotype networks for major clades were inferred with POPART 1.7 (Leigh & Bryant, 2015), using median joining networks (Bandelt, Forster, & Rohl, 1999). Bayesian analysis was conducted in BEAST 2.4.8 (Bouckaert et al., 2014), partitioned by codon position with the best-fitting model determined by MODELFINDER. The BEAST tree was time-calibrated using a relaxed clock approach based on secondary calibrations from Campanella et al. (2015). While fossil *Odontesthes* exist, they are not assigned to any particular species or clade (Dyer, 2006). We used the 95% highest posterior density (HPD) for the split between *Odontesthes* and *Basilichthys* as a normal distribution for our secondary calibration



**FIGURE 2** (a) Maximum likelihood cytochrome *b* phylogeny with ultrafast bootstrap values written on major nodes inferred in IQ-TREE. Numbers in parentheses indicate the number of individuals sampled. None of the species in the *Odontesthes argentinensis* species-group are found to be monophyletic; this group is marked with an asterisk at the base. *Odontesthes brevianalis* and *O. mauleanum* are not reciprocally monophyletic, but break into two distinct clades. Notably, the *O. mauleanum* populations LLA and PESC cluster with haplotypes of marine congeners. *Odontesthes gracilis* individuals are always interspersed among *O. regia* individuals. (b) Maximum likelihood ddRADseq phylogeny based on 151 tips and 2,211,938 bp, with ultrafast bootstrap values written on the nodes. A minimum of 75 individuals were present for each locus, and the full tree is shown with tip labels in Appendix S1: Figure S4. Population codes can be found in Appendix S1: Table S2, and on the map in Figure 1b. Branch lengths are shown in substitutions per site. Numbers in parentheses indicate the number of individuals sampled. The *O. argentinensis* species-group is again marked with an asterisk, but most species in this group now form distinct clades in contrast to mtDNA haplotypes. *Odontesthes brevianalis* and *O. mauleanum* do not cluster into distinct species, but individuals from the *O. mauleanum* Lake Llanquihue population (LLA) do not cluster with marine congeners as they do with mtDNA. *Odontesthes gracilis* is still interspersed among *O. regia* individuals [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

(mean = 20.86,  $SD = 1.0$ ), as well as the split of *O. nigricans* and *O. incisa* from all other *Odontesthes* species as a calibration (mean = 5.68,  $SD = 1.0$ ), but enforced no other constraints on the tree. We ran three independent runs for 100,000,000 generations on the Cipres Science Gateway ([www.phylo.org](http://www.phylo.org)), and assessed convergence in TRACER 1.6.0.

### 2.3 | ddRAD sequencing and matrix assembly

We followed the protocol developed by Peterson, Weber, Kay, Fisher, and Hoekstra (2012), modified to use the enzymes *MseI* and *PstI* and a 350–550-bp size selection. Genomic libraries for 164 *Odontesthes* samples representing 13 species (Appendix S1: Table S2) and five *Basilichthys microlepidotus* were prepared at the University of Puerto Rico Sequencing and Genomics Facility. Pooled samples were sequenced on a half-lane of a HiSeq 4000

at the University of Chicago Genomics Facility. Sequences were demultiplexed, filtered and assembled into RAD loci using IPYRAD version 0.7.2 (Eaton, 2014). We used the default parameters for de novo assembly of ddRAD data, with a minimum sequence depth of 6 for base-calling and a cluster threshold of 0.85; reads with more than five low-quality base calls were discarded. For our complete data set, we only retained loci that were present for at least 77 individuals, which included outgroup *Basilichthys microlepidotus* samples. Additional matrices were generated removing the outgroup samples, enforcing a minimum of 75 individuals per locus, and 100 individuals per locus.

### 2.4 | Phylogenomics

We used both concatenation and multispecies coalescent approaches for phylogenetic inference. Concatenated ddRAD loci



were analysed under maximum likelihood using IQ-TREE (Nguyen et al., 2015) and the GTR + G model, and 1,000 UFBoot replicates to assess branch support (Hoang et al., 2018). Unlinked single nucleotide polymorphisms (SNPs; one per locus) were analysed under the multispecies coalescent model in SVDQUARTETS (Chifman & Kubatko, 2014) available in PAUP\* 4.0a164 (Swofford, 2019) using all possible quartet combinations.

## 2.5 | Biogeography

To reconstruct the biogeographical history of *Odontesthes* with our phylogenetic resolution of the group, we first generated an ultrametric tree using only one tip per species scaled to time using TREEPL1.0 (Smith & O'Meara, 2012), an implementation of the penalized-likelihood approach (Sanderson, 2002). We used secondary calibrations from the 95% HPDs from the fossil-calibrated analysis of Campanella et al. (2015) for the group at three points: the divergence of *Basilichthys microlepidotus* from all *Odontesthes* 20.0–23.04 million years ago (Ma), the divergence of *O. incisa* from all other *Odontesthes* species in our ddRAD phylogeny 3.27–6.93 Ma, and the divergence between *O. argentinensis* and other freshwater lineages in the Paraná and Uruguay rivers 1.81–0.68 Ma. The time-calibrated tree was necessary to reconstruct the biogeography of the group, but we are not testing the effect of any particular major geological event; for example, the sustained uplift of the Andes mountains ~12 Ma (Garzzone et al., 2008) took place well before the inferred origin of extant *Odontesthes* species.

We defined five areas that *Odontesthes* currently occupies (Figure 1a): eastern Pacific Ocean, eastern Pacific freshwater drainages, Patagonia (restricted to only the Atlantic-draining portion east of the Andes), western Atlantic Ocean, and western Atlantic freshwater drainages (excluding Patagonia, but including the Pampas region, La Plata, Paraná and Uruguay rivers, Patos and Tramandaí lagoon systems in Brazil). Although there is no geographical barrier between Atlantic-draining Patagonia and warmer freshwater habitats to the northeast, it is probably too cold for the freshwater representatives of the *O. argentinensis* species-group, some of which have temperature-dependent sex determination (Cussac et al., 2009; Strussmann, Moriyama, Hanke, Cota, & Takashima, 1996). We restricted dispersal between nonadjacent areas, so that dispersal could not occur directly between the Pacific Ocean and Atlantic freshwater drainages, Pacific and Atlantic freshwater drainages, and Pacific freshwater drainages and Patagonia, where the Andes mountain range creates a physical barrier. Additionally, ancestors could only inhabit two of the five areas at one time, although no modern *Odontesthes* species occupies more than one of these ranges. Reconstruction was performed in the BIOGEOBEARS R package (Matzke, 2013a) under all available models (Matzke, 2013b, 2014), with model selection performed using the Akaike information criterion (AIC). R code is available on Figshare (Hughes, Cardoso, & Sommer, 2019).

## 2.6 | Admixture analysis with FASTSTRUCTURE and Patterson's *D*

To examine possible nuclear admixture in our samples with ddRAD-derived SNP data, we ran clustering analyses using FASTSTRUCTURE (Raj, Stephens, & Pritchard, 2014) on individuals from two clades where species were not monophyletic in our *cytb* data set (Figure 2a). The first clade was the *O. argentinensis* species-group, represented in our ddRAD data set by *O. argentinensis* ( $n = 18$ ), *O. bonariensis* ( $n = 15$ ), *O. ledae* ( $n = 13$ ) and *O. mirinensis* ( $n = 11$ ) (Figure 2b). The species *O. humensis* and *O. perugiae* also fall in this clade, but because we were only able to obtain one individual for each of these, we excluded them from this analysis. The second group included *O. regia* ( $n = 26$ ), *O. gracilis* ( $n = 3$ ), *O. smitti* ( $n = 7$ ), *O. brevianalis* ( $n = 9$ ) and *O. mauleanum* ( $n = 34$ ). We excluded a few samples with high missing data. Due to the hierarchical nature of these groups, we decomposed each of these two initial groups into subgroups until the subgroup contained only individuals of one species, or the optimal value of  $K$  was 1 as determined by the chooseK.py script packaged with FASTSTRUCTURE. For each data set and each value of  $K$ , we ran 10 replicates, and tested values of  $K$  between 1 and 6.

We calculated Patterson's  $D$  for the two groups above, where mtDNA suggested introgression. This statistic is commonly known as the ABBA–BABA test (Durand, Patterson, Reich, & Slatkin, 2011). This test uses biallelic SNPs based on a four-taxon pectinate tree, including one outgroup taxon to determine which allele is ancestral (the “A” allele), and which is derived (“B”), with the lineages typically denoted as (Outgroup,(P3,(P2,P1))). The  $D$ -statistic is calculated based on the number of patterns that conflict with the tree (the “ABBA” and “BABA” patterns). These patterns should occur in equal frequencies if incomplete lineage sorting is the only process acting, resulting in  $D = 0$ . But if introgression is occurring between P3 and either P1 or P2, then there will be an excess of either “ABBA” or “BABA” patterns. We used DSUITE, a tool that calculates  $D$ -statistics from VCF files and assesses significance using jackknifing (Malinsky, 2019). Under this framework, we calculated  $D$  for two separate clades, with *Odontesthes hatcheri* samples used as the outgroup population in both analyses. For the clade composed of primarily Pacific-drainage taxa, we treated all samples of marine species (*O. smitti*, *O. gracilis* and *O. regia*) as a single population, given that FASTSTRUCTURE did not differentiate amongst these species, but used the Lake Llanquihue (LLA) *O. mauleanum* population as “P2” and the Lake Calafquen *O. mauleanum* population as “P1.” In the *O. argentinensis* species-group, *O. argentinensis* represented “P3,” *O. bonariensis* “P2,” and *O. mirinensis* plus *O. ledae* samples were used for “P1.”

## 3 | RESULTS

### 3.1 | mtDNA

One representative from each new haplotype sequence has been deposited on GenBank (MK983245–MK983397). Sequence

alignments and NEWICK tree files are available on Figshare (<https://doi.org/10.6084/m9.figshare.11413209>). MODELFINDER supported different substitution models for each of the three partitions: K2P + I + G for the first codon position, HKY + I + G for the second and TN + G for the third. Maximum-likelihood analysis supported the monophyly of the genus *Odontesthes* and distinguished seven well-supported clades (99%–100% bootstrap support; Figure 2a), but relationships among clades are not resolved with confidence (<95%, as the recommended interpretation of the UFBoot method). Haplotype networks are shown in Appendix S1: Figure S1. Most haplotypes from freshwater *Odontesthes brevianalis* or *O. mauleanum* were contained in a single well-supported haplogroup that showed no segregation of haplotypes between species (Figure 2a; Appendix S1: Figure S1). However, individuals from a few freshwater populations in southern Chile, Lake Llanquihue (LLA) and its tributary the Pescado River (PESC) carry divergent mtDNA haplotypes that are closely related to the marine species *O. regia* and *O. smitti* (Figure 2a; Appendix S1: Figure S1). *Odontesthes gracilis* nests within *O. regia* haplotypes (Figure 2a; Appendix S1: Figure S1), but *O. smitti* haplotypes collected from the southwestern Atlantic (MADR and MDP; Figure 1b) are monophyletic (Figure 2a). The divergence of sequences from Lake Llanquihue and the Pescado River from its sister clade formed by the *O. regia-smitti* haplogroup dated to the Pleistocene (mean age 744,000 years ago, 95% HPD 1.2 million to 154,700 years ago; Appendix S1: Figure S2). Sequences obtained from Patagonian *O. hatcheri* clustered into a distinct haplogroup, with the exception of some hybrid individuals that carried *O. bonariensis* haplotypes, where this species has been introduced into Patagonia (Conte-Grand et al., 2015; Rueda et al., 2017). Another clade includes haplotypes from species associated with the Atlantic Basin (marine *O. argentinensis*, and freshwater *O. bonariensis*, *O. ledae*, *O. mirinensis*, *O. perugiae* and *O. piquava*) that did not sort according to species boundaries (Figure 2a; Appendix S1: Figure S1).

### 3.2 | ddRADseq species phylogeny

Using the de novo assembly pipeline in IPYRAD, we assembled three ddRAD matrices with different amounts of missing data. In matrices without *Basilichthys microlepidotus* samples, we rooted the trees on *O. incisa*, which is the first branch of *Odontesthes* in all analyses that include the outgroup (Appendix S1: Figure S2). Resulting phylogenies based on the three matrices produced nearly identical results between concatenation and multispecies coalescent SVDQUARTETS analyses (Appendix S1: Figures S3–S7), resolving relationships with confidence for major lineages (Figure 2b), although relationships among individuals within these groups differed. Not all morphospecies were monophyletic, namely *O. ledae*, *O. mirinensis*, *O. mauleanum*, *O. brevianalis* and *O. gracilis* (Figure 2b; Appendix S1: Figures S3–S7).

In agreement with the mtDNA genealogy, the ddRAD phylogenies resolve *O. incisa* as the sister group of all other species in the genus (no ddRAD data were obtained for *O. nigricans* due to the

poor quality of DNA extractions from our available specimens). The remaining *Odontesthes* species split into two groups, mostly corresponding to geography. One is a primarily Pacific Basin clade from localities west of the Andes that contains the two freshwater Chilean species *O. brevianalis* and *O. mauleanum* and the marine *O. regia* and *O. gracilis*, plus *O. smitti* from Atlantic localities (Figures 1b and 2b). *Odontesthes mauleanum* and *O. brevianalis* do not form well-supported separate clades in either concatenation or multispecies coalescent trees, but some structure grouping individuals from the same collection localities was observed (Figure 4b). Individuals from LLA that carried the marine *O. regia/gracilis* mtDNA haplotype (Figure 2a) were grouped by the RAD data with other freshwater populations of *O. mauleanum* and *O. brevianalis* with high support (Figure 2b). *Odontesthes gracilis* individuals never formed a monophyletic group. The second major clade is in the Atlantic Basin, containing *O. hatcheri* as the sister to the *O. argentinensis* species group (Figure 2b). In stark contrast to the *cytb* genealogy, which does not differentiate the morphological species (Figure 2a), analyses of genome-wide ddRADseq data clearly delineate *O. argentinensis* and *O. bonariensis* into monophyletic groups, except for *O. ledae* and *O. mirinensis*, although just one individual each of *O. humensis* and *O. perugiae* was available.

### 3.3 | Biogeography

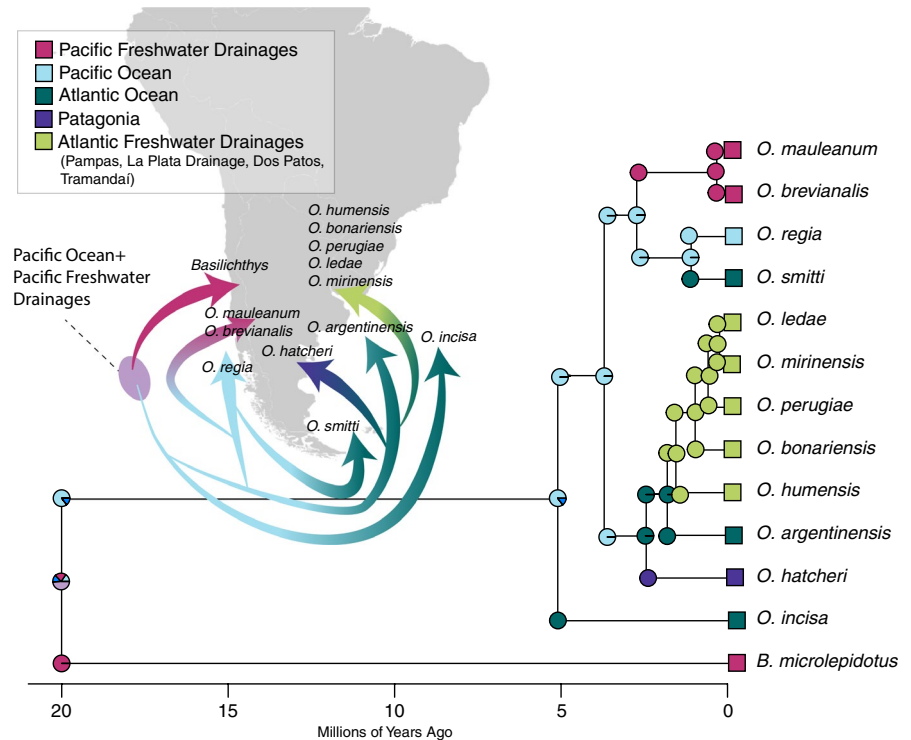
AIC scores selected the best-fit DIVA-like + J model available in BIOGEOBEARS, a likelihood implementation of the parsimony-based DIVA model (Ronquist, 1997), with the addition of the “jump dispersal” parameter “J” (Matzke, 2013b, 2014). All models with the “J” parameter had similar AIC scores, and resulted in highly similar biogeographical reconstructions, inferring a southeastern Pacific Ocean origin for all extant *Odontesthes*, three separate transitions of the Atlantic Ocean, and three separate transitions into freshwater environs (Figure 3).

### 3.4 | Admixture analysis of nuclear SNPs with FASTSTRUCTURE and Patterson's D

Among members of the *O. argentinensis* species-group, which share indistinguishable mtDNA haplotypes (Figure 2a), FASTSTRUCTURE indicated two genetic clusters, one containing all *O. argentinensis* individuals, and the other cluster contained the three remaining freshwater species, *O. bonariensis*, *O. mirinensis* and *O. ledae* (Figure 4a). Analysing this second cluster separately, *O. bonariensis* formed a separate group from the two remaining Brazilian species, but *O. ledae* and *O. mirinensis* did not form two separate clusters when analysed alone. There was no evidence of nuclear admixture in individual population assignments.

When all individuals from *O. regia*, *O. smitti*, *O. gracilis*, *O. brevianalis* and *O. mauleanum* were included in our FASTSTRUCTURE analysis, the optimal value of *K* was 2 (Figure 4b), and individuals from the *O. mauleanum* population Lake Llanquihue (LLA) did not show

**FIGURE 3** Biogeographical reconstruction from BIOGEOBEARS under the DIVA-like + J model, placing the origin of all modern *Odontesthes* in the Pacific Ocean, with three separate colonization events of the Atlantic Ocean, and three freshwater transitions: one to freshwater Pacific drainages, and two separate invasions of Atlantic freshwater drainages [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



evidence of nuclear admixture with marine congeners, despite what their mtDNA signal suggests (Figure 2a). Analysing these two clusters separately, the three marine species *O. regia*, *O. smitti* and *O. gracilis* did not form separate genetic clusters. Among *O. brevianalis* and *O. mauleanum* individuals, there were three clusters: a population for all Lake Calafquen (LCAL) individuals, a population with all Lake Llanquihue (LLA) individuals, and individuals from all remaining populations (RMAU, HUI, TOP, REL; Figure 4b).

The *D*-statistic calculated for the *O. argentinensis* species-group was greater than zero, although not substantially so at  $D = 0.056$  ( $p = .0013$ ), suggesting weak introgression between *O. argentinensis* and *O. bonariensis* (Figure 4c). The *D*-statistic using *O. hatcheri* again as an outgroup, *O. regia*, *O. smitti* and *O. gracilis* individuals as P3, LLA *O. mauleanum* population individuals as P2, and *O. mauleanum* individuals from LCAL as P1 was slightly larger at  $D = 0.132$  ( $p < .0000001$ ), suggesting some introgression between the marine individuals and the LLA population.

## 4 | DISCUSSION

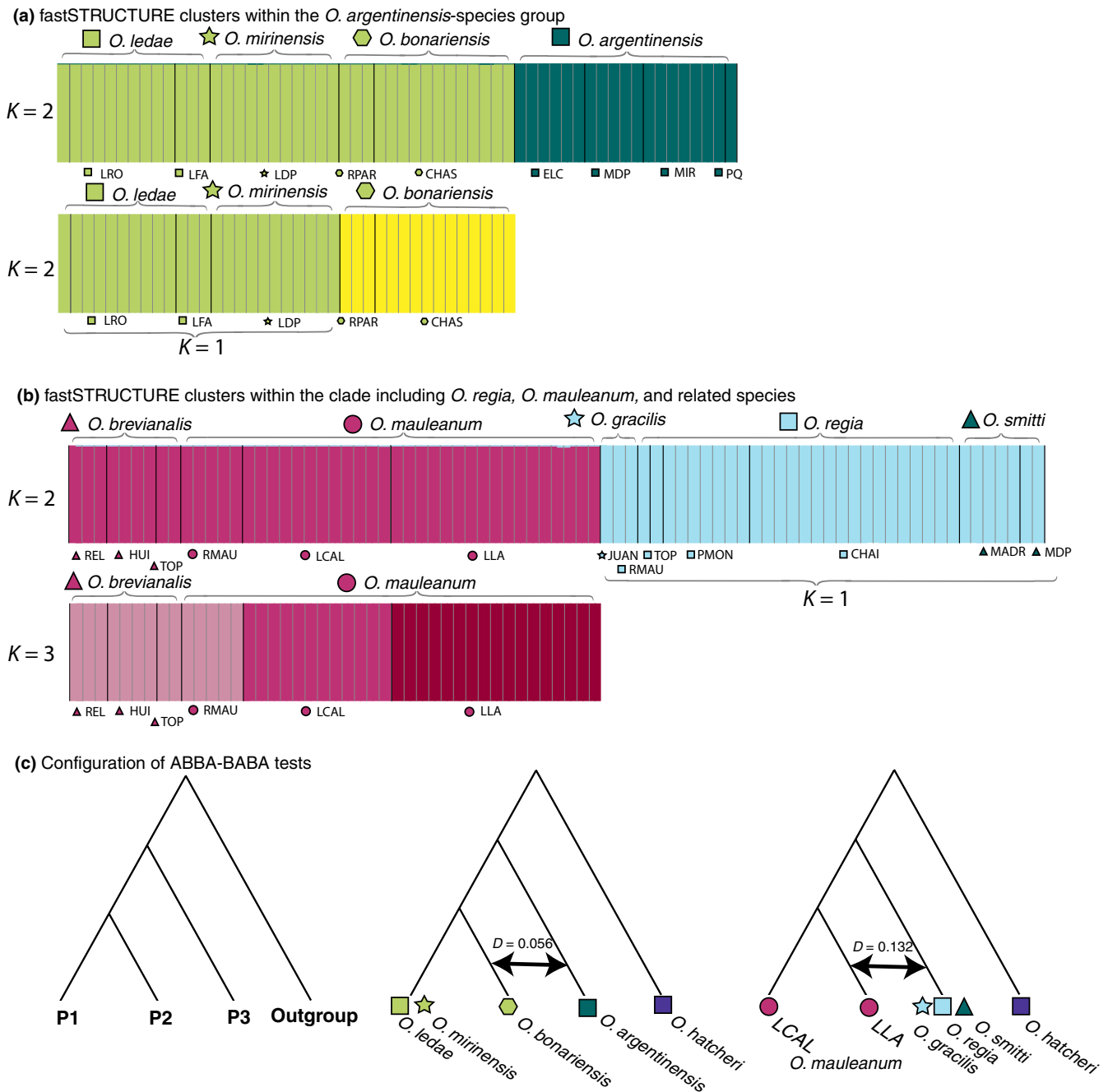
### 4.1 | Resolution of species boundaries and phylogeny

Until now, morphological and mtDNA hypotheses have been the primary resource available to understand the evolution South American silversides. Previous studies (Campanella et al., 2015; García et al., 2014; González-Castro et al., 2016, 2019) failed to separate many nominal species, especially in the Atlantic Basin

(Figure 1c,d), and to confidently establish phylogenetic relationships in the *Odontesthes argentinensis* species-group. In contrast, genomic ddRAD data show strong support for the current taxonomy with a few exceptions and confidently establish phylogenetic relationships. Most morphologically described species were identified as monophyletic, with a few notable exceptions. The *O. argentinensis* species-group, indistinguishable with mtDNA, mostly sorted into monophyletic groups where multiple individuals per species could be obtained, except for the two Brazilian species *O. ledae* and *O. mirinensis*, which FASTSTRUCTURE also did not identify as separate genetic clusters. Genetic differentiation between *O. regia* and *O. gracilis* is not supported by the RAD data (Figure 2b), suggesting that the species in the Juan Fernandez islands (*O. gracilis*) is not genetically different from the common species distributed along the coast of Chile (*O. regia*). Additional sampling to enable explicit assessment of species boundaries is necessary before recommending a taxonomic decision to classify *O. gracilis* Steindachner 1898 as a junior synonym of *O. regia* Humboldt 1821, but notably FASTSTRUCTURE did not distinguish between the three marine species *O. gracilis*, *O. regia* and *O. smitti*. *Odontesthes mauleanum* and *O. brevianalis* are not reciprocally monophyletic, although they were difficult to distinguish in the field. Broader geographical sampling is needed to confidently assess these species boundaries; FASTSTRUCTURE suggested some population structure but did not clearly separate *O. brevianalis* and *O. mauleanum* populations into distinct clusters.

Our results are similar to the molecular phylogeny proposed by Campanella et al. (2015) (Figure 1d), but differ significantly from the hypothesis based on morphology (Figure 1c), especially regarding the implied transitions between marine and freshwater





**FIGURE 4** (a) Distrupt plots generated from FASTSTRUCTURE results for the *Odontesthes argentinensis* species-group. (b) Distrupt plots generated from FASTSTRUCTURE results for the clade of *Odontesthes* that includes *O. brevipennis*, *O. mauleanum*, *O. smitti*, *O. regia* and *O. gracilis*. (c) ABBA-BABA tests for introgression for the *O. argentinensis* species-group and the clade containing three marine species (*O. smitti*, *O. regia* and *O. gracilis*) and two freshwater populations of *O. mauleanum* (LLA and LCAL). LLA individuals possessed marine haplotypes, which suggested introgression between marine individuals and this population. *Odontesthes hatcheri* was used as an outgroup in both cases [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

habitats. The morphological hypothesis placed all marine species except *O. argentinensis* in a derived position, suggesting a recent invasion of the marine environment by freshwater species. The phylogenetic framework supported by the RAD data (Figure 2b), although not complete, sheds new light on the processes underlying the diversification of these fishes and their radiations into freshwater habitats.

## 4.2 | Silverside habitat transitions and biogeography

The phylogenomic hypothesis has significant implications for this group's biogeography. Ancestral area reconstruction (Figure 3) places the origin for all species of *Odontesthes* in the eastern Pacific Basin, unsurprising given that the sister group to *Odontesthes* and *Basilichthys*, *Atherinopsini*, is a northeastern Pacific marine clade

(Bloom, Unmack, Gosztanyi, Piller, & Lovejoy, 2012; Bloom et al., 2013; Campanella et al., 2015; Dyer, 1997). This Pacific origin implies at least three separate events of colonization of the Atlantic, and it supports three independent invasions of freshwater habitats by marine ancestors. One of these inferred invasions triggered a species radiation into the La Plata River Basin that currently extends over much of the Pampas region and freshwater habitats ranging through inland and coastal water habitats of Uruguay and southern Brazil (Figure 3), encompassing nine described species. Our results suggest strongly that the early radiation of freshwater species—represented in our study by *O. bonariensis*, *O. humensis* and *O. perugiae*—transpired along the major Paraná and Uruguay river basins and the shallow lakes in the Pampas region in the Buenos Aires province of Argentina and Uruguay, and only recently reached the coastal lagoon systems in southern Brazil (represented in this study by *O. ledae* and *O. mirinensis*). *Odontesthes hatcheri* populations in Patagonian lakes and rivers east of the Andes are probably from a separate, earlier transition to fresh water. The third freshwater clade (*O. brevianalis* and *O. mauleanum*) is found west of the Andes, in rivers and lakes flowing towards the Pacific Ocean, sister to marine *O. regia* and *O. smitti*.

The pattern of evolution of freshwater species from marine ancestors implied by the RAD phylogeny does not support a previously proposed ecological model of speciation involving divergence with gene flow in southwestern Atlantic populations (Beheregaray & Sunnucks, 2001; Beheregaray et al., 2002; García et al., 2014). According to this model, marine populations of *O. argentinensis* entering the coastal lagoons and brackish estuaries along the coastal plain of Uruguay (Mirim), southern Brazil (dos Patos, Tramandaí) and Argentina (Mar Chiquita) gave rise repeatedly to phenotypically variable species flocks, similar to well-documented marine-to-freshwater transitions in marine stickleback fishes in Asia, Europe and North America (Bell & Foster, 1994). In sticklebacks, independently derived freshwater populations are nested in divergent marine clades (Fang, Merilä, Ribeiro, Alexandre, & Momigliano, 2018). The RAD phylogeny for *Odontesthes*, in contrast, strongly supports a single group containing freshwater species sister to the marine *O. argentinensis* (Figure 2b). Species in the coastal lagoon systems were not directly derived from ancestral *O. argentinensis* stock, but derived from other freshwater populations in the “*O. perugiae* group” (Figure 3). This single transition to freshwater from a marine ancestor in a particular geographical area is a repeated pattern in marine-derived freshwater fishes, including clingfishes (Conway, Kim, Rüber, Espinosa Pérez, & Hastings, 2017), anchovies (Bloom & Lovejoy, 2012), drums and pufferfishes (Bloom & Lovejoy, 2017), although silversides at large have colonized freshwater habitats in the Americas many times (Bloom et al., 2013; Campanella et al., 2015).

### 4.3 | mtDNA introgression and hybridization

Secondary contact between the established freshwater species and their marine relatives seems to be frequent in brackish and

estuarine habitats where environmental gradients are gradual and form extensive habitats, enabling the formation of hybrids exhibiting intermediate phenotypes. Lack of genetic differentiation among species in mtDNA markers may be the consequence of hybridization, as is probably the case with the *O. argentinensis* species-group (García et al., 2014). The numerous estuaries and coastal lagoons that have formed on the southwestern Atlantic coastline from northern Argentina to southern Brazil provide ample contact zones for marine and freshwater species that could homogenize mtDNA, with many of the freshwater species in the *O. argentinensis* species-group being sympatric as well. Intermediate phenotypes between marine and freshwater species in the Mar Chiquita coastal lagoon system have been documented, but were interpreted as incipient speciation events (González-Castro et al., 2016, 2019). A signature for nuclear introgression among *O. argentinensis*, *O. bonariensis*, *O. mirinensis* and *O. ledae* is weak according to the ABBA-BABA test, but broader geographical sampling across the complex coastline they inhabit would probably clarify how these interactions play out in local systems. Additionally, selection may play a significant role in limiting the flow of nuclear alleles between marine and freshwater, where vastly different physiological mechanisms are needed for survival.

Natural hybrids may occur among broadly sympatric species or can be confined to particular contact areas, and can be detected through the observation of intermediate phenotypes or discovery of introgressed mtDNA haplotypes or nuclear alleles cutting across the presumed contact area (Funk & Omland, 2003; Harrison, 1990; Willis et al., 2014). Possible hybrid silversides have been inferred in estuarine areas where marine and freshwater species come into secondary contact (González-Castro et al., 2016), but it is unclear whether these hybrid zones are persistent or ephemeral. The fitness of hybrid individuals in the area of secondary contact is an important factor determining the stability of hybrid zones (Barton & Hewitt, 1985). Hybrids may be maintained when they have higher fitness than either parental type under intermediate ecological conditions, but alternatively, they may be ephemeral when the hybrid zone is maintained through a balance between hybrid inferiority and continuous invasion of parental types into the area (Moore & Buchanan, 1985). The latter scenario may lead to conditions of “genetic tension” among loci coding for important physiological traits.

A recent study comparing gene expression profiles in gills of wild-caught *O. bonariensis* (freshwater) and *O. argentinensis* (marine) revealed significant differences between these two species for more than 3,000 transcripts, some of them coding for osmoregulatory/ion transport and immune system functions (Hughes et al., 2017). Additionally, more than 1,000 transcripts with nonsynonymous SNPs in the coding sequences were found to be different between the species. These genetic disparities between species probably reflect adaptive responses to critical challenges in relation to transitions from marine to freshwater conditions. The silverside hybrid zones reported in nature may therefore represent genetic tension zones rather than persistent hybrid swarms where incipient species may be forming, but detailed studies of these hybrid zones are necessary to confirm this hypothesis. If a genetic tension zone limits the

flow of nuclear alleles, it does not seem to affect the flow of mtDNA haplotypes among the *O. argentinensis* species group (Figure 2a), perhaps due to their relative neutrality.

Steep gradients between marine and freshwater habitats are more typical in southern Argentina and Chile, where rivers originating in the Andes drain straight into the ocean, providing scant opportunities for coexistence and hybridization. Freshwater species in Patagonia (*O. hatcheri* primarily in Argentina and *O. brevianalis* and *O. mauleanum* in Chile) show sharply distinct haplogroups from their marine relatives, a pattern not found in the *O. argentinensis* species-group where secondary contact zones abound (Figure 2a). However, we report an exceptional case suggesting a recent mitochondrial capture of marine haplotypes characteristic of *O. regia* and *O. smitti* by a freshwater population of *O. mauleanum* in Lake Llanquihue. We hypothesize based on these biological results that this pattern could have been caused by mitochondrial capture of the marine haplotype following a marine incursion into freshwater habitats, creating a temporary secondary contact zone between *O. mauleanum* and *O. regia*. This haplotype appears to be common in Lake Llanquihue, a large lake in the southern Chilean Lake District, only ~22 km away from Reloncaví Sound to the south. Strong selection on nuclear loci and smaller population sizes in freshwater lakes could have erased this signal, although the ABBA-BABA test suggested some nuclear introgression between marine individuals and the Llanquihue population compared to the higher-elevation Lake Calafquen. Lake Llanquihue was probably covered by the Llanquihue glacier at least three times, between 70,000 years ago and finally during the last glacial maximum 14,000 years ago (Clapperton, 1994; Harrison & Glasser, 2011), but the origin of the introgressed haplotypes appears to be older, suggesting that it might be related to the end of the coldest Patagonian glaciation ~700,000 years ago, or the largest Patagonian glaciation 1.2 Ma (Ruzzante et al., 2008), although we do not know of geological evidence that points to marine incursions during these glaciation cycles. The signal of this secondary contact and introgression would not have been apparent without the mtDNA data, highlighting its continued importance in the genomic era.

## 5 | CONCLUSIONS

Our newly generated genome-wide ddRAD data provided remarkable resolution to the phylogenetic relationships of *Odontesthes* silverside fishes (Figure 2b), which was critical to understanding the history of habitat shifts, biogeography and introgression events in this group. This new hypothesis implies three separate transitions to freshwater habitats (Figure 3), one in southern Chile, one in the Atlantic drainages of Patagonia, and one in the lower Paraná and Uruguay river basins, spawning a species radiation that now occupies coastal fresh waters from the Pampas region in Buenos Aires province, Argentina, across Uruguay, to southern Brazil in the dos Patos and Tramandaí lagoon systems. Mitochondrial *cytb* data were mostly uninformative for assessing phylogeny and species boundaries (Figure 2a), but uniquely highlight the contrasting histories of

introgression between marine and freshwater congeners on both the western and the eastern coasts of southern South America. Mitochondrial data support secondary contact between *O. mauleanum* and *O. regia* in or near the Lake Llanquihue locality in southern Chile (Figure 2a). In contrast, the relatively homogenous *cytb* haplotypes found across species in the *O. argentinensis* species group suggest ongoing gene flow and complex scenarios of speciation-with-gene flow across habitats (García et al., 2014; González-Castro et al., 2016, 2019) that should be investigated with deeper population sampling to understand these dynamics more clearly to ascertain if incipient speciation is taking place.

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## AUTHOR CONTRIBUTIONS

L.C.H., G.O., R.B-R., E.M.H. and L.R.M. designed the study. Y.P.C., M.C., R.C., L.R.M., M.G-C., V.C., E.M.H. and G.M.S. provided tissues. L.C.H., J.A.S. and R.C. performed laboratory work. L.C.H., J.A.S. and R.C. analysed the data. L.C.H., Y.P.C., R.B-R. and G.O. wrote the manuscript. All authors contributed to the final draft of the manuscript.

## DATA AVAILABILITY STATEMENT

Cytochrome *b* sequences are accessioned on GenBank with numbers MK983245–MK983397. Raw reads for ddRAD sequences are archived under NCBI BioProject PRJNA546013. Data matrices, NETWORK tree files and R code are available for download on Figshare <https://doi.org/10.6084/m9.figshare.11413209>.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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