







Diagnostic locus for identification of silverside hybrids (*Odontesthes humensis* and *Odontesthes bonariensis*) in a natural population

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ABSTRACT

Hybridization is a natural phenomenon that occurs more often in fish than in other vertebrates. The use of nuclear and mitochondrial molecular markers provides valuable results in the detection of these events. The aim of this study was to investigate the occurrence of interspecific hybrids in natural populations of silverside. The samples of *Odontesthes humensis*, *Odontesthes bonariensis*, and individuals that were morphologically different from pure species were collected in the Mangueira lagoon, located in southern Brazil. Result: Six tetranucleotide microsatellite loci were synthesized and tested. The UFPEL_OH3 locus proved to be diagnostic for the detection of silverside hybrids, and it was possible to distinguish between pure and hybrid species. The mitochondrial marker gene *cytb* synthesized from conserved *Odontesthes* sequences in the GenBank genetic database showed no differences in the genetic sequence of the samples, needing further studies to confirm the hypothesis.

Keywords: cytochrome b; hybridization; microsatellite marker; fish.

Locus diagnóstico para identificação de híbridos de peixe-rei (*Odontesthes humensis* and *Odontesthes bonariensis*) em população natural

RESUMO

A hibridação é um fenômeno natural que ocorre mais frequentemente em peixes do que em outros vertebrados. Para detectá-la, são utilizados marcadores moleculares nucleares e mitocondriais, os quais fornecem resultados valiosos. O objetivo deste estudo foi investigar a ocorrência de híbridos interespecíficos em populações naturais de peixe-rei. As amostras de *Odontesthes humensis*, *Odontesthes bonariensis* e de indivíduos morfologicamente diferentes das espécies puras foram coletadas na lagoa Mangueira, localizada no Sul do Brasil. Foram sintetizados e testados seis *loci* microssatélites tetranucleotídicos. O *locus* UFPEL_OH3 mostrou-se um diagnóstico para detectar híbridos de peixe-rei, possibilitando a distinção de espécies puras de híbridadas. O marcador mitocondrial gene *cytb*, sintetizado com base nas sequências conservadas de *Odontesthes* no banco de dados genéticos do GenBank, não apresentou diferenças na sequência genética das amostras, portanto, são necessários mais estudos para confirmar a hipótese de hibridação.

Palavras-chave: citocromo b; hibridação; marcador microssatélite; peixe.

INTRODUCTION

Interspecific natural crossing occurs more frequently in fish than in other vertebrates, and although there is an assumption that hybridization has little influence on speciation and evolution, hybrids may be involved in the emergence of new ones, strains, and gene flow. Accurate detection of hybrids using molecular methods depends on the markers used and the degree of differentiation between parent species (Hashimoto et al., 2013; Porto-Foresti et al., 2013; Hasselman et al., 2014). Hybridization can, to some extent, threaten biodiversity by competing in various ways with the native parental lineage (Hashimoto et al., 2010; Hashimoto et al., 2011); however, it can generate diversity by increasing the adaptive potential of individuals mixed with new genetic variations (Prado et al., 2011).

According to Abdul-Muneer (2014), to determine the level of conservation of the diversity and genetic structure of natural populations, a most used tool are genetic markers, with the microsatellite ones being commonly used in ecology and evolution, as they detect genetic differences between two or more individuals in a population

and between populations. Microsatellites, as they are randomly distributed throughout the genome, present a high degree of polymorphism and ease of detection, which is why they are ideal for studying the genetic diversity and evolution of species. New markers have been used, such as the restriction enzyme-associated DNA (RAD) markers, which provide genome-wide data with the combination of mtDNA. The combination of both is a powerful tool to resolve phylogenetic relationships among species in the face of past or ongoing gene flow across species boundaries (Hughes et al., 2020).

In the 1990s, when microsatellite markers were first described, a powerful means of reporting genetic variation at nuclear loci was discovered. Allendorf (2017) stated that with microsatellite markers, it is possible to detect bottlenecks that occurred in the past, thus enabling the estimation of the current effective population size from a single sample, exposing the richness of loci that markedly improved the ability to estimate the genetic and population characteristics in different species, which constitute important parameters for the establishment of conservation strategies. Currently, studies in population genetics allow detecting regions of the genome that are affected by natural selection, such as characteristics related to adaptations to the environment, hybridization, demographic depression, and inbreeding, as well as anthropogenic factors (Hohenlohe et al., 2021).

In turn, the analysis of the mitochondrial genome, either alone or in combination with other nuclear markers, such as microsatellites, is widely used in population structure elucidation studies, as it presents maternal inheritance and the repertoire of encoded genes is extremely conserved (Aziz et al., 2015; Satoh et al., 2016). Mitochondrial markers are widely used in fish studies, and a large number of universal primers are available to amplify different genes from the mitochondrial genome of many fish species, as they appear not to undergo recombination and evolve about 10 times faster than the nuclear genome (Kochzius, 2009).

The silverside or pejerrey, *Odontesthes humensis* (de Buen, 1953) and *Odontesthes bonariensis* (Valenciennes, 1835) belong to the Atheriniformes order, but the phylogenetic relationships among families within the order Atheriniformes have been revised, according to the new classification belonging to Ovalentaria (Betancur-R et al., 2017; Campanella et al., 2015). Silverside is an important fishing resource in the various regions where they are distributed, whether in commercial, sport, or aquarium fisheries. It can be found in rivers and freshwater lakes, the marine environment, and estuaries, even at low altitudes to very high areas such as Lake Titicaca. They occur in several countries in South America, such as Brazil, Uruguay, Argentina, Peru, and Chile (Eschmeyer, 2013; Campanella, 2015; Wingert, 2017).

To investigate the occurrence of interspecific hybrids in natural populations of silverside, primer sets were developed for the amplification of six microsatellite regions from the genomic library of *O. humensis* and primers were also designed for amplification of a fragment of the mitochondrial gene cytochrome b (gene *cytb*) from conserved regions of *Odontesthes* sp. contained in the GenBank database.

MATERIALS AND METHODS

The animals evaluated in this study were collected from local licensed fishermen; therefore, there is no need for an ethics committee for animal experimentation for this activity.

Sampling locations. For better evaluation and use of the material, fragments were obtained from two biological sources of the same animal: muscle and fin (200–300 mg). The samples were provided by artisanal fishermen at the time of landing at different points of Lagoa Mangueira between the coordinate's latitude 33°6'0.75"S and longitude 52°43'56.96"W.

A total of 75 randomly chosen specimens were used, with 20 animals of the species *O. humensis*, 20 animals of the species *O. bonariensis*, and 35 animals that were morphologically different from the pure species (Figure 1).

First, the identification and interspecific differentiation were carried out through the morphological analysis (Bemvenuti, 2002).

DNA extraction. Total genomic DNA extraction was performed following the sodium chloride protocol proposed by Lopera-Barrero et al. (2008), with modifications. To check the integrity of the DNA obtained, the samples were subjected to horizontal electrophoresis with 1% agarose gel and 1x SB buffer for 40 min at 120 V. For this, an aliquot of 7 µL of DNA was used, and the same was stained with 1.1 µL of GelRed (Biotium, USA) and 1.1 µL of 5x loading buffer, as well as Gene Ruler DNA Ladder Mix (Life Science Fermenta) as a reference for molecular weight estimation.

Primer design. The microsatellite primers designed for this study were selected from the results obtained by Tavares et al. (2014) who detected, through the genomic library of *O. humensis*, the “Best PALs” (bPALs), which are SSR loci that present long repeat units (4mers, 5mers, and 6mers) in the populations of *O. humensis*. In that study, a total of 167 bPALs were generated.



Figure 1. Silverside (A) *O. humensis*; (B) hybrid; (C) *O. bonariensis*.

From the analysis of this library, primer sets were designed for the amplification of six tetranucleotide loci, which were synthesized by the company Integrated DNA Technologies (IDT) and then validated by the PCR amplification technique on a 1% agarose gel. The criteria for the choice of primers were based on the presence of tetranucleotide-only SSR motifs, maximum temperature difference between the primers of 5°C, and the size of the motif sequence ranging from 21 to 25 bp.

Gene *cytb*. The mitochondrial sequences of gene *cytb* were obtained by scanning the conserved sequences of *Odontesthes* sp. from the NCBI GenBank database. After aligning the gene sequences, conserved regions among the different species of *Odontesthes* were analyzed for primer design. The following criteria were used to choose the primers: difference between forward and reverse primers of less than 3°C, GC content between 40 and 60%, annealing temperature between 55°C and 65°C, and primers with a size between 18 and 25 nucleotides. The set of primers that best fit the established criteria was selected for synthesis by the IDT Company. Finally, the primers were validated by the PCR amplification technique and the amplicon was sequenced to confirm the gene sequence.

The edited sequences and alignment were performed with the aid of the MEGA program (ClustalW alignment), with the parameters Pairwise alignment and Multiple alignment, and after that, an UPGMA phylogenetic tree was built with the Bootstrap parameters with 10,000 resampling, following the MCL model.

PCR and Sequencing. For the PCR, the enzyme Taq DNA polymerase and 10× PCR reaction buffer containing MgCl₂ (Sigma-Aldrich), 100 mM dNTP (Thermo Scientific), and specific primers for the kingfish were used. A total of 35 cycles were performed by PCR, the initial denaturation temperature used was 94°C for 5 min, final denaturation at 94°C for 45 s, annealing temperature as established for each primer for 45 s, the phase of extension was 72°C for 45 s, and the final extension at 72°C for 8 min. The sequencing was carried out following the criteria established by the company MacroGen. The microsatellite and mitochondrial sequences were confirmed through amplicon sequencing by the company MacroGen, the mitochondrial sequence through alignment by the MEGA program and use of the BLAST tool (Basic Alignment Tool Sequence) for the mitochondrial sequences.

Polyacrylamide. Genotyping of microsatellite loci was performed by means of vertical polyacrylamide gel electrophoresis, consisting of 32 mL of 1xSB buffer solution, 8 ml of 30% polyacrylamide, 400 µL of 10% APS, and 40 µL TEMED with 1xSB running buffer. Each gel was loaded with two 100 bp molecular weight markers and 13 samples, totaling capacity for 15 samples. It was used for running in polyacrylamide gel buffer solution 1x SB and a constant voltage of 160 V for 1 h and 30 min, resulting in 120 mA. The revelation of the amplification of alleles was carried out in a transilluminator under UV light and registered by a photo documenter. The alleles were analyzed

through direct observation in the polyacrylamide gel, according to molecular weights.

RESULTS

Among the tested primers, the UFPEL_OH3 locus detected the presence of interspecific hybrids. The correlation between morphology and genetic profile was analyzed through DNA polymorphism within and between species, where the diagnostic primer (UFPEL_OH3) amplified the frequency band of 200 bp in *O. humensis* and 400 bp in *O. bonariensis*, and in hybrid individuals, there was a heterozygous amplification pattern, 200 and 400 bp in the same individual (Figure 2).

High polymorphism was detected in 12% polyacrylamide gel observation, which showed allele variation of 21 alleles in the diagnostic primer and 43 alleles referring to the 6 loci tested in the study population (Table 1).

A total of 236 conservation sites were found for the hybrids, as well as for the pure specimens collected and compared in the study, confirming that the studied mitochondrial sequence showed 100% identity among the species evaluated. When compared to the sequences from the GenBank database, *O. humensis*, *Odontesthes ledae*, *Odontesthes perugiae*, and *Odontesthes argentinensis* were genetically closest to the samples under study (Table 2).

The phylogenetic tree (Figure 3) shows the genetic proximity of the taxa. It can be observed that the samples used in this study are closely linked with a common ancestor, while the preserved samples of *Odontesthes* from the GenBank gene bank underwent cladogenesis, promoting genetic distance between species, corroborating the results found in the variation sites.

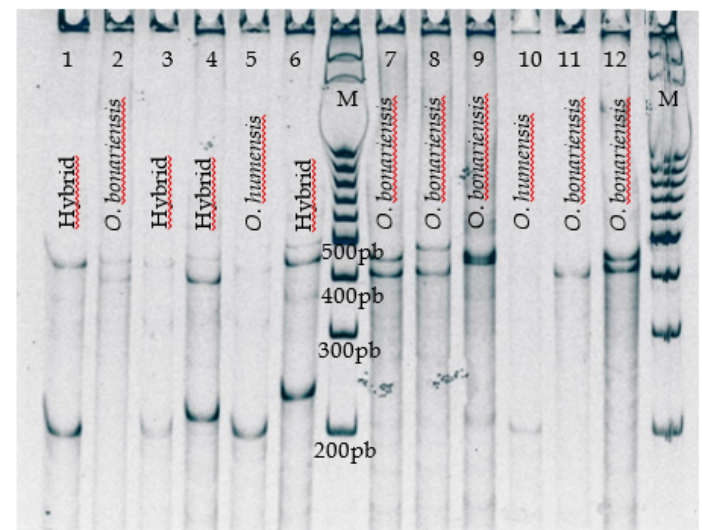


Figure 2. Electrophoretic profile records under UV light in a photodocumentary, in 12% polyacrylamide gel, exposing the polymorphism of the locus UFPEL_OH3 in *O. bonariensis*, *O. humensis*, and hybrids.

Table 1. Primer sequence, annealing temperature (Tm²°C), number of alleles per locus (N° alleles), number of heterozygotes (Het), and number of homozygotes (Homo).

Loci	Primer sequence 5'–3'	Tm ² °C	N° alleles	Het			Homo		
				HB	BO	HU	HB	BO	HU
UFPEL_OH1	F: TGTGAGACAGGTGTGAGC R: TTCCATACAAGCTGTACGC	55.0	12	18	7	3	7	12	15
UFPEL_OH2	F: GTGTGTTGAAGTCTTTTCAGC R: TGTATGAGTTGCCCTTTTGG	54.0	15	19	14	6	12	4	11
UFPEL_OH3	F: GACAGGTTGGACACATTGG R: AGTAACAGAGACCCTCATCC	53.2	21	20	8	14	10	8	5
UFPEL_OH4	F: CCTTCATCTAGTCGCTCCC R: CAGTCGACTTGAACAGTAGG	55.0	14	12	11	10	20	8	7
UFPEL_OH5	F: CTAAACTTCCTCAGTCCACC R: GTCTCTTTGCTCAAGCTGC	55.0	8	0	0	0	31	9	18
UFPEL_OH6	F: CGATGACCACTGGGATAGG R: AAGAGAGCAGTGGTGTAAAGC	54.0	7	3	0	1	23	19	18

Table 2. Sites of conservation of mitochondrial sequences from the samples under study (*O. humensis*, 1–5; hybrid, 2–5; *O. bonariensis*, 1–5) compared with *Odontesthes* sequences from the GenBank genetic database.

Samples under study	Sites of conservation	Sequences from the GenBank genetic database	Variation sites
<i>O. humensis</i> 1	236	<i>O. hatcheri</i> (GQ352668.1)	13
<i>O. humensis</i> 2	236	<i>O. bonariensis</i> (GQ352667.1)	51
<i>O. humensis</i> 3	236	<i>O. incisa</i> (GQ352666.1)	51
<i>O. humensis</i> 4	236	<i>O. smitti</i> (GQ352665.1)	51
<i>O. humensis</i> 5	236	<i>O. argentinensis</i> (GQ352664.1)	51
Hybrid 2	236	<i>O. nigricans</i> (KM400719.1)	13
Hybrid 3	236	<i>O. mauleanum</i> (KM400716.1)	11
Hybrid 4	236	<i>O. regia</i> (KM400715.1)	15
Hybrid 5	236	<i>O. gracilis</i> (KM400714.1)	14
<i>O. bonariensis</i> 1	236	<i>O. brevianalis</i> (KM400713.1)	14
<i>O. bonariensis</i> 2	236	<i>O. humensis</i> (KM400712.1)	0
<i>O. bonariensis</i> 3	236	<i>O. perugiae</i> (KM400711.1)	1
<i>O. bonariensis</i> 4	236	<i>O. ledae</i> (KM400710.1)	0
<i>O. bonariensis</i> 5	236	<i>O. argentinensis</i> (KM400708.1)	3

DISCUSSION

Hybridization is a natural phenomenon known for a long time to affect many fish species (Rueda et al., 2016). The use of nuclear (microsatellites) and mitochondrial (gene *cytb*) molecular markers provides valuable results in the detection of hybridization events and the identification of hybrids. Genetic characterization is essential, as hybrids can present morphological and biological characteristics that are different from each other.

Tejedor (2001) reported the occurrence of natural hybridization between the taxa *O. bonariensis* and *O.*

argentinensis in Lagoa Grande da Argentina; Rueda et al. (2016) confirmed the occurrence of hybrids between *O. bonariensis* and *O. hatcheri* in Patagonian lakes in Chile and Argentina; and Hughes et al. (2020) detected the presence of some hybrid individuals carrying the *O. bonariensis* haplotypes, where these species were introduced in Patagonia, as well as hybrids of others silverside species in South America. In the region of the lake under study, there are no studies mentioning the occurrence of interspecific hybrids of silverside and there is no information on whether these species can result in fertile hybrids and in the viability of backcrossing. In this sense,

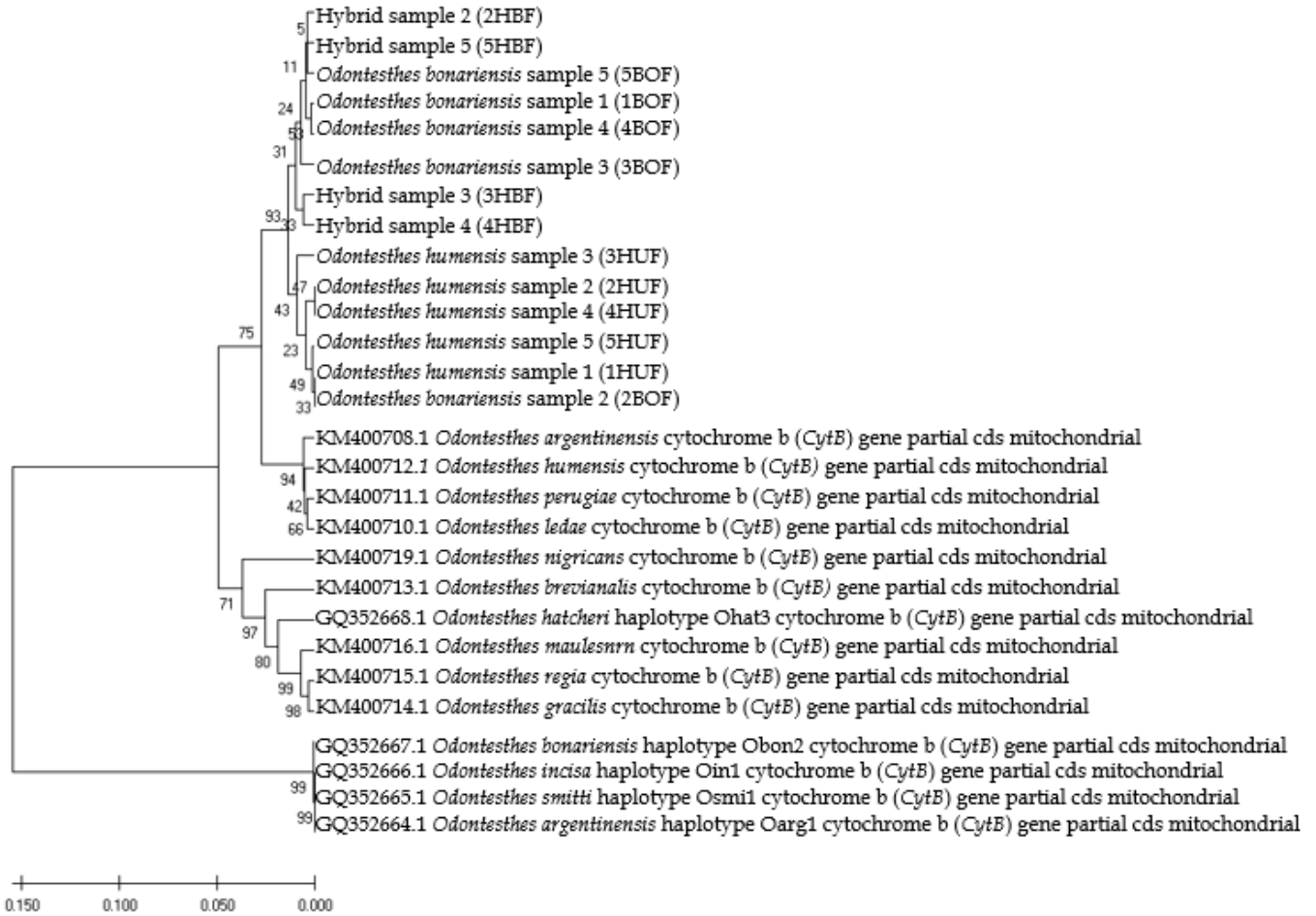


Figure 3. Phylogenetic tree showing the genetic distances between species of the genus *Odontesthes* from the GenBank database and from samples collected in Lagoa Mangueira, RS, Brazil.

the application of molecular genetic techniques constitutes an important tool for identifying stocks and the particular characteristics of this population are essential to start any monitoring program to help preserve the genetic integrity of pure species (Porto-Foresti et al., 2013).

According to Hasselman et al. (2014), anthropogenic disturbances in the habitat are the main drivers of hybridization, with the collapse of reproductive isolation among incipient species that recently diverged in sympatry or introgression among allopatric species. The study by Garcia et al. (2014) with five species of *Odontesthes* corroborates Hasselman's statement and the results of this study. The genetic proximity measured by the mitochondrial sequence suggests the common maternal lineage in individuals, but the microsatellite marker UFPEL_OH3 served as a diagnostic tool, suggesting that speciation events may be occurring in the environment.

The results of this study serve as a basis for the characterization and monitoring in the detection of the natural hybridization of

kingfish in a natural environment, assisting in the development of appropriate management and exploitation programs for these hybrids in artisanal fisheries, and providing a more refined compilation of statistical data.

CONCLUSION

From the results of microsatellite markers (nuclear), it was possible to distinguish between pure and hybrid species, the locus UFPEL_OH3 being considered the diagnostic primer for *Odontesthes*, but the evaluation of the gene *cytb* fragment did not show sites of variation between individuals, needing further studies to confirm the hypothesis.

CONFLICT OF INTERESTS

Nothing to declare.

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AUTHOR'S CONTRIBUTIONS

Souza, D.M.: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. Freitas, S.F.: Conceptualization, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing. Tavares, R.A.: Conceptualization, Formal Analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. Moreira, H.L.M.: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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