



## OCCURRENCE OF SERRASSALMIDAE HYBRID FISH IN THE AMAZON RIVER BASIN, BRAZIL

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**Abstract:** This study reports a case of occurrence of hybrid fish in the Amazon River basin, Brazil and attentive on to possible risks promoted with from crossbreeding between two species on local biodiversity. The specimen was collected in February/2016, and was evaluated showing dubious characteristics indicating the possibility of the occurrence of hybridization. Thus, fin specimen fragments were sampled and the genetic analyzes were performed. Our results indicated that the studied sample had a heterozygous electrophoretic profile revealing itself as a hybrid between *Piaractus brachypomus* and *Colossoma macropomum* being the second species the maternal parent.

**Keywords:** genetic conservation; genetic markers; hybrid occurrence; crossbreeding; Neotropical ichthyofaunal.

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### INTRODUCTION

Interspecific hybridization can be defined as natural or human-induced crosses between different species. Interspecific hybridization occurs from six to ten percent of animal species in nature (Mallet 2005), being the phenomenon quite common in fishes, especially among freshwater species (Epifanio & Nielsen 2000, Scribner *et al.* 2000). In this group factors such as external fertilization, competition for spawning habitat and disproportion in the sexual relation of the parental

species are considered responsible agents for the greater occurrence of hybridization (Scribner *et al.* 2000).

Divergent views on the factors involved in hybridization make the topic widely discussed. According to Willis *et al.* (2012), hybridization is a natural phenomenon related to the speciation and diversification of individuals. So, when natural crossings occurs at random, during evolutionary history of species diversification, it may be considered as an important mechanism for the evolution of organisms, given the possibility of

emergence of genotypic combinations that could establish new evolutionary lineages (Arnold & Hodges 1995). On the other hand, hybridization events have also been treated as “genetic contamination” (Millar *et al.* 2012), since gene flow from innumerable interspecific crosses could lead to loss of genetic individuality of the species. Thus, the occurrence of hybridization in natural environment can both lead to the appearance of conditions conducive to the establishment of new gene clusters and individual adaptation, as well as the local extinction of populations and species (Dowling & Secor 1997, Allendorf *et al.* 2001, Willis *et al.* 2012, Mirimin *et al.* 2014).

An aggravating factor in the fish species hybridization process is the practice of interspecific crosses in captivity, which over time has become a common and growing activity in the Brazilian aquaculture scenario (Brasil 2017). So, when it comes genetic contamination and the problems of hybridization, the process should be included as a possible threat to the species conservation, especially when resulting from human interferences, as the artificial production of hybrids in captivity or the escapees and releases of hybrid fishes in the wild. The activity is commercially interesting in fish farming because the hybrid individuals resulting from this process have peculiar biological characteristics and may even express better performance than the average of the parental lineages in several aspects (Bartley *et al.* 2000, Helfman *et al.* 2009). This improvement is known as heterosis or hybrid vigor and production aims to implement desirable characteristics to fish such as increased growth rate, decrease in nutritional requirements, disease resistance and better meat quality (Bartley *et al.* 2000, Hashimoto *et al.* 2011). In this sense, only until 2014, about 17 hybrid forms were already reported as being of common use for this purpose, being produced from 12 species of fish of the orders Characiformes and Siluriformes (Hashimoto *et al.* 2010, Alves *et al.* 2013, Moro *et al.* 2013).

Among the Serrassalmidae family, tambaqui (*Colossoma macropomum* (Cuvier, 1816)), pirapitinga (*Piaractus brachypomus* (Cuvier, 1818)), pacu (*Piaractus mesopotamicus* (Holmberg, 1887)) and their hybrids, are among the most cultivated fish in the national fish culture, highlighting the region north, northeast and center-west from

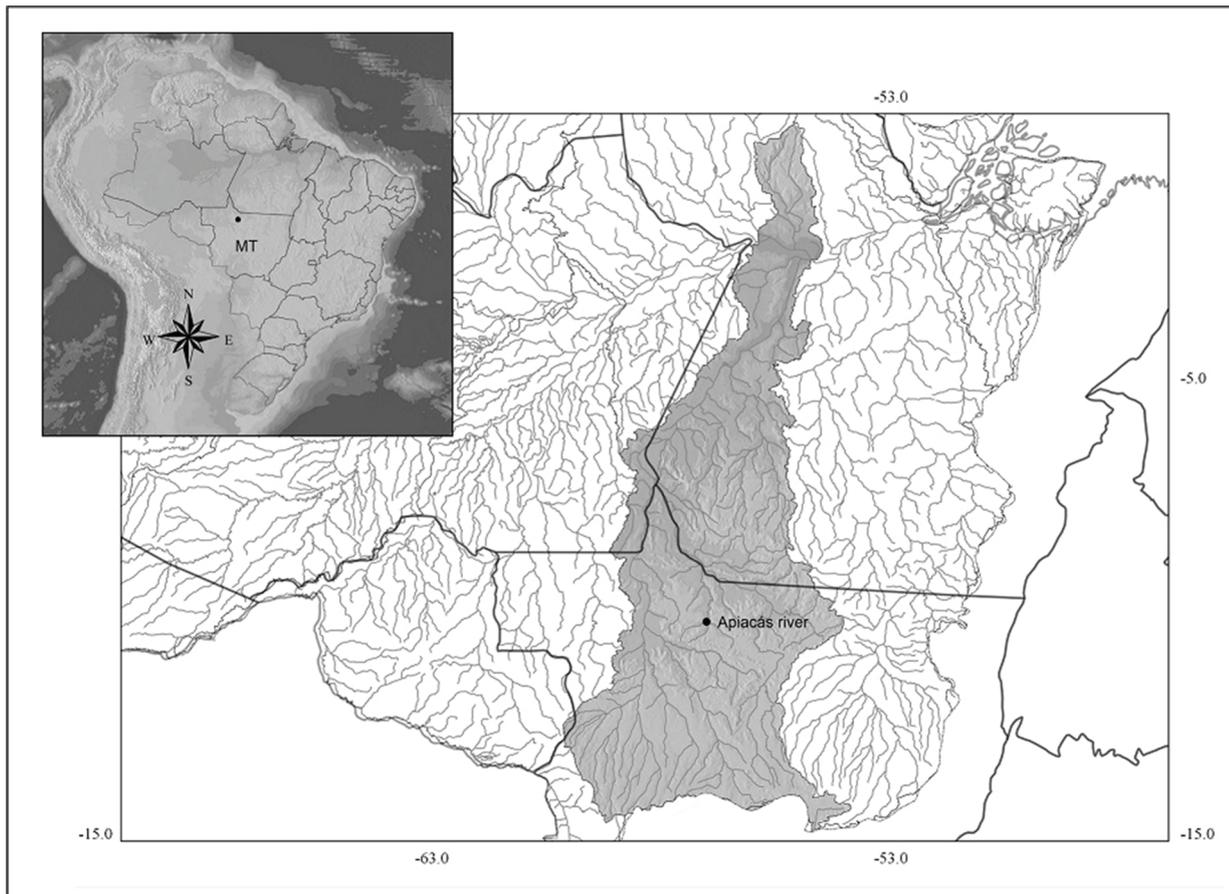
Brazil (IBAMA 2009). In view of the commercial representativeness of hybrids in fish farming and, on the other hand, the problems arising from the introduction of these individuals into the natural environment, diverse preventive measures of great importance should be taken with the purpose of avoiding escapes from fish farms (Azevedo-Santos *et al.* 2015).

According to Hashimoto *et al.* (2010), the monitoring of hybrids in the natural environment or even in crop stocks is not simple, because the occurrence of hybridization events, especially with regard to secondary and advanced effects, such as the occurrence of introgression and backcrossing with local populations, may result in individuals morphologically similar to the parental (Allendorf *et al.* 2001, Porto-Foresti *et al.* 2013), hindering the morphological differentiation.

The use of molecular markers that help in the identification of parental lines and products resulting from hybridization are valuable tools, once they make it possible to detect and study these biological events, both in the culture conditions and in wild (Scribner *et al.* 2000, Mourão *et al.* 2017). The present study aims to report the occurrence of a hybrid individual found in natural environment in the Apiacás River, a component of the Teles Pires River basin, which currently suffers different types of anthropic pressure as a consequence of local development. It is considered that the results reported here are of ecological importance since, despite the increasing impact and changes registered, the region still presents characteristics favorable to the implementation of conservation measures.

For the present study, a specimen initially identified as *P. brachypomus* (“pirapitinga”), voucher LBP 21582, belonging to the collection of the Laboratório de Biologia e Genética de Peixes (UNESP, Botucatu, São Paulo) was used. The specimen was collected in February/2016 in the Apiacás River (10°21'00.7" S, 56°58'56.7" W), municipality of Alta Floresta, state of Mato Grosso, part of the upper Teles Pires basin, drainage of the Tapajós River (Figure 1).

Morphological analyses of this fish, based on the identification key proposed by Queiroz *et al.* (2013), showed dubious characteristics, especially the absence of rays in the adipose fin, indicating the possibility of the occurrence of hybridization.



**Figure 1.** Map indicating the occurrence location of the fish sample collected in the natural environment in the Apiacás River ( $10^{\circ}21'0.74''S$   $56^{\circ}58'56.70''O$ ). Grey region highlights the Tapajós River basin in the central part of South America. MT = state of Mato Grosso, Brazil. Note: scale: 1 degree ~ 111.12 Km

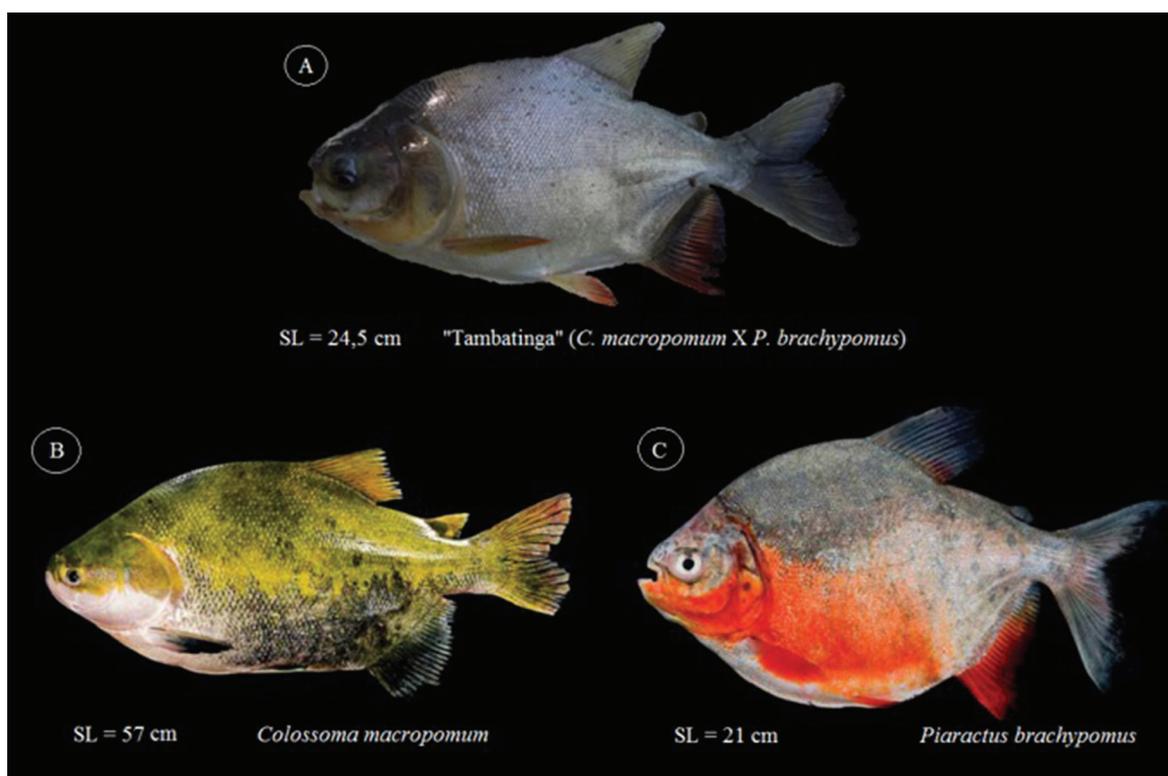
Thus, fin specimen fragments were sampled and stored in the tissue bank of the Laboratório de Genética de Peixes (UNESP, Bauru, São Paulo), where the genetic analyzes were performed. For a better evaluation of the possible hybridization event, tissue samples from three species of Serrassalmidae (Figure 2) (*Piaractus mesopotamicus* (Holmberg, 1887), *P. brachypomus* and *Colossoma macropomum*), were used as controls, and were obtained from the Centro Nacional de Pesquisa e Conservação de Peixes Continentais/Instituto Chico Mendes da Conservação de Biodiversidade, municipality of Pirassununga, state of São Paulo.

DNA extraction was performed based on the protocol of the commercial kit “Wizard Genomic DNA Purification Kit - Promega” and their quantification was done through the equipment Qubit 3.0 Fluorometer. Genetic identification of the individuals was performed through a PCR-Multiplex technique using multiple primers for the markers of type PCR (Polymerase Chain Reaction)-Multiplex technique using multiple primers for the

markers  $\alpha$ -tropomyosin genes (TROP – nuclear) and cytochrome C oxidase subunit I (COI – mitochondrial) (Table 1). The methodology was performed using the primers listed in Table I and under the conditions described by Hashimoto *et al.* (2011).

Amplifications by PCR-Multiplex (Polymerase Chain Reaction- Multiplex) were performed in a total volume of 25 $\mu$ l with 200 $\mu$ M of each dNTP (dATP, dTTP, dGTP and dCTP), 1.5 mM of MgCl<sub>2</sub>, 1 x Taq DNA buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 0.5 units of Taq DNA Polymerase (Invitrogen), 0.1 $\mu$ M of each primer and 10-50ng of DNA. All reactions were performed in thermocycler (Mastercycler personal, Eppendorf) under conditions of 5 minutes of denaturation at 95°C, 35 cycles for: TROP (95°C for 30 sec, 60°C for 30 sec and 72°C for 10 sec); COI (95°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec) and 7 min of final extension at 72°C.

The genetic identification of the species was based on the observation of the species-specific



**Figure 2.** Images of the individual and analyzed species: A) “Tambatinga” hybrid (*C. macropomum* X *P. Brachypomuns*); B) *Colossoma macropomum*; and C) *Piaractus brachypomuns*. Note: Images B and C obtained from Queiroz *et al.* (2013) with the permission of the authors..

electrophoretic profile for each of the molecular markers, visualized on 1.5% agarose gel stained with Nancy (Sigma) (0.0225 $\mu$ l/ml) and photographed in transluminator (Loccus Biotechnology) under ultraviolet light.

Genetic analyzes of the TROP (nuclear) revealed diagnostic bands of 270 bp for *P. mesopotanicus* (“pacu”), 130 bp for *P. brachypomus* (“pirapitinga”) and 170 bp for *C. macropomum* (“tambaqui”). The study sample presented a heterozygous electrophoretic profile, with bands of 130 bp and 170 bp, corresponding to an interspecific hybrid

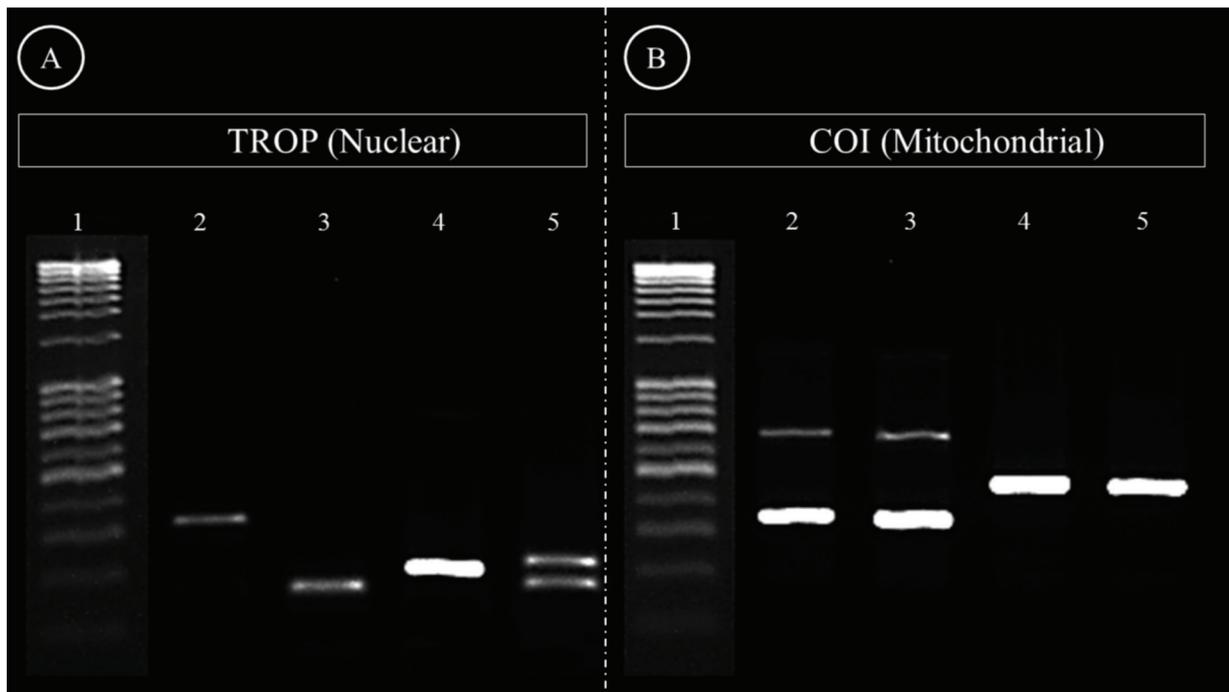
between *P. brachypomus* and *C. macropomum* (Figure 3A).

Genetic analysis of the COI (mitochondrial) revealed diagnostic bands in the 300 bp and 610 pb samples for *P. mesopotamicus* (“pacu”) and *P. brachypomus* (“pirapitinga”) and 430 bp for *C. macropomum* (“tambaqui”). The study sample presented a band of 430 bp, revealing *C. macropomum* (“tambaqui”) as its maternal parent (Figure 3B).

Two determining factors were considered decisive in this particular study: 1) the dubious

**Table 1.** Sequences of the primers and polymerase chain reaction (PCR) conditions used to amplify given gene fragments.

Primers	5' --- Sequence of primers --- 3'	Reference
TROP Cm R	ATACAACAATGCCATCGCT	Hashimoto <i>et al.</i> 2011
TROP Pb R	TTGACTTTATGCCACACAAAT	Hashimoto <i>et al.</i> 2011
TROP Pm R	CTTCAGCTGGATCTCCTGA	Hashimoto <i>et al.</i> 2011
TROP Serra F	GAGTTGGATCGGGCTCAG	Hashimoto <i>et al.</i> 2011
COI Cm R	AGCAAGATGGAGTGAGAAAATA	Hashimoto <i>et al.</i> 2011
COI Pb R	CTGTCAGAAGTATAGTAATTCCG	Hashimoto <i>et al.</i> 2011
COI Piaractus R	GAAGGAAGGATGGGGGTAGG	Hashimoto <i>et al.</i> 2011



**Figure 3.** Electrophoresis analysis of multiplex-PCR using species-specific primers for the nuclear TROP (A) and mitochondrial COI (B). 1) Ladder; 2) *Piaractus mesopotamicus*; 3) *Piaractus brachypomus*; 4) *Colossoma macropomum*; and 5) possible hybrid sample “Tambatinga”.

morphology between *P. brachypomus* and *C. macropomum* presented by the studied specimen, based on the identification of the specific key (Queiroz *et al.* 2013); and 2) the presence of important fish farming companies located in this region of the State of Mato Grosso (Brasil 2017). This region, are currently considered the third largest fish producer in the country, including the production of hybrid forms of round fish such as “tambatinga (Brasil 2017). In this way, genetic analyzes with molecular markers of nuclear and mitochondrial origin were performed to evaluate the morphological differences presented by the studied specimen.

The hybrid “Tambatinga” has great commercial importance, since it represents one of the main sources of production of the national aquaculture (IBAMA 2009). It is also worth noting that, according to IBAMA (2009), the production of this hybrid has even exceeded that of its parent species *P. brachypomus*. However, even with their production and commercial representativeness, there are still few studies evaluating the effects of their presence in natural environments, as well as the effects on wild populations of parental lineages (Hashimoto *et al.* 2012).

In general, the technology needed to promote hybridization among species of Serrasalmidae is

considered well established and widely used in fish farming systems (Hashimoto *et al.* 2014). Thus, the fish culture of this group varies among different regions of Brazil and is considered to be simpler and feasible than the production of other hybrids and pure species (Hashimoto *et al.* 2014). However, there are still few studies on the performance, genetic and ecological impacts that hybridization and hybrid products may have on the environment (Hashimoto *et al.* 2010).

In this sense, in addition to reporting the occurrence of this hybrid in nature, this work aims to highlight possible risks promoted by individuals from crossbreeding between two species on local biodiversity. According to Silva *et al.* (2009), reproductive aspects are highlighted since the occurrence of a sterile hybrid in a natural environment, although it does not have a reproductive capacity, can compete for food resources, territorial resources or even breeding sites. In the latter case, according to the same authors, a great loss could occur in relation to the recruitment of the species, since the eggs of an active native female could have contact with infeasible semen of a sterile hybrid male, affecting the reproductive performance of the species. In the case of the hybrid product being fertile, the genetic impact on the native species could also act to reduce

population size, since the genes of the hybrid products would be incorporated into the genome of the natural species promoting introgression processes (Silva *et al.* 2009). In this case, depending on adaptive ability and reproductive capacity, the hybrid could increase its distribution area along the basin and raise its population density, jeopardizing the viability of pure species (Silva *et al.* 2009).

Hashimoto *et al.* (2010) reported that viability studies were performed only in hybrids of *P. mesopotamicus* x *C. macropomum* and *Pseudoplatystoma corruscans* x *Pseudoplatystoma reticulatum*. In this sense, Ribeiro *et al.* (1995) and Toledo-Filho *et al.* (1998) found that although these animals are profitable in aquaculture because they present a hybrid vigor, they can also be fertile and able of reproducing with their parental species, and its occurrence in the environment is considered an imminent threat to the wild communities.

In this work, we highlight that although some authors consider that hybridization can occur naturally (Epifanio & Nielsen 2000, Scribner *et al.* 2000), the various anthropic actions, with consequent “genetic contamination” of the wild stocks of ichthyofauna, have been promoting an imbalance and acceleration in these occurrences. This contamination may be due to the morphological similarity between non-native individuals, hybrid and pure strains, which causes mixtures in the formation of seedlings in fish farms (Toledo-Filho *et al.* 1994), a fact corroborated by Hashimoto *et al.* (2014), who observed erroneous labeling when analyzing more than 900 individuals from Brazilian fish farms.

In addition to the morphological similarity between the groups, the situation may be aggravated, since native and hybrid individuals may present similar ecological relations, presenting related reproductive behaviors. This can lead to unsuccessful crosses with parental species, resulting in unviable descendants, or presenting agonistic behavior as territorial dispute, interfering in the reproductive dynamics of pure species (Toledo-Filho *et al.* 1994). Moreover, they may promote backcrossing or genetic introgression in the original ichthyofauna, an observation corroborated by Prado *et al.* (2017) and Sales *et al.* (2018).

In this way, considering the threats of presence of hybrids may offers to the natural environment

and our record of one hybrid in the Apicás River, considered as an environment still preserved and wild, the problem of leaks or releases in natural environments can become even more dangerous to the ecological balance, as already evidenced in systems of culture in tank network (Azevedo-Santos *et al.* 2011) including Brazil. This form of production, although economically productive, may cause several impacts to the environment. Considering the historical relationship between aquaculture and species introduction, our study investigated the potential of cage aquaculture in spreading non-native species into hydroelectric reservoirs. We interviewed 19 fish farmers in Furnas Reservoir, Grande River basin. All producers have grown exclusively Nile tilapia (*Oreochromis niloticus*). Once the expansion and growth of this type of activity traces increasingly sharp perspectives of high production and increased possibility of leaks. So, the premise of stablishing well-defined rules for the management of species in the environment should also be considered as a preventive way of create conditions for the conservation of native species (Moyle & Light 1996, Vermeij 1996).

Despite the strong evidence that the introduction is from anthropogenic actions, the possibility that the individual being studied is not a product of escape from fish farming should be considered, since the eventual cross-species occurrence is relatively common among fish in natural conditions (Utsunomia *et al.* 2014, Cruz *et al.* 2015).

The results of the present study warn of the increasing production of fish hybrids and suggest that the practice be monitored and supervised by responsible environmental agencies, since several reports of escape have been recorded (Hashimoto *et al.* 2010, Prado *et al.* 2011, Gomes *et al.* 2012, Prado *et al.* 2012, Vaini *et al.* 2014, Yabu *et al.* 2018). It also stresses that molecular identification tools that can aid in research and conservation programs are of great importance and applicability and should be routinely used. It is considered that such information may assist in the monitoring the environment and of stocks, preserving the conditions and maintenance of wild stocks of fish species.

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