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Gomes, Fátima; Schneider, Horacio; Barros, Claudene; Sampaio, Dioniso; Hashimoto, Diogo; Porto-Foresti, Fábio; Sampaio, Iracilda

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Innovative molecular approach to the identification of *Colossoma macropomum* and its hybrids

FÁTIMA GOMES¹, HORACIO SCHNEIDER¹, CLAUDENE BARROS², DIONISO SAMPAIO¹,
DIOGO HASHIMOTO³, FÁBIO PORTO-FORESTI³ and IRACILDA SAMPAIO¹

¹Instituto de Estudos Costeiros, Laboratório de Genética e Biologia Molecular, Universidade Federal do Pará,
Campus de Bragança, Alameda Leandro Ribeiro, s/n, 68600-000 Bragança, PA, Brasil

²Centro de Estudos Superiores de Caxias, Universidade Estadual do Maranhão, Laboratório de Genética e Biologia Molecular,
Praça Duque de Caxias, s/n, 65604-380 Caxias, MA, Brasil

³Departamento de Ciências Biológicas, Faculdade de Ciências, Universidade Estadual Paulista (UNESP),
Campus de Bauru, Rua Engenheiro Luiz Edmundo Coube, 14-01, 17033-360 Bauru, SP, Brasil

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ABSTRACT

Tambaqui (*Colossoma macropomum*) is the fish species most commonly raised in the Brazilian fish farms. The species is highly adaptable to captive conditions, and is both fast-growing and relatively fecund. In recent years, artificial breeding has produced hybrids with Characiform species, known as “Tambacu” and “Tambatinga”. Identifying hybrids is a difficult process, given their morphological similarities with the parent species. This study presents an innovative molecular approach to the identification of hybrids based primarily on Multiplex PCR of a nuclear gene (α -Tropomyosin), which was tested on 93 specimens obtained from fish farms in northern Brazil. The sequencing of a 505-bp fragment of the Control Region (CR) permitted the identification of the maternal lineage of the specimen, all of which corresponded to *C. macropomum*. Unexpectedly, only two CR haplotype were found in 93 samples, a very low genetic diversity for the pisciculture of Tambaqui. Multiplex PCR identified 42 hybrids, in contrast with 23 identified by the supplier on the basis of external morphology. This innovative tool has considerable potential for the development of the Brazilian aquaculture, given the possibility of the systematic identification of the genetic traits of both fry-producing stocks, and the fry and juveniles raised in farms.

Key words: pisciculture, Multiplex PCR, mitochondrial DNA, genetic bottleneck.

INTRODUCTION

A number of native Brazilian fishes have considerable potential for aquaculture, although for most species, the lack of a reliable scientific and technological database impedes the development of adequate husbandry and the full economic potential of the

species (Ostrensky et al. 2008). The “Tambaqui” (*Colossoma macropomum*), which is native to Amazon and Orinoco basins (Lima and Goulding 1998), is the most widely-farmed native fish in Brazil, and is found practically throughout the whole country (IBAMA 2008). This species is renowned for its excellent adaptation to captive conditions, even in polyspecific arrangements, as well as for

Correspondence to: Horacio Schneider
E-mail: horacio@ufpa.br

its rapid growth, high fecundity and the excellent flavor of its meat (Lima and Goulding 1998).

The Tambaqui has also been targeted in artificial hybridization programs over the past few decades, and has been crossed with a number of different species with the aim of producing sterile individuals or more productive breeds than either of the parent species (Bartley et al. 2001). Given their considerable fecundity, the female Tambaqui is normally preferred for this procedure, and it is normally mated with males of two other characiforme species, *Piaractus mesopotamicus* (“Pacu”) and *Piaractus brachipomus* (“Pirapitinga”). This produces hybrids known as “Tambacu” and “Tambatinga”, respectively. This hybridization aims to combine the characteristics of different species to produce an economically more viable organism which is, in particular, faster growing, and more resistant to disease and low temperatures (Senhorini et al. 1988, Fontes et al. 1990).

The marked similarities between the hybrids and their parent species hampers the identification of these animals based on morphology, or even their behavior or ecology (Scribner et al. 2001). Given this problem, the genetic characterization of stocks that have passed through some form of artificial manipulation has become increasingly important at fish farms where selective breeding is practiced (Toledo-Filho et al. 1994, Porto-Foresti et al. 2006). Given this, the development of reliable genetic markers has provided valuable insights, and has become an important tool in pisciculture, capable of generating data on endogamy, genetic variability and relatedness, as well as providing a basis for the identification of hybrids and their lineages in breeding programs (García de Leon et al. 1998, Liu and Cordes 2004).

Multiplex PCR is one of the most important modern molecular tools used in pisciculture for the identification of species and the differentiation of pure and hybrid specimens. This technique is a variation of the traditional PCR (Polymerase Chain Reaction), which allows the simultaneous amplification of a

number of different target regions using different pairs of primers, and was developed by Chamberlain et al. (1988). In Brazil, Multiplex PCR has been applied successfully to the identification of native elasmobranch fishes (sharks and rays) based on the analysis of muscle tissue or fins (De-Franco et al. 2009, Mendonça et al. 2009, 2010), as well as the differentiation of pure and hybrid specimens raised in fish farms (Hashimoto et al. 2009).

Another widely-used technique in fishery genetics is DNA sequencing, which can be applied to both mitochondrial and nuclear regions, and has been used successfully to solve questions related to phylogeny, population structure and species identification. Given its accelerated mutation rate, mitochondrial DNA has been widely used for the analysis of genetic variability at the population level. Santos et al. (2007) recorded high levels of genetic diversity in five native populations of *Colossoma macropomum* from the main channel of the Amazon River using DNA sequences of the mitochondrial Control Region. The parameters established in this study are important for the development of comparative studies of genetic variability in fry-producing stocks.

In the present study, these two complementary molecular approaches were combined for the analysis of native Tambaquis and specimens obtained from fish farms in northern Brazil. The Multiplex PCR focused on the nuclear α -Tropomyosin gene, while the DNA sequencing involved the mitochondrial Control Region. This combination of techniques permitted the identification of all the pisciculture specimens analyzed as pure Tambaqui, Tambacu or Tambatinga, as well as the determination of their maternal mitochondrial lineage. Only two haplotypes were identified, indicating a high level of endogamy in the fry-producing stocks of the northern Brazil. This reinforces the need for the renewal and diversification of these stocks in order to guarantee the genetic variability of the Tambaquis produced in the Brazilian fish farms.

MATERIALS AND METHODS

SAMPLES

A total of 93 samples were obtained from fish farms located in the Brazilian states of Pará (Augusto Corrêa, Bragança, Capitão Poço, Castanhal, Marabá, Santarém, and Viseu) Rondônia (Pimenta Bueno), Amapá (Macapá), and Piauí (Paulistana). The identification of the maternal mitochondrial DNA lineage was based on samples of native Tambaquis from Lago Grande in Santarém (Pará) and mitochondrial haplotypes of Pirapitinga (*Piaractus brachypomus*, AF283958) and Pacu (*Piaractus mesopotamicus*, AF283959) retrieved from Genbank. For multiplex analyses, additional samples of *P. brachypomus* and *P. mesopotamicus* were obtained from the Fish Genetics Laboratory (LaGenPe) at São Paulo State University in Bauri (UNESP-Bauri).

At the fish farms, the person who provided the specimens was asked to identify the animal (as pure Tambaqui, Tambacu or Tambatinga), and this classification was recorded for later comparison with the genetic identification. The tissue samples (fragments of muscle or fin) were preserved in absolute ethanol until analysis, in either the Genetics or Molecular Biology Laboratory of the Coastal Studies Institute of the Federal University of Pará (for the sequencing of mitochondrial DNA) and the LaGenPe at UNESP-Bauri (for the Multiplex PCR).

DNA EXTRACTION

Total DNA was isolated using the phenol/chloroform extraction protocol and precipitation with ethanol (Sambrook and Russel 2001). The specimens were visualized by electrophoresis in 1% agar gel, colored with ethidium bromide.

PCR AND DNA SEQUENCING

The identification of the maternal mitochondrial lineage was based on a fragment of DNA from the Control Region. This segment was amplified

by Polymerase Chain Reaction (PCR), using the primers D-loop L1 (5'-CCTAACTCCCAAA-GCTAGGTATTC-3', Santa Brígida et al. 2007) and D-loop H1, 5'-TGTTTATCACTGCTGRRTT-CCCT-3' (designed for this paper). This pair of primers amplifies the whole Control Region (approximately 1100 base pairs or bps), but only some 500 bps were used for the present analysis.

The PCR reactions were conducted in a total volume of 25 µl containing the following reagents: 2.5 µl of (10X) buffer, 4 µl of DNTP (1.25 mM), 1 µl of MgCl₂ (50 mM), 1.0 to 2.0 µl of DNA (50–100 ng/µl), 0.25 µl of each primer and 0.2 µl of Taq DNA polymerase (5 U/µl), topped off with purified water in order to complete the final reaction volume. The program used for the PCR reactions consisted of initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C (30 seconds), hybridization at 57°C (1 minute), and extension at 72°C (2 minutes), and a final extension at 72°C for 7 minutes.

The products of this amplification were sequenced immediately using the ABI Prism TM Dye Terminator Cycle Sequencing Reading Reaction kit (Applied Biosystems), followed by electrophoresis in an ABI 3130 automatic sequencer. The DNA sequences were edited and aligned in BIOEDIT 5.0.6 (Hall 1999), and the species from which the mitochondrial lineage was derived was identified through comparisons with the sequences available for the Tambaqui, Pacu, and Pirapitinga available in GenBank (*Colossoma macropomum* DQ480074, *Piaractus mesopotamicus* AF283959, and *Piaractus brachypomus* AF283958). The new sequences generated in the present study were deposited in GenBank under the accession codes JN581982-83.

MULTIPLEX PCR

The identification of specimens and the definition of their pure or hybrid status was finalized using

Multiplex PCR for the analysis of a nuclear marker – the α -Tropomyosin gene – based on a protocol adapted from Hashimoto et al. (2009). The species-specific primers that discriminate *C. macropomum*, *P. mesopotamicus*, and *P. brachypomus* were developed by the LaGenPe (UNESP-Bauru). The sequence of each primer is as follows: Tropo Serra F (5'-GAGTTGGATCGGGCTCAG-3'), Tropo Cm R (5'-ATACAACAATGCCATCGCT-3'), Tropo Pm R (5'-CTTCAGCTGGATCTCCTGA-3'), and Tropo Pb R (5'-TTGACTTTATGCCACACAAAT-3'). Tropo Serra F is a universal primer, and thus recognizes the DNA of the three species, whereas the reverse primers are specific to the three species, Tropo Cm R to *C. macropomum*, Tropo Pm R to *P. mesopotamicus* and Tropo Pb R to *P. brachypomus* (Figure 1).

The Multiplex PCR reaction was conducted in a total volume of 20 μ l containing the following reagents: 2.0 μ l of (10X) buffer, 1.5 μ l of DNTP (1.25 mM), 0.75 μ l of $MgCl_2$ (50 mM), 1.0 to 2.0 μ l of DNA (50–100 ng/ μ l), 0.3 μ l of each primer (except for CmR, for which a volume of 0.8 μ l was used) and 0.2 μ l of Taq DNA polymerase (5 U/ μ l), topped off with purified water in order to complete the final reaction volume (reagents from Invitrogen, USA). The program used for the PCR reactions consisted of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C (30 seconds), hybridization at 59°C (30 seconds) and extension at 72°C (5 seconds), followed by final extension at 72°C for 7 minutes. The size of the DNA fragments was determined using a low DNA mass ladder (Invitrogen), following electrophoresis in 1.5% agar gel colored with Ethidium Bromide and visualization under UV light. Photographs were taken with a 5.1 megapixel Olympus CAMEDIA, C-5060 digital camera.

MITOCHONDRIAL DNA SEQUENCES

A segment of 505 bps of the Control Region was obtained from the 93 specimens obtained from fish farms in the Brazilian states of Pará, Amapá,

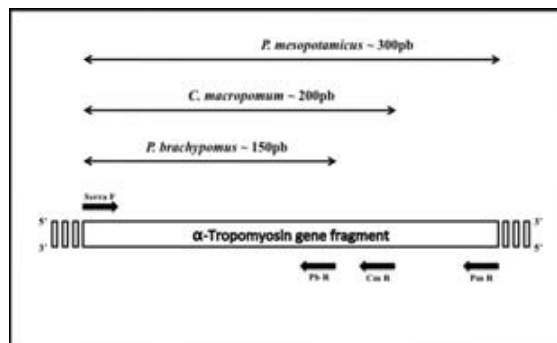


Figure 1. Schematic diagram of the primers used for the multiplex PCR analysis of the nuclear α -Tropomyosin gene. Primer Serra F (forward) is universal and reverse primers are species specific for *Colossoma macropomum* (Cm R), *Piaractus mesopotamicus* (Pm R), and *P. brachypomus* (Pb R).

Piauí, and Rondônia. These specimens presented low haplotypic diversity, with only two haplotypes being identified from the 93 sequences. These two haplotypes (Hap1 and Hap2) differ in only three nucleotides (Figure 2), and while Hap2 is exclusive to Santarém, Hap1 was found at all the other farms.

The sequences obtained in the present study were compared with those available in GenBank for *Colossoma macropomum* (DQ480074), *Piaractus mesopotamicus* (AF283959) and *Piaractus brachypomus* (AF283958), and a high degree of similarity (99%) was found with sequence DQ480074, i.e., Tambaqui, *C. macropomum*. The differences between the sequence of this species and those of the other two species were considerable. The sequences of haplotypes Hap1 and Hap2 differ from that of *P. mesopotamicus* (AF283959) at 57 and 55 sites, respectively, a divergence of approximately 11%, and from *P. brachypomus* (AF283958) at 59 and 57 sites (difference of 11.5%). The almost complete similarity between the DNA sequences of the specimens collected in the present study and those of GenBank sequence DQ480074 and the native specimen from Santarém confirms emphatically that the maternal lineage of all the specimens is derived from Tambaqui, *C. macropomum*.

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1 111111111 111111111 111222222 222222233 333333333 44444
4488991 133344445 566666677 888022245 555667803 444456788 44469
2356878365 7089012492 3123578356 3458123600 1582850083 5789079148 34597
C. macropomum CCGTATACGT AGACGTTGTA GTACACAAA AGACTCGATC CCCCAAGCTT CCCGGAGCTG TAGTT
Hap1 ..... T.....T.. .....
Hap2 ..... .A.....C. .... T.....
P. mesopotamicus AAACGAT.AA TACATACTAT AACATGTT.T GTGT.AA.CT .AT.TTA.CA TTAAT.ATC. CTA.A
P. brachypomus AAA.GATTAA TACATACTAT .ACATGTTTT GTGTCAAC.. .ATTTA.CA TTAATGATCA CTACA

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Figure 2. Variable sites in the mitochondrial Control Region haplotypes identified in *Colossoma macropomum*, *Piaractus mesopotamicus* and *P. brachypomus* (sequences obtained from GenBank), farmed specimens of *C. macropomum* (Hap1 and Hap2, Genbank JN581982-83) and a wild specimen of *C. macropomum* obtained from Lago Grande near Santarém, Pará (Genbank JN590242).

MULTIPLEX PCR

The multiplex amplification of the nuclear DNA of the α -Tropomyosin gene revealed different electrophoretic patterns for the three species (Tambaqui, Pacu, and Pirapitinga). For Pacu (*P. mesopotamicus*) a band of approximately 300 bps was obtained, whereas for Tambaqui (*C. macropomum*) this band had around

200 bps, decreasing to approximately 150 bps in Pirapitinga (*P. brachypomus*). Of the 93 specimens analyzed here, 42 had a heterozygous pattern, with two diagnostic bands, indicating the presence of hybrids.

As each band in the multiplex PCR corresponds to an allele inherited from one of the parents, the electrophoretic profile of a hybrid Tambacu (female *C. macropomum* x male *P. mesopotamicus*) contains

TABLE I
Morphological and molecular identification of the specimens analyzed in the present study.

Origin	Collection date	Number of specimens	Origin of the fry	mtDNA haplotype	Identification by:	
					The supplier	Multiplex PCR (number of specimens)
Augusto Correa/Pará	April 2008	7	Castanhal	Tambaqui – Hap1	Tambaqui	Tambacu (7)
Bragança/ Pará	July 2008	19	Castanhal	Tambaqui – Hap1	Tambaqui	Tambaqui (13), Tambacu (6)
Capitão Poço/Pará	April 2008	10	Castanhal	Tambaqui – Hap1	Unidentified hybrids*	Tambacu (5), Tambatinga (5)
Castanhal/Pará	June 2008	7	Castanhal	Tambaqui – Hap1	Tambaqui	Tambatinga (5), Tambacu (2)
Santarém/Pará	June 2008	26	Santarém	Tambaqui – Hap2	Tambaqui*	Tambaqui (26)
Viseu/Pará	May 2008	9	Castanhal	Tambaqui – Hap1	Tambatinga	Tambatinga (9)
Marabá/Pará	April 2009	4	Castanhal	Tambaqui – Hap1	Tambacu	Tambaqui (3), Tambacu (1)
Pimenta Bueno/Rondônia	August 2008	5	Porto Velho	Tambaqui – Hap1	Tambaqui	Tambaqui (5)
Paulistana/Piauí	July 2007	4	Unknown	Tambaqui – Hap1	Tambaqui	Tambaqui (2), Tambacu (2)
Macapá/Amapá	September 2009	2	Castanhal	Tambaqui – Hap1	Tambaqui	Tambaqui (2)

* Juvenile fishes.

a 200-bp band, corresponding to the allele inherited from the mother, and a 300-bp band, corresponding to that inherited from the father. Similarly, in the hybrid Tambatinga (female *C. macropomum* x male *P. brachypomus*), the 200-bp and 150-bp bands correspond to the allele inherited from the mother and father, respectively (Figures 3 and 4).

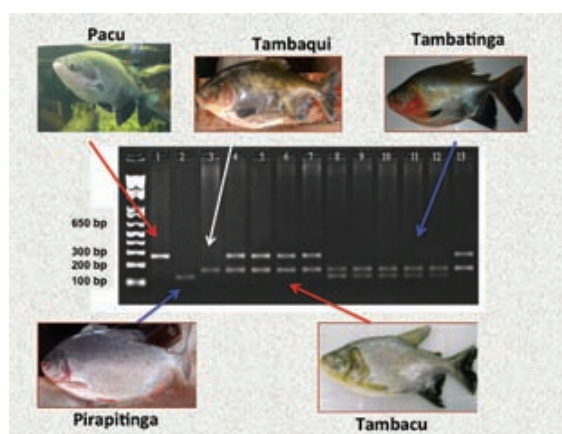


Figure 3. Agar gel (1.5%) for the PCR of the α -Tropomyosin gene in (1) Pacu, (2) Pirapitinga and (3) Tambacui specimens. Samples 4-13 were obtained from a fish farm in Capitão Poço, Pará (Brazil), showing the double banding representative of hybrid Tambacu (specimens 4, 5, 6, 7, and 13) and Tambatinga (8, 9, 10, 11, and 12). M = 1Kb Plus DNA Ladder. Except for Pacu, which the picture was retrieved from the internet (<http://pescariamadora.blogspot.com/2011/06/peixes-de-agua-doce-pacu.html>), all remaining pictures were taken by the authors of this study.



Figure 4. Agar gel (1.5%) for the PCR of the α -Tropomyosin gene in (1) Pacu, (2) Pirapitinga and (3) Tambacui, and of specimens obtained from a fish farm in Santarém, Pará (Brazil) (4-13). M = 1Kb Plus DNA Ladder.

Only 23 of the fishes were identified as hybrids by the persons who supplied the specimens, whereas the combined analysis of the mitochondrial Control Region and the nuclear α -Tropomyosin

gene identified 42 hybrids, 23 Tambacus and 19 Tambatingas. Suppliers not only misidentified hybrids as pure Tambaquis, but also in one case pure Tambaquis were identified as hybrids, re-emphasizing the difficulty of differentiating the specimens based on external morphology (Table I).

DISCUSSION

The Tambaqui (*Colossoma macropomum*) is the native fish species most commonly found in fish farms in Brazil, where artificial hybridization with other characiform species has generated a number of hybrids, in particular Tambacu and Tambatinga, which are also found throughout the country. The genetic monitoring of this process is important not only for the identification of specimens, but also for the evaluation of the genetic variability of fry-producing stocks.

The present analysis was based on a robust genetic tool consisting of the sequences of a fragment of mitochondrial DNA (used to identify the maternal lineage) and multiplex PCR of a nuclear marker, which enabled the differentiation of pure specimens from hybrids. The combination of these two approaches allowed us to characterize the genetic traits of the sample of 93 specimens obtained from ten fish farms located in four northern Brazilian states.

The mitochondrial DNA of animals is inherited from a single parent, that is, the mother in most eukaryotes (Moritz et al. 1987, Parker et al. 1998). In the present case, the sequences of the mitochondrial Control Region confirmed that all the specimens, both pure and hybrids, have the maternal lineage of Tambaqui (*C. macropomum*). The nuclear markers, by contrast, are inherited from both parents and have been widely and successfully used in studies of fish species for the analysis of gene flow within and between species, as well as for interspecific hybridization. Multiplex PCR of mitochondrial genes has been successfully

used for the identification of the Brazilian shark and ray species (De-Franco et al. 2009, Mendonça et al. 2009, 2010), and for the differentiation of pure and hybrid specimens produced in fish farms (Hashimoto et al. 2009). De-Franco et al. (2009) used multiplex PCR of cytochrome oxidase subunit I (COI) to differentiate the guitarfish species *Rhinobatos horkelli*, *R. percellens* and *Zapteryx brevirostris*. Mendonça et al. (2009) presented an effective system of multiplex PCR and PCR-RFLP techniques, which was used to differentiate the shark species *Rhizoprionodon lalandii* and *R. porosus*. In a subsequent study Mendonça et al. (2010) developed a multiplex system for COI that permitted the differentiation of nine species of Lamniform and Carcharhiniform sharks.

In the case of Tambaqui and its hybrids analyzed in the present study, it is important to note that, while it is inherited from both parents, multiplex PCR of the nuclear marker α -Tropomyosin would not be sufficient, on its own, to identify the parent species involved in the hybridization, given that other types of hybrids, such as the “Paqui”, produced by crossing a female pacu with a male tambaqui, are now common. The multiplex PCR banding pattern is identical in *Tambacus* and Paquis – both have one diagnostic band of 300 bps and one of 200 bps – making these hybrids indistinguishable when using only this technique. As shown in the present study, this problem can be overcome by using the mitochondrial DNA sequences in order to identify the maternal lineage.

The mitochondrial haplotype variation observed in the farmed specimens analyzed in the present study was unexpected low. The presence of only two haplotypes in the 93 specimens obtained from the 10 fish farms located in four Brazilian states indicates that all the individuals were descended from only two females. In the specific case of the Santarém farm, the only unit at which haplotype 2 was recorded, the fry were obtained

locally from the Pará State Agriculture Ministry (SAGRI). The fry from the Pimenta Bueno farm in Rondônia (Hap1) were obtained locally, in the state capital Porto Velho. The specimens from Paulistana (Hap1) were from an artificial lake, although their origin was not known. Specimens from the other farms, in Amapá and Pará (both Hap1) were acquired from the same facility in Castanhal, Pará.

This reduced mitochondrial diversity in the Tambaqui stocks might be a result of the history of the farming of the species in Brazil, which began in the 1980s (Guimarães 1999). As in most other domesticated species, this process tends to provoke an accentuated genetic bottleneck, given that the descendants of each generation are selected for the establishment of the breeding stock of subsequent generations. Our results are consistent with such a bottleneck, especially in the case of the 67 specimens from Rondônia, Piauí, Amapá, and Pará, which presented only the Haplotype 1. As each female Tambaqui normally is used only once or few times a year for the production of fry (Kubitza 2004) and the specimens analyzed in this study were obtained in different months (Table I), they possibly were not all descendants of the same mother. The overall results, then, indicate a high rate of inbreeding in the breeding stock of female Tambaqui.

The haplotypic diversity in wild *C. macropomum* is high, as indicated by the study of Santos et al. (2007), who found 47 different haplotypes in 48 specimens representing five different sites within the geographic range of the species. Given this variation in wild populations, if the genetic variability of the breeding females was representative of that found in the wild, much more distinct mitochondrial haplotypes might have been expected in the present study. This situation is worrying, and means that the fry-producing centers must understand the need for the introduction of new breeding females from the wild, in order to avoid the possibility of an eventual collapse in the genetic composition of farmed stocks in Brazil.

A second question that demands attention is the difficulty in identifying organisms produced through artificial breeding programs, which hampers the reliable identification of specimens. The additional hybrids identified in the present study through molecular analyses, and the consequent reduction in the number of pure specimens, indicates that many fish farmers are raising hybrids they presume to be pure Tambaquis. This is understandable, considering the marked morphological similarities among the different forms, but it may have serious economic consequences, given the distrust it may provoke between the producers of farmed fish and their consumers.

The correct identification of the products of pisciculture reinforces the trust of the consumer and strengthens the fish farming market. The genetic tools developed in the present study represent a major advance for the monitoring of the genetic variability of Tambaqui and its hybrids, which are now farmed throughout Brazil.

ACKNOWLEDGMENTS

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RESUMO

O Tambaqui (*Colossoma macropomum*) é a espécie de peixe mais comumente cultivada em pisciculturas no Brasil. A espécie é altamente adaptada às condições de cativeiro, apresentando rápido crescimento e alta fecundidade. Nos últimos anos tem ocorrido o cruzamento artificial entre

espécies de Characiformes, produzindo os híbridos “Tambacu” e “Tambatinga”. A identificação de híbridos é uma tarefa difícil, em virtude da grande similaridade morfológica entre as espécies parentais. O presente estudo apresenta uma abordagem molecular inovadora para identificação de híbridos com base em PCR Multiplex de um gene nuclear (α -Tropomiosina), em que foram testados 93 espécimes obtidos em pisciculturas da região norte do Brasil. O sequenciamento de um fragmento de 505 pares de bases da Região Controle permitiu a identificação da linhagem materna de todos os espécimes como sendo de *C. macropomum*. Inesperadamente foram encontrados apenas dois haplótipos nas 93 amostras, um baixíssimo índice de diversidade para populações cultivadas de Tambaqui. A PCR multiplex identificou 42 híbridos, em contraste aos 23 híbridos citados pelos fornecedores dos peixes, em uma identificação realizada com base na morfologia. Esta ferramenta inovadora possui um grande potencial para o desenvolvimento da aquicultura brasileira dada a possibilidade de identificação sistemática das características genéticas tanto das matrizes produtoras de alevinos quanto dos alevinos e juvenis criados em fazendas.

Palavras-chave: piscicultura, PCR Multiplex, DNA mitocondrial, “Gargalo-de-garrafa” genético.

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