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Original article

Molecular assessment of *Gymnotus* spp. (Gymnotiformes: Gymnotidae) fishing used as live baitfish in the Tietê River, Brazil

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The capture of live bait for sport fishing is an important activity for fishing communities. The main species used for this purpose are members of the genus *Gymnotus*, which comprises numerous species of cryptic nature that are difficult to identify based on external morphology. The aims of this work were to identify through partial sequences of the COI gene *Gymnotus* species fished in the Jacaré-Guaçu River, SP, and to develop a molecular diagnostic approach using PCR-RFLP to identify these species. Partial COI sequences were compared to those of other species deposited in GenBank. The sequences were assessed in the NEBCutter program to determine restriction sites in the sequence and the enzymes to be tested. Phenetic analysis performed by Neighbor-Joining method showed that the specimens sampled belong to two species preliminary identified here as *G.* cf. *sylvius* and *G.* cf. *cuia*, with *G.* cf. *sylvius* accounting for 95.2% of the individuals sampled. The enzymes NlaIII and SacI generated fragments that allowed distinguishing the *Gymnotus* species using PCR-RFLP. This analysis can be used to accurately identify these species, which is fundamental for monitoring *Gymnotus* fishing and assessing the conservation of this genetic resource.

Keywords: Molecular identification, PCR-RFLP, Tuvira.

A captura de iscas-vivas para a pesca esportiva constitui uma atividade importante em comunidades de pescadores. As principais espécies utilizadas para este propósito pertencem ao gênero *Gymnotus*, o qual compreende inúmeras espécies de natureza críptica que dificulta a identificação baseada na morfologia externa. Os objetivos deste trabalho foram identificar através de sequências parciais do gene COI, espécies de *Gymnotus* capturadas no Rio Jacaré-Guaçu, Ibitinga, SP, e desenvolver um diagnóstico molecular por meio de PCR-RFLP. Sequências parciais de COI foram comparadas com outras espécies depositadas no GenBank. As sequências foram analisadas no Programa NebCutter para determinar os sítios de restrição e definir as enzimas a serem testadas. A análise fenética pelo método de Neighbor-Joining mostrou que os espécimes pertencem a duas espécies identificadas preliminarmente aqui como *G.* cf. *sylvius* e *G.* cf. *cuia*, sendo que *G.* cf. *sylvius* representou 95,2% dos indivíduos amostrados. As enzimas NlaIII e SacI geraram fragmentos que permitiram discriminar as espécies por meio de PCR-RFLP. Esta análise pode ser usada na identificação precisa destas espécies, fundamental na proposição de monitoramento da pesca de *Gymnotus* na região e para medidas adequadas de conservação.

Palavras-chave: Identificação molecular, PCR-RFLP, Tuvira.

Introduction

Species of *Gymnotus* Linnaeus, 1758 are an important fishing resource for fishermen communities and the principal source of commercial live bait for sport fishing (Sousa *et al.*, 2017). The genus *Gymnotus* is a member of the family Gymnotidae (order Gymnotiformes). This genus consists of many species, represented by 43 valid species in six clades (Tagliacollo *et al.*, 2016), with wide distribution in the continental waters of South and Central America, except in Chile and Belize (Albert, 2001).

The identification of species in this genus based on morphological characters is sometimes challenging due to the morphological conservatism among species

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and high phenotypic plasticity within species. Many efforts have been made based on studies of morphology, cytogenetics, molecular, and electrical signals to elucidate the taxonomy of electric fishes, including *Gymnotus* (Alves-Gomes *et al*., 1995; Albert, Crampton, 2003; Fernandes *et al.*, 2005; Margarido *et al*., 2007; Maxime, Albert, 2009; Scacchetti *et al*., 2011; Milhomem *et al.*, 2012; Tagliacollo *et al*., 2016; Sousa *et al.*, 2017; Craig *et al.*, 2017, 2018).

The existence of cryptic species in combination with poor-quality data from landing records for biomass estimation and catch quotas, may imperil the long-term survival of given fish genetic resources (Hilsdorf, Hallerman, 2017). In addition, worldwide concerns on the indiscriminate use of live baitfish, the ecological risks of introducing alien species, and the depletion of local species, are on the agenda of fish diversity conservation in many countries (Drake, Mandrak, 2014; Sousa *et al.*, 2017). Therefore, the correct identification of species is one of the first steps towards successfully establishing conservation and management measures to protect local baitfish species. Hebert *et al.* (2003) proposed the use of a fragment of approximately 650bp of the mitochondrial cytochrome c oxidase subunit 1 (COI or COX1) as a global identification system for most animal species presently known as the DNA barcoding.

Supported by this background, we use partial sequences of COI to identify *Gymnotus* spp. captured as live baitfish and develop a rapid and low-cost molecular methodology to identify unequivocally the species being caught to help ensure the sustainability of *Gymnotus* fisheries and preserve the biodiversity of species.

Material and Methods

Collection and Sampling Site. Taxon sampling was carried out between October 2015 and March 2016 along the Jacaré-Guaçu River, a tributary of the Tietê River, southeast region of Brazil (Fig. 1). Eight individuals from four fishing sites were collected in two season periods, in a total of 64 individuals. All specimens were photographed, labeled and a small piece of muscle or fin clips were taken from each fresh fish and stored in 95% ethanol at -20°C. Voucher specimens were deposited in the Ichthyology Collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP), SP, Brazil, receiving the register numbers MZUSP 124930 and MZUSP 124931.

The procedures carried out in this study were in accordance with the ethical standards of the Colégio Brasileiro de Experimentação Animal (COBEA) and were approved by the Comitê de Ética em Experimentação Animal do Instituto de Pesca (CEEAIP) protocol number 01/2016.

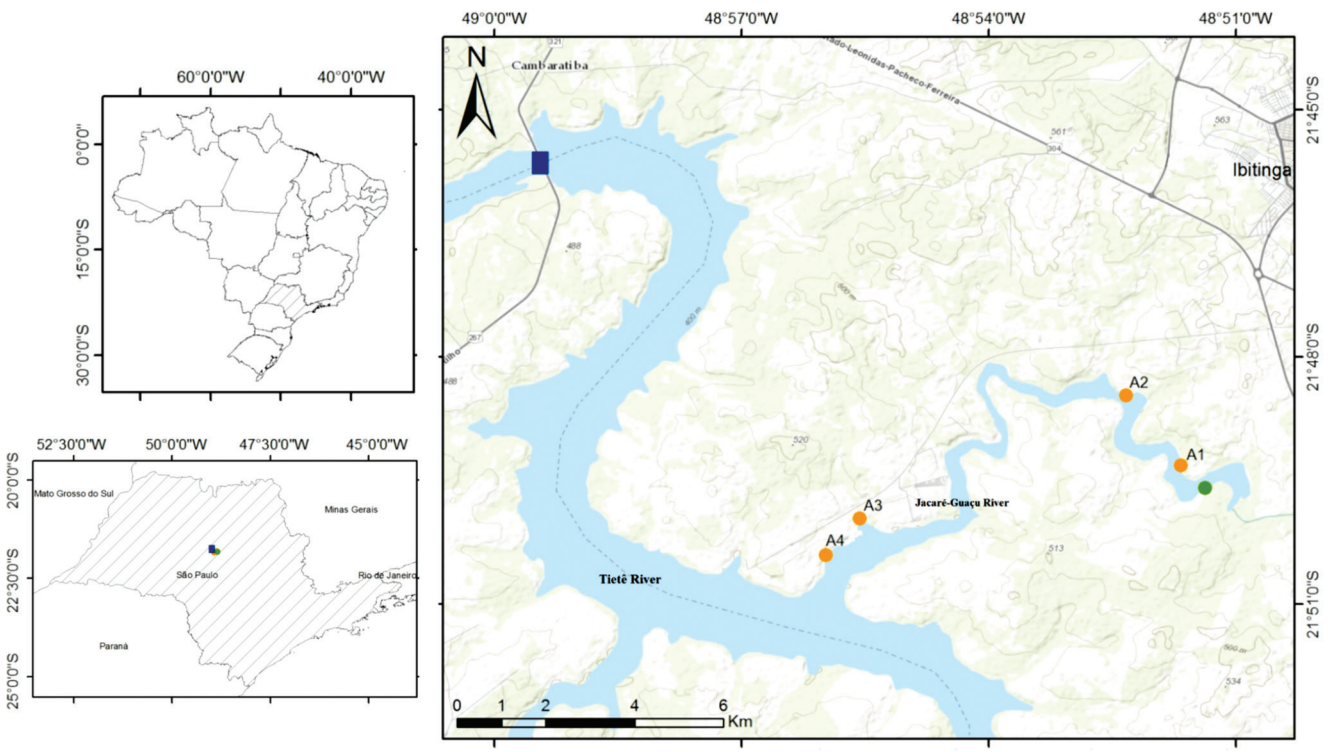

Fig. 1. Map of the study area. Characterization of the catchment sites of *Gymnotus* spp. in the Jacaré-Guaçu River, southeastern Brazil. Orange dots (A1-A4) = collection sites; green dot = embarkation and disembarkation; blue rectangle = Ibitinga dam; lines on the diagonal = boundary of the State of São Paulo. 1: 100,000 (UTM projection), WGS Datum 1984. Elaborated by Luís Campanha, modified by Lilian P. Faria Pereira.

DNA extraction, PCR and Sequencing. Total genomic DNA was extracted following the protocol of saline extraction method (Aljanabi, Martinez, 1997). Partial sequences of the cytochrome c oxidase 1 (COI or Cox1) gene have been amplified by the polymerase chain reaction (PCR) using the universal primers FishF1 (5 ' – TCA ACC AAC CAC AAA GAC ATT GGC AC-3 ') and FishR1 (5 ' – TAG ACT TCT GGG TGG CCA AAG AAT CA-3') (Ward *et al*., 2005). PCR was conducted for final volume of 50 µL containing 1x buffer solution, 0.2 mM of each DNTP, 25 mM $MgCl_2$, 0.2 µM of each primer (sense and anti-sense), 1.0 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen™, Carlsbad, CA, USA) and 0.5 – 2 ng of DNA. The amplification reaction was conducted in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) with an initial cycle of denaturation at 94°C for 2 min, followed by 35 cycles (94°C for 40 S, 58°C for 1 min, 72°C for 1 min), and the final extension at 72°C for 7 min.

PCR products were purified with CleanSweep™ PCR Purification Reagent (Applied Biosystems™), according to the protocol provided by the manufacturer. For the sequencing reactions the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems ™) was used. Sequencing was performed on the ABI 3730 DNA Analyzer (Applied Biosystems™).

Sequence Analysis. The COI partial sequences of all samples were edited in the Chromas V.2.6 program. The Codon Code Aligner V 1.5.2 program (Codon Code Corporation, Dedham, Massachusetts, United States) was used for quality evaluation and obtaining the consensus sequence (contig) from the forward and reverse sequences.

The sequences were compared to the sequences of other species of *Gymnotus* deposited in GenBank (Tab. **S1**) for the identification of similarity using the MegaBLAST program (Zhang *et al.*, 2000; Morgulis *et al.*, 2008). The number of haplotypes of the partial sequences of the COI gene was determined in the DnaSP program V.5.10 (Rozas *et al.*, 2003). Sequences from each haplotype were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (accession numbers: MN167125, MN167126, MN167127, MN167128, MN167129, MN167130, MN167131).

The sequences were aligned with the ClustalW command (Thompson *et al.*, 1994) implemented in the MEGA 6 program (Tamura *et al.*, 2014). Neighbor-Joining (NJ) based on Kimura two Parameter (K2P) genetic distance were used to analyze the phenetic relationship between the samples, as recommended by Barcode of Life. The bootstrap re-sampling analysis was performed using 1,000 replicates (Nei, Kumar, 2000) and the genetic divergence was calculated by the K2P distances (Kimura, 1980).

PCR-RFLP. *Gymnotus* partial COI sequences amplified herein, and those retrieved from GenBank were assessed using the NEBCutter program (Vincze *et al.*, 2003). The diagnostic enzymes were used to distinguish among the *Gymnotu* sampled individuals.

Digestion was carried out at 10 µL final volume, including 1.0 µL of endonuclease (10 U.mL-1) (BioLabs), 1 µL of 10x NEBuffer 4 (50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 1mM dithiothreitol (DTT), pH 7.9, 25ºC; Biolabs) and 5 µL of the PCR product. Solutions were incubated for 1-2 h at 37°C in the thermal block. The resulting sizes of restriction fragments were determined in 3.0% agarose gel electrophoresis by comparison with a standard DNA molecular weight marker. Images were digitalized for further analysis of the banding patterns.

Results

Molecular Identification. Out of 64 sequences examined only two showed poor quality and could not be used. After editing, the sequences were 585 bp long. Of the 62 successfully sequenced samples, 59 showed similarity of 99 to 100% with the species *G. sylvius* Albert & Fernandes-Matioli, 1999 and the other three sequences presented a best identity with *G. inaequilabiatus* (Valenciennes, 1839) when compared with other GenBank sequences in the MegaBLAST program. However, a species level match was not made, presumably due to misidentifications of the species names in GenBank.

The bootstrap values indicate branch statistical supports. Analysis showed that specimens from the fishing in the Jacaré-Guaçu River grouped in two different clades: one with sequences of *G. sylvius* (clade A) and another one with sequences of *G. inaequilabiatus* (clade C) (Fig. 2). The first one comprising 95.2% of the individuals sampled and the second one 4.8%. Specimens grouped in clade A yielded 5 haplotypes (Photos **S2**, **S3**, **S4**, **S5** and **S6**) with 4 polymorphic sites (n = 59) and in clade C, 2 haplotypes (**S7** and **S8**) with 3 polymorphic sites (n = 3).

Considering a recent revision of Craig *et al.* (2017) with the description of seven subspecies of *G. carapo* Linnaeus, 1758, and a revision of Craig *et al.* (2018) of *G. carapo* and *G. tigre* Albert & Crampton, 2003 clade we preliminary identified here two species: *G.* cf. *sylvius* and *G.* cf. *cuia* Craig, Malabarba, Crampton & Albert, 2018. Species of *G.* cf. *sylvius* were collected in the four fishing sites and *G.* cf. *cuia* in A2, A3 and A4.

The intraspecific genetic divergence for *G.* cf. *sylvius* ranged from 0.0 to 0.6% and for *G.* cf. *cuia* from 0.0 to 0.9%. The divergence between *G.* cf. *sylvius* and *G.* cf. *cuia* ranged from 4.7 to 5.6% (Tab. 1).

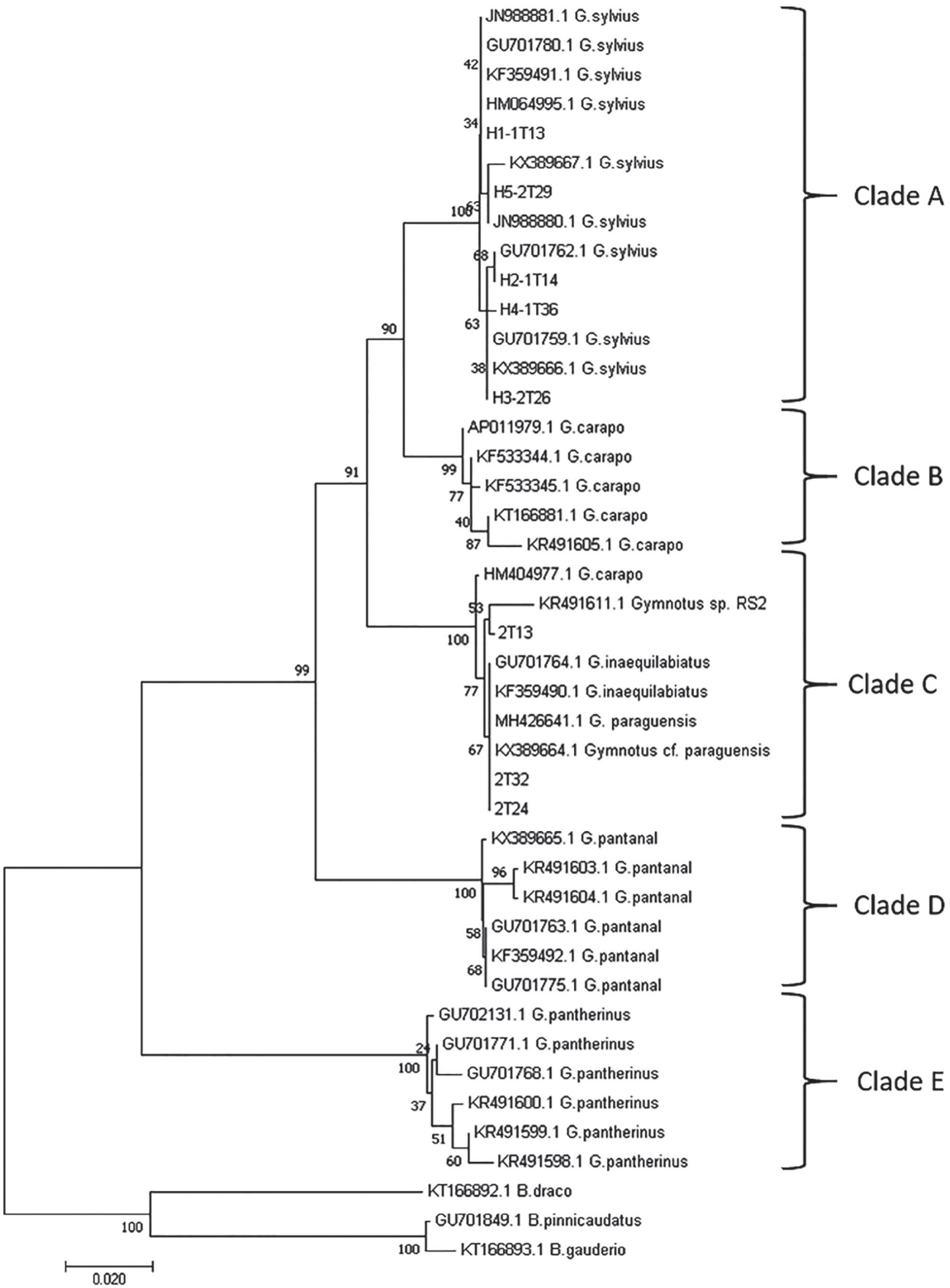

Fig. 2. Neighbor-joining dendrogram of the COI partial sequences of *Gymnotus* spp. (1T13, 2T29, 1T14, 1T36, 2T26, 2T13, 2T32 and 2T24) collected in the Jacaré-Guaçu River, State of São Paulo, and obtained from GenBank, constructed from the distances of Kimura (K2P). The bootstrap values are indicated on the branches.

PCR-RFLP. Based on the *in silico* analysis, we determined the enzymes that can be used for the diagnosis of the *Gymnotus* species present in the Jacaré-Guaçu River. The enzymes that allowed differentiation between the species were NlaIII (5 '... CATG' ... 3') and SacI (5' ... GAGCT'C ... 3 '). These two enzymes generated fragments that allowed also to differentiate the species *G. pantanal* Fernandes, Albert, Daniel-Silva, Lopes, Crampton & Almeida-Toledo, 2005 and *G. pantherinus* (Steindachner, 1908), in places where these two species occur in sympatry with *G.* cf. *sylvius* and *G.* cf. *cuia* (Tabs. 2–3).

COI cleavage patterns using NlaIII and SacI endonucleases are depicted in the Figs. 3–4.

Tab. 1. Genetic divergence by parameter K2P for the analyzed species.

	G. cf. *sylvius*	*G. carapo*	*G.* cf. *cuia*	*G. pantanal*	*G. pantherinus*
*G.*cf. *sylvius*	0.0 - 0.6%	2.6 - 4,0%	4.7 - 5.6%	6.8 - 7.1%	13.0 - 14.8%
G. carapo	-	0.3 - 1.5%	4.6 - 6.5%	6.6 - 8.0%	12.7 - 15.0%
G. cf. *cuia*	-	-	0.0 - 0.9%	7.2 - 8.4%	14.8 - 17.7%
G. pantanal	-	-	-	0.0 - 1.0%	13.1 - 15,8%
G. pantherinus	-	-	-	-	0.2 - 1.6%

Tab. 2. Size of fragments generated by *Nla*III for *G.* cf. *sylvius*, *G.* cf. *cuia*, *G. pantherinus* and *G. pantanal*.

Fragments	*G.* cf. *sylvius*	*G.* cf. *cuia*	*G. pantherinus*	*G. pantanal*
1	306	189	97	306
2	382	193	591	382
3		306		

Tab. 3. Size of fragments generated by *Sac*I for *G.* cf. *sylvius*, *G.* cf. *cuia*, *G. pantherinus* and *G. pantanal*.

Fragments	*G.* cf. *sylvius*	*G.* cf. *cuia*	*G. pantherinus*	*G. pantanal*
1	234	160	Does not cut	Does not cut
2	454	528		

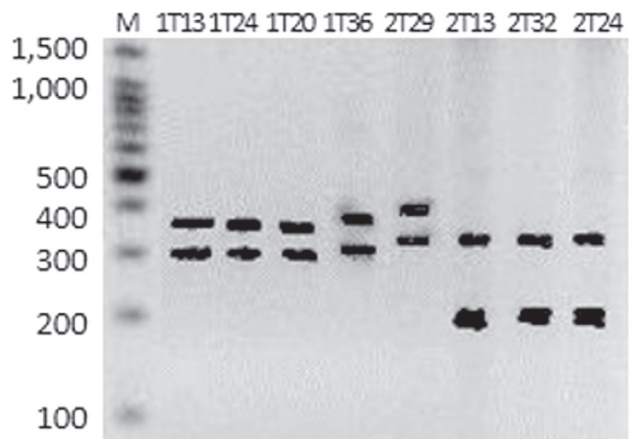

Fig. 3. Agarose gel electrophoresis (3.0%) of NlaIII restriction fragments obtained from COI partial PCR amplification. *Gymnotus* cf. *sylvius* (1T13, 1T24, 1T20, 1T36, 2T29) and *G.* cf. *cuia* (2T13, 2T32 and 2T24). M- 100 bp molecular weight marker (Sinapse Inc). Numbers at fragments indicate estimated molecular weight.

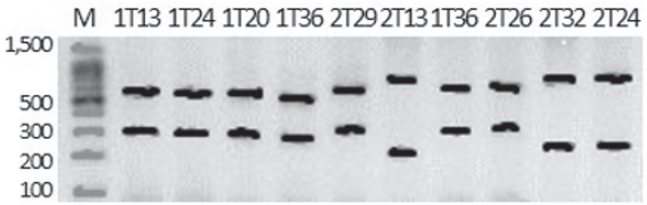

Fig. 4. Agarose gel electrophoresis (3.0%) of NlaIII restriction fragments obtained from COI partial PCR amplification. *Gymnotus* cf. *sylvius* (1T13, 1T24, 1T20, 1T36, 2T26, 2T29) and *G.* cf. *cuia* (2T13, 2T32 and 2T24). M- 100 bp molecular weight marker (Sinapse Inc). Numbers at fragments indicate estimated molecular weight.

Discussion

Successful conservation plans need precise species identification (FAO, 2013). Thus, genetic markers have been applied widely for traceability of fish and fish product as well as for the species identification and populations to enforce fisheries regulation for the conservation of fisheries resources (Ogden, 2008). Partial sequences of COI obtained in this study demonstrated that the *Gymnotus* species caught as live bait by artisanal fisher community in the Jacaré-Guaçu River belong to two distinct species - *G.* cf. *sylvius* and *G.* cf. *cuia*.

Species of *Gymnotus* known to occur in the Upper Paraná basin include: *G. carapo australis* Craig, Crampton & Albert, 2017, *G. cuia*, *G. inaequilabiatus*, *G. pantanal*, *G. pantherinus*, *G. paraguensis* Albert & Crampton, 2003 and *G. sylvius* (Craig *et al.*, 2017; Craig *et al.*, 2018). Of these species, *G. carapo australis*, *G. cuia*, and *G. pantanal* are ecologically abundant and *G. inaequilabiatus*, *G. pantherinus*, *G. paraguensis* and *G. sylvius* are not abundant. *Gymnotus inaequilabiatus* is often misidentified or mistaken for other species, despite the presence of multiple salient external diagnostic characters (Maxime, Albert, 2014) that place it in the *G. tigre* clade rather than the *G. carapo* clade. Further, *G. inaequilabiatus* is unique among congeners in its large adult body size, growing to one-meter total length.

We found inconsistent taxonomic identifications when comparing the sequences of the specimens classified here as *G.* cf. *sylvius* and *G.* cf. *cuia* with the sequences available in GenBank. Three sequences presented better identity with *G. inaequilabiatus* (clade C), but also grouped with *G. paraguensis*, *G.* cf. *paraguensis*, *G. carapo* and *Gymnotus* sp. (Fig. 2). Our sequences of *G.* cf. *cuia* presented also 99% of identity with *Gymnotus* new sp. RS2 that, according to Tagliacollo *et al.* (2016), belongs to *G. carapo* clade and not to *G. tigre clade*, which includes the species *G. inaequilabiatus* and *G. paraguensis* (Maxime, Albert, 2014; Craig *et al*., 2018). It is possible that the specimens identified as *G. inaequilabiatus* in previous studies based on Graça, Pavanelli (2007) and Ota *et al.* (2018) may be actually *G. cuia*. These discordances were probably caused by misidentification of specimens used in previous studies,

once new data from literature, as the recent revisions of Scacchetti *et al.* (2011), Craig *et al.* (2017, 2018) and Utsunomia *et al.* (2018) are now available to elucidate this complex group.

Despite of these considerations, we demonstrated that at least two different species are fished as live bait in Jacaré-Guaçu River. These two species, preliminarily identified here as *G.* cf. *sylvius* and *G.* cf. *cuia*, presented 4.7% to 5.6% of genetic divergence (Tab. 1) and they can easily be discriminated by PCR-RFLP method developed in this work. This technique has been proven to efficiently identify different taxa for distinct purposes (Wolf *et al.*, 1999; Bieliková *et al.*, 2010; Schmidt *et al.*, 2015). The PCR-RFLP technique developed herein showed that the *Nla*III and *Sac*I endonucleases cut patterns clearly distinguished *G. pantanal* and *G. pantherinus* species where they occur in sympatry with *G.* cf. *sylvius* and *G.* cf. *cuia*, as shown in Tabs. 2 - 3. The data demonstrated that the most frequent species was *G.* cf. *sylvius* corresponding to 95.2% of the fished specimens. Fernandes-Matioli *et al.* (2000) also observed *Gymnotus* populations of different species living in sympatry in some watersheds of Southeastern Brazil and always one of the species occur at a significantly lower frequency. The sequences alignment and analysis of the haplotypes retrieved from GenBank database allowed a carefully examination of polymorphic restriction sites to detect variation among species, excluding sites with intra-specific variation.

The identification of *Gymnotus* species fished for live bait is extremely important for the management of this fishery resource and as a result to determine proper fisheries regulation. The current fishing legislation in the Paraná River Basin refers to *Gymnotus* species as *G. carapo* and determines the minimum capture size of 20 mm (IBAMA, 2009). Thus, the present outcomes show the need for legislation change to include other *Gymnotus* being caught as live bait. The minimum capture size depends on the captured species, since each species has its reproduction cycle. Sousa *et al.* (2017) also suggested a revision of capture legislation in the Pantanal of Mato Grosso do Sul, because it recognizes two *Gymnotus* species. However, these authors found three species of *Gymnotus* commercialized as live bait in the region.

The use of live bait has been concerning worldwide due to risks of introduction of invasive alien species by sport anglers and the ecological impact on the native biodiversity once this live bait species become established (Sá *et al.*, 2017). At the same time, even local fish species being indiscriminately used as live bait can impact the long-term survival of local species population. Species identification and management through legal monitoring is central to maintain sustainable fishing by local communities. The molecular surveillance tool developed herein allows the implementation of a rapid and low-cost diagnostic system by PCR-RFLP to identify *Gymnotus* species in the sampling fishing area of this study, which can be applied to yield seasonal *Gymnotus* fishing data and support a reliable fishing regulation to use this fisheries resources sustainably.

Acknowledgments

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