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When You Get What You Haven't Paid for: Molecular Identification of "Douradinha" Fish Fillets Can Help End the Illegal Use of River Dolphins as Bait in Brazil

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Abstract

The fishery for *Calophysus macropterus*, an Amazonian necrophagous catfish, is highly detrimental to river dolphins and caimans, which are deliberately killed for use as bait. In the Brazilian Amazon, this fishery has increased over the last decade, in spite of the rejection of scavenger fishes by Brazilian consumers. It was suspected that *C. macropterus* fillets were being sold in Brazilian markets, disguised as a fictitious fish (the "douradinha"). We collected 62 fillets from "douradinha" and other suspiciously named fish from 4 fish-processing plants sold at 6 markets in Manaus, in the Brazilian Amazon, and sequenced the cytochrome *b* gene to identify fillets to species. Sixty percent of fillets labeled "douradinha" or with other deceptive names were actually *C. macropterus*. Six other fish species of low commercial value were also found. The presence of dolphin tissue in the stomach contents of *C. macropterus* was confirmed by mtDNA control region sequencing. Our results formed the scientific basis for a moratorium on the fishing and fraudulent selling of *C. macropterus*, issued by the Brazilian Ministries of the Environment and Fisheries. Exposure of this fraud via the mass media can help end the illegal use of dolphins as bait in Brazil.

Resumen

La pesca del bagre carroñero Amazónico *Calophysus macropterus* es altamente perjudicial para los delfines de río y caimanes, que son sacrificados deliberadamente para emplear su carne como carnada. A pesar de la aversión de los consumidores brasileños por peces carroñeros, la pesca de *C. macropteurs* ha aumentado en la última década en la Amazonia Brasileña, creando la sospecha de que filetes de *C. macropterus* están siendo vendidos en mercados brasileños como si fueran de otro pescado: la "douradinha". Colectamos 62 filetes de "douradinha" y otros pescados de nombre sospechoso en 4 frigoríficos, vendidos en 6 mercados de Manaus, en la Amazonia Brasileña, y secuenciamos el gene citocromo *b* para identificar la especie de los filetes. Sesenta por ciento de los filetes vendidos como "douradinha" u otros nombres engañosos de hecho eran *C. macropterus*. Otras seis especies de pescado de bajo valor comercial también fueron encontradas en los filetes. La presencia de tejido de delfín en contenidos estomacales de *C. macropterus* fue confirmada molecularmente. Nuestros resultados dan soporte científico a la moratoria de pesca y venta fraudulenta de *C. macropterus*, firmada por los Ministerios Brasileños del Ambiente y de la Pesca. La exposición de este fraude en los medios masivos de comunicación puede ayudar a detener el uso ilegal de delfines como carnada en Brasil.

Subject areas: Conservation genetics and biodiversity, Molecular systematics and phylogenetics
Key words: *Calophysus macropterus*, conservation genetics, forensics, Inia geoffrensis, piracatinga, Sotalia fluviatilis

Amazonian river dolphins were traditionally respected and feared because local folklore attributed magical powers to them (da Silva and Best 1996; Slater 2001). Incidental capture and intentional killing by fishermen did occur, but Amazonian legends offered some protection to the "boto" or pink river dolphin (*Inia geoffrensis*) and the tucuxi (*Sotalia fluviatilis*). The early 2000s marked the start of a change in this traditional protection, as the first reports appeared on the use of dolphins and caimans as bait in the fishery for *Calophysus macropterus* (Pimelodidae), a scavenger catfish known as "piracatinga" in Brazil, and "mota" or "zamurito" in Colombia and Venezuela (Estupiñán et al. 2003; Silveira and Viana 2003). This catfish largely replaced, either explicitly or implicitly, the overfished "capaz" *Pimelodus grosskopfii*, which is considered a delicacy in Colombia (Gómez et al. 2008; Salinas et al. 2014).

The use of dolphins and caimans as bait to catch *C. macropterus* began in Brazil (Estupiñán et al. 2003; Silveira and Viana 2003) but seems to have spread to neighboring countries (Aya et al. 2010; Tavera et al. 2010; Trujillo et al. 2010a; Diniz 2011). Both Amazonian river dolphins have been used as bait, but fishermen prefer the boto, because it yields more fish per carcass (Estupiñán et al. 2003; Brum 2011; da Silva et al. 2011; Iriarte and Marmontel 2013). Although by-caught dolphins are also used as bait, intentional killing has become widespread (Estupiñán et al. 2003; Trujillo et al. 2010a; da Silva et al. 2011; Diniz 2011; Alves et al. 2012; Brum and da Silva 2013), and mortality has increased to threatening levels. Using fish landing reports and the fish yield of a dolphin carcass, da Silva et al. (2011) estimated that approximately 1650 dolphins are killed each year in a single area in the Brazilian Central Amazon. In the Mamirauá Sustainable Development Reserve, minimum-count surveys of artificially marked botos were conducted from 1994 to 2007, and a decrease of about 10% per year was observed after 2000 (da Silva et al. 2011). In the same area, apparent survival declined in the interval 2001–2011 in comparison to 1994–2000, and dolphin harvest was estimated to be at three times the sustainable level (Mintzer et al. 2013). These studies demonstrate that intentional killing for use as bait is a major threat to river dolphin populations.

Initially, all *C. macropterus* produced from Brazil was exported, either legally or illegally, to Colombia (Estupiñán et al. 2003; Gómez et al. 2008). Brazilian consumers are disgusted by scavenger fishes such as *C. macropterus*, which is locally known as "the water vulture." In spite of this, a local production chain was firmly established by 2008 (da Silva et al. 2011). Thus, we hypothesized that the "douradinha," a fish which appeared in Brazilian markets around 2008 and which did not correspond to any known Amazonian fish species, might be *C. macropterus* disguised as a fictitious fish. Douradinha are conveniently not identifiable morphologically, because they are exclusively sold processed, as frozen fillets.

In recent decades, fisheries and wildlife forensics have greatly benefited from molecular approaches (e.g., Baker and Palumbi 1994, Roman and Bowen 1998, Marko et al. 2011). We used molecular tools to assign douradinha fillets to known species, and to identify *C. macropterus* stomach contents. Our goals were 1) to provide scientific proof of the fraudulent mislabeling of *C. macropterus*, a crime against consumers according to Brazilian law (Article 66 of Federal Law Nº. 8.078/90); 2) if proven, to inform consumers about the true identity of douradinha, to diminish its sales and relieve pressure on dolphin and caiman populations; and 3) to create consumer awareness about the illegal killing of river dolphins for bait, and increase public pressure for ending the practice.

Materials and Methods

Fish with 3 suspicious names not corresponding to known Portuguese common names were analyzed: "douradinha," "douradinho," and "piratinga." Additionally, on 2 occasions, packages of fish were labeled "dourada," but the salesperson assured the buyer that they contained "douradinha" (Table 1). Fish fillets originated from 4 fish-processing plants in Manacapuru and Manaus and were sold frozen, in packages with 6 to 20 individual fillets. They were acquired in 2 supermarket chains, 3 street markets, and a fish shop in the city of Manaus, Amazonas State, from October 2011 to June 2012. Sixty-two fillets from 9 packs were sequenced. Packs and fillets were photographed, and samples were labeled according to fish-processing plant and batch number. Two *C. macropterus* vouchers were also sequenced. One of them was collected in the Mamirauá Reserve, Central Amazon, and the other was acquired unprocessed from the Central de Abastecimento (CEASA) Market of Manaus. Both specimens were deposited in the ichthyological collection of Instituto Nacional de Pesquisas da Amazônia, under codes 46736 and 46735.

Stomach contents from 2 *C. macropterus* collected in the Mamirauá Reserve were also analyzed. A fragment of muscle was removed from each stomach and preserved in ethanol.

Total DNA was extracted from fillets through the salting-out protocol (Miller et al. 1988). Although controlling for potential sources of contamination is paramount in studies that may be used in a legal context, in our case samples were high quality (having been frozen while fresh) and were collected from a central portion

Table 1. Sampling of frozen "douradinha" fillets in Manaus, State of Amazonas, Brazil

Fish-processing plant	City	Price per kg (US$)	Market	Name	Purchase date	Number of batches / collected samples / sequenced samples
Frigopesca	1	6.86	Carrefour	Douradinha[a]	10/23/2011	1 / 8 / 8
Friolins	2	3.67	Feira do CIGS[c]	Dourada[b]	10/29/2011	3 / 52/ 22
J. Carneiro	2	6.12	Repropesca	Dourada[b]	10/29/2011	1 / 17 / 9
Peixão	1	5.22	Supermercado Veneza	Douradinho	10/25/2011	2 / 38 / 18
—	1	—	Feira do Coroado[c]	Douradinha	10/29/2011	1 / 4 / 2
—	1	4.86	Feira do Peixe[c]	Douradinha	06/22/2012	1 / 4 / 3

City: location of the processing plants or open markets. 1: Manaus, 2: Manacapuru.
[a]The pack was labeled "douradinha", but market label and invoice both stated "piratinga".
[b]Despite the existence of a fish named "dourada" (*Brachyplatystoma juruense*), seller assured the buyer that fillets were "douradinha."
[c]Artisanal street markets.

of any given fillet, where quantities of target DNA vastly outnumbered any potential contamination. The complete cytochrome *b* gene was amplified through polymerase chain reaction (PCR) using the primers CytbSiluF and CytbSiluR (Villa-Verde et al. 2012), in 20-µL reactions containing approximately 20–100 ng of DNA template, 1U Taq, 1× enzyme buffer, 200 µM dNTP, 2.5 mM $MgCl_2$, 1 µg/µL bovine serum albumin, and 0.5 µM of each primer. Amplification thermal conditions were as follows: 4 min at 94 °C; 35 cycles of 45 s at 93 °C, 45 s at 53 °C, and 45 s at 72 °C; and 5 min of final extension at 72 °C. All PCR experiments included negative controls.

The DNA of stomach contents was isolated using standard phenol-chloroform extraction (Sambrook et al. 1989). The mitochondrial control region was amplified by PCR using primers RCPb-F and RCPb-R (Cunha et al. 2014), in 20-µL reactions with reagent concentrations and negative controls as described above. Cycle sequencing was performed as follows: 3 min at 93 °C; 30 cycles of 1 min at 92 °C, 1 min at 48 °C, and 1 min at 72 °C; and 5 min of final extension at 72 °C.

PCR products were purified using the Agencourt AMPure XP kit (Beckman Coulter), and both strands were sequenced in an ABI3500 genetic analyzer using BigDye chemistry (Applied Biosystems) with the same primers as those used for amplification. Sequences were edited in SeqMan 7 (DNAStar Inc.) and deposited in GenBank.

DnaSP 5 (Librado and Rozas 2009) was used to define haplotypes. A similarity search using the BLASTn algorithm (Altschul et al. 1990) was conducted in GenBank. Identifications were considered successful when they had identity over 99% ($E < 0.01$). For phylogenetic analyses, sequences were visually aligned in MEGA 5 (Tamura et al. 2007). Cytochrome *b* sequences ($N = 76$) from most pimelodid species ($N = 57$) and from 9 siluriform species, available in GenBank, were included in the analysis. A neighbor-joining tree of cytochrome *b* haplotypes was built in MEGA using the Kimura 2-parameter evolution model (K2P, Kimura 1980), and 10 000 bootstrap replicates were conducted to assess node confidence. The relationships among confirmed *C. macropterus* haplotypes were visualized in a median-joining network built with Network (Bandelt et al. 1999; www.fluxus-engineering.com).

Control region sequences from stomach contents were also identified using a BLASTn search in GenBank and through phylogenetic analyses using the neighbor-joining algorithm.

Data Archiving

In compliance with the data archiving policy (Baker 2013), all novel sequences have been deposited in GenBank under accession numbers KM268907-KM268913 and KP241036.

Results

Identity of "Douradinha" Fillets

The mtDNA cytochrome *b* gene was successfully amplified and sequenced for all 62 fillets collected from markets and from the 2 *C. macropterus* vouchers. Similarity searches and phylogenetic analyses showed that about 60% of fillets labeled "douradinha" or with other deceptive names were actually from *C. macropterus* (Figures 1 and 2). Six other pimelodid species were also identified: *Hypophthalmus* cf. *edentatus* (21%), *Pinirampus pirinampu* (11.3%), *Hypophthalmus* cf. *marginatus* (3.2%), *Pimelodina flavipinnis* (3.2%), *Ageneiosus ucayalensis* (1.6%), and *Oxydoras sifontesi* (1.6%) (Figure 2). One sequence (FP01) had 99% similarity to an undescribed pimelodid species (GenBank JQ624020). *Calophysus macropterus* was observed in packs from all fish-processing plants and in all street market samples. However, the frequency of *C. macropterus* sold varied from 100% (3 different batches from the same processing plant, $N = 22$; and all fillets from street markets) to 0 (1 batch from another processing plant, which was entirely *Hypophthalmus* cf. *edentatus*, $N = 13$).

Calophysus Macropterus Haplotype Analyses

Analysis of *C. macropterus* cytochrome *b* sequences ($N = 32$) revealed 26 polymorphic sites, which defined 7 haplotypes. The most common haplotype was observed in individuals sold by all fish-processing plants and street markets and included the voucher obtained in the CEASA market as well as a *C. macropterus* sequence deposited in GenBank (JF898528) (Figure 3). The other haplotypes were rare.

A highly divergent *C. macropterus* haplotype was found in a sample from one of the processing plants, in a batch of 8 fillets, which contained another *C. macropterus* haplotype and 4 other fish species.

Calophysus Macropterus Stomach Contents

Control region sequences obtained from both stomach content samples were 592 bp and of good quality. Similarity and phylogenetic analyses identified both samples as belonging to *Inia geoffrensis* (Figure 4). A haplotype network built using all *I. geoffrensis* sequences available in GenBank revealed that one of the sequences was a new haplotype, genetically closest to a haplotype from the Brazilian and Colombian Amazon (reported by Banguera-Hinestroza et al. 2002 and Gravena et al. 2014). The new haplotype was deposited in GenBank (KP241036). The other sample had a haplotype already observed in the Brazilian Amazon, more specifically in the Mamirauá Reserve (haplotype MM3 of Hollatz et al. 2011; Supplementary Figure S1 online).

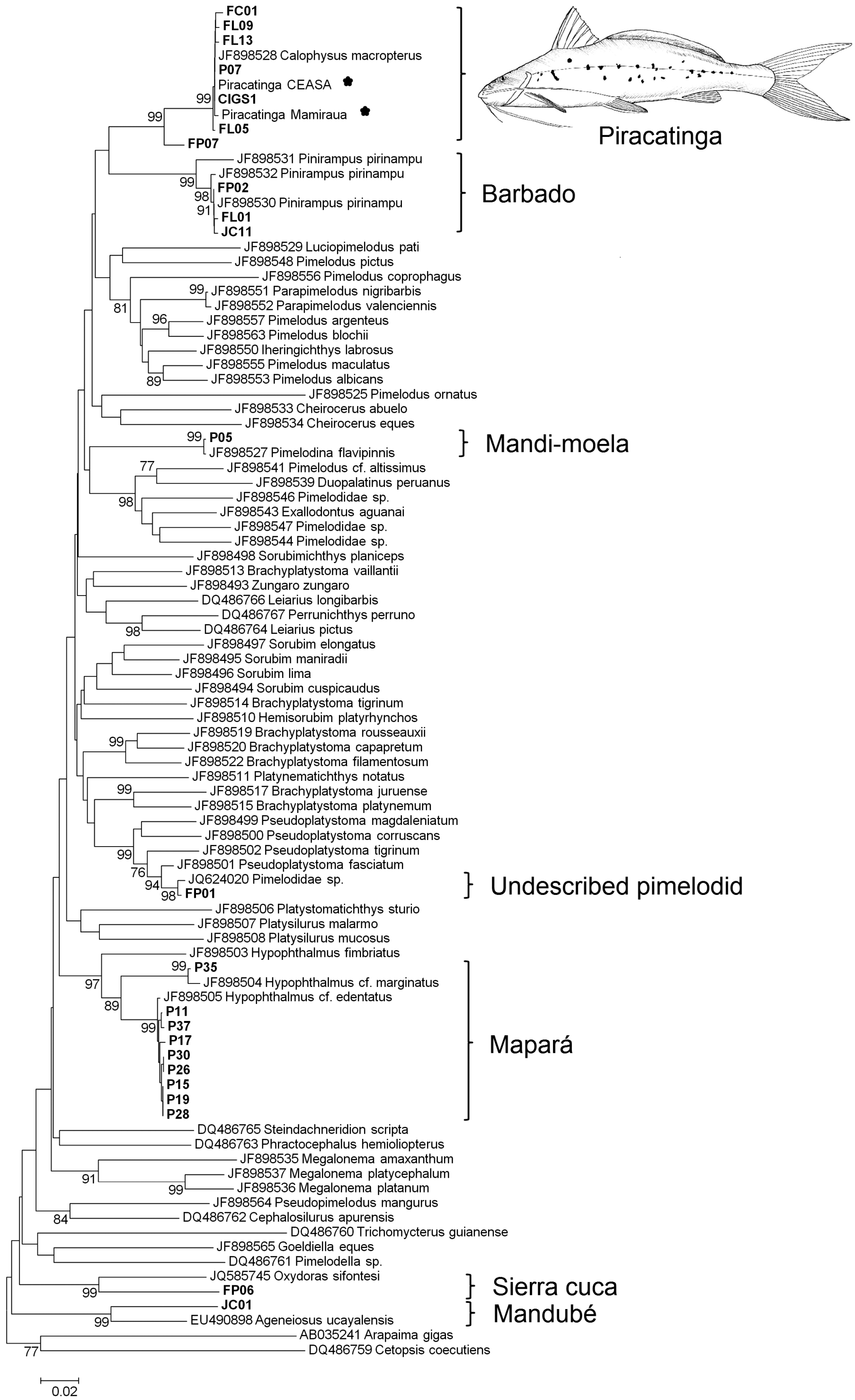

Figure 1. Phylogenetic tree (NJ/K2P) of the cytochrome *b* gene of Pimelodidae species, including the haplotypes found in "douradinha" samples (in bold letters) and voucher sequences from potential source species. Stars indicate *C. macropterus* vouchers sequenced in this study. Numbers above nodes correspond to bootstrap values (10 000 replicates). Brackets refer to pimelodid species found among "douradinha" fillets, with their common names. *Calophysus macropterus* drawing from Agudelo et al. (2000).

Discussion

"Douradinha" Hides *C. macropterus*

Our results showed that 60% of fish sold as "douradinha" or under other suspicious names were *C. macropterus*. The fact that several different new names (Table 1) were used for *C. macropterus* strongly suggests that fish-processing plants are intentionally deceiving consumers and not simply replacing an unfortunate name ("piracatinga" comes from the indigenous Tupi "pirá" and "catinga", which jointly mean "stinky fish", Dias 1858) with a more attractive one,

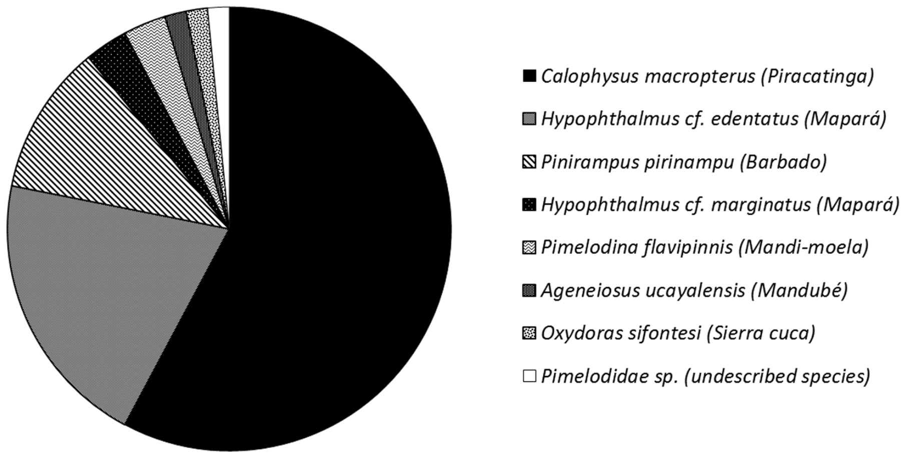

Figure 2. Frequency of the 8 fish species identified among "douradinha" fillets (*N* = 62). Common names are between parentheses (note that 2 species of genus *Hyphophtalmus* have the same common name).

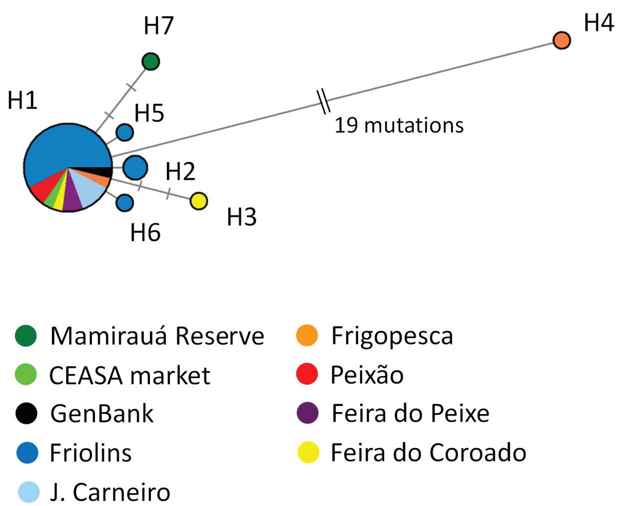

Figure 3. Median-joining haplotype network of the cytochrome *b* gene of *C. macropterus*. Colors represent the processing plants, markets or site (in the case of Mamirauá) from which samples were collected (see Table 1). The individual from GenBank had unknown sampling locality. Circle size is proportional to frequency. Branch length reflects molecular distance: each stretch of branch accounts for one mutation.

as has been done in the "rebranding" of other fish species formerly scorned by consumers. All 4 fish-processing plants, which include the largest processing plants from Manaus and Manacapuru (State of Amazonas), sold *C. macropterus* under fictitious names. This fraud was also observed in 3 street markets. Fraud in the trade of catfish seems to be common in Brazil, as supported by Carvalho et al. (2011), who found a similarly high rate of mislabeling (80%) in "surubim" (*Pseudoplatystoma* spp.).

The fillet packs analyzed in this study were bought in supermarket chains, open markets, and a fish store, confirming the existence of a retail market for *C. macropterus* in Brazil. Moreover, the owner of one processing plant stated that "douradinha" is the fish he offers in public bids to the State of Amazonas, because of its low price. Thus, it is very likely that public schools, hospitals, penitentiaries, and the army are important consumers of *C. macropterus*. There are reports of "douradinha" being sold to other Brazilian regions (northeast, central-west, and southeast; Flores et al. 2008; da Silva VMF, personal observation). To date, we have not found "douradinha" fillets being sold outside Manaus, but our sampling effort in other cities was only opportunistic.

Calophysus macropterus markets in Colombia and Brazil are different but also share some similarities. While in Colombia *C. macropterus* is sold fraudulently as "capaz" (*Pimelodus grosskopfii*), in Brazil new common names were invented for the species. In both countries, there is little consumption of *C. macropterus* at fishery or landing sites (e.g., Leticia in Colombia and Tefé in Brazil), and the important markets are large cities (Bogotá, Cali, Girardot, and Melgar in Colombia, Gómez et al. 2008; Salinas et al. 2014; and Manaus in Brazil, this study). In Brazil, *C. macropterus* is one of the cheapest river fish meats available (about US$5/kg; Table 1). At fishing or landing sites, fishermen receive approximately US$0.50 to US$1.00/kg, depending on seasonal availability of *C. macropterus* (Brum 2011; Iriarte and Marmontel 2013; Brum et al. 2013). Hence, both fishermen and processing plant owners need large numbers of *C. macropterus* to make their businesses profitable, which results in higher hunting pressure on dolphins.

Another observed similarity between the Colombian and Brazilian markets is the average price of *C. macropterus* (Salinas et al. 2014; and this study). The upper threshold for the price in Colombian markets is probably regulated by the availability of the preferred *Pimelodus grosskopfii* (sold at US$12/kg; Salinas et al. 2014).

In Colombia, molecular identification confirmed that *C. macropterus* is being sold as "capaz" (*P. grosskopfii*) and revealed that two-thirds of this fish collected in supermarkets came from the Amazon basin and one-third from the Orinoco (Salinas et al. 2014). As in Brazil, other fish species are also involved in the fraud, but at a lower frequency; *C. macropterus* constituted more than 90% of the fish sampled in Colombia (Salinas et al. 2014), compared with 60% in Brazil (this study).

Beltrán-Pedreros et al. (2011) and Salinas et al. (2014) raised another concern: high mercury concentration in *C. macropterus* meat, which could lead to serious public health issues if this fish is ingested frequently and in large quantities. Average total mercury concentrations in Brazil and Colombia were higher than the limit set by the World Health Organization (0.5 µg/g; Beltrán-Pedreros et al. 2011; Salinas et al. 2014). Therefore, risk to human health is another important reason to prohibit trade in *C. macropterus*.

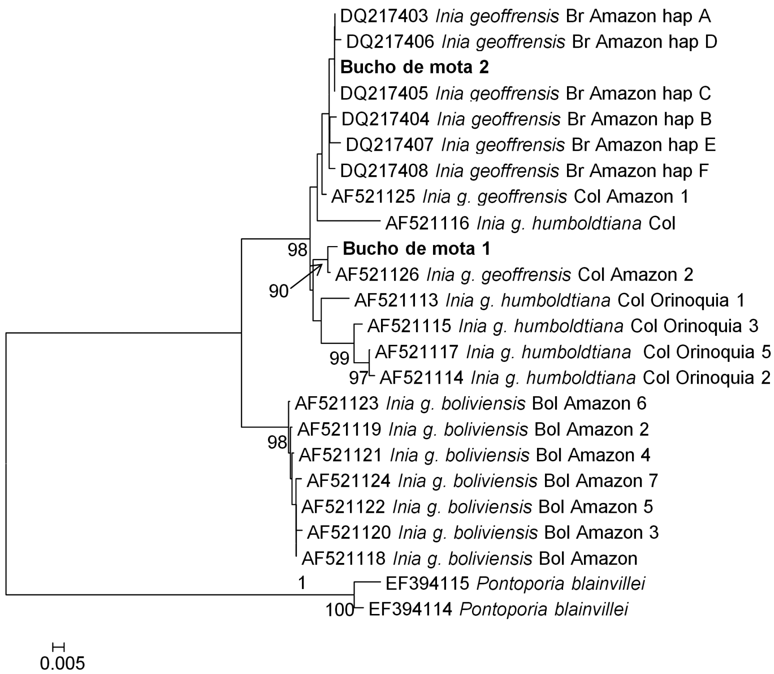

Figure 4. Phylogenetic tree (NJ/K2P) of the mitochondrial control region of *Inia* spp. haplotypes, including the 2 haplotypes found in *C. macropterus* stomach contents (in bold letters, "Bucho de mota" means "Piracatinga stomach"). Numbers above nodes correspond to bootstrap values (10000 replicates). Bol, Bolivia; Br, Brazil; Col, Colombia. Sequences of *Pontoporia blainvillei* were used as outgroup.

Finally, our molecular data confirmed the presence of *I. geoffrensis* in the stomachs of *C. macropterus*. The control region sequences indicated that the individuals used as bait were from the Central West Amazon, where the fishery of *C. macropterus* using botos as bait is best documented (Brum 2011; Brum et al. 2013; Iriarte and Marmontel 2013).

Implications for Conservation

The fishery for *C. macropterus* is highly detrimental to river dolphins, which are used as bait. This activity yields reasonable income with considerably less effort than other fisheries or working options in the Amazon (Brum 2011; Iriarte and Marmontel 2013), and the lack of control by government agencies contributes to its spread. A new occupation, the dolphin hunter, has emerged to supply fishermen with bait (Estupiñán et al. 2003; Brum 2011; Alves et al. 2012; Brum and da Silva 2013). Thus, during the last decade, the fishery for *C. macropterus* has spread in volume and geographic extent. The intentional killing of dolphins has reached unsustainable levels and has become a serious threat (da Silva et al. 2011; Mintzer et al. 2013).

In order to tackle this problem, and before dolphin populations are irreversibly depleted, researchers have proposed urgent actions (Estupiñán et al. 2003; Flores et al. 2008; Trujillo et al. 2010b; Barreto et al. 2011; Iriarte and Marmontel 2013), which include a ban on the *C. macropterus* fishery and media campaigns to create consumer awareness about the fraud and its consequences for dolphins. In Brazil, where dolphin hunting and its use as bait is more intense, only the regulation or control of fishing and selling of *C. macropterus* is likely to reduce the threat to dolphins. Brazilian law prohibits the capture, killing, and harassment of all cetaceans (Federal Law Nº. 7.643/87), so there is a legal basis to close the fishery for *C. macropterus* at least provisionally. However, controlling fishing and the illegal killing of dolphins in the vast Amazon territory is very difficult, especially with the limited funding and personnel available to Brazilian environmental agencies. Therefore, it is important to decrease demand for *C. macropterus*, in order to reduce smuggling from Brazil and also eliminate the incipient Brazilian internal market.

The molecular identification of "douradinha" fillets will be fundamental in the implementation of any moratorium on the fishery for *C. macropterus*. Until proper regulation is developed, "douradinha" fillets must be unambiguously identified, thus requiring the use of molecular techniques. As shown by our results, the assignment of fillets to species by their commercial names is not straightforward, as "douradinha," "dourada," "piratinga," etc., all hide *C. macropterus* as well as other fish species. Moreover, once those names become exposed as false, processing plants may start using other fictitious names to disguise illegal sales of *C. macropterus*. Therefore, both during a moratorium and after, molecular identification will be the most effective way to ensure that *C. macropterus* is not being illegally sold.

Molecular tools can also be usefully applied to routinely identify *C. macropterus* stomach contents, especially if the fishery is allowed using alternative baits. The long, good-quality sequences we obtained from 2 stomachs demonstrates that molecular identification is feasible. A diagnostic kit that can detect and discriminate all dolphin and caiman species illegally used as bait is being validated (Cunha HA, unpublished data) and may be an important tool for the control of the *C. macropterus* fishery.

In December 2013, our evidence of fraud in the selling of "douradinha" was presented to the Amazon State Public Prosecutor, who recommended the ban of this activity. As a result, the Brazilian Ministry of the Environment and Ministry of Fisheries issued a 5-year moratorium on the fishery and trade of *C. macropterus*, to take effect in January 2015 (Instrução Normativa Interministerial n° 6, of July 17th, 2014). Only subsistence catch and consumption (up to 5 kg/day) will be allowed until researchers and government agents devise alternative baits and appropriate fishery regulations and mechanisms of control.

Molecular confirmation of fraud will also be needed to convince consumers, and to help draw media attention. Raising consumer awareness about the fraudulent selling of *C. macropterus* and its adverse impact on river dolphin populations is likely to reduce impacts (Teisl et al. 2002; van der Ploeg et al. 2011), and the most effective way may be via media campaigns (Teisl et al. 2002; Howe et al. 2012). Consumers tend to have a strong negative reaction to this type of fraud, in which they are misled to consume a fish they find disgusting. But the most important aspect in this case is that it involves the killing of botos, which are charismatic animals. Highlighting results from the present study in the media may influence public opinion, eradicate the internal market and the national demand for the "douradinha," and ultimately force Brazilian authorities to take other necessary actions to stop the illegal and detrimental use of river dolphins as bait.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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