The fishing and illegal trade of the angelshark: DNA barcoding against misleading identifications

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ABSTRACT

Morphological identification in the field can be extremely difficult considering fragmentation of species for trade or high similarity between congeneric species. In this context, the shark group belonging to the genus *Squatina* is composed of three species distributed in the southern part of the western Atlantic. These three species are classified in the IUCN Red List as endangered, and they are currently protected under Brazilian law, which prohibits fishing and trade. Molecular genetic tools are now used for practical taxonomic identification, particularly in cases where morphological observation is prevented, e.g., during fish processing. Consequently, DNA barcoding was used in the present study to track potential crimes against the landing and trade of endangered species along the São Paulo coastline, in particular *Squatina guggenheim* (n = 75) and *S. occulta* (n = 5), as well as the Brazilian guitarfish *Pseudobatos horkelii* (n = 5). DNA barcoding revealed the continuous fishing and trafficking of these protected species, thus giving clear evidence that the current conservation models and methods of monitoring are not working.

1. Introduction

The Elasmobranchii subclass of Chondrichthyes currently consists of 509 shark species and 630 rays (Weigmann, 2016). However, this biodiversity should be greater owing to the continuous description of new species in recent years (Borsa, 2016). Out of this total, more than 90% is listed on the IUCN Red List with about 16.5% in the threatened categories and at least 40% listed as "Data Deficient" (IUCN, 2017). Such statistics call for the immediate collection of biological data toward formulating and implementing novel plans for the conservation of these species.

Several characteristics make this group especially susceptible to overexploitation, such as longevity, late sexual maturation, low fecundity and long gestation periods (Stevens et al., 2000; Dulvy et al., 2008; Field et al., 2009). Consequently, many Elasmobranch species are impacted by artisanal fisheries, as well as recreational and industrial fishing for meat, fins, liver oil, or cartilage (Vannuccini, 1999). Early on, the lower commercial values of these species tended to limit direct exploitation (Walker, 1999; Molina and Cooke, 2012). Nowadays, however, estimates indicate that about 100 million sharks are caught annually, even when excluding illegal, unreported and unregulated catches (Liu et al., 2013).

Even with growing awareness of the vulnerability of most species of sharks and rays to exploitation (Castro, 1987; Camhi, 1998), attempts to manage species of this group have largely failed, essentially by the lack of basic information on catches, landings, and commercial sales. The correct management of any species, including Elasmobranch, is based on precise identification (Oliver et al., 2015; Davidson et al., 2016). In this case, however, morphological identification is made very difficult by the common fishing practice of cutting off the head, fins and tail, which are deposited at sea, to increase the space needed for storage and to preserve the meat longer (De Franco et al., 2012).

Among exploited sharks, angelsharks comprise the second most threatened Elasmobranch family globally, and they stand out in many regions of the world (Dulvy et al., 2014; Meyers et al., 2017). The angelshark genus *Squatina* (Squatinidae) comprises 22 extant,

morphologically homogeneous, benthic species (Vooren and Da Silva, 1992; Compagno, 2005; Castro-Aguirre et al., 2006; Last and White, 2008), which inhabit continental shelves and upper slopes down to 500 m (Compagno, 2005). They are moderately sized (total length about 1–2 m) and globally distributed in temperate to tropical seas (Compagno, 2005; Last and White, 2008). While some species occur over a wide geographic range, most are restricted to a smaller area (Compagno, 2005). Among the species distributed in the coastal waters of southeastern Brazil and Patagonian central Argentina (Cousseau and Figueroa, 2001; Vooren and Klippel, 2005), the spiny angelshark *Squatina guggenheim*, the Argentine angelshark *S. argentina*, the Hidden angelshark *S. occulta* and the Atlantic angelshark *S. dumeril* are frequently landed and marketed, despite conservation laws controlling local fisheries. The morphological identification of such phylogenetically closely related species is particularly difficult, and Vaz and de Carvalho (2013) have only recently reviewed the taxonomy of the genus and validated three of these species, especially samples with morphological traits related to angelsharks.

Given these results, the development of molecular tools applied to species identification has gained in importance, especially for the quantification of exploited natural populations, evaluation and inspection of the trade of species under government protection, and the certification of processed products, adding value to their commercialization (Ogden, 2008; Migone and Howlett, 2012; Maralit et al., 2013; Helyar et al., 2014). Studies using genetic identification of fishery products are appearing in the literature with more frequency, and some of them have been directed to the shark group (e.g., Clarke et al., 2006; Sebastian et al., 2008; De Franco et al., 2012; Maduna et al., 2017).

In view of the capture and commercialization of Elasmobranch species worldwide, even legally protected species, the usual method of fish processing that prevents morphological identification, and the natural difficulties encountered when distinguishing a large number of species, the present study aimed to use DNA barcoding to track potential crimes against the landing and trade of endangered species along the São Paulo coastline.

2. Material and methods

2.1. Sampling

The samples were obtained between 2015 and 2016 from industrial fishing boats that use bottom trawls and from regional fish distribution markets in the coastal regions of São Paulo, Brazil (Fig. 1). Muscle fragments were collected from 85 carcasses declared by fishermen and traders only as cação (shark) in the cities of Ubatuba (13), Santos (15), Praia Grande (1), Peruíbe (1) and Cananéia (55). Researchers were able to identify morphological traits and relate them to angelsharks. Collected material was preserved in 95% ethanol and stored at −20 °C at the Laboratório de Genética Pesqueira e Conservação (GenPesC) at the Universidade Federal de São Paulo (UNIFESP), Campus Baixada Santista.

2.2. DNA extraction, amplification and sequencing

Genomic DNA extraction was done with the NucleoSpin® Tissue kit (Macherey-Nagel), following the manufacturer's instructions. Amplification of the mitochondrial cytochrome oxidase *c* subunit I gene (COI) was performed using the enzyme Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) with the following primers: Forward - FishF1: 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3' and Reverse - FishR1: 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' (Ward et al., 2005). After amplification, PCR products were subjected to enzymatic purification using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific). Sequencing followed the BigDye® Terminator v3.1 Cycle Sequencing kit protocol (Thermo Fisher Scientific), and the sequences were generated by the ABI PRISM® 3100 Genetic Analyzer (Thermo Fisher Scientific) automatic sequencing platform.
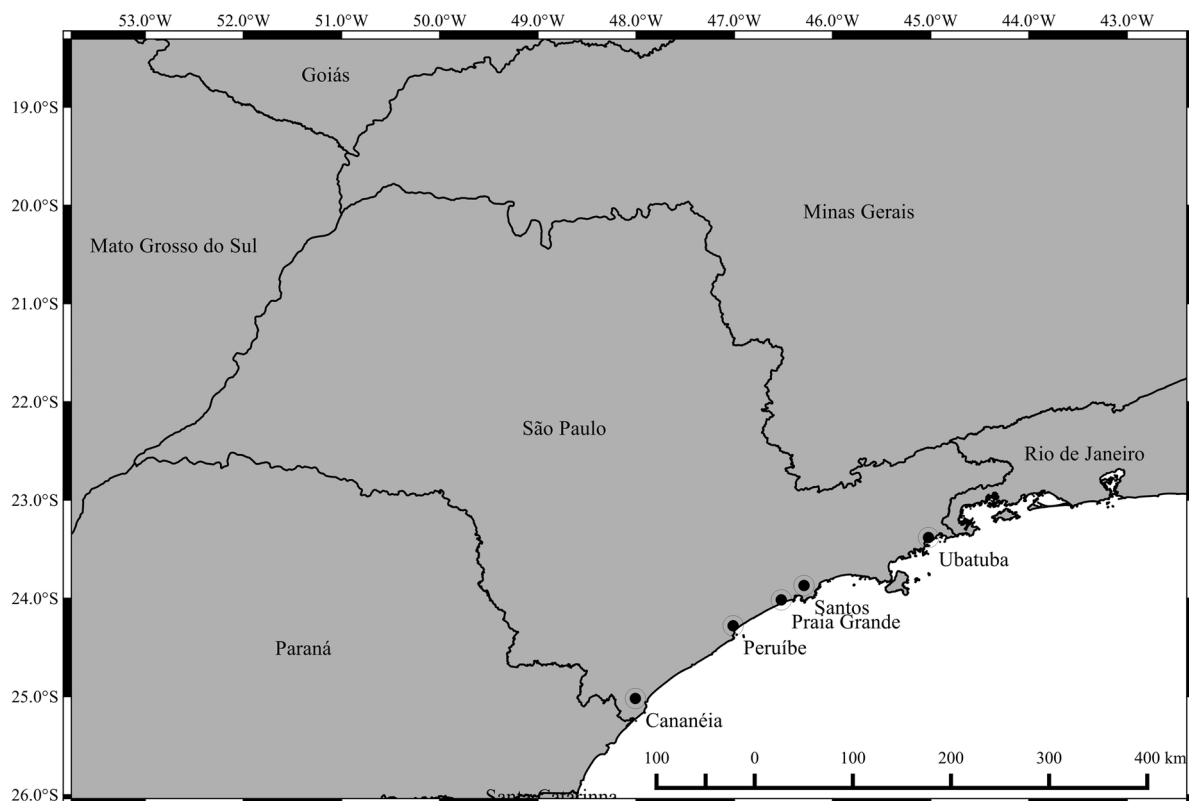

Fig. 1. Sampling by geographic location in the State of São Paulo, Brazil. Black dots represent the algelsharks sampling localities.

Table 1
Angel shark species sampled along the São Paulo coast, Brazil. *n*, number of samples collected per region.

| São Paulo region	*n*	*Squatina guggenheim*	*S. occulta*	*Pseudobatos horkelii*
Ubatuba	13	9	4	–
Santos	15	9	1	5
Praia Grande	1	1	–	–
Peruíbe	1	1	–	–
Cananeia	55	55	–	–
Total	85	75	5	5

2.3. Analysis and species identification

The analyses were performed in two steps. First, we compared the similarity of sequences generated individually with the species sequences morphologically identified and available in the Barcode of Life Online Database (BOLD) (http://www.boldsystems.org/) (Ratnasingham and Hebert, 2007) and the Basic Local Alignment Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from GenBank® (Benson et al., 2017). Only sequences with more than 99% similarity to the sequences deposited in these databases were considered.

Next, the evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987) with1000 bootstrap replications shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter (K2P) method (Kimura, 1980) as a model of DNA sequencing evolution to distinguish species found in this study from those in global databases and to give greater support to the identifications. To construct the phylogenetic tree, the generated sequences and sequences extracted from the BOLD

and GenBank databases were aligned through the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004), using Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar et al., 2016) to conduct sequence alignment and infer phylogenetic trees. As an external group, sequences deposited in the BOLD database for the Goblin shark (*Mitsukurina owstoni*) were selected, and another two species, the Atlantic angelshark *Squatina dumeril* and the Southern guitarfish *Pseudobatos percellens*, were utilized to reinforce the molecular identification between closely related species.

3. Results

The species of the 85 carcasses were identified and denominated as cação (shark) by fishermen and merchants of the São Paulo coast. In the genetic analysis, high similarity (99.73% to 100%) was obtained between the DNA sequences generated and the specific standard sequences available in the databases. In all samples, three species of Elasmobranchs were identified (Table 1), most of which belonged to the Spiny angelshark *Squatina guggenheim* (n = 75, 88.23%), the Hidden angelshark *Squatina occulta* (n = 5, 5.88%), and the Brazilian guitarfish *Pseudobatos horkelii* (Last et al., 2016) (n = 5, 5.88%), recently renamed from the genus *Rhinobatos*. The COI DNA sequences were submitted to the GenBank database under the accession numbers MF795326 to MF795410. The analysis involved 30 sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All gaps and missing data were eliminated. The final dataset consisted of a total of 651 base pairs in the final dataset (Fig. 2).
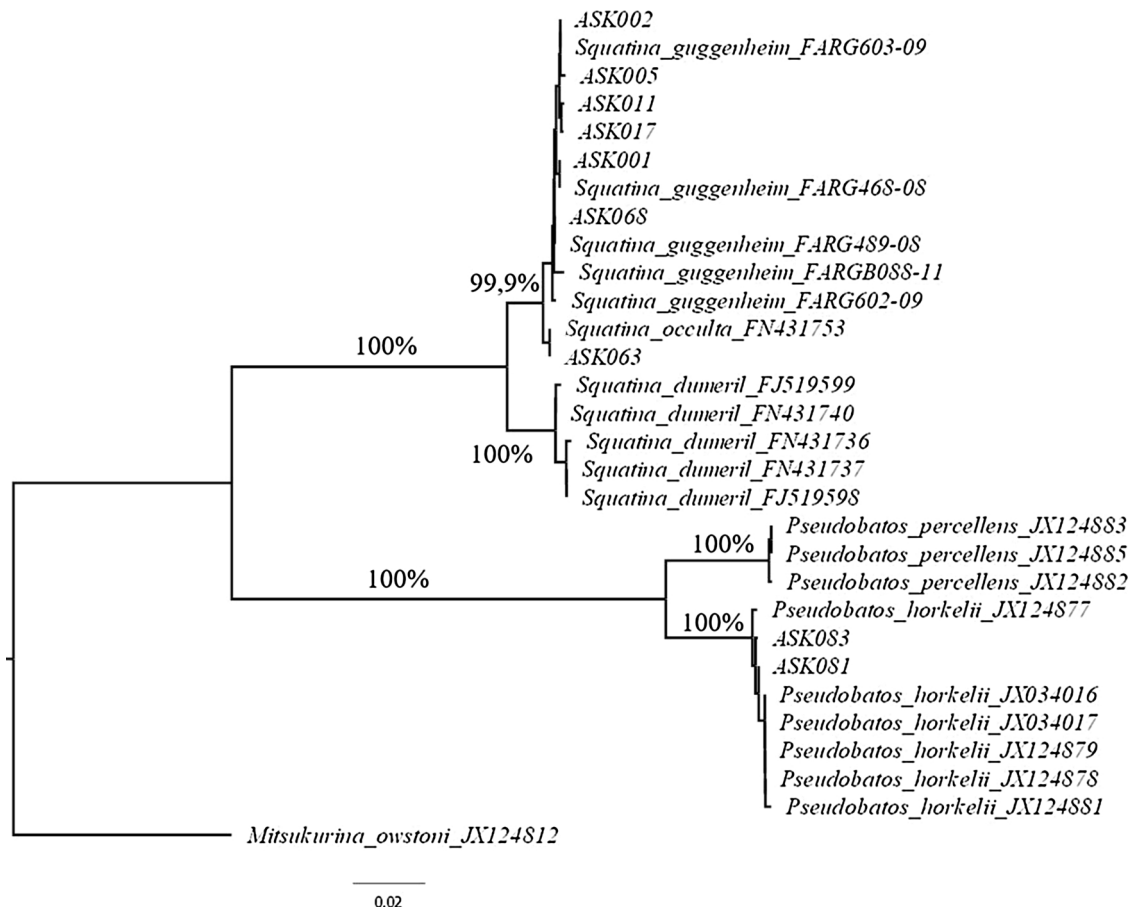
Fig. 2. Molecular Phylogenetic Tree by the Neighbor-Joining Method. ASKXXX code refers to the studied sequenced algelshark individuals. Other species and codes refer to GenBank and BOLD sequences. The percentage in the branches refer to the bootstrap confidence levels for phylogenetic trees.

4. Discussion

This study revealed the capture and commercialization of three species of Elasmobranchs, the angelsharks *Squatina guggenheim* and *S. occulta* and the Brazilian guitarfish *Pseudobatos horkelii*, all registered on the Brazilian List of Endangered Species – Fish and Aquatic Invertebrate. According to Normative Instruction No. 005 of May 21, 2004, revalidated by Ministerial Order Nº 445 of December 17, 2014, the capture, transportation, storage, handling, processing, and trade of these species are completely prohibited in Brazil.

In addition to the threatened species status imposed by the Brazilian government, *S. guggenheim* is listed as "Endangered" (EN) on the IUCN Red List, underscoring the significant fishing pressure on this species throughout its area of incidence (Chiaramonte and Vooren, 2007). Angelsharks are heavily fished in southern Brazil, and significant declines have been documented. An angelshark bottom gillnet fishery commenced around 1990, and at present, large numbers of angelsharks are caught this way (Villwock de Miranda and Vooren, 2003). According to research trawl surveys of the outer shelf in the years 1986/87 and 2001/02, the abundance of the Spiny angelshark in southern Brazil had decreased by 85%, which was attributed to recruitment overfishing. Furthermore, pupping and nursery areas in Brazil occur in shallow inshore waters at depths of < 30 m. Intensive fishing by gillnet and trawl in these nursery areas results in additional pressure on gravid females and juveniles of the species (Silva 1996, Vooren and Klippel, 2005).

Squatina occulta is still listed on the IUCN Red List as "Not Evaluated" (NE). However, its taxonomic status remains controversial (Solé-Cava et al., 1983; Solé-Cava and Levy, 1987; Vooren and da Silva, 1992; Soto, 2001). Based on the IUCN Red List, the species appears as a synonym of *S. punctata*, which is classified as "Endangered" (Musick and Kyne, 2007), even though genetic analysis has validated the species (Furtado-Neto and Carr, 2002; Stelbrink et al., 2010; Falcão et al., 2014). In our phylogenetic tree, *S. occulta* appears as a distinct species and sister group of *S. guggenheim* in concordance with Stelbrink et al. (2010).

Although erroneously related to angelshark carcasses, sampling of the species *P. horkelii* signals the extent of environmental crimes that have been continuously practiced in this region of Brazil. This species is endemic to the southwestern Atlantic. Its distribution center in southern Brazil is subject to trawling in coastal waters, even though it relies on these regions for breeding and nursery areas. Given the reported decline, with the abundance of this species having decreased by > 80% since 1986, and continued fishing pressure in its area of occurrence, the species is rated as "Critically Endangered" (CR) by the IUCN Red List. As pointed out by estimates, without proper protection, this ray could be extinct in about ten years (Lessa and Vooren, 2016). Although protected in Brazil, its capture seems to be continuing, despite laws against this practice and the denunciations coming from researchers and environmentalists. In an evaluation performed by De Franco et al. (2012) using genetic markers to identify species of guitarfish captured and traded in southeastern Brazil, the exploitation of *P. horkelii* was verified as comprising about 56% of rays exploited in Brazil. In the present study, we highlighted a weakness in the current evaluation models in place for controlling fishing in Brazil. In large part, this limitation results from management practices that fail to provide a reasonable record of catches with species-specific data, in turn confounding attempts to prevent illegal fishing and trade of endangered species. De Franco et al. (2012) also noted this same failure with respect to Brazilian guitarfish exploitation.

Worldwide, fish products are processed for commercialization in different ways, thus hindering visual inspection of morphological characters essential for taxonomic identification of captured species (Bornatowski et al., 2013). In Brazil, molecular techniques have been used to identify species of the genus *Squatina* (Falcão et al., 2014) and guitarfish (De Franco et al., 2012). In addition, studies have used molecular markers to identify several species of Elasmobranchs (Mendonça et al., 2010; Ribeiro et al., 2012; Domingues et al., 2013), as well as identify fraudulent labeling of different species of fish (Carvalho et al., 2015) and species threatened with extinction (Rodrigues et al., 2016).

In Brazilian markets, Elasmobranchs are usually sold as "cação", one of their popular trade names. This generalized and nonspecific labeling also complicates efforts to curb consumption of endangered species (Bornatowski et al., 2013). The mitigation of this practice could be done through the use of modern techniques of genetic identification, in particular DNA barcoding to identify Elasmobranchs at species level. This would result in correct identification before sales in supermarkets (Bornatowski et al., 2013).

It is crucial for Brazil to resume its data collection system for all of its fisheries, including data on landings, in order to ensure efficient policymaking and effective management and conservation. To ensure sustainable fishing and inhibit crimes of exploitation, we need to (i) formulate species-level statistics using modern molecular tools, (ii) maintain and continually update lists of endangered species, and (iii) improve the implementation of long-term conservation and management programs.

In conclusion, crimes against the environment, including such practices as the capture and trade of protected species and fraudulent labeling, need to be directly tackled, along with increasing public awareness. This can be directly achieved through the use of techniques like DNA barcoding, which has proven to be a powerful tool in the monitoring and conservation of traded and natural resources. As such, it should be proposed as a standard procedure for identification of species in Brazilian fish landings and in the surveillance of commercial fraud along the production chain through the final delivery of fish products to consumers.

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