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Original Article

Molecular identification of sharks from the genus *Sphyrna* (Elasmobranchii: Chondrichthyes) in Maranhão Coast (Brazil)

Identificação molecular de tubarões do gênero *Sphyrna* (Elasmobranchii: Chondrichthyes) na Costa do Maranhão, Brasil

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Abstract

Sharks of the genus *Sphyrna* are under intense exploitation globally. In Brazil's northern coast, this genus represents a high proportion of fisheries landings and comprises four species. However, due to difficulty of specific identification when specimens are landed, most of the records are limited to the genus level. Here we analyzed the effectiveness of ITS2 (Internal Transcribed Spacer 2 of rDNA) fragment length protocol (Abercrombie et al., 2005) for identifying hammerhead shark species, comparing with the analysis of COI (Cytochrome oxidase subunit I) and ITS2 sequences. We evaluated samples of muscle tissue acquired in the main fishing ports of Maranhão: Carutapera, Raposa e Tutóia. Sampling was conducted between March 2017 to March 2018 and complemented with material deposited in collection (2015). COI results indicated the occurrence of endangered species which are prohibited to be landed. These include *Sphyrna mokarran* (67%), *S. lewini* (15%), *S. tudes* (3%), and *S. tiburo* (15%). For the ITS2 marker, we investigated the optimization of the protocol developed by Abercrombie (2005) for to improve the use in this geographical area througout design of a new primers.

Keywords: Elasmobranchii, Sphyrnidae, COI, ITS2, forensic identification.

Resumo

Os tubarões do gênero *Sphyrna* estão sob intensa exploração em todo o mundo. No litoral norte do Brasil, esse gênero representa grande proporção dos desembarques pesqueiros e compreende quatro espécies. Porém, devido à dificuldade de identificação específica no momento do desembarque dos espécimes, a maioria dos registros limita-se ao nível do gênero. Aqui analisamos a eficácia do protocolo baseado no comprimento de fragmentos de ITS2 (Abercrombie et al., 2005) para identificar espécies de tubarão-martelo, comparando com a análise das sequências COI e ITS2. Foram avaliadas amostras de tecido muscular adquiridas nos principais portos pesqueiros do Maranhão: Carutapera, Raposa e Tutóia. A amostragem foi realizada entre março de 2017 a março de 2018 e complementada com material depositados em coleção (2015). Os resultados do COI indicaram a ocorrência de espécies ameaçadas cujo desembarque é proibido. Estes incluem Sphyrna mokarran (67%), S. lewini (15%), S. tudes (3%) e S. tiburo (15%). Para o marcador ITS2, investigamos a otimização do protocolo desenvolvido por Abercrombie (2005) para melhorar o uso nesta área geográfica através do desenho de novos primers.

Palavras-chave: Elasmobranchii, Sphyrnidae, COI, ITS2, identificação forense.

1. Introduction

Sharks (Superorder Selachimorpha) are key components for the structure and balance of food chains in estuarine, marine, and oceanic ecosystems (Libralato et al., 2006; Dulvy et al., 2014). These animals can be meso or apex predators (Heithaus et al., 2012) and play a big role in the structural maintenance of food webs, as well as the energy flow across trophic levels (Baum and Worm, 2009; Fogliarini et al., 2021; Cruz et al., 2021). Nevertheless, several species are exploited by humans worldwide, and are considered the most endangered group of marine fishes in the world (Davidson and Dulvy, 2017), and the second most endangered vertebrates in the world (Dulvy et al., 2021).

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Overfishing is having a profound impact on elasmobranchs, mostly due to their life history features that comprise slow growth, low fecundity, and long lifespans. In the last 60 years, industrial, artisanal, and recreational fishing for sharks increased globally, which led to significant declines in shark populations (Freire et al., 2015). Furthermore, most shark catches are incidental, but the economic value of their fins and meat have been on opposite trends, with the meat value increasing steadily in the last decade (Molina and Cooke, 2012).

Brazil is among the main countries involved in elasmobranch fishing and is currently the greatest importer of shark meat in the world (Dent and Clarke, 2015; Barreto et al., 2017). About 33% of the elasmobranch species occurring in Brazil are under some level of extinction threat according to the Brazilian Red List of endangered species (Barreto et al., 2017). Among these are all the species of hammerhead sharks recorded along the Brazilian Amazon coast.

Indeed, hammerhead sharks, genus *Sphyrna*, are among the most widely caught sharks due to their wide distribution and the high value of their fins (Bezerra et al., 2016). This scenario led the Brazilian Ministry of Environment to include *Sphyrna* species in the Ordinance 445/2014, which prohibits their capture, retention, landing, trade, and consumption within the Brazilian territory (Brasil, 2014). Four species of hammerhead sharks have been recorded along the Brazilian Amazon coast. Two of those are large coastal-oceanic (*Sphyrna lewini* and *S. mokarran*), and two are small to medium-sized coastal-estuarine species (*S. tiburo* and *S. tudes*), which present high rates of catch and trade in Maranhão state's fishing ports (Lessa et al., 1995, 1999).

However, the record of fisheries data for these species is hampered since morphological identification at the species level requires specimens to be landed whole. In fact, they are landed without head and fins and quickly sold to previously assigned buyers. Additionally, they are further sold to local consumers under the generic name of *cação* (Bornatowski et al., 2018; Cruz et al., 2021). This activity enables the capture of threatened species and hampers the identification of specimens at the species level, thus preventing environmental agencies from effectively applying laws that prohibit the capture of specific species (Feitosa et al., 2018; Cruz et al., 2021).

Over the last decades, molecular methods have been increasingly applied to solve such elasmobranch identification in landing and trade situations (Abercrombie et al., 2005; Clarke et al., 2006; Chuang et al., 2016; Silva, 2017; Bernardo et al., 2020; Feitosa et al., 2018). In this sense, the cytochrome oxidase subunit I (COI) gene has been consolidated as the "barcoding" fragment applied to sharks, as well as for other vertebrates (Hebert et al., 2003). Furthermore, a few molecular markers that do not require sequencing, for example ITS2 fragment length evaluation, have been proposed for widespread use to identify fisheries landings production, since these are faster and cheaper than those that require sequencing.

Abercrombie et al. (2005) developed a multiplex reaction to amplify regions of the ITS2 nuclear DNA gene, which produce different patterns band in agarose gels for *Sphyrna lewini* and *S. mokarran*. Therefore, our study aims to identify specimens of *Sphyrna* landed on fishing ports in Maranhão state, where is recorded two more species of the genus: *S. tiburo* and *S. tudes*. To achieve our goals, we compare the barcoding (COI) and band pattern (ITS2) methods to identify hammerhead species, improving the ITS2 protocol to comprise *S. tudes* and *S. tiburo*. We also analyze their applicability to the fisheries monitoring in Brazilian Amazon coast, one of the global elasmobranch conservation hotspots (Dulvy et al., 2014).

2. Materials and Methods

Shark muscle tissue samples were obtained under the SISBIO license nº 48601 in the period between March 2017 and March 2018. The cities sampled comprise the main areas of shark landings in the state of Maranhão Tutoia (Parnaíba River delta/Maranhão's east coast) (N=25), Raposa (Gulf of Maranhão) (N=15), and Carutapera (Maranhão's west coast) (N=6) (Figure 1). Samples

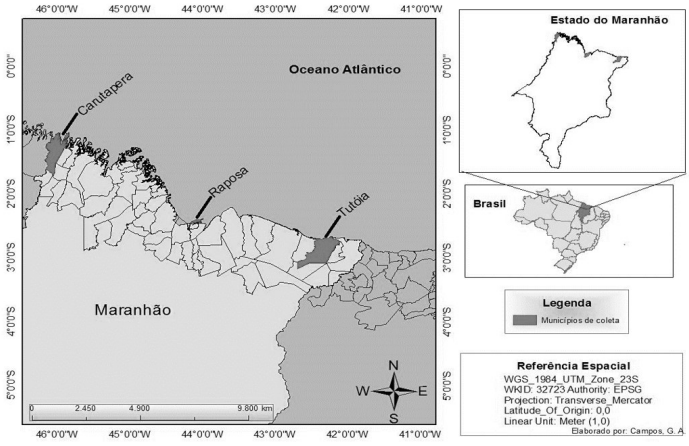

Figure 1. Location of the main fisheries landing sites in Maranhão state's coast.
Source: Núcleo Geoambiental – UEMA (2018).

collected were deposited in the Maranhão Fauna Tissue and DNA Collection (CoFauMA) from the State University of Maranhão (UEMA). Additionally, we used samples collected in 2015 and deposited CoFauMA as voucher species and positive controls (1 for each species, N = 4). Shark tissue samples were collected in fish markets and fishing vessels, comprising semi-industrial and artisanal fisheries. Such vessels use drift and bottom gillnets, as well as longlines operating in coastal shallow areas. Since these fishing vessels operate throughout the coast, the landing sites do not correspond to the place where specimens were caught.

All tissue samples were stored in sterile vials with 95% ethanol at –20 °C until further processing. We used 20 mg of tissue from each sample for DNA extraction, which was done with the Wizard Genomic DNA purification kit following manufacturer's protocol. We evaluated extracted DNA quality in 1% agarose gel stained with Gel Red (BIOTIUM). Then, we used the extracted DNA to carry out the Polymerase Chain Reactions (PCR) to amplify COI and ITS2 genes. For COI, we used the FISH R1 and FISH F1 primers (Geraghty et al., 2013). For ITS 2 sequences, FISH5.8SF and FISH28SR was used (Abercrombie et al., 2005). After amplification, we purified samples with PEG 20% and sequenced amplicons through Sanger sequencing (Sanger et al., 1977) using the DYEnamicTM dye terminator kit and the MegaBACE 1000 automatic sequencer (GE HealthCare). Following Abercrombie et al. (2005) for the ITS2 fragments comparison in electrophoresis we used the GtHH1123F and ScHH401F as forward primers and FISH28SR as reverse primer in the same mixture reaction. PCR products were visualized in agarose gel at 1% and stained with Gel Red using a 200pb ladder. Furthermore, we used a negative control in each reaction to detect potential contaminations, and positive controls as well as.

Sequences obtained were corrected manually using the MEGA software version 7.0 (Kumar et al., 2015) and submitted to the BOLD systems (2023) and NCBI GenBank platforms (NCBI, 2023a) to verify the level of similarity to those deposited in the databases. We further used the MEGA software to calculate Kimura-2-paremeters (K2P) genetic distances between and within species with COI sequences.

To primer design were used the Primer-BLAST in NCBI (2023b). The used parameters were PCR product size (Min: 70 / Max: 700); Primer melting temperatures (Tm): Min: 58.0 / Opt: 62.0 / Max: 66.0 / Max Tm difference: 4; Database: nr; Organism: Sphyrna (taxid:7822); Primer Size: Min: 18 / Opt: 20 / Max: 24; Primer GC content (%): Min: 40.0 / Max: 60.0; the other parameters were default. To *in sílico* PCR we used BLAST (NCBI, 2023c).

3. Results and Discussion

3.1. ITS2 fragments

We obtained three band patterns for the ITS2 amplicons from *Sphyrna* species. These comprise 800 bp bands (columns 1 through 5) and other with approximately 450 bp (Columns 6 and 7) (Figure 2). Such results corroborate the band patterns described by Abercrombie et al. (2005), which established a pattern of 782 bp for *S. lewini* and 445 bp for *Sphyrna mokarran* using the same primers. However, we only obtained a single band pattern of 1000 bp for the *S. tudes* and *S. tiburo* samples.

To improve the use of the ITS2 fragment protocol in this geographical area, we further sequenced ITS2 amplicons and used as a template for the design of primers for *S. tudes* and *S. tiburo*. Retrieved primers were evaluated by *in silico* PCR disclosing that there was no genetic variability enough to avoid the cross-amplification between species (see Supplementary Material). The primer 2 (Table 1), however, could be useful to improve the protocol. This pair of primers presents positive amplification for four species: *S.tiburo, S.tudes, S.media* and *S.corona.* The close phylogenetic relationship of these species were already showed by Lim et al. (2010), and explain the our results. The amplification of *S. corona,* however, should not be considered in our approach to the Amazon Coast, as the species is restricted to the Pacific Coast. Primer 2 can be used to validate that a sample belongs to *S.tiburo/tudes/media* (*S. media* was not catched in Maranhao Coast, but it's recorded in Amazon Coast) when the result is negative for *S. lewini* or *S.mokarran,* avoiding the incorrect interpretation of false negatives, especially when accessing meat processed or other parts of the body. Therefore, we conclude that ITS2 fragments could not be efetivelly for

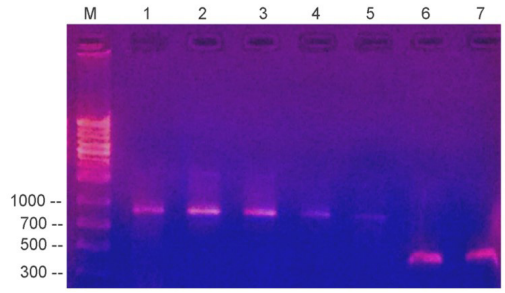

Figure 2. ITS2 band patterns for *Sphyrna* species. Columns 1 through 5 are *Sphyrna lewini*, and columns 6 and 7 are *S. mokarran*.

Table 1. Primer pair for ITS2 amplification in *Sphyrna* from Amazon Coast.

Template species	Sequence (5'->3')	Start - Stop	Tm	GC%	Product length	Positive amplification
S. tudes	GGGACCGTGTTTGGCTTGTG (F)	321-340	62.07	60	121	*Sphyrna tudes*
	TGCTCTCTCACTCCATTGCCA (R)	441-421	61.45	52		*Sphyrna corona*
						Sphyrna media
						Sphyrna tiburo

Table 2. Kimura-2-paremeter (K2P) distances between hammerhead shark species in Maranhão based on COI sequences.

Species	*S. lewini*	*S. mokarran*	*S. tiburo*	*S. tudes*
S. lewini	0.0118			
S.mokarran	0.1033	0.0055		
S.tiburo	0.1095	0.1103	0.0067	
S.tudes	0.0947	0.1191	0.0728	0.000

all *Sphyrna* species from Amazon Coast, being partially useful, as it correctly identify *S. lewini* and *S. mokarran*.

3.2. COI barcoding

Regarding the effectiveness of the COI gene for specimen identification, we obtained sequences of approximately 600 bp (N= 48) that, when compared to databases aiming to identify species, demonstrated similarity indices above 98%.

Genetic distances of the fragments were also compared, yielding differences between 0.07 to 0.11 between species (Table 2). Therefore, the COI gene effectively identified four *Sphyrna* species in the Brazilian northern coast and yielded genetic distances within the range delimited for DNA barcoding (Hebert et al., 2003), which confirms the effectiveness of this gene to identify sharks, as demonstrated in previous studies (Feitosa et al., 2018; Bernardo et al., 2020).

S. lewini showed the high genetic diversity with up to 0.01 distance between samples (Table 2), thus indicating a potential population structuring in the region. The existence of intraspecific genetic subgroups has already been reported for this species, one of the most widely distributed in the world (Elizondo-Sancho et al., 2022; Grobler et al., 2023).

During the last three decades, several studies have been carried out in Maranhão state, mainly focused on the reproductive biology (Lessa et al., 1999; Lessa, 1997) and population dynamics (Almeida et al., 2011; Almeida, 2008; Barreto et al., 2016; Lessa et al., 1998, 1999, 2009; Lessa and Nóbrega, 2000; Lessa, 1986) of sharks. Recently, Martins et al. (2018) studied the supply chain of the shark fin trade in Northern Brazil and Feitosa et al. (2018) used molecular methods to identify sharks fished along the same area. Both studies point *Sphyrna* as one of the most frequent taxa landed by fisheries in Maranhão state.

Although we obtained samples from hammerhead sharks in all sampling points, Tutoia comprised 55% of our samples. The frequency of occurrence for each species according to COI sequences were 67% for *S. mokarran*, 15% for *S. lewini* and *S. tiburo* respectively, and 3% for *S. tudes*.

The high frequency of *S. mokarran* is concerning since this species is highly vulnerable to fishing pressure due to its life history features, including slow sexual maturity and habitat use. Gallagher et al. (2014) . It reproduces every two years and occupies larger areas than any other hammerhead species (Stevens and Lyle, 1989; Denham et al., 2010; Hammerschlag et al., 2011).

Lessa et al. (1991) describes the occurrence of *S. tudes* captured near estuaries in Maranhão state's coast. However, we do not discard that its population decline might be the reason for its low frequency among our samples, since *S. tiburo* has similar habits but was captured more frequently.

These inferences are important indications for the need to reassess the legal instruments of protection for hammerhead sharks, especially at the international level. CITES (2023) only includes *S. lewini* and *S. mokarran* in its Appendix II, which are considered species not at extinction risk, but that could become threatened. These species are classified by IUCN as threatened, which considers *S. tudes* and *S. tiburo* as least concern. Brazilian laws (Brasil, 2014) indicate that *S. tiburo* (EN), Endangered, *S. tudes*, *S. mokarran* and *S. lewini* are critically endangered.

4. Conclusion

We validate COI barcoding as effective to identify all four *Sphyrna* species occurring in Northern Coast of Brazil proving that a single genetic marker can be used with this goal. ITS2 fragments can be used as more cheap and easy marker to detect *Sphyrna* biological tissues, being able to differentiate *S. lewini* and *S. mokarran* and a group formed by the other species from the Amazon Coast. Regarding catches in Maranhao Coast, *S. mokarran* was more frequently captured in our study, what must be consider in action plans to protect the sharks.

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Supplementary Material

Supplementary material accompanies this paper.
Supplementary Data

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