O uso do DNA barcoding para identificar barbatanas de tubarão comercializadas ilegalmente no Brasil

The use of DNA barcoding to identify illegally traded shark fins in Brazil

Carvalho, CBV[1]; Freitas, JM[1]

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RESUMO: A demanda por barbatanas de tubarão tem aumentado nos últimos anos, estimulando o comércio ilegal e técnicas de captura predatórias que ameaçam a sobrevivência das populações naturais. As barbatanas de tubarão são normalmente removidas imediatamente após a captura e o corpo do animal é jogado de volta ao mar, o que impede a identificação morfológica da espécie. Quando a identificação morfológica está comprometida, a identificação genética pode ser usada para associar amostras desconhecidas com amostras de referência por meio da comparação de sequências de genes mitocondriais. Neste estudo, nós usamos sequências de 650 pares de base da subunidade I do gene citocromo c oxidase (COI) associadas com o Barcode of Life Database (BOLD) para identificar uma carga de barbatanas de tubarão apreendidas pela Polícia Federal do Brasil em 2011. Nós conseguimos associar com sucesso 25 das 26 amostras encaminhadas para o laboratório com três espécies diferentes, sendo elas *Prionace glauca, Isurus oxyrinchus* e *Sphyrna zygaena*. Embora as três espécies não estejam atualmente protegidas pelas leis brasileiras, este estudo reforça a utilidade da ferramenta do DNA barcoding na casuística forense.

PALAVRAS-CHAVE: DNA barcoding; COI; Identificação; Comércio ilegal; Barbatanas de tubarão.

[1]Área de Perícias em Genética Forense, Instituto Nacional de Criminalística, Departamento de Polícia Federal.
Endereço para correspondência: SAIS QD 07 LT 23, CEP 70610-200, Brasília, DF. E-mail: benigno.cbvc@dpf.gov.br

INTRODUCTION

The demand for shark fins has increased in the last years, stimulating the international illegal trade and predatory capture techniques that threatens the survival of natural populations in various regions of the planet[1, 2]. Estimates suggest that a number between 26 and 73 million sharks are harvested annually to support the global shark fin industry[3].

During the shark fishing for commercial purposes the fins are usually removed immediately after the catch and the body of the animal is thrown back into the ocean, preventing the morphological identification of the species[4]. When morphological identification is compromised, the genetic identification can be used to try to associate unknown samples to a reference sample by comparing sequences of mitochondrial genes that vary between species[5].

One of the most commonly used mitochondrial genes for species identification is the subunit I of cytochrome c oxidase (COI). Based on approximately 650 base pair sequences of this gene a universal system for cataloging and identifying animal species, named DNA barcoding, has been proposed[6, 7]. Sequences of specimens with known identity that accomplish some quality criteria are being uploaded in the Barcode of Life Database (BOLD), an international publicly available database which can be used for species identification[8]. DNA barcoding has been used successfully to identify illegal and fraudulent animal products, including sharks[9, 10].

Shark fishing with commercial purposes is permitted in Brazil, however, this activity must be practiced in accordance with some regulations, including the limitation of the fins weight up to 5% of the carcasses weight and the prohibition of capture of any of the 12 protected shark species in the Brazilian coast[11]. The transportation and trading of protected species is considered an environmental crime in Brazil and the perpetrator can be punished with imprisonment. This paper shows how the technique of DNA barcoding was used to identify a cargo of shark fins seized by the Brazilian Federal Police in 2011.

MATERIAL AND METHODS

In 2011 the Brazilian Federal Police seized a 20 kg cargo of shark fins in an airport of São Paulo, Brazil. The material was being transported without any documentation, raising doubts about the legality of its origin and destination. After initial assessment and separation based on size and shape, 26 samples were sent to the DNA laboratory of the National Institute of Criminalistics.

Approximately 3 mm^3 tissue fragments were collected from each sample for DNA extraction. After overnight digestion in extraction buffer with DTT and Proteinase K, DNA was extracted using standard phenol chloroform procedures and purified with Amicon® Ultra (Millipore). Fragments of approximately 650 bp from the 5› region of the COI gene were amplified using FishF1 and FishR1 primers[12]. The PCR were performed in 25 µl reaction tubes containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 mM of each primer and 1 µl DNA (DNA not quantified). The cycling parameters employed were 11 min at 94, followed by 35 cycles of 94 for 30 s, 54 ° for 30 seconds and 72 ° for 1 min. Amplification products were purified using Exo-SAP-IT® (USB) and sequenced in both directions using Big Dye Terminator kit v1.1 (Life Technologies). The extension products were again treated with enzyme alkaline phosphatase and purified by ethanol precipitation. Capillary electrophoresis was performed in an ABI 3130 genetic analyzer (Life Technologies).

Sequences were assembled and had their quality assessed with SeqScape v2.6 (Life Technologies) software. Consensus sequences were searched in BOLD Species Level Barcode Records database using the identification engine (www. boldsystems.org). BOLD identifies an unknown specimen to species level when there is less than 1% sequence divergence between the query sequence and the reference sequence.

RESULTS

DNA extraction was successful for 25 of the 26 samples sent to the laboratory. Good quality 650 bp sequences were obtained from the 25 samples whose DNA extraction was possible. Analysis of the nucleotide sequences showed no signs of heteroplasmy and its translation into amino acids sequences did not reveal the presence of stop codons or pseudogenes.

All sequences were compatible with chondrichthyan species. Nineteen sequences resulted in 100% similarity matches. All the other matches were > 99.51%. Twenty sequences were identified by BOLD as *Prionace glauca*, the blue shark. Two samples were matched to the shortfin mako *Isurus oxyrinchus*, however, BOLD do not provided a species level match, presenting other non-congeneric species as a possible candidate. The last three samples were matched to the

smooth hammerhead shark *Sphyrna zygaena* but, again, BOLD do not provided a species level match, presenting two other non-congeneric species as possible candidates (Table 1).

TABLE 1. Results of BOLD identification engine (Species Level Barcode Records database), on March 2013, for best and 2nd best matched species and similarity for each sequence produced in this study

Sample	Best matched species	% Similarity	2nd Best matched species	% Similarity
1	*Prionace glauca*	100	*Carcharhinus falciformis*	96.66
2	*Sphyrna zygaena*	100	*C. zygaena*[1]/ *C. leiodon*[2]	99.85
3	*P. glauca*	100	*C. falciformis*	96.64
4	*P. glauca*	100	*C. falciformis*	96.64
5	*Isurus oxyrinchus*	100	*P. glauca*[2]	99.85
6	*P. glauca*	100	*C. falciformis*	96.65
7	*P. glauca*	100	*C. falciformis*	96.65
8	*P. glauca*	100	*C. falciformis*	96.65
9	*P. glauca*	99.8	*C. falciformis*	96.25
10	*S. zygaena*	99.69	*C. zygaena*[1]/ *C. leiodon*[2]	99.53
11	*P. glauca*	100	*C. falciformis*	96.65
12	*P. glauca*	100	*C. falciformis*	96.65
13	*P. glauca*	100	*C. falciformis*	96.64
14	*P. glauca*	100	*C. falciformis*	96.25
15	*P. glauca*	99.63	*C. falciformis*	96.05
16	*P. glauca*	100	*C. falciformis*	96.25
17	*P. glauca*	100	*C. falciformis*	96.65
18	*P. glauca*	100	*C. falciformis*	96.64
19	*P. glauca*	100	*C. falciformis*	96.65
21	*P. glauca*	99.83	*C. falciformis*	96.44
22	*P. glauca*	100	*C. falciformis*	96.65
23	*S. zygaena*	99.51	*C. zygaena*[1]/ *C. leiodon*[2]	99.22
24	*P. glauca*	100	*C. falciformis*	96.65
25	*P. glauca*	100	*C. falciformis*	96.44
26	*I. oxyrinchus*	99.53	*P. glauca*[3]	99.52

[1]Species represented in BOLD by one sequence (not published). The species "*Carcharhinus Zygaena"* does not exist[15].
[2]One sequence (not published) clustered together with the best matched species.
[3]Two sequences (not published) clustered together with the best matched species.

DISCUSSION

Since the mean COI sequence divergence between congeneric species of sharks and rays is 7.48%, the gene is useful to discriminate most species of the group[13]. Besides, validation studies have shown that the COI gene allows accurate identification of species where an appropriate reference database is used[5]. In March 2013, BOLD contained 822 species of elasmobranchs with barcodes in its database. There are records of occurrence of 81 shark species in the Brazilian coast[14] and 68 (84%) of them are represented in BOLD with barcodes. We consider that at least for shark species found in Brazilian coast BOLD is representative and may be used for identification in most cases.

BOLD results were consistent, with most sequences being identified to species level. Sequences with best matched species *I. oxyrinchus* and *S. zygaena* also resulted in few non-congeneric species being listed as possible candidates. The species "*Carcharhinus zygaena"*, presented as a possible candidate when *S. zygaena* was the best

matched species, does not exist[15] and it is probably a typo. The other species that were presented as possible candidates, *C. leiodon* and *P. glauca*, are neither morphologically nor genetically similar to *S. zygaena* and *I. oxyrinchus*, and this result was unexpected. Although *C. leiodon* and *P. glauca* are represented by a large number of sequences in BOLD (25 and 67 sequences, respectively) only one or two non-published sequences representing them were clustered together with the best matched species. We consider that these sequences represent misidentified or genetically divergent specimens.

The species *P. glauca*, *I. oxyrinchus* and *S. zygaena* have a worldwide distribution[15] and are frequently caught by fishermen along the Brazilian coast. The three species are not considered threatened in Brazil and, therefore, their capture and transport in accordance with current regulations is allowed, but the lack of documentation is an offense usually punished with fines. Although shark fishing data for Brazil are very limited, there is evidence that North Atlantic populations of these three species are in decline due to overexploitation[16]. Further studies to assess the conditions of local populations of these and other shark species should be conducted in order to ensure sustainable fishing.

CONCLUSION

In the present case, we report the identification of 25 samples of a seized shark fin cargo. Although we did not identify any protected species by the Brazilian legislation, three different species were successfully identified using sequences of the subunit I of cytochrome c oxidase gene (COI). These results corroborate the DNA barcoding as a valuable tool for species identification in forensic casework and illegal animal trading. Techniques and tools that help to fight the illegal trade of shark fins must be improved and widespread in order to preserve the natural populations of sharks worldwide.

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ABSTRACT: The demand for shark fins has increased in the last years, stimulating the illegal trade and predatory capture techniques that threaten the survival of natural populations. Shark fins are usually removed immediately after the catch and the body of the animal is thrown back into the ocean, preventing the morphological identification of the species. When morphological identification is compromised, genetic identification can be used to associate unknown samples to a reference sample by comparing sequences of mitochondrial genes. In this study we used sequences of 650 base pair of the subunit I of cytochrome c oxidase gene (COI) associated with the Barcode of Life Database (BOLD) to identify a cargo of shark fins seized by the Brazilian Federal Police in 2011. We have successfully matched 25 of 26 samples sent to the laboratory to three different species, *Prionace glauca, Isurus oxyrinchus* and *Sphyrna zygaena.* Although none of them are currently protected by Brazilian laws, this study reinforces the utility of DNA barcoding in forensic casework.

KEYWORDS: DNA barcoding; COI; Identification; Illegal trade; Shark fins.

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