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DNA barcode reveals the illegal trade of rays commercialized in fishmongers in Brazil

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ABSTRACT

Sequences of the mitochondrial gene COI (DNA Barcode) were used to identify species of rays and skates commercialized in fishmongers in Brazil. The comparisons of the obtained sequences with previously published data available in NCBI and BOLD showed that the fish products corresponded to four species, *Hypanus dipterurus*, *Potamotrygon motoro*, *Paratrygon ajereba* and *Gymnura altavela*, the last of which is classified as vulnerable according to the IUCN Red List and therefore should not be marketed in accordance with the MMA ordinance 445/2015.

1. Introduction

Rays and skates are cartilaginous fish that have the body flattened dorso-ventrally, plus six or seven pairs of gill slits and developed pectoral fins fused to the head [1,2]. Along with sharks, they are a major market target for their fins, meat, gillrakers and liver oil [3]. Despite the decrease in the shark and ray landings since the peak in 2003, it is not known if this reduction is caused due to the rise of sustainable fisheries or by population declines [4–6]. In Brazil, 24 species of rays are threatened with extinction according to the Brazil Red Book of Threatened Species of Fauna [7], which are forbidden from being marketed through the MMA ordinance 445/2015, that prohibits fishing and trading of 475 threatened species of fish. Furthermore, due to non-characterization and the grouping of all species of rays and skates in a single NCM (Mercosur Common Nomenclature), the identification based on a morphological analysis is often compromised. In this way, genetic identification represents a solid alternative for classifying biological products that have been processed or non-characterized. DNA Barcoding is currently the most widespread technique for the identification of commercialized biological material, for it is a species-specific molecular identification system based on the mitochondrial DNA sequence Cytochrome Oxidase Subunit I (COI) and requires only a small fragment of tissue [8,9].

2. Material and methods

52 individual fragments of muscle labelled as rays/skates obtained from fishmongers in markets in the city of São José do Rio Preto, São Paulo State, Brazil were analysed and were stored in the collection of the Laboratório de Genética de Peixes, UNESP - Bauru/SP.

DNA extraction was conducted using the procedures from the commercial kit "Wizard Genomic DNA Purification Kit" (Promega) with some modifications (the biological material was left in a water bath at 60 °C overnight, using the kit solution and adding protein K enzyme – 10 ng/ml). Fragments of approximately 650 base pairs (bp) of the COI gene were amplified by PCR (Polymerase chain reaction) by testing different combinations of the primers COI FishF1 Forward (5′ ACGCCTGTTTATCAAAAACAT-3′), COI FishR1 Reverse (5′ CCGGTCTGAACTCAGATCACGT-3′), COI FishF2 Forward (5′-

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TCGACTAATCATAAAGATATCGGCAC-3′) and COI FishR2 Reverse (5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) [10]. PCR reactions were conducted in a thermo cycler - ProFlex PCR System (Applied Biosystems) at a total volume of 25 μl, including 10–30 ng/μl of genomic DNA, 1X Taq buffer, 150 μl of each dNTP, 1,5 m of MgCl2, 0,5U of Taq polymerase (Invitrogen) and with a concentration of 10 nmol/μl of each pair of primers. The cycling conditions were an initial denaturation of 95 °C for 5 min followed by 35 cycles at 94 °C for 30 s; 54 °C for 45 s; 68 °C for 1 min and a final extension of 72 °C for 5 min. Before the sequencing, PCR products were purified using the enzyme kit "Exo-SAP IT" (USB Corporation). PCR sequencing reaction was done using the "Big Dye Terminator v.3.1 Cycle Sequencing Ready Reaction" (Applied Biosystems) kit using the same primers used before. Two PCR reactions was done for each sample, one using forward and the other using reverse primers to increase reliability. The solution for each reaction contained 10–30 ng/μl of genomic DNA, 1X BigDye buffer, 5X Bigdye and 0,1–0,4 μl of primer, with a total volume of 7 μl. The cycling conditions were an initial denaturation of 96 °C for 2 min followed by 35 cycles at 96 °C for 30 s; 50 °C for 15 s and 60 °C for 4 min.

Samples were sequenced in an ABI 31-30-Genetic Analyser (Applied Biosystems) and sequences were aligned and edited using Geneious 4.8.5 [11]. Sequences were compared to data available in databanks, using the BOLD Species Level Barcode (http://v4.boldsystems.org/) and NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov). DNA integrity and PCR products were visualized in agarose gel 2% stained with Nancy-520 (Sigma) and observed in transilluminator under UV light.

Table 1
Genetic identification of samples using the COI gene and their conservation status.

Samples ID	Primer	Species (% of identification)	Genetic Similarity (%)		Conservation Status (IUCN)	Conservation Status (Brazilian Red List)
	Combinations		BOLD	NCBI		
10.129	FishF1-FishR2	*Gymnura altavela* 6%	99.04	98.00	VU	CR
10.130	FishF1-FishR2		98.29	98.00	VU	CR
10.131	FishF1-FishR2		97.98	97.00	VU	CR
10.531	FishF1-FishR2	*Hypanus dipterurus* 10%	–	94.00	DD	DD
10.535	FishF2-FishR1		98.67	99.00	DD	DD
10.536	FishF1-FishR2		100.00	98.00	DD	DD
10.537	FishF1-FishR2		98.52	98.00	DD	DD
10.542	FishF1-FishR2		100.00	99.00	DD	DD
10.122	FishF2-FishR1	*Potamotrygon motoro* 19%	100.00	97.00	DD	LC
10.123	FishF1-FishR2		97.44	98.00	DD	LC
10.126	FishF1-FishR2		100.00	97.00	DD	LC
10.127	FishF1-FishR2		98.15	97.00	DD	LC
10.128	FishF1-FishR2		99.17	99.00	DD	LC
10.109	FishF2-FishR1		100.00	98.00	DD	LC
10.110	FishF2-FishR1		99.32	98.00	DD	LC
10.111	FishF2-FishR1		99.00	98.00	DD	LC
10.114	FishF2-FishR1		98.96	98.00	DD	LC
10.131	FishF2-FishR1		98.92	98.00	DD	LC
10.124	FishF1-FishR2	*Paratrygon ajereba* 65%	–	97.00	DD	CR
10.125	FishF1-FishR2		100.00	–	DD	CR
10.107	FishF2-FishR1		100.00	–	DD	CR
10.112	FishF2-FishR1		100.00	–	DD	CR
10.113	FishF2-FishR1		99.38	100.00	DD	CR
10.116	FishF2-FishR1		100.00	100.00	DD	CR
10.117	FishF2-FishR1		–	98.04	DD	CR
10.118	FishF2-FishR1		100.00	–	DD	CR
10.119	FishF2-FishR1		100.00	100.00	DD	CR
10.121	FishF2-FishR1		100.00	99.37	DD	CR
10.122	FishF2-FishR1		97.44	98.00	DD	CR
10.123	FishF2-FishR1		–	95.00	DD	CR
10.124	FishF2-FishR1		100.00	–	DD	CR
10.125	FishF2-FishR1		100.00	100.00	DD	CR
10.126	FishF2-FishR1		100.00	100.00	DD	CR
10.127	FishF2-FishR1		100.00	100.00	DD	CR
10.132	FishF2-FishR1		99.68	100.00	DD	CR
10.136	FishF2-FishR2		100.00	100.00	DD	CR
10.137	FishF1-FishR2		100.00	100.00	DD	CR
10.138	FishF2-FishR2		–	100.00	DD	CR
10.139	FishF2-FishR1		100.00	100.00	DD	CR
10.142	FishF2-FishR1		100.00	100.00	DD	CR
10.145	FishF2-FishR1		100.00	99.00	DD	CR
10.147	FishF2-FishR1		99.22	99.00	DD	CR
10.149	FishF2-FishR1		97.83	98.00	DD	CR
11.782	FishF2-FishR2		–	100.00	DD	CR
11.791	FishF2-FishR2		–	100.00	DD	CR
11.796	FishF2-FishR2		–	100.00	DD	CR
12.688	FishF2-FishR2		–	100.00	DD	CR
12.691	FishF2-FishR2		–	100.00	DD	CR
12.702	FishF2-FishR2		–	100.00	DD	CR
12.703	FishF2-FishR2		–	98.28	DD	CR
12.708	FishF2-FishR2		–	100.00	DD	CR
12.724	FishF2-FishR2		–	98.60	DD	CR

F: forward, R: reverse, VU: vulnerable, DD: data deficient.

3. Results

It was possible to obtain high quality DNA and to identify all the 52 samples studied in this work as four different species (Table 1). Three primer combinations (FishF1-FishR2, FishF2-FishR1 and FishF2-FishR2) showed the best results. Among all samples (Table 1), 6% (N = 3) corresponded to the marine skate *Gymnura altavela* (Linnaeus, 1758) (Gymnuridae), commonly known as Spiny Butterfly; 10% (N = 5) to the marine ray *Hypanus dipterurus* (Jordan & Gilbert, 1880) (Dasyatidae), also known as Diamond Stingray; 19% (N = 10) identified as *Potamotrygon motoro* (Müller & Henle, 1841) (Potamotrygonidae), popularly as Ocellate River Stingray; and the majority of samples (65%, N = 34) identified as *Paratrygon ajereba* (Müller & Henle, 1841) (Potamotrygonidae), also known as Manzana Ray. Genetic identity was ≥97% for BOLD and NCBI for all sequences, except for the individuals 10.531 and 10.531 with 94% and 95% of similarity for NCBI, respectively (Table 1). According to the IUCN (www.iucnredlist.org) data (Table 1), *G. altavela* is actually considered a vulnerable (VU) species while for the other species no information was available (DD - data deficient).

4. Discussion and conclusion

The genetic strategies proposed in this work showed highly efficient results for the identification of commercialized biological tissues of rays. Species identification based on a fragment of the COI gene, DNA barcoding, represent a relatively simple and efficient tool for food authentication and conservation purposes, widely applied to identify and to provide COI sequences for ray species around the world [12–15].

A vulnerable ray species, *Gymnura altavela* was identified as being commercialized, which is common for the group [16,17]. Besides this threat status, its current population trend is classified as “decreasing” [18], and this species is listed Critically Endangered (CR) by the Brazil Red Book of Threatened Species of Fauna [7], indicating that the species may need more protection measures to avoid that its state of threat becomes more severe. The results obtained highlight the necessity of tighter inspections and stricter enforcement laws of marketed rays and skates, in addition to the need for further studies about the conservation status of their species, especially since three species identified here are still without conservation status (data deficient – DD) and public IUCN data indicates that 76,19% of the four genus of South American freshwater rays are placed in this category [18]. It is worth emphasizing that species within this group may be in an advanced process of extinction as in this group there is not adequate information to make an assessment of their risk of extinction, based on their distribution and/or population status [18].

Declaration of competing interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Raul B. Camacho-Oliveira: Data curation, Formal analysis, Methodology. **Cahique M. Daneluz:** Methodology, Formal analysis. **Fernanda D. do Prado:** Formal analysis. **Ricardo Utsunomia:** Formal analysis. **Fausto Foresti:** Writing - review & editing, Resources. **Fábio Porto-Foresti:** Writing - review & editing, Resources, Supervision, Project administration.

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