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A multiplex PCR approach for the molecular identification and conservation of the Critically Endangered daggernose shark

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ABSTRACT: The daggernose shark *Isogomphodon oxyrhynchus* is an inshore tropical species endemic to the coastal waters off northeastern South America with limited distribution and restrictive biological traits. According to the IUCN, this species is Critically Endangered owing to a dramatic population decline over the past 10 yr. Here we implemented a multiplex PCR methodology based on species-specific primers for the precise identification of samples from the daggernose shark (body parts and fins). The effectiveness of the method was verified through application for analysis of unidentified shark samples collected in fish markets and a worldwide inventory of over 57 shark species. This reliable molecular system of identification combines the advantages of low cost and high throughput and may be used locally to monitor the capture and trade of this Critically Endangered shark species.

KEY WORDS: Genetic identification · ITS2 · Multiplex PCR · Sharks · DNA forensics

INTRODUCTION

The daggernose shark *Isogomphodon oxyrhynchus* is a tropical inshore species with a restricted natural range, inhabiting shallow waters of the western Atlantic from Trinidad to northern Brazil (Compagno 1984; our Fig 1). Its distribution is associated with hot, humid climates and highly turbid waters where mangroves are the dominant system (Lessa et al. 1999). The species has biological parameters that limit population growth, including low fecundity (2 to 8 pups), late sexual maturity, long gestation period (1 yr) and a biennial reproductive cycle. *I. oxyrhynchus* is under intense fishing pressure across its entire range, being caught incidentally in floating gillnets by artisanal fisheries. Limited available data from demographic analyses suggest that the *I. oxyrinchus* population has been decreasing at ~18% per year with huge declines (>90%) resulting over the past 14 yr (Santana & Lessa 2002, Lessa et al. 2006). Considering its biological traits, and the fact that it is over-exploited by fisheries in its restricted occurrence area, the daggernose shark is listed as Critically Endangered by the IUCN (Lessa et al. 2006), as a species that needs urgent management and conservation to prevent it from becoming extinct.

Correct statistical data from fisheries is of utmost importance for designing conservation measures. However, this task is hampered by the fact that the finning process, where fins and/or body parts of shark species are removed, makes correct species identification difficult. Genetic profiling has thus become a widely used method for forensic identification of marine species for the purposes of trade monitoring and fisheries management (Heist 2005, Ogden et al. 2009). For instance, genetic profiling has been used to quantify global fin landings, detect illegal captures and identify the geographic origin of

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Fig. 1. Geographical distribution (black shading) of the daggernose shark *Isogomphodon oxyrhynchus* on the northeastern coast of South America

traded shark parts (reviewed in Shivji 2010). In this context, several studies report efforts to develop molecular markers for the correct identification of endangered shark species in fisheries landings (Chapman et al. 2003, Feldheim et al. 2010).

Several markers such as cytochrome oxidase subunit I (COI), cytochrome B and 16S have proven useful as a DNA barcoding method to identify body parts landed from fisheries. However, due to the lengthy sequencing procedures involved, such methodologies are still expensive and time consuming (Dudgeon et al. 2012). Thus, the validation of less expensive, reliable and easy-to-use tools for monitoring shark exploitation in developing countries is required.

Among the diverse PCR-based methods so far developed, a multiplex PCR-based method designed by Pank et al. (2001) has been very useful and enabled correct genetic identification of several shark species worldwide (Shivji et al. 2002, Chapman et al. 2003, Abercrombie et al. 2005, Magnussen et al. 2007, Pinhal et al. 2012). This rapid, low-cost and highly reproducible methodology requires only PCR for species identification, without additional DNA sequencing or cleavage by enzymatic processing. It employs a combination of universal and species-specific primers (SSPs) of the nuclear ribosomal DNA internal transcribed spacer 2 (ITS2) locus in a single-reaction, producing a unique diagnostic profile for each target species. Although several SSPs had already been developed prior to our study, these were only applicable to a few of the many shark species known worldwide.

Therefore, taking into account the restrictive biological traits and the endangered status of daggernose sharks in the wild, we designed and validated a triplex PCR-based assay for the genetic identification of daggernose shark by-products from fisheries. Our assay expands the repertoire of SSPs available and can be implemented for fast and accurate identification of this target species in order to assist in both local conservation and trade monitoring.

MATERIALS AND METHODS

Shark sampling and DNA extraction

Daggernose shark samples were collected from 10 wild specimens caught by artisanal and commercial fisheries along the north coast of Brazil from 2009 to 2014. The 10 specimens were morphologically identified and confirmed by specialists. Fins and muscle samples were preserved in 95% ethanol and kept at −20°C for long-term storage.

Total genomic DNA was isolated following the phenol-chloroform protocol (Sambrook & Russel 2006) with modifications and stored at −20°C until use. The DNA integrity was accessed by 1% agarose gel stained with GelRed (Uniscience) and quantified with NanoDrop-1000 (Thermo Scientific).

For diagnostic validation, the putative species-specific daggernose primer was also tested against 57 non-target shark species provided by colleagues from Brazil, USA and Australia. Data for both target and non-target shark species, including geographic source and sample sizes are presented in Table 1. The non-target species tested for primer specificity control included species closely related to daggernose sharks and commonly caught by fisheries worldwide (in some cases by the same local fisheries), as well as other shark species from several distinct oceanic areas.

PCR amplification and sequencing of ITS2 locus

The whole sequence of the ITS2 region was amplified using the universal primers FISH5.8SF and FISH28SR following the PCR conditions described by Pank et al. (2001). Amplified segments were visual-

Table 1. Inventory of target and non-target shark species tested with daggernose shark species-specific primers (SSPs) in triplex PCR assays. Geographic ocean basin origins of the shark test species are shown, together with the number of individuals of each species tested from each geographic region. The target species is shown in **bold**

Species	Geographic origin (number tested)
ORDER CARCHARHINIFORMES	
Isogomphodon oxyrhynchus (daggernose shark)	Atlantic (10)
Rhizoprionodon lalandei (Brazilian sharpnose)	Atlantic (5)
Rhizoprionodon porosus (Caribbean sharpnose)	Atlantic (5)
Rhizoprionodon terranovae (Atlantic sharpnose)	Atlantic (5)
Rhizoprionodon longurio (Pacific sharpnose)	Pacific (6)
Rhizoprionodon acutus (milk shark)	Pacific (5)
Rhizoprionodon taylori (Australian sharpnose)	Pacific (5)
Rhizoprionodon oligolinx (grey sharpnose)	Indian (2)
Sphyrna mokarran (great hammerhead)	Atlantic (3); Pacific (2)
Sphyrna lewini (scalloped hammerhead)	Atlantic (5); Pacific (3)
Sphyrna zygaena (smooth hammerhead)	Atlantic (5); Pacific (3)
Sphyrna tiburo (bonnethead)	Atlantic (5)
Sphyrna tudes (golden hammerhead)	Atlantic (5)
Eusphyra blochii (winghead)	Pacific (1)
Loxodon macrorhinus (sliteye)	Atlantic (6)
Galeocerdo cuvier (tiger)	Atlantic (5); Pacific (3)
Triaenodon obesus (whitetip reef)	Pacific (5)
Prionace glauca (blue shark)	Atlantic (7); Pacific (4)
Carcharhinus altimus (bignose)	Atlantic (3); Pacific (2)
Carcharhinus longimanus (oceanic whitetip)	Atlantic (2); Pacific (3)
Carcharhinus signatus (night)	Atlantic (5)
Carcharhinus plumbeus (sandbar)	Atlantic (2); Pacific (3)
Carcharhinus obscurus (dusky)	Atlantic (2); Pacific (3)
Carcharhinus limbatus (blacktip)	Atlantic (3); Pacific (3)
Carcharhinus falciformis (silky)	Atlantic (2); Pacific (2); Indian (1)
Carcharhinus porosus (smalltail)	Atlantic (5)
Carcharhinus galapagensis (Galapagos)	Pacific (5)
Carcharhinus leucas (bull)	Atlantic (8); Pacific (3)
Carcharhinus isodon (finetooth)	Atlantic (5)
Carcharhinus acronotus (blacknose)	Atlantic (5)
Carcharhinus perezi (Caribbean reef)	Atlantic (5)
Carcharhinus amboinensis (pigeye)	Pacific (2); Indian (3)
Carcharhinus brachyurus (bronze whaler)	Atlantic (2); Pacific (3)
Carcharhinus tilstoni (Australian blacktip)	Pacific (5)
Carcharhinus sorrah (spot-tail)	Índian (2); Pacific (4)
Carcharhinus amblyrhynchos (gray reef)	Pacific (5)
Negaprion brevirostris (lemon)	Atlantic (5)
Negaprion acutidens (sicklefin lemon)	Pacific (5)
Mustelus norrisi (smoothhound)	Atlantic (2)
Mustelus canis (smooth dogfish)	Atlantic (6)
Mustelus californicus (grey smoothhound)	Pacific (1)
Triakis semifasciata (leopard)	Pacific (1)
Scyliorhinus sp. (catshark)	Atlantic (6)
Apristurus profundorum (deep-water catshark)	Atlantic (1)
ORDER LAMNIFORMES	
Isurus oxyrinchus (shortfin mako)	Atlantic (6); Pacific (3)
Isurus paucus (longfin mako)	Atlantic (2); Pacific (2)
Lamna nasus (porbeagle)	Atlantic (2); Pacific (3)
Carcharodon carcharias (white shark)	Atlantic (2); Indian (3); Pacific (3)
Alopias vulpinus (thresher)	Atlantic (1)
Alopias superciliosus (bigeye thresher)	Atlantic (5)
Carcharias taurus (sand tiger)	Atlantic (2); Indian (2); Pacific (2)
ORDER ORECTOLOBIFORMES	
Ginglymostoma cirratum (nurse)	Atlantic (10)
Nebrius ferrugineus (tawny nurse)	Pacific (1)
ORDER SQUALIFORMES	
Squalus acanthias (spiny dogfish)	Atlantic (4)
Centrophorus squamosus (leafscale gulper)	Atlantic (1)
ORDER SQUATINIFORMES	
Squatina californica (Pacific angel)	Pacific (2)
ORDER HEXANCHIFORMES	
Hexanchus griseus (sixgill)	Pacific (2)
Heptranchias perlo (sharpnose sevengill)	Atlantic (1)

ized on a 1.2% agarose gel stained with GelRed under UV light. The products were purified with the illustra ExoProStar 1-Step kit (GE Healthcare Life Sciences) according to the manufacturer's protocol.

Sequencing reactions were performed with the BigDye Terminator v3.1 kit (Applied Biosystems), according to the manufacturer's protocol. The cycling profile comprised an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 s and extension at 60°C for 1 min. The products were gel purified using the DyeEx 2.0 Spin Kits (Qiagen), and sequencing was carried out on an Applied Biosystems 3130 DNA analyzer (Applied Biosystems). The final ITS2 locus

sequence obtained was analyzed with the software Geneious (version 4.8.5, Biomatters; Drummond et al. 2009).

Primer design and multiplex PCR

Complete ITS2 locus sequences obtained from the daggernose sharks were aligned with GenBank sequences of closely related species using Geneious (as above). Several putative species-specific primers (SSPs) were designed for daggernose shark based on the nucleotide differences detected between the target species and closely related non-target species sequences using Geneious (Drummond et al. 2009) and OligCalc (Kibee 2007). The putative SSPs were designed as reverse primers to, and initially tested against, closely related species in a PCR triplex format including the SSP with the forward and reverse universal ITS2 primers (Pank et al. 2001). Two amplification products are expected from the target species with this triplex PCR: a species-characteristic-sized PCR amplicon generated by the reverse SSP in conjunction with the forward ITS2 universal primer, and a positive control amplicon generated by the 2 ITS2 universal primers (Fig. 2). In contrast, the PCR product from other species is expected to yield only the positive control amplicon, due to the fact that no annealing site of the SSP is present in the genomic DNA of the non-target species (Pank et al. 2001).

Several inspections of temperature and extension time were applied to the triplex-PCR for standardization. This resulted in the following optimized conditions: denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 2.5 min, and a 5 min final extension at 72°C. PCR reactions consisted of 30 ng of DNA, 400 nmol of each primer, 2.5 mM of $MgCl_2$, 1 × PCR buffer, 200 µM dNTPs and 1 unit of Platinum Taq DNA polymerase (Invitrogen). Amplifications were carried out in a PTC-200 (MJ Research) thermal cycler and checked through a 1.2% agarose gel stained with GelRed under UV light. Eight closely related species of *Carcharhinus* were utilized for the initial test of the SSPs designed. Then, the putative SSP that consistently amplified the correct-sized fragment for the daggernose shark and presented no amplification in the 8 closely related species (see Table 2 for primer sequence) was chosen for further tests against 49 additional non-daggernose shark species, totaling 57 species and 279 individuals (see Table 1 for additional information), representative of the wide variety of species that are known to be commonly taken by fisheries. This test further confirmed the specificity of the primer which exclusively amplified in the presence of daggernose shark DNA.

Market screening

The daggernose shark SSP developed in this study and SSPs developed by others for non-daggernose shark species (Pank et al. 2001, Chapman et al. 2003, Abercrombie et al. 2005, Pinhal et al. 2012) were successfully used for screening shark body parts from markets. All samples were tested against all assays. A total of 51 samples of unknown species were sourced from the fish markets in Bragança, PA, on the north coast of Brazil (an area within the distribution range of the daggernose shark) and screened

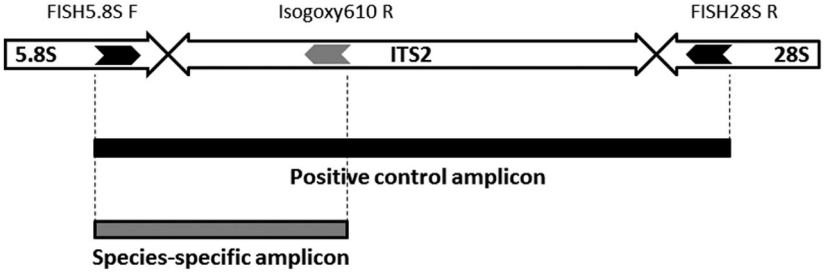

Fig. 2. Schematic representation of the shark nuclear 5.8S and 28S ribosomal RNA genes and ITS2 locus showing the annealing sites and orientation of primers of the universal shark primers (black) and species-specific primer for *Isogomphodon oxyrhynchus* (gray) used in triplex PCR assay. Black and gray bars represent the spatial coverage of the 2 amplicons expected to be produced using this combination of 3 primers when tested against target DNA. In contrast, only the positive control amplicon would be produced in the absence of the tar get species. Figure adapted from Shivji et al. (2002)

Table 2. Shark universal and daggernose shark species-specific primer (SSP) used in the triplex assay. Note that primers FISH5.8S-F and FISH28S-R were developed previously by Pank et al. (2001)

Primer	Sequence
FISH5.8S-F	5′-TTA GCG GTG GAT CAC TCG GCT CGT-3′
FISH28S-R	5′-TCC TCC GCT TAG TAA TAT GCT TAA ATT CAG C-3′
Isogoxy-610-ITS2	5′-CGG CCC CCT CCT GGC TG-3′

against all assays. Furthermore, the common names of the samples given by the sellers at fish markets were recorded for comparison with the results of our screening analysis, with the aim of verifying the accuracy of identifications made by the sellers. After identification by SSPs, the ITS2 locus of the 51 samples obtained for screening was sequenced, as described above. Sequences were matched against 10 daggernose shark ITS2 sequences acquired in the present study and to ITS2 sequences from another 22 shark species available from GenBank.

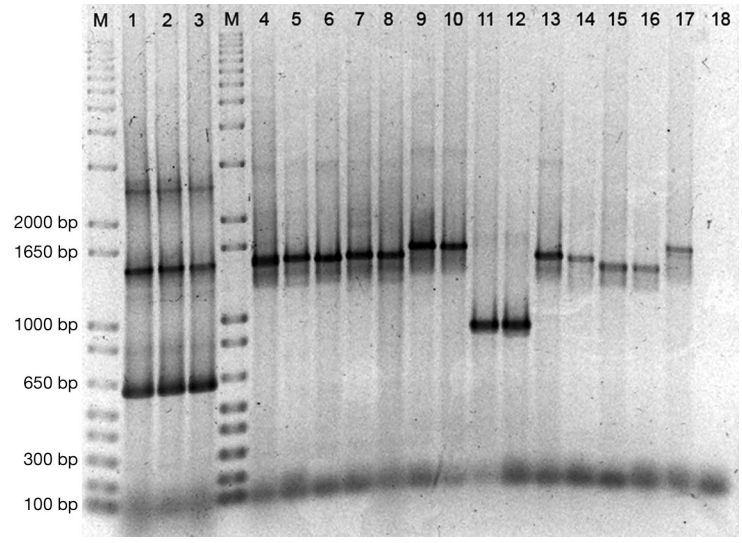

Fig. 3. Agarose gel image showing results of PCR using 2 universal shark primers and the species-specific primer for daggernose shark *Isogomphodon oxyrhynchus*. Lanes 1 to 3 show triplex-PCR amplification products of the target species *Isogomphodon oxyrhynchus*. Lanes 4 to 17 show triplex-PCR amplification products from non-target species (4: *Carcharhinus porosus*; 5: *Carcharhinus obscurus*; 6: *Carcharhinus acronotus*; 7: *Carcharhinus falciformis*; 8: *Carcharhinus leucas*; 9: *Rhizoprionodon porosus*; 10: *Rhizoprionodon lalandei*; 11: *Sphyrna tiburo*; 12: *Sphyrna lewini*; 13: *Prionace glauca*; 14: *Galeocerdo cuvier*; 15: *Carcharodon carcharias*; 16: *Isurus oxyrhynchus*; 17: *Ginglymostoma cirratum*). Line 18 is the negative control. Lanes labeled 'M' contain the molecular size-standard 1 kb plus

RESULTS

Evaluating the species-specific primer

The triplex PCR assay developed for daggernose sharks was effective for species diagnosis. The ITS2 locus control amplicon recovered for this species was about 1450 bp. The ITS2 sequences recovered presented low intraspecific variability for the 10 samples analyzed (99.6% of average similarity).

The amplicon generated by the SSP was 610 bp and was specific for the identification of daggernose shark body parts, whereas the control amplicon of non-target species was variable, ranging from 800 to 1650 bp (Fig. 3). The low-frequency appearance of a third high molecular weight amplicon (~4000 bp) on the target species was due to the amplification of 2 tandem repeats of the locus 45S rDNA, and does not interfere with the accuracy and effectiveness of the current methodology.

Market screening

Of the 51 samples acquired at the market, 4 were listed by fisherman and sellers as body parts from daggernose sharks. However, our molecular screening showed that these 4 samples were not from daggernose sharks, but came from other sympatric shark species. This suggests that the statistical data based on landing and identification by sellers (who are also the fishers) lead to errors, which could affect fisheries management. Market samples identified included 29 from the genus *Rhizoprionodon* (20 *R. porosus* and 9 *R. lalandei*), 7 blue sharks *Prionace glauca*, 6 shortfin mako sharks *Isurus oxyrhinchus*, 3 scalloped hammerhead sharks *Sphyrna lewini* and 6 from the genus *Carcharhinus* (4 *C. porosus*, 1 *C. obscurus* and 1 *C. falciformis*). Posterior sequencing of all shark samples obtained from the markets confirmed beyond doubt their identification made using SSPs (data available from the authors on request).

DISCUSSION

The daggernose shark SSP only amplified reliably for daggernose shark. Furthermore, the large dataset evaluated (289 total samples) confirmed its potential species-diagnostic utility. The ITS2 locus has been proved to be an excellent marker for genetic identification of shark species, capable of distinguishing clearly among closely related congeneric species (Pank et al. 2001, Shivji et al. 2002, Chapman et al. 2003, Pinhal et al. 2012). Moreover, the ITS2 locus belongs to the 45S ribosomal DNA tandem repeats (Lewin 2004), indicating the existence of a large number of primer annealing sites, which facilitates PCR amplification even with minor amounts of template DNA (Chapman et al. 2003, Mukherjee et al. 2007). The ITS2 region has been shown by diverse

studies to have enough resolution for species identification (Yao et al. 2010, Doukakis et al. 2011, Marino et al. 2015). All the data obtained for this region showed that ITS2 presents a low intraspecific polymorphism and high interspecific variability even for closely related species (Pinhal et al. 2012), which confirms the potential of ITS2 locus to be used for the development of SSPs. Related to our data, we performed 2 ClustalW alignments, showing (1) the low level of intraspecific variability for daggernose shark ITS2 loci (see Supplement 1 at www.int-res.com/articles/suppl/n032p169_supp.pdf), and (2) the high interspecific variability among shark species (Supplement 2; URL as above). All these results confirm the ability of this methodology to correctly identify daggernose shark body parts landed in bays and sold in markets worldwide. Our methodology permits the genetic identification of the species in one single tube reaction with no need for extra sequencing steps or restriction enzyme treatment (e.g. PCR-RFLP method), and is faster and less expensive than DNA barcoding. All these characteristics are critical for introducing molecular approaches to wildlife monitoring in developing countries. Nevertheless, it is important to point out that DNA barcoding provides, in most cases, unambiguous identification of which shark species the body part belongs to. The multiplex PCR-based methodology determines only whether the sample was acquired from the target species or not. Thus, the choice of method will be determined by the aims and scope of the study at hand.

The genetic identification of samples from the market was different from the identification of the corresponding body parts by fishermen and sellers. Market screening using molecular genetics provides a powerful tool to confirm the identity of shark species and to trace sampled individuals back to their original area of capture (Chapman et al. 2009). However, the results of our study indicate that information on shark landings provided by fishermen and sellers is not reliable and may therefore be inadequate for management of shark species. The daggernose shark is not listed in CITES Appendices I or II; however, given its Critically Endangered classification, it should be listed in Appendix I. In 2009, it was included in 'Anexo 1' of the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA). This listing prohibits the commercialization of the entire or body parts of this species in Brazil. However, there have been no records of this species from fisheries monitoring since 2009, suggesting that its conservation status is worse than indicated in official trade reports from IBAMA. This absence from the statistics could be the result of (1) errors in fisheries records; (2) lack of management in the area where the species occurs; (3) failure to record sharks caught and discarded at sea; and/or (4) the finning process, which leads to errors in, and absence of, records since the sharks' bodies are missing parts and thus also morphological characteristics needed for correct species identification. However, the considerable doubt that even moderate exploitation pressure can be sustained highlights the need for reliable information about the current exploitation level of this species to inform planning, and management and conservation strategies.

CONCLUSIONS

Shark species are being overexploited around the world and the exploitation rate is unsustainable (Worm et al. 2013). Therefore, efforts to improve trade monitoring and fisheries management are crucial for species conservation. In the current study, we presented a simple low-cost and efficient tool that can be easily applied for identification of body parts from the daggernose shark *Isogomphodon oxyrhynchus*, a Critically Endangered species under intense fishing pressure across its natural distribution area. Considering that the occurrence of this species is primarily restricted to marine areas belonging to developing countries, the low cost of this PCR-based methodology allows its implementation as a straightforward routine for trade monitoring and fisheries management.

Data accessibility. Daggernose shark ITS2 sequence: GenBank accession nos. KU556288 to KU556297.

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