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Application of multiplex PCR approaches for shark molecular identification: feasibility and applications for fisheries management and conservation in the Eastern Tropical Pacific

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Abstract

Here we describe the application of new and existing multiplex PCR methodologies for shark species molecular identification. Four multiplex systems (group ID, thresher sharks, hammerhead sharks and miscellaneous shark) were employed with primers previously described and some designed in this study, which allow for species identification after running PCR products through an agarose gel. This system was implemented for samples (bodies and fins) collected from unidentified sharks landed in the port of Buenaventura and from confiscated tissues obtained from illegal fishing around the Malpelo Island Marine Protected Area, Pacific Coast of Colombia. This method has allowed reliable identification, to date, of 407 samples to the genus and/or species levels, most of them (380) identified as the pelagic thresher shark (*Alopias pelagicus*). Another seven samples were identified as scalloped hammerhead sharks (*Sphyrna lewini*). This is an easy-to-implement and reliable identification method that could even be used locally to monitor shark captures in the main fishing ports of developed and developing countries.

Keywords: cytochrome oxidase I, molecular identification, multiplex PCR, Pacific Coast of Colombia, sharks

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Introduction

Sharks fisheries have experienced rapid growth around the world because of increased demand for shark products, particularly in Asian countries (Abercrombie *et al.* 2005; Clarke *et al*. 2004; Shivji *et al.* 2005). Exploitation of some shark species and populations to unsustainable levels, including illegal shark fining activities, is an increasing concern for fisheries managers and conservation organizations around the world (Clarke *et al*. 2006). Some researchers have presented data on estimates of numbers and composition of the market for shark fins in Hong Kong, which comprise around 50% of the global fin trade (Baker 2008; Clarke *et al*. 2006). From these data, there is concern about the decline of shark populations worldwide being affected not only by direct trade but also from habitat destruction and bycatch (Clarke *et al.* 2004; Gilman *et al*. 2007). To monitor these populations, there is an increased need for tools that are reliable, fast and easy to implement that aid in the monitoring of worldwide shark exploitation. Molecular genetic techniques, including DNA barcoding, have proved an extremely useful tool for this task in a variety of taxa. An increasing number of manuscripts have presented results on the application of genetic identification techniques for various sharks species, using samples from fins and other body parts (Abercrombie *et al.* 2005; Clarke *et al.* 2006; Holmes *et al.* 2009; Shivji *et al.* 2005; Ward *et al.* 2008). However, most of these techniques are based on sequencing of ITS2 and cytochrome oxidase I (COI) fragments (Abercrombie *et al.* 2005; Holmes *et al.* 2009; Shivji *et al.* 2005), which increases the time and cost of analyses, especially when trying to identify high volumes of samples. Here we present a new methodology, similar to that described by Abercrombie *et al.* (2005), for shark species identification based on COI multiplex PCR and detection on agarose gels.

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Methodology

Multiplex PCR assay design

Two new multiplex PCR methodologies were designed to assist in the identification of shark species in the

Eastern Tropical Pacific (ETP) and complement existing protocols. Previous studies utilizing multiplex PCR for shark identification (e.g. Shivji *et al.* 2002 and Abercrombie *et al.* 2005) focused primarily upon variation within the nuclear ITS2 region. Unfortunately, there is a paucity of sequence data available of this gene region for most shark species and nuclear DNA occurs in much lower quantity than mitochondrial DNA. Recent focus on genetic barcoding (Ward *et al.* 2005; Wong *et al.* 2009) has produced detailed information on both inter- and intraspecific variation within most shark species for the mitochondrial cytochrome oxidase subunit I gene (COI). Because of these data and higher copy number per cell of mitochondrial DNA, we chose to focus upon this region for assay development. As multiplex PCR methods are limited in how many taxa can be identified per assay, we chose to complement existing methods using a dichotomous key approach. The initial multiplex was designed to separate samples into phylogenetically coherent groups. With focus upon common groups within the ETP, we designed the multiplex PCR to categorize the sample into four groups including the hammerhead sharks (*Sphyrna* spp.), thresher sharks (*Alopias* spp.), mako sharks (*Isurus* spp.) and carcharhinid sharks (*Carcharhinus* spp., *Galeocerdo cuvier* and *Prionace glauca*). All available reference COI sequences from species of medium to large sharks that are present in the ETP (*Alopias pelagicus*, *A. superciliosus*, *A. vulpinus*, *Carcharhinus albimarginatus*, *C. brachyurus*, *C. falciformis*, *C. galapagensis*, *C. leucas*, *C. limbatus*, *C. longimanus*, *C. obscurus*, *C. plumbeus*, *C. porosus*, *C. signatus*, *Isurus oxyrinchus*, *I. paucus*, *Nasolamia velox*, *Negaprion brevirostris*, *Prionace glauca*, *Sphyrna lewini*, *S. mokarran* and *S. zygaena*) were downloaded from GenBank and used to design both group- and thresher shark-specific primers. In all cases, multiple sequences were available, spanning a broad geographic range, allowing for incorporation of intraspecific variability in sequence composition. Primers were designed so that all had similar Tm's, resultant products were of sufficiently different size so as to be easily separated on an agarose gel and that specific primers incorporated diagnostic nucleotides at the 3′ end to minimize amplification in nontarget taxa. Diagnostic nucleotides sites were chosen so that all animals within a distinct grouping (i.e. species, genus and order) shared this character while all animals outside of the desired group possessed a different character at this site. This multiplex PCR design approach has worked well in the design of other PCR-based identification methods (e.g. Shivji *et al.* 2002 and Hyde *et al.* 2005).

PCR cycling conditions were adjusted and queried using both target and nontarget DNA so as to eliminate false-positive results. Assays were screened using DNA from multiple species (*Alopias pelagicus*, *A. superciliosus*, *A. vulpinus*, *C. falciformis*, *C. galapagensis*, *C. limbatus*, *C. longimanus*, *C. obscurus*, *Isurus oxyrinchus*, *I. paucus*, *Prionace glauca*, *Sphyrna lewini* and *S. zygaena*) to test for the occurrence of false negatives and false positives. In all cases, the correct taxonomic group or species were identified using the appropriate assay with no amplification in nontarget species (see Figs 1 and 2). The PCR methodologies of Shivji *et al.* (2002) and Abercrombie *et al.* (2005) have been previously validated, so the application of these methods followed their protocols.

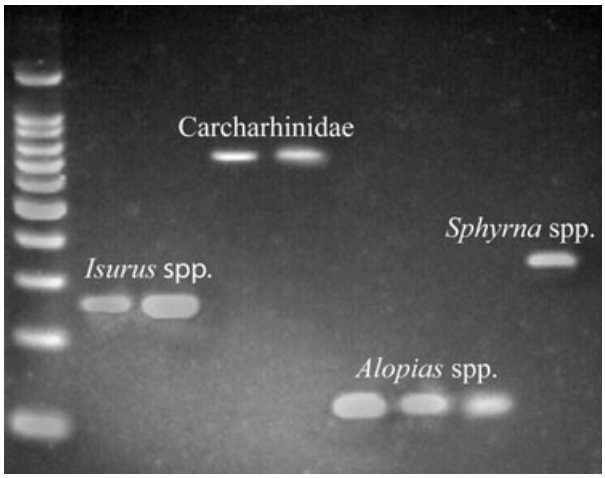

Fig. 1 Identification gel for the initial multiplex (shark group ID) separating samples into phylogenetically coherent groups.

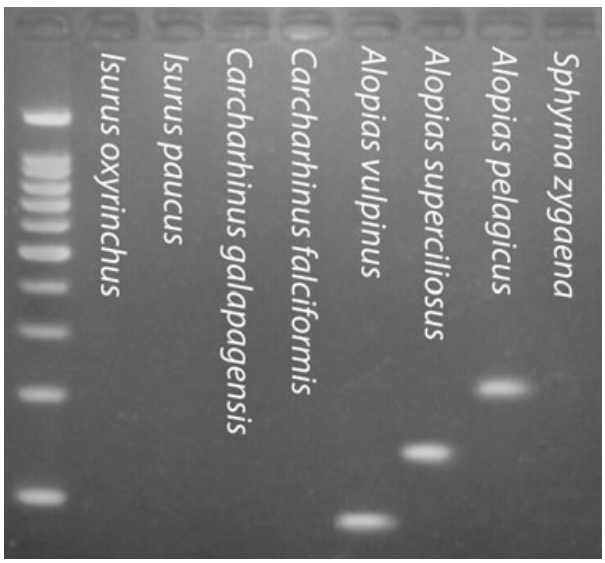

Fig. 2 Identification gel for the multiplex separating the three thresher shark species (thresher shark ID).

Multiplex PCR primers (Table 1b and Fig. 2) were designed to separate the three thresher shark species (*A. pelagicus*, *A. superciliosus* and *A. vulpinus*). The

Table 1 Primers for shark species identification by multiplex PCR, a) shark group ID; b) thresher shark ID; c) hammerhead shark ID; and d) miscellaneous shark ID. (P) refers to pelagic thresher shark, (C) refers to common thresher shark, (BE) refers to bigeye thresher shark, (LF) refers to longfin mako sharks and (SF) refers to shortfin mako shark

Primer name	Primer sequence (5′-3′)	PCR product size	Reference
(a)			
Fish Cox 1F	TCWACCAACCACAAGAYATYGGCAC	650 bp	Modified from Ward *et al*. (2005)
Thresher R (P)	TAGAAGTGATCCTGGCTGTCCTAA	119 bp	This study
Thresher R (C)	TAGAAGTGATCCGGGTTGTCCTAA	119 bp	This study
Thresher R(BE)	TAGGAGTGATCCGGGCTGGCCTAA	119 bp	This study
Hammer R	GCTTCTACYCCAGCRGAAGCT	334 bp	This study
Mako R (SF)	GCTATGTCTGGTGCTCCGATC	253 bp	This study
Mako R (LF)	CCATATCGGGTGCACCGATC	253 bp	This study
Carcharhinidae R	ACATGATAAAGGATTGGATCTCCTCCA	673 bp	This study
(b)			
Universal thresher F	AGCTGGRGTTGAAGCYGGAG		This study
Common thresher R	TCCAGCATGTGCTAGATTTCCC	76 bp	This study
Bigeye thresher R	TTGATGAGATACCTGCTAAATGAAGC	129 bp	This study
Pelagic thresher R	GTTTGATATTGGGAGATTGCAGGG	198 bp	This study

Primer name	Primer sequence (5′-3′)	Reference
(c)		
Fish 5.8 S-F	TAGCGGTGGATCACTCGGCTCGT	Shivji *et al*. (2002)
Fish 28 S-R	TCCTCCGCTTAGTAATATGCTTAAATTCAGC	Shivji *et al*. (2002)
GtHH123F	AGCAAAGAGCGTGGCTGGGGTTTCGA	Abercrombie *et al*. (2005)
ScHH401F	GGTAAAGGATCCGCTTTGCTGGA	Abercrombie *et al*. (2005)
SmHH630F	TGAGTGCTGTGAGGGCACGTGGCCT	Abercrombie *et al*. (2005)
(d)		
Fish 5.8 S-F	TAGCGGTGGATCACTCGGCTCGT	Shivji *et al*. (2002)
Fish 28 S-R	TCCTCCGCTTAGTAATATGCTTAAATTCAGC	Shivji *et al*. (2002)
Longfin mako	CCTCAACGACACCCAACGCGTTC	Shivji *et al*. (2002)
Shortfin mako	AGGTGCCTGTAGTGCTGGTAGACACA	Shivji *et al*. (2002)
Blue shark	AGAAGTGGAGCGACTGTCTTCGCC	Shivji *et al*. (2002)
Silky shark	ACCGTGTGGGCCAGGGTC	Shivji *et al*. (2002)
Porbeagle	GTCGTCGGCGCCAGCCTTCTAAC	Shivji *et al*. (2002)
Dusky/Galapagos shark	GTGCCTTCCCACCTTTTGGCG	Shivji *et al*. (2002)

method of Abercrombie *et al*. (2005) was applied to separation of the common large hammerhead species (*S. lewini*, *S. mokarran* and *S. zygaena*). For separation of the mako sharks (*I. oxyrinchus* and *I. paucus*) and some carcharhinid taxa (*C. falciformis*, *C. galapagensis/C. obscurus* and *P. glauca*), we used the method of Shivji *et al*. (2002).

Samples, DNA extraction and PCR

Samples were obtained from landings of artisanal fisheries in Buenaventura, Pacific Coast of Colombia, and from fishing boats found fishing illegally in the Marine Protected Area off the Santuario de Flora y Fauna de Malpelo in the Colombian Pacific, for a total of 458 skin and muscle samples collected between August 2009 and April 2010 (Fig. 3). A set of samples, identified morphologically by experts in the field (NOAA), were used as positive controls for molecular species identification.

DNA was extracted following the protocol of Hyde *et al*. (2005) using 200 μL of 10% Chelex (BioRad) and heating at 60 °C for 20 min, followed by heating at 103 °C for 25 min followed by a brief centrifugation and storage at 4 °C until multiplex PCR analysis.

Each 10-μL PCR was composed of HotStar Master Mix (Qiagen) or the Maxima® HotStart PCR Master Mix (Fermentas), CoralLoad loading buffer (Qiagen), 0.25 μM of each primer for the respective assay (Table 1) and 1.0 μL of DNA extract. All four multiplex PCRs were amplified with the following conditions: an initial denaturation

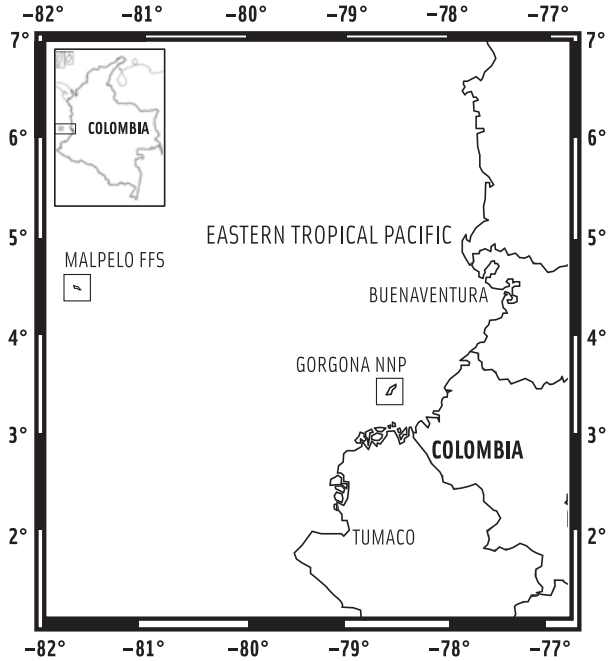

Fig. 3 Geographic location of Buenaventura and Malpelo Island, Pacific Coast of Colombia, where samples from this study were obtained.

step at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, annealing at 63 °C for 1 min and 70 °C for 1.5 min. One half of the PCR amplification product (5 μL) was run on a 2% agarose gel for 30 min including a 100-bp size standard for each row. Agarose gels were stained with ethidium bromide, and products were visualized using an UV transilluminator. The size of the resultant products were determined by comparison with a 100-bp DNA ladder, and these sizes were used to assign categorical identification based upon the product sizes expected for the individual assay (Table 1).

Results and discussion

A total of 407 samples were successfully identified to the genus and/or species level using the multiplex method presented here. This result represents a success rate of 89%. Fifty-one samples (11%) did not amplify successfully. This was likely due to DNA degradation or due to the fact that they belonged to other species out of the scope of the study. For these 51 samples, we attempted to amplify and sequence a 650-bp fragment of the COI gene using the primers and protocols described by Ward *et al.* (2005). We were unsuccessful in getting PCR products for these in three independent PCR runs.

Using the initial group specific multiplex PCR, 407 samples amplified successfully. Of these, 96% represented the genus *Alopias* (n = 391), commonly known as thresher sharks. Using the thresher shark-specific multiplex PCR, 367 samples were identified as *A. pelagicus*, the pelagic thresher shark. We were unsuccessful in amplifying twenty-four samples already identified as *Alopias* spp. using this multiplex PCR. We proceeded to amplify them for sequencing using the universal COI primers of Ward *et al.* (2005). Of these 24 samples, we successfully amplified, sequenced and identified thirteen of these as *A. pelagicus*, for a total of 380 *A. pelagicus* identified in the total sample. The other eleven samples were not successfully amplified, likely due to low DNA quality. No primer site mutations were found in these sequences, so failure to amplify using the *Alopias* multiplex PCR was likely due to low DNA quality.

Eleven samples were identified as belonging to the genus *Sphyrna*, representing only 2.7% of the total sample successfully analysed. Five of these eleven were identified as *Sphyrna lewini*, the scalloped hammerhead shark. The other six samples were not identified as either *S. zygaena* or *S. mokarran*, the species for which primers have been developed in the past. As before, we attempted to amplify these failed samples using universal COI primers. We successfully amplified, sequenced and identified four of these six samples, two as *S. zygaena* and two as *S. lewini*. The other two samples were not successfully amplified, likely due to low DNA quality.

Only three samples, <0.7% of the total sample successfully analysed, were identified as being a carcharhinid shark. Sequencing of the COI gene identified these samples as tiger sharks, *Galeocerdo cuvier*, confirming the presence of carcharhinid sharks in the sample analysed.

From this initial study, it appears that *Alopias pelagicus* is the most exploited shark species in this area of the Colombian Pacific. *Alopias pelagicus* is a pelagic shark, characterized by having a k reproductive strategy, implying low reproductive rate and late sexual maturation and considered as Vulnerable by the IUCN (Camhi *et al.* 2008). Because of this and the general lack of knowledge on this species, it is important to alert management authorities in Colombia and abroad to undertake research on total landed numbers and capture effort for this species in the main ports along the ETP, where this shark species appears to be an important resource for local communities (Beltrán 2001). Understanding the biology, stock structure, migration patterns and genetic diversity of this species in the ETP and throughout the species range will be crucial to implement management strategies that promote sustainable use of this resource in the near future.

The multiplex PCR approaches presented in this study were designed for species found in the ETP, but they will probably work on shark species found in other ocean basins. Validation of these methodologies for other regions in the near future will be very appropriate.

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Data accessibility

All sequences generated from this study were submitted to GenBank as accession numbers JN315423–JN315445. Please refer to Table S1 (Supporting Information) for a detailed description of each sequence submitted to GenBank.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1 Sample list, sample ID and accession numbers for sequences generated in this study

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