Genetic Identification of Pelagic Shark Body Parts for Conservation and Trade Monitoring

MAHMOOD SHIVJI,* SHELLEY CLARKE,† MELISSA PANK,* LISA NATANSON,‡ NANCY KOHLER,‡ AND MICHAEL STANHOPE§

*Guy Harvey Research Institute, Oceanographic Center, Nova Southeastern University, 8000 North Ocean Drive, Dania Beach, FL 33004, U.S.A., email mahmood@nova.edu
†T.H. Huxley School, Imperial College, London SW7 2BP, United Kingdom
‡National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 28 Tarzwell Drive, Narragansett, RI 02882, U.S.A.
§Evolutionary Bioinformatics, GlaxoSmithKline Pharmaceuticals, 1250 South Collegeville Road, UP1345, Collegeville, PA 19426–0989, U.S.A.

Abstract: *The conservation and management of sharks on a species-specific basis is a pressing need because of the escalating demand for shark fins and the recognition that individual shark species respond differently to exploitation. Difficulties with the identification of many commonly fished sharks and their body parts has resulted in a global dearth of catch and trade information, making reliable assessment of exploitation effects and conservation needs for individual species nearly impossible. We developed and tested a highly streamlined molecular genetic approach based on species-specific, polymerase-chain-reaction primers in an eight-primer multiplex format to discriminate simultaneously between body parts from six shark species common in worldwide pelagic fisheries. The species-specific primers are based on DNA sequence differences among species in the nuclear ribosomal internal transcribed spacer 2 locus. The primers and multiplex format accurately and sensitively distinguished samples from each of three lamnid (*Isurus oxyrinchus*, Isurus paucus*, and* Lamna nasus*) and three carcharhinid (*Prionace glauca*, Carcharhinus obscurus*, and* Carcharhinus falciformis*) species from all but one other shark species encountered in the North Atlantic fishery. Furthermore, the three lamnid primers were robust enough in their discriminatory power to be useful for species diagnosis on a global scale. Preliminary testing of dried fins from Asian and Mediterranean commercial markets suggests that our genetic approach will be useful for determining the species of origin of detached fins, thus allowing the monitoring of trade in shark fins for conservation assessment. Our approach will also facilitate detection of products from protected and other at-risk shark species and may prove useful as a model for development of the high-throughput, genetic, species-diagnosis methods typically required in conservation and management contexts.*

Identificación Genética de las Partes del Cuerpo de un Tiburón Pelágico para la Conservación y Monitoreo de su Comercialización

Resumen: *La conservación y manejo de tiburones fundamentado a nivel de especie es una necesidad imperativa debido a la creciente demanda de aletas de tiburón y el reconocimiento de que las especies individuales de tiburones responden de manera distinta a la explotación. Las dificultades para la identificación de muchos tiburones capturados comúnmente, así como de partes de su cuerpo, han resultado en una escasez global de información sobre capturas y comercialización, haciendo casi imposible el poder realizar evaluaciones de los efectos de la explotación y de las necesidades de conservación. Desarrollamos y evaluamos un método altamente estilizado de genética molecular basado en detonadores de la reacción en cadena de la polimerasa, especie-específicos, en un formato múltiple de ocho detonadores para discriminar simultáneamente entre las partes del cuerpo de seis especies de tiburones provenientes de pesquerías pelágicas mundi-*

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*ales comunes. Los detonadores especie-específicos están basados en diferencias en las secuencias de ADN entre especies del locus espaciador 2 nuclear, ribosomal, transcrito. Los detonadores y el formato múltiple distinguen muestras con precisión y sensitividad de cada uno de los tres lámnidos (*Isurus oxyrinchus*,* Isurus paucus *y* Lamna nasus*) y tres especies de carcarínidos (*Prionace glauca*,* Carcharhinus obscurus *y* Carcharhinus falciformis*) especies todas encontradas en las pesquerías de Norteamérica, excepto una. Mas aún, los detonadores de los tres lamnidos fueron lo suficientemente robustos en su poder discriminante como para ser usados para el diagnóstico de especies a escala mundial. Las pruebas preliminares de aletas secas de los mercados comerciales de Asia y el Mediterráneo sugieren que nuestro método genético puede ser útil para determinar la especie de origen de las aletas separadas, permitiendo así usar el monitoreo de las aletas de tiburón para evaluaciones de conservación. Nuestro método también podría facilitar la detección de productos provenientes de especies protegidas o en riesgo y podría resultar útil como un modelo para el desarrollo de métodos genéticos de alto rendimiento para el diagnóstico de especies, métodos típicamente requeridos en los contextos de conservación y manejo.*

Introduction

Conservation and effective management of the world's exploited shark populations have become issues of considerable concern on an international scale as a result of greatly expanded commercial fishing efforts over the past two decades (Bonfil 1994; Weber & Fordham 1997; Food and Agriculture Organization [FAO] 1998, 2000; National Marine Fisheries Service [NMFS] 2001). The status of many of the world's shark populations is poorly known, hampering the development and implementation of appropriate conservation measures aimed at sustaining populations over the long term. This lack of knowledge stems from several political and biological factors, including traditionally low levels of management interest and research funding, the general public's misconception of sharks as man-eaters and therefore not deserving of conservation, and logistical difficulties associated with studying these animals. The population status of pelagic sharks—defined here as species most commonly, but not exclusively, encountered in offshore and oceanic habitats—in particular is extremely poorly known. This is likely due, in addition to the factors identified above, to the offshore habitat of these species, their typically highly mobile nature resulting in the routine crossing of political boundaries, and what appear to be complex life-history and migratory characteristics.

The recent international attention being directed at shark conservation and management stems from the realization that sharks, with life-history characteristics more similar to those of mammals (e.g., slow growth, late reproductive maturity, relatively few young) than of teleost fishes, are unlikely to respond well to the expanded fishing pressure they are experiencing (FAO 1998, 2000). The increased exploitation of sharks over the past decade is reflected in the escalating quantities of shark fins exported to Asian markets to feed the growing demand for shark-fin soup, a delicacy served when entertaining or celebrating with guests. The much higher price garnered by fins than by the flesh of most shark species has resulted in an escalation by several nations in the practice of "finning," whereby only the fins from a shark are kept for market and the rest of the animal is discarded at sea (Rose 1996; Camhi 1999). Pelagic sharks, which are caught in directed fisheries and as incidental catch in offshore long-line fisheries targeting tuna and billfishes, are now commonly exploited for their fins worldwide (Rose 1996; Weber & Fordham 1997).

In response to concerns about the sustainable health of shark populations on a global scale, the United Nations Food and Agriculture Organization (FAO) developed an International Plan of Action for the Conservation and Management of Sharks that calls for all member nations participating in shark fisheries to develop and implement their own national plans of action aimed at ensuring the conservation and management of shark stocks (FAO 1998). To comply with the FAO plan directive, the United States has recently developed its own National Plan of Action for the Conservation and Management of Sharks (National Marine Fisheries [NMFS] 2001).

A major and recurring recommendation in both the FAO and U.S. plans is the collection of data on shark catch, landings, and the assessment of shark stocks on a species-specific basis (FAO 1998, 2000; NMFS 2001). This recommendation has its roots in (1) the historical absence of reliable data on shark catch and trade on a species-specific basis, making robust stock assessments and identification of overfished and potentially threatened populations and species nearly impossible in most cases and (2) the recognition that individual species differ in their life-history characteristics and therefore in their susceptibility to exploitation (Bonfil 1994; Smith et al. 1998; Castro et al. 1999).

A major obstacle to obtaining data on shark catch and trade and implementating conservation and management plans on a species-specific basis, however, is the difficulty of accurately identifying many commonly exploited species. Shark species within the economically

important families Carcharhinidae and Lamnidae (hereafter referred to as carcharhinids and lamnids, respectively), for example, can differ only slightly in their morphology and are often confused with each other (Castro 1993; Bonfil 1994; Castro et al. 1999). The species-identification problem is exacerbated by the practice widespread in commercial fisheries of removing the head, tail, and most fins from the landed sharks while at sea to minimize storage needs and prevent spoiling. This removal of the primary species-distinguishing characteristics leaves shark carcasses (known as "logs") that are often challenging for fishery management and enforcement personnel to identify accurately.

Species-specific conservation and management difficulties have been further compounded by the rapidly expanding trade in shark fins. Accurate identification of species based on morphological characteristics of detached shark fins is difficult in most cases (a possible exception is blue shark [*Prionace glauca*] fins [S.C., personal observations]) (Vannuccini 1999), and only preliminary morphological keys exist (Nakano & Kitamura 1998; Anonymous 1999). These species-identification difficulties have been a major factor contributing to the worldwide scarcity of species-specific records on shark catch and trade (Bonfil 1994; Castro et al. 1999). There is a pressing need to solve this problem so that effective conservation and management of sharks can proceed on a species-specific basis (FAO 1998; NMFS 2001).

The DNA–based methods for species identification, although widely and routinely employed in biomedical and agricultural practices (Clapp 1993; Hendolin et al. 2000; Zarlenga et al. 2000), have not been implemented widely in fish management and conservation. This is partly due to the perception that DNA methods, although accurate, are slow and expensive and therefore unlikely to be suitable in monitoring and enforcement contexts where large numbers of samples need to be screened relatively quickly. Routine adoption of DNA identification methods for fish conservation and management therefore awaits the availability of simpler, quicker and more easily implemented approaches.

For sharks, the genetic identification methods developed thus far have been based on conventional polymerase-chain reaction (PCR) amplification of mitochondrial or nuclear loci, followed by subsequent restriction-endonuclease digestion of the amplicons to yield species-diagnostic restriction-fragment-length-polymorphism patterns (Martin 1993; Shivji et al. 1996; Heist & Gold 1999). These methods, although effective for species identification, require multiple steps (PCR followed by restriction-endonuclease digestion with multiple enzymes) and are therefore relatively slow, labor intensive, and expensive.

We present an alternative, accurate, sensitive, quick, and easy-to-implement genetic approach for identification of pelagic shark species. The approach is highly streamlined and therefore likely to be amenable to routine use in conservation and management contexts. Species diagnosis is achieved by PCR only, without the need for further manipulation of the amplicons by restriction-endonuclease digestion, sequencing, or DNA hybridization steps. Our approach relies on the use of multiple species-specific PCR primers that can be used in a single-reaction-tube, multiplex setting to simultaneously discriminate between six shark species (three lamnids: porbeagle (*Lamna nasus*), shortfin mako (*Isurus oxyrinchus*), and longfin mako (*Isurus paucus*); and three carcharhinids: blue (*Prionace glauca*), dusky (*Carcharhinus obscurus*) and silky (*Carcharhinus falciformis*) commonly encountered in North Atlantic and worldwide pelagic fisheries. These six species are also frequently utilized in the global fin market (Shivji & Clarke, unpublished data).

The U.S. National Marine Fisheries Service (NMFS) has recently prohibited landings of dusky and longfin mako sharks from the U.S. Atlantic fishery (NMFS 1999). The dusky shark has also been categorized as vulnerable to extinction in North America by the American Fisheries Society (Musick et al. 2000) and, depending on the population, is in the vulnerable or lower risk–near threatened category of the World Conservation Union's (IUCN) 2000 Red List of Threatened Animals (www.redlist.org). All six shark species are widely distributed globally (Compagno 1984). For five of the six species (porbeagle samples outside the northwest Atlantic were unavailable for testing), our PCR primers may be suitable for identification of animals from globally distributed populations.

Methods

Sources of Shark Tissue Samples

We collected tissue samples (reference samples) used for DNA sequencing and species-specific PCR primer design during fishery-independent surveys of shark population abundance and tagging conducted by the NMFS off the U.S. Atlantic coast. Shark tissue samples (test samples) that we used for testing the diagnostic utility of our species-specific primers were collected from the Atlantic, Mediterranean, and Pacific by us or other shark experts who identified the whole animals. Table 1 contains the shark reference and test species analyzed and their geographic origins and sample sizes. Dried shark fins were obtained by us from the Hong Kong fin market and from the FAO as part of a separate study.

The reference samples we used for DNA sequencing consisted of white muscle. Test samples were fin and muscle tissues. All tissues, with the exception of dried fins, were kept in SED preservative (saturated NaCl, EDTA, DMSO; Seutin & White 1991) or 95% ethanol at room tem-

Table 1. Shark reference and test species tested using the six species-specific primers in triplex and octaplex-PCR formats, and their geographic origins.

Species	*Geographic origin* (n)*
Reference samples	
Isurus paucus (longfin mako)	NW Atlantic (1)
Isurus oxyrinchus (shortfin mako)	NW Atlantic (1)
Lamna nasus (porbeagle)	NW Atlantic (1)
Carcharhinus obscurus (dusky)	NW Atlantic (1)
Carcharhinus falciformis (silky)	NW Atlantic (1)
Prionace glauca (blue)	NW Atlantic (1)
Test samples	
longfin mako	NW Atlantic (5)
longfin mako	W Pacific (3)
shortfin mako	NW Atlantic (8)
shortfin mako	NE Pacific (3)
shortfin mako	W Pacific (9)
porbeagle	NW Atlantic (17)
dusky	NW Atlantic (12)
dusky	SW Pacific (3)
dusky	W Pacific (6)
silky	NW Atlantic (13)
silky	W Pacific (8)
blue	NW Atlantic (8)
blue	Mediterranean (24)
blue	NE Pacific (6)
blue	W Pacific (5)
blue	SW Pacific (3)
Alopias vulpinus (thresher)	NW Atlantic (6)
Alopias superciliosus (bigeye thresher)	NE Pacific (1)
	W Pacific (6)
Alopias pelagicus (pelagic thresher)	NE Pacific (2)
	W Pacific (2)
Carcharodon carcharias (white)	NW Atlantic (4)
	NE Pacific (2)
	Indian Ocean (4)
Lamna ditropis (salmon)	NE Pacific (17)
Carcharias taurus (sandtiger)	NW Atlantic (3)
Carcharhinus altimus (bignose)	NW Atlantic (5)
Carcharhinus longimanus (oceanic whitetip)	NW Atlantic (1)
	NE Pacific (2)
	W Pacific (3)
Carcharhinus signatus (night)	NW Atlantic (2)
Carcharhinus plumbeus (sandbar)	NW Atlantic (5)
	Central Pacific (2)
Carcharhinus limbatus (blacktip)	NW Atlantic (8)
Carcharhinus leucas (bull)	NW Atlantic (6)
Carcharhinus brevipinna (spinner)	NW Atlantic (5)
	W Pacific (3)
Carcharhinus isodon (finetooth)	NW Atlantic (5)
Carcharhinus acronotus (blacknose)	NW Atlantic (6)
Carcharhinus perezi (caribbean reef)	NW Atlantic (3)
	SW Atlantic (3)
Carcharhinus amboinensis (java)	SW Pacific (2)
Carcharhinus tilstoni (australian blacktip)	SW Pacific (2)
Carcharhinus sorrah (spot-tail)	SW Pacific (2)
Carcharhinus amblyrhynchos (grey reef)	Central Pacific (2)
Negaprion brevirostris (lemon)	NW Atlantic (2)
	SW Atlantic (2)
Negaprion acutidens (sicklefin lemon)	SW Pacific (2)
Galeocerdo cuvier (tiger)	NW Atlantic (3)
	W Pacific (3)
Triaenodon obesus (whitetip reef)	Central Pacific (2)
Rhizoprionodon terranovae (atlantic sharpnose)	NW Atlantic (6)
Sphyrna mokkaran (great hammerhead)	NW Atlantic (3)
	SW Pacific (1)
Sphyrna lewini (scalloped hammerhead)	NW Atlantic (3)
	W Pacific (3)
Sphyrna zygaena (smooth hammerhead)	NW Atlantic (2)
	NE Pacific (3)
	W Pacific (3)

Continued

Table 1. Continued

**Number of animals tested from each geographic location.*

perature for short-term storage or at 4° C for long-term storage. Dried fins were stored at room temperature.

DNA Extraction, PCR Amplification, and DNA Sequencing

Genomic DNA was extracted from 25 mg of tissue with the QIAmp Tissue Kit (QIAGEN Inc., Valencia, California) and stored at −20° C until use. A PCR fragment (hereafter referred to as a positive control amplicon) of approximately 1350 bp from the lamnid sharks (longfin mako, shortfin mako, porbeagle) and 1470 bp from the carcharhinid sharks (blue, dusky, silky) containing the entire nuclear ribosomal DNA internal transcribed spacer (ITS2) region plus portions of the flanking 5.8S (approximately 160 bp) and 28S (approximately 60 bp) ribosomal RNA genes were amplified from the six shark species by standard PCR, employing the shark universal primers FISH5.8SF (forward primer 5′-TTAGCGGTG GATCACTCGGCTCGT-3′) and FISH28SR (reverse primer 5′-TCCTCCGCTTAGTAATATGCTTAAATTCAGC-3′). Following PCR amplification, both strands of the positive control amplicon were sequenced according to standard protocols on an automated sequencer, with internal sequencing primers designed as necessary. Reference sequences for this locus for the six species were deposited in GenBank under the following accession numbers: porbeagle, AF515444; shortfin mako, AF515442; longfin mako, AF515443; blue, AF515441; silky, AF513986; and dusky, AY033819.

Design of Species-Specific Primers and the Multiplex PCR Assay

We aligned the ITS2 sequences obtained from reference shark samples with the alignment program ClustalX (Thompson et al. 1997) and manually adjusted the align-

ment using the sequence-editing program GeneDoc (Nicholas & Nicholas 1997).

Five to seven PCR primers putatively specific for each of the six pelagic species were designed based on nucleotide-sequence differences in the ITS2. Each primer was first tested individually on geographically widespread samples (from both Atlantic and Pacific Oceans when possible; Table 1) of the species for which the primer was designed (hereafter referred to as the target species). To test each primer, we used a combination of three primers simultaneously—one putatively species-specific primer plus the shark forward and reverse universal primers—in multiplex (triplex) PCR reactions (Fig. 1). Our a priori measure of primer success in species identification was the expectation that a three-primer, multiplex combination containing, for example, the primer for shortfin mako would produce two amplicons when used to amplify the target-species (i.e., shortfin mako) genomic DNA: (1) a 1350-bp positive-control amplicon generated from the two shark universal primers and (2) a smaller 771-bp amplicon diagnostic for shortfin mako generated from the forward primer specific to the shortfin mako and the shark universal reverse primer (Fig. 1). In contrast, this combination of primers, when tested against genomic DNA from any other (nontarget) shark species would produce only the positive-control amplicon because of failure of the primer specific to shortfin mako to anneal to DNAs from these other species. Analogous test results would be expected for the other five shark species when a three-primer combination is used that includes a different species-specific primer.

Amplifications were initially performed in a Mastercycler Gradient (Eppendorf Inc., Westbury, New York) thermal cycler with the temperature gradient option to determine the most stringent annealing temperature (range used: 55°–68° C) that could be used for each putatively species-specific primer to amplify its target species. Total amplification-reaction volumes were 50 μL and contained 1 μL of the extracted genomic DNA, 12.5 pmol of each primer, 1X PCR buffer (QIAGEN Inc.), 40 μM dNTPs, and 1 unit of HotStar Taq DNA Polymerase (QIAGEN Inc.). The PCR thermal cycling profile we used was 94° C initial heating for 15 minutes to activate the DNA polymerase, followed by 35 cycles of 94° C for 1 minute, 58°–68° C (gradient) for 1 minute, 72° C for 2 minutes, and a 5-minute final extension step at 72° C. Completed reactions were kept at 4° or −20° C until checked by gel electrophoresis on 1.2% agarose gels. Each primer that successfully amplified its target species at an annealing temperature of 65° C or higher was subsequently tested at 65° C (all other PCR conditions as described above) for its amplification performance against various nontarget shark species typically or occasionally encountered in the U.S. Atlantic pelagic fishery. For those nontarget species that are globally distributed, these primers were also tested on Pacific representatives where available (Table 1) to verify that potential population-level sequence polymorphisms in the ITS2 locus would not reduce the diagnostic performance of the primers. To further evaluate the discriminatory power of the three carcharhinid primers in worldwide pelagic-shark fisheries, they were also tested against closely related, nontarget carcharhinid species endemic to the Indo-Pacific, when samples were available (Table 1). These amplifications were performed in Mastercycler Gradient (gradient option off) or MJ Research PTC-100 (MJ Research, Inc., Waltham, Massachusetts) thermal cyclers.

From these amplification tests, we selected the one "optimized" primer for each of the six pelagic species that demonstrated the best performance in the triplex PCR assay according to the following criteria: (1) the primer demonstrated the greatest species specificity in its amplification behavior at the high-stringency annealing temperature of 65° C and (2) the primer produced a

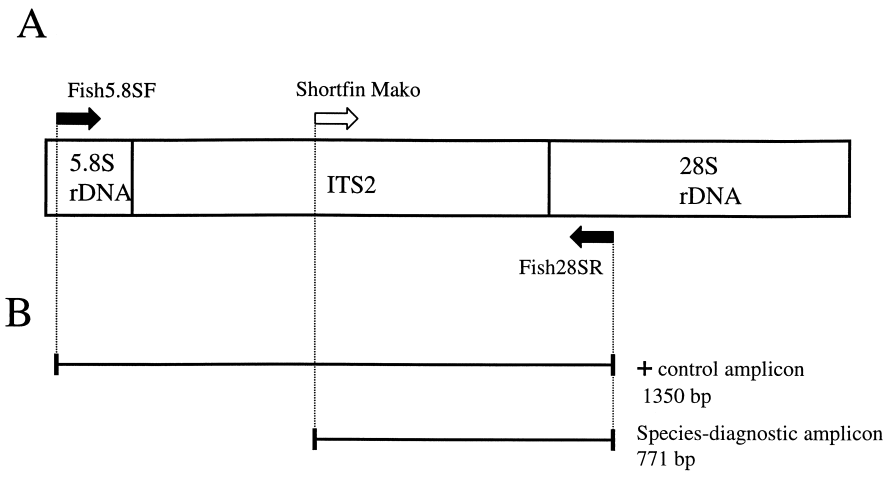

Figure 1. (a) Schematic representation of the shark nuclear 5.8S and 28S ribosomal RNA genes and ITS2 locus showing relative annealing sites and orientation of primers used in the triplex–PCR assays. The shark universal primers (FISH5.8SF and FISH28SR) are shown as solid arrows. The shortfin mako shark primer (open arrow) is an example of a species-specific primer. (b) Spatial coverage of the two amplicons expected to be produced using this combination of three primers when tested against shortfin mako DNA.

species-specific amplicon that was diagnostic in size for each of the six species.

To further streamline the species-identification assay, these optimized primers were evaluated for their diagnostic performance in a more extensive multiplex (octaplex) PCR reaction that used all eight primers simultaneously (i.e., the two-shark, universal forward and reverse primers and all six species-specific primers) in a single-tube amplification reaction. Shark test samples analyzed (target and nontarget species) were the same as above (Table 1). The PCR conditions for the octaplex reactions were as described above, with the following modifications: annealing temperature was 65° C for all species, and HotStar Taq DNA polymerase was used in two amounts, 0.5 and 1 unit per reaction, in separate trials to assess the effect of the amount of DNA polymerase on the performance of the eight-primer octaplex assay.

To test the primers and multiplex approach for their utility in amplifying DNA from dried fins obtained from the Hong Kong market, we used the octaplex assay on 12 fins designated by the fin traders as blue shark, 8 fins designated as "mako" shark (species not identified), and 55 fins designated as silky shark. The 31 dried fin samples obtained from the FAO were identified as part of a blind test by the octaplex assay, with the exception that the primer for longfin mako was replaced by a primer for the thresher shark (*Alopias vulpinus*; primer details to be published separately) because we suspected the presence of thresher shark fins in the samples based on the Mediterranean location of the fin fishery.

Results

Testing each Species-Specific Primer in the Three-Primer Multiplex Assay

The ITS2 loci in the three lamnid and three carcharhinid sharks we sequenced ranged from 1123 to 1136 bp and 1233 to 1269 bp in size, respectively. Sequence divergence (p-distance, expressed as absolute percent difference between sequences) between each pair of species ranged from 11% to 15% among the lamnids, 9% to 13% among the carcharhinids, and 55% to 58% between the lamnids and carcharhinids. Annealing sites for each of the six optimized species-specific PCR primers were internal to the forward and reverse shark universal primers (Fig. 2).

In the three-primer triplex assays, each species-specific primer demonstrated complete species specificity (porbeagle, shortfin mako, longfin mako, silky, blue primers) or nearly complete species specificity (dusky primer, see below) species specificity when tested against target and nontarget species (Fig. 3). The sequence of each species-specific primer is given in Table 2. Each of the six species-specific primers amplified a diagnostic-sized amplicon from its target species (Fig. 3). As expected, the coamplification of a positive control amplicon occurred in all cases in longfin mako, shortfin mako, porbeagle, and dusky sharks. Co-amplification of the positive control amplicon from blue and silky sharks was inconsistent, however, typically occurring in lower yields, when the blue and silky primers were included in the PCR (see discussion section on the ramifications of this inconsistency). In the case of all nontarget species, only the positive control amplicon was amplified (Fig. 3; remaining gels not shown). The only exception to the complete species specificity of each primer was observed with the dusky primer, which amplified both dusky and oceanic whitetip DNA.

Testing the Eight-Primer Octaplex Assay

Combining the six pelagic shark primers with the two shark universal primers in an eight-primer, single-tube amplification reaction at 65° C annealing temperature consistently yielded a species-diagnostic-sized amplicon from geographically widespread samples of each of the six pelagic species (Fig. 4). Each amplicon had a size (Table 2; size inferred from the ITS2 sequences of reference animals) easily distinguishable from that of the other amplicons on a 1.2% agarose gel.

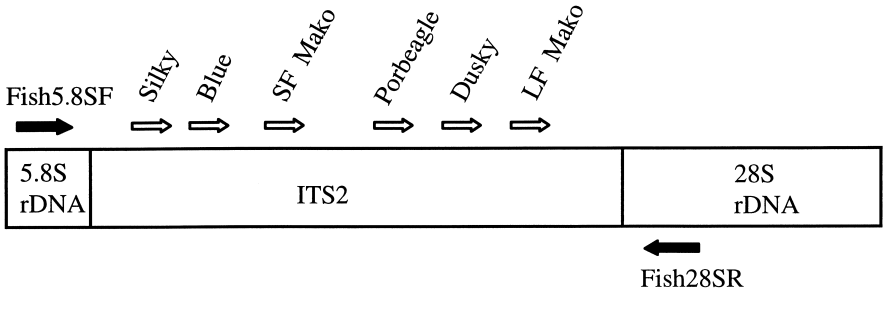

Figure 2. Schematic representation of the shark nuclear 5.8S and 28S ribosomal RNA genes and ITS2 locus showing relative annealing sites and orientation of primers used in the octaplex–PCR assays. Shark universal primers (FISH5.8SF and FISH28SR) are shown as solid arrows. The six species-specific primers are shown as open arrows. Abbreviations: SF Mako, shortfin mako; LF Mako, longfin mako.

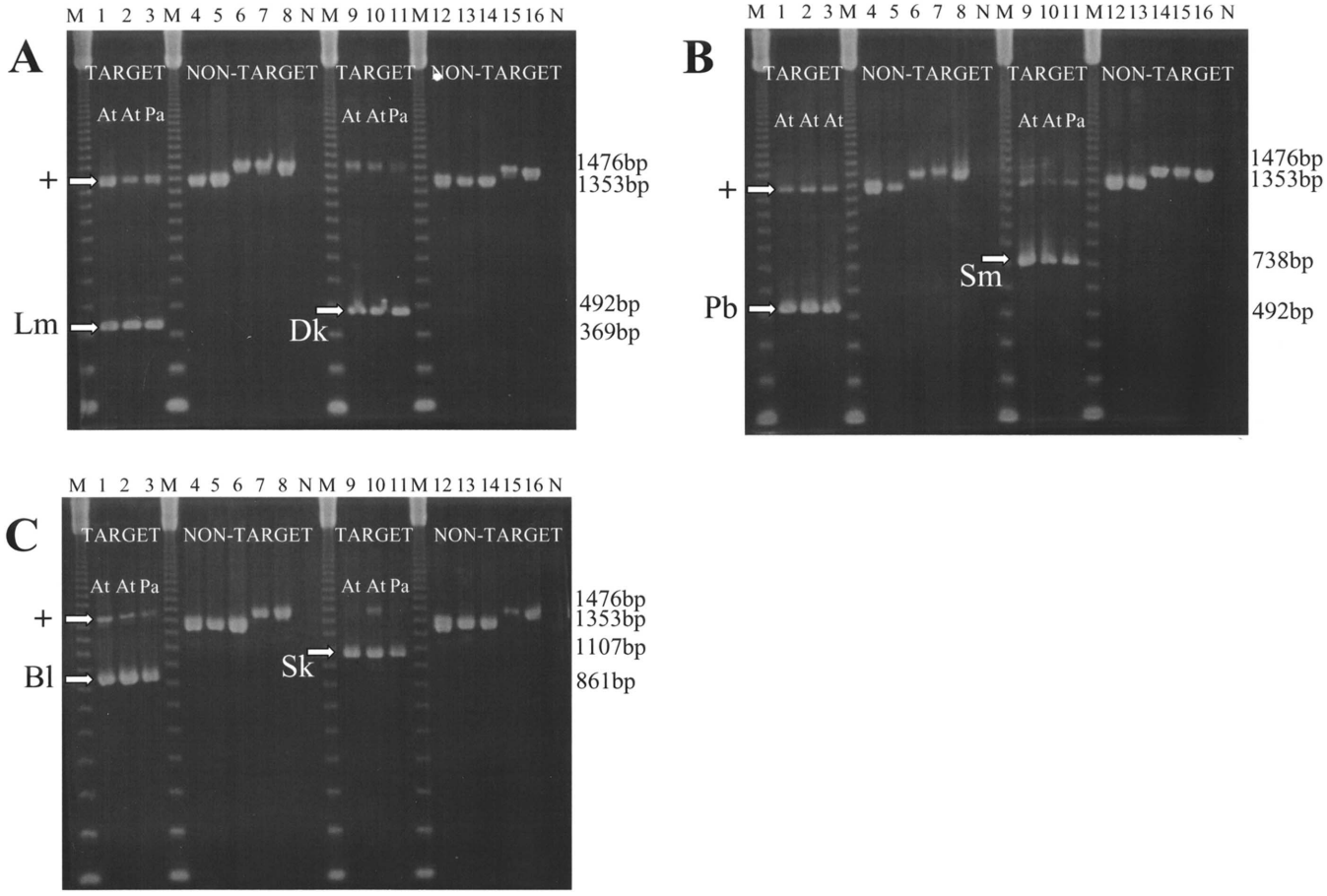

Figure 3. Results of amplification reactions with the three-primer multiplex combination of two shark universal primers and one species-specific primer. (a) Testing specific primers for longfin mako and dusky sharks against their target species (lanes 1–3 and 9–11, respectively) and nontarget species (lanes 4–8 and 12–16, respectively). Geographic origin of the target animals: At, Atlantic; Pa, Pacific. Nontarget species: lane 4, porbeagle; 5, shortfin mako; 6, silky; 7, dusky; 8, blue; 12, porbeagle; 13, shortfin mako; 14, longfin mako; 15, silky; 16, blue. Arrows labeled Lm and Dk show the longfin mako and dusky species-specific amplicons, respectively. Arrow labeled + indicates the positive control amplicon. Lanes labeled N contain the negative control reactions (no shark DNA). Lanes labeled M contain the molecular size standard (Gibco-Life Technologies 123 bp ladder standard). Sizes (bp) of individual molecular size-standard bands spanning the species-specific and positive control amplicons are indicated to the right of the gel picture. (b) Testing of specific primers for porbeagle and shortfin mako sharks against their target and nontarget species. Nontarget species: lane 4, longfin mako; 5, shortfin mako; 6, silky; 7, dusky; 8, blue; 12, porbeagle; 13, longfin mako; 14, silky; 15, dusky; 16, blue. Arrows labeled Pb and Sm show the porbeagle and shortfin mako species-specific amplicons, respectively. All other annotation is as in figure A. (c) Testing specific primers for blue and silky sharks against their target and nontarget species. Nontarget species: lane 4, porbeagle; 5, shortfin mako; 6, longfin mako; 7, silky; 8, dusky; 12, porbeagle; 13, shortfin mako; 14, longfin mako; 15, dusky; 16, blue. Arrows labeled Bl and Sk show the blue and silky species-specific amplicons, respectively. All other annotation is as in figure A.

The amount of DNA polymerase used in the reaction had a clear influence on the amplification yield of the positive control amplicon. In the presence of target species, 0.5 units of enzyme per octaplex–PCR reaction produced consistent and clearly recognizable amplification of the species-diagnostic amplicon, but usually no amplification of the positive control amplicon (gels not shown). Increasing the amount of DNA polymerase to one unit per reaction produced both the species-diagnostic and positive control amplicons from target species in the case of longfin mako, shortfin mako, porbeagle, and dusky sharks. As with the triplex assay, however, coamplification of the positive-control amplicon was inconsistent in the case of blue and silky sharks, usually occurring in low yields (Fig. 4). With nontarget species, only the positive-control amplicon was amplified in the octa-

Table 2. Species-specific primer sequences and size of the species-diagnostic amplicon produced.

Shark species	*Primer sequence*	*Amplicon size (bp)*
Longfin mako primer	5′-CCTCAACGACACCCAACGCGTTC-3′	418
Dusky primer*	5′-GTGCCTTCCCACCTTTTGGCG-3′	480
Porbeagle primer	5′-GTCGTCGGCGCCAGCCTTCTAAC-3′	554
Shortfin mako primer	5′-AGGTGCCTGTAGTGCTGGTAGACACA-3′	771
Blue primer	5′-AGAAGTGGAGCGACTGTCTTCGCC-3′	929
Silky primer	5′-ACCGTGTGGGCCAGGGTC-3′	1085

**Dusky primer sequence reported previously by Pank et al. (2001).*

plex assay, regardless of the amount of DNA polymerase used.

Overall, for the sample sizes and geographic origins tested here, the octaplex assay proved 100% accurate and sensitive in its ability to discriminate among samples of known identity of the six pelagic shark species. No false-positive amplifications occurred, with the exception of the dusky primer also amplifying DNA from its congener, the oceanic whitetip shark.

Testing Dried Shark Fins

The octaplex assay corroborated the Hong Kong trader's designation of blue shark (Ya jian chi) fins in all cases (n = 12). Of the eight fins designated as "mako" (Qing lian chi) by the traders, seven fins were genetically identified as belonging to shortfin mako. Analysis of the one "mako" fin resulted in amplification of only the positive-control amplicon, indicating that the fin was not from a shortfin or longfin mako or any of the four remaining species tested with the octaplex assay. Of the 55 fins designated as silky shark (Wu yang chi) by fin traders, 45 fins were genetically typed as silky, with the remaining 10 fins producing only the positive-control amplicon. The 31 FAO shark-fin samples were identified with 100% accuracy in the blind test (D. Bartley, personal communication), with 24 samples turning out to be blue sharks and 7 thresher sharks.

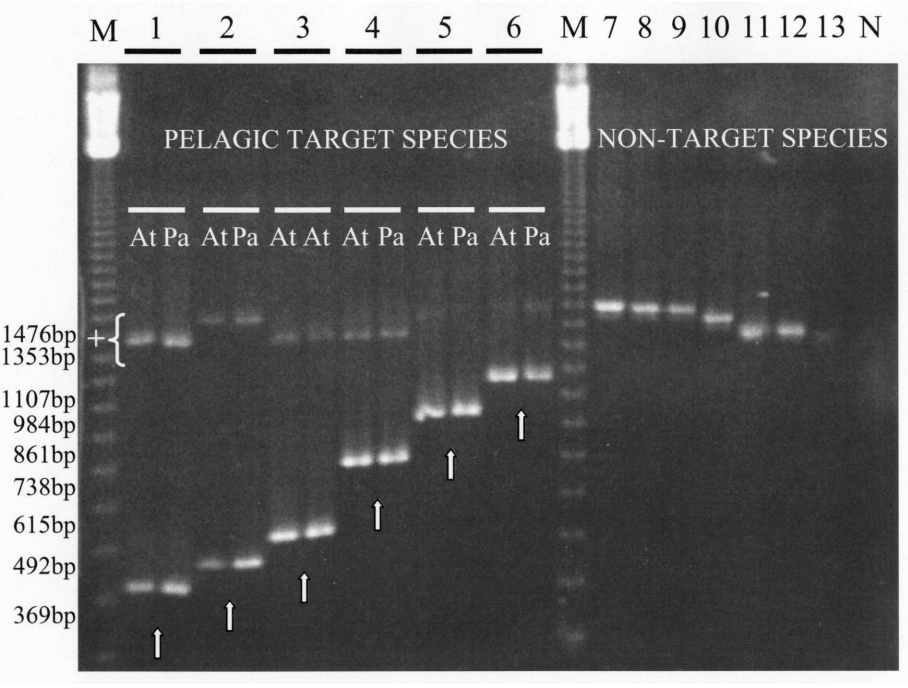

Figure 4. Results of octaplex–PCR testing on pelagic-shark target and nontarget species. Lanes 1–6 show octaplex–PCR amplification products from the six target species: 1, longfin mako; 2, dusky; 3, porbeagle; 4, shortfin mako; 5, blue, 6, silky. Geographic origin: At, Atlantic; Pa, Pacific. The species-diagnostic amplicons are indicated by arrows. Lanes 7–13 show octaplex–PCR amplification products from nontarget species: 7, night; 8, bignose; 9, sandbar; 10, tiger; 11, thresher; 12, pelagic thresher; 13, bigeye thresher. The + indicates the positive control amplicons. Lane labeled N contains the negative-control reaction (no shark DNA). Lanes labeled M contain the molecular size-standard (Gibco-Life Technologies 123 bp ladder standard). Sizes (bp) of individual, molecular size-standard bands spanning the species-diagnostic and positive control amplicons are indicated to the left of the gel picture.

Discussion

The predominant recent approaches to vertebrate species identification for ecological, conservation, management, and forensic questions involve either PCR amplification of a specific locus followed by restriction endonuclease analysis (the PCR–RFLP approach; e.g., Innes et al. 1998; Heist & Gold 1999; Lindstrom 1999; Asensio et al. 2000; Gharrett et al. 2001) or phylogenetic reconstructions to determine relationships between DNA sequences from unknown samples and known species (the phylogenetic approach; e.g., Baker & Palumbi 1994; Malik et al. 1997; Palumbi & Cipriano 1998; Dizon et al. 2000). Both approaches, although generally robust and effective for species identification, are relatively time consuming and expensive, requiring downstream analysis of the amplified products after the PCR step (i.e., restriction endonuclease analysis and DNA sequencing, respectively).

We present an alternative, efficient approach to species identification which requires only PCR without additional downstream manipulation of the amplified products. Given international concerns about the ecological health of exploited shark populations, we demonstrate the utility of this method for species identification of shark body parts, including dried fins.

The ITS2 locus was targeted for species discrimination based on our observations (Pank et al. 2001; Shivji et al. unpublished data) that this locus is highly conserved within shark species, but sufficiently variable even between congeners to provide species-diagnostic nucleotide polymorphisms. Furthermore, the ITS2 is part of a class of repetitive elements in eukaryotes (Lewin 2000), thus providing an abundant target for primer annealing and enhancing the efficiency of PCR amplification.

Multiplex Amplification Format

The initial three-primer triplex amplifications (i.e., one species-specific and two shark universal primers) were structured to allow inclusion of an internal positive control in each PCR reaction. The rationale behind this approach is described in detail by Pank et al. (2001). To recapitulate briefly, the internal positive control was included to prevent the complete absence of any amplification (due, for example, to inhibitory substances in the starting DNA or to errors in setting up the reaction) from being interpreted as the absence of the target species (i.e., a false-negative result). Inclusion of an internal positive control also reduced costs of the assay by circumventing the need for separate positive-control reactions aimed at verifying the integrity of the PCR chemical components and DNA quality.

The typically low-yield (or occasionally visually undetectable) coamplification of the positive-control amplicon when silky and blue sharks were the target species deserves comment. We are not certain of the reason for this occurrence but have also observed it with other shark species when the annealing site of the species-specific forward primer is relatively close to the annealing site of the shark universal 5.8SF primer (Fig. 2; Pank et al. 2001). We speculate that this phenomenon may result from some form of primer competition, possibly for DNA polymerase binding, between the closely annealed forward primers. This putative "primer-proximity" competitive interaction is suggested by the observation that the positive control amplicon was amplified efficiently from both silky and blue shark DNA in all instances when their respective species-specific primers were absent from the multiplex reaction (Fig. 3).

This low-yield or inconsistent amplification of the positive control amplicon does not detract from the diagnostic utility of the multiplex assay for silky and blue sharks. As indicated above, the internal positive control is included in the assay only for the purpose of preventing false-negative results from being interpreted as the absence of the target species. In fact, its coamplification along with the species-diagnostic amplicon is quite unnecessary for species identification because identification of the unknown sample can be reliably accomplished simply by scoring for the appearance of a species-diagnostic amplicon by itself. Absence of any amplification signals a problem with the integrity of the reaction. In this context, the internal positive control achieved its goal remarkably well in all cases, amplifying robustly in the absence of the target species (Figs. 3 & 4).

Combining the eight primers in an octaplex–PCR assay further streamlines the identification process by requiring only a single PCR amplification to distinguish among the six shark species simultaneously, instead of individual tests of the unknown samples with each species-specific primer in separate reactions. For this octaplex assay to work reliably, the species-specific and shark universal primers had to be designed to function under stringent PCR conditions (65° C annealing temperature) to (1) prevent or reduce primer-primer annealing when relatively large numbers of primers are mixed together and (2) maintain species-specific primer annealing to the genomic DNA. The latter is especially important in cases where the sequence of the species-specific primer differs from its orthologous sequence in closely related, nontarget species by only one or two nucleotides (Pank et al. 2001).

A second requirement for the octaplex–PCR assay to work efficiently was that each of the six species-diagnostic amplicons needed to be sufficiently different in size from one another and the positive control amplicon needed to be easily identifiable on an agarose gel. To accommodate this requirement, we were careful to design primers that annealed to the ITS2 locus sufficiently far apart to yield unambiguously scorable bands on visual inspection of the gel (Fig. 4), circumventing the need for more sophisticated analysis of band size by special-

ized software. This design provides the advantage of increasing speed and simplicity of gel interpretation and therefore rapidity of the assay.

Applications to Shark Conservation and Management

Our species-specific primers have been developed primarily for use in the conservation and management of multinational shark fisheries in the North Atlantic. The six shark species in this study form a reasonably cohesive group in that they are most commonly encountered offshore either as bycatch in the tuna and swordfish fisheries (i.e., shortfin and longfin makos, blue, silky, dusky; Castro 1993; Bonfil 1994; Buencuerpo et al. 1998; Beerkircher 2000) or in the pelagic, directed fishery (porbeagle, shortfin mako; NMFS 2001). In an applications context, we envision use of this suite of eight primers as a first attempt to determine the species identity of unknown samples obtained from North Atlantic pelagic fisheries, because there is a high probability that the unknown samples belong to one of these six species.

But we recognize that, depending on the region, other species such as the oceanic whitetip, night, thresher, and bigeye thresher sharks can also be common in the Atlantic pelagic fishery (Castro 1993). Furthermore, because there is overlap in some species' distributions, several other mostly nearshore shark species are also occasionally encountered in offshore fisheries (Castro 1993; Bonfil 1994; Beerkircher 2000). In recognition of this possibility, we made a considerable effort to develop primers for each of the six pelagic species which are species specific (or nearly species-specific in the case of the dusky primer) when tested against nontarget species that might also be encountered, frequently or occasionally, in the North Atlantic fishery.

For two reasons, we are confident that the suite of six primers we have developed will provide a reliable means of accurately identifying longfin mako, shortfin mako, porbeagle, dusky, silky, and blue shark tissues obtained from the North Atlantic pelagic fishery. First, we have verified the species specificity of each primer against 21 other shark species that might be encountered in the North Atlantic pelagic fishery. Second, even though we have not tested these primers against all possible shark species found in the North Atlantic, it is unnecessary to do so for the following reasons. As non-coding regions, ribosomal ITS loci are typically highly variable at the DNA sequence level (Miller et al. 1996; Harris & Crandall 2000). In sharks, the ITS2 is highly divergent between taxa above the genus level (Shivji et al. this paper and unpublished data), making it extremely unlikely that these three lamnid and three carcharhinid primers will amplify DNA from more distantly related shark species, especially at the high PCR stringency conditions we used. Closely related nontarget species (all other lamnids and most carcharhinids, including congeners) that occur in the North Atlantic (Compagno 1984) have been tested and, with the one exception (i.e., oceanic white-tip), do not cross amplify with the six pelagic-shark primers. Other nontarget species, including a few carcharhinids not included in the set of species tested, occupy different habitats and are unlikely to be encountered in the pelagic swordfish-tuna and directed shark fisheries (Buencuerpo et al. 1998; Beerkircher 2000).

The observation that the dusky primer, although nearly species-specific, also amplifies its congener, the oceanic whitetip, can lead to some ambiguity in the discrimination of samples from these two species because the latter is also commonly encountered in the pelagic fishery. This potential ambiguity is ameliorated, however, by the fact that the dusky and oceanic whitetip carcasses are relatively easily distinguished from each other by morphology, and the oceanic whitetip shark's large, paddle-like fins with rounded white-tips are unmistakable (Castro 1993).

Our primers also prove useful for identification of dried shark fins. Trade in shark fins, although widespread, is poorly documented on a species-specific level (Vannuccini 1999). Consequently, reliable quantitative assessment of the current level and impact of the shark-fin harvest on the status of individual pelagic shark species is impossible. Our dried-fin test results suggest that it should be possible to determine the species identity of the fins using our methods and to begin to apply them to the problem of monitoring trade in these products. Our preliminary surveys of dried fins in the Hong Kong market suggest that, at least for blue, silky, and possibly shortfin mako sharks, the fin traders we surveyed were reasonably accurate in their assessment of the fins' species of origin. Interestingly, 9 of the 10 fins misidentified as "silky" came from a single fin trader. The trader and fin sample sizes we have surveyed thus far, however, are too small to permit firm conclusions about the large-scale accuracy of identifications made by traders. We are currently developing species-specific primers for additional shark species that figure prominently in the global shark trade (Vannuccini 1999), and we are investigating the concordance between fin trader-based (visual) and genetics-based identifications.

Utility of the Six Pelagic Shark Primers for Global Scale Surveys

In light of the global distribution of the six pelagic species, we asked whether these primers would also amplify their target species from globally widespread areas, including the Atlantic and Indo-Pacific oceans. In the case of the porbeagle, we were unable to obtain test samples from outside the northwestern Atlantic and therefore could not address this issue. For the other five species, however, we had representatives from globally widespread areas (Table 1), and our results indicate that

the primers will successfully amplify individuals of the target species from as far apart as the western Atlantic and western Pacific ocean basins (Figs. 3 & 4). These results suggest that low intraspecific variation exists in the ITS2 locus in globally distributed populations of at least five of the six species.

Due to difficulties in obtaining samples from all appropriate nontarget shark species worldwide, we have been unable to test every one of the six pelagic-shark primers for its species specificity against every closely related nontarget species in the Indo-Pacific. This raises the question of whether all six primers are as robustly species-specific on a global scale as they are for North Atlantic populations. We found the three lamnid-species primers robustly species specific in tests against other closely related nontarget taxa likely to be encountered in Indo-Pacific pelagic fisheries (i.e., other lamniform sharks: salmon shark, white shark, common thresher, bigeye thresher, and pelagic thresher). Furthermore, the high ITS2 sequence divergence between taxa above the genus level and the high stringency PCR conditions used makes it extremely unlikely that these lamnid primers will amplify more distantly related species not already tested. Consequently, we suggest that the shortfin and longfin mako primers will be reliable for identification of these two species on a global scale. The porbeagle primer may be useful on a global scale as well. The only caveat is that, because we have not tested this primer on porbeagle samples outside the northwestern Atlantic, there is a small (but unlikely) possibility that sufficient intraspecific variation exists in the primer ITS2 annealing site within this species on a global scale to reduce the primer's diagnostic utility outside the northwest Atlantic.

The global-scale utility of the three carcharhinid primers is more uncertain because there are several carcharhinid species in the Indo-Pacific against which we have not tested these primers. Despite this uncertainty, for two reasons these primers may turn out to be of diagnostic utility worldwide after all. First, our results show each of these three carcharhinid primers is species-specific even when tested against 21 other carcharhinid species (including 16 congeners from the genus *Carcharhinus*; Table 1), with only one cross-species amplification occurring (i.e., dusky primer also amplifying the oceanic whitetip). Although we cannot discount the possibility of some cross-species amplification occurring on a global scale with the 14 remaining Indo-Pacific *Carcharhinus* species not tested in this study, the very low incidence of cross-species amplification observed thus far even with congeners suggests that these primers will likely continue to demonstrate species specificity when tested against most, if not all, of the remaining *Carcharhinus* species. Second, in the practical application of identification of sharks caught in pelagic fisheries, most of the carcharhinid species not yet tested are unlikely to be encountered frequently in these fisheries because they typically occupy nearshore habitats (Compagno 1984). These two factors taken together will reduce the potential for misidentification of sharks caught in worldwide pelagic fisheries when this suite of primers is used.

Use of the six species-specific primers either individually or in various combinations of one to six primers in a multiplex–PCR format provides an efficient way to achieve accurate and rapid identification of pelagic-shark body parts, including dried fins. Furthermore, the streamlined octaplex approach may prove useful as a model for development of rapid species-diagnosis assays for a wide diversity of taxa that are hunted or require conservation action.

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