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Genetic tracking of basking shark products in international trade

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Abstract

Mounting evidence that sharks are being over-fished to supply shark fin markets is causing widespread concern about the sustainability of these practices. The basking shark *Cetorhinus maximus*, whose fins command high market prices, has proven especially sensitive to exploitation. To prevent further population declines, this species is now protected in the territorial waters of several countries, and is listed on Appendix II of the Convention on International Trade in Endangered Species (CITES) requiring monitoring of trade in its products by all parties to CITES. Tracking trade in basking shark products, however, is often hampered by difficulties in identifying shark products to species of origin. Here, we present the development and application of a streamlined genetic forensics assay that does not require DNA sequencing to identify basking shark products. The dual-primer, species-specific polymerase chain reaction strategy provides diagnostic redundancy for robustness in legal venues. It is also effective for identifying basking shark products regardless of geographic origin, an important consideration, given the global distribution of the species and international sourcing of fins to the trade. Application of the assay confirmed the presence of basking shark fins in the Hong Kong and Japan markets, and indicated an apparent relationship between the Chinese fin trader category 'Nuo Wei Tian Jiu' and fins from basking sharks. The assay was also used in a law enforcement investigation to document illegal trade in basking shark fins in the United States where this species is prohibited from harvest and trade. These trade detections suggest that the high market value of basking shark fins is continuing to drive the exploitation, surreptitious and otherwise, of this highly threatened species, underscoring the need for improved trade monitoring. The streamlined assay developed here can assist in monitoring and conservation on a worldwide scale.

Introduction

The basking shark *Cetorhinus maximus* (Gunnerus, 1745), is the single member of the lamniform family Cetorhinidae, and the second largest fish species in the world. Despite its circumglobal distribution, the species has proven exceptionally sensitive to exploitation (Compagno, 2001). Long periods spent surface feeding (Sims & Quayle, 1998) make basking sharks easy targets for harpoon fisheries. In addition, their life history (i.e. long-lived, slow to mature and few young produced at a time; Compagno, 2001) has made population recovery after exploitation extremely tenuous. Indeed, there are several historical examples of the rapid collapse of small-scale fisheries after only short periods of harvesting, followed by long periods of low population numbers (Anon., 2002; Pauly, 2002). Although basking sharks were historically fished mainly for liver oil, meat and hide, the recently burgeoning market for shark fins includes a demand for basking shark fins, with single, large fins fetching up to US $57 000 (Clarke, 2004*a*).

The population collapses and low recovery potential from exploitation have prompted implementation of protective measures for basking sharks by several countries. The species is currently fully protected within territorial waters of the United Kingdom, Malta and continental United States, and partially protected in New Zealand where directed fishing is prohibited. The November 2005 listing on Appendices I and II of the Convention on Migratory Species (CMS, 2006) is aimed at strengthening international conservation by requiring that treaty member states implement strict legislation to ban fishing and trade in this species. The World Conservation Union (IUCN) lists the status of basking shark populations as 'Vulnerable' throughout their range and 'Endangered' in the heavily fished north Pacific

and north-east Atlantic (Fowler, 2005). From an international trade regulatory perspective, the species is listed on Appendix II of the Convention on International Trade in Endangered Species (CITES) of wild fauna and flora, requiring strict regulation and tracking of trade by signatory states to avoid levels of utilization incompatible with its survival (CITES: http://www.cites.org/eng/resources/species.html). Criteria for listing marine fish species on CITES (FAO, 2001) suggest that given the basking shark's life-history data and historical fishery collapses, it falls within the lowest productivity category and could qualify for Appendix I listing if the population declined to $\leq 20\%$ of the historic baseline.

Despite regional and international harvest moratoria and trade regulations for basking sharks, there are concerns that high market value, especially for their fins, is promoting overexploitation. Two factors are largely responsible for the current inability to assess the extent of basking shark exploitation and trade in its products: first, most nations do not collect information on the volume of their shark fisheries by individual species. Second, and more importantly from a trade tracking and regulation enforcement perspective, accurate species identification of basking shark products remains difficult for the non-expert (Anon., 2006). Customs inspection and fisheries enforcement personnel not practiced in identifying whole shark fins may confuse the smaller basking shark fins (e.g. second dorsal, pelvic, anal fins), and even first dorsal and pectoral fins from smaller basking sharks, with fins from other large lamnid sharks and wings (discs) from large batoids. Along these lines, at the port of Hong Kong (the world's largest fin trade center), available species identification materials are not always circulated to inspection officials or placed in intelligence databases (Clarke, 2004*a*). In addition, processed shark products (e.g. meat, cartilage) that can contain tissues from multiple species are likely to require DNA forensic identification to detect the presence of regulated taxa (Hoelzel, 2001). Given the identification problems amidst international commerce where most shark products traded involve unregulated species, molecular genetic tools for forensic identification are being increasingly examined in terms of their practicality for implementation of CITES and other protective regulations.

To assist in DNA-based identification of basking shark products, Hoelzel (2001) published a forensic assay that was based on DNA sequencing using a species-specific primer from the mitochondrial cytochrome *b* gene. Because of the dependence of this test on DNA sequencing, and the need for rapid, cost-effective assay methods for high-volume screening in increasingly widespread international regulatory regimes, we have extended this species-specific primer approach to eliminate the need for sequencing. Here, we report the development and extensive diagnostic validation of two basking shark species-specific PCR primers derived from the nuclear ribosomal internal transcribed spacer 2 (ITS2) locus. We subsequently apply these primers in a streamlined multiplex PCR electrophoretic assay to investigate the international shark fin trade and assist US law enforcement activities.

Materials and methods

Shark samples

Tissues from 44 globally distributed, basking shark reference individuals (north-west Atlantic: $n = 17$, north-east Atlantic: $n = 11$; Caribbean: $n = 1$, Mediterranean: $n = 3$, Indian Ocean: $n = 1$, south-west Pacific: $n = 8$, Tasman sea: $n = 1$, south-east Pacific: $n = 2$) were used for the development of our basking shark-specific primers. These samples were collected by experienced researchers from unambiguously identified whole basking sharks.

For diagnostic validation, each primer set was tested for amplification performance on DNA from 80 non-basking shark species (hereafter non-target species; 1–61 individuals per species), including closely related taxa common in global shark fisheries (Table 1). All eight extant shark orders (Compagno, 2001) were represented in our non-target testing. Tissues tested (fins, white muscle and liver) were stored in 95% ethanol or dried (fins only).

PCR amplification and DNA sequencing of the ITS2 locus

Genomic DNA was extracted from *c*. 25 mg of all tissue types and species using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). We chose the nuclear locus ITS2 based on studies (Pank *et al*., 2001; Shivji *et al*., 2002; Chapman *et al*., 2003; Abercrombie, Clarke & Shivji, 2005) demonstrating sufficient divergence to distinguish closely related species, but also very high conservation within species on a global scale. PCR amplification of the 44 basking shark reference samples with the shark ITS2 universal primers FISH5.8SF and FISH28SR (Table 2) produced a fragment *c*. 1400 bp in length (hereafter the positive control amplicon) containing the entire ITS2 region and short portions of the flanking 5.8S (*c*. 160 bp) and 28S (*c*. 60 bp) ribosomal RNA genes. Partial 5.8S rDNA-ITS2 region sequences (111 bp from the 3′ end of the 5.8S rRNA gene and 375 bp from the 5′ end of the ITS2) were obtained using standard automated sequencing from 10 globally distributed, reference animals (one each from the north-west Atlantic, Caribbean sea, Indian Ocean, south-east Pacific and Mediterranean, two from the north-east Atlantic and three from the south-west Pacific). Negative controls (i.e. all PCR components without template DNA) were run alongside all PCR reactions.

Designing and testing species-specific primers

We aligned the partial ITS2 sequences of the 10 reference basking sharks with homologous sequences from nine lamniform sharks (Lamnidae: *Isurus oxyrinchus*, *Isurus paucus*, *Lamna ditropis*, *Lamna nasus*, *Carcharodon carcharias*; Alopiidae: *Alopias vulpinus*, *Alopias pelagicus*, *Alopias superciliosus*; Odontaspididae: *Carcharias taurus*) (Shivji *et al*., 2002; Chapman *et al*., 2003; Abercrombie, 2004) using the program ClustalX (Thompson *et al*., 1997) with subsequent

Table 1 Target (bold) and non-target shark species tested in triplex and quadruplex PCR formats

Species	Common name	A	P	I	O	[a]Approximately ITS2 amplicon size (bp)
Lamniformes						
Cetorhinus maximus	**Basking shark**	**28**	**10**	**1**	**5**	**1400**
Isurus oxyrinchus	Short-fin mako	11	29			1350
Isurus paucus	Long-fin mako	7	4			1350
Lamna ditropis	Salmon shark		31			1350
Lamna nasus	Porbeagle	27	17	1		1350
Carcharodon carcharias	White shark	16	22	18		1350
Alopias vulpinus	Thresher	22	11			1350
Alopias superciliosus	Bigeye thresher	9	21			1350
Alopias pelagicus	Pelagic thresher		6			1350
Carcharias taurus	Sand tiger	20	12	29		1350
Odontaspis ferox	Smalltooth sand tiger		1			1350
Pseudocarcharias kamoharai	Crocodile shark		1			1350
Carcharhiniformes						
Carcharhinus altimus	Bignose	6				1470
Carcharhinus longimanus	Oceanic whitetip	3	9			1470
Carcharhinus signatus	Night	4				1470
Carcharhinus plumbeus	Sandbar	6	4			1470
Carcharhinus limbatus	Blacktip	12				1470
Carcharhinus obscurus	Dusky	5	5			1470
Carcharhinus falciformis	Silky	6	4			1470
Carcharhinus melanopterus	Blacktip reef		1			1470
Carcharhinus porosus	Smalltail	2				1470
Carcharhinus galapagensis	Galapagos		11			1470
Carcharhinus leucas	Bull	6	4			1470
Carcharhinus brevipinna	Spinner	6	4			1470
Carcharhinus isodon	Finetooth	10				1470
Carcharhinus acronotus	Blacknose	10				1470
Carcharhinus perezi	Caribbean reef	10				1470
Carcharhinus amboinensis	Pigeye		2	1		1470
Carcharhinus brachyurus	Bronze whaler		2			1470
Carcharhinus tilstoni	Australian blacktip		2			1470
Carcharhinus sorrah	Spot-tail		2	1		1470
Carcharhinus amblyrhynchos	Gray reef		10			1470
Carcharhinus dussumieri	Whitecheek			3		1470
Carcharhinus hemiodon	Pondicherry		2			1470
Negaprion brevirostris	Lemon	10				1470
Negaprion acutidens	Slcklefin lemon		2			1470
Galeorhinus galeus	School	2				1450
Sphyrna mokarran	Great hammerhead	7	3			860
Sphyrna lewini	Scalloped hammerhead	5	5	7		860
Sphyrna zygaena	Smooth hammerhead	2	9			860
Sphyrna tiburo	Bonnethead	10				860
Sphyrna tudes	Golden hammerhead	1				860
Eusphyra blochii	Winghead		2			860
Galeocerdo cuvier	Tiger	5	3	2		1450
Triaenodon obesus	Whitetip reef		1			1470
Rhizoprionodon terranovae	Atlantic sharpnose	10				1500
Rhizoprionodon acutus	Milk		2			1500
Rhizoprionodon taylori	Australian sharpnose		1			1500
Rhizoprionodon porosus	Caribbean sharpnose	5				1500
Rhizoprionodon oligolinx	Grey sharpnose	2				1500
Prionace glauca	Blue shark	5	5			1470
Mustelus norrisi	Smoothhound	2				1500
Mustelus henlei	Brown smoothhound	2				1500
Mustelus canis	Smooth dogfish	2				1500
Mustelus californicus	Grey smoothhound		1			1500

Table 1. Continued

Species	Common name	A	P	I	O	[a]Approximately ITS2 amplicon size (bp)
Loxodon macrorhinus	Sliteye	1				1500
Triakis semifasciata	Leopard		2		2	1500
Scyliorhinus retifer	Chain catshark				1	1350
Apristurus profundorum	Smalleye catshark	2				1400
Cephaloscyllium ventriosum	Swell		2			1450
Paragaleus randalli	Slender weasel shark			1		1500
Nasolamia velox	Whitenose	1				1500
Orectolobiformes						
Ginglymostoma cirratum	Nurse	10				1650
Nebrius ferrugineus	Tawny nurse		1			1150
Orectolobus ornatus	Ornate wobbegong		1			1650
Squaliformes						
Squalus acanthias	Spiny dogfish	5				1250
Squalus cubensis	Cuban dogfish	5				1200
Deania calceus	Birdbeak dogfish	2				1200
Dalatias licha	Kitefin	1				1200
Isistius brasiliensis	Cookiecutter shark	1				1200
Etmopterus spinax	Velvet belly	1				1100
Etmopterus pusilius	Smooth lantern	2				1200
Centrophorus granulosus	Gulper	1				1200
Centrophorus squamosus	Leafscale gulper	1				1200
Heterodontiformes						
Heterodontus francisci	Horn shark		2			1300
Squatiniformes						
Squatina californica	Pacific angel		2			1250
Hexanchiformes						
Hexanchus griseus	Sixgill		4			1100
Hexanchus vitulatus	Bigeye sixgill	1				1000
Heptranchias perlo	Sharpnose sevengill		1			1150
Pristiophoriformes						
Pristiophorus nudipinnis	Shortnose sawshark				1	1150
Pristiophorus japonicus	Japanese sawshark				1	1100

[a]Amplicon size refers to the size of the PCR fragment generated by the two universal primers.
Numbers of individuals tested and their ocean basin origins are shown for each species. A, Atlantic; P, Pacific; I, Indian; O, other.

Table 2 Shark universal (FISH) and basking shark *Cetorhinus maximus* (BSK) species-specific primer sequences used in the triplex and quadruplex PCR assays

Primer	Sequence
FISH5.8SF[a]	5′-TTAGCGGTGGATCACTCGGCTCGT-3′
FISH28SR[a]	5′-TCCTCCGCTTAGTAATATGCTTAAATTCAGC-3′
BSK328F	5′-TCTCGGCCTCCGGGCGAACGAATGAGA-3′
BSK503F	5′-AAGATGCGGCACGCTGTTGGGCACGC-3′

[a]FISH5.8SF and FISH28SR primer sequences reported previously by Pank *et al.* (2001).

manual adjustment with the editing program GeneDoc (Nicholas & Nicholas, 1997). Based on nucleotide differences between the basking shark and the aforementioned species, nine ITS2 forward primers putatively specific for basking shark were designed and tested for amplification reliability and species specificity against the 44 reference basking sharks and 80 non-target species at a 65 °C annealing temperature. A multiplex (triplex) PCR strategy (Pank *et al.*, 2001) was used for initial evaluation of primer performance. This triplex PCR consisted of one putatively species-specific, basking shark forward primer, and the two shark universal forward and reverse ITS2 primers. Our a priori expectation for a successful diagnostic primer was that the triplex combination would produce two amplicons when used to amplify target species DNA: an *c.* 1400 bp positive control amplicon generated from the two universal primers, and a smaller amplicon diagnostic for the basking shark generated from the basking shark-specific forward primer and the universal reverse primer. In contrast, when tested against genomic DNA from non-target shark species, this triplex combination would produce only the positive control amplicon due to failure of the basking shark-specific primer to anneal to DNA from non-target species. After verifying optimal performance in triplex PCR, that is consistent amplification of all 44 reference basking sharks and species specificity against all non-target species at 65 °C annealing temperature, two species-specific primers

(BSK328F and BSK503F; Table 2) were selected for further diagnostic trials (see next section).

Amplifications were performed in Mastercycler Gradient (Eppendorf Inc., Westbury, NY, USA) and MJ Research PTC-100/200 thermal cyclers. All amplification reaction volumes (basking and non-target species) were 50 μL and contained *c.* 30–80 ng of genomic DNA, 12.5 pmol of each universal primer (FISH5.8SF and FISH28SR), 4.2 pmol of the BSK328F primer and 1.25 pmol of the BSK503F primer (determined after optimization), 1 × PCR buffer (1.5 mM $MgCl_2$; Qiagen Inc.), 40 μM dNTPs, and 1 U of HotStar Taq™ DNA polymerase (Qiagen Inc.). The PCR thermal cycling profile was: 94 °C for 15 min to activate the hot start DNA polymerase, followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 2 min and a 5-min final extension at 72 °C.

Combining species-specific primers in a quadruplex PCR assay

To further streamline the assay while simultaneously increasing diagnostic power, we designed a multiplex PCR involving four primers (quadruplex PCR), and tested it for diagnostic robustness. The quadruplex combination consisted of the shark universal primers and both basking shark species-specific primers (Fig. 1). Our expectation for a positive diagnostic result (i.e. basking shark DNA present) was the co-amplification of three fragments (see Fig. 1 for expected amplicon sizes). A negative result (i.e. basking shark DNA absent) was expected to yield only the positive control amplicon. All thermal cycling conditions were those used in the triplex PCR.

Screening market fins

Nineteen dried shark fin samples obtained from Hong Kong ($n = 14$) and Japan ($n = 5$) markets as part of a broader fin trade study (Clarke *et al.*, 2006) were tested with the validated quadruplex assay. Hong Kong fin traders categorized the fins as 'Nuo Wei Tian Jiu' (pinyin romanization 'Norway Nine Heavens'). Based on interviews with the traders, we hypothesized that these fins were derived from basking sharks. Fins identified as derived from basking sharks by the quadruplex PCR assay were subsequently sequenced (486 bp as above) to confirm their species origin and as a further check of the accuracy of the species-specific primers.

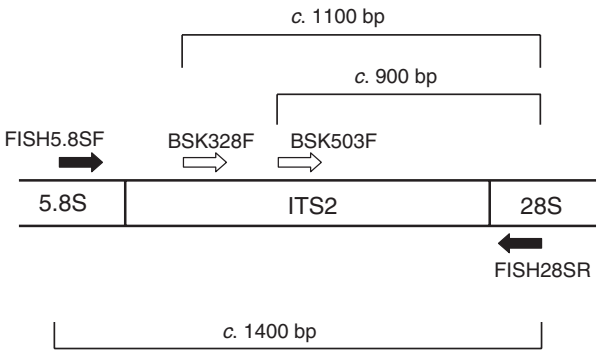

Figure 1 Schematic representation of the nuclear 5.8S and 28S ribosomal RNA genes and ITS2 locus showing relative annealing sites and orientation of primers used in the quadruplex PCR assay. Shark universal primers are shown as solid arrows. The basking shark *Cetorhinus maximus* species-specific primers are shown as open arrows. Brackets indicate amplicons sizes expected from PCR of basking shark DNA.

Law enforcement investigation

At the request of the US National Oceanic and Atmospheric Administration Office of Law Enforcement (NOAA OLE), we applied the quadruplex assay to determine the species origin of two dorsal fins confiscated in a law enforcement investigation. The two fins were each part of complete fin sets (each set comprising one dorsal, two pectoral, one lower caudal) found in the possession of a major US seafood dealer.

Results

ITS2 sequence characteristics

Sequences of the 486 bp 5.8S rDNA-ITS2 region were identical in the 10 globally distributed reference basking sharks, the two law enforcement case fins and 11 of the 14 Asian market fins [we were unable to sequence five of the Asian market fin samples: three fins (one from Hong Kong and two from Japan) did not yield amplifiable DNA; two fins, although amplifiable to permit unambiguous species diagnosis by quadruplex PCR, proved perplexingly intractable to sequencing]. Three of the Asian market fins sequenced differed from the ITS2 consensus sequence at one (two fins) and two (one fin) nucleotide positions. The four sequence variants are available from GenBank (accession no. EF194106, EF194107, EF194108 and EF194109). All 26 ITS2 sequences (reference animals and fin samples) contained a GA dinucleotide microsatellite at least 11 repeats long.

Performance of diagnostic primers

In triplex PCR (gel results not shown), both the BSK328F and BSK503F primers consistently produced species-diagnostic amplicons of *c.* 1100 and 900 bp, respectively, from all 44 reference basking sharks. Only the positive control amplicon, ranging in size from 860 to 1500 bp depending on species, was generated from the 80 non-target species, with no instances of false-positive results (i.e. appearance of basking shark diagnostic amplicons). The fact that the universal ITS2 primers (FISH5.8SF and FISH28SR) amplify the ITS2 locus from sharks has been demonstrated previously by sequencing the PCR products from multiple shark species (Pank *et al.*, 2001; Shivji *et al.*, 2002; Chapman *et al.*, 2003; Abercrombie, 2004; Abercrombie *et al.*, 2005;

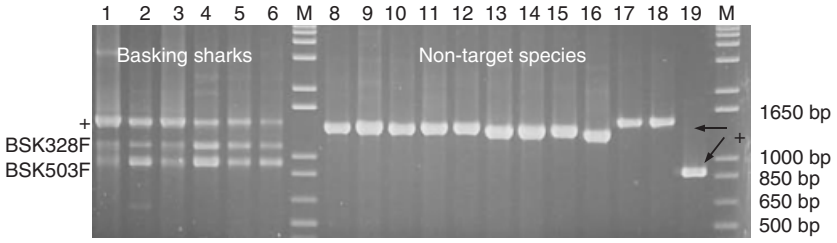

Figure 2 Quadruplex PCR results from six basking shark *Cetorhinus maximus* reference animals (lanes 1–6) sampled from a worldwide distribution, nine non-target lamniform species (lanes 8–16) and three non-target carcharhiniform species (lanes 17–19). Basking sharks: lane 1, north-west Atlantic; lane 2, Caribbean; lane 3, Meditteranean sea; lane 4, Indian Ocean (South Africa), lane 5, south-west Pacific Ocean (New Zealand); lane 6, south-east Pacific Ocean (Chile). Non-target species: lane 8, shortfin mako; 9, longfin mako; 10, white; 11, porbeagle; 12, salmon shark; 13, sand tiger; 14, thresher; 15, bigeye thresher; 16, pelagic thresher; 17, sandbar; 18, silky; 19, scalloped hammerhead. Species diagnostic (BSK328F and BSK503F) and positive control (+) amplicons indicated. M represents the molecular size standard.

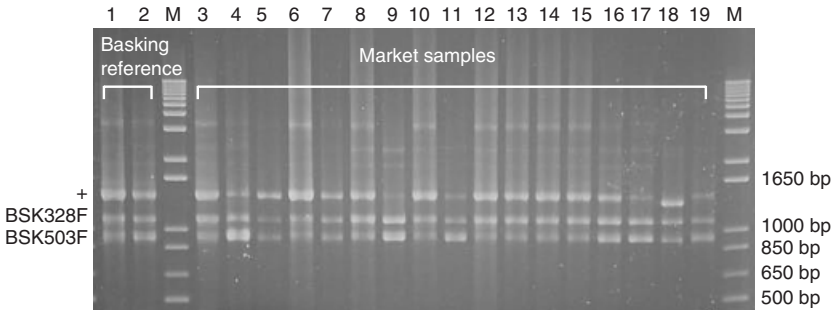

Figure 3 Quadruplex PCR analysis of 17 dried fin samples from the international trade. Lanes 1–2, basking shark *Cetorhinus maximus* reference animals (lane 1, north-west Atlantic; lane 2, south-west Pacific); Lanes 3–14, Hong Kong market fins, lanes 15–17, Japanese market fins; lanes 18–19, NOAA law enforcement case fins. Species diagnostic (BSK328F and BSK503F) and positive control (+) amplicons indicated. M represents the molecular size standard.

this study; some of these sequences are available in GenBank).

Combining the two species-specific primers in a quadruplex PCR assay resulted in the consistent, simultaneous amplification of two distinct, species-diagnostic amplicons from all 44 reference basking sharks (Fig. 2). The positive control amplicon was co-amplified from 40 of these samples, albeit in variable yields as assessed qualitatively by band intensity. There were no false-negative results (i.e. only the positive control amplicon being produced from the target species). Testing the quadruplex assay on the 80 non-target species resulted in amplification of only the ITS2-positive control amplicon, which was clearly distinguishable from the two basking shark diagnostic amplicons (Fig. 2). No false-positive amplifications (i.e. production of the basking shark diagnostic amplicons) were detected from any of the non-target species.

Detecting basking sharks fins in international trade and trade name-taxon concordance

The quadruplex PCR assay diagnosed 13 of the 14 'Nuo Wei Tian Jiu' fins from the Hong Kong and three of the five fins from the Japan markets as derived from basking sharks (Fig. 3). Owing to differences in yield and quality of DNA extracted from dried fins, template DNA amount used for amplifications had to be optimized, ranging from 30 to 800 ng in most cases, but also required dilution of the original DNA preparation (1:10 and 1:200 with sterile distilled water) in a few cases.

The quadruplex PCR assay identified the two NOAA law enforcement investigation samples tested as derived from basking sharks (Fig. 3). This primer-based diagnosis was confirmed by DNA sequencing.

Discussion

Declining reagent costs and improved technological throughput have made DNA sequencing a valuable approach for forensic identification of wildlife parts in some contexts. However, sequencing remains comparatively expensive in terms of required infrastructure (high cost of automated sequencers and labor-saving robotics and their maintenance) to be practical for routine monitoring of wildlife trade. This is especially true where large volumes of products (e.g. shark fins and other fishery products) may be passing through trade routes and need to be screened quickly to prevent adding substantial economic costs to legitimate commerce. Also importantly, large-scale DNA

sequencing appears unlikely to become practical in the near future for routine trade monitoring and fisheries enforcement in most developing countries due to high infrastructure costs amidst economic resource limitations. Given the broadly international nature of the shark trade (around 85 countries supply the Hong Kong fin market; Clarke, 2004*a*), we suggest that streamlined, comparatively inexpensive molecular shark species identification methods are needed for practical implementation of conservation-oriented regulations.

Here, we have demonstrated the accuracy and efficiency of a quadruplex PCR assay simultaneously utilizing two species-specific primers to identify products (fins and meat) from basking sharks. The worldwide diagnostic utility of this assay is demonstrated by its ability to identify all 44 reference animals regardless of geographic origin, and the fact that the assay successfully identified 18 basking shark fin samples obtained from globally provisioned markets in three countries. The multiplex strategy permits rapid species diagnosis with a single PCR without the need for downstream DNA sequencing or restriction enzyme analysis, thus eliminating any post-PCR analysis steps and associated costs (equipment, reagents and labor). Furthermore, with its comparatively small equipment needs (thermal cycler, power supply and gel electrophoresis apparatus), this assay may prove more adaptable to field conditions (e.g. screening in ports in mobile labs or on-board smaller fisheries enforcement vessels) than current automated sequencing equipment.

Using either species-specific primer in triplex PCR provided consistent diagnostic identification. However, because trade in basking shark products is illegal in some countries, there is greater potential for regulatory violations to be assessed in legal venues where the reliability of the genetic assay may be questioned. With this possibility in mind, we designed the species-specific primers to produce clearly different-sized diagnostic amplicons to allow multiplexing both primers in a single PCR. This strategy provides diagnostic redundancy, essentially eliminating the already minimal likelihood of failure to detect the target species due to rare mutations in any single primer-annealing site (note: we did not observe such instances despite the globally widespread reference sample set). The robustness of the assay is further enhanced by the inclusion of both universal primers in the multiplex PCR, thereby providing an internal control in both the triplex and quadruplex assays to prevent false-negative results, that is complete failure of the DNA to amplify as being interpreted as absence of the target species. Complete amplification failure can occur due to inhibitory substances in the template DNA or due to operator error in PCR setup.

Although inclusion of both universal primers should theoretically also generate a positive control amplicon from the target species, we found this outcome to be inconsistent in multiplex PCR (i.e. variable yields and occasional absence of this amplicon), likely due to primer competition dynamics (Shivji *et al.*, 2002; Chapman *et al.*, 2003; Abercrombie *et al.*, 2005), or possibly sample DNA degradation in some cases. Indeed, by reducing the concentration of both species-specific primers relative to the universal primers in the PCR (see 'Materials and methods'), we generally obtained greater yields of the positive control from the target species, arguing for a role of primer competition dynamics. We emphasize, however, that the multiplex assay has been designed to achieve unambiguous identification of target species DNA without requiring co-amplification of the positive control. Production of the positive control amplicon is required only from non-target species to prevent false-negative results.

A potential limitation of this assay for consistently detecting all types of basking shark products is the relatively large size of the PCR amplicons (*c.* 900 and 1100 bp) needed for diagnosis. Depending on age, storage conditions and type of processing (e.g. cooking), the DNA in some shark products may be too degraded to amplify such large amplicons. The three Asian market fins that did not yield PCR amplicons likely had DNA too degraded to produce the required amplicon sizes (up to 18 μL of the genomic DNA preparation from these three fins was undetectable by eye on an agarose gel). This amplicon size limitation notwithstanding, we have found that most uncooked shark tissue types, including market-derived dried fins (Clarke *et al.*, 2006) and even cartilage pills (J. E. Magnussen and M. S. Shivji, unpubl. data), are amenable to multiplex PCR testing using this general ITS2-based method.

Despite the higher level of international trade monitoring required by the CITES Appendix II listing, only eight import/export cases of basking shark products have been officially reported post-listing. These included 5538 kg of fins (number of animals unknown) exported from Norway in 2005, and 126 fins (estimated to be from 29 animals) exported from New Zealand between 2003 and 2005 (Anon., 2006). These official reports from only two countries, although suggesting a substantial number of animals traded, almost certainly represent a significant underestimation of worldwide basking shark exploitation. This is because most countries do not keep track of their shark landings and exports by species, some countries have filed reservations to the CITES Appendix II listing (Clarke, 2004*a*) exempting them from reporting requirements and a significant amount of the export may be surreptitious given the protected status of basking sharks in several countries.

Our unambiguous identification of several basking shark fins in a fin market survey, and documentation of contemporary illegal trade in this species in a country (US) with among the most regulated and enforced shark fisheries in the world, is consistent with the notion that the high market value of basking shark fins will continue to drive exploitation and trade surreptitiously and otherwise. This continued market demand raises urgent concerns about the true extent of exploitation of this species, which is likely to be greater than reflected by official trade reports to CITES.

Because of considerable doubt whether even moderate exploitation pressure on basking sharks can be sustained (Compagno, 2001), and its ramifications for further erosion of already low genetic diversity worldwide (Hoelzel *et al.*, 2006), reliable information on the current level of

exploitation is essential for planning effective management and conservation strategies. Given the near worldwide absence of shark landings records by species, improved tracking of trade in products by species through major market centers can provide an alternative means of gauging relative extraction rates for species of concern (Clarke, 2004*b*). An observed concordance between the name 'Nuo Wei Tian Jiu' used by traders and basking shark DNA provides a useful link for assessing the presence of basking shark fins in Hong Kong auction records. However, the presence also of cryptically labeled basking shark fins in these auctions (S. C. Clarke, pers. obs.) also suggests that tracking solely by trade name from official records will be insufficient, and additional means of identification will be necessary to assess the full extent of trade.

We suggest that the relatively 'low-technological' equipment requirements of the assay presented here (compared with available genetic technologies), its simplicity of use, diagnostic accuracy (with built in redundancy) and ability to identify basking shark DNA regardless of geographic source make it a useful conservation and law enforcement tool to track the direction (exporting and importing countries) and extent (legal and surreptitious) of trade in this threatened species on a worldwide basis.

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