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A streamlined, bi-organelle, multiplex PCR approach to species identification: Application to global conservation and trade monitoring of the great white shark, *Carcharodon carcharias*

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Abstract

The great white shark, *Carcharodon carcharias*, is the most widely protected elasmobranch in the world, and is classified as Vulnerable by the IUCN and listed on Appendix III of CITES. Monitoring of trade in white shark products and enforcement of harvest and trade prohibitions is problematic, however, in large part due to difficulties in identifying marketed shark parts (e.g., dried fins, meat and processed carcasses) to species level. To address these conservation and management problems, we have developed a rapid, molecular diagnostic assay based on species-specific PCR primer design for accurate identification of white shark body parts, including dried fins. The assay is novel in several respects: It employs a multiplex PCR assay utilizing both nuclear (ribosomal internal transcribed spacer 2) and mitochondrial (cytochrome *b*) loci simultaneously to achieve a highly robust measure of diagnostic accuracy; it is very sensitive, detecting the presence of white shark DNA in a mixture of genomic DNAs from up to ten different commercially fished shark species pooled together in a single PCR tube; and it successfully identifies white shark DNA from globally distributed animals. In addition to its utility for white shark trade monitoring and conservation applications, this highly streamlined, bi-organelle, multiplex PCR assay may prove useful as a general model for the design of genetic assays aimed at detecting body parts from other protected and threatened species.

Abbreviations: CITES – Convention on International Trade in Endangered Species of Wild Fauna and Flora; Cyt *b* – Cytochrome b; FAO – United Nations Food and Agriculture Organization; ITS2 – Internal Transcribed Spacer 2; IUCN – International Union for Conservation of Nature; PCR – Polymerase Chain Reaction; RFLP – Restriction Fragment Length Polymorphism

Introduction

Effective trade monitoring and enforcement of moratoria on regulated or protected species amidst legal trade in similar species is of widespread concern in aquatic wildlife management (Baker and Palumbi 1994; DeSalle and Birstein 1996; Malik et al. 1997; Dizon et al. 2000; Roman and Bowen 2000; Hoelzel 2001). Species protection efforts developed for conservation and management purposes can be compromised when there are only slight morphological differences between the products of protected

and legally harvested species, potentially allowing significant levels of exploitation and trade of the protected species to go undetected. This problem exists for the conservation of the globally distributed great white shark, *Carcharodon carcharias* (hereafter referred to as white shark).

In response to ongoing targeted and incidental capture of this species in fisheries worldwide, its strongly K-selected life-history strategy, and some evidence of local population declines, the white shark has become one of the first sharks for which widespread international protection has been sought (Francis 1996; Compagno et al. 1997; Wintner and Cliff 1999; Environment Australia 2001a). The species is currently listed on Appendix III of CITES by Australia, requiring permitting and monitoring of trade, and proposals have been put forward by Australia and the U.S.A. to move this species onto Appendix I (CITES 2000; 2002). It is also listed as "Vulnerable" on the 2002 International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Category VU A1cd + 2cd) (Compagno et al. 1997; www.redlist.org). As a result of legislative protection, the capture of white sharks and trade in their derivatives (e.g., fins, skin, jaws, flesh) is prohibited within and across the borders of Australia, South Africa, Namibia, Malta, and California and the Atlantic states of the U.S.A. (Environment Australia 2001a; NMFS 2001; Compagno 2001). Although these conservation measures make the species the most widely protected elasmobranch in the world, there remains a demand for white shark body parts (Compagno et al. 1997; Environment Australia 2001a; Compagno 2001).

International and domestic monitoring of trade in white sharks and enforcement of capture and trade prohibitions remain extremely difficult, however, in large part due to substantial difficulties associated with identification of processed shark carcasses (known as "logs" or "barrels") and other marketed shark body parts such as dried fins and filets to species (Rose 1996; Compagno et al. 1997; Vannuccini 1999; FAO 2000; Environment Australia 2001a; Environment Australia 2001b). In some cases, shark products (e.g., meat, shark cartilage pills) may even consist of a mix of tissues from multiple species, further exacerbating species identification problems (Vannuccini 1999; Hoelzel 2001; J. Magnussen and M. Shivji, unpublished data). These identification difficulties greatly undermine species-specific conservation and management efforts (FAO 2000; Hoelzel 2001; Pank et al. 2001; Smith and Benson 2001; Shivji et al. 2002).

To solve similar species-identification problems important in conservation and management, genetic analyses have been successfully used to accurately identify body parts from many exploited marine taxa (Martin 1993; Malik et al. 1997; Baker et al. 1998; Innes et al. 1998; Palumbi and Cipriano 1998; Heist and Gold 1999; Dizon et al. 2000). Although such analyses have sometimes revealed extensive accidental or surreptitious trade in prohibited species (Malik et al. 1996; Palumbi and Cipriano 1998), they are not yet widely employed for routine monitoring of catches or trade in marine organisms. This is largely because currently used genetic approaches (i.e., mainly PCR-RFLP analysis and phylogenetic analysis of DNA sequences) typically require multiple-step, post-PCR processing, including restriction endonuclease digestion or DNA sequencing, and are considered too time consuming and expensive for use in routine monitoring or large-scale market survey contexts. Such approaches are also of limited value when multiple species may be present in a single product (Hoelzel 2001). The routine application of molecular genetic techniques to the monitoring of marine wildlife trade urgently awaits the development of robust approaches that are faster and easier to implement (Palumbi and Cipriano 1998; FAO 2000; Shivji et al. 2002).

To facilitate conservation efforts and monitoring of trade in white shark body parts, we report here a novel, highly streamlined, and sensitive molecular diagnostic assay employing species-specific PCR primers from two organelles simultaneously to achieve accurate identification of white shark body parts from throughout the species' global range.

Materials and methods

Shark samples

Tissues from 53 white shark individuals (the target species) used for development and testing the diagnostic utility of our putative white shark, species-specific primers (both ITS2 and cyt *b* primers) were collected from across the global range of the species to ensure worldwide applicability of our assay (Figure 1). All but one of these animals were reliably identified (as whole animals) by fisheries personnel and/or experienced researchers. One dried fin sample, identified by fin traders as originating from a white shark,

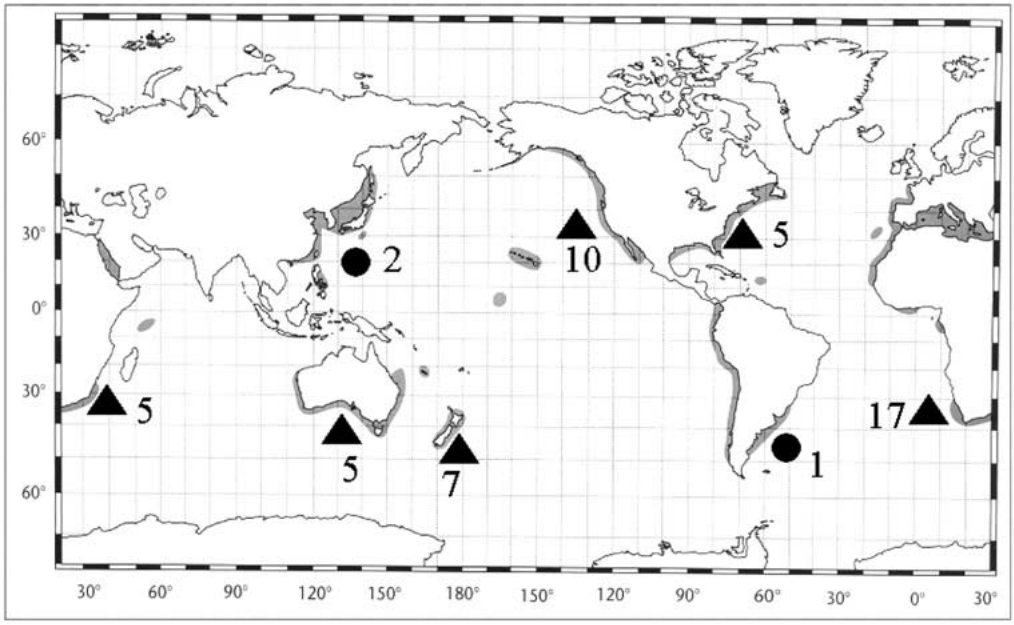

Figure 1. Global distribution of white sharks and sampling locations. Shaded areas represent the currently known distribution of this species. ▲ indicates sampling locations where white sharks receive legislative protection. ● indicates locations where legislative protection does not currently exist. Numbers next to symbols represent sample sizes for each geographic location. Map adapted from Last and Stevens (1994).

was collected in the Hong Kong shark fin market (S. Clarke, pers comm.). The capture location of this animal is unknown.

Tissue types used for diagnostic testing included fins, white muscle, liver and dried vertebrae with small bits of soft-tissue still attached. Diverse tissue types were used to ensure adequacy of our assay for identifying various types of white shark products. All tissues were stored frozen or in 95% ethanol.

The amplification performance of each putative, white shark species-specific primer we designed was also tested on DNA from 68 non-white shark species (non-target species), including most species in the sister orders Lamniformes and Carcharhiniformes, comprising the majority of sharks exploited worldwide (Table 1). Shark species most likely to be misidentified as white sharks either as whole animals, processed carcasses, or body parts, are in the family Lamnidae (lamnid sharks; Order Lamniformes): shortfin mako (*Isurus oxyrinchus*), longfin mako (*Isurus paucus*), porbeagle (*Lamna nasus*), and salmon shark (*Lamna ditropis*) (Castro 1993; Environment Australia 2001b). We therefore tested our putatively white shark-specific primers against 11–23 individuals of each of these four sister lamnid species, attempting where possible to use animals from both the Atlantic and Indo-Pacific (Table 1). Overall, the primers were tested on non-target species representing all eight extant shark orders (Compagno 1999).

PCR amplification and DNA sequencing of the ITS2 locus

Genomic DNA was extracted from 25 mg of all tissue types and species using the DNeasy Tissue Kit (QIAGEN Inc.). We chose ITS2 as the nuclear locus for shark species discrimination based on our studies (Pank et al. 2001; Shivji et al. 2002; D. Abercrombie, J. Magnussen, J. Nielsen, M. Shivji, unpublished data) that show this locus to be highly conserved within shark species, but also sufficiently divergent to allow discrimination between closely related species.

An approximately 1340 bp fragment (hereafter referred to as the positive control amplicon) containing the entire nuclear ribosomal DNA ITS2 region plus short portions of the flanking 5.8S (approx. 160 bp) and 28S (approx. 60 bp) ribosomal RNA genes was amplified by PCR from a reference female white shark individual, using the shark ITS2 universal primers FISH5.8SF (forward primer 5′-TTAGCGGTGGATCACTCGGCTCGT-3′) and FISH28SR (reverse primer 5′-TCCTCCGCTTAGTAATATGCTTAAATTCAGC-3′) (Pank et al. 2001). Following amplifi-

Table 1. Shark test species and their geographic origins. (n) represents the number of individuals of each species tested from each geographic region

Species	Geographic origin (n)
Order Lamniformes	
Isurus oxyrinchus (shortfin mako)	Atlantic (10)
	Pacific (13)
Isurus paucus (longfin mako)	Atlantic (5)
	Pacific (6)
Lamna nasus (porbeagle)	Atlantic (20)
Lamna ditropis (salmon shark)	Pacific (21)
Alopias vulpinus (thresher)	Atlantic (17)
Alopias superciliosus (bigeye thresher)	Atlantic (17)
	Pacific (3)
Alopias pelagicus (pelagic thresher)	Pacific (4)
Carcharias taurus (sandtiger)	Atlantic (13)
Odontaspis ferox (smalltooth sandtiger)	Pacific (1)
Cetorhinus maximus (basking shark)	Atlantic (5)
Order Carcharhiniformes	
Prionace glauca (blue shark)	Atlantic (3)
	Pacific (3)
Carcharhinus altimus (bignose)	Atlantic (5)
Carcharhinus longimanus (oceanic whitetip)	Atlantic (3)
	Pacific (3)
Carcharhinus signatus (night)	Atlantic (2)
Carcharhinus plumbeus (sandbar)	Atlantic (4)
	Pacific (3)
Carcharhinus limbatus (blacktip)	Atlantic (6)
Carcharhinus obscurus (dusky)	Atlantic (4)
	Pacific (2)
Carcharhinus falciformis (silky)	Atlantic (3)
	Pacific (3)
Carcharhinus melanopterus (blacktip reef)	Pacific (1)
Carcharhinus porosus (smalltail)	Atlantic (1)
Carcharhinus galapagensis (Galapagos)	Pacific (6)
Carcharhinus leucas (bull)	Atlantic (3)
	Pacific (3)
Carcharhinus brevipinna (spinner)	Atlantic (5)
	Pacific (1)
Carcharhinus isodon (finetooth)	Atlantic (3)
Carcharhinus acronotus (blacknose)	Atlantic (6)
Carcharhinus perezi (Caribbean reef)	Atlantic (5)
Carcharhinus amboinensis (Java)	Pacific (2)
Carcharhinus brachyurus (bronze whaler)	Pacific (3)
Carcharhinus tilstoni (Australian blacktip)	Pacific (2)
Carcharhinus sorrah (spot-tail)	Pacific (2)
Carcharhinus amblyrhynchos (grey reef)	Pacific (5)
Negaprion brevirostris (lemon)	Atlantic (6)
Negaprion acutidens (sicklefin lemon)	Pacific (2)
Galeocerdo cuvier (tiger)	Atlantic (3)
	Pacific (3)
Triaenodon obesus (whitetip reef)	Pacific (1)
Rhizoprionodon terranovae (Atlantic sharpnose)	Atlantic (6)
Rhizoprionodon acutus (milk)	Pacific (2)
Rhizoprionodon taylori (Australian sharpnose)	Pacific (1)
Rhizoprionodon porosus (Caribbean sharpnose)	Atlantic (2)
Sphyrna mokarran (great hammerhead)	Atlantic (5)
	Pacific (1)
Sphyrna lewini (scalloped hammerhead)	Atlantic (3)
	Pacific (3)
Sphyrna zygaena (smooth hammerhead)	Atlantic (1)
	Pacific (5)
Sphyrna tiburo (bonnethead)	Atlantic (6)
Eusphyra blochii (winghead)	Pacific (1)
Galeorhinus galeus (school)	Pacific (3)
Mustelus norrisi (smoothhound)	Atlantic (2)
Mustelus canis (smooth dogfish)	Atlantic (4)
Triakis semifasciata (leopard)	Pacific (2)
Scyliorhinus retifer (chain catshark)	Location unknown (1)
Apristurus profundorum (smalleye catshark)	Atlantic (2)
Cephaloscyllium ventriosum (swell)	Pacific (2)
Order Orectolobiformes	
Ginglymostoma cirratum (nurse)	Atlantic (3)
Nebrius ferrugineus (tawny nurse)	Pacific (1)
Rhincodon typus (whale)	Pacific (2)
Orectolobus ornatus (ornate wobbegong)	Pacific (1)
Order Squaliformes	
Squalus acanthias (spiny dogfish)	Atlantic (4)
Squalus cubensis (Cuban dogfish)	Atlantic (1)
Deania calceus (birdbeak dogfish)	Atlantic (2)
Dalatias licha (kitefin)	Atlantic (1)
Etmopterus spinax (velvet belly)	Atlantic (1)
Etmopterus pusilius (smooth lantern)	Atlantic (2)
Centrophorus granulosus (gulper)	Atlantic (1)
Centrophorus squamosus (leafscale gulper)	Atlantic (1)
Order Heterodontiformes	
Heterodontus francisci (horn)	Pacific (2)
Order Squatiniformes	
Squatina californica (Pacific angel)	Pacific (2)
Order Hexanchiformes	
Hexanchus griseus (sixgill)	Pacific (2)
Heptranchias perlo (sharpnose sevengill)	Pacific (1)
Order Pristiophoriformes	
Pristiophorus nudipinnis (shortnose sawshark)	Location unknown (1)

cation, both strands of the ITS2 positive control amplicon were sequenced using standard protocols on an ABI 377 sequencer (GenBank accession number AY198335). Negative control reactions (i.e., all PCR components without template DNA) were always run alongside all the PCR reactions described in this study.

Design and testing of ITS2 and cyt b species-specific primers

ITS2 primer design. The ITS2 sequence from the reference white shark was aligned with homologous sequences (Shivji et al. 2002; M. Shivji, unpub-

lished data) from its sister taxa in the family Lamnidae (shortfin mako; longfin mako; salmon shark; porbeagle shark), and three forward ITS2 primers putatively specific for white shark designed based on nucleotide differences between the white shark and its confamilial species.

Each putatively species-specific primer was tested for its amplification reliability and species-specificity against globally widespread white shark samples and 68 non-target species (Table 1) at the stringent annealing temperature of 65 °C. Amplifications were performed employing the general multiplex (triplex) PCR strategy detailed by Pank et al. (2001). The white shark triplex reaction consisted of one putatively species-specific, white shark forward primer, plus the two shark forward and reverse universal ITS2 primers (Figure 2A). Our *a priori* expectation for a successful, white shark, diagnostic primer was that the 3-primer triplex combination would produce two amplicons when used to amplify target species genomic DNA: an approximately 1340 bp positive control amplicon generated from the two shark universal primers, and a smaller amplicon diagnostic for white shark generated from the white shark-specific forward primer and the shark universal reverse primer. In contrast, this combination of primers, when tested against genomic DNA from any other (non-target) shark species would produce only the larger positive control amplicon due to failure of the white shark-specific primer to anneal to DNA from non-target species.

Total amplification reaction volumes were 50 uL, and contained 1 uL of the extracted genomic DNA, 12.5 pmol of each primer, 1X PCR buffer (QIAGEN Inc.), 40 uM dNTPs, and 1 unit of HotStar Taq^{TM} DNA Polymerase (QIAGEN Inc.). The PCR thermal cycling profile employed was: 94 °C initial heating for 15 min to activate the hot start DNA polymerase, followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 minute, 72 °C for 2 min, and a 5 min final extension step at 72 °C. Amplifications were performed in Mastercycler Gradient (Eppendorf Inc.) or MJ Research PTC-100 (MJ Research Inc.) thermal cyclers. From the above trials, we selected one ITS2 "optimal" primer (GWSITS2F: 5′-GCTGGAGTTCATTCTCCGTGCTG-3′) that amplified all 53 globally distributed white sharks and was species-specific at 65 °C annealing temperature.

Cyt b *primer design.* Cyt *b* sequences for the white shark and its four lamnid sister taxa were obtained from GenBank ((Martin and Palumbi 1993; Naylor et al. 1997; accession numbers white shark, L08031; shortfin mako, L08036; longfin mako, L08037; porbeagle, L08038; salmon shark, U91438), and aligned for primer design. Multiple primers, both putatively white shark species-specific and universal lamnid primers (i.e., universal primers that anneal to cyt *b* from all lamnid sharks) were designed and tested for their amplification performance. From the above trials, we selected an "optimal", cyt *b* reverse primer (GWScb-R: 5′-AGTCAGAACTAGTATGTTGGCTACAAGAAT-3′) that when used in PCR with a universal lamnid cyt *b* forward primer (LAM 499F; 5′-GCTTCTCAGTAGACAACGCCACCCT-3′) amplified all 53 globally distributed white sharks and was species-specific against all 68 non-target taxa at 65 °C annealing temperature. Relative annealing locations for the cyt *b* universal and species-specific primers are shown in Figure 2B.

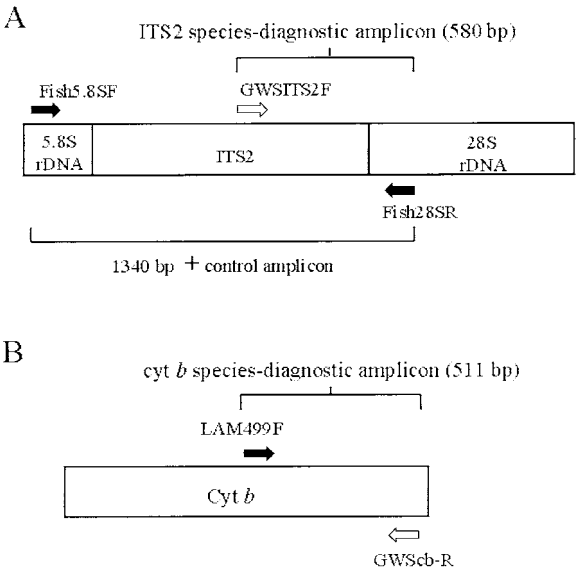

Figure 2. Schematic representation of relative annealing sites and orientation of primers used to identify white sharks. Primers denoted by open arrows are species-specific. Solid arrows represent universal primers. **A**. Primers associated with the nuclear ribosomal ITS2 locus and spatial coverage of the two amplicons expected from PCR of white shark DNA. **B**. Primers associated with the mitochondrial cyt *b* locus and spatial coverage of the single amplicon expected from PCR of white shark DNA.

Design and testing of the bi-organelle, pentaplex PCR assay

A multiplex PCR assay involving five primers simultaneously (pentaplex PCR) was tested for its diagnostic robustness. This pentaplex combination

included: the three ITS 2 primers – two universal primers (FISH5.8SF and FISH28SR) and the white shark species-specific primer (GWSITS2F); and the two cyt *b* primers – the universal lamnid forward primer (LAM499F) and the reverse, white shark species-specific primer (GWScb-R). With the exception of the additional cyt *b* primer pair, PCR amplification conditions were the same as those used for the ITS2 triplex PCR. Reaction products were checked by electrophoresis on 1.5% agarose gels.

Our *a priori* expectation for a positive (i.e., white shark) diagnostic result with this bi-organelle pentaplex assay was the amplification of two species-specific amplicons: (1) an ITS2 diagnostic sized amplicon (580 bp), and (2) a cyt *b* diagnostic sized amplicon (511 bp). Based on our previous experience using high-density (>3 primers) multiplex reactions in this format (Pank et al. 2001; Shivji et al. 2002), we also expected some amplification of the positive control amplicon generated by the universal ITS2 forward and reverse primers (1340 bp), albeit at relatively low yields. A negative diagnostic result (i.e., DNA from non-target species) was expected to yield only one, larger, positive control amplicon corresponding to the entire ITS2 locus, approximately 860–1500 bp in size depending on shark species tested (due to variability in ITS2 size in the various species).

Screening pooled samples

Some types of shark products may consist of multiple species combined together, greatly diminishing or complicating the ability to detect individual species in the mixture using RFLP, phylogenetic or direct sequence comparison methods. We therefore assessed the diagnostic utility and sensitivity of our bi-organelle, pentaplex PCR assay for screening multiple, pooled shark samples, as may be found in such products.

Twenty-three different combinations, each consisting of pooled DNAs from 10 different shark species, were constructed for screening with the bi-organelle pentaplex assay. Each combination comprised 1μl DNA from one white shark and 1 μl DNA from each of nine non-target species pooled together in a single PCR tube. Each of the 23 combinations was screened in triplicate (i.e., 69 trials in total). The DNA combinations were organized to include both closely related (i.e., all four lamnids and several lamniform species) and more distantly related (i.e., other orders) shark species in the mixtures. These trials ultimately included DNA from a representative of almost all of the non-target species in at least one of the reactions. To assess the incidence of false positive amplifications under the pooled DNA conditions, 8 additional ten-species mixtures containing DNA from non-target species only were screened (in triplicate; 24 trials total) with the bi-organelle pentaplex assay. With the exception of the pooled DNA templates, all amplification conditions were as described above.

Results

The reference white shark ITS2 locus was 1122 bp in size, and contained a 50 bp compound microsatellite repeat ([GA]12[CA]4[GA]2GG[CA]3[GA]3). Sequence divergence (JC model [Jukes and Cantor 1969]) between the white shark and each of its four sister lamnid species ranged from 5.5–7.4%. Attempts to compare the white shark ITS2 sequence to 19 carcharhinid shark ITS2 sequences (Pank et al. 2001; Shivji et al. 2002; M. Shivji, unpublished data) resulted in only very approximate alignments because of difficulty assigning homology, with sequence divergences in excess of 55%.

ITS2 and cyt b primer testing

The final (optimal) white shark ITS2 and cyt *b* primers selected demonstrated complete species specificity with respect to the 68 non-target species tested. For brevity, gel documentation results for individual locus primer testing are not shown but are available upon request from the corresponding author. In the ITS2 triplex primer assay, the white shark-specific ITS2 primer consistently produced a species-diagnostic amplicon of approximately 580 bp from all 53 globally distributed white sharks tested. Co-amplification of the positive control amplicon occurred at lower yields compared to the species-diagnostic amplicon. For non-target species, only the positive control amplicon was produced in every case, even from the most closely related (confamilial) species (i.e., there were no false positive amplifications). The cyt *b* primer pair consistently amplified a single, species-diagnostic amplicon of 511 bp from all 53 white sharks, and did not amplify any of the non-target species.

Bi-organelle, pentaplex PCR

Combining the three ITS2 and two cyt *b* primers together in a pentaplex PCR assay resulted in

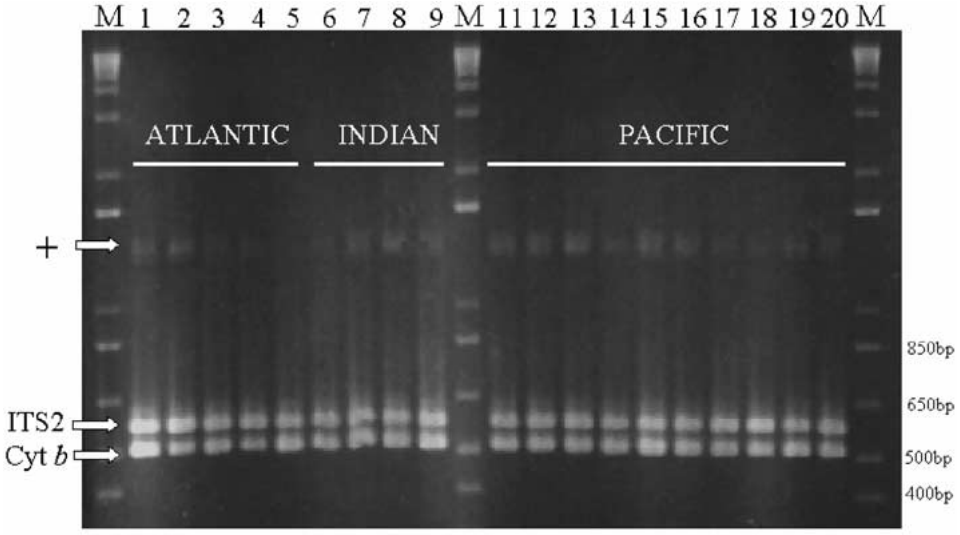

Figure 3. Amplification results using the bi-organelle pentaplex assay on DNA from 19 globally distributed white sharks. Lanes 1–3, NW Atlantic (USA); lane 4, SW Atlantic (Brazil); lane 5, SE Atlantic (South Africa); lanes 6–9: Indian Ocean (South Africa); lanes 11–12, NW Pacific (Japan, Taiwan); lanes 13–16: NE Pacific (USA); lanes 17–20: SW Pacific (Australia, New Zealand). Arrows indicate species-diagnostic (ITS2 and cyt *b*) and positive control (+) amplicons. Lanes labeled M represent the molecular size standard (Gibco-Life Technologies 1 Kb Plus ladder).

the consistent amplification of two distinct species-diagnostic amplicons from all 53 white sharks (Figure 3). There were no instances of false negative results in replicate pentaplex PCR trials. However, the co-amplification of the ITS2 positive-control amplicon when using this pentaplex reaction mixture on white shark DNA was inconsistent. Under the reaction conditions used here, the positive-control amplicon either occurred at very low yields, or was visually undetectable based on ethidium bromide stained gels (see discussion section for ramifications of this inconsistent positive-control amplification).

In the case of the 68 non-target species, the bi-organelle pentaplex assay amplified only the ITS2 positive control amplicon in every case. All the non-target ITS2 positive control amplicons, including the smaller ITS2 (approximately 860 bp) from hammerhead sharks, were clearly distinguishable from the two white shark species-diagnostic amplicons (Figure 4). No false-positive amplifications were detected with any of the non-target sharks tested.

Screening pooled samples

The bi-organelle pentaplex PCR assay proved sufficiently sensitive to detect DNA from a single white shark in all 23 ten-species mixtures. Both white shark diagnostic amplicons (ITS2 and cyt *b*) were produced in triplicate trials from 20 of the 23 mixtures. For 3 mixtures, however, one out of the three triplicates per mixture failed to produce any amplicons (i.e., 3 of the 69 total trials [4.3%] demonstrated complete absence of any amplification). White shark species-diagnostic amplicons were not produced from the 8 mixtures containing combinations of only non-target species (i.e., no false positive results were observed). In 23 of the 24 trials, only the positive-control ITS2 amplicons were produced as expected, appearing as a short "smear" of amplicons representing ITS2 loci of different sizes, depending on species composition of the pooled DNA mixture (Figure 5). For one of these 8 mixtures, one of the triplicate trials (i.e, one out of 24 total trials; 4.2%) demonstrated complete absence of amplification. In trials where hammerhead shark DNAs were included in the non-target species mixture, the smaller ITS2 amplicons from the hammerheads were clearly distinguishable by size from the white shark diagnostic amplicons (Figure 5).

Discussion

Utilizing species-specific PCR primers is currently the most efficient DNA-based approach for identifying wildlife tissues in forensic applications because annealing specificity of such primers circumvents the

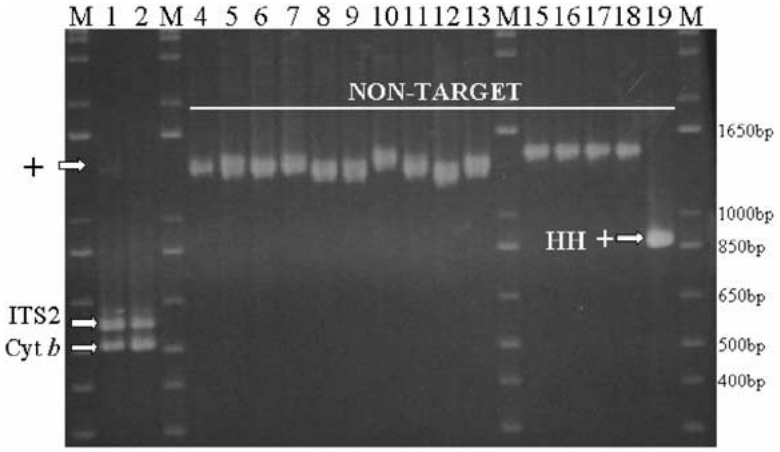

Figure 4. Amplification results using the bi-organelle pentaplex assay on DNA from 2 white sharks (lanes 1 and 2), 10 non-target lamniform species (lanes 4–13) and 5 non-target carcharhiniform species (lanes 15–19). Non-target species: lane 4, shortfin mako; 5, longfin mako; 6, porbeagle; 7, salmon shark; 8, smalltooth sandtiger; 9, sandtiger; 10, basking; 11, thresher; 12, bigeye thresher; 13, pelagic thresher; 15, bull; 16, tiger; 17, dusky; 18, sandbar; 19, great hammerhead. HH+ indicates smaller hammerhead size positive control amplicon. All other annotations as in Figure 3.

need for further characterization of PCR amplicons by restriction endonuclease digestion or DNA sequence analysis. In legal contexts and other strongly disputed enforcement cases, primers derived from multiple loci may be desirable to enhance the diagnostic accuracy of the detection assay, both by providing a "backup" diagnosis from more than one locus and by reducing the likelihood of detection failure due to intraspecific sequence variation at the primer annealing sites for any single primer.

Here, we demonstrate the efficacy of a multiplex, species-specific, PCR primer strategy based on loci from two organelles with different evolutionary dynamics to identify tissues from a shark species targeted for global conservation efforts. The bi-organelle pentaplex PCR assay reported here is highly streamlined, simple-to-perform, and reliably and efficiently discriminates white sharks from at least 68 other commercially exploited shark species, including all other members of the family Lamnidae. The ability of this assay to identify white sharks from throughout their global distribution suggests low intraspecific variation at these loci, a finding consistent with our observations of ITS2 loci from other shark species (Pank et al. 2001; Shivji et al. 2002. M. Shivji, unpublished data) and the relatively slow rate of shark cyt *b* evolution reported by Martin et al. (1992).

Although co-amplification of the positive control ITS2 amplicon from the target species was inconsistent (very low yields or undetectable) in the pentaplex PCR setting, this inconsistency does not detract from the functional utility of the assay. This is because the ITS2 universal forward and reverse primers are both included in the triplex ITS2 and pentaplex reactions to provide an internal positive control, ensuring that amplification of a positive control amplicon always occurs in the *absence* of the target species. This feature of the assay is meant to prevent false negative results, i.e., the complete absence of amplification (due, for example, to inhibitory substances in the starting DNA or errors in setting up the reaction) from being interpreted as the absence of the target species. In this context, the internal positive control worked exceptionally well, consistently amplifying only the whole ITS2 locus in the absence of white shark DNA. When screening an unknown sample for the presence of white shark DNA, detection of the target species requires only amplification of the species-diagnostic amplicons; co-amplification of the ITS2 positive control is unnecessary to diagnose the target species (Pank et al. 2001; Shivji et al. 2002).

We are uncertain about the reason for low-yield or sometimes lack of amplification of the ITS2 positive control in the triplex and pentaplex reactions in the presence of white shark DNA. We speculate that this occurrence may have to do with some form of primer competition, possibly for DNA polymerase binding to several annealed primers (see Pank et al. 2001 and Shivji et al. 2002 for a more detailed explanation). Importantly, the positive control amplicon was consistently amplified from all 68 non-target

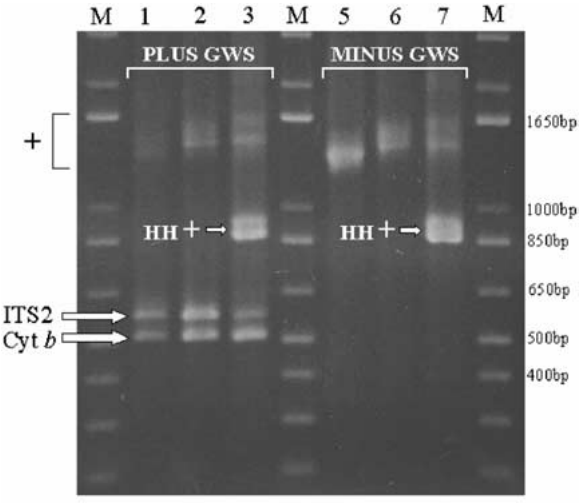

Figure 5. Amplification results from pooled DNA sample screening: Lanes 1–3 are ten-species DNA mixtures (one white shark plus nine non-target species). Non-target DNAs are: Lane 1, shortfin mako, longfin mako, porbeagle, salmon shark, thresher, bigeye thresher, pelagic thresher, sandtiger, basking; Lane 2, sandbar, bignose, dusky, Galapagos shark, blacktip, spinner, Caribbean reef shark, blue, silky; Lane 3, Java shark, great hammerhead, scalloped hammerhead, smooth hammerhead, smooth dogfish, Australian blacktip, spot-tail, gray reef, tiger shark). Lanes 5–7 are the same pooled sample reactions repeated, with white shark DNA substituted by shortfin mako DNA. HH+ indicates smaller hammerhead shark size positive control amplicons. GWS represents great white sharks. All other annotations as in Figure 3.

shark species, with no cases of false positive results (i.e., production of white shark specific amplicons) observed in replicate testing.

For a false positive result to occur, the non-target shark DNA would have to be very similar in sequence to white shark DNA at both the ITS2 and cyt *b* primer annealing sites. While false positive results could theoretically occur with as yet untested shark species, we believe this possibility exceedingly remote for two reasons: First, neither species- specific primer annealed to DNAs from the white shark's four most closely related sister species in the family Lamnidae; it is highly unlikely, therefore, that either primer would anneal to DNA from phylogenetically more distant species at the stringent annealing temperature (65 °C) used in this assay. As indicated earlier, sequence divergence in the ITS2 locus between white shark and even species in its sister order Carcharhiniformes is large enough to preclude meaningful sequence alignments. Second, the use of two species-specific primers simultaneously for diagnosis makes it is even less likely that both primers would anneal to DNA from any single species not yet tested. The novel use of primers from two loci simultaneously in one PCR greatly bolsters the functional reliability of this type of assay, without comprising the overall efficiency of the procedure.

In addition to its accuracy, this assay also demonstrated considerable sensitivity, always correctly detecting the presence or absence of white shark DNA amongst a pooled sample of up to ten different shark species in one PCR reaction. However, the approximately 4% of trials in which there was complete amplification failure suggests that pooled sample tests should be run at least in duplicate, and emphasizes the importance of incorporating a positive control in the assay. We caution also that false negatives are possible in a pooled sample analyses; i.e., the presence of white shark may go undetected despite the production of a positive control amplicon, if the white shark DNA is degraded to the point of being unamplifiable by PCR. We note, however, there were no instances of false negatives in all our pooled sample trials (69 trials total) using different types and condition of white shark tissues, indicating that false negative outcomes are likely to be infrequent.

Overriding the small potential for false negatives in pooled sample analyses are the considerable time and cost advantages accruing from reducing, by up to an order of magnitude, the number of PCR reactions needed. Pooling samples may be a practical option in high volume screening contexts, as is often required in trade monitoring or regulation enforcement. Only those pooled sets that produce a positive diagnostic result (i.e., both white shark diagnostic amplicons) would need further screening if it were necessary to determine which of the individual samples in the set were derived from white sharks. The highly sensitive detection capability of the assay may also prove useful to identify white sharks in mixed-species products such as cartilage pills or mixed-species meat. The processed and powdered cartilage found in pills yields DNA suitable for analysis by this assay (J. Magnussen and M. Shivji, unpublished data).

Applications to conservation and management

We envision the diagnostic primers and PCR strategy we have developed will be useful in several white shark management and trade-monitoring contexts. The utility of this assay for identifying a range of tissue types (muscle, liver, fins) likely to occur in trade of globally distributed animals will contribute to worldwide conservation efforts for this species. One direct

benefit of the accuracy of the assay is its applicability to cases involving illegal marketing of white shark parts, solving the current problem of basing such cases on typically weaker circumstantial evidence regarding species identification (Compagno et al. 1997).

The streamlined nature of the pentaplex approach, its sensitivity, and ease of application makes it potentially applicable to larger scale surveys of the various types of shark products in international trade. Management and conservation organizations could use this assay to conduct genetic surveillance to maximize the likelihood of identifying any illegal white shark products circulating amongst trade in legal shark species. For example, since valuable fins or fin-sets from white sharks can be mislabeled as belonging to several other large bodied sharks (especially other lamnids), the assay could be used to screen all suspect fins (e.g., above a prescribed size) sampled in port or in the market-place or before export, to ensure that they originate from legally harvested species (Environment Australia 2001b). Additionally, it is often possible to identify processed lamnid shark carcasses ("logs" or "barrels") to family level based upon the presence of caudal keels and other morphological characters (Castro 1993; Environment Australia 2001b). Because small to moderate sized white shark carcasses can be misidentified or purposefully misrepresented as one of its similar but legal to harvest sister species, it may be useful to establish mandatory screening of all lamnid shark carcasses in areas where white sharks are protected. While this assay has direct applicability to current and future white shark conservation and trade monitoring efforts, the bi-organelle, multiplex PCR feature of the assay may prove useful as a model (with appropriate choice of diagnostic loci) for the development of genetic surveillance procedures for traded wildlife products in general.

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