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Rapid and Simultaneous Identification of Body Parts from the Morphologically Similar Sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) Using Multiplex PCR

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Abstract: Many commercially exploited carcharhinid sharks are difficult to identify to species owing to extensive morphological similarities. This problem is severely exacerbated when it comes to identifying detached shark fins, and the finless and headless shark carasses typically sold in markets. To assist in the acquisition of urgently needed conservation and management data on shark catch and trade, we have developed a highly streamlined approach based on multiplex polymerase chain reaction (PCR) that uses species-specific primers derived from nuclear ribosomal ITS2 sequences to achieve rapid species identification of shark body parts. Here we demonstrate the utility of this approach for identifying fins and flesh from two globally distributed, morphologically very similar carcharhinid sharks (*Carcharhinus obscurus* and *Carcharhinus plumbeus*) intensively targeted in fisheries worldwide, and often confused for each other even as whole animals. The assay is conducted in a 4-primer multiplex format that is structured to simultaneously achieve the following efficiency and cost-reduction objectives: it requires only a single-tube amplification reaction for species diagnosis, it incorporates an internal positive control to allow detection of false-negative results, and it is novel in that it allows species identification even when DNAs from two species are combined in the same tube during the PCR reaction. The latter innovation reduces the required effort for screening a set of unknown samples by 50%. The streamlined approach illustrated here should be amenable for use in a shark conservation and management context where large numbers of samples typically need to be screened; the approach shown may also provide a model for a rapid diagnostic method applicable to species identification in general.

Key words: *Carcharhinus,* fins, genetic identification, ITS2, multiplex PCR, sharks.

INTRODUCTION

Industrial-scale shark fisheries have experienced tremendous growth worldwide over the past two decades. Both directed shark fishing and the enormous shark bycatch that

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occurs in the tuna and billfish fisheries have resulted in unprecedented exploitation pressure on many shark populations (Bonfil, 1994; Castro et al., 1999). Unlike most teleost fishes, sharks are extremely susceptible to population crashes resulting from overfishing, with the history of shark fisheries providing several examples of rapid declines in catch rates and fishery collapses after relatively short but intensive exploitation (Anderson, 1990; Castro et al., 1999).

The sensitivity of sharks to overexploitation is due to their life-history characteristics, which are more similar to those of large mammals than teleost fishes. Fertilization is internal, and most sharks demonstrate slow growth, delayed maturation, long reproductive cycles and life spans, and low fecundity (Castro et al., 1999). Most sharks also function as top predators in marine ecosystems and are relatively less abundant than many of the teleost fishes that are also the target of large-scale fisheries. These characteristics combine to lower the reproductive potential of most shark species, and make recoveries from overexploitation extremely slow (Bonfil, 1994; Musick, 1999).

For those few shark species for which longer-term catch data are available, there is increasing indication that most are in severe decline (Castro et al., 1999). These observations, coupled with the historical failure of several shark fisheries and the generally K-selected life-history strategies of most sharks, have led to widespread concerns that similar declines are occurring worldwide in industrial-scale, mostly unmanaged, shark fisheries (Bonfil, 1997; Weber and Fordham, 1997; Camhi, 1998, 1999; Castro et al., 1999). Shark fisheries on the high seas and in coastal regions of most countries have been historically unmanaged or only semimanaged for several reasons, including the low economic value generally accorded to shark products. More recently, however, with changes in market price driven by increased demand for shark fins for the Asian delicacy shark fin soup and cartilage products for pharmaceutical applications, shark exploitation has increased tremendously, prompting calls for urgent implementation of management and conservation measures.

Effective management and conservation worldwide of the typically multispecies shark fisheries will require collection of biological and fishery information on a species-specific basis. This requirement stems from the different life-history characteristics of individual shark species, resulting in differential sensitivity of each species to intensive exploitation (Heist and Gold, 1998; Castro et al., 1999). A major impediment to obtaining shark fishery catch data and implementing management programs on a species-specific basis, however, is the significant problem of accurate species identification of many commonly targeted species (Bonfil, 1994; Castro et al., 1999). Many requiem sharks in the large and economically important family Carcharhinidae, for example, are only subtly different in their morphology, requiring a "practiced" eye to discriminate accurately (Branstetter, 1982; Castro, 1993). The identification problem is exacerbated by the common fishery practice of removing the head, tail, and most fins from landed sharks while still at sea to reduce required storage space for the animal. This practice removes the major morphological identifying characters, leaving shark carcasses that are difficult for even fishery management personnel to identify accurately. Further obstacles for species-specific management have arisen with the recent burgeoning demand and high price for shark fins, which have escalated the fishing practice of keeping only the fins and discarding the rest of the animal. Accurate identification of the species-of-origin of detached shark fins is extremely difficult in most cases, requiring careful examination by experts.

These species identification difficulties have been a major factor contributing to the worldwide scarcity of species-specific records on shark catch (Bonfil, 1994; Castro et al., 1999). Even in those countries where some attempt is made to document shark catch, the statistics typically lump the multispecies catch together as generic "sharks" or "large sharks versus small sharks" (Bonfil, 1994, 1997; Castro et al., 1999). In the United States, for example, which along with New Zealand and Australia is one of few countries with active commercial shark fishery management programs (Weber and Fordham, 1997), the species identification difficulties have forced management based on aggregate species groups instead of individual species (NMFS, 1993).

Two species, the dusky shark (*Carcharhinus obscurus*) and the sandbar shark (*Carcharhinus plumbeus*), provide a good illustration of the problem of shark species identification and the importance of implementing species-specific management and conservation. Both species have overlapping, cosmopolitan distributions in tropical and warm temperate waters and are the target of fisheries worldwide for their meat and fins (Compagno, 1984). Even as intact animals, the two species are similar enough to be commonly confused for each other (Castro, 1983, 1993), with the problem considerably worsening when dealing with carcasses or fins. Both species are listed in the "Lower Risk–Near Threatened" category of the International Union for

Conservation of Nature Red List of Threatened Animals. The dusky shark is of particular concern because it is a slow-growing species with an estimated intrinsic rebound potential from fishing pressure that is one of the lowest of the large sharks (Smith et al., 1998). Severe declines due to overfishing have been documented for this species in North American waters (Castro et al., 1999). In reaction to these declines, the United States National Marine Fisheries Service (U.S. NMFS), the primary federal agency responsible for fisheries management, has listed the dusky shark as a prohibited species in its newly proposed Atlantic shark fisheries management plan (NMFS, 1999). Given the difficulties of reliably discriminating sandbar and dusky sharks and their body parts on morphological grounds and the higher price obtained for sandbar sharks in the market, it is believed that dusky sharks are frequently misclassified as sandbar in various catch and tag records (Natanson, 1990; Castro, 1993). This problem may manifest itself in fisheries' landing records, and catch statistics on these two species are believed unreliable.

Development of effective conservation planning for sandbar and dusky sharks and enforcement of the proposed dusky shark prohibition are critically dependent on the availability of alternative methods for accurate discrimination of carcasses and fins from these two species. Molecular genetic methods, which have proved enormously useful for species and strain discrimination in a wide variety of organisms (Clapp, 1993), can also be used for discriminating shark species (Martin, 1993; Shivji et al., 1996; Heist and Gold, 1998). The methods developed so far for sharks are based on standard polymerase chain reaction (PCR) amplification of mitochondrial or nuclear loci followed by restriction fragment length polymorphism (RFLP) analysis of the amplified locus to yield species-diagnostic DNA banding patterns on a gel. These methods, although effective for shark species discrimination, are comparatively labor intensive and expensive. Routine application of genetic methods in fish conservation and management is still relatively scarce and awaits the availability of simpler, more rapid, and easily implemented methods. We report here on the development of a rapid, single-reaction-tube, 4-primer multiplex PCR approach to reliably and simultaneously discriminate fins and other tissues from globally distributed dusky and sandbar sharks. To further streamline the approach for improved screening efficiency and reduced costs, we have extended the utility of this method to the identification of DNAs from two shark species combined together in the same amplification reaction.

Table 1. Geographic Origin and Number of Shark Tissue Samples Analyzed

Species	Geographic origin (n)*
Reference samples	
Carcharhinus plumbeus (sandbar shark)	U.S. Atlantic (1)
Carcharhinus obscurus (dusky shark)	U.S. Atlantic (1)
Test samples	
Sandbar shark	U.S. Atlantic (15)
Sandbar shark	Gulf of Mexico (6)
Sandbar shark	Pacific (Hawaii) (13)
Dusky shark	U.S. Atlantic (13)
Dusky shark	S. Pacific (Australia) (4)
Dusky shark	W. Pacific (6)
Blind test	
Sandbar sharks	Chesapeake Bay, U.S. Atlantic (13)
Dusky sharks	Chesapeake Bay, U.S. Atlantic (2)

*The values in parentheses represent the number of animals from each geographic location.

Materials and Methods

Shark Tissue Samples

Dusky and sandbar shark tissue samples (reference samples) used for DNA sequencing and species-specific PCR primer design were collected by us during fishery-independent shark population abundance and tagging surveys of coastal sharks conducted by the U.S. NMFS off the U.S. Atlantic coast. Dusky and sandbar tissue samples (test samples) that we used for verifying the diagnostic utility of our species-specific primers were collected from the Atlantic and Pacific by us, or shark expert colleagues who identified the whole animals. Blind samples (samples whose species identity was unknown to us) were collected and identified by D. Grubbs (Virginia Institute of Marine Sciences, U.S.A.) as part of a fishery-independent shark survey. Table 1 shows the geographic origin and sample sizes of the shark samples analyzed.

Reference samples used for DNA sequencing consisted of white muscle. Test samples were fin, heart, white muscle, or liver tissues. Blind samples were all fin tissues. All tissues were kept in SED (saturated NaCl, ethylenediaminetetraacetic acid, and dimethylsulfoxide) preservative at room

temperature for short-term storage or at 4°C for long-term storage.

DNA Extraction, PCR Amplification, and DNA Sequencing

Genomic DNA was extracted from 25 mg of tissue using the QIAmp Tissue Kit (Qiagen Inc., Valencia, Calif.), and stored at −20°C until use. An approximately 1490-bp fragment (hereafter referred to as the positive control amplicon) containing the entire nuclear ribosomal DNA internal transcribed spacer (ITS2) region plus short portions of the flanking 5.8S (160-bp) and 28S (60-bp) ribosomal RNA genes was amplified from both dusky and sandbar sharks by standard PCR, employing the shark universal primers FISH5.8SF (forward primer 5′-TTAGCGGTGGATCACTCGGCTCGT-3′) and FISH28SR (reverse primer 5′-TCCTCCGCTTAGTAATATGCTTAAATTCAGC-3′). We have focused on the ITS2 locus as a genetic marker for shark species discrimination because of the demonstrated utility of ITS loci for species discrimination in other organisms (Collins and Paskewitz, 1996; Taylor and Bruns, 1999), as well as our studies (unpublished data) that show this locus to be highly conserved within shark species, but also sufficiently divergent to allow discrimination between closely related species. Following PCR amplification, both strands of the positive control amplicon were sequenced using standard protocols on an ABI 373A sequencer, with internal sequencing primers designed as necessary. Reference sequences for this locus have been deposited in GenBank under the following accession numbers: AY033819, AY033820.

Species-Specific Primer Design and Multiplex PCR

Dusky and sandbar shark ITS2 sequences were aligned with the aid of the sequence editing program ESEE (Cabot and Beckenbach, 1989). PCR primers putatively specific for dusky and sandbar sharks were designed on the basis of nucleotide differences between the two species.

The dusky and sandbar primers were first each tested separately on the shark test samples (dusky, $n = 23$; sandbar, $n = 34$) for their utility in discriminating the two species. The testing consisted of using a combination of 3 primers simultaneously (i.e., the shark forward and reverse universal primers plus either the dusky or sandbar primer) in high-stringency multiplex PCR reactions. Our a priori measure of primer success in species discrimination was the expectation that a 3-primer multiplex combination containing, for example, the sandbar-specific primer would produce 2 amplicons when used to amplify the target species (i.e., sandbar) genomic DNA: a 1490-bp positive control amplicon generated from the two shark universal primers, and a smaller amplicon diagnostic for sandbar generated from the sandbar-specific forward primer and the shark universal reverse primer. In contrast, this combination of primers, when tested against nontarget (i.e., dusky) genomic DNA would produce only the positive control amplicon owing to failure of the sandbar-specific primer to anneal to dusky DNA. Analogous results would be expected for a 3-primer combination that included a dusky-specific primer.

All amplifications were performed using the Mastercycler Gradient (Eppendorf Inc.) thermal cycler. Total reaction volumes were 50 μl, and contained 1 μl of the extracted genomic DNA, 12.5 pmol of each primer, 1× PCR buffer (Qiagen Inc.), 40 μM dNTPs, and 1 U of HotStar *Taq* DNA Polymerase (Qiagen Inc.). The PCR thermal cycling profile used was 94°C initial heating for 15 minutes to activate the DNA polymerase, followed by 35 cycles of 94°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes, and a 5-minute final extension step at 72°C. Completed reactions were kept at 4°C or −20°C until checked by gel electrophoresis on 1.2% agarose gels after ethidium bromide staining.

After testing the utility of the dusky and sandbar primers individually, we designed and evaluated the performance of a 4-primer multiplex PCR assay for species discrimination. This approach used all 4 primers simultaneously (i.e., the two shark universal forward and reverse primers and both dusky and sandbar species-specific primers) in a single tube amplification reaction. Test samples used and amplification conditions were as described above. The accuracy and reliability of the 4-primer multiplex PCR approach was further tested by evaluating its performance in a blind test on fin tissue samples from 15 sharks provided to us as being either "sandbar or dusky."

Screening Combined DNA Samples from Two Animals

Since availability of streamlined identification protocols is necessary for routine implementation of genetic techniques in fishery conservation and management practices, we evaluated the performance of the 4-primer multiplex PCR approach for screening combined DNA samples from two different animals in the same amplification reaction. For this exercise, we pooled and tested genomic DNAs from two animals of the same species (i.e., two different sandbars or

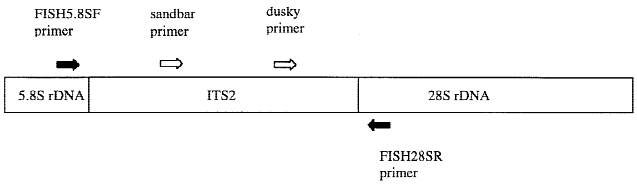

Figure 1. Schematic representation of the shark nuclear 5.8S and 28S ribosomal RNA genes and ITS2 locus showing relative annealing sites and orientation of the shark universal primers (solid arrows: FISH5.8SF and FISH28SR) and species-specific primers (open arrows: sandbar and dusky).

two different duskys) as well as different species (i.e., a sandbar and a dusky) in different combinations of individual sharks. With the exception of combining DNAs from two animals in each PCR reaction, all reaction conditions were identical to those described above.

Results

Testing Each Species-Specific Primer Individually

The ITS2 locus in dusky and sandbar sharks is approximately 1268 bp in size, with a sequence divergence of 4% between species. Annealing sites for both species-specific PCR primers were positioned internal to the forward and reverse shark universal primers, in places that were expected to generate unambiguously diagnostic-sized amplicons for each species (Figure 1). In the 3-primer multiplex assay, both dusky and sandbar species-specific primers performed as expected, amplifying both the species diagnostic-sized amplicon and the positive control amplicon from the target species; for nontarget species, the 3-primer assay amplified only the positive control amplicon (Figure 2, A and B). An unexpected result, however, was that in some cases of testing the sandbar-specific primer on sandbar sharks, the positive-control amplicon amplified with relatively low efficiency (Figure 2, B, lanes 1–10). This low efficiency of positive control amplification from sandbar sharks was sporadic in repeated trials. Primer sequences for dusky and sandbar that proved consistently reliable for discriminating the two species from widely separated geographic areas (Atlantic and Pacific Oceans) are given in Table 2, along with the sequence of the nontarget species at the orthologous position in the ITS2.

Testing Both Species-Specific Primers Together

The dusky and sandbar species-specific primers, when combined with the two shark universal primers in a single-tube, 4-primer, multiplex PCR assay, accurately and reliably distinguished the two species in tests screening one shark sample at a time (Figure 3). As in the case of the 3-primer amplifications employing a single species-specific primer, the amplification efficiency of the positive control amplicon from sandbar sharks was often relatively low under the PCR conditions used. One dusky shark individual from the southern Pacific produced a dusky-diagnostic-sized amplicon, as expected, but also a second faint amplicon consistent in size with that of a sandbar-diagnostic amplicon (Figure 3, lane 19).

Blind tests using the 4-primer assay on fin tissue from the 15 unknown animals from the U.S. Atlantic identified 13 animals as sandbar and 2 as dusky with 100% accuracy (D. Grubbs, personal communication; gel photographs not shown). Overall, for the combined set of test samples and blind samples (dusky, $n = 25$; sandbar, $n = 47$) evaluated here, the assay proved 100% accurate and reliable in its ability to distinguish between the two species, with no false positives or negatives occurring.

Identifying Combined DNAs from Two Shark Individuals

Combining DNAs from two different animals into a single amplification reaction did not adversely affect the dynamics of the multiplex PCR assay. The 4-primer assay unambiguously identified the species present in each reaction in repeated trials. Pooled DNAs from two individuals of the same species (i.e., 2 duskys or 2 sandbars) generated a single, correct, species-diagnostic-sized amplicon (Figure 4, lanes 1–8); pooled DNAs from two individuals of the different species (i.e., 1 dusky and 1 sandbar) generated the two amplicons diagnostic for dusky and sandbar sharks (Figure 4, lanes 9–12). Under these combined DNA conditions, however, amplification of the positive control amplicon occurred at very low efficiency in the case of the pooled sandbar-sandbar samples (Figure 4, lanes 5–8) and dusky-sandbar samples (Figure 4, lanes 9–12).

Discussion

The power of DNA analysis methods for identifying cryptic organisms and parts of organisms has been demonstrated unequivocally in a large number of studies. Despite the proven effectiveness of genetic methods, however, their implementation in the context of marine fisheries manage-

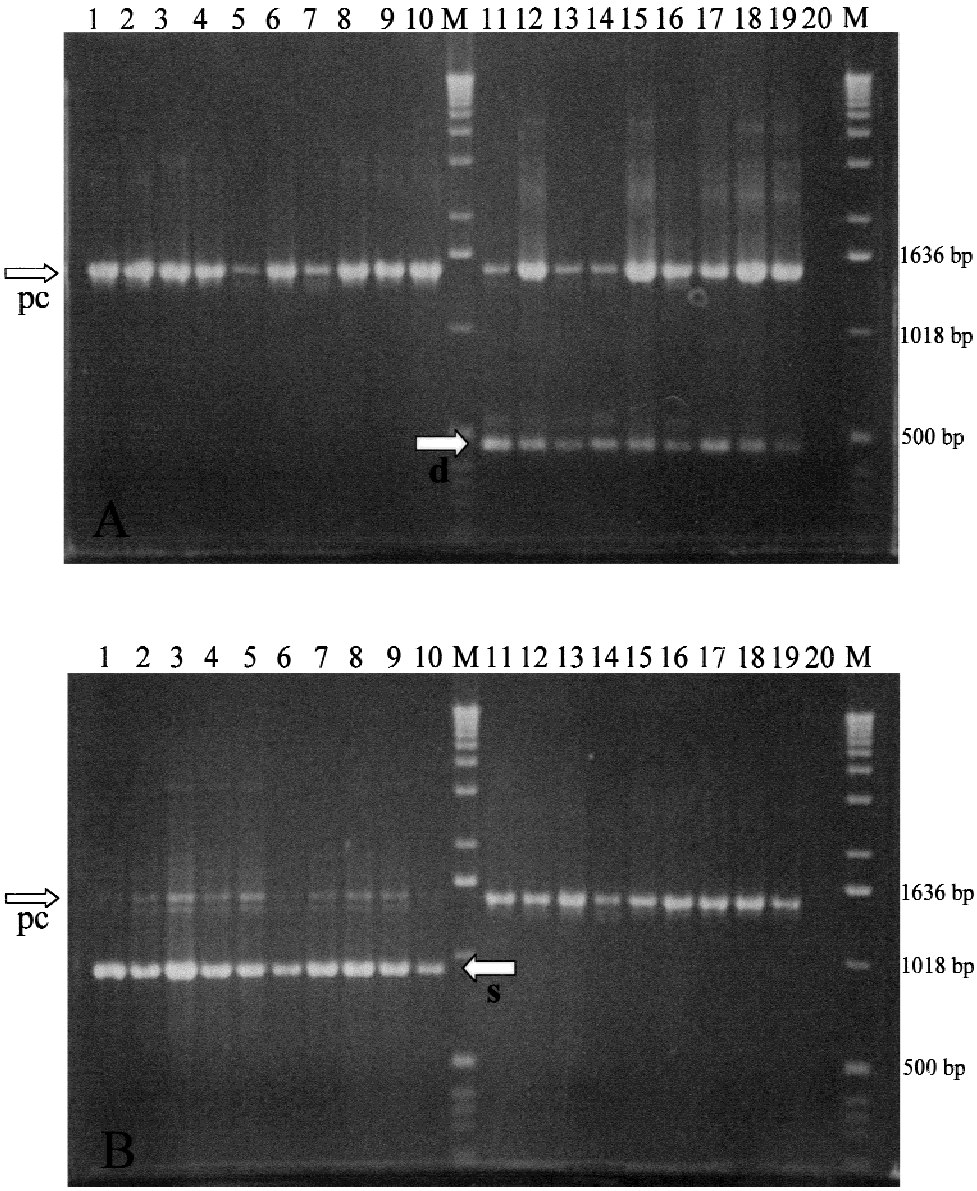

Figure 2. Results of 3-primer multiplex amplification reactions using a combination of 2 shark universal primers and 1 species-specific primer. **A:** Testing the dusky-specific primer against sandbar shark (lanes 1–10) and dusky shark (lanes 11–19) individuals. Lanes 1–6 represent sandbar sharks from the U.S. Atlantic and Gulf of Mexico; lanes 7–10 represent sandbar sharks from the Pacific. Lanes 11–15 represent dusky sharks from the U.S. Atlantic; Lanes 16–19 represent dusky sharks from the Pacific. **B:** Testing the sandbar-specific primer against sandbar shark (lanes 1–10) and dusky shark (lanes 11–19) individuals. Geographic origins of the sharks same as in A. Lanes 20 contain the negative control reactions (no shark DNA). Arrows labeled *pc* show the approximately 1490-bp positive control amplicon; arrows labeled *d* and *s* show the dusky and sandbar species-specific amplicons, respectively. Lanes M contain the molecular size standard (Gibco-Life Technologies, 1-kb ladder).

ment and conservation is still rare. This is largely because most current methods are still considered too labor intensive or expensive for application in arenas requiring screening of hundreds to thousands of samples (Palumbi and Cipriano, 1998). In light of widespread concerns about the ecological health of exploited shark populations, we have focused on the development of genetic identification methods that will lend themselves to routine screening of large numbers of shark carcasses and fins.

Our results demonstrate that the use of more than two primers in a multiplex PCR format targeting the ribosomal ITS2 locus is a rapid and reliable method for discriminating two globally widespread, intensively harvested, morphologically similar species that are problematic when it comes to

Table 2. Species-Specific Primer Sequences and Sequence of the Nontarget Species at the Orthologous Location in the ITS2*

Primer or orthologue	Sequence (5′–3′)
Dusky-specific primer sequence	GTGCCTTCC**CACC**TTTTG**GCG**
Sandbar ITS2 orthologue	GTGCCT**GCCACCG**TTTTG**CAC**
Sandbar-specific primer sequence	AAGTGGAGCGACTGTCTGC**A**GGT**C**
Dusky ITS2 orthologue	AAGTGGAGCGACTGTCTGC**T**GGT**G**

*Differences in sequence between the two species are indicated in boldface type.

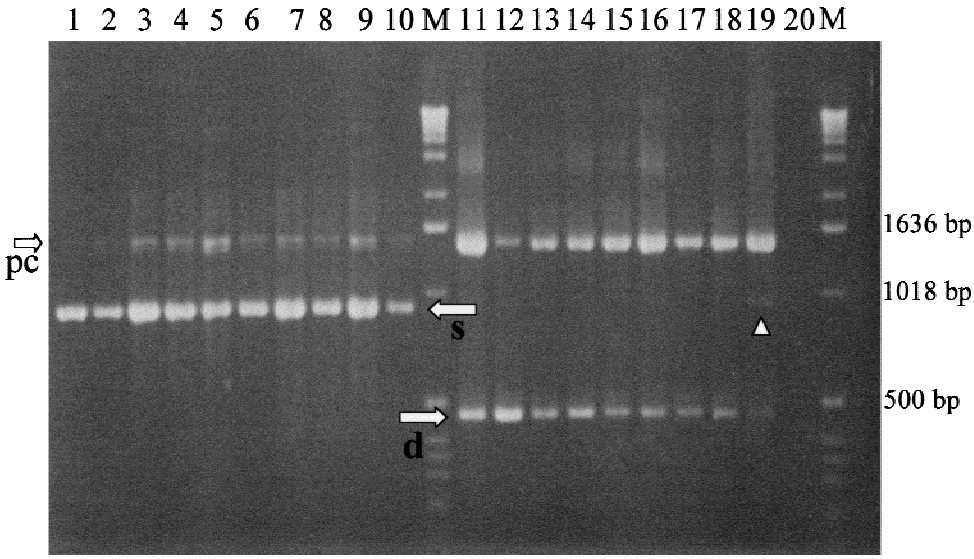

Figure 3. Results of 4-primer multiplex amplification reactions using a combination of 2 shark universal primers, the dusky-specific primer and the sandbar-specific primer. Lanes 1–10: sandbar sharks. Lanes 11–19: dusky sharks. Geographic origin of sharks and labeled arrows are the same as in Figure 2. The open triangle (△) shows the low-yield, sandbar-size amplicon generated from one dusky shark individual from the South Pacific. Lanes M contain the molecular size standard.

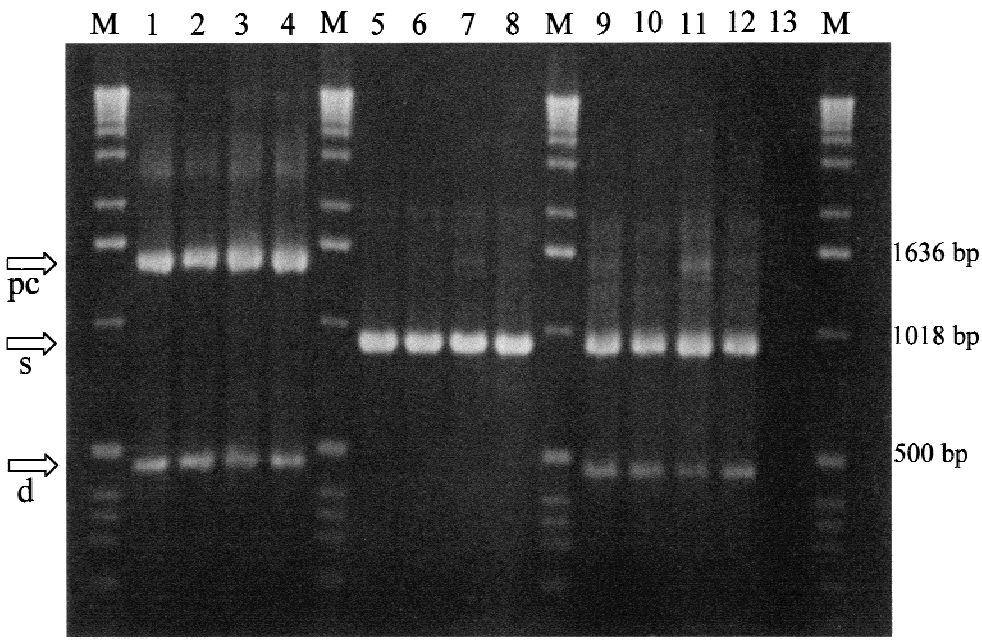

Figure 4. Results of testing combined DNAs from 2 shark samples in the same reaction using the 4-primer multiplex PCR format (primer combinations are the same as in Figure 3). Lanes 1–4 each represent combinations of 2 different dusky shark individuals pooled together; lanes 5–8 each represent combinations of 2 different sandbar shark individuals pooled together; lanes 9–12 each represent combinations of different dusky and sandbar shark individuals pooled together. Lane 13 is the negative control (no shark DNA). Arrow labeled *pc* shows the positive control amplicon; arrows labeled *d* and *s* represent the dusky and sandbar species-specific amplicons, respectively. Lanes M contain the molecular size standard.

species identification. The ITS2 locus in sharks may be particularly amenable for species diagnosis for several reasons. First, this locus appears to be highly conserved within species, even in the case of geographically distant populations. This high level of conservation is suggested by the observation that our dusky and sandbar primers amplified the correct, species-diagnostic-sized amplicon from dusky and sandbar sharks collected from as far apart as the U.S. Atlantic and Pacific. Second, this locus is sufficiently variable, even between closely related congeneric species, to allow

design of species-specific PCR primers. Third, the ITS2 locus forms part of the ribosomal DNA repeats and provides an abundant target for primer annealing and PCR amplification, thus enhancing the efficiency of genetic detection methods.

Primer Design Considerations

Important prerequisites for a genetic assay applicable to routine and widespread use in management and conservation are accuracy, speed, and reliability. Several key design considerations were necessary to achieve a robust diagnostic assay with these properties. First, the primers had to have an estimated melting temperature high enough to permit their use under high-stringency PCR conditions. We found that a high-stringency reaction was necessary for achieving primer specificity to genomic target sequence and avoiding primer-to-primer annealing problems. The importance of high-stringency conditions for ensuring primer annealing and amplification specificity is illustrated by the case of the sandbar-specific primer, whose sequence differs from the dusky ITS2 genomic DNA sequence at the orthologous position by only 2 bases over the primer's 24-base annealing site (see Table 2). Despite this small sequence difference, the sandbar primer proved species-specific at the high-stringency PCR conditions employed here, failing to amplify DNA from any dusky sharks with the single exception of a dusky sample from the South Pacific.

A possible explanation for the above observation is that this dusky individual contained a nucleotide polymorphism in a small proportion of its ITS2 repeat units, such that the sandbar primer was able to anneal and amplify a PCR fragment at low efficiency. This is quite possible given only two nucleotide position differences between the sandbar primer and dusky sequence at its orthologous position in the ITS2. Intra-individual sequence heterogeneity in ITS repeats has been observed in some organisms (Harris and Crandall, 2000). This putative ITS2 polymorphism may not be common in the South Pacific dusky population, however, given that the sandbar primer did not amplify the other three Australian dusky sharks tested. In general, we have found in other trials (unpublished results) that with properly designed primers and PCR conditions, species-specific primer annealing can be consistently achieved even if the primer sequence differs from the nontarget species orthologous sequence by only a single base positioned at the 3′ end of the primer.

The second key consideration for a practical assay was the development of species-specific primers that would produce different-sized diagnostic amplicons that could be easily distinguished. This was achieved by designing primers that annealed to the ITS2 locus sufficiently far apart so as to amplify unambiguously scoreable, species-diagnostic bands. The clearly distinct sizes of the dusky and sandbar amplicons increases the speed of gel interpretation by requiring only simple visual observation for species identification, circumventing the need for more sophisticated analysis of band size by specialized software.

The success of PCR amplifications is widely acknowledged to be sensitive to various qualitative factors, including the presence of polymerase-inhibiting substances present in the starting DNA (Higuchi, 1989), and simple errors in setting up the amplification reactions. Because complete reaction failure (i.e., absence of any amplification) is not uncommon in PCR, a third important design consideration we undertook was to ensure the reliability of the amplification reaction itself. We addressed this issue by incorporating an internal positive control in the assay, whereby both forward and reverse shark universal primers were always included along with the species-specific primers in each reaction. In the 3-primer assay, for example, this strategy allows verification of the integrity of PCR components by checking for the appearance of the internal positive control amplicon, which would be expected to amplify in the absence of the target species. In this context, a reaction result showing the complete absence of both a species-diagnostic amplicon and the internal positive control amplicon would signal a reaction failure, as opposed to the absence of the target species.

The Multiplex PCR Assays

Increasing the number of PCR primers from the standard 2 per reaction to 3 and 4 per reaction when screening single sharks at a time did not diminish the specificity and diagnostic performance of the primers. Furthermore, even though amplification efficiency of the positive control amplicon was low when using the sandbar-specific primer with sandbar DNA, this did not detract from the diagnostic utility of the multiplex assay for the following reason: the internal positive control was included in the assay only to prevent the complete absence of any amplification (e.g., from PCR reaction failure) from being interpreted as the absence of a particular species (i.e., a false-negative result). In this regard, the internal positive control achieved its goal remarkably well in all cases, amplifying robustly in the ab-

sence of the target species (Figure 2, A and B). Species could be reliably identified simply by scoring for the appearance of a species-diagnostic amplicon; the presence or absence of an accompanying positive control amplicon was irrelevant to the diagnosis.

The reason for the low efficiency of positive control amplification when using the sandbar-specific primer with sandbar DNA is not clear. We speculate that it may have resulted from some form of competition, possibly for DNA polymerase binding, between the sandbar-specific primer and the 5.8SF shark universal primer, whose annealing sites are relatively close to each other. This putative "primer proximity" competitive interaction is suggested by the observation that the positive control amplicon was amplified efficiently from both sandbar and dusky DNAs in all instances in the absence of sandbar-specific primer (Figure 2, A).

The robustness of the 4-primer multiplex assay for unambiguous species identification is further substantiated by the fact that it was not necessary to optimize any of the PCR components when increasing the number of primers from 2 to 4. All 4 primers were added in equal amounts, the genomic DNA concentrations were unaltered, and the concentrations of dNTPs, magnesium, and *Taq* polymerase were kept the same as those used in standard 2-primer PCR amplifications. The simplicity and diagnostic effectiveness of the 4-primer assay offers the advantage that dusky and sandbar shark carcasses and tissues can be discriminated in one step by performing a single-tube amplification reaction, rather than testing each sample with each species-specific primer separately.

We have further streamlined the species identification process by demonstrating that the 4-primer assay can be used to identify combined DNAs from two shark individuals at a time. In this case, the DNA from two individuals of the same or different species were pooled together and tested, again without optimization of PCR components. The 4-primer assay was able to unambiguously determine the identity of the pooled DNAs, even if they were from different species. To ensure the effectiveness of the assay under these combined DNA conditions, we pooled DNAs from different combinations of individual sharks, but noted no variation in the diagnostic effectiveness of the assay. In this situation, however, the problem of low amplification efficiency of the positive control amplicon seemed to be exacerbated when sandbar DNA was included in the pooled mixture. The near absence of the positive control amplicon in these cases, however, did not detract from the diagnostic utility of the assay for correctly identifying combined DNA samples (see above). The ability to identify combined samples from two animals at the same time offers the considerable advantage of reducing the work of screening a set of samples by 50%, thereby increasing the practicality of this method for use in contexts requiring identification of large numbers of unknown samples.

Our multiplex PCR method based on ITS2 sequence polymorphisms provides a robust way to achieve accurate identification of sandbar and dusky shark body parts. The multiplex assay is much more streamlined, and therefore faster and cheaper than the heretofore developed shark genetic identification methods that require the additional steps of restriction endonuclease digestion of PCR amplicons and screening of a single animal at a time (Martin, 1993; Shivji et al., 1996; Heist and Gold, 1998). The simplicity and rapidity of the assay should facilitate its routine implementation in management and conservation plans for these two species. The ability of this assay to correctly identify dusky and sandbar sharks from regions in the Atlantic and Pacific indicates it may be suitable for use on a global scale. The transfer of this technology to fishery conservation and management practice will, for the first time, allow the collection of reliable catch and trade data for these two conservation-requiring taxa on a species-specific basis. Furthermore, the streamlined methodology illustrated here may prove useful as a model for development of rapid species diagnosis assays for a wide diversity of taxa.

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