Fisheries Research 210 (2019) 81–88

Contents lists available at ScienceDirect

Fisheries Research

journal homepage: www.elsevier.com/locate/fishres

Identification of shark species in commercial products using DNA barcoding

Rosalee S. Hellberg*, Rachel B. Isaacs, Eduardo L. Hernandez

Chapman University, Schmid College of Science and Technology, Food Science and Nutrition, One University Drive, Orange, CA 92866, United States

ARTICLE INFO

Handled by Dr. J Viñas

Keywords:
DNA barcoding
Elasmobranchs
Shark cartilage
Shark meat
Species identification

ABSTRACT

Sharks are harvested globally and sold in a variety of commercial products. However, they are particularly vulnerable to overfishing and many species are considered protected or endangered. The objective of this study was to identify species in various commercial shark products and to assess the effectiveness of three different DNA barcoding primer sets. Thirty-five products were collected for this study, including fillets, jerky, soup, and cartilage pills. DNA barcoding of these products was undertaken using two full-length primer sets and one mini-barcode primer set within the cytochrome *c* oxidase subunit (COI) gene. Successfully sequenced samples were then analyzed and identified to the species level using sequence databases and character-based analysis. When the results of all three primer sets were combined, 74.3% of the products were identified to the species level. Mini-barcoding showed the highest success rate for species identification (54.3%) and allowed for a wide range of identification capability. Six of the 26 identified products were found to be mislabeled or potentially mislabeled, including samples of shark cartilage pills, shark jerky, and shark fin soup. Six products contained species listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendices and 23 products contained near-threatened, vulnerable or endangered species according to the International Union for the Conservation of Nature (IUCN) Red List. Overall, this study revealed that a combination of DNA barcoding primers can be utilized to identify species in a variety of processed shark products and thereby assist with conservation and monitoring efforts.

1. Introduction

Sharks are harvested worldwide both in targeted fisheries and as bycatch in other fishing operations (Bräutigam et al., 2015). There is a wide diversity of shark products on the global marketplace, including meat, fins, skin, oil, and cartilage (S. Clarke, 2004; Dent and Clarke, 2015). The greatest consumer demand is for shark meat and fins; however, other shark products are not recorded separately in trade statistics, making them difficult to track. Sharks are particularly vulnerable to overfishing due to their late maturity, relatively long gestation periods, and low fecundity (Bräutigam et al., 2015). Many populations of sharks and rays are considered threatened or endangered: close to 20% of the 1,038 species of sharks and rays assessed by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species have been categorized as Critically Endangered, Endangered, or Vulnerable, and another 12% have been categorized as Near Threatened (Bräutigam et al., 2015). Furthermore, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has 13 Appendix II listings for sharks and rays, meaning that international trade of these organisms must be controlled through the use of export permits (CITES, 2018). For proper enforcement of CITES, it is essential that customs agents are able to identify these species in globally traded shark products.

Intact, unprocessed shark specimens can often be identified to the species level by expert taxonomists using morphological indicators (Hanner et al., 2016; Marshall and Barone, 2016). Some shark fins can be identified in this way as well; however, extensive training is required and identification can be problematic due to species that are similar in appearance and the focus on at-risk species. In order to overcome these challenges, a number of DNA-based analyses have been developed for the identification of shark species (reviewed in Dudgeon et al., 2012; Hanner, et al., 2016; Rodrigues-Filho et al., 2012). These methods are largely based on the use of polymerase chain reaction (PCR) for amplification of universal or species-specific DNA regions. Several multiplex species-specific PCR assays have been developed to assist with shark conservation efforts and monitoring of international trade (Abercrombie et al., 2005; Chapman et al., 2003; Clarke et al., 2006; Shivji et al., 2002; Shivji et al., 2005). These studies have revealed trade of shark fins from protected species such as white shark (*Carcharodon carcharias*) and hammerhead sharks (*Sphyrna* spp.). While species-specific PCR assays are favored for the rapid identification of known target species, a universal approach, such as DNA barcoding, is advantageous

* Corresponding author.
E-mail address: hellberg@chapman.edu (R.S. Hellberg).

https://doi.org/10.1016/j.fishres.2018.10.010
Received 17 April 2018; Received in revised form 4 October 2018; Accepted 11 October 2018
Available online 24 October 2018
0165-7836/

in applications where a wide range of species is possible.

DNA barcoding is a sequencing-based technique that utilizes universal primers targeting a short, standardized genetic region for the identification of species (Hebert et al., 2003). The standard target for DNA barcoding of animal species is a ~650 bp region of the mitochondrial gene coding for cytochrome *c* oxidase subunit I (COI). Because of campaigns such as the Fish Barcode of Life Initiative (http://www.fishbol.org/), DNA barcoding is supported by a large database of sequence information to assist with species identification. DNA barcoding of elasmobranchs has been investigated in numerous studies and has proven to be effective in identifying a wide range of species (Bineesh et al., 2017; Doukakis et al., 2011; Ward et al., 2008; Wong et al., 2009). This method has also been utilized to reveal mislabeling of shark products, as well as trade of threatened and endangered shark species (Asis et al., 2016; Barbuto et al., 2010; Cardeñosa et al., 2017; Holmes et al., 2009; Liu et al., 2013; Moore et al., 2014; Naaum Amanda and Hanner, 2015; Sembiring et al., 2015; Steinke et al., 2017). However, it can be challenging to recover the full-length DNA barcode from products that have undergone extensive processing as the DNA is often degraded and highly fragmented (Fields et al., 2015; Shokralla et al., 2015). To address this, Fields et al. (2015) developed a mini-barcoding assay for shark species identification that targets a shorter 110–130 bp region within the full-length COI barcode. This assay was shown to be effective in identifying sharks to the species or genus level in 100% of processed fins tested and 62% of shark fin soup samples. These results indicate potential use of the shark mini-barcoding assay for species identification in other highly processed shark products, such as shark cartilage supplements.

The objective of this study was to use DNA barcoding to identify shark species in commercial products and to compare the effectiveness of three different barcoding methods: shark mini-barcoding, fish full barcoding, and mammalian full barcoding.

2. Materials and methods

2.1. Sample collection

A total of 35 commercial shark products were collected for this study. The products were purchased online and from restaurants or retail outlets in Orange and Los Angeles Counties, CA, USA. A variety of products were collected, including shark jerky (n = 3), shark fin soup (n = 1), shark cartilage pills (n = 29), and fresh or grilled shark fillets (n = 2). Following collection, each product was assigned a sample number and catalogued. Products were then held at their recommended storage temperatures until DNA extraction. DNA was extracted from perishable items within two days of collection.

2.2. DNA extraction

Sterile forceps were used to sample tissue from the jerky, soup (ceratotrichia), and fillet samples. Cartilage pills in capsule form were twisted open and the powder was poured directly into a sterile microcentrifuge tube for weighing, while tablets (solid form) were broken up with sterile forceps and then placed into a sterile microcentrifuge tube. DNA was extracted from ~25 mg of each sample using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), Spin-Column protocol, according to the manufacturer's instructions, with modifications made to the final elution step. DNA was eluted using pre-heated (37 °C) AE buffer at a volume of 60 µl for cartilage pill samples and 100 µl for all other samples. A reagent blank negative control with no sample added was included with each set of DNA extractions.

2.3. PCR

DNA extracts from each sample underwent PCR using three different primer sets (Table 1): a shark mini-barcode primer set (Fields et al., 2015) and two full-barcode primer sets ('fish full barcode' and 'mammalian full barcode') used in a previous study on shark species identification (Wong et al., 2009). With the exception of Shark COI-MINIR, all primers included M13 tails to facilitate DNA sequencing (Table 1). Amplification of shark mini-barcodes was carried out with the following reaction mixture: 25 µl HotStar Taq Master Mix (2X) (Qiagen), 22 µl of molecular-grade sterile water, 1 µl of 10 µM C_FishF1t1 (Table 1), 1 µl of 10 µM Shark COI-MINIR (Table 1), and 1 µl of template DNA. Fish and mammalian full barcodes were amplified using the following reaction mixture: 25 µl HotStar Taq Master Mix (2X) (Qiagen), 23 µl of molecular-grade sterile water, 0.5 µl of 10 µM forward primer cocktail (Table 1), 0.5 µl of 10 µM reverse primer cocktail (Table 1), and 1 µl of template DNA. A no-template control (NTC) with molecular-grade sterile water instead of DNA was included alongside each set of reactions. PCR was carried out using a Mastercycler nexus Gradient Thermal Cycler (Eppendorf). The cycling conditions for shark mini-barcoding were: 95 °C for 15 min; 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min; and a final extension step at 72 °C for 5 min. The cycling conditions for fish full barcoding were: 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. The cycling conditions for mammalian full barcoding were: 95 °C for 15 min; 5 cycles of 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min; 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min; and a final extension step at 72 °C for 10 min.

2.4. PCR product confirmation and DNA sequencing

Confirmation of PCR products was achieved using 2.0% agarose E-Gels (Life Technologies, Carlsbad, CA) run on an E-Gel iBase (Life Technologies). A total of 16 µl of sterile water and 4 µl of PCR product were loaded into each well (Hellberg et al., 2014). Each sample with a visible PCR product on the agarose gel was purified with the QIAquick PCR Purification Kit using a Microcentrifuge (Qiagen), according to the manufacturer's instructions. Purified PCR products were sequenced at the GenScript facility (Piscataway, NJ) with M13 primers. Mini-barcode products were only sequenced in one direction using the forward M13 primers, as described in Fields et al. (2015), while all full-barcoding products were sequenced bi-directionally (Ivanova et al., 2007). DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a 3730xl Genetic Analyzer (Life Technologies).

2.5. Sequencing results and analysis

Raw sequence data was assembled and edited using Geneious R7 [(Biomatters, Ltd., Auckland, New Zealand) (Kearse et al., 2012)]. The resulting sequences were trimmed to the appropriate full-barcode (652–658 bp) or mini-barcode (127 bp) regions. Trimmed sequences with < 2% ambiguities were queried through the Barcode of Life Database (BOLD) Animal Identification Request Engine (http://www.boldsystems.org/), Species Level Barcodes. Any sequences that could not be identified to the species level in BOLD were next queried in GenBank with the Nucleotide Basic Local Alignment Search Tool (BLASTn; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The top species matches were recorded. Sequences with multiple top species matches and/or secondary matches with ≥ 98% genetic similarity were next examined using character-based analysis, as described in Wong et al. (2009). The conservation status of each identified species was determined using the IUCN Red List of Threatened Species (http://www.iucnredlist.org/).

3. Results

3.1. Species identification using DNA barcoding

DNA barcodes were obtained from at least one primer set for 26 of the 35 commercial shark products tested in this study (Fig. 1). DNA

Table 1
Details for the PCR primer sets and M13 tails used in this study (Messing, 1983).

Primer set	Primer cocktail	Primer name	Primer sequence (5'-3')[a]	Ratio in Cocktail	Barcode length	Reference
Shark mini-barcode	C_FishF1t1	VF2_t1	**TGTAAAACGACGGCCAGT**CAACCAACCACAAAGACATTGGCAC	1	127 bp	Ivanova, et al. (2007)
		FishF2_t1	**TGTAAAACGACGGCCAGT**CGACTAATCATAAAGATATCGGCAC	1		
	N/A	Shark COI-MINIR	AAGATTACAAAAGCGTGGGC	N/A		Fields, et al. (2015)
Fish full barcode	C_FishF1t1	VF2_t1	**TGTAAAACGACGGCCAGT**CAACCAACCACAAAGACATTGGCAC	1	652 bp	Ivanova, et al. (2007)
		FishF2_t1	**TGTAAAACGACGGCCAGT**CGACTAATCATAAAGATATCGGCAC	1		
	C_FishR1t1	FishR2_t1	**CAGGAAACAGCTATGAC**ACTTCAGGGTGACCGAAGAATCAGAA	1		
		FR1d_t1	**CAGGAAACAGCTATGAC**ACCTCAGGGTGTCCGAARAAYCARAA	1		
Mammalian full barcode	C_VF1LFt1	LepF1_t1	**TGTAAAACGACGGCCAGT**ATTCAACCAATCATAAAGATATTGG	1	658 bp	Ivanova, et al. (2007)
		VF1_t1	**TGTAAAACGACGGCCAGT**TCTCAACCAACCACAAAGACATTGG	1		
		VF1d_t1	**TGTAAAACGACGGCCAGT**TCTCAACCAACCACAARGAYATYGG	1		
		VF1i_t1	**TGTAAAACGACGGCCAGT**TCTCAACCAACCAIAAIGAIATIGG	3		
	C_VR1LRt1	LepRI_t1	**CAGGAAACAGCTATGAC**TAAACTTCTGGATGTCCAAAAAATCA	1		
		VR1d_t1	**CAGGAAACAGCTATGAC**TAGACTTCTGGGTGGCCRAARAAYCA	1		
		VR1_t1	**CAGGAAACAGCTATGAC**TAGACTTCTGGGTGGCCAAAGAATCA	1		
		VR1i_t1	**CAGGAAACAGCTATGAC**TAGACTTCTGGGTGICCIAAIAAICA	3		
M13	N/A	M13F (−21)	**TGTAAAACGACGGCCAGT**	N/A	N/A	Messing (1983)
	N/A	M13R (−27)	**CAGGAAACAGCTATGAC**	N/A	N/A	

[a]Shaded portions indicate M13 tails.

barcodes were recovered from 100% of the jerky, fillet, and soup products, but only 69% of the 29 shark cartilage pill samples. The one shark cartilage tablet collected for this study failed PCR with all three primer sets, while 20 of the 28 capsules collected were sequenced by at least one method (Table 2). The shark mini-barcoding primer set was the most successful at identifying shark or other fish species in the products tested, with identification success in 19 of the 35 products (Fig. 1). The mammalian full-barcoding primer set allowed for species identifications in 16 of the 35 products; however, only 10 of the products were identified as shark or other fish species. The remaining six products were identified as wild rice (*Oryza rufipogon*). The fish full-barcoding primer set was the least successful and was only able to identify species in 3 of the commercial shark products.

In cases where one sequence matched multiple species with a genetic similarity of ≥ 98%, character analysis was applied (Wong et al., 2009). The use of character analysis allowed for five of the shark cartilage products (S19, S22, S26, S31, and S35) sequenced across the mini-barcode region to be identified to species level. Character analysis also reduced the number of secondary species matches obtained for three other samples (S21, S27, and S33) sequenced across the mini-barcode region. For example, the mini-barcode sequence for S33 showed a top species match with 99.12% genetic similarity to spot-tail

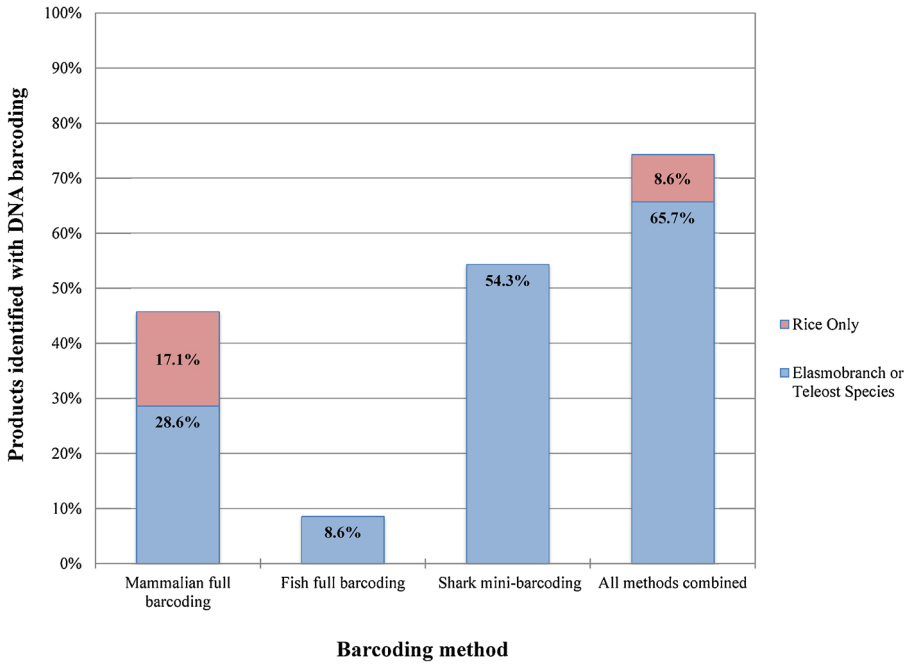

Fig. 1. Percentage of commercial shark products (n = 35) identified through DNA barcoding with three different primer sets.

Table 2
Species identified in the 26 commercial shark products successfully sequenced by at least one of the primer sets tested in this study. Products found to be mislabeled or potentially mislabeled are shown in boldface.

Sample ID	Sample description	Identified species		
		Fish full barcode	Mammalian full barcode	Shark mini-barcode
S01	Mako shark steak, grilled	Failed PCR	Shortfin mako (*Isurus oxyrinchus*)	Shortfin mako (*Isurus oxyrinchus*)
S02	Mako shark jerky	Failed PCR	Shortfin mako (*Isurus oxyrinchus*)	Shortfin mako (*Isurus oxyrinchus*)
S05	**Shark cartilage capsules**	**Winter skate (*Leucoraja ocellata*)**[a]	**Failed PCR**	**Failed sequencing**
S08	Shark cartilage capsules	Failed PCR	Wild rice (*Oryza rufipogon*)[a]	Failed PCR
S09	**Shark's fin soup**	**Failed sequencing**	**Delagoa threadfin bream (*Nemipterus bipunctatus*)**[b]	**Red bigeye (*Priacanthus macracanthus*)**[b]
S10	Thresher shark fillet, fresh/frozen	Failed PCR	Pelagic thresher (*Alopias pelagicus*)	Failed PCR
S11	Shark jerky	Failed PCR	Pelagic thresher (*Alopias pelagicus*)	Failed PCR
S12	**Mako shark jerky**	**Failed PCR**	**Thresher (*Alopias vulpinus*)**	**Thresher (*Alopias vulpinus*)**
S13	Shark cartilage capsules	Failed PCR	Failed PCR	Spot-tail shark (*Carcharhinus sorrah*)[a]
S14	Shark cartilage capsules	Failed PCR	Failed sequencing	Spot-tail shark (*Carcharhinus sorrah*)[a]
S16	**Shark cartilage capsules**	**Winter skate (*Leucoraja ocellata*)**[a]	**Failed sequencing**	**Failed PCR**
S17	Shark cartilage capsules	Failed PCR	Failed PCR	Tope shark (*Galeorhinus galeus*)
S18	Shark cartilage capsules	Failed PCR	Wild rice (*Oryza rufipogon*)[a]	Failed PCR
S19	**Shark cartilage capsules**	**Failed PCR**	**Wild rice (*Oryza rufipogon*)**[a]	**Silky shark (*Carcharhinus falciformis*)**[c]
S21	Shark cartilage capsules	Failed sequencing	Tope shark (*Galeorhinus galeus*)	Spot-tail shark (*Carcharhinus sorrah*)[a]
S22	Shark cartilage capsules	Failed sequencing	Blackspot shark (*Carcharhinus sealei*)[b]	Spot-tail shark (*Carcharhinus sorrah*)[a,c]
S23	Shark cartilage capsules	Failed PCR	Failed sequencing	Spot-tail shark (*Carcharhinus sorrah*)[a]
S26	**Shark cartilage capsules**	**Failed PCR**	**Wild rice (*Oryza rufipogon*)**[a]	**Silky shark (*Carcharhinus falciformis*)**[c]
S27	Shark cartilage capsules	Failed sequencing	Wild rice (*Oryza rufipogon*)[a]	Blacktip reef shark (*Carcharhinus melanopterus*)[a]
S28	Shark cartilage capsules	Failed sequencing	Failed sequencing	Blue shark (*Prionace glauca*)
S30	Shark cartilage capsules with dogfish shark	Failed PCR	Wild rice (*Oryza rufipogon*)[a]	Failed PCR
S31	Shark cartilage capsules	Failed PCR	Failed sequencing	Silky shark (*Carcharhinus falciformis*)[c]
S32	Shark cartilage capsules	Tope shark (*Galeorhinus galeus*)	Tope shark (*Galeorhinus galeus*)	Tope shark (*Galeorhinus galeus*)
S33	Pacific Ocean shark cartilage capsules	Failed sequencing	Tope shark (*Galeorhinus galeus*)	Spot-tail shark (*Carcharhinus sorrah*)[a]
S35	Shark cartilage capsules	Failed PCR	Failed PCR	Spot-tail shark (*Carcharhinus sorrah*)[ac]
S36	Shark cartilage capsules	Failed PCR	Failed PCR	Spot-tail shark (*Carcharhinus sorrah*)[a]

[a] Sequence had secondary species matches with ≥ 98% genetic similarity that could not be ruled out with character analysis.
[b] Top species match was < 98% genetic similarity.
[c] Species identification included the use of character analysis.

Table 3
Conservation status of the elasmobranch species detected in commercial products tested in this study.

Elasmobranch species	Common name	CITES Listing	IUCN Red List status	Number of products containing species
Leucoraja ocellata	Winter skate	Not listed	Endangered	2
Alopias pelagicus	Pelagic thresher	Appendix II (October 2017)	Vulnerable	2
Alopias vulpinus	Thresher	Appendix II (October 2017)	Vulnerable	1
Galeorhinus galeus	Tope shark	Not listed	Vulnerable	4
Isurus oxyrinchus	Shortfin mako	Not listed	Vulnerable	2
Carcharhinus sorrah	Spot-tail shark	Not listed	Near Threatened	8
Carcharhinus falciformis	Silky shark	Appendix II (October 2017)	Near Threatened	3
Carcharhinus melanopterus	Blacktip reef shark	Not listed	Near Threatened	1
Prionace glauca	Blue shark	Not listed	Near Threatened	1
Carcharhinus sealei	Blackspot shark	Not listed	Near Threatened	1

shark (*Carcharhinus sorrah*) and a secondary species match to night shark (*Carcharhinus signatus*) with 98.92% genetic similarity. However, character analysis revealed that the sequence did not contain one of the nucleotides determined to be diagnostic for night shark.

Despite the use of character analysis, eight of the samples sequenced with mini-barcoding continued to have at least one secondary species match with genetic similarity ≥ 98% (Table 2). This occurred with seven samples containing spot-tail shark and one sample containing blacktip reef shark (*Carcharhinus melanopterus*). In most cases, the secondary matches were to other *Carcharhinus* spp. These results are consistent with previous DNA barcoding research that has reported less than 1% genetic divergence among some members of the *Carcharhinus* genus (Ward, et al., 2008). Five products sequenced with the shark mini-barcode (S21, S22, S33, S35, and S36) showed equivocal BOLD matches (99.1–100%) to both spot-tail shark and blacktip shark (*Carcharhinus limbatus*). Upon further examination, it was found that each sample matched numerous published entries for spot-tail shark and only one entry for blacktip shark, which was an Early-Release sequence and not publicly accessible. When the sequences were queried in GenBank, they all matched spot-tail shark with no equivalent match to blacktip shark. Therefore, these samples were determined to be spot-tail shark.

None of the shark species detected with mammalian full barcoding showed multiple species matches with ≥ 98% genetic similarity. All of the samples identified as wild rice showed secondary matches in BOLD to other plant species, such as meadow grass (*Poa annua*) and ryegrass (*Lolium rigidum*). The two samples identified as winter skate (*Leucoraja ocellata*) with full fish barcoding (S05 and S16) each showed a secondary match to one sequence labeled as little skate (*Leucoraja erinacea*). However, upon further investigation, it was found that this sequence (BOLD Sample ID JF894896) was misidentified and is actually derived from winter skate (Coulson et al., 2011).

Mammalian full barcoding generated barcodes for two samples (S09 and S22) that did not show a species match with ≥ 98% genetic similarity in BOLD. Therefore, these samples were instead identified with GenBank. Sample S09, labeled as “Shark’s Fin Soup,” was identified as delagoa threadfin bream (*Nemipterus bipunctatus*) with 94% genetic similarity, and sample S22, a bottle of shark cartilage capsules, was identified as blackspot shark (*Carcharhinus sealei*) with 96% genetic similarity. In both cases, the sequence quality was relatively low, with < 23% high quality (HQ) bases. Similarly, the mini-barcode primer set generated a barcode for the shark fin soup sample (S09) with a low HQ score (9.9%) that did not show a species match with ≥ 98% genetic similarity in BOLD. The top species match for this sample in GenBank was red bigeye (*Priacanthus macracanthus*) with 90% genetic similarity.

3.2. Mislabeled products

Among the 26 samples for which sequences were obtained, 5 samples (19%) were determined to be mislabeled and one was considered to be potentially mislabeled. The five mislabeled samples claimed to be manufactured in the United States and consisted of one “mako shark” jerky product (S12) identified as thresher shark (*Alopias vulpinus*); two shark cartilage pill products (S05, S16) containing undeclared winter skate and no shark species; and two shark cartilage pill products (S19 and S26) containing undeclared rice ingredients in addition to shark species. Another shark cartilage product (S27) that tested positive for rice in addition to shark contained cellulose as an ingredient, which may have been the source of the rice. Therefore, this product was not considered to be mislabeled. The one sample of shark fin soup (S09) tested was determined to be potentially mislabeled due to the detection of teleost fish in the product instead of shark. Of note, the mislabeled jerky product (S12) was obtained from a different brand and online distributor as compared to the correctly labeled sample of mako shark jerky (S02). The two samples containing winter skate were sold under different commercial brand names but were purchased from the same online distributor and originated from the same manufacturer. In contrast, the two shark cartilage pill products identified as containing undeclared rice were purchased from different sellers and originated from different manufacturers.

3.3. Conservation status of identified species

Six of the commercial shark products tested in this study were found to contain CITES-listed shark species: silky shark (*Carcharhinus falciformis*) and thresher sharks [(*Alopias* spp.) (Table 3)]. However, it should be noted that the CITES listings for these species were not effective until after this study was completed (effective date: 4 October 2017). The three products containing thresher sharks consisted of two jerky samples and one fillet, while silky shark was detected in three shark cartilage pill samples. All 10 species of sharks and skate detected in this study appear on the IUCN Red List of Threatened Species (IUCN, 2017). These species were detected in 23 different commercial products, with some products found to contain multiple species (Table 2). Five of these species are considered to be near threatened, four are considered vulnerable, and one is considered endangered.

4. Discussion

4.1. Comparison of DNA barcoding methods

Using a combination of three DNA barcoding primer sets, species identification (including rice, teleost, and elasmobranch species) was possible in the majority (74.3%) of commercial shark products tested (Fig. 1). On an individual basis, shark mini-barcoding had the highest identification rate (54.3%), followed by mammalian full-barcoding (45.7%), and fish full-barcoding (8.6%). The three DNA barcoding primer sets proved to be complementary in that they allowed for a wide range of species to be identified. Despite the low success rate of the fish full-barcode primer set, it was the only method that enabled the identification of winter skate in shark cartilage pills (Table 2). Along these

lines, the other two primer sets also showed advantages for identification of certain shark species, such as spot-tail shark with mini-barcoding and pelagic thresher (*Alopias pelagicus*) with mammalian full barcoding. Mammalian full barcoding not only amplified shark species but also resulted in the detection of wild rice in products, indicating the universal nature of this primer set. However, it is important to note that any plant species identifications based on COI DNA barcoding must be verified using a plant-specific DNA barcoding assay, such as that used by Newmaster et al. (2013).

The mini-barcode was most effective for detecting species within the shark cartilage pills, demonstrating the benefits of using shorter barcodes on highly processed samples containing degraded DNA. The mammalian full barcode was more effective with lightly processed products likely due to the better DNA quality within these samples. Interestingly, there was only one instance in which all three primer sets were successful with the same product (S32), which was identified as tope shark (*Galeorhinus galeus*). In three cases (S21, S22, and S33), the use of multiple primer sets allowed for the identification of more than one shark species in shark cartilage pills. For example, mammalian full barcoding enabled the identification of tope shark in two cartilage pill samples (S21 and S33), while mini-barcoding enabled the identification of spot-tail shark in these products. With regards to CITES-listed species, shark mini-barcoding allowed for the identification of silky shark and thresher shark in products, but not pelagic thresher. On the other hand, mammalian full barcoding allowed for the identification of thresher and pelagic thresher but not silky shark. These results indicate potential complementary uses of these primer sets in identifying CITES-listed species, which require strict monitoring of trade by all member parties.

While all jerky, fillet, and soup products were identified to the species level, only 69% of the shark cartilage pill samples were successfully sequenced and identified. In comparison, Wallace et al. (2012) reported a success rate of only 20% for DNA barcoding of five animal product capsules. The one capsule (velvet antler) that was successfully sequenced by Wallace et al. (2012) failed with full-length DNA barcoding, but was recovered using a universal mini-barcode primer set. The reduced success with shark cartilage pills in the current study may have been due to several factors, including DNA degradation during processing, the presence of species that could not be amplified with the primer sets used, and/or the use of species mixtures. Because DNA barcoding primers are able to amplify a wide range of species, the presence of multiple species in a single product can lead to an unreadable electropherogram and sequencing failure. The presence of species mixtures may also explain the relatively low genetic similarity (94–96%) obtained for the top species matches for two samples: a sample of shark fin soup (S09) and a shark cartilage product (S22). Both samples had sequences with relatively low quality scores, which may have been a result of simultaneous amplification of multiple species in a single product.

4.2. Mislabeling of commercial products

Potential mislabeling was detected in a variety of product types, including jerky, soup, and shark cartilage supplements (Table 2). Species substitution was the most common type of mislabeling detected, followed by the use of undeclared fillers. As previously mentioned, the one sample of shark fin soup tested was found to be potentially mislabeled due to the detection of teleost fish instead of shark. One explanation for this finding is that the restaurant intentionally did not include shark in the product because it is illegal to sell shark fin in California under A.B. 376, Shark fins (2011). In contrast to these results, a large-scale survey on shark fin soup from U.S. restaurants detected a number of shark species, including tope shark, blue shark (*Prionace glauca*), and other *Carcharhinus* spp., with no reports of teleost fish species (Fields et al., 2015).

Among the product types tested, mislabeling was detected most frequently in the shark cartilage supplements. Out of the 20 supplements with a recoverable barcode, 20% were found to be mislabeled. Similarly, Wallace et al. (2012) reported 2 of 10 shark natural health products collected in North America to be mislabeled, including one sample of shark bones and one dried, shredded shark fin. Undeclared rice was detected in two of the shark cartilage products tested in the current study (S19 and S26). Rice is a common filler used in dietary supplements; however, additional testing of the shark cartilage products using plant-specific barcodes would be needed to confirm this detection. The presence of undeclared fillers has previously been reported in herbal products sold in North America (Newmaster et al., 2013). In comparison to the current study, which found undeclared fillers in 7% of shark cartilage supplements tested, Newmaster et al. (2013) reported the presence of undeclared fillers (rice or wheat) in 21% of herbal products tested. The presence of undeclared fillers such as these in a product can be a health risk for individuals with allergies.

Three bottles of shark cartilage pills were found to contain rice, with no shark species detected in the products (S08, S18, and S30). However, all of these samples included rice flour or rice powder in the ingredient list. Due to the possibility that these products contained shark DNA that could not be amplified by the methods used in this study, they were not considered to be mislabeled. One of the samples (S30) specifically stated that it contained dogfish shark, which is considered an acceptable market name for a number of species, including *Squalus* spp. (FDA, 2016). Dogfish from the *Squalus* genus was detected previously with the shark mini-barcoding method in a sample of shark fin soup (Fields, et al., 2015) and the authors predicted that the shark mini-barcoding assay described in their study would be capable of amplifying all or most shark species. However, the use of fillers, such as rice, can be problematic for DNA sequencing, as this can result in an unreadable mixed signal due to the simultaneous amplification of multiple species.

4.3. Conservation issues

This study revealed the presence of near threatened, vulnerable, and endangered elasmobranch species on the U.S. commercial marketplace. Many of these species are considered to be of concern because they are under heavy fishing pressure, targeted by unmanaged and unreported fisheries, and known to be exploited for their fins and meat (IUCN, 2017). However, it should be noted that sustainable fisheries do exist for some of these species in specific geographic regions. For example, the National Oceanic and Atmospheric Administration (NOAA) FishWatch considers U.S. wild-caught shortfin mako (*Isurus oxyrinchus*) to be sustainably managed and responsibly harvested (NOAA, 2017).

Winter skate, which was found in two products, was the only species detected in this study that is considered to be endangered by IUCN. This species inhabits shelf waters of the northwest Atlantic Ocean and it is primarily harvested for use in skate wings (Kulka et al., 2009). The IUCN considers this species to be endangered globally due to the observance of substantial declines in major areas of the species' range. However, according to NOAA FishWatch, winter skate that is wild-caught in the United States is considered to be sustainably managed and responsibly harvested (NOAA, 2017).

The most common species detected varied depending on the type of commercial product. For example, all of the jerky, steak, and fillet samples were found to contain shortfin mako, pelagic thresher or thresher. All three species are considered vulnerable according to the IUCN Red List and the latter two are CITES-listed. On the other hand, the majority of shark cartilage pills contained spot-tail shark, a near threatened species, with other commonly detected species being tope shark (vulnerable) and silky shark (near threatened and CITES listed).

Less frequently detected species include winter skate and two near threatened species (blue shark and blackspot shark). Previous studies reported the presence of blue shark in a sample of dried shark cartilage (Wallace et al., 2012) and basking shark (*Cetorhinus maximus*) in a cartilage pill product (Hoelzel, 2001). Similar to the results of the current study, Fields et al. (2015) primarily detected requiem sharks (*Carcharhinus* spp.) followed by tope (school) sharks, blue sharks, and spot-tail shark in dried processed fin samples from Hong Kong. These results support earlier reports that shark cartilage is utilized as a by-product of existing shark fisheries (Rose, 1996). Currently, shark cartilage is not separately recorded as part of global trade statistics and there is a lack of information on the quantities being traded and the exact species that are used.

5. Conclusions

This study revealed the effectiveness of DNA barcoding for the identification of species in commercial shark products. The three primer sets examined in this study proved to be complementary in their ability to identify a range of elasmobranch species. Shark mini-barcoding was found to be the most successful assay for identification of shark species in highly processed shark cartilage pills, while mammalian full barcoding was the most effective at identifying species in lightly processed products, such as fillets and jerky. This study also revealed the ability of these assays to detect trade of threatened and endangered species in commercial shark products, including several CITES-listed species, thereby facilitating conservation efforts and monitoring of international trade. While many of the shark species detected in this study have been reported in the global shark fin trade, this is the most extensive report to-date of shark species in commercial shark cartilage supplements. Many of the species identified in these supplements are known for being targeted in the commercial shark fin trade and the results indicate that they are also being used for shark cartilage production. Furthermore, this is the first report of the use of winter skate as a substitute for shark species in cartilage pill supplements. Although DNA barcoding was successful with lightly processed products, detection of species in shark cartilage pills was relatively challenging and may benefit from further optimization.

Acknowledgements

This work was supported in part by a grant from the National Science Foundation, Division of Earth Sciences, NSF-EAR #1359500. Additional funding support was received from Chapman University, Office of the Chancellor. These funding entities were not involved with the design or execution of the study.

References

Abercrombie, D.L., Clarke, S.C., Shivji, M.S., 2005. Global-scale genetic identification of hammerhead sharks: application to assessment of the international fin trade and law enforcement. Conserv. Gen. 6, 775–788.
Asis, A.M.J.M., Lacsamana, J.K.M., Santos, M.D., 2016. Illegal trade of regulated and protected aquatic species in the Philippines detected by DNA barcoding. Mitochondrial Dna Part A 27 (1), 659–666.
Barbuto, M., Galimberti, A., Ferri, E., Labra, M., Malandra, R., Galli, P., Casiraghi, M., 2010. DNA barcoding reveals fraudulent substitutions in shark seafood products: the Italian case of "palombo" (*Mustelus* spp.). Food Res. Int. 43 (1), 376–381.
Bineesh, K.K., Gopalakrishnan, A., Akhilesh, K.V., Sajeela, K.A., Abdussamad, E.M., Pillai, N.G.K., Basheer, V.S., Jena, J.K., Ward, R.D., 2017. DNA barcoding reveals species composition of sharks and rays in the Indian commercial fishery. Mitochondrial Dna Part A 28 (4), 458–472.
Bräutigam, A., Callow, M., Campbell, I.R., Camhi, M.D., Cornish, A.S., Dulvy, N.K., Fordham, S.V., Fowler, S.L., Hood, A.R., McClennen, C., Reuter, E.L., Sant, G., Simpfendorfer, C.A., Welch, D.J., 2015. Global Priorities for Conserving Sharks and Rays: A 2015–2025 Strategy.
Cardeñosa, D., Fields, A., Abercrombie, D., Feldheim, K., Shea, S.K.H., Chapman, D.D., 2017. A multiplex PCR mini-barcode assay to identify processed shark products in the global trade. PLoS One 12 (10) e0185368.
Chapman, D.D., Abercrombie, D.L., Douady, C.J., Pikitch, E.K., Stanhope, M.J., Shivji, M.S., 2003. A streamlined, bi-organelle, multiplex PCR approach to species identification: application to global conservation and trade monitoring of the great white shark, *Carcharodon carcharias*. Conserv. Gen. 4, 415–425.
CITES, 2018. Convention on International Trade in Endangered Species of Wild Fauna and Flora. (Accessed 7/10/2018). https://www.cites.org/eng.
Clarke, S., 2004. Shark Product Trade in Hong Kong and Mainland China and Implementation of the CITES Shark Listings. Hong Kong, China, TRAFFIC East Asia.
Clarke, S.C., Magnussen, J.E., Abercrombie, D.L., McAllister, M.K., Shivji, M.S., 2006. Identification of shark species composition and proportion in the Hong Kong shark fin market based on molecular genetics and trade records. Conserv. Biol. 20 (1), 201–211.
Coulson, M.W., Denti, D., Van Guelpen, L., Miri, C., Kenchington, E., Bentzen, P., 2011. DNA barcoding of Canada's skates. Mol. Ecol. Resour. 11 (6), 968–978.
Dent, F., Clarke, S., 2015. State of the Global Market for Shark Products. FAO Fisheries and Aquaculture, Rome, FAO, pp. 187 Technical Paper No. 590.
Doukakis, P., Hanner, R., Shivji, M., Bartholomew, C., Chapman, D., Wong, E., Amato, G., 2011. Applying genetic techniques to study remote shark fisheries in northeastern Madagascar. Mitochondrial DNA 22 (sup1), 15–20.
Dudgeon, C.L., Blower, D.C., Broderick, D., Giles, J.L., Holmes, B.J., Kashiwagi, T., Krück, N.C., Morgan, J.A.T., Tillett, B.J., Ovenden, J.R., 2012. A review of the application of molecular genetics for fisheries management and conservation of sharks and rays. J. Fish Biol. 80 (5), 1789–1843.
FDA, 2016. The Seafood List. (Accessed 7/26/17). https://www.accessdata.fda.gov/scripts/fdcc/?set=seafoodlist.
Fields, A.T., Abercrombie, D.L., Eng, R., Feldheim, K., Chapman, D.D., 2015. A novel mini-DNA barcoding assay to identify processed fins from internationally protected shark species. PLoS One 10 (2) e0114844.
Hanner, R.H., Naaum, A.M., Shivji, M.S., 2016. DNA-based authentication of shark products and implications for conservation and management. In: Hanner, R.H., Nauum, A.M. (Eds.), Seafood Authenticity and Traceability: A DNA-Based Perspective. Academic Press/Elsevier, San Diego, CA, USA, pp. 171–179.
Hebert, P.D.N., Cywinska, A., Ball, S.L., DeWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. R. Soc. B Biol. Sci. 270 (1512), 313–321.
Hellberg, R.S., Kawalek, M.D., Van, K.T., Shen, Y., Williams-Hill, D.M., 2014. Comparison of DNA extraction and PCR setup methods for use in high-throughput DNA barcoding of fish species. Food Anal. Method 7, 1950–1959.
Hoelzel, A.R., 2001. Shark fishing in fin soup. Conserv. Gen. 2 (1), 69–72.
Holmes, B.H., Steinke, D., Ward, R.D., 2009. Identification of shark and ray fins using DNA barcoding. Fisheries Res. 95, 280–288.
IUCN, 2017. The IUCN Red List of Threatened Species. Version 2017-1. (Accessed 7/26/17). http://www.iucnredlist.org.
Ivanova, N.V., Zemlak, T.S., Hanner, R.H., Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. Notes 7, 544–548.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649.
Kulka, D.W., Sulikowski, J., Gedamke, T., 2009. Leucoraja Ocellata. The IUCN Red List of Threatened Species 2009https://doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161631A5468670.en. e.T161631A5468670 (Accessed 7/26/17).
Liu, S.-Y.V., Chan, C.-L.C., Lin, O., Hu, C.-S., Chen, C.A., 2013. DNA barcoding of shark meats identify species composition and CITES-listed species from the markets in Taiwan. PLoS One 8 (11) e79373.
Marshall, L.J., Barone, M., 2016. SharkFin Guide: Identifying Sharks from Their Fins. FAO, Rome, Italy.
Messing, J., 1983. New M13 vectors for cloning. Methods Enzymol. 101, 20–79.
Moore, A.B.M., Almojil, D., Harris, M., Jabado, R.W., White, W.T., 2014. New biological data on the rare, threatened shark *Carcharhinus leiodon* (Carcharhinidae) from the Persian Gulf and Arabian Sea. Mar. Freshwater Res. 65 (4), 327–332.
Naaum Amanda, M., Hanner, R., 2015. Community engagement in seafood identification using DNA barcoding reveals market substitution in Canadian seafood. DNA Barcodes Berlin (Berlin) 3 (1), 74.
Newmaster, S.G., Grguric, M., Shanmughanandhan, D., Ramalingam, S., Ragupathy, S., 2013. DNA barcoding detects contamination and substitution in North American herbal products. BMC Med. 11 (1) e222.
NOAA, 2017. FishWatch. (Accessed 7/26/17). https://www.fishwatch.gov/.
Rodrigues-Filho, L.F., Pinhal, D., Sondre, D., Vallinoto, M., 2012. Shark DNA forensics: applications and impacts on genetic diversity. In: Caliskan, M. (Ed.), Analysis of Genetic Variation in Animals. InTech, Rijeka, Croatia, pp. 269–286.
Rose, D., 1996. An Overview of World Trade in Sharks and Other Cartilaginous Fishes. A TRAFFIC Network Report 106 p.
Sembiring, A., Pertiwi, N.P.D., Mahardini, A., Wulandari, R., Kurniasih, E.M., Kuncoro, A.W., Cahyani, N.K.D., Anggoro, A.W., Ulfa, M., Madduppa, H., Carpenter, K.E., Barber, P.H., Mahardika, G.N., 2015. DNA barcoding reveals targeted fisheries for endangered sharks in Indonesia. Fish. Res. 164, 130–134.
Shivji, M., Clarke, S., Pank, M., Natanson, L., Kohler, N., Stanhope, M., 2002. Genetic identification of pelagic shark body parts for conservation and trade monitoring. Conserv. Biol. 16 (4), 1036–1047.
Shivji, M.S., Chapman, D.D., Pikitch, E.K., Raymond, P.W., 2005. Genetic profiling reveals illegal international trade in fins of the great white shark, *Carcharodon carcharias*. Conserv. Gen. 6, 1035–1039.

Shokralla, S., Hellberg, R.S., Handy, S.M., King, I., Hajibabaei, M., 2015. A DNA mini-barcoding system for authentication of processed fish products. Sci. Rep. 5, 15894 Article number.
Steinke, D., Bernard, A.M., Horn, R.L., Hilton, P., Hanner, R., Shivji, M.S., 2017. DNA analysis of traded shark fins and mobulid gill plates reveals a high proportion of species of conservation concern. Sci. Rep. 7, 9505 Article number.
Wallace, L.J., Boilard, S.M.A.L., Eagle, S.H.C., Spall, J.L., Shokralla, S., Hajibabaei, M., 2012. DNA barcodes for everyday life: routine authentication of Natural Health Products. Food Res. Int. 49 (1), 446–452.
Ward, R.D., Holmes, B.H., White, W.T., Last, P.R., 2008. DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. Mar. Freshwater Res. 59 (1), 57–71.
Wong, E.H.K., Shivji, M.S., Hanner, R.H., 2009. Identifying sharks with DNA barcodes: assessing the utility of a nucleotide diagnostic approach. Mol. Ecol. Resour. 9, 243–256.