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Development of a DNA Mini-Barcoding Protocol Targeting COI for the Identification of Elasmobranch Species in Shark Cartilage Pills

Development of a DNA mini-barcoding protocol targeting *COI* for the identification of elasmobranch species in shark cartilage pills

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Abstract

Many elasmobranch (shark and ray) species are considered threatened and their identification in processed products is important for conservation and authentication purposes. However, identification of elasmobranch species in shark cartilage pills has proven difficult using existing methodologies. The objective of this study was to develop a DNA mini-barcoding protocol using a ~130 bp region of the cytochrome *c* oxidase subunit I (COI) gene for species identification in shark cartilage pills. A total of 22 shark cartilage products underwent DNA extraction in duplicate using the DNeasy Blood and Tissue Kit (Qiagen). The effectiveness of a clean-up step following DNA extraction was analyzed by comparing DNA purity values and polymerase chain reaction (PCR) amplification rates. Next, five different mini-barcode primer sets were compared based on amplification rates, and the three top-performing primer sets were used in DNA sequencing. The incorporation of a clean-up step following DNA extraction showed a slight advantage over DNA extraction alone, with a higher amplification rate (52.3% vs. 47.8%) and A_{260}/A_{230} value (3.3 vs. 0.6). The three primer sets selected for DNA mini-barcoding showed DNA sequencing rates of 54.5-65.9% among the 44 duplicate samples. When the results for all three primer sets were combined, 18 of the 22 shark cartilage products were identified to the species or genus level. On an individual basis, the best-performing primer set identified 16 of the 22 products to the species or genus level. Overall, the protocol developed in this study increased the identification rate for elasmobranches in cartilage products by more than 2-fold as compared to previous research.

*Keywords***:** DNA barcoding; DNA extraction; elasmobranch; shark cartilage; species identification

1. Introduction

The skeletons of sharks and other elasmobranch species are composed of cartilage, which is a connective tissue rich in proteins such as collagens and proteoglycans (Sim, et al., 2007). Shark cartilage has been investigated as a possible treatment for numerous medical conditions, including arthritis and cancer (Merly & Smith, 2015; Mondo, et al., 2014; Sim, et al., 2007). Due to its purported health benefits, shark cartilage is widely used as a dietary supplement. It is produced by separating the cartilage from the meat and drying it into a powder for use in capsules or tablets (Rose, 1996). Despite the numerous shark cartilage supplements available on the market, global trade information on these products, including the species used, is extremely limited (Clarke, 2004; Dent & Clarke, 2015).

Sharks and other elasmobranchs are especially vulnerable to overfishing due to biological factors such as low fecundity and long reproductive cycles (Bräutigam, et al., 2015; Ferretti, Worm, Britten, Heithaus, & Lotze, 2010). Among the 1,038 shark and ray species assessed by the International Union for the Conservation of Nature (IUCN), close to 20% are considered Critically Endangered, Endangered, or Vulnerable, and an additional 12% are categorized as Near Threatened (Bräutigam, et al., 2015). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) also lists 13 elasmobranch species or species groups in Appendix II, meaning that export permits are required for international trade of these animals (CITES, 2019). Shark cartilage pills have been reported to contain Near Threatened, Vulnerable, and Endangered elasmobranch species, including silky shark (*Carcharhinus falciformis*), a CITES-listed species (Hellberg et al. 2019). With growing concern over exploitation of global elasmobranch populations and a

lack of trade data, improved methods to identify and track species in shark cartilage supplements are needed.

DNA-based techniques, such as DNA barcoding and species-specific polymerase chain reaction (PCR), are commonly used to identify elasmobranch species when morphological indicators are lacking (Appleyard, White, Vieira, & Sabub, 2018; Dudgeon, et al., 2012; Hanner, Naaum, & Shivji, 2016; Rodrigues-Filho, Pinhal, Sondre, & Vallinoto, 2012). While species-specific PCR is preferable for targeted approaches, DNA barcoding is more appropriate for applications involving a wide range of species, such as shark cartilage supplements. DNA barcoding is a sequencing-based method for species identification that uses universal primer sets to target a short, standardized region of the genome (Hebert, Cywinska, Ball, & DeWaard, 2003). The most common genetic region targeted for the identification of animal species is a ~650 base pair (bp) region of the mitochondrial gene coding for cytochrome *c* oxidase subunit I (CO1). However, it has proven challenging to recover the full-length DNA barcode from highly processed samples due to DNA fragmentation (Fields, Abercrombie, Eng, Feldheim, & Chapman, 2015; Shokralla, Hellberg, Handy, King, & Hajibabaei, 2015).

An alternative approach for identification of processed samples is DNA mini-barcoding, which targets shorter regions of the full-length barcode. For example, Shokralla et al. (2015) developed a fish mini-barcoding system using the cytochrome *c* oxidase subunit I (COI) gene that enabled identification of 88.6% of processed fish products tested, as compared to 20.5% identification success with full barcoding. DNA barcoding and mini-barcoding techniques have been used to identify elasmobranch species in a range of commercial products, including dried shark fin, shark meat, and shark fin soup (Barbuto, et al., 2010; Cardeñosa, et

al., 2017; Chuang, Hung, Chang, Huang, & Shiao, 2016; Fields, et al., 2015; R.S. Hellberg, Isaacs, & Hernandez, 2019; O'Bryhim, Parsons, & Lance, 2017; Steinke, et al., 2017). A number of studies have also differentiated shark species utilizing DNA barcoding combined with character-based analysis, which is based on the presence or absence of specific nucleotides determined to be diagnostic for a given species (Fields et al. 2015; Velez-Zuazo et al. 2015; Wong et al. 2009).

DNA barcoding of shark cartilage supplements has proven challenging, likely due to factors such as DNA degradation during processing, the presence of PCR inhibitors, and the possibility of multiple species in one product (R.S. Hellberg, et al., 2019). Hellberg et al. (2019) utilized a combination of *COI* full and mini-barcoding to identify elasmobranch species in shark products, including cartilage supplements. Full-length barcoding of shark cartilage supplements enabled identification in only 20.7% of these products, as compared to 51.7% of supplements identified with shark mini-barcoding. Optimization of DNA extraction and amplification techniques may improve DNA barcoding performance, thereby enabling identification of species in a greater number of shark cartilage pills.

The objective of this study was to develop a mini-barcoding protocol for the identification of elasmobranch species in shark cartilage pills through optimization of DNA extraction, amplification, and sequencing techniques. The effectiveness of a clean-up step following DNA extraction was examined; five mini-barcoding primer sets were compared based on PCR amplification rates; and uni-directional sequencing was compared to bi-directional sequencing for the three top-performing primer sets.

2. Methods

2.1 Sample collection and preparation

A subset (n = 22) of the shark cartilage pills tested by Hellberg et al. (2019) were selected for use in this study. All products that failed PCR or sequencing in the previous study were selected (n = 9), along with samples that represented the range of species detected in shark cartilage pills (n = 13). Among the 13 samples previously identified, Hellberg et al. (2019) detected a single species in 11 products and a combination of two species in 2 products. A composite sample was made for each product by combining three pills. Pills in capsule form were twisted open to release the cartilage powder and tablets were ground together using a sterile mortar and pestle. The composite samples were stored at room temperature in sterile 50-mL Falcon tubes (Corning, Corning, NY).

2.2 Comparison of DNA extraction methods

DNA extraction was performed on ~25 mg of each composite sample using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), Spin-Column protocol. Sample lysis was carried out for 2 h at 56°C with pulse vortexing every 30 min. DNA was eluted with 60 µL of AE buffer pre-heated to 37°C. All samples were extracted in duplicate and a reagent blank negative control was included alongside each set of DNA extractions. The concentration and purity of each DNA extract was measured using a Biophotometer Plus (Eppendorf, Happauge, NY). All samples underwent PCR as described below with the original shark mini-barcode primer set (Table 1) used in Fields et al. (2015). Amplification results were determined by gel electrophoresis, as described below. The remaining portion of each duplicate DNA extract (~50 µL) underwent a clean-up step with the PowerClean Pro CleanUp Kit (Qiagen), according to the manufacturer's instructions, with the exception that AE buffer was used in the final elution step. The

concentration and purity of the DNeasy Kit plus PowerClean Kit DNA extracts were measured with a Biophotometer Plus. All samples underwent PCR using the original shark mini-barcode primers, followed by gel electrophoresis (as described below). The DNA concentration, DNA purity, PCR amplification rates, and PCR amplification consistency for samples extracted with the DNeasy Kit were compared to those of the samples extracted with the DNeasy Kit plus PowerClean Kit to determine the optimal method to be used for the PCR primer comparison studies.

2.3 PCR primer comparison

In order to compare PCR primer sets, DNA was extracted in duplicate from each sample using the DNeasy Blood and Tissue Kit plus the PowerClean Pro CleanUp Kit. Each DNA extract was then tested against five different primer sets (Table 1): the original shark mini-barcode primer set (Fields, et al., 2015), two variations of the shark mini-barcode primer set (V1 and V2, designed in the current study), and two primer sets (SH-D and SH-E) used in a previous study on fish species identification (Shokralla, et al., 2015). The V1 and V2 primer sets were designed based on comparison with an alignment of 1049 elasmobranch sequences obtained from GenBank (Accession numbers: FJ518910–FJ519800, FJ519802-FJ519959; Wong, et al., 2009). M13 tails were attached to the SH-D, SH-E, and forward shark mini-barcode primers to facilitate DNA sequencing (Table 1). PCR for the shark mini-barcode primer set and its variations (V1 and V2) was carried out using 12.5 µL HotStar Taq Master Mix (Qiagen), 10.5 µL of molecular-grade sterile water, 0.5 µL of 10 µM forward primer cocktail, 0.5 µL of 10 µM reverse primer, and 1.0 µL of template DNA. PCR for the SH-D and SH-E primer sets was carried out using the same preparation mix as the shark mini-barcode primers, except that 9.5 µL of sterile water, and 2.0 µL of template DNA were used. A no-template control (NTC)

containing sterile water in place of DNA was included in each set of reactions. Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and a Mastercycler Nexus Gradient Thermal Cycler (Eppendorf) was used to carry out PCR.

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 (R.S. Hellberg, et al., 2019). The same cycling conditions were used for the V1 and V2 shark mini-barcoding primer sets except that the annealing temperatures were 46ºC and 54ºC, respectively. These optimal annealing temperatures were determined based on the results of gradient PCR at a temperature range of 43-60°C followed by gel electrophoresis, as described below (Shokralla, et al., 2015). The cycling conditions for the SH-D primer set were: 95ºC for 15 min; 35 cycles of 94ºC for 40 s, 50ºC for 1 min, and 72ºC for 30 s; and a final extension step at 72ºC for 5 min. The cycling conditions for the SH-E primer set were: 95ºC for 15 min; 35 cycles of 94ºC for 40 s, 46ºC for 1 min, and 72ºC for 30 s; and a final extension step at 72ºC for 5 min.

2.4 Gel electrophoresis

PCR products (4 µL) were loaded onto 2% agarose E-Gels (Life Technologies, Carlsbad, CA) and run using the E-Gel 0.8-2.0% Program on an E-Gel iBase (Life Technologies) for 15 min (Rosalee S. Hellberg, Kawalek, Van, Shen, & Williams-Hill, 2014). The results were visualized with FOTO/Analyst Express (Fotodyne, Hartland, WI) and Transilluminator FBDLT-88 (Fisher Scientific, Waltham, MA) combined with FOTO/Analyst PCImage (version 5.0.0.0, Fotodyne). Samples with visible bands of the expected size following electrophoresis were considered positive for PCR amplification.

184 *2.5 DNA sequencing*

185 Primer sets with amplification rates equal to or greater than the original shark mini-
186 barcode primer set (shark mini-barcode V1 and V2) were further examined based on DNA
187 sequencing results. Samples that produced PCR amplicons visible with gel electrophoresis
188 underwent PCR clean-up using a 4-fold dilution of ExoSAP-IT, as described in Weigt et al.
189 (2012). The products were then submitted to GenScript (Piscataway, NJ) for DNA sequencing.
190 For bi-directional sequencing, samples were sequenced in both directions using the M13 forward
191 primer and the reverse primer. For uni-directional sequencing, only the sequence read from the
192 M13 forward primer was analyzed (Fields, et al., 2015). The resulting sequencing files were
193 assembled, edited, and trimmed to the mini-barcode region (127 bp) using Geneious R7
194 (Biomatters, Ltd., Auckland, New Zealand) (Kearse, et al., 2012). Sequences were only
195 considered acceptable if they had < 2% ambiguities and were ≥ 64 bp in length (at least 50%
196 coverage of the mini-barcode region). The resulting sequences were initially searched against
197 the Barcode of Life Database (BOLD) Animal Identification Request Engine
198 (http://www.boldsystems.org/), Species Level Barcodes (November 2018). Sequences that could
199 not be identified in BOLD were next searched against GenBank using the Nucleotide Basic
200 Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Top species
201 matches obtained through these databases were further examined by determining the source of
202 the entry and checking to see whether the entry was previously identified as a misidentified
203 specimen. Additionally, character-based analysis was applied where applicable to assist with
204 species identification (Fields, et al., 2015; Velez-Zuazo, Alfaro-Shigueto, Mangel, Papa, &
205 Agnarsson, 2015; Wong, Shivji, & Hanner, 2009). Sequences with equivalent top matches to

206 species from multiple genera at less than 98% similarity were not considered successful
207 identifications.

208 *2.6 Statistical analysis*

209 DNA concentrations, $A_{260/280}$ ratios and $A_{260/230}$ ratios for samples tested with the DNeasy
210 Kit alone and the DNeasy Kit plus PowerClean Kit were compared using the Wilcoxon signed-
211 rank test, with a pre-determined level of significance of $p < 0.05$. PCR amplification rates and
212 amplification consistency were compared for samples tested with the DNeasy Kit alone and the
213 DNeasy Kit plus PowerClean Kit using McNemar's test, with a level of significance of $p < 0.05$.
214 Results for PCR amplification, DNA sequencing, and species identification for the various
215 primer sets were compared using Cochran's Q test, with a level of significance of $p < 0.05$.
216 Statistically significant results were compared with Dunn's post-hoc test with the Bonferroni
217 correction (adjusted $p < 0.05$). The sequence lengths, quality scores (% HQ), and percent
218 ambiguities were compared across the various primer sets and sequencing methods tested using
219 the Kruskal-Wallis H test and Dunn's post hoc test with the Bonferroni correction (adjusted $p <$
220 0.05). All analyses were carried out with IBM SPSS Statistics 23 (Armonk, New York, USA).

221 **3. Results and Discussion**

222 *3.1 Comparison of DNA extraction methods*

223 As shown in Table 2, the average DNA concentration of the samples extracted with the
224 DNeasy Kit (69.6 ± 70.3 ng/ul) was significantly higher than that for samples extracted with the
225 DNeasy Kit plus PowerClean Kit (23.4 ± 13.1 ng/ul), according to a Wilcoxon signed-rank test
226 ($p < 0.05$). The average A_{260}/A_{280} ratios for samples extracted with the DNeasy Kit (1.7 ± 0.5)
227 and the DNeasy Kit plus PowerClean Kit (1.6 ± 0.4) were not significantly different and were
228 close to the optimal ratio of ~1.8 for pure DNA (Desjardins & Conklin, 2010). The A_{260}/A_{280}

ratios could not be determined for 7 of the 44 replicates tested with the DNeasy Kit alone because the A_{280} value was not within the measuring range. Paired samples with missing data were excluded from the statistical analysis, resulting in a total of 37 paired data points analyzed by the Wilcoxon signed-rank test. The average A_{260}/A_{230} ratio for samples extracted with the DNeasy Kit (0.6 ± 0.3) was significantly lower than that for 8 of 22 samples extracted with the DNeasy Kit plus PowerClean Kit (3.3 ± 3.0). The A_{260}/A_{230} ratio could not be determined for a portion of the 44 replicates tested with the DNeasy Kit alone (n = 3) and the DNeasy Kit plus PowerClean Kit (n = 14) because the A_{230} value was not within the measuring range. Paired samples with missing data were excluded from the statistical analysis, resulting in a total of 27 paired data points analyzed by the Wilcoxon signed-rank test. The lower A_{260}/A_{230} ratios for samples extracted with the DNeasy Kit indicate the presence of contaminants that were removed by the PowerClean Kit. These may have included residual guanidine from the silica column in the DNeasy Kit or carbohydrates in the shark cartilage pills, such as cellulose, that were carried over during extraction (Matlock, 2015).

PCR amplification rates for the two extraction methods were compared using the original shark mini-barcode primer set (for example, see Supplementary Figure S1). As shown in Table 2, use of the DNeasy Kit plus PowerClean Kit resulted in visible bands in the gel for 23 of the 44 duplicate samples for a success rate of 52.3%, as compared to a success rate of 47.8% with the DNeasy Kit alone. Samples extracted with the DNeasy Kit plus PowerClean Kit also showed greater agreement among duplicate samples (95.5%) as compared to the DNeasy Kit (77.3%). However, there were no significant differences between the two methods on the basis of PCR amplification rate or consistency (McNemar's test, $p > 0.05$).

Overall, the DNeasy Kit plus PowerClean Kit was determined to be the optimal method for extraction of DNA from shark cartilage pills. This was based on the significant increase in the A_{260}/A_{230} values obtained with this method combined with the higher PCR amplification rate and amplification consistency as compared to the DNeasy Kit alone. Although the DNeasy Kit plus PowerClean Kit led to a significantly reduced DNA concentration, the DNA yield was sufficient to allow for PCR amplification. These results indicate that the additional clean-up steps carried out with the PowerClean Kit allowed for the removal of PCR inhibitors such as carbohydrates and guanidine, thus improving PCR amplification rates and consistency.

3.2 PCR primer comparison

Out of the 44 duplicate samples tested with the five mini-barcode primer sets, 34 (77.3%) could be amplified with at least one primer set (Fig. 1). On an individual basis, the shark mini-barcode V1 and V2 primer sets were the most successful (68.2%), followed by the original shark mini-barcode primer set (56.8%). The amplification rate for the mini SH-D and mini SH-E primer sets (22.7%) was significantly lower than the rates obtained with the original shark mini-barcode, shark mini-barcode V1, and shark mini-barcode V2, according to a Cochran's Q test followed by Dunn's test with the Bonferroni correction ($p < 0.05$). The decreased amplification rates observed for the mini SH-D and SH-E primer sets were likely because they were not designed for the specific amplification of shark species, but rather for the universal amplification of processed fish species. On the other hand, the shark mini-barcode primer sets utilize a universal forward fish cocktail combined with a reverse primer specifically designed to amplify shark species (Table 1). Based on the results of PCR amplification, the original shark mini-barcode and the shark mini-barcode V1 and V2 primer sets were selected for use in DNA sequencing.

3.3 DNA sequencing

Out of the 44 duplicate samples sequenced with the three shark mini-barcode primer sets, 33 (75%) were sequenced with at least one primer set (Table 3). Sequencing rates for the individual primer sets and sequencing methods (uni-directional and bi-directional) were not significantly different according to Cochran's Q test ($p > 0.05$). The shark mini-barcode V2 primer set showed the highest sequencing rates of 65.9% and 63.6% for bi-directional and uni-directional methods, respectively. Sequencing rates for the other primer sets were all <60%. When comparing bi-directional sequencing with uni-directional sequencing, the former had a higher sequencing rate for two out of the three primer sets (V1 and V2).

The average sequence length (113 ± 16 bp) for all primer sets combined was close to the target sequence length of 127 bp and the average percent ambiguities was low, at 0.09 ± 0.33% (Table 3). The sequence lengths obtained with bi-directional sequencing for each primer set were significantly higher than those obtained with uni-directional sequencing, according to the Kruskal-Wallis H test and Dunn's post hoc test with the Bonferroni correction, adjusted $p < 0.05$). The bi-directional sequences showed a combined average of 0.18 ± 0.44% ambiguities, with no significant differences across the three primer sets.

As shown in Table 3, the sequencing quality scores had a combined average of 44.2 ± 30.6% HQ. Similar to the results for sequencing length, the average quality score was highest for the bi-directional results (54.3 ± 32.9% HQ) as compared to the uni-directional results (33.8 ± 24.1% HQ). However, the only statistical difference was that the quality score for the bi-directional shark mini-barcode (70.6 ± 17.1% HQ) was significantly higher than the other quality scores, according to the Kruskal-Wallis H test and Dunn's post hoc test with the Bonferroni correction (adjusted $p < 0.05$). The reduced quality of the sequences obtained with the shark

297 mini-barcode V1 and V2 primer sets is likely due to the use of degeneracies in the reverse
298 primers (Table 1), which may have resulted in co-amplification of other gene regions and/or
299 multiple mini-barcodes in products with mixed species.

300 Overall, bi-directional sequencing showed greater performance as compared to uni-
301 directional sequencing, with improvements in sequencing rates, sequence lengths, and quality
302 scores. When comparing the individual primer sets, the shark mini-barcode V2 primer set
303 allowed for the greatest sequencing rate, while the original shark mini-barcode primer set
304 allowed for the greatest sequence quality and length.

305 *3.4 Species identification*

306 The three shark mini-barcode primer sets were next evaluated for their ability to identify
307 elasmobranch species in the shark cartilage pills (Table 4). This analysis was focused on the
308 results of bi-directional sequencing because of the improved performance discussed above for
309 this method. Overall, 18 of the 22 shark cartilage products (81.8%) could be identified to the
310 species or genus level with at least one of the primer sets. On an individual basis, the shark
311 mini-barcode V2 primer set identified the greatest percentage of products (72.7%) to the species
312 or genus level, followed by the original shark mini-barcode primer set (63.6%), and the shark
313 mini-barcode V1 primer set (59.1%). These percentages are an improvement over the previous
314 identification rate (36.4%) reported by Hellberg et al. (2019) for the same set of 22 shark
315 cartilage products amplified uni-directionally with the original shark mini-barcode primer set.
316 The increased identification rates observed in the current study were likely due to a combination
317 of improvements made to the methodology, including the use of a composite sample for DNA
318 extraction, testing of duplicate samples, incorporation of the PowerClean Kit into the extraction
319 process, and the use of modified shark mini-barcode primer sets. The benefit of testing samples

in duplicate is illustrated by the fact that there were 3-5 samples per primer set for which only one of the duplicate samples could be identified (Table 4).

The modified primer sets (V1 and V2) used in the current study enabled the identification of two species that were not detected with the original shark mini-barcode primer set: winter skate (*Leucoraja ocellata*) and spiny dogfish (*Squalus acanthias*). These species are considered endangered and vulnerable, respectively, according to the IUCN Red List of Threatened Species (http://www.iucnredlist.org/). Although the samples identified as *L. ocellata* also matched a sequence labeled as little skate (*Leucoraja erinacea*), this sequence was previously reported to be misidentified and actually belong to winter skate (Coulson, et al., 2011; R.S. Hellberg, et al., 2019). The inability of the original shark mini-barcode primer set to detect *L. ocellata* or *S. acanthias* in the shark cartilage products is consistent with the results of Hellberg et al. (2019). In contrast, Fields et al. (2015) reported the ability to identify *S. acanthias* in shark fin soup using this primer set. For optimal identification of species in shark cartilage pills, it is recommended that samples be tested against all three primer sets described here (i.e., original shark mini-barcode, V1, and V2).

3.5 Shark mini-barcoding considerations

An important consideration with regard to DNA barcoding is the level of genetic similarity achieved between the query sequence and the reference sequences. Traditionally, a cut-off of >2% genetic divergence has been applied to differentiate fish species based on the full barcode (Handy, et al., 2011). However, many of the mini-barcode sequences obtained in the current study showed multiple species matches with ≥ 98% genetic similarity. This occurred most frequently with the *Carcharhinus* species, which are closely related and have been previously reported to show high genetic similarity across the DNA barcode region (R.S.

Hellberg, et al., 2019; Ward, Holmes, White, & Last, 2008). While the full-length DNA barcode can effectively differentiate many of these species (e.g., see Appleyard et al. 2018; Wong et al. 2009), the shorter amplicon generated with mini-barcoding showed reduced differentiation capability when relying on a distance-based approach. In some of these cases, the use of character-based identification keys developed in previous studies (Fields, et al., 2015; Velez-Zuazo, et al., 2015; Wong, et al., 2009) enabled differentiation (Table 4). For example, many samples showed equivalent genetic matches to multiple members of the *Carcharhinus* genus in BOLD and/or GenBank. When a set of diagnostic nucleotides published in Wong et al. (2009) was applied to these sequences, it allowed for the identification of silky shark, a CITES-listed species, in five of these samples.

In several instances, a secondary match showed > 99.22% genetic similarity to the top match in BOLD, corresponding to less than 1 nucleotide difference in a 127 bp sequence. These results were often due to the presence of an ambiguity or gap in the sequence of the secondary match and there is potential for a mis-identification to occur when additional consideration is not given to the secondary match. For example, one of the PS07 shark mini-barcode V2 sequences showed a top match (97.58%) to scalloped hammerhead shark (*Sphyrna lewini*) and a secondary match (97.32%) to spot-tail shark (*Carcharhinus sorrah*) in BOLD. However, character analysis revealed that the query sequence was not *S. lewini* due to the absence of diagnostic nucleotides described in Fields et al. (2015) and the sample was instead identified as *C. sorrah*.

The results discussed above support the use of character-based analysis in combination with genetic similarity values, as recommended in previous studies on shark DNA barcoding (Fields, et al., 2015; Velez-Zuazo, et al., 2015; Wong, et al., 2009). However, it should be noted that character-based keys are limited in species number, subject to change, and sometimes cannot

366 be used with the mini-barcode region targeted in this study. Therefore, confirmation of species
367 for enforcement purposes should include an attempt to sequence the full-length barcode and/or a
368 longer mini-barcode, such as that described in Cardeñosa et al. (2017). In cases where there is <
369 1 nucleotide difference between the primary and secondary species matches and character
370 analysis cannot be utilized, it may be more appropriate to report all species rather than relying
371 only on the top match.

372 Finally, it is important to note that shark cartilage pills are a highly processed product
373 with the possibility for species mixtures. While species were identified in the majority of
374 samples in the current study, standard DNA barcoding and mini-barcoding do not enable
375 simultaneous identification of multiple species in a single sample. Therefore, additional research
376 should be conducted to explore the use of mini-barcodes combined with next-generation
377 sequencing or PCR cloning to identify species in mixed samples.

378 **4. Conclusions**

379 This study describes the development of an effective method for the DNA-based
380 identification of elasmobranch species in shark cartilage pills. Overall, the combined results for
381 the three shark mini-barcode primer sets tested in this study allowed for species or genus-level
382 identification in 81.8% of shark cartilage products. This was a marked improvement over
383 previous research that was only able to identify 36.4% of these products to the species or genus-
384 level. The methodology described in the current study is expected to facilitate conservation
385 efforts and monitoring of international trade by providing an improved protocol to determine
386 whether shark cartilage pills are mislabeled and/or contain at-risk species. These improvements
387 may also serve to increase mini-barcoding identification rates for other highly processed
388 commercial shark products, such as shark fin soup. Future research should explore the utility of

shark mini-barcodes combined with next-generation sequencing and/or PCR cloning approaches to identify mixed species in shark products. Additionally, the ability of a longer mini-barcode to amplify in these products should be examined in order to improve differentiation of closely related species.

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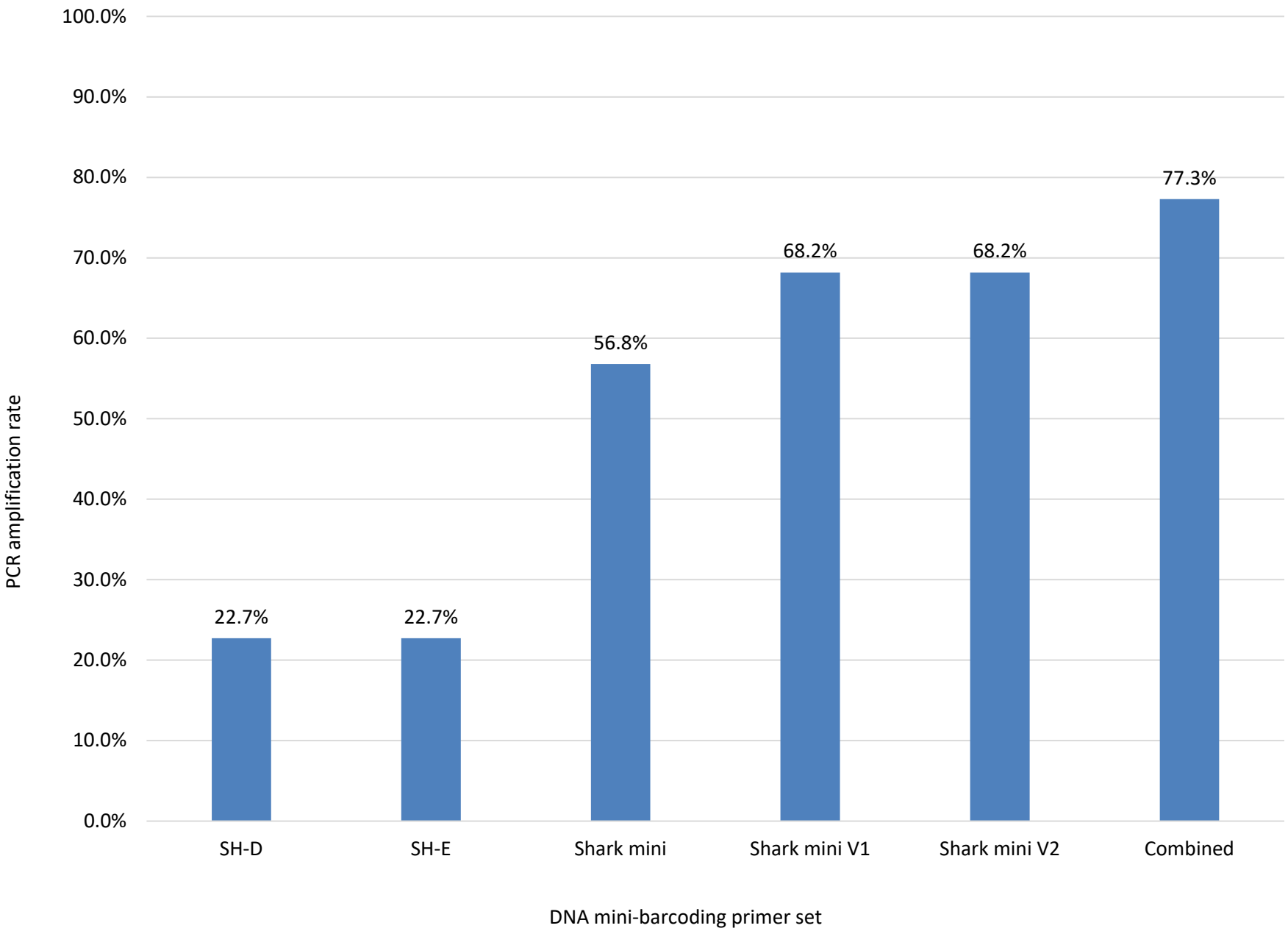
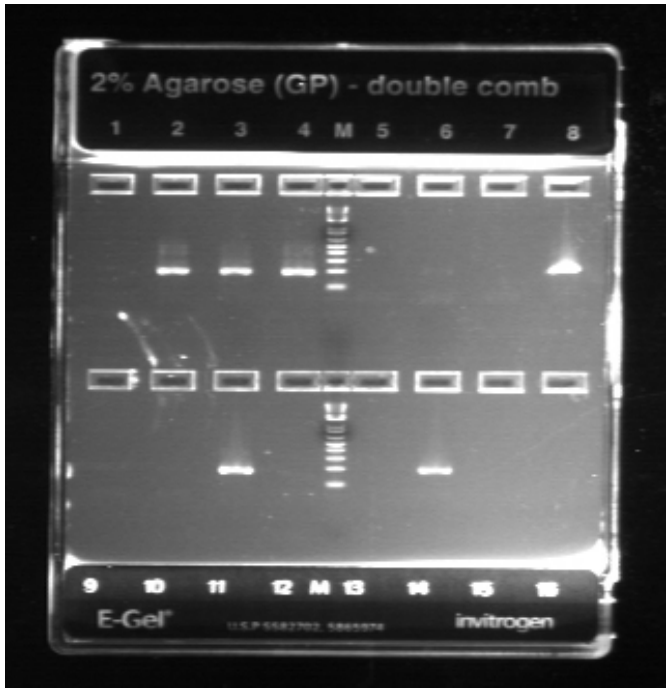
Figure Captions

Figure 1. PCR amplification rates obtained for duplicate shark cartilage samples (n = 44) tested with the five primer sets compared in this study. The 'Combined' column shows the results for all primer sets combined

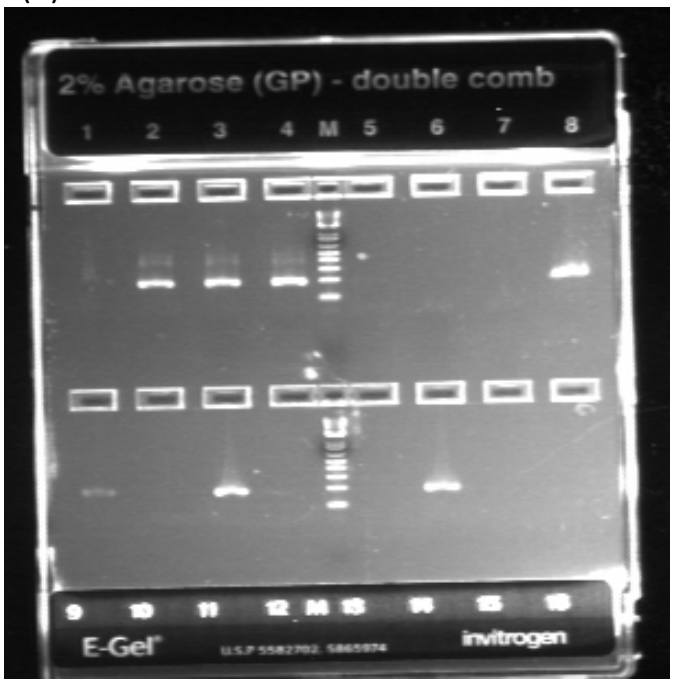
Supplementary Figure S1: Example of gel electrophoresis results measuring PCR amplification rates of the original shark mini-barcode combined with (a) DNeasy Kit alone and (b) DNeasy Kit plus PowerClean. Lane assignments are the same in each gel and are as follows: Lane 1 = PS20, Lane 2 = PS21, Lane 3 = PS22, Lane 4 = PS23, Lane 5 = PS24, Lane 6 = PS25, Lane 7 = PS27, Lane 8 = PS28, Lane 9 = PS29, Lane 10 = PS30, Lane 11 = PS32, Lane 12 = reagent blank, Lane 13 = non-template control, Lane 14 = positive control.


100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
PCR amplification rate
22.7%
22.7%
56.8%
68.2%
68.2%
77.3%
SH-D
SH-E
Shark mini
Shark mini V1
Shark mini V2
Combined
DNA mini-barcoding primer set

(a)



(b)



Supplementary Figure S1: Example of gel electrophoresis results measuring PCR amplification rates of the original shark mini-barcode combined with (a) DNeasy Kit alone and (b) DNeasy Kit plus PowerClean. Lane assignments are the same in each gel and are as follows: Lane 1 = PS20, Lane 2 = PS21, Lane 3 = PS22, Lane 4 = PS23, Lane 5 = PS24, Lane 6 = PS25, Lane 7 = PS27, Lane 8 = PS28, Lane 9 = PS29, Lane 10 = PS30, Lane 11 = PS32, Lane 12 = reagent blank, Lane 13 = non-template control, Lane 14 = positive control.

Table 1. Primer sequences for five primer sets compared in this study

Primer set	Primer name	Direction	Primer sequence (5'-3')[a]	Barcode length	Reference
Shark mini-barcode	VF2_t1	Forward	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC	127 bp	(Fields, et al., 2015; Ivanova, Zemlak, Hanner, & Hebert, 2007)
	FishF2_t1	Forward	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC		
	Shark COI-MINIR	Reverse	AAGATTACAAAAGCGTGGGC		(Fields, et al., 2015)
Shark mini-barcode_V1	VF2_t1	Forward	See above	127 bp	(Fields, et al., 2015; Ivanova, et al., 2007)
	FishF2_t1	Forward	See above		
	Shark_Mini_V1_R	Reverse	AAGATTATTACAAAAGCRTGRGC		Current study
Shark mini-barcode_V2	VF2_t1	Forward	See above	127 bp	(Fields, et al., 2015; Ivanova, et al., 2007)
	FishF2_t1	Forward	See above		
	Shark_Mini_V2_R	Reverse	AAGATTATTACRAADGCRTGRGC		Current study
Mini_SH-D	Mini_SH-D_F	Forward	CACGACGTTGTAAAACGACGGIACIGGITGRACIGTITAYCCYCC	208 bp	(Shokralla, et al., 2015)
	Mini_SH-D_R	Reverse	GGATAACAATTTCACACAGGGTRATICCIGCIGCIAGIAC		
Mini_SH-E	Mini_SH-E_F	Forward	CACGACGTTGTAAAACGACACYAAICAYAAAGAYATIGGCAC	226 bp	(Shokralla, et al., 2015)
	Mini_SH-E_R	Reverse	GGATAACAATTTCACACAGGCTTATRTTRTTTATICGIGGRAAIGC		

[a]Shaded portions of primer sequences indicate M13 tail

Table 2. Comparison of DNA extraction and PCR amplification results obtained for duplicate shark cartilage samples (n = 44) tested with the original shark mini-barcode primer set. DNA concentrations and absorbance ratios are expressed as the average ± standard deviation

	DNeasy Kit	DNeasy Kit plus PowerClean Kit
DNA concentration (ng/ul)	69.6 ± 70.3[a]	23.4 ± 13.1[b]
A_{260}/A_{280} ratio[c]	1.7 ± 0.5[a]	1.6 ± 0.4[a]
A_{260}/A_{230} ratio[d]	0.6 ± 0.3[a]	3.3 ± 3.0[b]
PCR amplification rate	47.8%	52.3%
PCR amplification consistency	77.3%	95.5%

[ab]A different superscript letter in the same row indicates a significant difference between extraction methods, according to the Wilcoxon signed-rank test ($p < 0.05$).

[c]Ratios could not be determined for 7 DNeasy Kit replicates because the A280 value was not within the measuring range

[d]Ratios could not be determined for 3 DNeasy Kit replicates and 14 DNeasy Kit + PowerClean Kit replicates because the A230 value was not within the measuring range

Table 3. Sequencing rates and quality parameters obtained for shark cartilage samples tested in duplicate (n = 44) with three shark mini-barcode primer sets

Primer Set	Sequencing method	Sequencing rate	Sequence length (bp)	HQ (%)	Ambiguities (%)
Shark mini-barcode	Uni-directional	56.8%	107 ± 11[a]	41.4 ± 15.4[a]	0.00 ± 0.00[a]
	Bi-directional	56.8%	127 ± 0[b]	70.6 ± 17.1[b]	0.16 ± 0.39[ab]
Shark mini-barcode V1	Uni-directional	54.5%	105 ± 17[a]	29.3 ± 24.6[a]	0.00 ± 0.00[a]
	Bi-directional	59.0%	118 ± 17[b]	47.9 ± 35.4[a]	0.31 ± 0.57[b]
Shark mini-barcode V2	Uni-directional	63.6%	104 ± 17[a]	30.9 ± 28.8[a]	0.00 ± 0.00[a]
	Bi-directional	65.9%	119 ± 14[b]	46.0 ± 36.7[a]	0.08 ± 0.32[ab]
Combined[c]		75%	113 ± 16	44.2 ± 30.6	0.09 ± 0.33

[ab]A different superscript letter in the same column indicates a significant difference, according to the Kruskal-Wallis H test and Dunn's post hoc test with the Bonferroni correction (adjusted $p < 0.05$).
[c]Sequencing results for all primer sets combined

Table 4. Species identified in the 18 shark cartilage products sequenced by at least one of the primer sets tested in this study. CITES-listed species are indicated with boldface. In cases where duplicate samples had different species identifications, the top matches are separated by a semicolon

Sample ID	Product description	Sequencing method	Primer Set		
			Shark mini-barcode	**Shark mini-barcode V1**	**Shark mini-barcode V2**
PS04	Shark cartilage capsules	Bi-directional	***Carcharhinus falciformis*[ab]**	N/A	***Carcharhinus falciformis*[ab]**
PS05	Shark cartilage capsules	Bi-directional	N/A	*Leucoraja ocellata*	*Leucoraja ocellata*
PS06	Shark cartilage tablets	Bi-directional	*Carcharhinus sorrah*[c]	*Carcharhinus melanopterus*[cd]; *Carcharhinus* spp.[d]	*Carcharhinus melanopterus*[a]
PS07	Shark cartilage capsules	Bi-directional	***Carcharhinus falciformis*[a]**; *Carcharhinus melanopterus*[c]	N/A	*Carcharhinus sorrah*[abcd]
PS08	Shark cartilage capsules	Bi-directional	***Carcharhinus falciformis*[ab]**	N/A	Carcharhinus spp.[ab]
PS13	Shark cartilage capsules	Bi-directional	*Carcharhinus sorrah*	*Carcharhinus sorrah*[c]	*Carcharhinus sorrah*; *Carcharhinus* spp.
PS15	Shark cartilage capsules	Bi-directional	***Carcharhinus falciformis*[a]**	***Carcharhinus falciformis*[a]**	*Carcharhinus melanopterus*[d]; ***Carcharhinus falciformis*[a]**
PS16	Shark cartilage capsules	Bi-directional	N/A	*Leucoraja ocellata*	*Leucoraja ocellata*
PS17	Shark cartilage capsules	Bi-directional	*Carcharhinus melanopterus*[b]	*Negaprion acutidens*[abcd]	N/A
PS19	Shark cartilage capsules	Bi-directional	***Carcharhinus falciformis*[a]**; *Carcharhinus melanopterus*	***Carcharhinus falciformis*[a]**; *Carcharhinus melanopterus*	***Carcharhinus falciformis*[a]**; *Carcharhinus melanopterus*
PS20	Shark cartilage capsules	Bi-directional	N/A	*Squalus acanthias*	*Squalus acanthias*
PS21	Shark cartilage capsules	Bi-directional	*Carcharhinus sorrah*[a]	*Carcharhinus sorrah*[abc]	*Carcharhinus sorrah*[cd]; *Carcharhinus* spp.
PS22	Shark cartilage capsules	Bi-directional	*Carcharhinus sorrah*	*Carcharhinus amblyrhynchoides*[bd]	*Carcharhinus melanopterus*[c]; *Carcharhinus sorrah*

PS23	Shark cartilage capsules	Bi-directional	*Carcharhinus sorrah*[c]	*Carcharhinus leucas*[d]; *Carcharhinus sorrah*[acd]	*Carcharhinus sorrah*[b]
PS28	Shark cartilage capsules	Bi-directional	*Prionace glauca*	*Prionace glauca*	*Prionace glauca*
PS29	Shark cartilage capsules	Bi-directional	*Carcharhinus melanopterus*[c]; *Carcharhinus sorrah*[c];	N/A	N/A
PS30	Shark cartilage capsules with dogfish shark	Bi-directional	N/A	N/A	*Carcharhinus* spp.[b]
PS32	Shark cartilage capsules	Bi-directional	*Galeorhinus galeus*	*Galeorhinus galeus*	*Galeorhinus galeus*

[a]Identification included the use of character analysis
[b]Identification was only successful for one of the duplicate samples
[c]Sequence had secondary species matches with ≥ 99.22% genetic similarity to the top species match that could not be ruled out with character analysis
[d]Top species match had < 98% genetic similarity to query sequence