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1   **Development of a DNA mini-barcoding protocol targeting *COI* for the identification of**

2   **elasmobranch species in shark cartilage pills**

3

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24 Abstract

25   Many elasmobranch (shark and ray) species are considered threatened and their

26 identification in processed products is important for conservation and authentication purposes.

27 However, identification of elasmobranch species in shark cartilage pills has proven difficult

28 using existing methodologies. The objective of this study was to develop a DNA mini-barcoding

29 protocol using a ~130 bp region of the cytochrome *c* oxidase subunit I (COI) gene for species

30 identification in shark cartilage pills. A total of 22 shark cartilage products underwent DNA

31 extraction in duplicate using the DNeasy Blood and Tissue Kit (Qiagen). The effectiveness of a

32 clean-up step following DNA extraction was analyzed by comparing DNA purity values and

33 polymerase chain reaction (PCR) amplification rates. Next, five different mini-barcode primer

34 sets were compared based on amplification rates, and the three top-performing primer sets were

35 used in DNA sequencing. The incorporation of a clean-up step following DNA extraction

36 showed a slight advantage over DNA extraction alone, with a higher amplification rate (52.3%

37 vs. 47.8%) and A_{260}/A_{230} value (3.3 vs. 0.6). The three primer sets selected for DNA mini-

38 barcoding showed DNA sequencing rates of 54.5-65.9% among the 44 duplicate samples. When

39 the results for all three primer sets were combined, 18 of the 22 shark cartilage products were

40 identified to the species or genus level. On an individual basis, the best-performing primer set

41 identified 16 of the 22 products to the species or genus level. Overall, the protocol developed in

42 this study increased the identification rate for elasmobranches in cartilage products by more than

43 2-fold as compared to previous research.

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

45 identification

46

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

69   lack of trade data, improved methods to identify and track species in shark cartilage

70   supplements are needed.

71   DNA-based techniques, such as DNA barcoding and species-specific polymerase

72   chain reaction (PCR), are commonly used to identify elasmobranch species when

73   morphological indicators are lacking (Appleyard, White, Vieira, & Sabub, 2018; Dudgeon, et

74   al., 2012; Hanner, Naaum, & Shivji, 2016; Rodrigues-Filho, Pinhal, Sondre, & Vallinoto,

75   2012). While species-specific PCR is preferable for targeted approaches, DNA barcoding is

76   more appropriate for applications involving a wide range of species, such as shark cartilage

77   supplements. DNA barcoding is a sequencing-based method for species identification that

78   uses universal primer sets to target a short, standardized region of the genome (Hebert,

79   Cywinska, Ball, & DeWaard, 2003). The most common genetic region targeted for the

80   identification of animal species is a ~650 base pair (bp) region of the mitochondrial gene

81   coding for cytochrome *c* oxidase subunit I (CO1). However, it has proven challenging to

82   recover the full-length DNA barcode from highly processed samples due to DNA

83   fragmentation (Fields, Abercrombie, Eng, Feldheim, & Chapman, 2015; Shokralla, Hellberg,

84   Handy, King, & Hajibabaei, 2015).

85   An alternative approach for identification of processed samples is DNA mini-barcoding,

86   which targets shorter regions of the full-length barcode. For example, Shokralla et al. (2015)

87   developed a fish mini-barcoding system using the cytochrome *c* oxidase subunit I (COI) gene

88   that enabled identification of 88.6% of processed fish products tested, as compared to 20.5%

89   identification success with full barcoding. DNA barcoding and mini-barcoding techniques

90   have been used to identify elasmobranch species in a range of commercial products,

91   including dried shark fin, shark meat, and shark fin soup (Barbuto, et al., 2010; Cardeñosa, et

4

92   al., 2017; Chuang, Hung, Chang, Huang, & Shiao, 2016; Fields, et al., 2015; R.S. Hellberg,

93   Isaacs, & Hernandez, 2019; O'Bryhim, Parsons, & Lance, 2017; Steinke, et al., 2017). A

94   number of studies have also differentiated shark species utilizing DNA barcoding combined

95   with character-based analysis, which is based on the presence or absence of specific

96   nucleotides determined to be diagnostic for a given species (Fields et al. 2015; Velez-Zuazo

97   et al. 2015; Wong et al. 2009).

98   DNA barcoding of shark cartilage supplements has proven challenging, likely due to

99   factors such as DNA degradation during processing, the presence of PCR inhibitors, and the

100   possibility of multiple species in one product (R.S. Hellberg, et al., 2019). Hellberg et al. (2019)

101   utilized a combination of *COI* full and mini-barcoding to identify elasmobranch species in shark

102   products, including cartilage supplements. Full-length barcoding of shark cartilage supplements

103   enabled identification in only 20.7% of these products, as compared to 51.7% of supplements

104   identified with shark mini-barcoding. Optimization of DNA extraction and amplification

105   techniques may improve DNA barcoding performance, thereby enabling identification of species

106   in a greater number of shark cartilage pills.

107   The objective of this study was to develop a mini-barcoding protocol for the

108   identification of elasmobranch species in shark cartilage pills through optimization of DNA

109   extraction, amplification, and sequencing techniques. The effectiveness of a clean-up step

110   following DNA extraction was examined; five mini-barcoding primer sets were compared based

111   on PCR amplification rates; and uni-directional sequencing was compared to bi-directional

112   sequencing for the three top-performing primer sets.

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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

138   concentration and purity of the DNeasy Kit plus PowerClean Kit DNA extracts were measured

139   with a Biophotometer Plus. All samples underwent PCR using the original shark mini-barcode

140   primers, followed by gel electrophoresis (as described below). The DNA concentration, DNA

141   purity, PCR amplification rates, and PCR amplification consistency for samples extracted with

142   the DNeasy Kit were compared to those of the samples extracted with the DNeasy Kit plus

143   PowerClean Kit to determine the optimal method to be used for the PCR primer comparison

144   studies.

145   *2.3 PCR primer comparison*

146   In order to compare PCR primer sets, DNA was extracted in duplicate from each sample

147   using the DNeasy Blood and Tissue Kit plus the PowerClean Pro CleanUp Kit. Each DNA

148   extract was then tested against five different primer sets (Table 1): the original shark mini-

149   barcode primer set (Fields, et al., 2015), two variations of the shark mini-barcode primer set (V1

150   and V2, designed in the current study), and two primer sets (SH-D and SH-E) used in a previous

151   study on fish species identification (Shokralla, et al., 2015). The V1 and V2 primer sets were

152   designed based on comparison with an alignment of 1049 elasmobranch sequences obtained

153   from GenBank (Accession numbers: FJ518910–FJ519800, FJ519802-FJ519959; Wong, et al.,

154   2009). M13 tails were attached to the SH-D, SH-E, and forward shark mini-barcode primers to

155   facilitate DNA sequencing (Table 1). PCR for the shark mini-barcode primer set and its

156   variations (V1 and V2) was carried out using 12.5 µL HotStar Taq Master Mix (Qiagen), 10.5

157   µL of molecular-grade sterile water, 0.5 µL of 10 µM forward primer cocktail, 0.5 µL of 10 µM

158   reverse primer, and 1.0 µL of template DNA. PCR for the SH-D and SH-E primer sets was

159   carried out using the same preparation mix as the shark mini-barcode primers, except that 9.5 µL

160   of sterile water, and 2.0 µL of template DNA were used. A no-template control (NTC)

161 containing sterile water in place of DNA was included in each set of reactions. Primers were

162 synthesized by Integrated DNA Technologies (Coralville, IA) and a Mastercycler Nexus

163 Gradient Thermal Cycler (Eppendorf) was used to carry out PCR.

164   The cycling conditions for shark mini-barcoding were: 95°C for 15 min; 35 cycles of

165 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

166 (R.S. Hellberg, et al., 2019). The same cycling conditions were used for the V1 and V2 shark

167 mini-barcoding primer sets except that the annealing temperatures were 46ºC and 54ºC,

168 respectively. These optimal annealing temperatures were determined based on the results of

169 gradient PCR at a temperature range of 43-60°C followed by gel electrophoresis, as described

170 below (Shokralla, et al., 2015). The cycling conditions for the SH-D primer set were: 95ºC for

171 15 min; 35 cycles of 94ºC for 40 s, 50ºC for 1 min, and 72ºC for 30 s; and a final extension step

172 at 72ºC for 5 min. The cycling conditions for the SH-E primer set were: 95ºC for 15 min; 35

173 cycles of 94ºC for 40 s, 46ºC for 1 min, and 72ºC for 30 s; and a final extension step at 72ºC for

174 5 min.

175 *2.4 Gel electrophoresis*

176   PCR products (4 µL) were loaded onto 2% agarose E-Gels (Life Technologies, Carlsbad,

177 CA) and run using the E-Gel 0.8-2.0% Program on an E-Gel iBase (Life Technologies) for 15

178 min (Rosalee S. Hellberg, Kawalek, Van, Shen, & Williams-Hill, 2014). The results were

179 visualized with FOTO/Analyst Express (Fotodyne, Hartland, WI) and Transilluminator FBDLT-

180 88 (Fisher Scientific, Waltham, MA) combined with FOTO/Analyst PCImage (version 5.0.0.0,

181 Fotodyne). Samples with visible bands of the expected size following electrophoresis were

182 considered positive for PCR amplification.

183

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}

10

229   ratios could not be determined for 7 of the 44 replicates tested with the DNeasy Kit alone

230   because the A_{280} value was not within the measuring range. Paired samples with missing data

231   were excluded from the statistical analysis, resulting in a total of 37 paired data points analyzed

232   by the Wilcoxon signed-rank test. The average A_{260}/A_{230} ratio for samples extracted with the

233   DNeasy Kit (0.6 ± 0.3) was significantly lower than that for 8 of 22 samples extracted with the

234   DNeasy Kit plus PowerClean Kit (3.3 ± 3.0). The A_{260}/A_{230} ratio could not be determined for a

235   portion of the 44 replicates tested with the DNeasy Kit alone ($n = 3$) and the DNeasy Kit plus

236   PowerClean Kit ($n = 14$) because the A_{230} value was not within the measuring range. Paired

237   samples with missing data were excluded from the statistical analysis, resulting in a total of 27

238   paired data points analyzed by the Wilcoxon signed-rank test. The lower A_{260}/A_{230} ratios for

239   samples extracted with the DNeasy Kit indicate the presence of contaminants that were removed

240   by the PowerClean Kit. These may have included residual guanidine from the silica column in

241   the DNeasy Kit or carbohydrates in the shark cartilage pills, such as cellulose, that were carried

242   over during extraction (Matlock, 2015).

243   PCR amplification rates for the two extraction methods were compared using the original

244   shark mini-barcode primer set (for example, see Supplementary Figure S1). As shown in Table

245   2, use of the DNeasy Kit plus PowerClean Kit resulted in visible bands in the gel for 23 of the 44

246   duplicate samples for a success rate of 52.3%, as compared to a success rate of 47.8% with the

247   DNeasy Kit alone. Samples extracted with the DNeasy Kit plus PowerClean Kit also showed

248   greater agreement among duplicate samples (95.5%) as compared to the DNeasy Kit (77.3%).

249   However, there were no significant differences between the two methods on the basis of PCR

250   amplification rate or consistency (McNemar's test, $p > 0.05$).

251   Overall, the DNeasy Kit plus PowerClean Kit was determined to be the optimal method

252   for extraction of DNA from shark cartilage pills. This was based on the significant increase in

253   the A_{260}/A_{230} values obtained with this method combined with the higher PCR amplification rate

254   and amplification consistency as compared to the DNeasy Kit alone. Although the DNeasy Kit

255   plus PowerClean Kit led to a significantly reduced DNA concentration, the DNA yield was

256   sufficient to allow for PCR amplification. These results indicate that the additional clean-up

257   steps carried out with the PowerClean Kit allowed for the removal of PCR inhibitors such as

258   carbohydrates and guanidine, thus improving PCR amplification rates and consistency.

259   *3.2 PCR primer comparison*

260   Out of the 44 duplicate samples tested with the five mini-barcode primer sets, 34 (77.3%)

261   could be amplified with at least one primer set (Fig. 1). On an individual basis, the shark mini-

262   barcode V1 and V2 primer sets were the most successful (68.2%), followed by the original shark

263   mini-barcode primer set (56.8%). The amplification rate for the mini SH-D and mini SH-E

264   primer sets (22.7%) was significantly lower than the rates obtained with the original shark mini-

265   barcode, shark mini-barcode V1, and shark mini-barcode V2, according to a Cochran's Q test

266   followed by Dunn's test with the Bonferroni correction ($p < 0.05$). The decreased amplification

267   rates observed for the mini SH-D and SH-E primer sets were likely because they were not

268   designed for the specific amplification of shark species, but rather for the universal amplification

269   of processed fish species. On the other hand, the shark mini-barcode primer sets utilize a

270   universal forward fish cocktail combined with a reverse primer specifically designed to amplify

271   shark species (Table 1). Based on the results of PCR amplification, the original shark mini-

272   barcode and the shark mini-barcode V1 and V2 primer sets were selected for use in DNA

273   sequencing.

274   *3.3 DNA sequencing*

275   Out of the 44 duplicate samples sequenced with the three shark mini-barcode primer sets,

276   33 (75%) were sequenced with at least one primer set (Table 3). Sequencing rates for the

277   individual primer sets and sequencing methods (uni-directional and bi-directional) were not

278   significantly different according to Cochran's Q test ($p > 0.05$). The shark mini-barcode V2

279   primer set showed the highest sequencing rates of 65.9% and 63.6% for bi-directional and uni-

280   directional methods, respectively. Sequencing rates for the other primer sets were all <60%.

281   When comparing bi-directional sequencing with uni-directional sequencing, the former had a

282   higher sequencing rate for two out of the three primer sets (V1 and V2).

283   The average sequence length (113 ± 16 bp) for all primer sets combined was close to the

284   target sequence length of 127 bp and the average percent ambiguities was low, at $0.09 \pm 0.33\%$

285   (Table 3). The sequence lengths obtained with bi-directional sequencing for each primer set

286   were significantly higher than those obtained with uni-directional sequencing, according to the

287   Kruskal-Wallis H test and Dunn's post hoc test with the Bonferroni correction, adjusted $p <$

288   0.05). The bi-directional sequences showed a combined average of $0.18 \pm 0.44\%$ ambiguities,

289   with no significant differences across the three primer sets.

290   As shown in Table 3, the sequencing quality scores had a combined average of $44.2 \pm$

291   30.6% HQ. Similar to the results for sequencing length, the average quality score was highest

292   for the bi-directional results ($54.3 \pm 32.9\%$ HQ) as compared to the uni-directional results (33.8

293    $\pm 24.1\%$ HQ). However, the only statistical difference was that the quality score for the bi-

294   directional shark mini-barcode ($70.6 \pm 17.1\%$ HQ) was significantly higher than the other quality

295   scores, according to the Kruskal-Wallis H test and Dunn's post hoc test with the Bonferroni

296   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

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320   in duplicate is illustrated by the fact that there were 3-5 samples per primer set for which only

321   one of the duplicate samples could be identified (Table 4).

322   The modified primer sets (V1 and V2) used in the current study enabled the identification

323   of two species that were not detected with the original shark mini-barcode primer set: winter

324   skate (*Leucoraja ocellata*) and spiny dogfish (*Squalus acanthias*). These species are considered

325   endangered and vulnerable, respectively, according to the IUCN Red List of Threatened Species

326   (http://www.iucnredlist.org/). Although the samples identified as *L. ocellata* also matched a

327   sequence labeled as little skate (*Leucoraja erinacea*), this sequence was previously reported to be

328   misidentified and actually belong to winter skate (Coulson, et al., 2011; R.S. Hellberg, et al.,

329   2019). The inability of the original shark mini-barcode primer set to detect *L. ocellata* or *S.*

330   *acanthias* in the shark cartilage products is consistent with the results of Hellberg et al. (2019).

331   In contrast, Fields et al. (2015) reported the ability to identify *S. acanthias* in shark fin soup

332   using this primer set. For optimal identification of species in shark cartilage pills, it is

333   recommended that samples be tested against all three primer sets described here (i.e., original

334   shark mini-barcode, V1, and V2).

335   *3.5 Shark mini-barcoding considerations*

336   An important consideration with regard to DNA barcoding is the level of genetic

337   similarity achieved between the query sequence and the reference sequences. Traditionally, a

338   cut-off of >2% genetic divergence has been applied to differentiate fish species based on the full

339   barcode (Handy, et al., 2011). However, many of the mini-barcode sequences obtained in the

340   current study showed multiple species matches with $\geq$ 98% genetic similarity. This occurred

341   most frequently with the *Carcharhinus* species, which are closely related and have been

342   previously reported to show high genetic similarity across the DNA barcode region (R.S.

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343   Hellberg, et al., 2019; Ward, Holmes, White, & Last, 2008). While the full-length DNA barcode

344   can effectively differentiate many of these species (e.g., see Appleyard et al. 2018; Wong et al.

345   2009), the shorter amplicon generated with mini-barcoding showed reduced differentiation

346   capability when relying on a distance-based approach. In some of these cases, the use of

347   character-based identification keys developed in previous studies (Fields, et al., 2015; Velez-

348   Zuazo, et al., 2015; Wong, et al., 2009) enabled differentiation (Table 4). For example, many

349   samples showed equivalent genetic matches to multiple members of the *Carcharhinus* genus in

350   BOLD and/or GenBank. When a set of diagnostic nucleotides published in Wong et al. (2009)

351   was applied to these sequences, it allowed for the identification of silky shark, a CITES-listed

352   species, in five of these samples.

353   In several instances, a secondary match showed > 99.22% genetic similarity to the top

354   match in BOLD, corresponding to less than 1 nucleotide difference in a 127 bp sequence. These

355   results were often due to the presence of an ambiguity or gap in the sequence of the secondary

356   match and there is potential for a mis-identification to occur when additional consideration is not

357   given to the secondary match. For example, one of the PS07 shark mini-barcode V2 sequences

358   showed a top match (97.58%) to scalloped hammerhead shark (*Sphyrna lewini*) and a secondary

359   match (97.32%) to spot-tail shark (*Carcharhinus sorrah*) in BOLD. However, character analysis

360   revealed that the query sequence was not *S. lewini* due to the absence of diagnostic nucleotides

361   described in Fields et al. (2015) and the sample was instead identified as *C. sorrah*.

362   The results discussed above support the use of character-based analysis in combination

363   with genetic similarity values, as recommended in previous studies on shark DNA barcoding

364   (Fields, et al., 2015; Velez-Zuazo, et al., 2015; Wong, et al., 2009). However, it should be noted

365   that character-based keys are limited in species number, subject to change, and sometimes cannot

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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

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389 shark mini-barcodes combined with next-generation sequencing and/or PCR cloning approaches

390 to identify mixed species in shark products. Additionally, the ability of a longer mini-barcode to

391 amplify in these products should be examined in order to improve differentiation of closely

392 related species.

393 **5. Acknowledgements**

397 **6. References**

398 Appleyard, S. A., White, W. T., Vieira, S., & Sabub, B. (2018). Artisanal shark fishing in Milne

399   Bay Province, Papua New Guinea: biomass estimation from genetically identified shark

400   and ray fins. *Sci. Rep., 8*(1), 6693.

401 Barbuto, M., Galimberti, A., Ferri, E., Labra, M., Malandra, R., Galli, P., & Casiraghi, M.

402   (2010). DNA barcoding reveals fraudulent substitutions in shark seafood products: The

403   Italian case of "palombo" (*Mustelus* spp.). *Food Res Int, 43*(1), 376-381.

404 Bräutigam, A., Callow, M., Campbell, I. R., Camhi, M. D., Cornish, A. S., Dulvy, N. K.,

405   Fordham, S. V., Fowler, S. L., Hood, A. R., McClennen, C., Reuter, E. L., Sant, G.,

406   Simpfendorfer, C. A., & Welch, D. J. (2015). Global Priorities for Conserving Sharks and

407   Rays: A 2015-2025 Strategy. Global Sharks and Rays Initiative.

408 Cardeñosa, D., Fields, A., Abercrombie, D., Feldheim, K., Shea, S. K. H., & Chapman, D. D.

409   (2017). A multiplex PCR mini-barcode assay to identify processed shark products in the

410   global trade. *PLoS ONE, 12*(10), e0185368.

411 Chuang, P. S., Hung, T. C., Chang, H. A., Huang, C. K., & Shiao, J. C. (2016). The species and

412   origin of shark fins in Taiwan's fishing ports, markets, and customs detention: A DNA

413   barcoding analysis. *PLoS ONE, 11*(1), e0147290.

414 CITES. (2019). *Convention on International Trade in Endangered Species of Wild Fauna and*

415   *Flora*. https://www.cites.org/eng (Accessed 4/12/2019).

416 Clarke, S. (2004). Shark Product Trade in Hong Kong and Mainland China and Implementation

417   of the CITES Shark Listings.

418 Coulson, M. W., Denti, D., Van Guelpen, L., Miri, C., Kenchington, E., & Bentzen, P. (2011).

419   DNA barcoding of Canada's skates. *Mol Ecol Resour, 11*(6), 968-978.

420 Dent, F., & Clarke, S. (2015). *State of the Global Market for Shark Products*. FAO Fisheries and

421   Aquaculture Technical Paper No. 590. Rome, FAO. 187 pp.

422 Desjardins, P., & Conklin, D. (2010). NanoDrop microvolume quantitation of nucleic acids. *J*

423   *Vis Exp, 45*, e2565.

424 Dudgeon, C. L., Blower, D. C., Broderick, D., Giles, J. L., Holmes, B. J., Kashiwagi, T., Krueck,

425   N. C., Morgan, J. A. T., Tillett, B. J., & Ovenden, J. R. (2012). A review of the

426   application of molecular genetics for fisheries management and conservation of sharks

427   and rays. *J Fish Biol, 80*(5), 1789-1843.

428 Ferretti, F., Worm, B., Britten, G. L., Heithaus, M. R., & Lotze, H. K. (2010). Patterns and

429   ecosystem consequences of shark declines in the ocean. *Ecology Letters, 13*(8), 1055-

430   1071.

431 Fields, A. T., Abercrombie, D. L., Eng, R., Feldheim, K., & Chapman, D. D. (2015). A novel

432   mini-DNA barcoding assay to identify processed fins from internationally protected shark

433   species. *PLoS One, 10*(2), e0114844.

434   Handy, S. M., Deeds, J. R., Ivanova, N. V., Hebert, P. D. N., Hanner, R., Ormos, A., Weigt, L.

435   A., Moore, M. M., Hellberg, R. S., & Yancy, H. F. (2011). *Single laboratory validated*

436   *method for DNA-barcoding for the species identification of fish for FDA regulatory*

437   *compliance* (Vol. 2017). Silver Spring, MD: United States Food and Drug

438   Administration.

439   Hanner, R. H., Naaum, A. M., & Shivji, M. S. (2016). DNA-Based Authentication of Shark

440   Products and Implications for Conservation and Management. In R. H. Hanner & A. M.

441   Nauum (Eds.), *Seafood Authenticity and Traceability: A DNA-based Perspective* (pp.

442   171-179). San Diego, CA, USA: Academic Press/Elsevier.

443   Hebert, P. D. N., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications

444   through DNA barcodes. *Proc. R. Soc. B Biol. Sci., 270*(1512), 313-321.

445   Hellberg, R. S., Isaacs, R. B., & Hernandez, E. L. (2019). Identification of shark species in

446   commercial products using DNA barcoding. *Fish Res, 210*(February), 81-88.

447   Hellberg, R. S., Kawalek, M. D., Van, K. T., Shen, Y., & Williams-Hill, D. M. (2014).

448   Comparison of DNA extraction and PCR setup methods for use in high-throughput DNA

449   barcoding of fish species. *Food Anal Methods, 7*(10), 1950-1959.

450   Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S.,

451   Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meinties, P., &

452   Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software

453   platform for the organization and analysis of sequence data. *Bioinformatics, 28*(12),

454   1647-1649.

455   Matlock, B. (2015). Assessment of Nucleic Acid Purity. *NanoDrop Spectrophotometers,*

456   *Technical Note 52646*.

457   Merly, L., & Smith, S. L. (2015). Pro-inflammatory properties of shark cartilage supplement.

458   *Immunopharmacol Immunotoxicol, 37*(2), 140-147.

459   Mondo, K., Glover, W. B., Murch, S. J., Liu, G. L., Cai, Y., Davis, D. A., & Mash, D. C. (2014).

460   Environmental neurotoxins beta-N-methylamino-L-alanine (BMAA) and mercury in

461   shark cartilage dietary supplements. *Food Chem Toxicol, 70*, 26-32.

462   O'Bryhim, J. R., Parsons, E. C. M., & Lance, S. L. (2017). Forensic species identification of

463   elasmobranch products sold in Costa Rican markets. *Fish Res, 186*, 144-150.

464   Rodrigues-Filho, L. F., Pinhal, D., Sondre, D., & Vallinoto, M. (2012). Shark DNA

465   Forensics: Applications and Impacts on Genetic Diversity. In M. Caliskan (Ed.),

466   *Analysis of Genetic Variation in Animals* (pp. 269-286). Rijeka, Croatia: InTech.

467   Rose, D. (1996). An Overview of World Trade in Sharks and Other Cartilaginous Fishes. A

468   TRAFFIC Network Report. 106 p.

469   Shokralla, S., Hellberg, R. S., Handy, S. M., King, I., & Hajibabaei, M. (2015). A DNA mini-

470   barcoding system for authentication of processed fish products. *Sci Rep, 5*, 15894.

471   Sim, J.-S., Im, A. R., Cho, S. M., Jang, H. J., Jo, J. H., & Kim, Y. S. (2007). Evaluation of

472   chondroitin sulfate in shark cartilage powder as a dietary supplement: Raw materials and

473   finished products. *Food Chem, 101*(2), 532-539.

474   Steinke, D., Bernard, A. M., Horn, R. L., Hilton, P., Hanner, R., & Shivji, M. S. (2017). DNA

475   analysis of traded shark fins and mobulid gill plates reveals a high proportion of species

476   of conservation concern. *Sci Rep, 7*, 9505.
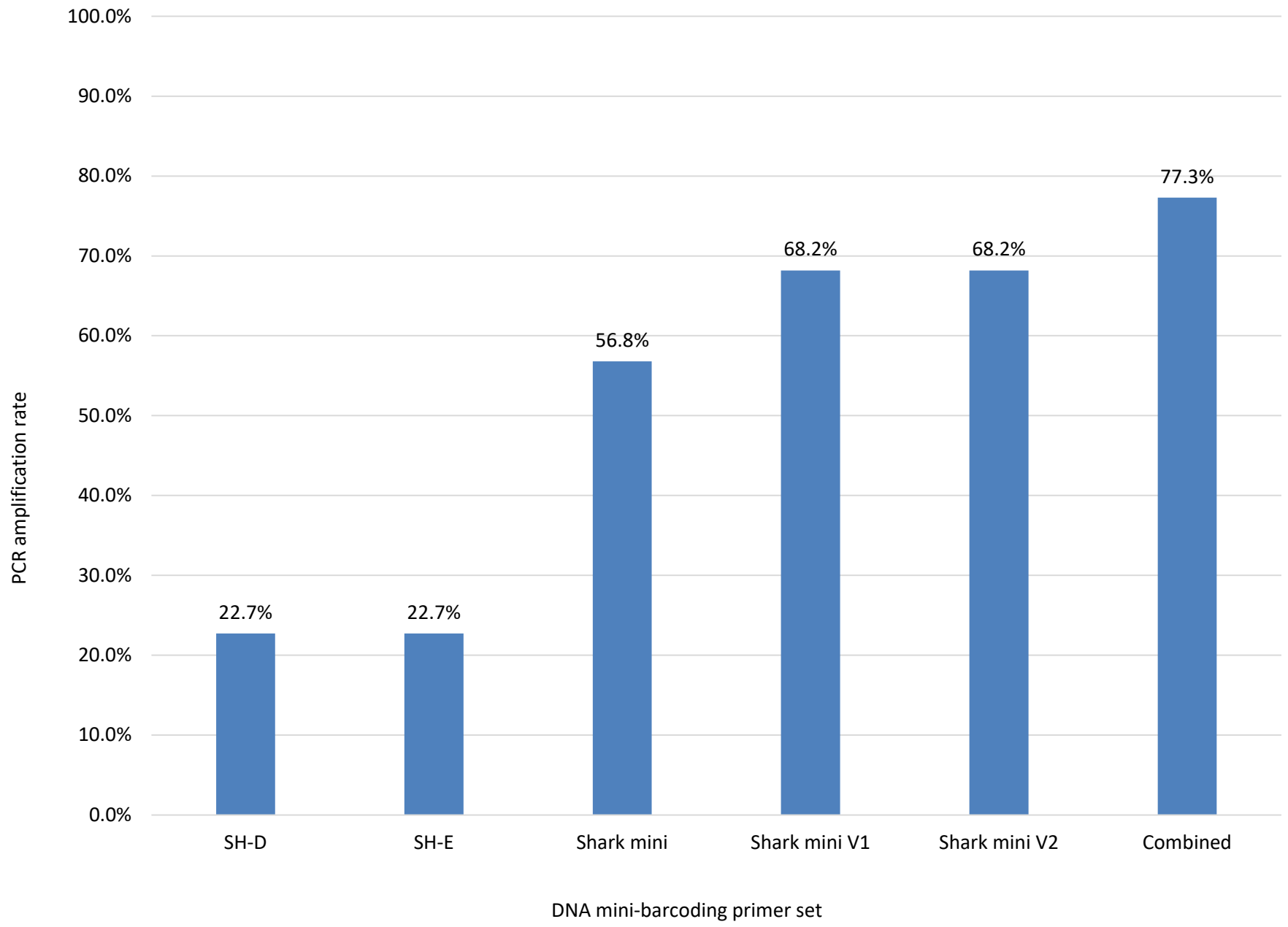
477   Velez-Zuazo, X., Alfaro-Shigueto, J., Mangel, J., Papa, R., & Agnarsson, I. (2015). What

478   barcode sequencing reveals about the shark fishery in Peru. *Fish Res, 161*, 34-41.

479   Ward, R. D., Holmes, B. H., White, W. T., & Last, P. R. (2008). DNA barcoding Australasian

480   chondrichthyans: results and potential uses in conservation. *Mar Freshwater Res, 59*(1),

481   57-71.

482   Weigt, L., Driskell, A., Baldwin, C., & Ormos, A. (2012). DNA barcoding fishes. In W. J. Kress

483   & D. L. Ericksono (Eds.), *DNA barcodes methods and protocols* (pp. 109-126). New

484   York: Humana Press.

485   Wong, E. H. K., Shivji, M. S., & Hanner, R. H. (2009). Identifying sharks with DNA barcodes:

486   assessing the utility of a nucleotide diagnostic approach. *Mol Ecol Resour, 9*, 243-256.
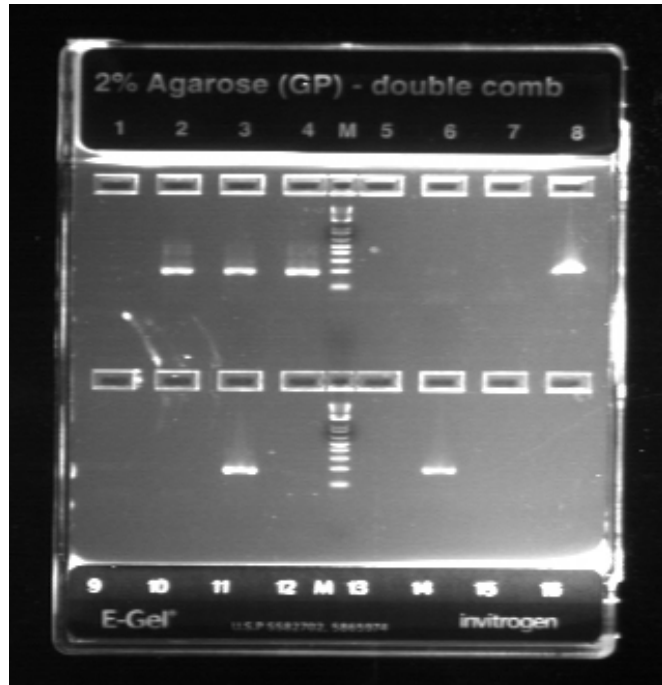
487

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.
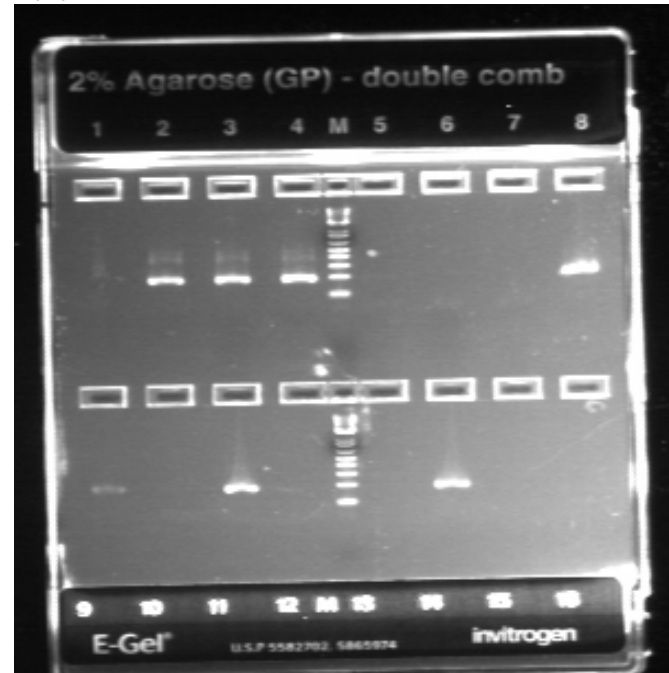
Supplementary Figure S1: Example of gel electrophoresis results measuring PCR amplification rates of the original shark mini-barcode combined with (a) DNeasy Kit alone and (b) DNeasy Kit plus PowerClean. Lane assignments are the same in each gel and are as follows: Lane 1 = PS20, Lane 2 = PS21, Lane 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	TGTAAAACGACGGCCAGTCAACCAACCA CAAAGACATTGGCAC	127 bp	(Fields, et al., 2015; Ivanova, Zemlak, Hanner, & Hebert, 2007)
	FishF2_t1	Forward	TGTAAAACGACGGCCAGTCGACTAATCAT AAAGATATCGGCAC		
	Shark COI-MINIR	Reverse	AAGATTACAAAGCGTGGGC		(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	AAGATTATTACAAAGCRTGRGC		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	CACGACGTTGTAAAACGACGGIACIGGITG RACIGTITAYCCYCC	208 bp	(Shokralla, et al., 2015)
	Mini_SH-D_R	Reverse	GGATAACAATTTCACACAGGGTRATICCIG CIGCIAGIAC		
Mini_SH-E	Mini_SH-E_F	Forward	CACGACGTTGTAAAACGACACYAAICAYA AAGAYATIGGCAC	226 bp	(Shokralla, et al., 2015)
	Mini_SH-E_R	Reverse	GGATAACAATTTCACACAGGCTTATRTTR TTTATICGIGGRAAIGC		

[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