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1 Evaluating manta ray mucus as an alternative DNA source for population genetics study:
2 underwater-sampling, dry-storage and PCR success

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12

13 Abstract

14 Sharks and rays are increasingly being identified as high-risk species for extinction,
15 prompting urgent assessments of their local or regional populations.

16 Advanced genetic analyses can contribute relevant information on effective population size
17 and connectivity among populations although acquiring sufficient regional sample sizes can
18 be challenging. DNA is typically amplified from tissue samples which are collected by hand
19 spears with modified biopsy punch tips. This technique is not always popular due mainly to a
20 perception that invasive sampling might harm the rays, change their behaviour, or have a
21 negative impact on tourism. To explore alternative methods, we evaluated the yields and PCR
22 success of DNA template prepared from manta ray mucus collected underwater and captured
23 and stored on a Whatman FTATM Elute card. The pilot study demonstrated that mucus can be
24 effectively collected underwater using a toothbrush. DNA stored on cards was found to be
25 reliable for PCR-based population genetic studies. We successfully amplified mtDNA ND5,
26 nuclear DNA RAG1, and microsatellite loci for all samples. As the yields of DNA with the
27 tested method were low, further improvements are desirable for assays that may require larger
28 amounts of DNA, such as population genomic studies using emerging next-gen sequencing.

29

30

31 Introduction

32

33 Sharks and rays are increasingly being identified as high-risk species for extinction,
34 prompting urgent assessments of their local or regional populations

35 (Dulvy et al. 2014a). The Reef Manta Ray *Manta alfredi* (Kreffft 1868) and the Giant Manta
36 Ray *M. birostris* (Walbaum 1792) are currently listed as Vulnerable by the International
37 Union for the Conservation of Nature (IUCN) Red List of Threatened Species in 2011
38 (Marshall et al. 2011a; Marshall et al. 2011b). Both species have been listed on Appendix I &
39 II of the Convention for Migratory Species (CMS) and both species were recently awarded
40 Appendix II listing on the Conventions on International Trade in Endangered Species of Wild
41 Fauna and Flora (CITES). These key conservation steps represent the first significant
42 movement to address reported global declines in manta rays (Vincent et al. 2013). Manta rays
43 have been described as having extremely conservative life history traits, representing one of
44 the least fecund elasmobranch species and with one of the lowest maximum intrinsic rates of
45 population increase of any studied Chondrichthyan (Couturier et al. 2012; Dulvy et al.
46 2014b).

47

48 A crucial knowledge gap still exists in the empirical understanding of their population
49 dynamics, structure, status and trends, which needs to be addressed for the implementation of
50 effective management (CITES 2013). DNA-based population studies can complement
51 logistically and financially challenging long-term field studies by providing insights into the
52 patterns of population structure, connectivity, and effective population sizes (Dudgeon et al.
53 2012; Schwartz et al. 2007).

54

55 Apart from samples taken from landed specimens, manta ray tissue samples are typically
56 collected underwater while SCUBA or free diving using hand spears with modified biopsy
57 punch tips (Figure 1). Tourism operators are often briefed about the importance of tissue
58 collection and cooperate with field researchers (Kashiwagi et al. 2012b). However, as manta
59 rays are a major attraction for tourism (O'Malley et al. 2013), such sampling activity has also
60 been discouraged in some areas where people fear that invasive sampling might harm the rays,
61 change their behaviour, or have a negative impact on tourism (Braithwaite 2010; Huntingford
62 et al. 2006; Rose et al. 2014). National Parks and protected areas may prohibit the use of
63 invasive sampling techniques for research on threatened species populations contained within
64 their boundaries and minimally invasive techniques to study these animals are constantly
65 being refined (Marshall & Pierce 2012). As such, the availability of alternative methods to
66 collect DNA from manta rays would facilitate an increase in sampling opportunities as a
67 matter of urgency.

68

69 Here we test the feasibility of the collection of body surface mucus from wild manta rays and

70 its effectiveness as a DNA source for PCR-based population genetics studies.
71 Epidermal cells in surface mucus have been successfully used in many studies for humans,
72 livestock, and wild animals (Gustavsson et al. 2009; Le Vin et al. 2011; McClure et al. 2009;
73 Prunier et al. 2012; Smith & Burgoyne 2004), but only a handful of studies exist that have
74 examined large marine fish (Hoolihan et al. 2009; Lieber et al. 2013). Lieber et al. (2013),
75 recently reported an ~75% PCR success rate using mucus from the Basking Shark *Cetorhinus*
76 *maximus* (Gunnerus 1765), stored in 99 % ethanol, in amplifying the high copy number
77 mitochondrial DNA (mtDNA) genes cytochrome c oxidase subunit 1 (CO1) and control
78 region (CR) and the nuclear ribosomal internal transcribed spacer 2 (ITS2) region. The
79 feasibility of using mucus from other sharks and rays has been largely unexplored,
80 particularly in regards to underwater collection, amplification of single copy nuclear genes
81 and microsatellites, and dry storage methods that may eliminate the needs for special shipping
82 considerations and freezers (Smith & Burgoyne 2004; Williams 2007). Here we report
83 preliminary results on the effectiveness of this technique, its limitations, and its applicability
84 to future manta ray research. We also review potential areas for improvement and future
85 directions.

86 87 Materials and methods

88
89 All procedures were conducted in accordance to the University of Queensland Animal Ethics
90 Committee approval number SBMS/206/11/ARC and Ecuadorian Ministry of the
91 Environment, research permits: 009RM-DPM-MA.

92
93 Mucus from eighteen *Manta birostris* was collected on SCUBA from Isla de la Plata in
94 Ecuador (1° 15 29.62S, 81° 4 25.96W) between 2 September and 20 September 2012. Samples
95 were obtained using a small toothbrush held in the diver's hand (Video 1) or mounted on an
96 extendable pole (Figure 2). For each sample, the dorsal surface of the ray was rubbed back
97 and forth or in a circular motion ~3-5 times whereupon the brush was placed into an
98 individual 50 ml plastic tube to prevent cross contamination. On dry land, approximately 120
99 μ l mucus was transferred from brush with a clean sterile cotton bud and then onto FTA™
100 Elute Cards and/or Indicating FTA™ Elute Cards (GE Healthcare) using three side-to-side
101 motions, 90° each way (Figure 3), spreading mucus and cells evenly to an area of
102 approximately 625 mm². These cards, which are impregnated with a chemical formula that
103 lyses cells and denatures proteins upon contact, are designed for room temperature storage
104 and shipment of DNA from biological samples for PCR analysis. The applied volume of
105 liquid samples is the recommended amount to avoid overloading the chemicals (GE
106 Healthcare). Cards were then air dried and placed in separate resealable plastic bags. Samples
107 were then transported via land and air as normal domestic and international postage and kept
108 at room temperature with desiccants until further analysis in the lab.

109

110 DNA for downstream analyses was prepared using the recommended simple protocol for
111 FTA™ Elute Cards that releases single stranded DNA (ssDNA) into water. Three squares (6
112 mm × 6 mm × 3) were cut out using a clean scalpel, washed by pulse-vortex in 1.5 ml of pure
113 water for 5 seconds, then placed in 300 µl of pure water and heated at 98 °C for 30 minutes.
114 At the end of the incubation step, tubes went through 60 times pulse-vortex at a rate
115 approximately one pulse/second. The cut-outs were removed from tubes and eluates were
116 stored at -20 °C until further analyses.

117

118 The quality and quantity of template DNA was assessed with commonly used
119 spectrophotometry (NanoDrop™ 1000, Thermo Scientific), fluorometry (Qubit™ ssDNA
120 Assay Kit, Invitrogen) and 1 % agarose gel electrophoresis. PCR success on mtDNA (ND5),
121 nuclear DNA (RAG1) and three microsatellite loci (MA09, MA14 and MA34) was assessed
122 using published protocols and 1~4 µl of template DNA in 12 ~ 20 µl reaction (Kashiwagi et al.
123 2012a; Kashiwagi et al. 2012b). PCR products were sequenced and genotyped and compared
124 with known types (Kashiwagi et al. 2012a; Kashiwagi et al. 2012b).

125

126 Results

127

128 Time between sampling and lab analyses ranged from 81 to 343 days.
129 Spectrophotometric measurements of the concentration of DNA templates ranged from 12.18
130 to 29.00 ng/µl (23.16 ± 4.05 ng/µl, mean \pm s.d., n = 18). Absorbance spectra lacked the
131 typical peak at wavelength 260 nm observable in DNA templates prepared from tissue
132 samples using a commercial DNA extraction kit (e.g. Qiagen DNeasy Kit). Instead, spectra
133 showed high absorbance around wavelength 230 - 240 nm, that was also present in blank (i.e.
134 card only) sample (Figure 4). Fluorometric measurements ranged from 0.0743 to 2.16 ng/µl
135 (0.589 ± 0.536 ng/µl, mean \pm s.d., n = 18). There was no visible band or smear with gel
136 electrophoresis loaded with 10 µl of samples. Samples concentrated approximately ten times
137 by both standard ethanol precipitation and vacuum drying also failed to show a band or smear.
138 PCR was successful for all five markers (ND5, RAG1 and three microsatellite loci) across all
139 samples. These PCR products were successfully sequenced and genotyped consisting with
140 known types.

141

142 Discussion

143

144 Our results demonstrate that DNA from manta ray mucus collected underwater and stored dry
145 on FTA™ Elute cards can be reliably used in PCR-based population genetic studies. To our
146 knowledge, this study is the first example involving underwater collection of mucus by
147 SCUBA divers. The advantages of the developed method include: (i) a reduction in sampling

148 gear, (ii) a significantly reduced impact on the sampled organism, (iii) an increased
149 acceptance as a sampling protocol in region of vibrant tourism, and (iv) reliable, dry, room
150 temperature storage of DNA without need for liquid reagents, refrigerator/freezer, and special
151 shipping considerations.

152

153 Capturing mucus from both species of Manta was easily achieved, but should only be
154 attempted by experienced field researchers that understand the behaviour of these animals.

155 Minimal to no reaction to sampling was noted in all samples taken from manta rays in

156 Ecuador by experienced field researchers (See Video 1). Good quality samples were obtained
157 on SCUBA and whilst free diving. Several collection tools were initially tested to trap mucus

158 from the dorsal and ventral surfaces of mantas including scouring pads, cotton buds, cotton
159 wool, and a small comb, but small disposable toothbrushes were found to be most effective.

160 Larger samples of mucus were always obtained using the toothbrush in the hand rather than
161 attached to an extendable pole, however both techniques resulted in adequate samples.

162 Researchers wanting to sample individual manta rays that cannot be approached closely

163 underwater or that are sampling manta rays at the surface from a boat may benefit from the

164 latter technique. Better quality mucus samples were taken with circular brushing motions and

165 from the dorsal surface rather than the ventral surface. The black pigment on the dorsal

166 surface of both species of manta sloughs off considerably (Coles 1916) tinting the mucus and

167 making it very obvious that a good quality sample was taken. This characteristic allows for

168 the confirmation of acquiring a good quality mucus sample during underwater collection and

169 also allows for confidence of mucus transfer to the FTA Elute Cards.

170

171 DNA yields from FTA Elute cards using the simple purification method were low judging

172 from the lack of visible DNA in gel electrophoresis and fluorometric measurements showing

173 that only three out of 18 templates were above 1 ng/μl. The low absorbance and the lack of

174 distinct peak at 260 nm in spectrometry also indicate that the calculated concentration may be

175 inaccurate. This is an expected result judging from the manufacturer's product information

176 (GE Healthcare Life Sciences 2012) and empirical findings (de Vargas Wolfgramm et al.

177 2009), which state that the single stranded DNA eluted from FTA Elute cards using the

178 simple protocol is often below the lower detection limit of the current spectrophotometer. As

179 such, we recommend fluometric quantitation as an important first step in downstream

180 analyses for avoiding genotyping errors by using too little copy number of template DNA

181 (Taberlet et al. 1996; Taberlet et al. 1999). Furthermore, it is safest to assume that the amount

182 of cell materials in a given volume of mucus is low. Therefore, it is important that the sampler

183 spread the mucus evenly and fully onto the card. Indeed, we observed variable PCR success

184 when preparing DNA template from three 3mm diameter punches, where the samples were

185 simply tapped onto the card directly from the brush (Maxwell and Christensen, unpublished

186 data).

187

188 We successfully sequenced *M. alfredi* using this method as well (Maxwell and Christensen,
189 unpublished data). We recommend that the potential utilization of mucus samples beyond the
190 basic PCR based assay be explored further because high quality and quantity of DNA will
191 likely become increasingly important for population genomic analyses with emerging
192 technological advancement in high throughput sequencing (Allendorf et al. 2010; Hohenlohe
193 et al. 2012; Narum et al. 2013). Higher yields may be possible by the use of special recovery
194 kit for FTA card (Mas et al. 2007; McClure et al. 2009; Stangegaard et al. 2011) or use of
195 alternative storage media (Allen-Hall & McNevin 2013; Ivanova & Kuzmina 2013; Lee et al.
196 2012). Whole genome amplification may be useful for generating suitable quantities of DNA
197 from minute amounts (Pinard et al. 2006). At the same time, presence of foreign DNA in the
198 mucus and its effect in downstream analyses should be investigated in the near future.

199

200 In conclusion, we demonstrated that mucus samples collected underwater can be effectively
201 used for PCR based population genetic studies in manta rays. This newly described method
202 may create new opportunities to study sensitive or threatened species in regions where tissue
203 sampling had been discouraged or prevented previously.

204 However, tissue sampling remains as the most preferred option for DNA sampling until more
205 conclusive testing on yields and presence of foreign DNA are completed and for additional
206 reasons that tissues are also useful for research applications such as fatty acid and stable
207 isotope analyses (Couturier et al. 2013a; Couturier et al. 2013b).

208

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215

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217

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322

323



Figure 1. Tissue sampling with a biopsy tip and a hand spear.



Figure 2. Mucus sampling with a toothbrush mounted on an extendable pole.

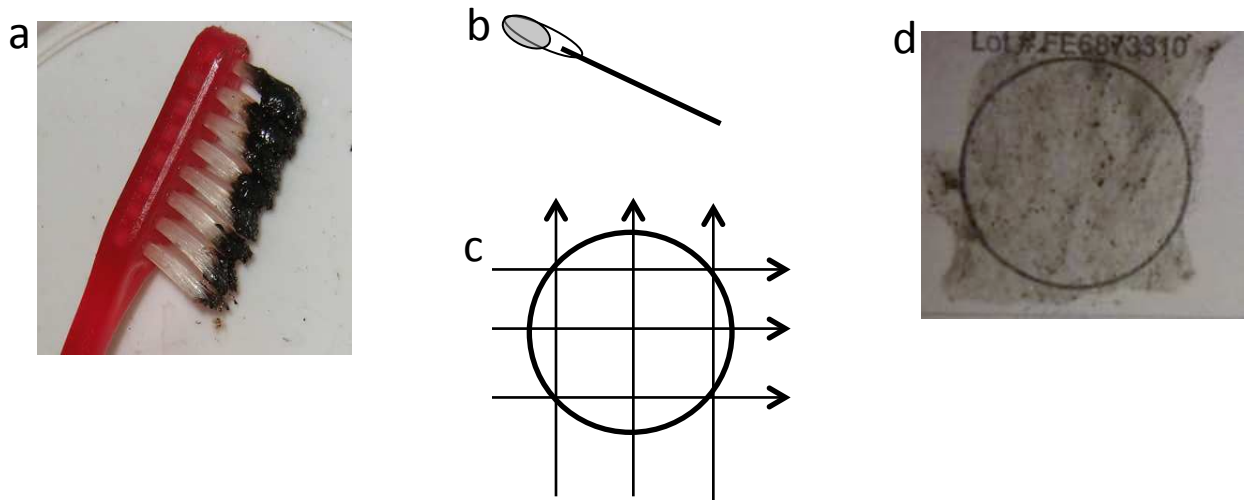


Figure 3. Application of mucus to FTA card (a) Black mucus collected on tooth brush, (b) Cotton bud with $\sim 120 \mu\text{l}$ of mucus, (c) Transferring mucus onto FTA card using three side-to-side motions, 90° each way, (d) FTA card with mucus sample.

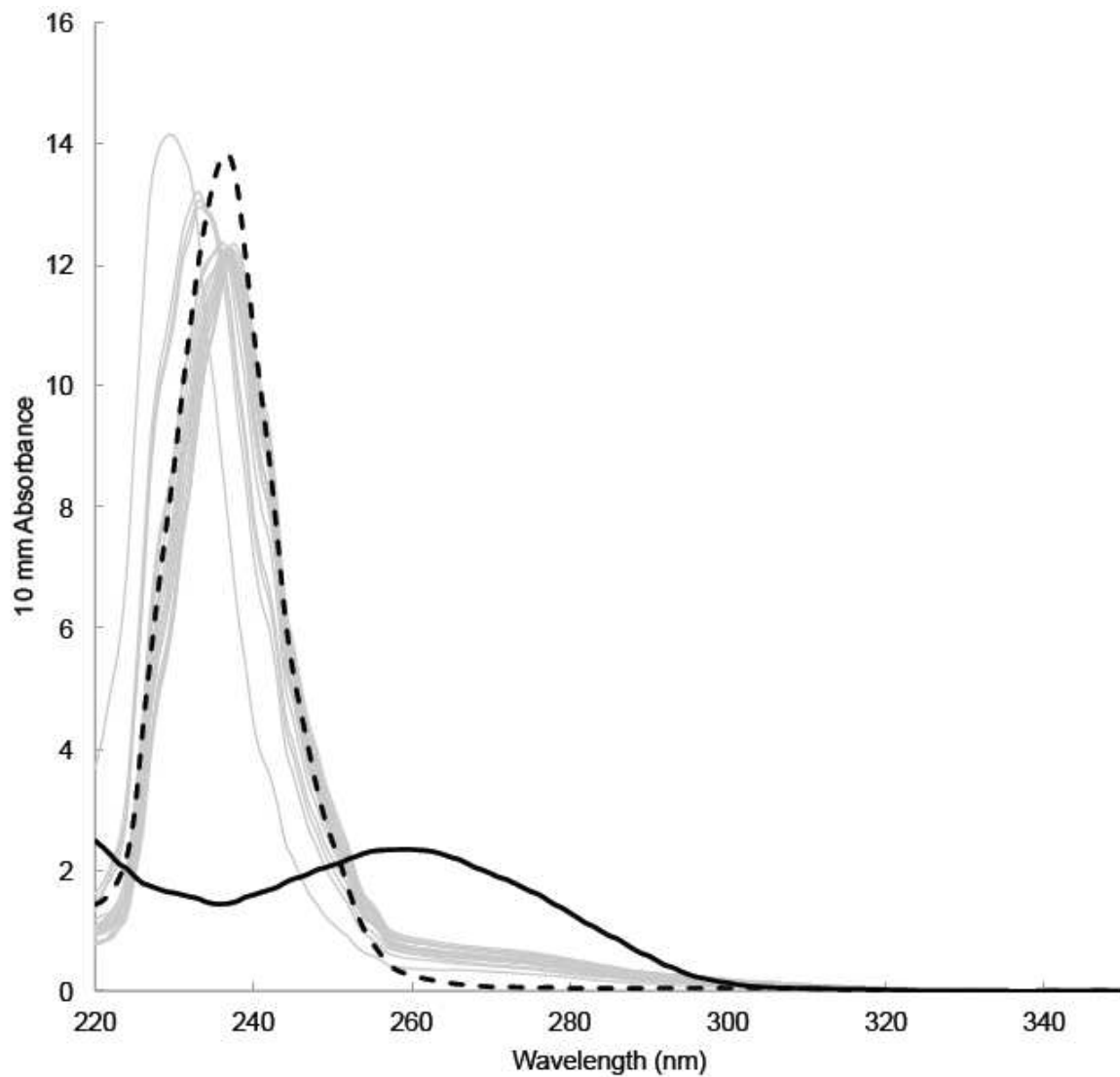


Figure 4. Absorbance spectrum of DNA prepared from mucus samples (grey lines), blank (i.e. card only, black dotted line) and a tissue sample with DNA extraction kit (black solid line) measured by NanodropTM.