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

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Abstract

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

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

Introduction

Sharks and rays are increasingly being identified as high-risk species for extinction, prompting urgent assessments of their local or regional populations (Dulvy et al. 2014a). The Reef Manta Ray *Manta alfredi* (Krefft 1868) and the Giant Manta Ray *M. birostris* (Walbaum 1792) are currently listed as Vulnerable by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species in 2011 (Marshall et al. 2011a; Marshall et al. 2011b). Both species have been listed on Appendix I & II of the Convention for Migratory Species (CMS) and both species were recently awarded Appendix II listing on the Conventions on International Trade in Endangered Species of Wild Fauna and Flora (CITES). These key conservation steps represent the first significant movement to address reported global declines in manta rays (Vincent et al. 2013). Manta rays have been described as having extremely conservative life history traits, representing one of the least fecund elasmobranch species and with one of the lowest maximum intrinsic rates of population increase of any studied Chondrichthyan (Couturier et al. 2012; Dulvy et al. 2014b).

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

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

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

its effectiveness as a DNA source for PCR-based population genetics studies.

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

Materials and methods

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

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

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

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

Results

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

Discussion

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

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

Capturing mucus from both species of Manta was easily achieved, but should only be attempted by experienced field researchers that understand the behaviour of these animals. Minimal to no reaction to sampling was noted in all samples taken from manta rays in Ecuador by experienced field researchers (See Video 1). Good quality samples were obtained on SCUBA and whilst free diving. Several collection tools were initially tested to trap mucus from the dorsal and ventral surfaces of mantas including scouring pads, cotton buds, cotton wool, and a small comb, but small disposable toothbrushes were found to be most effective. Larger samples of mucus were always obtained using the toothbrush in the hand rather than attached to an extendable pole, however both techniques resulted in adequate samples. Researchers wanting to sample individual manta rays that cannot be approached closely underwater or that are sampling manta rays at the surface from a boat may benefit from the latter technique. Better quality mucus samples were taken with circular brushing motions and from the dorsal surface rather than the ventral surface. The black pigment on the dorsal surface of both species of manta sloughs off considerably (Coles 1916) tinting the mucus and making it very obvious that a good quality sample was taken. This characteristic allows for the confirmation of acquiring a good quality mucus sample during underwater collection and also allows for confidence of mucus transfer to the FTA Elute Cards.

DNA yields from FTA Elute cards using the simple purification method were low judging from the lack of visible DNA in gel electrophoresis and fluorometric measurements showing that only three out of 18 templates were above 1 ng/µl. The low absorbance and the lack of distinct peak at 260 nm in spectrometry also indicate that the calculated concentration may be inaccurate. This is an expected result judging from the manufacturer's product information (GE Healthcare Life Sciences 2012) and empirical findings (de Vargas Wolfgramm et al. 2009), which state that the single stranded DNA eluted from FTA Elute cards using the simple protocol is often below the lower detection limit of the current spectrophotometer. As such, we recommend flurometric quantitation as an important first step in downstream analyses for avoiding genotyping errors by using too little copy number of template DNA (Taberlet et al. 1996; Taberlet et al. 1999). Furthermore, it is safest to assume that the amount of cell materials in a given volume of mucus is low. Therefore, it is important that the sampler spread the mucus evenly and fully onto the card. Indeed, we observed variable PCR success when preparing DNA template from three 3mm diameter punches, where the samples were simply tapped onto the card directly from the brush (Maxwell and Christensen, unpublished data).

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

In conclusion, we demonstrated that mucus samples collected underwater can be effectively used for PCR based population genetic studies in manta rays. This newly described method may create new opportunities to study sensitive or threatened species in regions where tissue sampling had been discouraged or prevented previously.
However, tissue sampling remains as the most preferred option for DNA sampling until more conclusive testing on yields and presence of foreign DNA are completed and for additional reasons that tissues are also useful for research applications such as fatty acid and stable isotope analyses (Couturier et al. 2013a; Couturier et al. 2013b).

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

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

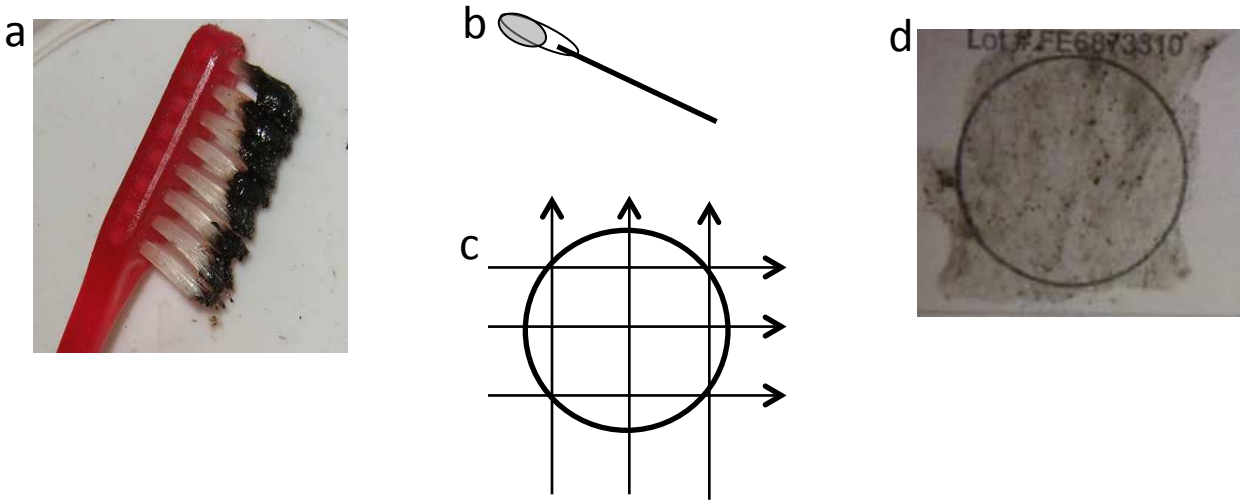

Figure 3. Application of mucus to FTA card (a) Black mucus collected on tooth brush, (b) Cotton bud with ~120 µl of mucus, (c) Transferring mucus onto FTA card using three side-to-side motions, 90° each way, (d) FTA card with mucus sample.

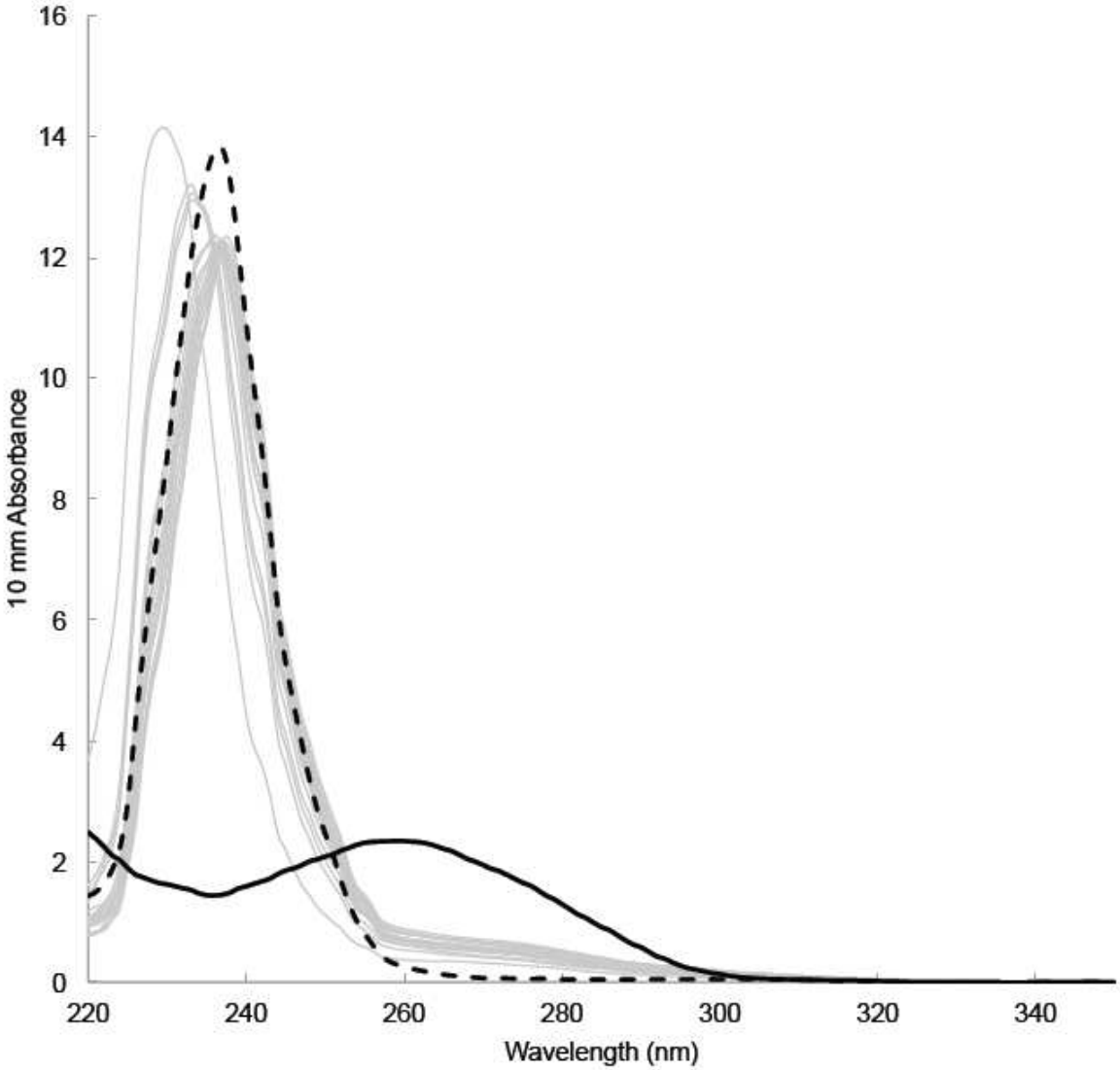

Figure 4. Absorbance spectrum of DNA prepared from mucus samples (grey lines), blank (i.e. card only, black dotted line) and a tissue sample with DNA extraction kit (black solid line) measured by Nanodrop™.