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PRIMER NOTE

Noninvasive methodology for the sampling and extraction of DNA from free-ranging Atlantic spotted dolphins (*Stenella frontalis*)

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Abstract

Genetic sampling and molecular investigations are important parts of studying wild populations. However, collecting tissues from free-ranging animals can be difficult or impractical. This study develops a sampling and extraction protocol for template DNA from faecal material collected in a marine environment from small cetaceans. DNA was extracted from faecal material of free-ranging Atlantic spotted dolphins (*Stenella frontalis*) and subsequently tested for its suitability in molecular investigations by amplifying both mitochondrial and nuclear DNA. The resulting mitochondrial sequences were found to closely match known *S. frontalis* haplotypes. Three microsatellite loci were amplified and fall within the expected size range for cetaceans. Mother and calf families previously assigned by observation were genetically confirmed using both mitochondrial haplotype and allele sharing between the mother and offspring. The protocol effectively collects and extracts dolphin DNA from faecal samples and enables species identification as well as confirmation of genetic relatedness and should be considered as a noninvasive alternative to current protocols.

Keywords: cetaceans, faeces, noninvasive genotyping, *Stenella frontalis*

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Introduction

Molecular genetic techniques have rapidly revolutionized the way natural populations are studied. The ease with which these techniques are applied to a wide variety of organisms and questions continue to improve. However, the collection of genetic material from free-ranging animals can prove challenging, particularly for agile open-water organisms such as cetaceans. Difficulty in collecting genetic material can be further exacerbated when traditional invasive techniques such as biopsy darting or blood sampling are impractical or are detrimental to ongoing studies. One response to these challenges has been the collection and use of faecal material, which contains sloughed intestinal cells of the host, as a noninvasive template in genetic investigations. This technique has proven successful in a number of terrestrial organisms such as birds (Broderick *et al*. 2003; Idaghdour *et al*. 2003), mountain lions (Ernest *et al*. 2000), lynx (Palomares *et al*. 2002), canids (Paxinos *et al*. 1997), bears (Taberlet *et al*. 1997; Wasser *et al*. 1997), barbary macaques (Lathuilliere *et al*. 2001), bonobos (Gerloff *et al*. 1995), chimpanzees (Bradley *et al*. 2000; Morin *et al*. 2001), gorillas (Bradley *et al*. 2000; Jensen-Seaman & Kidd 2001), and elephants (Fernando & Lande 2000; Okello *et al*. 2005). The use of faecal material for genetic sampling of aquatic animal groups has proven more challenging. However, for several semi-aquatic mammals such as river otters (Dallas *et al*. 2003) and seals (Reed *et al*. 1997), faecal material can be readily obtained terrestrially. The greatest challenge in the collection of substantial faecal material for genetic analysis lies in the fully aquatic mammals such as cetaceans and sirenians.

Among fully aquatic mammals, the difficulty of sample collection varies. Collecting faecal material from sirenians presents less of a challenge because adequate amounts of compact faecal matter float to the surface and may remain intact for several hours (Tikel *et al*. 1996). Concentrated samples can be collected even if the animal is no longer in the vicinity. In contrast, collecting faecal matter from small cetaceans such as dolphins is especially problematic since the animals defecate underwater in rapidly diffusing plumes

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as observed in this study and others (Parsons *et al*. 1999; Parsons 2001). Therefore, to obtain adequate amounts of faeces, researchers must be in the water when the animals defecate and immediately collect a sample before it disperses into an unusable state.

We describe here a technique for faecal DNA collection and extraction that was designed to support a long-term study of resident Atlantic spotted dolphins (*Stenella frontalis*) in the Bahamas (Herzing 1996; Herzing & Brunnick 1997; Herzing 1997; Herzing *et al*. 2003; Miles & Herzing 2003). This ongoing project examines the behavioural and acoustic aspects of Atlantic spotted dolphins in their natural environment. Therefore, any attempt to simultaneously sample genetic material must be noninvasive so that the integrity of the long-term data collection and ongoing studies is maintained. Prior to the development of the technique described here, previously published extraction techniques were attempted, however, they did not provide a reliable method for obtaining genomic DNA from *S. frontalis* samples. Therefore, the goal of this project was to develop a new laboratory protocol for the reliable extraction of genomic DNA from aquatic faecal samples of cetaceans, using *S. frontalis* as a model species.

Materials and methods

Sample collection

Faecal samples were collected from 15 individual Atlantic spotted dolphins (*Stenella frontalis*) on Little Bahama Bank in the northwestern Bahamas including three mother and calf families. Two family sets consisted of the mother and one calf, and the other set was comprised of the mother and two of her offspring. Prior to genetic analysis, mother and calf relationships were assigned based on the observed close association between a mother and calf in the first years of life (Herzing 1997).

Sampled dolphins were well separated from large groups and defecation was directly observed to make a positive identification of the individual and verified through underwater photographs and video. During behavioural encounters, snorkelers were in the water carrying sterile 100 mL air-filled, screw-top collection jars stored in a neoprene weight belt. Immediately following defecation (< 30 s), the snorkeler collected a portion of the faecal matter out of the water column (25 °C–31 °C, mean = 28 °C). Samples were collected only while suspended in the water column and never near the benthos.

Sample preservation and storage

After collection, the sample was brought to the research vessel for processing. The sample was allowed to settle out of suspension for 1–8 h at ambient temperature (29 °C– 32 °C) and out of direct sunlight. After settling, excess seawater was poured off and the remaining sample was preserved by adding approximately 20.0 mL of 20% dimethyl sulfoxide (DMSO) and 80% 5 M sodium chloride (NaCl) solution (Amos & Hoelzel 1991). The samples were stored at –20 °C on board the research vessel until transferred to the laboratory for long-term storage. Once in the laboratory, samples were divided into at least three 1.5-mL aliquots and transferred to 2.0 mL cryotubes for storage at –80 °C. Excess sample was either divided into additional 1.5 mL aliquots or kept in the collection cup and stored at –20 °C.

Cell lyses and digestion

Aliquots of samples were allowed to thaw at room temperature just prior to extraction. A portion of the faecal solution (500.0 μL) was suspended in 1.0 mL of 2× cetyltrimethyl ammonium bromide (CTAB) buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB) (Milligan 1992) and mixed on a rocking platform for 10 min. The samples were divided into two 2.0 mL microcentrifuge tubes so that each tube contained approximately 750.0 μL of sample. Samples were vortexed with 250.0 μL chloroform and allowed to settle for 2 min at 4 °C. Cellular debris was centrifuged out at 1400 × g for 15 min. The aqueous phase was recovered and transferred to a clean tube; 250.0 μL chloroform was again added, and the samples were briefly vortexed followed by a resting period of 2 min at 4 °C. An additional centrifugation at 1400 × g was carried out for 15 min to clear any remaining debris and the aqueous phase was recovered and transferred to a clean tube.

DNA precipitation and rehydration

To precipitate DNA, 50.0 μL (or approximately 10% of the total sample volume) 3 M sodium acetate and 1.0 mL (or approximately 2× the total sample volume) ethanol were added to the aqueous phase. Because faecal samples were dilute, 0.5 μL of 5.0 mg/mL glycogen (Ambion) was added to the aqueous phase. Glycogen is useful when working with dilute samples because it coprecipitates with the DNA and results in an enhanced DNA pellet. The samples were incubated at least 12 h at –80 °C, thawed, then centrifuged at 1400 × g for 10 min. The supernatant was decanted and 500.0 μL of cold 75% ethanol was added to the pellets and vortexed briefly. The samples were again centrifuged, the supernatant decanted and the pellets dried for approximately 10 min on medium heat in a SpeedVac System (Savant Instruments). DNA pellets were resuspended in 50.0 μL 8 mM NaOH. Rehydration in a weak base is recommended for isolated genomic DNA, which does not resuspend well in water or Tris buffers. All samples were allowed to solubilize at 4 °C for at least 30 min prior to visualization on a 1% ethidium bromide

(EtBr)-stained agarose gel. To reduce the pH of the resuspended DNA, samples were diluted 10-fold in dH_2O prior to subsequent analysis.

Because of the nature of the collection process, some of the collected samples were extremely dilute and contained small amounts of visible faecal material when collected. When extracting genomic DNA using the previously described method, the results were insufficient. In such cases, samples that were excessively dilute underwent an additional concentration protocol prior to extraction. Diluted sample (5.0 mL) was suspended in 5.0 mL of ethanol and mixed on a rocking platform for 10 min. The samples were allowed to precipitate overnight at –20 °C. After precipitation, the samples were gently centrifuged at 0.2 × g for 5 min. The samples were then processed according to the basic extraction procedure previously described. If the initial concentration effort failed to yield genomic DNA, a more intensive effort was made using the following steps. Rather than running two 2.0-mL tubes of faecal material, eight to 12 tubes were run through the extraction protocol. After the pellets of each tube were resuspended, the suspensions were combined and filtered through Montage PCR centrifugal filter devices following the manufacturer's recommended protocol (Millipore Corp.). The filtered samples were resuspended in 1× TE buffer and visualized on a 1% EtBr-stained agarose gel. This additional concentration process yielded usable DNA template when the standard protocol had not.

Mitochondrial analysis

The polymerase chain reaction (PCR) was used to amplify a fragment of the variable 5′ end of the control region of the mitochondrial genome using primers L15824 and H16265 (Rosel & Block 1996; Rosel *et al*. 1999). Reactions were carried out in 25.0 μL volumes consisting of 1 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers, 0.75 U *Taq* DNA polymerase (Fisher Scientific) and 1.0 μL of template DNA. The amount of template DNA or the concentration of primer was doubled in samples with low quality or quantity DNA. The cycle parameters were carried out as described by Adams & Rosel (2006). PCR products were cleaned using Montage PCR centrifugal filter devices and quantified using pGEM (Applied Biosystems) molecular sizing standard on a 1% EtBr-stained agarose gel. PCR products were cycle sequenced using a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems). The parameters consisted of 25 cycles of 10 s at 90 °C, 5 s at 50 °C and 4 min at 60 °C. Products were cleaned using Sephadex columns (Amersham Biosciences), dried and resuspended in formamide prior to loading on an ABI PRISM 310 genetic analyser (Applied Biosystems). Sequences were aligned by eye using SEQUENCHER version 4.6 (Gene Codes Corp.) and searched for matches in GenBank.

Microsatellite analysis

In addition to the mitochondrial DNA, three nuclear microsatellite markers were also amplified from the 15 individual samples (Table 1). Microsatellite amplifications were carried out in 25.0 μL reactions. Reactions for all three loci contained 0.06 M Tris-HCl (pH 8.5), 0.015 M $(NH_4)_2SO_4$, 0.2 mM dNTPs, 0.2 μM primers, 0.75 U *Taq* DNA polymerase (Fisher Scientific) and 1.0 μL template DNA. The concentration of $MgCl_2$ varied for each of the three loci: locus EV37 (Valsecchi & Amos 1996) contained 3.5 mM $MgCl_2$, D08 (Shinohara *et al*. 1997) contained 2.0 mM $MgCl_2$ and Ttr48 (Rosel *et al*. 2005) contained 2.5 mM $MgCl_2$. Cycle parameters for EV37 and D08 consisted of an initial denaturation of 2 min at 90 °C followed by 35–40 cycles of 30 s at 94 °C, 30 s annealing (58 °C EV37, 56 °C D08) and 30 s at 72 °C and a 5 min elongation at 72 °C. PCR parameters for Ttr48 consisted of an initial denaturation of 30 s at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C and a 10 min elongation at 72 °C. For samples that resulted in low quality or quantity DNA, the amount of template per reaction was doubled or the amount of primer was increased. All microsatellites were initially visualized on a 6% polyacrylamide gel stained with EtBr prior to sizing. DNA fragments were sized on ABI PRISM 310 genetic analyser using GENESCAN ANALYSIS 3.1 and GENOTYPER 2.1 (Applied Biosystems).

Results and Discussion

Results of the study indicate that successful genetic sampling can be achieved when faeces is collected within 30 s of observed defecation while the faecal material remains in a tight plume allowing for the capture of an optimum amount. Snorkelers that are capable of surface diving often have the best success in capturing samples as the depth of sample collection ranges from 1 to 9 m. Although the quantity of faeces captured will vary between organisms and situations, the extraction process can be applied to a wide range of initial collection amounts.

The quality of the extracted faecal DNA samples and their suitability for cetacean genetic analysis were tested using PCR-based amplifications with known cetacean specific markers. The first set of markers amplified a 351-bp fragment of the 5′ end of the mitochondrial control region. When all 15 sequences were aligned and compared, three haplotypes were found. The haplotypes were searched against the GenBank databases to determine the closest match. Two haplotypes matched sequences in GenBank with 100% accuracy (accession nos DQ060059 and DQ060054) and were derived from *Stenella frontalis* skin tissue (Adams & Rosel 2006). In 13 of the 15 sample sequences, DQ060059 was the most common haplotype ($n = 9$) followed by DQ060054 ($n = 4$). The remaining two individuals shared a

Table 1 Summary of mitochondrial haplotypes and microsatellite genotypes at three loci for *Stenella frontalis* ($n = 15$), M, mother; O, offspring; A, GenBank Accession no. DQ060054; B, accession no. DQ060059; C, novel haplotype from this study (GenBank Accession no. EF546440)

Sample	Haplotype	EV37	D08	Ttr48
BIGG	A	198/204	99/99	133/133
NAVE	A	198/198	99/99	131/131
MOHA	A	198/198	97/99	131/131
ROME	A	198/198	99/99	131/133
BRUS	B	198/198	99/99	131/131
EVER	B	198/198	99/99	131/131
PIGM	B	198/198	99/99	131/131
VENU	B	202/204	97/97	127/131
BARB (M)	B	198/204	97/99	127/131
BLOS (O)	B	198/198	99/99	129/131
FLYI (M)	B	198/204	97/99	127/131
KP (O)	B	204/204	97/99	131/131
FLAM (O)	B	198/204	99/99	131/131
MUGS (M)	C	198/198	99/99	131/133
MART (O)	C	198/204	99/99	131/131
Total number of alleles		3	2	4
Expected allele size range (bp)		178–224	103	128–142
Observed allele size range (bp)		198–204	97–99	127–133

single novel haplotype (GenBank Accession no. EF546440). This new haplotype differed because of a single-base transition from two additional *S. frontalis* haplotypes [accession nos DQ060057 and DQ060056 (Adams & Rosel 2006)]. It is clear that the sequence amplified from the faecal DNA was from *S. frontalis* and confirmed that this procedure works to extract the target DNA from sloughed intestinal cells of the host.

In addition to confirmation of species identity, mother and calf sets were also compared (Table 1). Since mitochondrial DNA is maternally inherited, the sequences of mother and offspring should provide a 100% match. In each of the three mother and calf sets, the sequences were identical between the mother and her respective offspring. Two of the mother and calf sets exhibited haplotypes matching DQ060059 and the third set exhibited the novel haplotype EF546440. The matching haplotypes within the mother and calf sets genetically confirmed relatedness, which supports the previous results based on observation.

Faecal DNA obtained using this protocol was also tested for its suitability for microsatellite analysis of *S. frontalis*. The microsatellite alleles found for the three loci all fell within the expected size range (Table 1). Locus EV37 was originally isolated from humpback whale (*Megaptera novaeangliae*) tissue and the observed allele size range was 178–224 bp (Valsecchi & Amos 1996). The allele size range for EV37 among the 15 *S. frontalis* samples was 198–204 bp with a total of three alleles. Loci D08 (Shinohara *et al*. 1997) and Ttr48 (Rosel *et al*. 2005) were designed in bottlenose dolphins (*Tursiops truncatus*) and the observed allele size was 103 bp and 128–142 bp, respectively. Size ranges for *S. frontalis* ranged from 97 to 99 bp with two alleles for D08 and from 127 to 133 bp with four alleles for Ttr48. In addition, in each of the three mother and calf sets, each offspring shared an allele with its mother at all three loci (Table 1), which indicates that the protocol works to extract template DNA that is suitable for microsatellite analysis. Microsatellite loci amplified from faecal DNA can be used to further genetically confirm observational data of relatedness between the mothers and calves.

The proposed technique works extremely well as a noninvasive method to collect and extract genetic material for use in molecular ecological studies of cetaceans. The protocol is relatively simple to execute and results in high-quality template DNA from faecal matter. Once template DNA has been obtained, both mitochondrial sequencing and microsatellite genotyping can be utilized to construct a genetic framework of the target population. Mitochondrial sequencing is a useful tool in cetacean species identity (Palumbi & Cipriano 1998), phylogenetics and population structure (O'Corry-Crowe *et al*. 1997) and hybrid identification (Willis *et al*. 2004). Microsatellites are a powerful tool that can be used to determine genetic relatedness such as paternity and sibship (Krützen *et al*. 2004), levels of genetic diversity (Adams & Rosel 2006), and genetic structure of populations and subpopulations (Hoelzel *et al*. 1998). In addition, while not the focus of this study, this noninvasive sampling methodology allows for diet analysis via the genetic identification of prey species in cetacean faecal matter (Jarman *et al*. 2004; Deagle *et al*. 2005;

Jarman *et al*. 2006). This protocol should be considered as an alternative to the noninvasive protocols in use today to support ongoing acoustic, behavioural, reproductive and social structure research efforts of cetaceans where invasive protocols could be detrimental.

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