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TECHNICAL NOTE

A thin soup: extraction and amplification of DNA from DMSO and ethanol used as preservative for cetacean tissue samples

Kelly M. Robertson · Jeremiah Minich · Amanda J. Bowman · Phillip A. Morin

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Abstract Two popular tissue preservatives, 100 % ethanol and 20 % salt saturated dimethyl sulfoxide (DMSO) solution were tested for the existence of amplifiable, free-floating DNA after 2–18 years of tissue storage. We found that short mtDNA fragments were consistently amplified and sequenced from DMSO preservative, while nDNA amplification was limited and inconsistent. Amplification of both mtDNA and nDNA failed most of the time for the ethanol samples.

Keywords DNA extraction · Preservative · DMSO · ETOH · mtDNA · nDNA

Long-term tissue sample collections form the basis for many genetic studies of both model and non-model organisms and are often accumulated over decades of research at substantial cost. The value of these samples, especially for rare species or those collected from remote geographic locations, is only growing as technology allows us to learn more about individuals, populations and species from stored pieces of tissue. Genomic technologies in particular are changing rapidly, with the potential for whole genomes to be sequenced from small pieces of tissue, even ancient bone fragments (Meyer et al. 2012).

The most common tissue preservation methods are storage in ethanol (70–100 %), 20 % salt saturated dimethyl sulfoxide (DMSO) solution (Michaud and Foran 2011; Amos 1997) or frozen without preservative. Recently, Shokralla et al. (2010), demonstrated that a preserved specimen can leak DNA into its preservative. They successfully obtained DNA from 1 ml of 95 % ethanol removed from vials of preserved insects and plants collected 7–10 year ago, and were able to generate sequences for mitochondrial, nuclear and plastid genes. Expanding upon their study, we examine the possibility of extending the life of samples by directly obtaining DNA from 100 % ethanol and 20 % salt saturated DMSO preservatives removed from vials that contained cetacean skin and muscle where DNA leakage may have occurred.

Seventeen samples from six cetacean species were chosen (Table 1). All samples were stored in their respective preservative at −20 °C for 2–18 years. Tissue samples were obtained from fishery bycatch, biopsies collected during research cruises, or from necropsies performed on stranded animals.

Of the 17 samples, three ethanol and four DMSO preservatives were tested using three different volumes: 10, 50 and 200 µl. The remaining 10 samples were extracted from a single volume (50 µl) of DMSO preservative that previously held false killer whale (*Pseudorca crassidens*) tissue. Each preservative volume was dried using a Thermo Savant DNA 120 SpeedVac (Thermo Scientific, Waltham, MA, USA) for approximately 1 h, reconstituted with 50 µl Milli-Q water, and vortexed for 10 s. In order to eliminate tissue cell contamination, samples were centrifuged at 10,000 rpm for 4 min. Approximately 50 µl of supernatant was then transferred to a new 1.7 ml tube.

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K. M. Robertson (✉) · A. J. Bowman · P. A. Morin
Marine Mammal and Turtle Division, Southwest Fisheries Science Center, National Marine Fisheries Services, National Oceanic and Atmospheric Administration, 8901 La Jolla Shores Dr., La Jolla, CA 92037, USA
e-mail: Kelly.Robertson@noaa.gov

J. Minich
Ocean Associates, Inc., 4007 N. Abingdon St., Arlington, VA 22207, USA

Table 1 Sample characteristics, preservative and amplification results from cetacean skin and muscle samples stored in DMSO and ethanol

Sample ID	Species	Tissue type	Preservative	Collection method	Years in preservative	Volume used (µl)	Nuclear DNA concentration (ng/µl)[a]	Dloop Seq	Sexing qPCR Assay	Microsatellites				
										KWM2bt	Sl1026t	TV5t	TtrRc11	Ttr58
3867	Short-beaked common dolphin	Skin	DMSO	Fishery bycatch	18	10	0.02	Failed	Failed					
3867						50	1.2	Yes	Yes					
3867						200[b]	–							
7709	Dall's porpoise	Muscle	DMSO	Stranding	14	10	0.78	Yes	Yes					
7709						50	1.8	Yes	Yes					
7709						200	10.26	Yes	Yes					
79755	Dall's porpoise	Skin	DMSO	Biopsy	2	10	0.03	Yes	Failed					
79755						50	0.04	Yes	Failed					
79755						200	–	Yes	Failed					
79748	Killer whale	Skin	DMSO	Biopsy	2	10	–	Yes	Yes					
79748						50	0.02	Yes	Yes					
79748						200	–	Yes	Yes					
28469	Short-beaked common dolphin	Skin	ethanol	Biopsy	9	10	–	Failed	Failed					
28469						50	–	Failed	Failed					
28469						200	0.02	Failed	Failed					
73688	Long-beaked common dolphin	Muscle	Ethanol	Stranding	3	10	–	Failed	Failed					
73688						50	–	Failed	Failed					
73688						200	–	Yes	Failed					
75688	Humpback whale	Skin	Ethanol	Biopsy	2	10	–	Failed	Failed					
75688						50	–	Failed	Failed					
75688						200	–	Failed	Failed					
16142	False killer whale	Skin	DMSO	Biopsy	12	50	0.12	Amplified[c]	Not tried	Yes	Yes	Yes	Yes	Failed
38071	False killer whale	Skin	DMSO	Biopsy	8	50	0.14	Amplified[c]	Not tried	Yes	Yes	Yes	Yes	Yes
38073	False killer whale	Skin	DMSO	Biopsy	8	50	–	Amplified[c]	Not tried	Yes	Failed	Failed	Yes	Failed
49098	False killer whale	Skin	DMSO	Biopsy	6	50	0.05	Amplified[c]	Not tried	Failed	Failed	Failed	Failed	Failed
53477	False killer whale	Skin	DMSO	Biopsy	5	50	–	Amplified[c]	Not tried	Failed	Failed	Failed	Failed	Yes
WGA														
16148	False killer whale	Skin	DMSO	Biopsy	12	50	0.36	Amplified[c]	Not tried	Failed	Failed	Failed	Failed	Failed
23316	False killer whale	Skin	DMSO	Biopsy	10	50	0.18	Amplified[c]	Not tried	Failed	Failed	Failed	Failed	Failed
23318	False killer whale	Skin	DMSO	Biopsy	10	50	0.05	Amplified[c]	Not tried	Failed	Failed	Failed	Failed	Failed
30072	False killer whale	Skin	DMSO	Biopsy	9	50	4.38	Amplified[c]	Not tried	Failed	Failed	Yes	Failed	Failed
30078	False killer whale	Skin	DMSO	Biopsy	9	50	4.59	Amplified[c]	Not tried	Failed	Failed	Failed	Failed	Failed

Scientific names are as follows: Short-beaked common dolphin (*Delphinus delphis*), Dall's porpoise (*Phocoenoides dalli*), Killer whale (*Orcinus orca*), Long-beaked common dolphin (*D. capensis*), Humpback whale (*Megaptera novaeangliae*) and False killer whale (*Pseudorca crassidens*)

[a] Based on nuclear locus BMI1 qPCR assay; Morin et al. (2007). For whole genome amplification (WGA) samples, the concentration is for gDNA prior to WGA

[b] Insufficient volume remaining for 200 µl extraction volume of sample 3867

[c] Sequencing not attempted

To purify the DNA from the supernatant, the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen Inc., Valencia, CA, USA) Animal Tissue Protocol was used beginning at Step 3. All samples were tested for PCR amplification and mtDNA sequencing of a 522 bp Dloop region (Martien et al. 2012) and compared to sequences previously generated from DNA extracted from tissue for the same sample. The nuclear DNA concentration was determined by a quantitative PCR (qPCR) assay using the nuclear locus BMI1 (Morin et al. 2007). Additional tests for nDNA included a qPCR sex identification assay using zinc finger (ZFX and ZFY) genes (Morin et al. 2005) or microsatellites (Table 1). Five of the ten false killer whale DMSO samples were tested for amplification of five

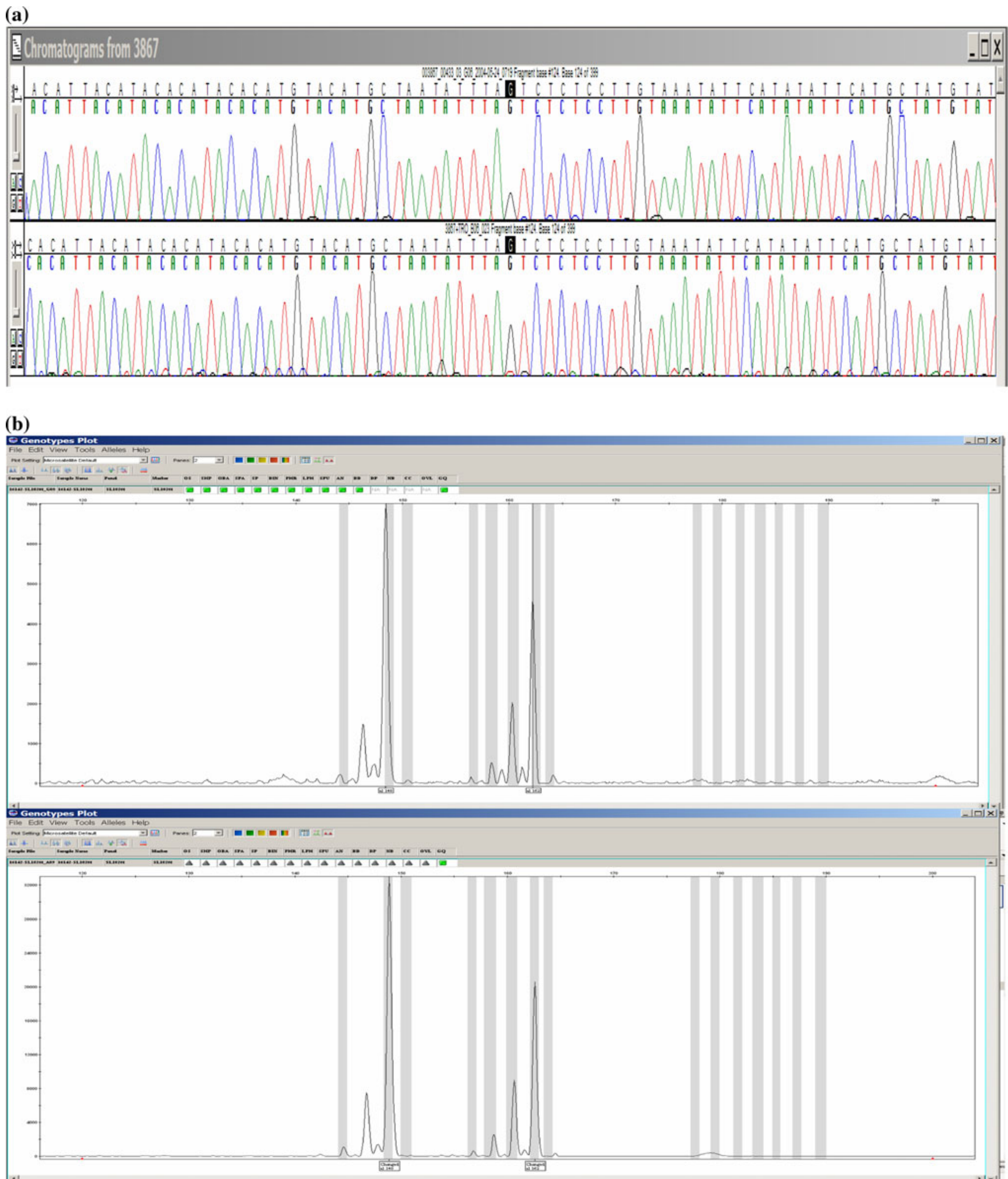

Fig. 1 Comparison of sequences and genotypes from preservative and tissue DNA. **a** Chromatographs from part of the Dloop sequence, sample 3867. **b** Microsatellite marker SL1026, sample 16142. In both cases, the *top figure* represents DNA from tissue and the *bottom figure* represents DNA from DMSO

microsatellite markers, KWM2bt, SL1026t, TV5t, TtrRc11, and Ttr58 (Supplementary Table 1), while the remaining five were subjected to whole genome amplification (WGA, REPLI-g® UltraFast Mini, Qiagen Inc.) and then tested for amplification of the five microsatellites. The genotypes were verified with genotypes

previously generated from DNA extracted from the same false killer whale tissue (unpublished data, SWFSC). Sequences and microsatellites were run on a 3730 Genetic Analyzer (ABI, Foster City, CA, USA). Sequences were aligned using Sequencher (Genecodes, Ann Arbor, MI) and allele sizes were determined using Genemapper v4.0 (ABI, Foster City, CA, USA).

All volumes of samples purified from DMSO yielded amplifiable mtDNA, except for one 10 µl sample (Table 1). The Dloop sequences resulting from the four DMSO samples matched sequences generated from DNA extracted from the corresponding tissues (Fig. 1a). The qPCR assay indicated that the concentration of nDNA was very low for all DMSO samples (Table 1). The single muscle sample in DMSO yielded the highest nDNA concentration at 10.26 ng/µl. The qPCR sexing was successful in three of the four samples.

Of the 50 genotypes attempted for the false killer whales, only 13 were successful and matched the genotypes generated using DNA extracted from corresponding tissue (Table 1; Fig. 1b). The WGAs amplified for mtDNA Dloop but performed poorly with microsatellites.

Only one of the three samples stored in ethanol (200 µl) yielded amplifiable DNA (Table 1). The Dloop sequence from this sample also matched its sequence generated from the corresponding tissue extract (Fig. 1a). Quantity of nDNA was virtually non-existent for the ethanol samples and all failed the sexing assay.

Our results demonstrate that we were able to obtain sufficient DNA to amplify mtDNA fragments from DMSO preservative, with less reliable results obtained from ethanol preservative. nDNA quantities were low to non-existent for both preservatives as indicted by the inconsistent qPCR nuclear assays and microsatellite processing. DMSO may have performed better because it makes cells permeable, allowing material to pass through cell membranes for better preservation (Sleutin et al. 1991), possibly resulting in greater DNA leakage. Ethanol dehydrates cells and causes precipitation of proteins (Flournoy et al. 1996), possibly resulting in lower DNA leakage, though the mechanisms are unclear and require more testing.

The single ethanol sample that yielded mtDNA was from muscle. Muscle also yielded the most nDNA from the DMSO sample. Muscle tissue is softer and more vascularized than skin tissue which is a tough, keratinized, multilayered surface (Ling 1974). It is possible that the higher DNA yield from muscle resulted from small amounts of blood cells that leaked into the preservative, although none was visible in the vial prior to our purification.

In contrast to the results of Shokralla et al. (2010), our ethanol samples performed poorly. They used 1 ml of preservative whereas our highest volume used was 200 µl. We were limited by our standard vial size which typically contains $\leq$1 ml of preservative. The yield of DNA from both DMSO and ethanol would probably increase by using a higher volume or by employing a DNA extraction procedure that would typically yield more DNA, e.g., salt precipitation (Miller et al. 1988). The sample size and tissue types tested here were small and do not encompass the full range of tissue/preservative possibilities. It is likely that with more testing of ethanol a higher percentage of samples will amplify for mtDNA, particularly from preservatives that contain soft tissues. Given these results and those of Shokralla et al. (2010), we recommend considering the importance of the sample prior to discarding its preservative and suggest that current or future methods for analyzing very small amounts of DNA may result in the remaining preservative being nearly as useful as the original tissue for amplifying short mtDNA fragments and possibly nDNA loci.

The ability to obtain DNA from preservative increases sample longevity and use, and demonstrates the importance of proper long term storage of tissues, and now, preservative. The application of this technique may be especially important for increasing sample sizes when samples are difficult to collect and for rare species, providing extra benefit for studies of species diversity and for conservation management.

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