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Brief Communication

MtDNA sequencing from zooplankton after long-term preservation in buffered formalin

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Abstract

Molecular genetic analysis of zooplankton has been slowed by the usual practice of preservation and storage of samples in dilute formalin solutions, which are not always adequately buffered for pH. We report here the determination of DNA sequences for *Meganyctiphanes norvegica* (Crustacea, Euphausiacea) preserved and stored in buffered formalin for up to 25 years. Specifically designed molecular protocols for DNA extraction and PCR amplification yielded valid sequence data for short (~100–200 bp) regions of the mitochondrial cytochrome *b* (mtCYB) gene for individual euphausiids. Critical aspects of our approach include: extended extraction and proteinase-K digestion to maximize DNA yield; use of protocols requiring short DNA fragments; design of species-specific PCR primers to minimize risks of contamination by exogenous DNA; and comparison with published DNA sequences for the same gene and species. We conclude that the yield of DNA and the success of subsequent molecular analyses depend primarily on the length of time the tissue has been exposed to formalin and the pH of the solution. Zooplankton samples intended for molecular analysis should preferably be preserved and maintained in ethanol or deep-frozen, but long-term storage in buffered formalin does not preclude some types of molecular genetic analysis.

1. Introduction

Formalin is an inexpensive, effective, and low-maintenance preservative, which maintains the morphological integrity of most classes of zooplankton. However, formalin-preservation of tissue has numerous direct and indirect impacts on the structure of DNA: covalent cross-linking, irreversible denaturation, modification, and fragmentation are all known chemical alterations of DNA exposed to formaldehyde (Chang and Loew, 1994, Chaw et al., 1980, Paäbo et al., 1989). In addition, unbuffered formalin, as well as buffered formalin after long storage, can be very acidic (Koshiba et al., 1993): pH values may range from 5 (at preservation) to 2 (after long storage; Bucklin, unpublished data). The consequences of preservation in formalin are almost certainly mediated by low pH, which fragments and denatures DNA (Kosel and Graeber, 1994).

We designed a study to demonstrate the feasibility of using formalin-preserved zooplankton collections for standard molecular population genetic analysis, using a species of euphausiid, *Meganyctiphanes norvegica* (Crustacea, Euphausiasea), for which we had earlier completed a population genetic study (Bucklin et al., 1997). The specimens chosen were collected during oceanographic cruises in 1975, 1982, 1997, and 1998, making them 25, 18, 3, and 2 years old at the time of analysis. The samples were preserved and archived by P.H. Wiebe (Woods Hole Oceanographic Institution) using the following protocol: preservation in 5–10% (by volume) formaldehyde, with immediate addition of sodium tetraborate to pH > 8.2, and rebuffering after 24 h of any sample with pH < 8.0. Sample pH was checked periodically and rebuffered as needed for 6 months, after which the sample pH was normally stable (P.H. Wiebe, personal communication). At the time of opening, sample pH was 7–8. DNA was purified from five individuals from each sample using a prolonged DNA extraction process modified from France and Kocher

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(1996) and Shiozawa et al. (1992) and including the following steps: (1) soak tissue for 24 h in Tris–HCl (10 mM)–EDTA (2 mM) buffer, pH 9.0 (TE9); (2) mince tissue and place in microcentrifuge tube; add 500 μl of TE9, 25 μl of 20% SDS, and 12.5 μl proteinase K (20 mg/ml); homogenize with micropestle; (3) incubate at 55 °C for 24 h with continuous mixing; (4) add 25 μl proteinase K (20 mg/ml), 25 μl of 20% SDS; incubate at 55 °C for 48 h with mixing; (5) proceed with a standard phenol purification (e.g., Bucklin, 2000); precipitate by adding 130 μl of 10 M ammonium acetate and 800 μl of 95% ethanol; and (6) resuspend in Tris (10 mM)–EDTA (2 mM) buffer, pH 7.6. Using this protocol, perceptible DNA was recovered from 17 out of 20 individuals, although in lower concentrations and with fewer larger fragments than DNA recovered from ethanol-preserved individuals (Fig. 1).

The purified DNA was evaluated by PCR amplification using one of two species-specific PCR primer pairs spanning 91 and 181 base-pair regions of mitochondrial cytochrome *b* (mtCYB). The primers were designed and named based on sequence data from 119 individuals of *M. norvegica* collected across its geographic range (Bucklin et al., 1997). The PCR protocol had long soak times: 94 °C (1 min); 52 °C (2 min); 72 °C (3 min) for 40 cycles; all experiments were carried out on a Perkin–Elmer TempCycler, Model 480. Each PCR reaction included negative controls for DNA extraction and PCR.

All 20 formalin-preserved individuals were analyzed for both mtCYB regions, regardless of apparent DNA yield. The shorter (91 bp) fragment of mtCYB was amplified and sequenced for 14 individuals, including one individual from the oldest (1975) sample. The longer (181 bp) fragment DNA was amplified and sequenced from eight of the same 20 individuals, but none from the 1975 sample (Table 1). Comparison of DNA sequences from formalin-preserved individuals with the most frequent haplotype among 119 ethanol-preserved specimens of *M. norvegica* collected from the Northwest and Northeast Atlantic Ocean (Bucklin et al., 1997), indicated that 21 sequences were identical to the most abundant haplotype from the previous study, and one sequence was distinguished by a unique base substitution at one site (Fig. 2). The finding of both identical and unique individuals among the formalin-preserved specimens suggested that the DNA sequences did not result from damage-induced sequence artifact or contamination.

In a related study, Bucklin et al. (unpublished) examined the effects of short-term (i.e., less than 1 month) storage in buffered and unbuffered formalin. The copepod, *Calanus finmarchicus*, was preserved in 5% buffered and unbuffered formalin; stored for 1, 3, 10, 23, and 40 days; and then moved to 95% ethyl alcohol. DNA was extracted using a standard protocol (see Bucklin, 2000) and was successfully amplified using species-specific PCR products for a 160 bp region of mt16S RNA. This study suggested that short-term exposure to unbuffered formalin does not destroy DNA, although these results were not confirmed by sequencing the PCR products. Bucklin et al. (unpublished) examined the feasibility of DNA recovery from zooplankton stored long-term in formalin that had not been effectively pH buffered. Using the long-duration extraction described here, no usable DNA was recovered from copepods collected during California Cooperative Fisheries Investigations (CalCOFI) surveys from 1949 to 1990. The archived samples were found to have formalin with pH 2–4 at the time of jar opening.

Our expectation is that, in most cases, molecular examination of zooplankton preserved and stored in formalin will require modified DNA extraction protocols and carefully designed molecular approaches, analogous

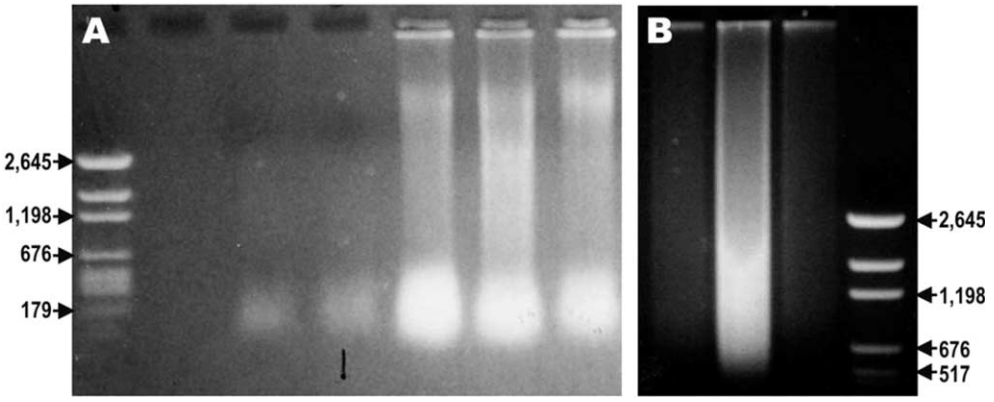

Fig. 1. DNA extracted from five ethanol-preserved euphausiids (A) and three formalin-preserved euphausiids (B), shown after electrophoresis on 1.4% agarose gels and photographed under ultraviolet light. DNA from ethanol-preserved tissue appeared to have higher yield and more large fragments, while formalin-preserved tissue appeared to yield less DNA and fewer large fragments. All individuals shown yielded usable DNA for PCR and sequencing of a region of mtCYB. The molecular weight marker indicates migration distances of fragments of the sizes indicated (in DNA basepairs).

Table 1
Collection information for zooplankton samples used in this study

Collection date	Cruise	Sample	Collection location		PCR/sequence		pH
			Latitude	Longitude	91 bp	181 bp	
December 1998	OC-334	MOC-002, net5	42° 38.82N	69° 43.13W	4	4	7
October 1997	EN-307	MOC-006, net6	42° 39.40N	69° 25.13W	4	1	7
April 1982	OC-118	MOC-162	39° 13.07N	69° 37.6W	5	3	8
August 1975	CH-125	Sample 171	39° 05.7N	70° 17.3W	1	0	7

A total of 20 individuals (five from each sample) of the euphausiid, *M. norvegica*, were analyzed by DNA extraction and PCR amplification of 91 and 181 bp fragments of mtCYB. PCRs and sequences were successful for the numbers of individuals shown (PCR/sequence); pH value of the sample at time of jar opening is indicated (pH).

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EtOH       TTT TTA GCA ATA CAC TAC ACA GCA AAT ATT GAT TTA GCT TTC TCC AGA GTA
52F-233R                                    .. ... ... ... ... ... ... ... ...

                                                                                               102
EtOH       GCA CAC ATT TGT CGA GAT GTT AAC TAT GGA TGA TTA CTA CGA ACT ATT CAT
52F-233R   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
92F-183R                   . ... ... ... ... ... ... ... ... .T. ... ... ...

                                                                                               153
EtOH       GCT AAC GGA GCT TCT TTC TTT TTT ATT TGT CTT TAT CTA CAC ACA GGA CGA
52F-233R   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
92F-183R   ... ... ... ... ... ... ... ... ... ... ... .

                                                                                           206
EtOH       GGG ATA TAT TAT GGA TCT TAT CTA TTT TTA GAG ACG TGA TCG ATT GGC GTA AT
52F-233R   ... ... ... ... ... ... ... ... ... ... ... .
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Fig. 2. Comparison of DNA sequence variation from alcohol- and formalin-preserved euphausiids, *M. norvegica*, for portions of the mtCYB gene. The most frequent mtCYB sequence among 119 alcohol-preserved individuals (EtOH; Bucklin et al., 1997) is compared to sequences determined for a 181 bp region (primers 52F-233R) for eight formalin-preserved individuals and a 91 bp region (primers 92F-183R) for 14 formalin-preserved individuals. The species-specific primers used in this study are shown in bold italic type and underlining. Identical sites are indicated by a period (.).

to preparation of ancient DNA (see Hummel, 2003; Schmerer, 2003). Protocols designed for purification of DNA from formalin-preserved tissues entail long-duration (i.e., days) extractions under conditions optimized for proteinase-K digestion of the tissue (Diaz-Cano and Brady, 1997; Kosel and Graeber, 1994; Savioz et al., 1997). Molecular analysis of small DNA fragments are most likely of success (Dubeau et al., 1986; Shibata et al., 1988; Kirby and Reid, 2001). Despite the disadvantage of low levels of variation for some gene portions, the existence of numerous copies of the mtDNA molecule in the tissue is almost certainly a distinct advantage for molecular studies of degraded or damaged DNA. It should be remembered that the primary pitfall of working with formalin-preserved tissue is contamination with exogenous DNA. The risk of contamination can be reduced by using species- or allele-specific PCR primers and stringent PCR amplification protocols (Chase et al., 1998; Weirich et al., 1997). These analyses require ultra-clean techniques, including use of new pipettors; sterile cotton-filled pipette tips; and ultra-clean reagents, glassware, plasticware, and workspace. We recommend working in a laboratory that has never been exposed to PCR products; a dedicated laboratory bench in a molecular laboratory risks contamination by air-borne PCR products. It is essential to include negative controls for all protocol steps: extraction, PCR, and sequencing procedures should include analysis of tubes carried through all protocol steps without added DNA. DNA sequences from formalin-preserved tissue should be compared to DNA sequences for the same gene and species (or population) determined from deep-frozen or ethanol-preserved samples, in order to evaluate patterns of sequence variation and to look for both identical and unique sequences from formalin-preserved specimens.

Samples that are preserved in unbuffered formalin, or not rebuffered during storage, should be checked for pH prior to molecular analysis. Low sample pH (i.e., <4) is likely to be an indicator of extensive DNA degradation and fragmentation. Analysis of tissue stored in formalin for longer than 1 year and samples with pH < 7 should focus on molecular analysis of 100–200 bp regions of DNA. The yield of usable DNA from formalin-preserved tissue can be maximized by buffering the formalin to maintain neutral pH (Kosel and Graeber, 1994; Koshiba et al., 1993); limiting storage time in formalin (since the DNA is increasingly fragmented with time;

Legrand et al., 2002; Pavelic et al., 1996); and storing the samples at 4 °C (Koshiba et al., 1993).

Molecular examination of formalin-preserved zooplankton will open the doors of archived oceanographic samples to population genetic and systematic examination. With appropriate molecular analysis, it will be feasible to examine the temporal structure of zooplankton assemblages, determine the persistence and variation in the frequencies of genetic types, and perhaps understand the impacts of climatic or anthropogenic changes in the ocean environment through correlation with shifts in the genetic makeup of key species. With wide-spread application of molecular genetic analysis of archived zooplankton samples, these types of studies can be carried out for ecosystems sampled as long as 100 years ago.

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