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Extraction and Amplification of Mitochondrial DNA from Formalin-Fixed Deep-Sea Mollusks

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The ability to extract, amplify and sequence DNA from various types of tissues including bone (4,6), minute larvae (7), formalin-fixed specimens (2,3) and dried museum specimens (1) has made it possible to address fundamental questions about evolution, phylogeny and the genetic structure of populations. A major problem in studying evolutionary patterns in the deep sea is the difficulty of obtaining amplifiable DNA from small macrofaunal organisms (usually about 1.0–5.0 mm). These species live at very low density, are collected from great depths (to 5000 m) and are typically fixed for 24–48 h in 10% buffered formalin and later transferred to 70% ethanol. The effects of fixation (3) and the very small amount of tissue in macrofaunal organisms have made it extremely difficult to obtain DNA sufficient for polymerase chain reaction (PCR). Though several methods have been published to extract DNA from formalin-fixed tissues (2,5,10), these have proved cumbersome with small amounts of tissue.

We describe methods to extract and amplify mtDNA from formalin-fixed deep-sea molluscan species by: (*i*) using a modification of a commercially available DNA extraction kit and (*ii*) developing species-specific primers. One of the greatest challenges we faced was extracting sufficient quantities of amplifiable DNA from minute amounts of tissue. Further, this material was extensively handled during the sorting process and prone to contamination.

To extract DNA, whole individuals were placed in microcentrifuge tubes with 200 µL of tissue lysis buffer ATL from the QIAamp® Tissue Extraction Kit (Qiagen, Chatsworth, CA, USA) and incubated for 24 h at 55°C. Then 5 µL of a 50 mg/mL solution of proteinase K and an additional 95 µL of lysis buffer were added and incubation continued at 55°C for another 72 h. The extraction then follows the manufacturer's instructions, except that buffer AL and ethanol were increased from 200 to 300 µL. DNA was eluted with one 200-µL aliquot of 10 mM Tris-HCl, pH 8.0.

Developing species-specific internal primers and working with small (<300-bp) mtDNA fragments were crucial steps for consistent amplification of DNA from formalin-fixed specimens. Reactions with the universal 16S primers, 16sar (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16sbr (5′-CTC CGG TTT GAA CTC AGA TC-3′) (8) resulted in the spurious amplification of human fragments. Because fresh material was not available for primer development, the following detailed strategy for targeting 16S rDNA was used: (*i*) a forward primer [Proto16F: 5′-A(A/T)-(A/G) (A/T)GA C(A/G)A GAA GAC CCT-3′] internal to 16sar was designed by aligning two protobranch bivalve species, *Nucula proxima* and *Solemya*

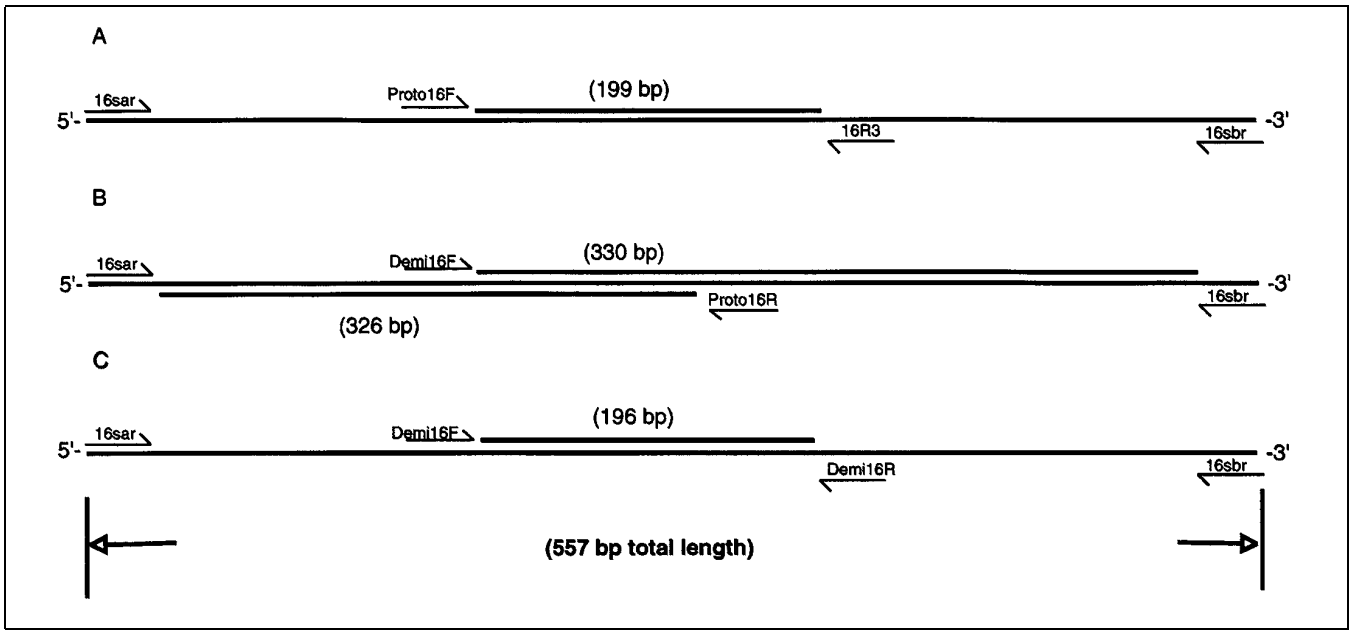

Figure 1. Diagram of the degenerate and species-specific primers developed relative to the universal primers 16sar and 16sbr used to amplify 16S rDNA fragments in *D. atacellana*. Primers used to target 16S in *D. atacellana* were developed as follows: (A) the degenerate primer Proto16F was designed from *N. proxima* and *S. velum*, and the degenerate primer 16R3 was designed from *N. proxima*, *S. velum* and *Homo sapiens* and used in the initial amplifications of *D. atacellana*; (B) the primer Proto16R was designed from sequence data and paired with 16sar, and the sequence data obtained were used to design the species-specific primer Demi16F; (C) Demi16F was then paired with 16sbr to amplify individuals of *D. atacellana*, and sequence information obtained from these reactions was used to design the reverse species-specific primer Demi16R. The primer set Demi16F and Demi16R was used in the collection-wide survey.

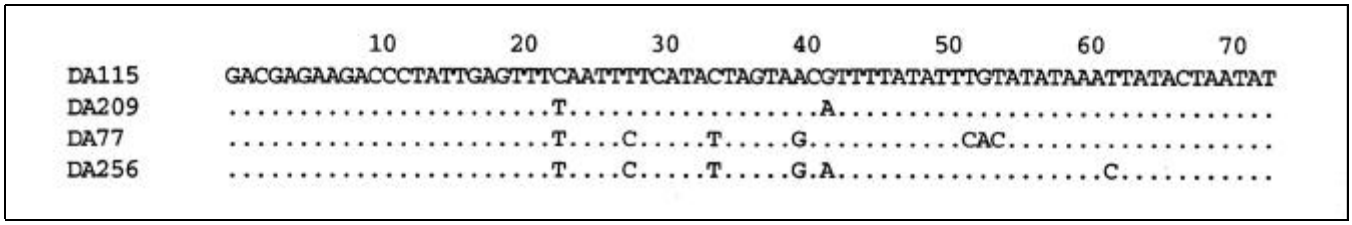

Figure 2. Alignment of 16S variable domain of four haplotypes observed in the protobranch bivalve *D. atacellana* amplified from DNA extracted from formalin-fixed DNA extractions. Individuals from independent collections of *D. atacellana* were extracted and PCR-amplified with the primers Demi16F and Demi16R. Products were sequenced and aligned. All point mutations observed are transitions.

velum; and (*ii*) a reverse primer [16R3: 5′-GCT GTT ATC CCT (A/G)(A/C/G/T)(A/G) GTA ACT-3′] internal to 16sbr was designed by aligning *N. proxima*, *S. velum* and *Homo sapiens*. Template DNA isolated from preserved deep-sea specimens of *Deminucula atacellana* was then amplified with Proto16F and 16R3 (annealing temperature 50°C).

Because we used degenerate primers at low annealing temperatures to target 16S, species-specific primers were then developed for *D. atacellana* by amplifying the whole 16sar and 16sbr fragments (557 bp) in two pieces, independent of Proto16F and 16R3 (Figure 1). A primer internal to Proto16F and 16R3 was designed [Proto16R: 5′-C(C/T)C (C/T)CA GTT GCC CCA ACT (A/C)AA-3′] from the DNA sequence of the 16S fragment amplified from *D. atacellana*. Template DNA from *D. atacellana* was then amplified with Proto16R and 16sar (annealing temperature 55°C), resulting in a 326-bp fragment. The fragments were sequenced, aligned and used to redesign a species-specific primer (Demi16F: 5′-GAC GAG AAG ACC CTA TTG AGT-3′) in the same area as Proto16F but extending it several bases at the 3′ end to select against contaminating human DNA. Template DNA from *D. atacellana* was then amplified with Demi16F and 16sbr (annealing temperature 60°C), resulting in a 330-bp fragment, then sequenced. Sequences were aligned and used to

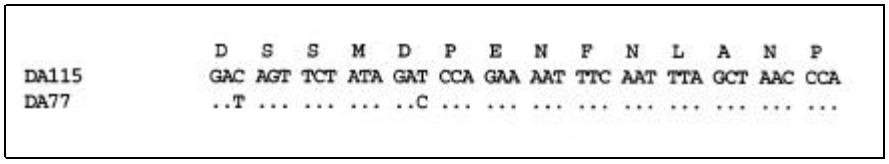

Figure 3. Alignment of two variable cytochrome b sequences from *D. atacellana*. Deduced amino acids are aligned beneath the nucleic acid sequence. Point mutations detected occur only in the third position of the codon.

redesign a primer [Demi16R: 5′-GAT TAC GCT GTT ATC CCT (A/G)TG-3′] conserved to *N. proxima* and *D. atacellana* in the same area as 16R3.

Once we had developed species-specific primers, DNA was amplified as follows: (*i*) 10 µL template (no dilution of stock DNA eluted from column), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton® X-100, 2.5 mM $MgCl_2$, 0.2 µM each dNTP, 20 pmol each primer and 1.0 U *Taq* DNA polymerase (Promega, Madison, WI, USA) were incubated with Taqstart™ Antibody (CLONTECH Laboratories, Palo Alto, CA, USA) following the manufacturer's protocol, and H_2O was added to 50 µL; (*ii*) reactions were layered with mineral oil and heated to 95°C for 2 min followed by 5 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, then 35–40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. After amplification, we confirmed and purified PCR products as follows: (*i*) running on 1.5% agarose gels, (*ii*) purification with the QIAquick™ PCR Purification Kit (Qiagen), (*iii*) sequencing with a Taq Dye Deoxy™ Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and (*iv*) ethanol precipitation followed by running on a Model 373 Automated DNA Sequencer (PE Applied Biosystems).

With these refinements, we have achieved consistent PCR amplification suitable for sequencing of short (196-bp) mtDNA fragments from 16S and cytochrome b (primers not shown) from nine independent collections of *D. atacellana* fixed in formalin nearly 30 years ago (9). Amplification success for 16S ranges from 47% to 100% within these collections and averages 71% over all collections for *D. atacellana*. Although we only amplify 196-bp fragments in our survey, amplifications of up to 300 bp have been achieved in 20% of the samples in a single round of PCR.

Because fresh tissues are not available for the species we work with, the integrity of the sequences was evaluated with alternative approaches. PCRs performed on DNA extractions from different days yield identical sequences. The 16S fragments from *D. atacellana* align well within a variable domain of the molecule (Figure 1). Haplotype diversity is low within collections, and sequence data show transition bias for both 16S and cytochrome b (Figure 2). Further, cytochrome b haplotypes vary only by point mutations in the third position of the codon (Figure 3). These observations suggest that formalin fixation or PCR-induced artifacts are unlikely and are consistent with what others have found (2).

Although others have reported success in working with formalin-fixed material, our samples, like most museum samples, are handled differently

(5). Most importantly, the volume of tissue available is extremely small, so attention to the efficiency of the extraction and amplification procedure is essential. A major advantage to our technique is the elimination of organic purification and ethanol precipitation, which minimizes the loss of DNA associated with these steps. Use of a silica-based purification procedure is also advantageous, as discussed elsewhere (4). Finally, sufficient PCR product is obtained from a single round of amplification and is suitable for sequencing, reducing the chance of cross-contamination associated with re-amplifying PCR products.

Although we only report the results for *D. atacellana*, using these procedures we have extracted, amplified and sequenced mtDNA fragments from 396 specimens and 14 molluscan species to date. Our methods enable us to address fundamental questions about the population biology of deep-sea mollusks from samples fixed in formalin over 30 years ago. Others working with limited amounts of material or requiring consistent amplification success might benefit from these methods.

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