

## ANCIENT DNA TECHNIQUES: APPLICATIONS FOR DEEP-WATER CORALS

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### ABSTRACT

The potential applications of ancient DNA (aDNA) techniques have been realized relatively recently, and have been revolutionized by the advent of PCR techniques in the mid 1980s. Although these techniques have been proven valuable in ancient specimens of up to 100,000 yrs old, their use in the marine realm has been largely limited to mammals and fish. Using modifications of techniques developed for skeletons of whales and mammals, we have produced a method for extracting and amplifying aDNA from sub-fossil (not embedded in rock) deep-water corals that has been successful in yielding 351 base pairs of the ITS2 region in sub-fossil *Desmophyllum dianthus* (Esper, 1794) and *Lophelia pertusa* (Linnaeus, 1758). The comparison of DNA sequences from fossil and live specimens resulted in clustering by species, demonstrating the validity of this new aDNA method. Sub-fossil scleractinian corals are readily dated using U-series techniques, and so the abundance of directly-dateable skeletons in the world's oceans, provides an extremely useful archive for investigating the interactions of environmental pressures (in particular ocean circulation, climate change) on the past distribution, and the evolution of deep-water corals across the globe.

Ancient DNA (aDNA) is classified as “any bulk or trace of DNA from a dead organism or parts of it”, or “any DNA that has undergone autolytic or diagenetic processes or fixation”, making this a wide discipline with respect to the types of material it encompasses (Herrman and Hummel, 1994). The general field of aDNA investigation began just prior to the advent of, and was revolutionized by polymerase chain reaction (PCR) technology, and interest has steadily grown during recent years (Herrmann and Humel, 1994; Pääbo et al., 2004). Ancient DNA techniques have been used successfully in multiple studies on terrestrial animals (Yang et al., 1996; Barnes et al., 2002; Huyen et al., 2003; Willerslev et al., 2003), plants (Willerslev et al., 2003), humans (Höss and Pääbo, 1993; Cattaneo et al., 1995; Prado et al., 1997; Cipollaro et al., 1998; Kalmár et al., 2000), large marine mammals (Pichler et al., 2001, B. Mcleod, Trent University, pers. comm.), vertebrates (Consuegra et al., 2002) and invertebrates (Geller, 1999) to explore past evolutionary and phylogeographic questions and provide conclusive identifications of fauna. These include samples of 200-yr-old wolves (Leonard et al., 2005), 4000-yr-old rats (Robins et al., 2005), 40,000-yr-old bears (Leonard et al., 2000; Pääbo, 2000) and woolly mammoth (Debruyne et al., 2003), and > 100,000-yr-old plants (Willerslev et al., 2003).

The quality of recoverable aDNA by specialized techniques varies as both a function of age of the material, and the environmental conditions under which the organism was preserved (Cooper, 1994; Willerslev et al., 2003; Robins et al., 2005). The maintenance of specimens at colder preservation temperatures (such as the permafrost) appear to yield better quantity and quality of aDNA (Pääbo, 2000). However, high levels of success have also been had in extracting aDNA from museum specimens and private collections that have had no special preservation or handling (Cooper,

1994; Greenwood et al., 1999; Pichler et al., 2001). Bone has also been determined to preserve higher quality DNA (yielding longer sequences) than ancient soft tissues (Cooper, 1994; Hagelburg, 1994). Many of the problems resulting in the unsuccessful amplification of target aDNA fragments have been overcome, but the amplification of ancient remains is problematic, mainly due to PCR inhibitory components and contamination inherent in older samples.

Mitochondrial DNA traditionally has been the main target of ancient investigations (Herrmann and Hummel, 1994; Yang et al., 1996; Rosenbaum et al., 1997; Noro et al., 1998; Barnes et al., 2002; Lambert et al., 2002; Willerslev et al., 2003), as it contains multiple copies of DNA per cell and so is easier to retrieve from older specimens than nuclear DNA (Cooper and Wayne, 1998; Greenwood et al., 1999). However, techniques have consistently improved to successfully extract sufficient quantity and quality of nuclear DNA for phylogenetic and population genetic purposes, opening up new avenues and questions for aDNA research (Greenwood et al., 1999; Leonard et al., 2000; Huyen et al., 2003).

One potential new area of opportunity in which aDNA could provide major new insights is that of deep-water corals. Deep-water scleractinians have been present throughout the world's oceans for millions of years and so have experienced many climatic changes and extinction events. Their modern distribution is controlled by a combination of these historical changes and by physical (e.g., ocean currents, suitability of substrate) and biological (e.g., recruitment, feeding) factors. Unravelling the relative importance of each of these controls is challenging, particularly in the past. The aragonitic skeleton of these corals is rich in uranium, making them suited to radiometric dating using the ingrowth of thorium (Cheng et al., 2000; Robinson et al., 2006) and as a result, the ages of each individual can be obtained back through hundreds of thousands of years (see Robinson et al., 2007). Combining aDNA with the absolute ages of ancient coral populations can provide a unique perspective on the changes of deep-water coral populations over time, covering times of climatic variability.

Specifically, sub-fossil scleractinian corals from NW Atlantic seamounts have been used to define major changes in intermediate and deep-ocean circulation that coincide with large swings in atmospheric climate since the Last Glacial Maximum (Adkins et al., 1998; Robinson et al., 2005). During this time the distribution of populations of scleractinians in the NW Atlantic has clearly changed (Robinson et al., 2007). The skeletons of these corals are found in abundance in the NW Atlantic (as well as many other areas of the world's oceans) through a large depth and geographic range, at temperatures of just a few °C. The question of how changes in oceanic circulation patterns have affected the historical migrations and present day distributions of scleractinian corals across seamount chains is key to understanding how modern climate change will affect deep marine benthic species in the future.

Robinson et al. (2007) used U-series dating of sub-fossil corals from seamounts to show patterns of historical population dynamics and possible migrations. By combining dating and ancient DNA techniques we hope to correlate times of climate change with corresponding population shifts. Our long range goal is to determine how changing ocean circulation patterns have affected populations of ancient scleractinians over tens of thousands of years. This information will enhance our understanding of the biogeography of modern coral populations. The first step in this study is to examine and develop aDNA techniques from sub-fossil scleractinian skeletons.

Here we present preliminary results from the initial extraction, amplification, and sequencing of sub-fossil aDNA from two species of deep-water scleractinian coral from the NW Atlantic: *Desmophyllum dianthus* (Esper, 1794) and *Lophelia pertusa* (Linnaeus, 1758).

## METHODS

**COLLECTIONS.**—Deep-water sub-fossil corals used in this preliminary study were collected from seamounts and ridges in the NW Atlantic (Table. 1) using specially designed scoops from the submersible DSV ALVIN (Fig. 1). These corals were scooped from the seafloor from areas where no live scleractinians were present (precluding recently dead material) and then placed in plastic crates on the front basket of the submersible. Corals were rinsed naturally as the vehicle surfaced through the water column, removing much of the sediment and debris, they were also washed with sterile freshwater on recovery. Samples were then sorted by (a) size (larger ones for more material); (b) amount of pitting and boring (less pitted individuals were selected to reduce chances of extraction of contaminant DNA); and (c) the amount of ferromanganese oxide coating (this metal-rich coating may inhibit extraction and/or PCR and so individuals with less coating were selected). Specimens selected for use in this study were then placed in the  $-20^{\circ}\text{C}$  freezer.

**CONTAMINATION PREVENTION.**—Contamination of extractions and PCR of aDNA molecules is an ever-present complication of aDNA studies, owing to both the high sensitivity of the methodology and the low concentration of genetic material present in older samples (Hedges and Schweitzer, 1995; Yang et al., 1997; Cooper and Poinar, 2000; Pääbo et al., 2004). We maintained contamination prevention standards deemed essential to all procedures. Sterile equipment and reagents solely dedicated to aDNA extraction and PCR procedures were used to prevent contamination of coral aDNA by other sources of DNA in the laboratory. A separate laboratory space was used for both extractions and PCR of aDNA and all equipment and surfaces were kept sterile by 70% bleach solution, 100% EtOH, UV irradiation (~1 hr) and autoclaving (where possible).

Sub-fossil material from two specimens of *D. dianthus* from Manning Seamount and one specimen of *L. pertusa* from the Lost City hydrothermal vent site (Table 1) were extracted using the procedure below. Live *D. dianthus* and *L. pertusa* collected from Lost City and Manning were extracted for comparisons to sub-fossil material. The scleractinian *Enallopsammia rostrata* (De Pourtalès, 1878) was used as an outgroup. All live specimens examined were

Table 1. Table of specimens used for this study. hv — hydrothermal vent; smt — seamount.

Species	Condition	Location/Latitude/Longitude	Depth (m)	Date collected
<i>Desmophyllum dianthus</i>	Sub-fossil	Manning (smt) 60°53'N, 38°20'W	1,656	13 June 2003
<i>Desmophyllum dianthus</i>	Sub-fossil	Manning (smt) 60°53'N, 38°20'W	1,656	13 June 2003
<i>Desmophyllum dianthus</i>	Live	Manning (smt) 60°53'N, 38°20'W	1,656	13 June 2003
<i>Desmophyllum dianthus</i>	Live	Lost City (hv) 30°70'N, 42°70'W	890	16 May 2003
<i>Lophelia pertusa</i>	Sub-fossil	Lost City (hv) 30°70'N, 42°70'W	798	27 May 2003
<i>Lophelia pertusa</i>	Live	Manning (smt) 60°53'N, 38°20'W	1,470	3 June 2003
<i>Enallopsammia rostrata</i>	Live	Muir (smt) 62°57'N, 33°77'W	1,525	8 June 2003

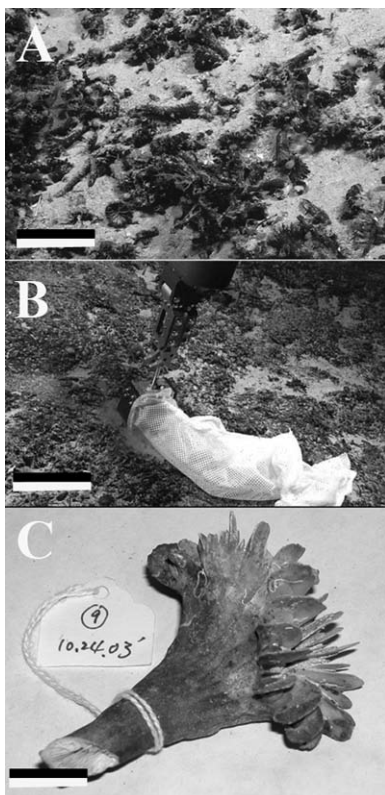


Figure 1. (A) Sub-fossil *Desmophyllum dianthus* in situ on the seafloor at Manning Seamount, collected by (B) scoops from the ROV HERCULES on the Corner Rise Seamounts. (C) A single *D. dianthus* from Manning Seamount prior to processing. Scale bars – (A) ~10 cm; (B) ~50 cm; and (C) ~1 cm

extracted and amplified in a different laboratory space and were prepared on a different (later) date than the sub-fossil extractions.

**SUB-FOSSIL MATERIAL PREPARATION.**—Large pieces of frozen skeleton (1–3 g) were separated from the main polyp or colony using a dremel tool under liquid nitrogen. These pieces were then washed several times with sterile water and placed under UV illumination for 30 min (with frequent turning) to sterilize the outer surfaces from extraneous DNA. The coral pieces were then scraped to remove the outer, irradiated surfaces. Material was then crushed under liquid nitrogen with a sterile pestle and mortar (separate for each sample), and ~500 mg was used for extractions. Any excess powder was frozen at  $-20^{\circ}\text{C}$  in microcentrifuge tubes for later use.

**DNA EXTRACTION AND PCR PROCEDURES.**—A modification of the BioSystems 101 GeneClean for Ancient DNA Kit with Lysis Matrix K tubes was used for sub-fossil extraction. Modifications were dependent on previous successes, and included a pre-incubation phase (washing in 0.1M EDTA, pH 8, 750  $\mu\text{L}$ , 12 hrs), vortexing with Lysis K for up to 1 hr, changing incubations times (12–48 hrs) and temperatures ( $45$ – $55^{\circ}\text{C}$ ) and eluting in 150  $\mu\text{L}$ . Qiagen DNEasy kit was utilized for live coral extractions using standard protocols. The nuclear ITS2 gene region was amplified using ITSFA and ITSRA primers (LeGoff-Vitry et al., 2004) using the following programs:

ITS2–1 cycle at  $95^{\circ}\text{C}$  (3 min),  $53^{\circ}\text{C}$  (1 min),  $72^{\circ}\text{C}$  (2 min); 4 cycles at  $94^{\circ}\text{C}$  (30 s),  $53^{\circ}\text{C}$  (1 min),  $72^{\circ}\text{C}$  (2 min); 35 cycles at  $94^{\circ}\text{C}$  (30 s),  $57^{\circ}\text{C}$  (1 min),  $72^{\circ}\text{C}$  (2 min); 1 cycle at  $75^{\circ}\text{C}$  (10 min).

A higher concentration of DNA template was used for sub-fossil PCR (between 5–10  $\mu\text{L}$ —as in H $\ddot{o}$ ss and P $\ddot{a}$ bo, 1993). Though this ITS region has shown intra-individual variation in some species of scleractinian (Diekman et al., 2001; Vollmer and Palumbi, 2004), it has been and is being used successfully in *D. dianthus* and *L. pertusa*, as well as other species of deep-water coral (LeGoff-Vitry et al., 2004, unpubl. data; K. Miller, U. of Tasmania, pers. comm., C. Morrison, USGS, pers. comm.). Although nuclear DNA is harder to obtain than mitochondrial markers (Cooper and Poinar, 2000), this region has been targeted for the later application of these data to population analysis. No positive controls were used in these samples to prevent contamination issues, though negative controls were run during each procedure.

Automated sequencing was performed at the Josephine Bay Paul Center at the Marine Biological Laboratory, Woods Hole, using an ABI 3730XL capillary sequencer. Sequences were assembled using AutoAssembler 2.1 and aligned using Clustal X and MacClade 4.06 software. Sequences were compared on the GenBank database using the BLAST algorithm. PAUP 4.0b10 was used for phylogenetic analysis (parsimony, likelihood, and distance-based methods) sequence divergence, and data matrixes. Both *D. dianthus* and *L. pertusa* sub-fossil specimens were re-extracted from original powder and were sequenced to assess sequence variation and contamination.

## RESULTS AND DISCUSSION

A 351bp fragment of the ITS2 region was successfully amplified in both sub-fossil *D. dianthus* from Manning Seamount and *L. pertusa* from the Lost City Hydrothermal Vent Field. When compared on BLAST, sequences most closely resembled *L. pertusa* ITS sequences (LeGoff et al., 2004). A neighbor-joining distance tree was constructed (Fig. 2) using 500 bootstrap replicates and revealed that sequences of sub-fossil specimens were most similar to their live counterparts, and least similar to the scleractinian outgroup (*E. rostrata*). These data suggest that the DNA recovered from sub-fossil specimens was likely obtained from the skeleton target species. Three sequences from LeGoff et al. (2004) were included in these analyses (ascension numbers AY257259, AY257332, AY257321), although no ITS sequence for *D. dianthus* are currently present in Genbank for comparison. The sub-fossil samples, when re-extracted and amplified, yielded sequences identical to the original extraction (after phylogram checking), demonstrating the noteworthy absence of contamination. On the second extraction, however, amplification of ITS2 was attempted three times in two of the *D. dianthus* samples before being successful. This is likely owing to the small concentration of aDNA extracted, as well as the possible presence of PCR inhibitors.

Given the inherent degradation of DNA in nucleic acids, it is unlikely that base-pair lengths of over 500bp are either possible or correct (Herrmann and Hummel, 1994; Rosenbaum et al., 1997; Cooper and Poinar, 2000), which is a limitation of this type of population study. However, methods and contamination procedures are continuously being improved (by other aDNA researchers as well as us) to increase the yield of DNA material recovered and to specifically identify small, highly variable regions within the cnidarian genome that may allow us to better understand the population dynamics of deep-water corals tens of thousands of years ago. Although we only examined three fossil specimens, multiple populations of deep-water corals are in the process of being extracted, with an extraction success rate of approximately 1 in 10 at present (Waller, unpubl. data).

This preliminary study has demonstrated the potential use of aDNA techniques on deep-water sub-fossil coral skeletons and thus will allow future population studies, as

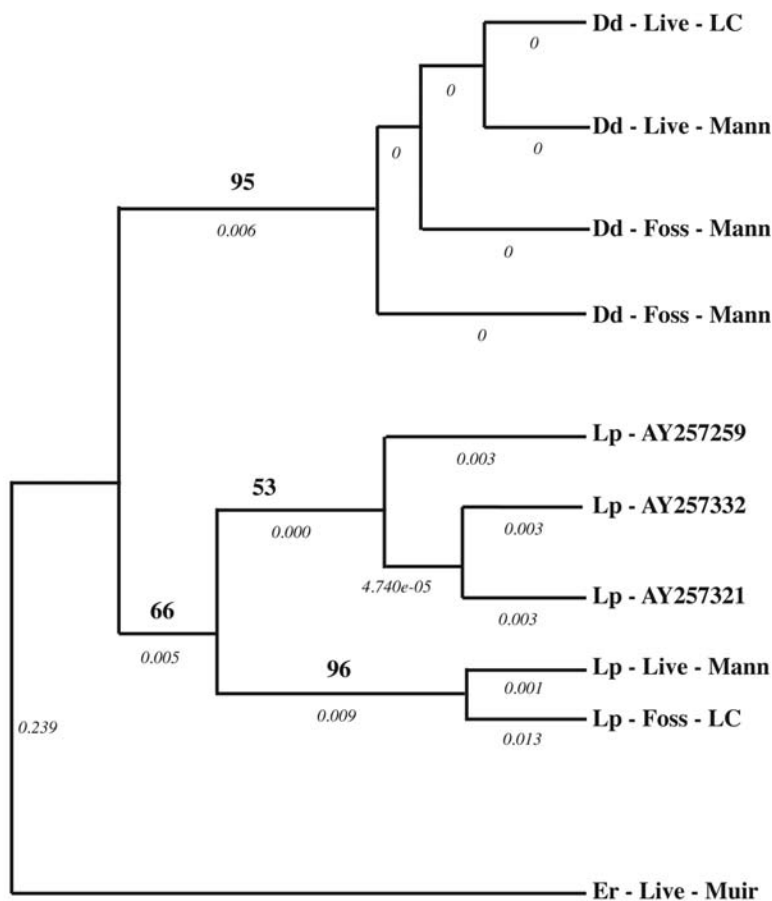


Figure 2. Neighbor-joining (Kimura 2 parameter) distance tree based on 351bp of the ITS2 region of fossil and live *Desmophyllum dianthus* (Dd) and *Lophelia pertusa* (Lp) and live *Enallopsammia rostrata* (Er) as the outgroup. Three *L. pertusa* sequences were taken from Genbank—ascension numbers in title. Fossil extractions and PCR have been repeated twice and yielded identical sequences on both occasions. Bold numbers are bootstrap values (500 replicates), numbers in italics are substitutions per nucleotide site. Mann—Manning Seamount, LC—Lost City Hydrothermal Vent Field.

well as the ability to begin evolutionary investigations using mitochondrial markers. This technique could also be applied to recently-deceased corals in both shallow and deep-water habitats for multiple purposes (e.g., assessing the role of colonization/extinction events, population analysis, evolutionary processes). Some of this work is beginning on larger population studies from wide age ranges on different seamounts in the NW Atlantic (see Robinson et al., 2007) to examine both population connectivity and the effects of climate change on ancient and recent populations of deep-water corals. This dating study has revealed that *D. dianthus* populations on these seamounts are modal in time (Robinson et al., 2007) and through the use of aDNA techniques we will be able to examine how (and if) populations both on a single seamount and across the seamount chains are connected through time. Through this combined approach we expect to gain an unprecedented view of how this species survives climate stress, either by shifting populations across seamounts onto refu-

gia seamount(s), and then becoming re-populated when favorable conditions return, or dying down to low levels and re-populating on single seamounts. At present, we know of no study which attempts this unique integration of dating and aDNA in the marine realm to reconstruct climate effects on populations with such detail.

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