

ANALYSIS OF ANCIENT DNA FROM FOSSIL CORALLINES (CORALLINALES, RHODOPHYTA)¹

*Jeffery R. Hughey*²

Division of Math, Science, and Health Professions, Hartnell College, 156 Homestead Ave., Salinas, California 93950, USA

Juan C. Braga, Julio Aguirre

Departamento de Estratigrafía y Paleontología, Universidad de Granada, 18002, Granada, Spain

William J. Woelkerling

School of Botany, La Trobe University, Bundoora, Victoria 3083, Australia

and Jody M. Webster

School of Earth and Environmental Sciences, James Cook University, Townsville, Queensland 4811, Australia

The field of molecular paleontology has recently made significant contributions to anthropology and biology. Hundreds of ancient DNA studies have been published, but none has targeted fossil coralline algae. Using regions of the SSU gene, we analyzed rDNA from fossil coralline algae of varying ages and states of preservation from Spain, Papua New Guinea (PNG), and the Great Barrier Reef (GBR). Specimens from PNG, GBR, and some localities from Spain did not contain endogenous ancient DNA. Reproducible sequence data were obtained from specimens ~550 years old from near Cadiz, Spain, and from rocky-shore deposits in Carboneras, Almeria Province of Spain (~78,000 years before present [YBP]). Based on BLAST searches and a phylogenetic analysis of sequences, an undescribed coralline alga belonging to the Melobesioidae was discovered in the Carboneras material as well as the following coralline genera: *Jania*, *Lithophyllum*, *Lithothamnion*, *Mesophyllum*, and *Phymatolithon*. DNA from fleshy brown and red macroalgae was also discovered in the specimens from Carboneras. The coralline algae identified using molecular techniques were in agreement with those based on morphological methods. The identified taxa are common in the present-day southeastern Spain littoral zone. Amino acid racemization, concentration ratios, and specific concentrations failed to show a correlation between biomolecular preservation and PCR amplification success. Results suggest that molecular investigations on fossil algae, although limited by technical difficulties, are feasible. Validity of our results was established using authentication criteria and a self-critical approach to compliance.

Key index words: amino acid racemization; ancient DNA; Corallinales; fossil algae; Rhodophyta; SSU

Abbreviations: Ala, alanine; Asp, aspartic acid; BLAST, basic local alignment search tool; D/L, dextrorotatory/levorotatory; GBR, Great Barrier Reef; Glu, glutamic acid; Gly, glycine; PNG, Papua New Guinea; Ser, serine; YBP, years before the present

The science of molecular paleontology is concerned with the retrieval, amplification, authentication, and analysis of DNA from ancient specimens (Marota and Rollo 2002). The field was introduced by Higuchi et al. (1984), Pääbo (1985), and Doran et al. (1986), who focused on the genetic investigation of human remains. Their apparent success, coupled with the advent of the polymerase chain reaction (Mullis and Faloona 1987), stimulated tremendous excitement over the possibility of using ancient DNA to address systematic, biogeographic, paleoecological, and evolutionary questions. Further research in the field swiftly followed on a variety of organisms (e.g., Cano et al. 1992). After the discipline's initial expansion, attempts were made to reproduce some of the more sensational findings but met with failure (e.g., Young et al. 1995). DNA sequences obtained from fossils older than 1 million years were shown to be artifactual (Hofreiter et al. 2001). In the case of the plant leaves, where *rbcl* gene sequences were reported from 17- to 20-million-year-old *Magnolia* and *Taxodium* impressions, it was concluded that the data were due to intralaboratory contamination (Wayne et al. 1999).

In an effort to minimize false claims, a set of nine authentication criteria were proposed (Cooper and Poinar 2000). A principal criterion is the estimation of biomolecular preservation via the analysis of amino acids (Poinar et al. 1996). Dextrorotatory/Levorotatory (D/L) racemization

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²Author for correspondence: e-mail jhughey@jafar.hartnell.edu.

ratios for aspartic acid (Asp) and alanine (Ala) are calculated for this determination. D/L Asp ratios lower than 0.12 indicate good biomolecular preservation and typically correlate with DNA retrievability (Poinar et al. 1996). D/L Ala ratios higher than Asp ratios indicate contamination from modern sources. Concentration ratios and specific concentrations, which compare fossil proportions and amounts of amino acids to a reference standard, are also calculated because they are indicative of biomolecular preservation (Klinken and Mook 1990). Strict adherence to this criterion and others, however, has been minimal. Some have argued that ancient DNA studies should be assessed by a more cognitive and self-critical approach (Gilbert et al. 2005).

In addition to understanding and applying criteria, knowledge of technical difficulties aids in determining the validity of ancient DNA results. There are four major technical guidelines: (1) contamination is rampant; (2) DNA degrades with time and is unlikely to survive for more than 1 million years; (3) DNA is rarely intact (>300 bp in length) in fossil materials; (4) ancient DNA, when present, occurs in minute quantities (Pääbo 1989, Lindahl 1993). Based on the application of these principles, the oldest verifiable samples to contain amplifiable DNA come from permafrost (Willerslev and Cooper 2005). These findings include the remains of a woolly mammoth found in Siberia, dated at >50,000 YBP (Höss et al. 1994); a bison >62,500 YBP (Gilbert et al. 2004); plant chloroplast DNA sequences obtained from ice cores up to 400,000 YBP (Willerslev et al. 2003); and bacterial sequences up to 600,000 YBP (Willerslev et al. 2004).

To date, no attempts to isolate ancient DNA from fossil algae have been reported. The oldest published DNA sequence obtained from an alga is from a 327-year-old herbarium specimen of *Sarcotialia stiriata* (Gigartinales, Rhodophyta) (Hughes et al. 2002). The purpose of this study was to determine whether fossil coralline red algae (Corallinales, Rhodophyta) contain amplifiable DNA and, if so, to determine (1) whether the DNA results correlate with microscopic identification and (2) the time period open to molecular genetic investigation. Coralline algae representing a range of ages (550–2.1 million YBP) and two preservational histories were analyzed (fossil coralline algae in deposits that remained submerged after formation vs. fossil corallines in subaerial rocks) from Spain, PNG, and the GBR. Following previous molecular studies on coralline algae (Bailey and Chapman 1996, 1998, Bailey 1999, Harvey et al. 2003), the 18S rRNA gene (SSU) was targeted. To authenticate our results, we adhered, where appropriate, to the current ancient DNA guidelines as proposed by Cooper and Poinar (2000), including the analysis of amino acids.

MATERIALS AND METHODS

DNA extraction, PCR, and cloning. Twenty-nine coralline fossils were analyzed in this study (Table S1, see the supplementary material). Specimens were pulverized using new mortars and pestles (Fisher Scientific, Pittsburgh, PA, USA) purchased exclusively for this project. Between usage, mortars and pestles were rinsed and cleaned with dishwashing soap, immersed in 5.25% sodium hypochlorite for 72 h, washed eight times with DNA-free water, autoclaved, and UV illuminated for 24 h. About 400 mg of coralline was ground under liquid nitrogen. The resulting powder was decalcified and digested for 30 h at 37°C under gentle agitation in 750 µL of extraction buffer containing 0.5 M EDTA (pH 8.0), 5% Sarkosyl Ultra reagents (Fluka BioChemika, Switzerland), and 20 µL of a 10 mg·mL⁻¹ solution of PCR grade Proteinase K (Roche, Indianapolis, IN, USA) (Krings et al. 1997). Following incubation, samples were centrifuged for 1 min at 4,000g, and the supernatant was removed and extracted with an equal volume of UltraPure phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen, Carlsbad, CA, USA). Supernatant was removed and concentrated to 100 µL using Microcon MW-30 columns following the manufacturer's instructions (Millipore, Bedford, MA, USA). DNA from concentrate was isolated using the MinElute Qiagen PCR Purification Kit (Qiagen, Valencia, CA, USA) following Yang et al. (1998). DNA was eluted with 70 µL of DNA-Free Elution Solution (QBIogene, Carlsbad, CA, USA). Immediately following elutions, to reduce DNA loss from binding to polypropylene or the denaturing of the DNA on polypropylene surfaces (Belotserkovskii and Johnston 1996), 6 µL of DNA extract was added to each 25 µL reaction containing HotStart Taq DNA Polymerase and Q-solution following the manufacturer's instructions (Qiagen). Primer pair names are based on relative annealing positions on 18S genes from published coralline algal sequences (Bailey and Chapman 1996, 1998, Bailey 1999, Harvey et al. 2003) (Table S2 in the supplementary material). Appropriate molecular behavior (Cooper and Poinar 2000) was determined using F61-R360 and F61-R464 primer pairs. This criterion proposes that authentic ancient DNA, because it is degraded, shows an inverse relationship between amplicon length and amplicon efficiency (Hofreiter et al. 2001). Generally, with ancient DNA, only short targets successfully PCR amplify. The amplification of large products is unusual and typically indicates contamination by exogenous DNA from modern sources (Cooper and Poinar 2000). Reactions were cycled in a PTC-150HB PCR MiniCycler (MJ Research, Watertown, MA, USA) using the following parameters: 95°C for 15 min, 42 cycles of 94°C for 30 s, 55°C for 60 s, 72°C for 60 s, and 72°C for 7 min. PCR products were cloned using the 2.1-TOPO® TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Eight or more clones per amplicon were sequenced by Rexagen Corp. (Seattle, WA, USA).

Authentication. Several of the authentication criteria proposed by Cooper and Poinar (2000) are specific to human studies, and therefore their implementation is unnecessary for the algae. Results in this study were confirmed through the following: (1) DNA extractions were performed in a medical science building never previously used for extracting, amplifying, or curating algae; (2) no modern DNA from corallines was extracted for this study, nor has the first author ever extracted DNA from a modern coralline; (3) extractions and amplification preparations were carried out in a physically isolated work area using a flow hood dedicated to the study of ancient DNA; (4) DNA extracts were maintained in a bleach-treated freezer in a separate wing of the building from the PCR machine; (5) multiple blank extractions were processed in parallel with the ancient corallines, and multiple negative controls were included in all PCR reactions; (6) DNA samples were tested for

appropriate molecular behavior (see explanation above); (7) results from the Carboneras material were confirmed by analyzing the same fossils twice; (8) small targets were amplified (<200 bp); (9) PCR products were cloned; (10) amino acid racemization, concentration ratios, and specific concentration results were determined in duplicate on a subset of the samples; (11) equipment, mortars and pestles, and tubes were illuminated with UV at least 24 h prior to use; and (12) commercial reagents (Taq Polymerase, primers, water, and buffers) were screened for modern coralline DNA prior to use by performing PCR reactions in quadruplicate with known DNA-free reagents.

At the onset of this project, DNA extracted from fossil coralline algae (FCA) materials from the fossil beach near Cadiz (~550 YBP) were separated from the other samples to avoid future carry-over contamination. Due to their young age, these specimens were more likely to contain larger quantities of intact DNA molecules compared to the other fossils. This likelihood was confirmed based on their PCR amplification intensity and results from the amino acid analyses. Prior to their exclusion, FCA specimens produced DNA sequences that were specific to *Lithophyllum*, *Lithothamnion*, and *Phymatolithon* (F59-R161, F61-R161, F100-R304). When the same primers were employed on the Carboneras material, they generated sequences that did not match the FCA specimens (Fig. S1 in the supplementary material). Although there was no evidence of contamination at that time, additional primers were synthesized (F240-R360, F288-R464, F464-R586) for use on the Carboneras and other specimens to avoid the possibility of future contamination.

Amino acid analysis. Analyses were carried out by MicroAnalytica LLC (Miami, FL, USA) following Poinar et al. (1996) and Klinken and Mook (1990). Three tests were performed: (1) D/L Asp and Ala ratios; (2) concentration ratios for Asp/glutamic acid (Glu), serine (Ser)/Glu, Ala/Glu; and (3) specific concentrations for Asp, Glu, Ser, glycine (Gly), and Ala. A 6-year-old herbarium specimen of *Bossiella plumosa* was shipped to MicroAnalytica for use as the reference standard. Representative specimens from the GBR and near Cadiz, Carboneras, and Roquetas de Mar, Spain, were analyzed; these included FCA-2, FCA-4.1, R5-2/4, R5-4/8, H2-2, H1-4, ELD-2, ELD-3, ELD-5, and ELD-7.

BLAST searches. The BLAST analyses were performed using default options in the nucleotide BLAST online program (Altschul et al. 1990). The program compares nucleotide sequences to sequence databases and reports identities (number of nucleotide differences between query and subject) as well as calculates a statistical significance for matches. The Nucleotide Collection (nr/nt) database was searched for all queries. Since most regions of the SSU gene contain synapomorphic and autapomorphic characters, sequence identity using the BLAST program can be accurately determined through the analysis of partial sequences (Venter et al. 2004, DeLong 2005).

Sequence analysis. Consensus sequences were determined from manually aligned amplicons using PAUP* 4.0.0b10 (Swofford 2002). DNA sequences were deposited in GenBank (Table S1). Genetic distances were calculated using PAUP* and represent uncorrected ("p") distances determined from sequence data published by Bailey and Chapman (1996, 1998), Bailey (1999), and Harvey et al. (2003). Sequences were initially assembled using the Clustal W 1.61 alignment program and then optimized manually in PAUP* using published parameters (Hughes et al. 2001). Following Harvey et al. (2003), *Audouinella dasyae* and *Meiodiscus spetsbergensis* were selected as outgroups for the maximum-parsimony analysis. Heuristic searches were carried out on 15 ancient SSU sequences and 30 modern corallines (Table S3 in the supplementary material) treating parsimony-informative characters as unordered and with equal weight. Uninformative

characters were excluded from analyses, and gaps were treated as missing. Trees were obtained via simple stepwise addition, holding one tree at each step during the addition sequence while swapping on best trees only using the tree-bisection-reconnection (TBR) branch-swapping algorithm with MaxTrees set to 20,000. Support for branches was assessed by calculating bootstrap proportion (BP) values (Felsenstein 1985) based on 1,000 resamplings. Bootstrap searches were Full Heuristic using the parameters described above. MulTrees and Collapse options were in effect, and the steepest descent option was off.

Age control and radiometric dating. The specimens from near Cádiz, Spain, were dated using C14 (by P. Grootes) in the "Leibniz Labor für Alterbestimmung und Isotopenforschung," Christian-Albrechts University, Kiel (Germany) laboratory. The conventional age was calculated according to Stuiver and Polach (1977), and calibrated ages were obtained using "CALIB rev 4.3" (Stuiver et al. 1998). The obtained calibrated age for the samples from near Cádiz is ~550 YBP (cal. AD 1427–1482). The specimens from Carboneras, Spain, were dated by C. Gallup at the Department of Geological Sciences in Duluth (MN, USA) by dating coralline skeletons with U/Th nonequilibrium techniques. The age of the sample corrected for detrital ²³⁰Th associated with detrital ²³²Th is 129,623 ± 1,020 YBP. High $\delta^{234}\text{U}$ values (272.3 ± 2.3 initial and 188.7 ± 1.5 present), however, suggest that they have been altered and that this dating is very likely older than the true age. One of the main ways uranium isotopic values become elevated is by alpha recoil effects (Thompson et al. 2003, Villemant and Feuillet 2003). If the dated coralline algae followed these trends, it would imply a true age of ~78,000 YBP for the sample. The common occurrence of *Strombus bubonius* in the bed from which corallines were sampled supports this dating for the Carboneras specimens, as a minimum age. The youngest deposits with this exotic tropical gastropod in the Quaternary rocks of southern Spain are at least from Oxygen Isotopic Stage (OIS) 5a (Zazo et al. 2003), which is dated at ~78,000 YBP (Lambeck and Chappell 2001). The age and stratigraphic framework of the samples collected from the fossil reefs in the Huon Gulf, PNG, and Ribbon Reef 5 in the northern GBR are summarized in Webster et al. (2004) and Braithwaite et al. (2004).

Microscopy. Corallines were identified to genus using a hand lens, Leica MZ12 stereomicroscope (Leica Microsystems, Wetzlar, Germany) and Nikon Labophot-2 compound microscope (Nikon, Melville, NY, USA). Generic concepts follow those of Woelkerling (1988), of Penrose, and of Womersley and Johansen in Womersley (1996) (also see Braga 2003).

RESULTS

Without exception, all water and negative controls failed to PCR amplify. DNA extracted from fossil corallines failed to generate PCR products when tested with the F61-R360 and F61-R464 primer pairs. Initial amplifications of fossil DNA were attempted using the F38-R100 primer pair. The BLAST analyses on cloned 18S rDNA sequences found similar or identical sequences in the nr/nt database for ascormycetes (Fungi), ciliophorans (Protista), *Nicotiana* (Embryophyta), basal angiosperms (Embryophyta), and corallines in one sample (FCA-2). A second attempt using the primer pair F59-R161 generated sequences similar or identical to a range of organisms in the database, including *Acidomyces* (Fungi, a contaminant of acid mine drainage; Baker

et al. 2004), *Cafeteria* (Bicosoecida), ciliophorans, *Cordyceps* (Fungi), *Euplotes* (Ciliophora), *Glomus* (Fungi), plasmodiophorids, various Protista, and *Schizochytrium* (Labyrinthulida) (Table 1). The third primer combination (F61-R161) produced sequences that were specific to the algae, Fungi, or to *Acidomyces*. Primer pairs F100-R304, F240-R360, F288-R464, and F464-R586 generated algal sequences and, in most cases, sequences identical to members of the Corallinales (Table 1).

Of the 29 specimens analyzed in this study (Table S1), SSU coralline sequences were obtained from

five. Two of the specimens came from near Cadiz, Spain (FCA-1 and 2, ~550 YBP). Sequences obtained from FCA-1 were identical to *Lithophyllum* and *Lithothamnion* (Table 1). FCA-2 generated sequences similar to *Phymatolithon* and identical to *Lithothamnion* using primers F59-R161 and F61-R161, and *Lithophyllum* with primers F100-R304 (Table 1). The other three samples came from rocky-shore deposits in Carboneras, from the Almeria Province of Spain (ELD-2, ELD-3, ELD-6, ~78,000 YBP). Depending on the primer pair, ELD-2 yielded sequences similar or identical to Haptophyceae,

TABLE 1. Amplification success and sequence homologies based on BLAST analysis for fossil coralline algae using different 18S primer pairs.

Sample name	Primer names					
	59-161	61-161	100-304	240-360	288-464	464-586
FCA-1	<i>Lithothamnion</i> (0 bp)	<i>Lithothamnion</i> (0-1 bp)	<i>Lithophyllum</i> (0-1 bp)	*	*	*
FCA-2	<i>Lithothamnion</i> (0-1 bp)	<i>Lithothamnion</i> (0-2 bp)	<i>Lithophyllum</i> (0-2 bp) <i>Phymatolithon</i> (3-6 bp)	*	*	*
ELD-2	<i>Schizochytrium</i>	Haptophyceae (1 bp) Phaeophyceae (0 bp)	Corallinales (10-13 bp)	<i>Jania</i> (1-3 bp)	Corallinoideae (0 bp) <i>Mesophyllum</i> (0 bp) <i>Lithophyllum</i> (0-2 bp)	<i>Caloglossa</i> (1-2 bp) Corallinales (0 bp) <i>Lithophyllum</i> (0-2 bp) <i>Lithophyllum</i> (0 bp)
ELD-3	<i>Euplotes</i> , <i>Schizochytrium</i>	<i>Acidomyces</i> (0 bp)	-	-	-	<i>Lithophyllum</i> (0 bp)
ELD-4	<i>Acidomyces</i> , ciliophoran Laminariales (0 bp)	Laminariales (0 bp)	-	-	-	-
ELD-5	Ascomycete	Sargassaceae (0-1 bp)	-	-	-	-
ELD-6	<i>Cordyceps</i> , Fungi Zygnematales	Chlorophyta (1-2 bp)	-	-	-	Corallinales (0 bp)
ELD-7	-	Fungi	-	-	-	-
2.1 Million	<i>Acidomyces</i>	-	-	-	-	-
RAM1	Ascomycete, Protist	-	-	-	-	-
45B	<i>Cordyceps</i>	<i>Cordyceps</i>	-	-	-	-
276	Erythropeltidiales	Erythropeltidiales	-	-	-	-
	-	<i>Eunotia</i> Dictyotales (0 bp)	-	-	-	-
276B	<i>Cordyceps</i>	-	-	-	-	-
78B	<i>Cordyceps</i>	-	-	-	-	-
165	<i>Cordyceps</i>	-	-	-	-	-
197	<i>Cordyceps</i>	-	-	-	-	-
7	-	Fungi	-	-	-	-
121	-	<i>Cordyceps</i> , Fungi	-	-	-	-
172	-	<i>Cordyceps</i>	-	-	-	-
H1-3	-	-	-	-	-	-
H1-4	-	-	-	-	-	-
H2-2	-	-	-	-	-	-
H2-21	-	-	-	-	-	-
H3-1	-	-	-	-	-	-
H3-2	-	-	-	-	-	-
R5-2/4	-	-	-	-	-	-
R5-4/7	-	-	-	-	-	-
R5-4/8	-	-	-	-	-	-
R5-5/1	-	-	-	-	-	-

- indicates failure to amplify; * indicates the reaction was not attempted; (# bp) indicates the number of base pair differences in comparison to sequences deposited in GenBank. In cases where sequences are indistinguishable at the lower taxonomic rank, the higher taxonomic rank is listed.

Phaeophyceae, *Caloglossa*, *Jania*, *Lithophyllum*, *Mesophyllum*, Corallinales, and Corallinoideae. ELD-2 also generated a sequence that differed from known Melobesioideae by >10 nucleotides using primers F100-R304 (Fig. S1). ELD-3 sequences were identical to *Lithophyllum*. DNA amplified from ELD-6 was similar to Chlorophyta and Corallinales. ELD-4 and ELD-6 fossils contained sequences that were indistinguishable from modern representatives of the Laminariales and Sargassaceae, respectively.

A specimen (#276) from a submerged (~1145 m) coral reef off PNG (~230,000 YBP, Webster et al. 2004) contained amplifiable DNA identical to modern Dictyotales (Table 1). Another sample from PNG, #45B (~130,000 YBP, Webster et al. 2004), contained DNA that was similar to the Erythropeltidales. Six specimens (H1-3, H1-4, H2-2, H2-21, H3-1, H3-2) from Roquetas de Mar, Almeria Province, and four from Ribbon Reef 5 from the northern GBR in northeastern Australia failed to amplify

corallines. The specimen from Rambla de las Amoladeras, Spain (RAM1, ~2.1 million YBP), failed to amplify any coralline algae.

Phylogenetic analysis of 15 partial ancient SSU sequences and 30 modern coralline sequences yielded 20,000 most-parsimonious trees of 909 steps (Fig. 1). Bootstrap support for most branches was poor. Sequences identified from BLAST searches as belonging to *Lithothamnion*, *Lithophyllum*, and *Phymatolithon* (FCA 1 and 2, F59-R161, F61-R161, F100-R304) from fossils from near Cadiz, Spain, clustered with modern sequences from these same genera. Likewise, sequences obtained from ELD-2 (F240-R360, F288-R464, F464-R586) and ELD-3 (F464-R586) from Carboneras, Spain, and identified through BLAST searches as *Jania*, *Mesophyllum*, and *Lithophyllum*, all grouped with their respective genera. Other sequences in the phylogenetic analysis went unresolved and swapped from branch to branch (ELD-2 Carboneras F288-R464, F464-R586;

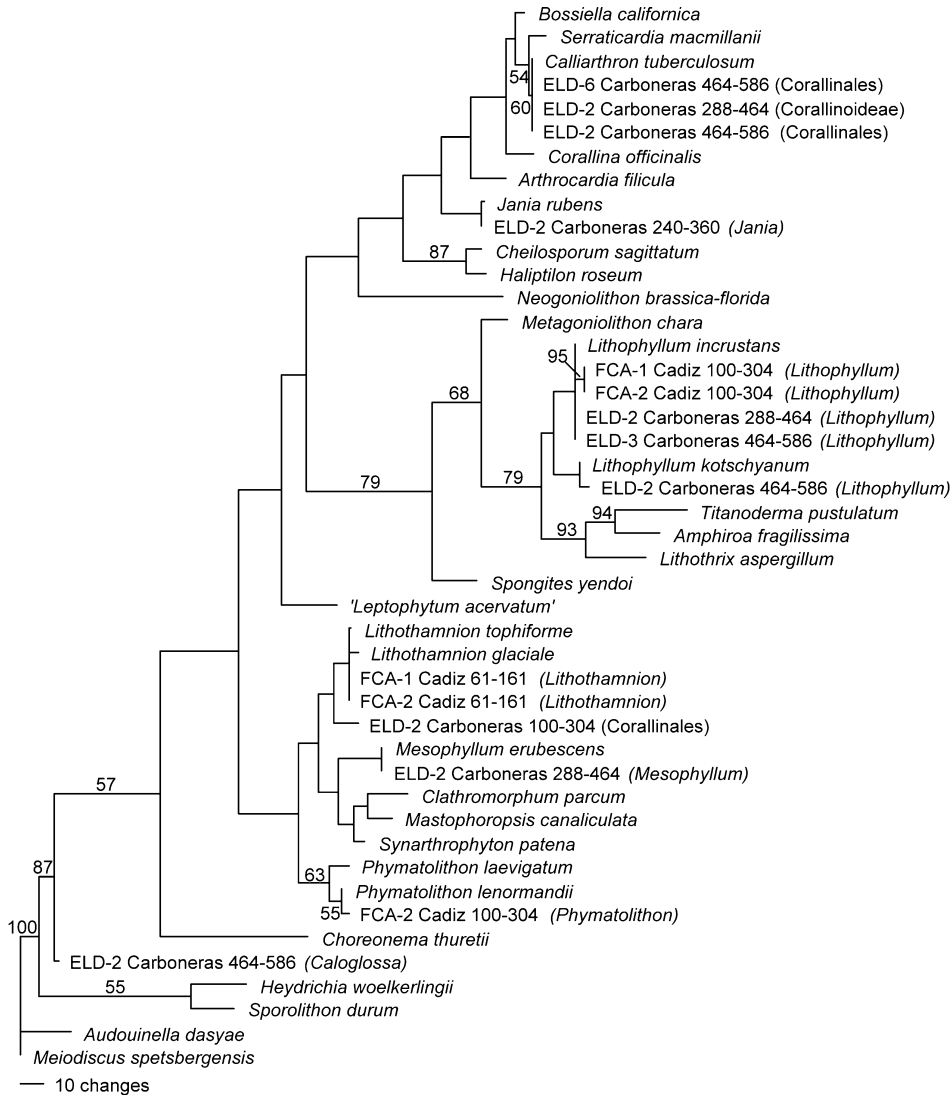


FIG. 1. One of 20,000 equally parsimonious trees resulting from an analysis of 18S rDNA sequences from 15 fossils and 30 modern corallines (909 steps). Abbreviated fossil names are given along with the locality, primers employed, and BLAST results in parentheses. Parsimony bootstrap values based on 1,000 replicates are shown on branches supported by >50%.

ELD-6 Carboneras F464-R586). The ELD-2 sequence that differed from known Melobesioideae occupied a sister position to *Lithothamnion* in the Hapalidiaceae (Fig. 1). Analysis of uncorrected ("p") genetic distances of the ELD-2 sequence showed that it differed from *Mesophyllum* by 0.08144, from *Synarthrophyton* by 0.08180, from *Clathromorphum* by 0.08957, from *Phymatolithon* by 0.09024, from *Lithothamnion* by 0.09513, from '*Leptophytum*' by 0.09850 (generic designation following Bailey and Chapman 1998), and from *Mastophoropsis* Woelkerling by 0.10847. Genetic distances between genera in the Melobesioideae range from 0.04912 (*Lithothamnion* vs. *Phymatolithon*) to 0.1002 (*Lithothamnion* vs. *Leptophytum*). Analysis of the ELD sequence versus taxa outside the Hapalidiaceae finds that *Heydrichia woelkerlingii* in the Sporolithaceae shows a moderately close genetic distance of 0.10396.

Microscopic examination of fossil specimens from near Cadiz and Carboneras, Spain, resulted in the identification of *Amphiroa*, *Lithophyllum*, and *Neogoniolithon* (Table 2). *Lithophyllum* was identified in both

TABLE 2. Identification of fossil coralline algae based on 18S rDNA sequences and morphological characters using microscopy.

Sample name	DNA sequences	Microscopy
FCA-1 (Cadiz)	<i>Lithophyllum</i> , <i>Lithothamnion</i>	<i>Lithophyllum</i>
FCA-2 (Cadiz)	<i>Lithophyllum</i> , <i>Lithothamnion</i> , <i>Phymatolithon</i>	<i>Lithophyllum</i>
ELD-2 (Carboneras)	<i>Jania</i> , <i>Mesophyllum</i> , Corallinoideae, unknown Coralline	<i>Neogoniolithon</i>
ELD-3 (Carboneras)	<i>Lithophyllum</i>	<i>Lithophyllum</i> , <i>Neogoniolithon</i>
ELD-4 (Carboneras)	Laminariales	No identifiable taxa
ELD-5 (Carboneras)	Sargassaceae	<i>Amphiroa</i> , <i>Lithophyllum</i> , <i>Neogoniolithon</i>
ELD-6 (Carboneras)	Corallinaceae	<i>Neogoniolithon</i>

TABLE 3. Amino acid racemization, concentration ratios, and specific concentrations for representative fossil coralline algae analyzed in this study.

Sample name	D/LAsp	D/LAla	Asp/Glu	Ser/Glu	Ala/Glu	Asp (%)	Glu (%)	Ser (%)	Gly (%)	Ala (%)	Combined average (%)
Modern ref.	0.027	0.057	1.18	0.71	1.08	100	100	100	100	100	100
FCA-2	0.203	0.065	4.20	0.75	1.00	18	5	5	7	5	8
FCA-4.1	0.222	0.064	4.22	0.76	0.95	16	4	5	5	4	7
R5-2/4	0.375	0.337	3.79	0.21	0.64	10	3	1	3	2	4
R5-4/8	0.319	0.341	4.80	0.27	0.86	10	2	1	3	2	4
H2-2	0.374	0.433	2.49	0.36	1.16	1	1	0	1	1	1
H1-4	0.222	0.204	1.28	0.48	1.01	1	1	1	1	1	1
ELD-2	0.485	0.634	2.42	0.22	1.02	1	1	0	1	1	1
ELD-3	0.342	0.227	1.54	0.51	1.10	0	0	0	0	0	0
ELD-5	0.439	0.617	2.73	0.23	1.08	0	0	0	0	0	0
ELD-7	0.450	0.666	2.92	0.29	1.02	0	0	0	0	0	0

Ala, alanine; Asp, aspartic acid; D/L, dextrorotatory/levorotatory; Glu, glutamic acid; Gly, glycine; Ser, serine.

FCA 1 and 2 (near Cadiz) using microscopy. Comparatively, the molecular method identified three genera, *Lithophyllum*, *Lithothamnion*, and *Phymatolithon*. Examination of thin sections of the Carboneras material indicated the presence of *Neogoniolithon*, *Lithophyllum*, and *Amphiroa*. *Jania*, *Lithophyllum*, *Mesophyllum*, and an undescribed coralline alga were revealed using molecular techniques.

Amino acid analysis was performed on one reference standard and 10 fossil corallines (Table 3). The D/L Ala ratio (0.057) from the standard was about two times that of Asp (0.027). D/L Asp ratios for the fossils were 7.5–18 times the reference standard and 1.1–11.7 times Ala. D/L racemization ratios for Asp ranged from 0.203 for FCA-2 to 0.485 for ELD-2. Ala ratios ranged from 0.064 for FCA-4.1 to 0.666 for ELD-7. Concentration ratios similar to the standard were found in H1-4 and ELD-3 for Asp/Glu, and FCA-2 and FCA-4.1 for Ser/Glu; with the exception of the R5-2/4 and R5-4/8, all were similar to the reference for Ala/Glu. Specific concentration results based on Asp, Glu, Ser, Gly, and Ala were consistent across all samples but, compared to the reference, showed very low combined averages. FCA (7%–8%) and R5 (4%) samples contained a higher percentage of amino acids, whereas H1 and H2 (1%), ELD-2 (1%), and ELD-3 (0%), ELD-5 (0%), and ELD-7 (0%) showed lower concentrations.

DISCUSSION

Fossil coralline DNA was isolated in five of the 29 samples analyzed in this study. Specimens from an emergent fossil beach at Roquetas de Mar, Spain, representing samples 1,405–6,930 years in age, all failed to amplify. In addition, samples from the subsurface in the GBR and submerged in the Huon Gulf, PNG, failed to yield results. It is likely that after-death processes, such as endogenous nuclease activity, microbial decomposition, and exposure to open air, seawater, or freshwater, which are destructive to biomolecules, are responsible for the degradation of these samples. Reproducible

results were observed in the fossils from emergent outcrops from young specimens near Cadiz (~550 YBP) and old fossils from Carboneras (~78,000 YBP). To the best of our knowledge, the corallines from Carboneras are some of the oldest successfully analyzed fossils. The time frame open to molecular genetic investigation in the corallines appears to be similar to that observed for animal bone (Hofreiter et al. 2001). The presence of red and brown algal DNA sequences in the subaerial fossil corallines from Carboneras was not anticipated. If representatives of the Florideophyceae, Laminariales, and Sargassaceae are truly present, how were these remains from unmineralized thalli preserved? Since these taxonomic groups contain intertidal species, it is possible that the corallines acted to biocement the algae. It is also possible that the preservational history from this site may have played a role in coralline integrity. Preservation of fossils in general is enhanced by isolation from the conditions reigning at the sea bottom or on the surface of the land. The fossil corallines from Carboneras occur attached to hard substrates and were partly embedded in micrite mud that fills the empty spaces left by coralline growths. Early filling by micrite mud of voids around coralline thalli and rapid lithification of this mud may have helped to exceptionally preserve not only coralline plants but also the organic matter from fleshy algae trapped in the impermeable mud.

Our data suggest that unlike mammal bone, D/L Asp and Ala ratios in corallines do not correlate with DNA preservation. For instance, specimens from Carboneras that successfully PCR-amplified generated higher ratios than specimens from the GBR and Roquetas de Mar, which failed to amplify (Table 3). In addition, whereas in bone the Asp ratios (<0.05) are much higher than Ala ratios (<0.009) (Klinken and Mook 1990), the Asp ratios in six of the 11 coralline samples were lower. One of these six included the modern coralline reference, where the Ala ratio (0.057) was approximately two times the Asp ratio (0.027). Normally, if Ala ratios are higher than Asp numbers, this indicates a recent and rather substantial incursion of modern materials into a fossil system (Poinar 2002). However, in this case, since the reference standard shows the same trend, it appears as though a reversed trend is exhibited by the algae. In addition, Asp and Ala D/L ratios were much higher in the coralline fossils (Asp, 0.203–0.450; Ala, 0.064–0.666). At this time we are unable to provide an explanation to account for these observed racemization differences.

Concentration ratios were also calculated to determine if amino acids are useful at predicting DNA preservation. If fossils show similar proportions of amino acids, compared to a reference standard, then they are considered to be well preserved. As with the D/L ratios, the concentration ratios for Asp/Glu,

Ser/Glu, and Ala/Glu failed to support a correlation (Table 3). Specific concentrations for Asp, Glu, Ser, Gly, and Ala were also determined. Results from this test represent the percentage of amino acids in the sample relative to the reference standard. A combined average value of 100%, for the specific concentrations, suggests similar preservation to modern material. The higher the combined average, the more superior the preservation. Based on this analysis, the best candidates for DNA preservation were the specimens from near Cadiz, Spain (7%–8%); inferior candidates were those from Carboneras (0–1%). Since the specimens from Roquetas de Mar, Spain (1%), and the GBR (4%) fell within this range, yet did not PCR amplify, it is apparent that specific concentrations also failed to identify the best candidates for ancient DNA retrieval.

As is evident from the BLAST analyses on SSU sequences obtained from the fossil samples, many organisms coamplify or selectively amplify over the coralline algae. The use of primer pairs F38-R100 and F59-R161 resulted in the amplification of plants, protists, and fungi (Table 1). It is likely that the DNA of these organisms comes from modern terrestrial specimens embedded in the fossils. A solution to this problem is to design coralline specific primers, a technique that was utilized in this study, but with varied success. One of the disadvantages of targeting the SSU gene is in its universality in eukaryotes. Another is the sensitivity of the PCR technique, which theoretically requires only one intact molecule for polymerization. A third difficulty is dealing with contamination from exogenous sources. Whereas the probabilities are rare for modern macroalgal contaminants to establish on corallines collected on land (near Cadiz and Carboneras), recent growth of modern contaminants on submerged collections is probably not uncommon. For this reason, we believe the sequences obtained from 276 and 45B from PNG are likely the result of recent or nearly recent algal colonization on these two specimens. Investigations of a drowned –250 m reef in Papua New Guinea indicate that deepwater coralline algal growth can persist up to 25 ka after the drowning of the shallow-water reef complex (Riker-Coleman et al. 2004). The sequences obtained from 276 and 45B match Dictyotales and Erythropeltidales, two orders known to contain representatives that occur at greater ocean depths (Peckol and Ramus 1992, Abbott 1999).

The advantage of using the SSU gene is in its organization. In coralline algae, it is approximately 1,750 nucleotides in length (+ or –100 bp), of which about 280 (15%) are phylogenetically informative (Bailey and Chapman 1996). The variation occurs in short hypervariable regions (10–30 bp) that are flanked by conserved stretches every 60–80 bp. This type of organization allows for the PCR amplification of degraded DNA. Once amplified,

cloned, and sequenced, the data can be analyzed using a BLAST analysis or aligned and phylogenetically identified.

Phylogenetic analysis showed that some of the ancient SSU sequences contained a sufficient number of synapomorphic characters to resolve them within the modern Corallinales (Fig. 1). The unique ELD-2 sequence (F100-R304) occupied an unsupported sister group relationship to *Lithothamnion* in the Melobesioideae (Fig. 1). Genetic distances between ELD-2 and extant coralline genera, based on a 182 nucleotide portion of the SSU gene, are comparable to those found between currently recognized genera in the Melobesioideae. Phylogenetic analysis and genetic distances suggest that the ELD-2 sequence may represent a previously undescribed coralline genus. Some of the ancient coralline sequences lacked an adequate number of synapomorphies to establish a firm position on the tree. These data highlight the difficulty of using partial DNA sequences to accurately assign taxonomic identities based on phylogenetic methods (Freshwater and Rueness 1994).

There are three possible explanations to account for the uniqueness of the ELD-2 sequence (F100-R304) from Carboneras. The first is that the polymorphisms are artifactual. DNA has been shown to be susceptible to damage and, therefore, is likely to contain a high proportion of substitutions (Höss et al. 1996, Hofreiter et al. 2001). However, alignment of the sequences against other corallines indicates that the same polymorphisms also occur in other Hapalidiaceae, suggesting that they are not damage induced (Figs. S1 and S2 in the supplementary material). The second explanation is that the sequence may be the result of the recombination of overlapping, short DNA templates. This phenomenon, known as "Jumping PCR," was first described by Higuchi et al. (1988) and later coined by Pääbo et al. (1990). The possibility of the same jumping event occurring in multiple clones from both ELD-2 extractions, which were performed 1 year apart, seems unlikely. The third possibility is that the sequence is authentic. Adherence in this study to ancient DNA criteria supports the latter explanation. Further molecular studies of this undescribed coralline using melobesioid-specific primers are needed.

Coralline genera identified from fossil samples using microscopy were in agreement with those determined from SSU sequences (Table 2). Comparison of morphological and molecular techniques on the material from near Cadiz, Spain, showed that DNA methods resolved two additional genera (Table 2). Evaluation of the Carboneras data indicated concordance between methodologies. Molecular analysis of the ELD-2 (Carboneras) specimens led to the identification of two genera; however, no morphologies could be attributed to the undescribed coralline alga. All identified taxa are

common on the southeastern coast of Spain (Calvín Calvo 1995) and, in general, in the western Mediterranean (Bressan and Babbini 2003).

In conclusion, consideration of the criteria proposed by Cooper and Poinar (2000) supports the conclusion that endogenous ancient DNA was successfully extracted and analyzed from ancient corallines, and possibly from red and brown algal fossils. Specimens ~550 years old from near Cadiz, Spain, and ~78,000 YBP from Carboneras, Almería Province of Spain, yielded verifiable results. A number of observations sustain this statement. The molecular data were in agreement with the morphological identifications. The sequences generated from coralline fossils from near Cadiz, Spain, indicated the presence of three genera; while those from Carboneras, Spain, revealed three coralline genera and an undescribed melobesioid. The potential application of ancient DNA techniques in discovering new taxa, studying mutation rates over time, and examining ecological changes in reef communities seems highly plausible on coralline fossils within hundreds of years, but also likely on exceptionally well-preserved rocky-shore deposits from the last 80,000 YBP.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Alignments of modern and fossil coralline SSU gene sequences generated using primer pair F100-R304 (177–194 nucleotides in length).

Figure S2. Alignments of modern and fossil coralline SSU gene sequences generated using primer pair F464-R586 (96–98 nucleotides in length).

Table S1. Specimens analyzed in this study.

Table S2. Primers used in this study. Primers 5' → 3'.

Table S3. List of species and 18S rDNA GenBank accession numbers used in the present study.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00462.x>.

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