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Fossil DNA in Cretaceous Black Shales: Myth or Reality?

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In a recent paper in this journal, Inagaki *et al*. (2005) reported the recovery of fossil DNA derived from past microbial communities from Cretaceous marine sediments deposited approximately 112 million years ago as a black shale interval known as Oceanic Anoxic Event (OAE)-1b. Although the recovery of fossil DNA to reconstruct past microbial communities is not entirely new—there are reports of its recovery from Holocene lake and marine sediments (Coolen and Overmann, 1998; Coolen *et al*., 2004a,b, 2006a,b) and Pleistocene marine sediments (Inagaki *et al*., 2001)—the presumed Cretaceous age of the fossil DNA derived from past microbial communities recovered by Inagaki *et al*. (2005) is unprecedented. If genuine, this would represent a large step in molecular paleontology. It would allow researchers to reconstruct ancient microbial communities at an unprecedented resolution and, thus, evaluate in much more detail the evolution of microbial life. Microbes are the key players in the biogeochemical cycling of elements during the evolution of life on Earth together with terrestrial higher plants since their evolution in the Ordovician.

In his commentary on Inagaki *et al*. (2005) in the same issue of *Astrobiology*, Hoehler (2005) outlines the potential of this approach and its pitfalls. He also delineates how this work could potentially relate to our understanding in an evolutionary context of the so-called Tree of Life, based on the ribosomal RNA gene present in all extant organisms: "Although the genome of every organism represent, at some level, a key to its evolutionary origin, we seemingly lack the genetic equivalent of skeletal fossils that would provide historical support for our inferences." Despite several critical notes and warnings, Hoehler (2005) believes that fossil DNA in sediments can aid in reconstructions of past microbial communities, though probably not at the same level as offered by the tools of modern genomics.

Both Inagaki *et al*. (2005) and Hoehler (2005) disregard one important piece of information in molecular paleontology that can be used to reconstruct ancient microbial communities: molecular fossils. These are fossil organic molecules derived from specific microbial lipids characteristic for groups of organisms. They are known since the early work of Alfred Treibs, who was able to show that fossil porphyrins are derived from chlorophyll (Treibs, 1936). In modern molecular paleontology, molecular fossils have been used, for instance, to determine the advent of the oxygenation of the Earth's early atmosphere (Brocks *et al*., 1999) and recently to calibrate the molecular clock of the genetic evolution of the diatoms (Sinninghe Damsté *et al*., 2004). In our work to determine whether fossil DNA could provide clues to ancient microbial communities, we always use molecular fossils as a reference point. Microbial lipids and their diagenetic products that were formed during settling of organic matter in the water column and subsequent sediment burial are far more stable than DNA and thus can serve as control for the more specific, though quantita-

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tively less reliable, preserved DNA. We have used this approach to show a good match between the quantitative sedimentary record of okenone, a specific pigment of various species of purple sulfur bacteria, and that of its 16S rDNA (Coolen and Overmann, 1998); of chlorobactene, a specific pigment of green sulfur bacteria, and its DNA (Coolen *et al*., 2006a); of long-chain alkenones, specific lipids of haptophyte algae, and haptophyte 18S rDNA (Coolen *et al*., 2004a, 2006b); and of a specific sterol derived from Group 1 methanotrophic bacteria and its 16S rDNA (Coolen *et al*., 2004b). These studies all indicated that rDNA in sediments is (partly) derived from the microbial community thriving in the water column in much the same way as the molecular fossils and, thus, allows for the reconstruction of ancient microbial communities with an unprecedented resolution. At the same time, these studies of Holocene (i.e., younger than 10 kiloyears) sulfidic sediments also indicated that the fossil DNA in the surface sediments is subjected to breakdown into smaller [less than 600 basepairs (bp) long] fragments, despite the dark, cold, and anoxic conditions (Coolen and Overmann, 1998; Coolen *et al*., 2006b), a process that ultimately will result in the complete destruction of fossil genetic information. In a recent study of Holocene sulfidic Black Sea sediments, a plausible contemporary equivalent of the Cretaceous OAE black shales studied by Inagaki *et al*. (2005), we quantified the number of 458-bp-long copies of *Emiliania huxleyi* 18S rDNA in various extracted DNA-size classes by means of quantitative polymerase chain reaction. After only 2,000 years of deposition, 70% of the 458-bp-long fossil *E. huxleyi* 18S rDNA copies were present in the extracted DNA size classes smaller than 4 kbp, and 10% was present in the DNA size class smaller than 700 bp (Coolen *et al*., 2006b). In this respect, the finding of ~900-bp-long fossil bacterial rDNA fragments in >100-Ma-old black shales by Inagaki *et al*. (2005) is remarkable and begs the question: Are the fossil genetic signals they recovered genuine?

Extraordinary findings require extraordinary evidence. For such a remarkable finding as reported by Inagaki *et al*. (2005), it would have been scientifically sound to compare their results with more conventional methods of reconstructing the ancient microbial communities, *i.e*., the analysis of molecular fossils, a technique that does not rely on polymerase chain reaction amplification of minute amounts of material. Unfortunately, Inagaki *et al*. (2005) did not make such comparisons, and their extraordinary findings are *not* confirmed by more conventional molecular paleontological techniques. However, the OAE-1b event has been thoroughly studied by molecular paleontologists, which has generated a generally consistent picture that is in strong contrast to the one obtained by the paleogenomics approach.

Characterization of the OAE-1b interval from three different sites—Ocean Drilling Program Site 1049C from the western Tethys, and two sites in the central Tethys: the Vocontian Basin in southeast France [close to the site where the samples used by Inagaki *et al*. (2005) were obtained] and the Ionian Basin (Greece)—all reveal that the organic matter is for a substantial part derived from pelagic marine Crenarchaeota (Kuypers *et al*., 2001, 2002; Tsikos *et al*., 2004), a group of Archaea that, in the present-day ocean, forms 20% of the picoplankton (Karner *et al*., 2001). This was evident from the presence of a specific membrane and other lipids of these Archaea. The archaeal lipids were carbon isotopically enriched relative to molecular fossils derived from algae and bacteria, an observation that enabled the researchers to attribute the substantial increase in the ^{13}C content of sedimentary organic matter in the OAE-1b interval to a contribution of up to 80% of marine, non-thermophilic Archaea. In fact, this OAE-1b interval was associated with a massive expansion of marine Archaea (Kuypers *et al*., 2001). In this respect, the absence of any archaeal rDNA sequences in this OAE-1b interval, as reported by Inagaki *et al*. (2005), is enigmatic and in our view strongly suggests that the fossil DNA is not genuine. Inagaki *et al*. (2005) hypothesize that archaeal DNA may be less stable than bacterial DNA but do not provide any basis for this assumption. If this were the case, the fossil DNA would provide a very biased view of the ancient microbial community.

Inagaki *et al*. (2005) reported that a number of 16S rRNA gene clones of oceanic sulfate reducing bacteria within the δ-Proteobacteria predominated at the OAE-1b interval. These were phylogenetically closely (up to almost 99% sequence similarity) related to environmental clones related to sulfate-reducing bacteria recovered from cold seep environments in which methane is anaerobically oxidized. Phylogenetic and lipid studies of the microbial communities thriving at such cold seeps in the present-day ocean have

provided a detailed fingerprint. Specifically, more general [*i.e.*, *iso* and *anteiso* C_{15} and C_{17} fatty acids (Pancost *et al*., 2000)] and highly specific [glycerol mono- and diethers (Hinrichs *et al*., 2000; Pancost *et al*., 2001)] lipids of sulfate-reducing bacteria participating in the microbial consortium capable of anaerobic oxidation of methane have been identified. They all have one feature in common: they are substantially depleted (*i.e.*, −70% to −100%) as a consequence of the fact that isotopically depleted biogenic methane serves as the sole carbon source of these prokaryotic ecosystems. This distinct signal has also been retrieved from ancient deposits reflecting cold seeps, such as carbonates (Peckmann and Thiel, 2004). If the 16S rDNA sequences of the sulfate-reducing bacteria recovered from the OAE-1b interval studied by Inagaki *et al*. (2005) are genuine, one would expect to find isotopically depleted molecular fossils derived from such bacteria. Detailed biomarker analyses from the three sections described above have failed to do so (Kuypers *et al*., 2001, 2002; Tsikos *et al*., 2004). Moreover, despite the dominance of archaeal molecular biomarkers in these sections, they are not depleted but enriched in ^{13}C. Sulfate-reducing bacteria in present-day cold seep settings are always accompanied by methanotrophic Archaea that produce lipids that are even more depleted than those of the sulfate-reducing bacteria. Hence, data on molecular fossils are in strong disagreement with the paleogenetic information provided by Inagaki *et al*. (2005).

In our investigations of the OAE-1b interval, we also noted that the organic matter of the Vocontian Basin is in a more advanced state of thermal maturity than that derived from the Ocean Drilling Program Site 1049C (Kuypers *et al*., 2002). This has resulted in the thermal destruction of intact core membrane lipids of Archaea in the Vocontian Basin black shales. Although the degree of thermal maturity may vary from site to site in a basin, depending on the burial history, the finding of intact DNA in black shales from the Vocontian Basin (Inagaki *et al*., 2005) is at least remarkable. DNA is not likely to be resistant to subsurficial burial at elevated temperatures for tens of millions of years.

Another observation against the genuine character of the retrieved sequences is the sometimes close relatedness of the retrieved sequences to those derived from present-day settings. In some cases, the sequences differ only by slightly more than 1% (Inagaki *et al*., 2004). One would expect to see a substantially larger difference if the retrieved gene sequences indeed represent those produced by the bacteria thriving during deposition of the OAE-1b interval 112 million years ago. Although rRNA genes are considered to be conservative, a recent accurate estimate of the molecular clock rate of this gene in diatoms is 1% per 14 million years (Sinninghe Damsté *et al*., 2004). With this molecular clock rate one would thus expect fossil DNA sequences of 112 million years to be 8% different from those of extant organisms. It is likely that molecular clock rates vary between different organisms, but a factor of 8 seems to be too high.

In summary, we believe that the claim of Inagaki *et al*. (2005) should be examined by analysis of molecular fossils from the black shales studied by these authors before the existence of fossil DNA in Cretaceous black shales can be accepted. The jury is still out. We also believe that much more work on fossil DNA in sediments much younger than Cretaceous has to be undertaken to assess, in as complete a manner as possible, the potential and potential pitfalls of the analysis of fossil DNA in sedimentary sequences. Unrealistic claims of fossil DNA in old sediments will have a negative effect on the development of this emerging new field in Earth Sciences.

ABBREVIATIONS

bp, basepairs; OAE, Oceanic Anoxic Event.

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