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Putative ancient microorganisms from amber nuggets

Patricia Veiga-Crespo, Lucía Blasco, Margarita Poza, Tomás G. Villa*

Department of Microbiology, Faculty of Pharmacy, University of Santiago de Compostela, Santiago de Compostela, Spain

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Summary. Evolutionary microbiology studies based on the isolation of ancient DNA and/or microbial samples are scarce due to the difficulty of finding well preserved biological specimens. However, amber is a fossil resin with natural preserving properties for microbial cells and DNA. The visualization by transmission electron microscopy of different microorganism-like specimens found in amber nuggets from both the Miocene and the Cretaceous periods was accompanied by studies of ancient DNA obtained from the nuggets. After the design of specific primers based on the present sequences of both genes in *Saccharomyces cerevisiae*, the ancestral AGP2 sequence from the Miocene, as well as the 18S rRNA from the Cretaceous, were amplified. [**Int Microbiol** 2007; 10(2):117-122]

Key words: *Saccharomyces cerevisiae* · amber · Miocene · Cretaceous · microfossils · micropaleontology

Introduction

Evolution has always been a field of intensive research in biology, and many laboratories worldwide focused their research on the evolution of a wide range of organisms. However, studies exploring evolution within the microbial world are hampered by the difficulty of finding well preserved, reliable samples of ancient microorganisms. The emergence of molecular biology techniques, especially PCR, has facilitated a new approach to micropaleontology. Over the last 20 years, a relatively large amount of information regarding ancient microorganisms has been obtained from the fossil record, and several kinds of molecular-biology-based analyses have been carried out. Nonetheless, our knowledge of the evolution of most microorganisms remains highly deficient.

Amber provides a near-perfect matrix for preserving biological samples for millions of years, and it excludes contamination with other sources of DNA. Amber is the fossilized state of natural resins secreted by conifers and *Leguminoseae* families as a natural defense mechanism. Under extreme conditions, resin undergoes a process of fossilization, originating amber. In the course of fossilization, insects and microorganisms sometimes become embedded within the resin, thus allowing preservation of samples for million of years while avoiding contamination with actual biota. Moreover, because of the physical attributes of amber [3,8,12], DNA from the entrapped biological specimens preserved within it can be readily extracted and PCR-amplified [3,4,16]. At present, ancient DNA contained in amber may have been at least partially degraded due to the effects of exogenous agents [2,7,12], including water and the action of free radicals and UV light, and endogenous processes such as base conversion by hydrolytic deamination. However, in the recovery of ancient DNA that has been degraded to a certain extent, there are certain advantages, including the difficulty in obtaining long-chain amplicons, which facilitates the detection of contamination with current DNA [7]. Initial studies addressed the direct cloning of fossil DNA, but this soon fell into disuse and was replaced by PCR amplification, thus avoiding the undesired DNA repair that occurs in direct cloning. The strict previous criteria of authenticity are necessary in order to obtain good results in this type of work.

The Miocene (30 million–5 million years ago) is a quite interesting geological epoch of the Neogene period from the biological point of view. In fact, it was during this period that

***Corresponding author:** T.G. Villa
Department of Microbiology, Faculty of Pharmacy
University of Santiago de Compostela, Campus Sur
15782 Santiago de Compostela, Spain
Tel. +34-981563100 (ext. 14949). Fax +34-981594631
E-mail: mpvilla@usc.es

America and Europe separated and the explosion of mammalian species occurred, they definitively replacing the large reptiles [www.triptico.com/misc/geolo.html]. The Cretaceous period (145–65 million years) was also interesting because it was when the last great extinction occurred and when the ancestors of current species evolved. Therefore, both the Cretaceous and the Miocene must be considered in micropalaeontology studies. The present study describes the visualization, by transmission electron microscopy, of different ancient microorganisms found in amber samples. These paleomicroorganisms probably represent ancient forms of current microbial species, and may be representatives of species that may have become extinct.

AGP2 gene, which codes for a plasma membrane carnitine carrier [15] (an arabinogalactan protein), is a genomic gene not essential for the survival of the yeast, whereas the 18S rRNA gene is an essential one. Both genes were selected in this study in order to compare their evolution.

Material and methods

Amber samples. We used samples from different geographical areas and geological eras: (i) amber nuggets from Santiago de los Caballeros Mountain (Dominican Republic), dated stratigraphically as Miocene samples (15–30 million-year old) [16]; (ii) amber nuggets from Simojovel Area (Chiapas, Mexico), dated stratigraphically as Miocene samples (15–30 million-year old); and, (iii) amber from Hukawng Valley (Burma/Myanmar) (Poinar, personal communication), dated stratigraphically as Cretaceous samples (107 million-year-old). In order to minimize the risk of contamination with current microorganisms, fissureless amber samples were used. Since the secretion of resin in plants is a gradual process, the amber nuggets were polished so as to analyze only the internal layers, thus ensuring that only fossils from the appropriate geological time were studied. Amber nuggets with insects trapped in their centers were chosen and polished aseptically as close to the arthropod as possible in order to avoid contamination with present-day microorganisms. The amber nuggets from the Dominican Republic strongly resembled ants, whereas the entrapped ones in Chiapas samples were identified as members of the termites group. The samples from the Cretaceous period probably contained ancient ants.

Processing of amber nuggets for transmission electron microscopy. Sterilization of the amber surfaces and the procedure to control for contamination were as previously described by Veiga-Crespo et al. [16]. The validation criteria for the selection of samples were: (i) samples from the same nuggets had to show similar results, and (ii) microorganisms visualized by transmission electron microscopy had to be present in successive ultramicrotome sections of the amber resin. The amber stones were initially screened for the presence of insects using an Olympus SZ-CTV lens in an optical microscope (Nikon AFX-II). Subsequently, the samples were prepared for transmission electron microscopy according to the method of Wier et al. [17]. We cut them with a Leika MZ6 microtome (Leika Ultracut UCT). The specimens were then examined under a CM12 transmission electron microscope (Philips).

Ancient DNA studies. After sterilization of the amber surfaces, the nuggets were treated as previously described for extracting ancient DNA [18]. Internal oligonucleotides for the AGP2 (forward: GTTTTGTATCTG CTTATTTCCATTT; reverse: GTCATTTGGCGAACAAACAATC) and 18S rRNA genes (forward: TATCTGGTTGATCCTGCCAGT; reverse: TCCTT GGATGTGGTAGCCGT) (both from *Saccharomyces cerevisiae*) as well as universal oligonucleotides to bacterial 16S rRNA (forward: CGTAGGCG-GATTAGTAAGTTAAA; reverse: CACGACACGAGCTGACGACA) were designed.

The amplification reaction mixture was as follows: 1 U *Taq* polymerase (Takara Shuto), 2 ng BSA/ml (Promega), 0.5 mM each oligonucleotide (Invitrogen), 2 mM $MgCl_2$ (Takara Shuto), 0.2 mM dNTPs mix (Takara Shuto), 50 ng fossil DNA, Taq polymerase buffer (Takara Shuto) and deionized sterile water to a final volume of 50 µl. The program of PCRs was: 1 cycle of 5 min at 94ºC, 35 cycles of 1 min at 94ºC, 30 s at 65ºC and 1 min at 72ºC. The process was completed with one cycle of 10 min at 72ºC. The obtained products were sequenced by Sistemas Genómicos SL (Valencia, Spain).

The DNA sequences were validated according to previously described criteria [16]. Sequence analyses were carried out with the BLAST 2.0 application [1] obtained from the US National Center for Biotechnology Information. Phylogenetic relationships were analyzed by the ClustalW method, included in the Vector NTI Advance Suite 9.0 (Informax).

Results and Discussion

Amber contents. Transmission electron microscope images confirmed that the arrangement of the insects' organs had been well-preserved in amber and showed a variety of microorganisms in the samples (Fig. 1).

Ancient microorganisms present in Miocene samples from the Dominican Republic. The most important finding in the Miocene samples from the Dominican Republic was the presence of yeast-like-budding microfossils, possible bacteria. The fossil putative bacteria were 0.5–1 µm long and 0.3–0.5 µm wide, and appeared as round or elongated cells, showing monopolar and slightly lateral budding. There was also evidence for cell walls. These microfossils were clearly smaller than extent forms of budding yeast. They were located close to the body of the insect and in groups, with no other types of microorganisms near them. This observation corroborated our previous finding of the presence of ancient yeast DNA in samples from different parts of the same amber nuggets [16]. Based on the electron microscopy images and the amplified ancient yeast DNA we postulate that the amber-entrapped microorganisms were an ancestor of current *S. cerevisiae*. The amber samples also contained other microorganisms: cocci-like cells smaller than current cocci, which were located at a distance from the budding bacteria specimens.

Analysis of the different bacteria-like organisms depicted in the transmission electron microscopy images confirmed the results obtained from studies of 16S rRNA of ancient DNA. Although PCR reactions were conducted in high stringency conditions, the high number of amplified sequences showed the high degree of conservation of the 16S rRNA

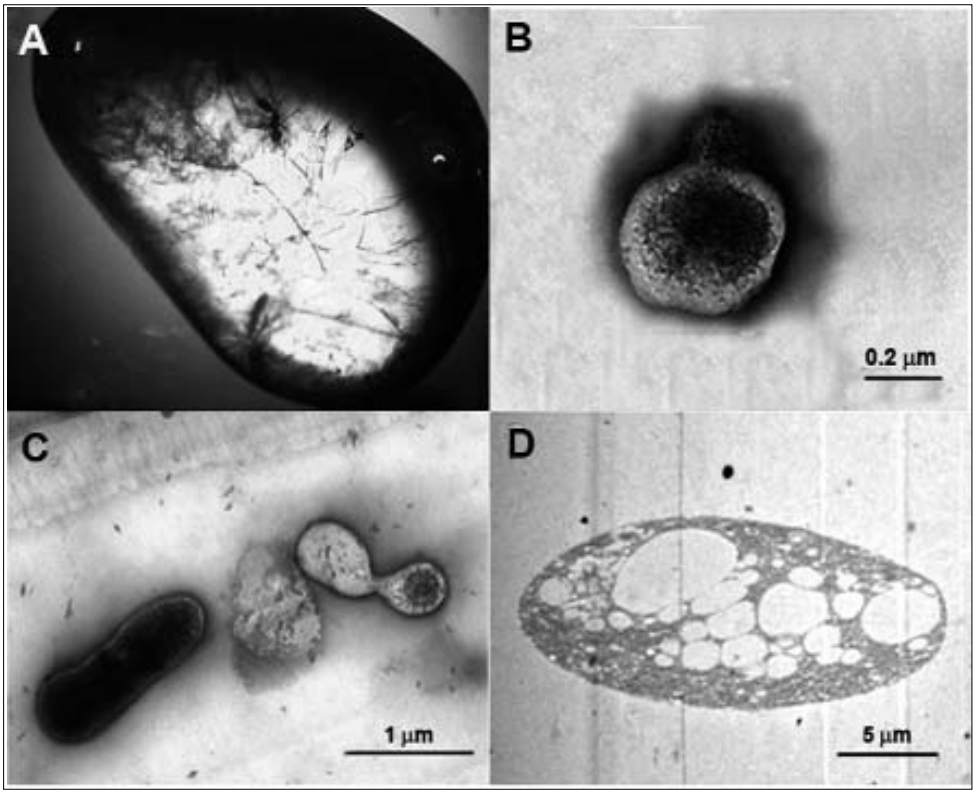

Fig. 1. (**A**) Entrapped insect in Miocenic samples from the Dominican Republic. (**B, C**) Miocenic amber from Chiapas sample showing budding-like bacteria. (**D**) Sample of Cretaceous amber from Burma showing a protozoan-like microorganism.

gene and the great diversity of bacteria in the samples. Furthermore, amplification of this gene confirmed the microscopy findings regarding the presence of different bacterial specimens in the samples (Fig. 2). It was impossible, however, to identify the bacteria at the species level, due to the high degree of conservation of these genes in current bacteria and to the short sequences amplified from the DNA obtained from the amber-embedded specimens.

Ancient microorganisms present in Miocene samples from Chiapas. Images depicting a diverse collection of microorganisms were obtained from the Chiapas samples. The insects preserved in the nuggets from Chiapas seemed to be members of the *Termitidae* family (data not shown). In these samples, the embedded microorganisms consisted of a *Bacillus*-like cell, or possibly a cell surrounded by capsule-like material, and two types of budding-bacteria-like microorganisms. The diameter of the *Bacillus*-like cell was ca. 200 nm. Some of the budding-bacteria-like microorganisms were small (Fig. 1B) while others were much larger (Fig. 1C).

Ancient microorganisms present in Cretaceous samples from Burma/Myanmar. The microorganisms from the Cretaceous samples were of particular relevance since they could provide insight into microbial life immediately before the last great Cretaceous extinction. The transmission electron microscopy images revealed a large number of *Paramecium*-like organisms ca. 20 µm that contained many internal organelles and/or vacuoles (see Fig. 1D). *Bacillus*-like microorganisms were also seen in these samples, although they were smaller than those in the Miocene samples. The cells were surrounded by slime-like material.

Different microorganisms containing hexagonal inclusions resembling magnetosomes have been visualizad in the course of the present study [11]; in future studies, EDX (TEM with energy-dispersive X-ray) analysis should be carried out.

Possible causes of the differences observed between the analyzed samples. The differences in the types of microorganisms found in samples from the same geologic time can be explained in terms of the different geographic areas the amber nuggets were collected, their different geological characteristics, and the correspondingly different types of insects. Amber nuggets from the Dominican Republic and the Chiapas region of Mexico presumably originated from different types of trees and thus, most likely, from distinct insect populations. Additionally, the amber may

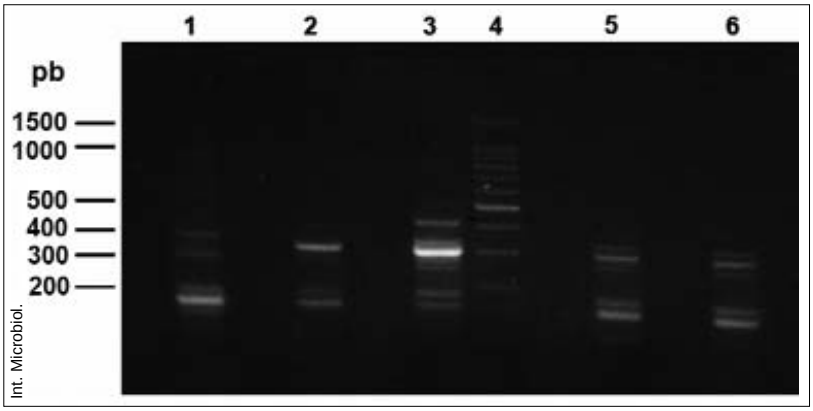

Fig. 2. Amplification of the 16S rRNA gene. Lanes 1–3, samples from the Dominican Republic; lane 4 100-bp DNA markers; lanes 5, 6: samples from Chiapas.

have formed either on the trunk of trees or on the soil, which would also explain the variation in microbial contents. The extinct leguminous tree *Hymenaea protera* migtht be the producer of the resins that originated the amber of the Dominican Republic in the Miocene [13], whereas the origin of the resins in the Chiapas region was the tree *Hymenaea mexicana* sp. nov [14].

The different geological conditions of these areas must also be considered. There are two areas in the Dominican Republic that contain major amber deposits: the northern area of Santiago de los Caballeros, including a fault-bounded unit designated La Toca Block, and tçhe eastern area of Cordillera Oriental. Paleogeographically, both areas are part of the same sedimentary basin, which was disrupted by movements along major faults [10]. The amber nugget analyzed in this study was obtained from the upper subunit of the La Toca Block formation [9]. The Chiapas amber from the Oligocene/Miocene was discovered after mining activity in the Simojovel area. The deposits of this region have been assigned to the planktonic foraminiferal zones of *Globigerina ciperoensis* and *Globorotalia kugleri*, an interval within zones N3 and N4 in the Cenozoic Planktonic Foraminiferal Zone sequence. The main amber mine of Burma/Myanmar is located in the Hukawng Valley, where the sedimentary rocks are considered to date from the Cretaceous period [5]. Another explanation for the different types of ancient microorganisms present in the amber nuggets may be the different paleoclimates of the respective time periods. The climatic conditions of the Cretaceous were wet and hot, with a temperature ca. 12ºC higher than the current average temperature. The atmospheric conditions showed high levels of CO_2 and water vapor. In the initial phase (M1; 30–25 million-year old) of the Miocene period, cooler temperatures prevailed all over the Earth. During the Middle-Miocene, the weather was much warmer and thus propitious for the growth of thermophilic evergreens, which produced resins that later fossilized to form amber [6].

Phylogenetic analysis. Unfortunately, the primers used in our studies of ancient microbial DNA from these samples, including those for 16S rRNA genes, did not allow positive species-specific identifications to be made based on the sequences of the amplified DNA (Fig. 2). However, the data obtained from the amplified genes did allow a limited phylogenetic analysis, as discussed below.

Miocene samples. The sequence of the *AGP2* gene from the Miocene samples obtained from the Dominican Republic (AY917137) was determined. BLAST analysis showed that the most similar current sequence was contained in the *AGP2* gene of *S. cerevisiae*. Similarity studies revealed that the homology of the Miocene *AGP2* gene with the cur-

Table 1. Similarities of AGP2 genes

	Saccharomyces cerevisiae	*Candida albicans*	Miocene AGP2
Saccharomyces cerevisiae	100	30	54
Candida albicans		100	32
Miocene AGP2			100

Table 2. Similarities of 18S rRNA

	Cretaceous 18S rRNA	*Saccharomyces cerevisiae*	Miocene 18S rRNA
Cretaceous 18S rRNA	100	27	43
Saccharomyces cerevisiae		100	64
Miocene 18S rRNA			100

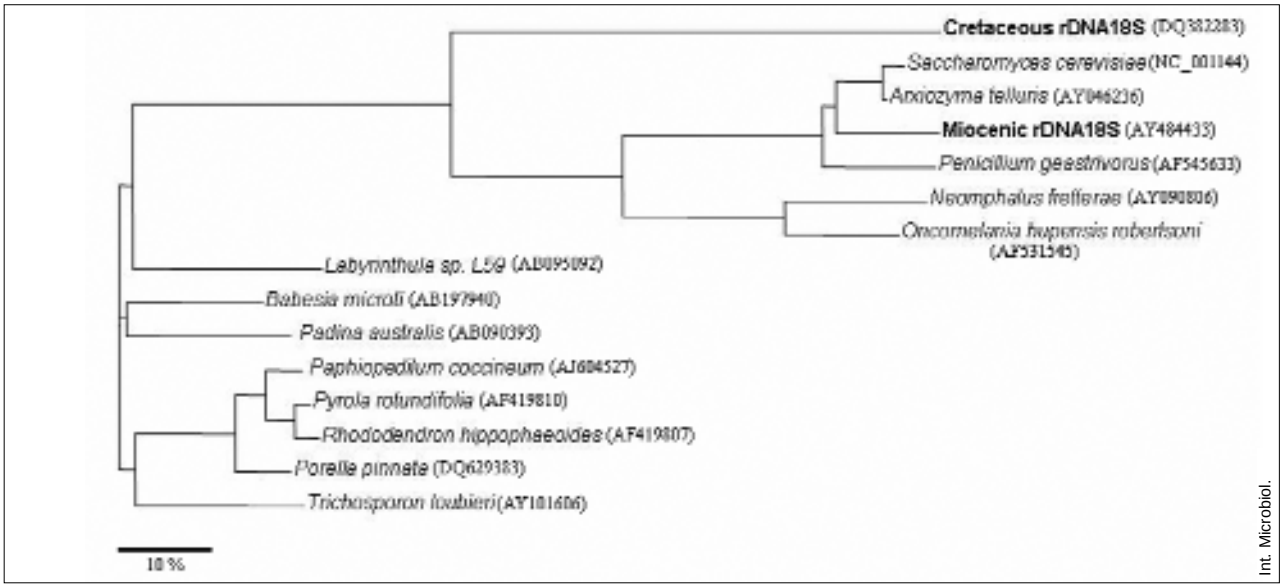

Fig. 3. Phylogenetic relationship of 18S rRNA genes.

rent sequence of *S. cerevisiae* was higher than that with the sequence of the *AGP2* gene from *C. albicans* (Table 1). This result, which reinforces the findings of our previous work with ancient yeast DNA, allows to conclude that ancient DNA from microorganism present in these samples belonged to an ancestor of the current *S. cerevisiae* rather than to a budding bacterium [16].

The degree of conservation of the *AGP2* gene (54%) differed from that of other ancestral sequences of housekeeping genes from yeast, such as the *PGU1* gene (69%). This may reflect the different selective pressures exerted on the yeast genome over time. With respect to phylogenetic relationships, the ancient microorganism seemed to be more closely related to *S. cerevisiae* than to *C. albicans*. However, the greatest hindrance in this analysis was that databases contain little information about the sequences of *AGP2* genes from different organisms, which hampered a more exhaustive phylogenetic analysis.

Cretaceous samples. While the 18S rRNA gene (GenBank accession no. DQ382283) of ancient yeast could be amplified, the age of the samples increased the difficulty in obtaining positive reactions for a number of other genes. In fact, degradation of DNA from the Cretaceous period did not allow us to obtain any large fragment. Nonetheless, as expected, the sequencing results showed high similarity between the current-DNA amplicon and the DNA sequence from ancient yeast. Despite the high degree of conservation of these two genes, conservation between Miocenic 18S rRNA and the sequences of *S. cerevisiae* was even higher (Table 2).

The phylogeny of the 18S rRNA gene from the Cretaceous to the present was also analyzed (Fig. 3). The *S. cerevisiae* sequence suggests that current yeast taxa should have diverged at an earlier time than represented by the sequence present in the Miocene samples. This result highlights the ancestral character of the new sequence and is consistent with the data regarding sequence divergence.

Transmission electron microscopy images obtained in this study should allow us to choose and design more specific primers for the amplification of ancient DNA in future studies. This, in turn, would allow a more accurate identification as well as a more precise analysis of the phylogenetic relationships between ancient microorganisms and their current counterparts. While this and other work addressing the study of amber-preserved microorganisms is, in many aspects, preliminary, the results are nonetheless relevant and contribute to expanding the field of micropaleontology.

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