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REVIEW

Microbial Cretaceous park: biodiversity of microbial fossils entrapped in amber

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Abstract Microorganisms are the most ancient cells on this planet and they include key phyla for understanding cell evolution and Earth history, but, unfortunately, their microbial records are scarce. Here, we present a critical review of fossilized prokaryotic and eukaryotic microorganisms entrapped in Cretaceous ambers (but not exclusively from this geological period) obtained from deposits worldwide. Microbiota in ambers are rather diverse and include bacteria, fungi, and protists. We comment on the most important microbial records from the last 25 years, although it is not an exhaustive bibliographic compilation. The most frequently reported eukaryotic microfossils are shells of amoebae and protists with a cell wall or a complex cortex. Likewise, diverse dormant stages (palmeloid forms, resting cysts, spores, etc.) are abundant in ambers. Besides, viral and protist pathogens have been identified inside insects entrapped in amber. The situation regarding filamentous bacteria and fungi is quite confusing because in some cases, the same record was identified consecutively as a member of these phylogenetically distant groups. To avoid these identification errors in the future, we propose to apply a more resolute microscopic and analytical method in amber studies. Also, we discuss the most recent findings about ancient DNA repair and bacterial survival in remote substrates, which support the real possibility of ancient DNA amplification and bacterial resuscitation from Cretaceous resins.

Keywords Amber · Cretaceous · Protists · Bacteria · Fungi · Pathogen

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Introduction

Amber is the fossilized resin produced from the trunk and the roots of certain trees, mainly of the genera *Agathis* (Araucariaceae) and *Hymanea* (Leguminoseae; Poinar 1992; Penney 2006). The most abundant amber deposits correspond to the Cretaceous, but only a small number of them are highly fossiliferous (Poinar 1992; Martínez-Delclòs et al. 2004). Due to their distinct biological origin, fossilized resins are complex mixtures of diverse compounds, such as terpenoids and phenolic derivates, which are only partially identified (Anderson and Crelling 1995). Amber acts as a natural embedding agent and preserves its trapped life more completely than other type of fossilization (Poinar 1998). The exact mechanism/s involved in biological preservation by fossilized resins are unknown; some authors have stated that it might be due to the extensive polymerization of biomolecules in fossil tissue by nonen-

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zymatic processes leading to chemical and mechanical fixations (Bada et al. 1999; Briggs 1999).

Traditionally, the paleobiological data regarding the species included in amber have focused on arthropods. Most studies do not contain data conducive to quantitative analysis but they tend to be exacerbated the further one goes back in geological time (Penney 2006). A small number of them are dedicated to unicellular biological systems, mainly to certain groups of eukaryotic microorganisms with an exoskeleton, such as foraminiferida, radiozoa, and diatoms (Armstrong and Brasier 2006). However, it is well known that microorganisms are the most ancient cells and they represent key phyla in the elucidation of cell evolution and Earth history (Cavalier-Smith 2006). The paucity of studies on microbial fossils might have three different causes; in the first place, their small size, which means they can only be evaluated by means of modern microscopical methodologies, specially adapted to these hard substrates. At present, only a few researchers in the world can process these samples appropriately. In the second place, due to the intrinsic difficulties of locating and studying microfossils in amber, they have been almost ignored by paleontologists, and, finally, paleontologists studying amber microfossils usually specialize in macroorganisms (without a microbiological background). So, according to some authors (Loeblich and Tappan 1964; Porter and Knoll 2000; Foissner and Schiller 2001), it is quite usual to find misinterpretations of microbial data.

We present here a critical review of microbial records found in different amber deposits worldwide, mainly from Cretacic, although some other important findings from other periods such as the Eocene–Oligocene are also included. Likewise, we discuss microbial identification difficulties, which are revealed by habitual reidentifications of the same record by the same or different authors. We propose the use of modern microscopic and analytical methods to avoid these errors, together with a more authoritative taxonomic identification by specialists. Finally, we analyze the molecular identification of ancient microorganisms and their possible resuscitation.

Protists

Paleomicrobiological studies of terrestrial and freshwater protists are extremely rare compared with those of microfossils from marine ecosystems. Nevertheless, the kingdom Protista is the best represented in ancient amber and the reported biodiversity is high in comparison with other microbial groups. Records of flagellata are rather scarce and most of them are represented by microfossils similar to extant photosynthetic genera. In Lower Cretaceous Spanish amber from Peñacerrada II (Ascaso et al. 2005; Martín-Gonzalez et al. 2008), we have found at least two distinct euglenids (Excavata: Euglenozoa: Euglenida), which showed some morphological features similar to the current genera *Euglena* and *Phacus*, and they have been considered the earliest fossils of free-living Excavata identified with confidence (Cavalier-Smith 2006). These genera also have their osmotrophic counterpart at present. Using scanning electron microscopy in backscattered electron mode (SEM-BSE; Ascaso et al. 2003), we have been able to observe the internal organization of cells with some detail, due to the fact that these particular microfossils have undergone two successive mechanisms of fossilization; pyritization, and, later, inclusion in the resin. By this means, we can detect the potential arrangement of chloroplasts in the pyritized cytoplasm (Fig. 1a, b). The right identification of this fossilized *Euglena* is corroborated by the characteristic movement of metaboly, exhibited by some cells inside the amber nugget, indicating the existence of unfavorable environmental conditions during entrapment in the resin (Martín-González et al. 2008). Records of Euglenozoa are not frequent in amber; they have also been reported in only two more recent deposits (Cenomanien, Upper Cretaceous) from Schliersee (Germany; Schönborn et al. 1999) and Écommoy (France; Breton and Tostain 2005). Two different morphotypes of small coccacean microalgae or photosynthetic flagellates (Chlorophyta) were also found inside diverse amber samples from Cretaceous. The most common was very similar to the extant genus *Chlamydomonas* (Schönborn et al. 1999; Martín-González et al. 2008). The other one was identified as *Chloromonas* sp. (Schönborn et al. 1999). It must be noted that all of these flagellates present in their actual life cycles transitory dormant stages, which resist unfavorable environmental conditions, particularly desiccation. Finally, numerous minute (2–5×3–6 μm) oval and spherical cells were observed in the German Mesozoic amber. They were assigned to the genera *Chlorella* and *Chloricystis*, respectively (Schönborn et al. 1999). As far as we know, the unique heterotrophic flagellates found in amber were identified as *Monas* and *Dinobryon*, respectively (Waggoner 1994a, b). We have also detected in Peñacerrada's amber numerous minute cells that would also be interpreted as crysomonads, but we cannot demonstrate it due to the total absence of singular morphological features in these protists. Average sizes (3–10×8–20 μm) of these flagellate (Fig. 1c, d) are bigger than those cells from Schliersee. We do not think that these microinclusions could be air bubbles entrapped in amber, because a red autofluorescence was observed, by confocal microscopy, inside the cells, when laser generated the excitation beam (for technical data, see Ascaso et al. 2003, 2005). It is known that air bubbles do not have autofluorescence, a property exhibited by some molecular compounds from biological materials.

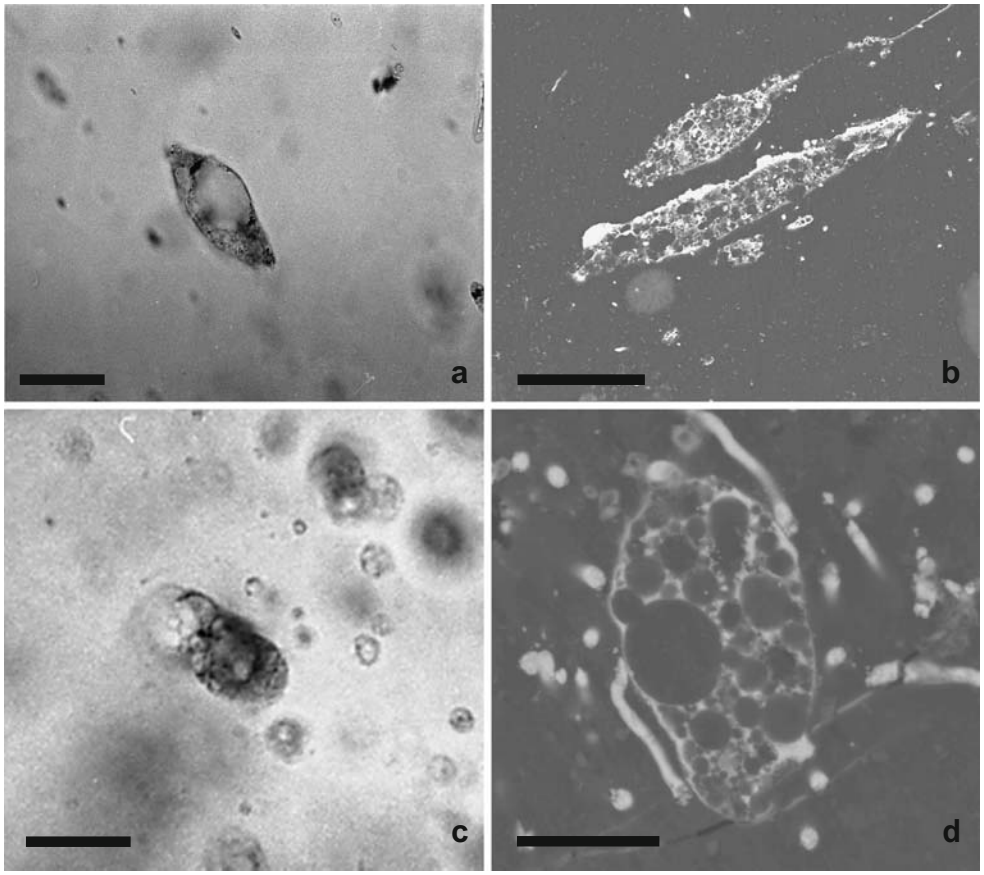

Fig. 1 Microfossils of flagellates found in Cretaceous amber samples from Peñacerrada (Alava, Spain). **a** General view by using optical microscopy of a *Euglena*-like flagellate. *Bar* 20 μm. **b** SEM-BSE micrograph of pyritized fusiform euglenids. *Bar* 50 μm. **c** A small flagellate, probably crysomonad, observed by optical microscopy. *Bar* 20 μm. **d** A SEM-BSE image of an oval crysomonad flagellate. *Bar* 10 μm. Note the abundant vesicles throughout the cellular cytoplasm, which might be due to the sulfide generation by sulfate-reducing bacteria during the pyritization

Earliest microfossils of testate amoebae identified with confidence correspond to the vase-shaped microfossils found in Chuar Goup, in Grand Canyon (Porter and Knoll 2000). Testate amoebae were the best represented group of protists inside fossilized resins. However, the number of records was remarkably lower than that found in other ancient habitats, such as Quaternary sediments and peats (Medioli et al. 1990; Charman et al. 2000; Charman 2001). In amber, most described microfossils correspond to amoebae with a shell built of xenosomes, that is, a proteinaceous material with agglutinated mineral particles from the environment (Amoebozoa: Testacealobosia). The earliest record of a thecamobien was found in a sample of Italian Triassic amber (220 Mya); this morphotype was identified as *Centropyxis hirsuta*, although in this case, the test had not spines like the same current species (Schmidt et al. 2006). Few distinct species of the same freshwater genus and other genera of testate amoebae with xenosomes (*Cyclopyxis*, *Nebela*, *Phryganella*, *Pontigulasia*) have been reported from several Mesozoic amber deposits in the USA (Waggoner 1996a), Spain (Martín-González et al. 2008), and Germany (Schönborn et al. 1999; Schmidt et al. 2004). Reports of fossilized testate amoebae with a shell made of idiosomes (Testaceafilosea: Rhizaria: Cercozoa) are very scarce. In Spanish Lower Cretaceus amber, we have found three morphotypes with some similarities to the extant genera *Assulina, Cyphoderia* and *Trinema* (Fig. 2b, c). The shell of current *Assulina* and related genera (such as *Euglypha* and *Trinema*) represents the highest level of complexity of testate amoebae. In this case, the siliceous exoskeleton elements (idiosomes) are elaborated and secreted by their own cells, so these amoebae may be able to carry out a biomineralization process. It was the first time that filopodia were preserved, indicating that the cell was alive when it was entrapped in the resin. Only one more record of this testate group, which was included in the genus *Cyphoderia*, has been published to date (Waggoner 1996b).

With regard to testate amoebae, we have detected two main differences, with paleoecological relevance, in samples from the Peñacerrada deposit with regard to other highly fossiliferous amber deposits. No Spanish fossilized testate amoebae have shown any spine in the shells. In studies from present environments, some authors have suggested that spineless forms occur in drier habitats than spined forms, since there are no spines in most species occurring in dry mosses (Schönborn 1992; Booth 2001). Another remarkable observation is that we have not found in the Cretaceous amber from Peñacerrada a very frequent extant genus of testate amoebae, such as *Arcella*, *Hyalosphenia*, or *Amphitrema*, with chitinous or proteinaceous tests, which are regarded as less resistant structures. On the contrary, amoeba

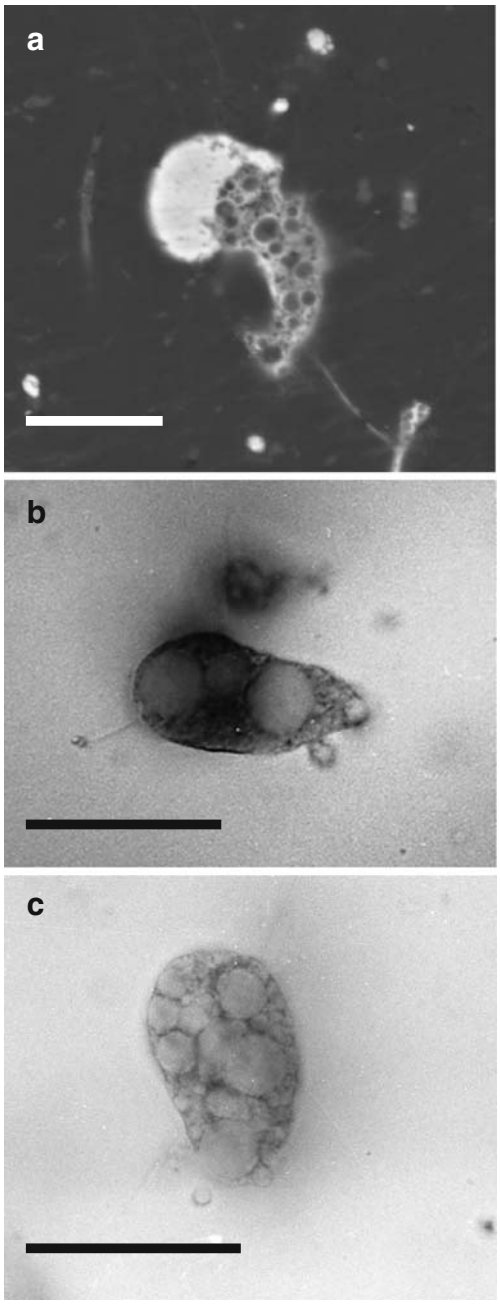

Fig. 2 Fossilized naked and testate amoebae entrapped in Peñacerrada's amber. **a** SEM-BSE picture of a naked monopodial amoeba *Hartmannella*-like. *Bar* 10 μm. **b** Optical micrograph of a shell, similar to the present genus *Centropyxis*. *Bar* 20 μm. **c** General appearance of a fossilized *Trinema*-like testa. *Bar* 20 μm

tests made by robust xenosomes (*Centropyxis*) or idiosomes with skeletal plates, mostly of siliceous (*Assulina*, *Trinema*, *Cyphoderia*), are predominant.

Although testate amoebae are now commonly used in paleoenvironmental studies, little is known of their taphonomy (Mitchell et al. 2008). Differential preservation of shells in testate amoebae is accepted at present, but opinions have diverged. Traditionally, the shells with platelets (idiosomes) have been considered as the most resistant to extreme environmental conditions and microbial degradation due to their biomineralization (Charman et al. 2000). However, the existing experimental data on amoeba shell decomposition are confusing. In forest litter, Lousier and Parkinson (1981) observed high decomposition of shells built from idiosomes and lower decomposition for species with shells made by xenosomes. In peatlands, Swindles and Roe (2007) showed that the resistance at acid pHs could be different even within the same genera. Furthermore, according to Mitchell et al. (2008), the microenvironmental gradients had an important role in determining the predominant preserved shell type. Besides the differential preservation of shells, another unknown and controversial aspect of ancient testate amoebae is the degree to which fossilization affects shell morphology. Some authors suggested that both distortion and perhaps size changes in shells are possible, and some reduction in the numbers and density of xenosomes in fossil specimens might be possible (Charman 1999; Charman et al. 2000).

Unlike testate amoebae, the oldest fossils of naked amoebae have been reported in amber. However, naked amoebae, without a protective shell, are not well preserved inside amber. At present, there are only four reliable findings of these protists. The three oldest ones were reported from Peñacerrada's deposits involving both monopodial and polypodial morphotypes. The first report was of a big polypodial amoeba, quite similar to the common genus *Amoeba* (Ascaso et al. 2005). The fossilized monopodial amoeba is small, with a patent hyaloplasm but absent uroid. These features were ascribed to the extant genus *Hartmannella* (Fig. 2a; Martín-González et al. 2008). Finally, the last morphotype corresponded to a "floating form", with radiating pseudopodia. It is very difficult to distinguish one amoeba species from another on the basis of this conformation, since it can be adopted by both monopodial and more usually by polypodial naked amoebae. For this reason, we did not assign this morphotype to a specific current genus of naked amoebae, although by its size and appearance, it is similar to the present genus *Deuteramoeba*. It must be pointed out that the "floating form" is not present in soil amoebae, indicating that Peñacerrada's amber deposit was an aquatic environment in Lower Cretaceous, corroborating the previous geological data on this amber deposit area (Alonso et al. 2000). Two additional records of naked amoebae have been published to date; Poinar et al. (1993b) found in the Upper Cretaceous amber from southern Germany, which was first erroneously considered from Triassic and was later redacted by Schmidt et al. (2001) in Late Cretaceous (Cenomanian), a polypodial amoeba with a filose uroid which showed affinities with the extant genera *Trichamoeba*, *Polychaos*, and *Thecamoeba*. This fossil is considered as a new genus and has been named *Triassamoeba alpha* (Poinar et al. 1993b). Finally,

Waggoner (1993) described in the Middle Cretaceous amber from Kansas numerous small spherical structures with a thick wall that recall the resting cysts of the extant genus *Naegleria.*

The last phylum with representative morphotypes entrapped in fossil resins corresponds to ciliated protozoa. Ciliates are heterotrophic microorganisms with nuclear dimorphism. Cilia, the main structures involved in nutrition and locomotion, have not been well preserved in amber over geological time. Therefore, we cannot appreciate in the records the oral or somatic infraciliature/ciliature arrangements, which are the morphological traits with highest taxonomical value in ciliatology. The alteration of cilia was probably due to the easily biodegradable protein content of these structures and their extracellular location. Small oval or spherical ciliates are the predominant fossils in Cretaceous ambers. They were assigned to the extant genera *Cyrtolophosis*, *Pseudoplatyophya*, *Mykophagophrys*, *Tetrahymena*, and *Nassula* (Poinar et al. 1993a; Waggoner 1994a; Schönborn et al. 1999). The main morphological characteristics used for these identifications were the size and shape of the body, the location of the oral apparatus, and the contractile vacuole. Other additional features, used in some cases, were the macronuclear location and the partially extruded extrusomes (Schönborn et al. 1999). We have also observed *Cyrtolophosis*-like cells and *Prorodon* inside Spanish amber (Fig. 3a–c; Ascaso et al. 2005). Other colpodids, a complex and extremely diverse taxa of soil ciliates, were also represented by other fossils, identified with more confidence as *Paracondylostoma*, *Bryometopus* (Schönborn et al. 1999), and the reniform *Colpoda* (Martín-González et al. 2008). From molecular data using small subunit rDNA as a molecular clock, it has been calculated that most colpodids evolved about 180 Mya (Wright and Lynn 1997), that is, before the formation of the most representative amber deposits. At least three different reports of the well-known genus *Paramecium* have been recorded from two distant Cretaceous amber deposits. In Schliersee resin (Cenomanian), two different species have been observed; one of them with a shape and size similar to those of several current species (Poinar et al. 1993a) and another one, which is somewhat smaller than present species, has been named *Paramecium triassicum* (Schönborn et al. 1999). We have also detected, at both optical and electron (SEM-BSE) microscopical levels, the presence of some ciliates with a *Paramecium aurelia*-like morphotype in Spanish amber from Peñacerrada (Fig. 3c; Fig. 2b in Ascaso et al. 2005). Finally, Veiga-Crespo et al. (2007) described a protozoan embedded in Burma/Myanmar which was tentatively identified as *Paramecium*. However, according to the size and the shape of the body displayed in the present

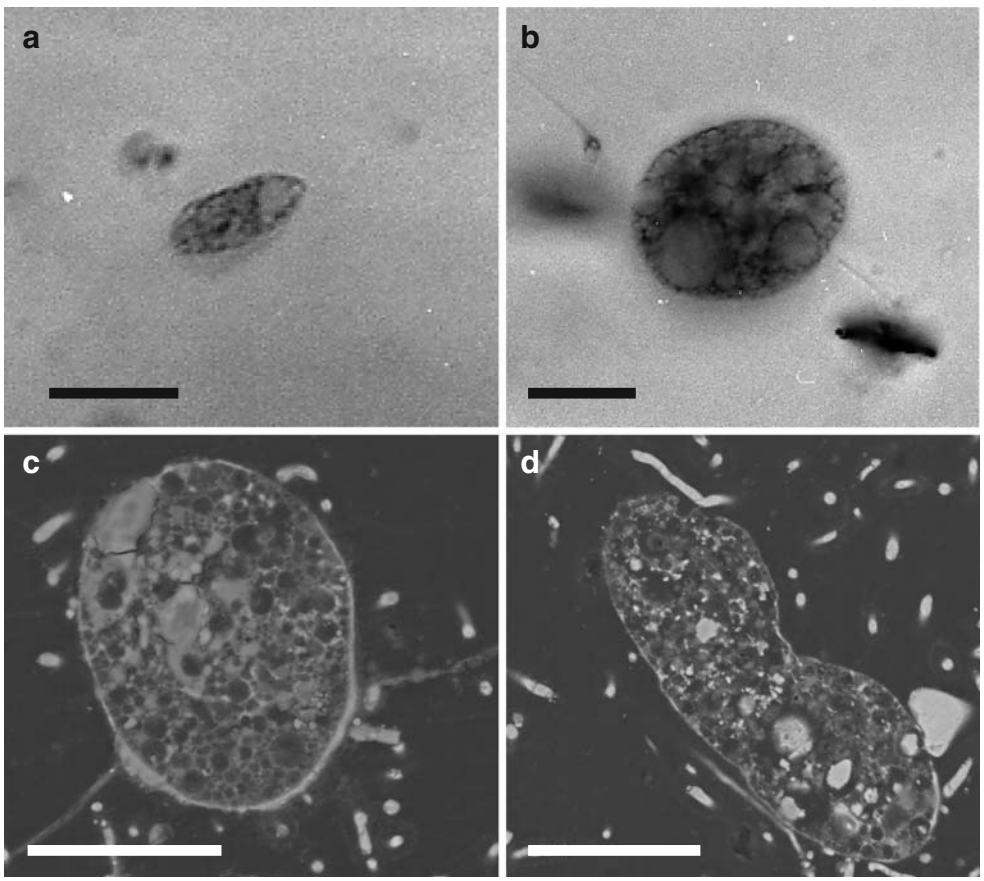

Fig. 3 Fossilized ciliates. **a** Small pyriform ciliate, similar to the present genera *Tetrahymena* and *Cyrtolophosis. Bar* 20 μm. **b** Optical and **c** SEM-BSE micrographs of an oval ciliate, quite similar to the current genus *Prorodon. Bars* 20 μm. **d** Pyritized microfossil similar to the present genus *Paramecium* observed by SEM-BSE. *Bar* 20 μm

species, this record most likely corresponds to a species of the genera *Tetrahymena* or *Cyrtholophosis*.

The microcoenosis of protists from diverse Cretaceous amber deposits do not present very remarkable differences with regard to their composition, despite locations of deposits being quite distant. In fact, statigraphy, geological characteristics, and paleoclimatical conditions are radically different in some cases although a few common traits can be found. The amber of Schliersee is from Late Cretaceous (Cenomanian, 99–93 Mya) and it is located in Bavaria (southern Germany). The sediment input was transported by small rivers into a shallow marine environment. Near the amber pieces coalified plant debris always co-occurs (Schmidt et al. 2004). Amber nuggets of Ellworth (Kansas) had been found at a cliff exposure along the Smooky Hill river, in a shale overlain by several inches of lignine (Langenheim et al. 1965; Waggoner 1996a). The Cenomanian amber of Écommoy (Sarthe, France) is a litter amber, formed in terrestrial to freshwater (occasionally briny) environment, in a marsh behind the coast (Breton and Tostain 2005). Finally, the Moraza–Peñacerrada sites (Peñacerrada I and II deposits) are included in Albian in the Basco-Cantabrian basin. Amber deposits have always been associated with coal layers and pyrite nodules. Numerous cysts of dinoflagellates have appeared in several samples along the statigraphic sections, providing additional evidence of marginal marine sedimentology (Alonso et al. 2000; Delclòs et al. 2007). Inside some amber nuggets, some bubbles containing sodium chloride have been detected (personal observations). These findings support the existence, in remote times, of an appropriate environment for developing sulfate-reducing bacteria, the main microorganisms involved in the pyritization present in many of the records found in these Spanish deposits.

In conclusion, protists seem to be the better-preserved group of eukaryotic microorganisms in ambers, because they were found in all fossiliferous deposits. Alternatively, the existence of a large number of protist records in comparison with other microbial groups might also be due to the fact that the morphological features are more useful for identifying protists than other microorganisms.

Fungi and bacteria

It is little short of amazing that microfossils are described as members of these phylogenetically distant microbial groups. In our opinion, two main problems are the origin of the controversial results regarding bacterial and fungal identification. In the first place, most researchers studying biological inclusions in amber are paleontologists or paleobiologists (animals or plants), most of them without experience or specialization in microorganisms. Thus, identifications are mainly based on morphological observations under optical microscopy, which would explain the strange confusions between filamentous fungi and bacteria. In the second place, ultrastructural analysis is absent in almost all paleomicrobial studies. As has been noted by Peñalver et al. (2007), the amber nuggets of several deposits, including some Spanish Cretaceous ambers, frequently present an intricate group of branched filamentous structures. Three different interpretations have been given to explain the nature of these and other filamentous formations. Some authors have suggested that they are not biological inclusions but a type of degradation or amber alterations (Peñalver et al. 2007). However, most reports state the biological nature of these filaments. Waggoner (1996a), therefore, described, in Middle Cretaceous amber (Ellsworth, Kansas), fossil filaments without septa but with some tips slightly swollen. All of these filaments appeared surrounded by light brown translucent hyaline tubular sheaths. Several sheaths had a refringent appearance, which was interpreted as empty structures. Considering these features resemble those of present sheathed bacteria, they were identified as *Leptothrix*-like bacteria (Waggoner 1996a). Quite similar pictures have previously been reported by the same author (Waggoner 1994c) in Dominican amber, although in this case, the dark blue–black filaments were identified as an actinobacteria, *Nocardioformis dominicatus* n.sp. Studying Écommoy amber deposits (Cenomanian) with light microscopy, French researchers (Breton and Tostain 2005) identified these inclusions as a new fossil cyanobacterium, which was named *Palaeocolteronema cenomanensis*. It was made of uniseriated trichomes not apically tapering, which often have dichotomous ramifications. The cells were 1.5–2.5 μm wide and the length was one or two times longer than wide. Lateral hormogonies with a short and thin peduncle were observed (Breton and Tostain 2005; Breton 2007). Unfortunately, no micrograph of this cyanobacterium has been published to date; only handmade drawings by the authors have been reported (Breton and Tostain 2005; Breton 2007). In German Cretaceous amber from Schliersee, filiform and long-branched inclusions were assigned successively to diverse biological groups, such as "fungus-like organism" (Poinar 1992), filamentous branching green algae similar to the extant genus *Trentepohlia* (Poinar et al. 1993a), filaments of sheathed bacteria (Poinar et al. 1993a), and hyphae of the fungus *Palaeodikaryomyces baueri* (Dörfelt and Schäfer 1998; Schönborn et al. 1999). In short, the filamentous inclusions in nuggets from the same amber deposits were identified as fungi, microalgae, and sheathed bacteria. We have not been able to corroborate if the German amber nuggets studied by the aforementioned authors were the same or not. However, more recently, during a new reexamination of the same amber samples, a

large number of branched filiform inclusions of about 10 μm in diameter and up to 2 mm in length were observed. This new observation, published by two of the aforementioned authors (Schmidt and Schäfer 2005) was made by optical microscopy and SEM. After morphological analysis of these fossils and later comparison with modern genera of sheathed bacteria, they concluded this time that the filaments corresponded to a species of the extant genus *Leptothrix*. However, the X-ray microanalysis of inclusions revealed the absence of ferric and manganese oxides encrusted in the sheaths, a typical trait of *Leptothrix*, a genus of β-proteobacteria which prefers oligotrophic and metal-rich aquatic environments (Spring 2006; Takeda et al. 2005). The authors named this microfossil *Leptotrichites resinatus* n.g. n. sp., due to the fact that it was structurally similar to the genus *Leptothrix* but located inside an ancient fossilized resin. Similar but inverse systematic change was published with a fossil from Baltic Tertiary amber. The same record was identified firstly as a cyanobacterium, *Rosaria succina* (Dörfelt et al. 2000) and, later, it was considered as a fungus and renamed *Metacapnodium succinum* (Rikkinen et al. 2003).

In amber from Peñacerrada, it is quite frequent to observe mycelium mats formed by hyaline hyphae with many vesicular structures. Most hyphae were 0.75–1 μm in width, and bifurcated branches may be observed in some cases. The hyphal system appeared to be coenocytic because no septa were detected (Fig. 4a). In other samples, using SEM-BSE associated to energy dispersive X-ray spectroscopy micro-analysis, we have also detected tabicate fungal hyphae (Fig. 4b). Only in one amber sample have we reported a dark mycelium made up of hyphae 1.3–5 μm in width. The hyphae seem to be septate and cystidia-like; gloeocystidia and refractive intercalary structures were also noted (Ascaso et al. 2005). These structures occur in extant basidiomycetes. We have not identified the fungal genus because we have never observed sporangia, conidia, or other morphological structures, which afford an undoubted characterization (Ascaso et al. 2005). Recently, after applying SEM-BSE and low temperature scanning electron microscopy (LTSEM) techniques adapted to amber (Ascaso et al. 2003), we have been able to obtain new micrographs, which support the fungal nature of these filaments, at least in amber from Peñacerrada (Fig. 4c,d). In Fig. 4d, particularly, we can distinguish the moulds of some hypha fragments and isolated cocci–bacilli bacteria.

Over the last 30 years, other papers have reported the presence of different mycelia entrapped in amber samples from Cretaceous and Tertiary deposits. Most descriptions are supported by a few morphological characteristics and,

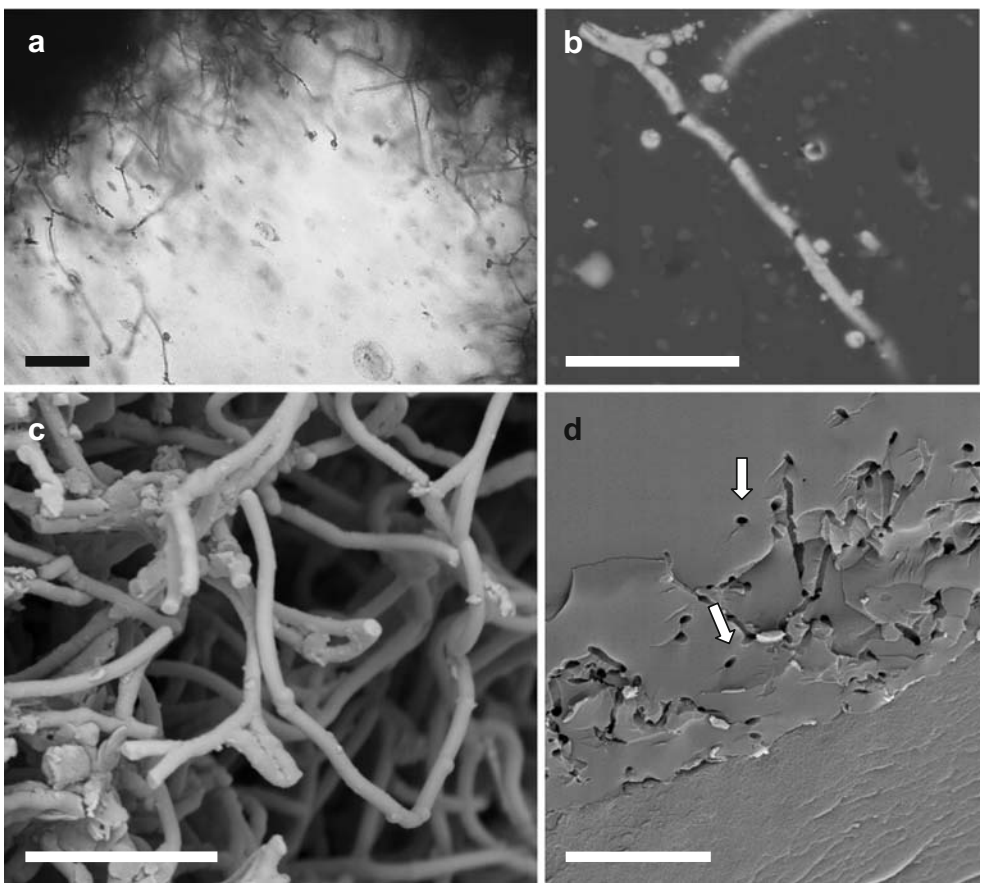

Fig. 4 Different views of fungal hyphae, using diverse microscopy methodologies. **a** Hyphae of a filamentous fungi observed by optical microscopy. *Bar* 25 μm. **b** Pyritized septate hypha observed by SEM-BSE. *Bar* 10 μm. **c** Three-dimensional view of a fossilized mycelium inside amber, by SEM-SE. *Bar* 5 μm. **d** LTSEM micrograph, showing the vestiges of cocci–bacilli bacteria (*arrows*) and coenocytic hyphae. *Bar* 10 μm

in most cases, the arrangement of asexual spores in conidia, sporangia, or other structures with relevant taxonomical features have not been observed in detail.

We have therefore preferred not to make any comment on the proposed identification (Poinar and Thomas 1982, 1984; Rikkinen and Poinar 2001; Thomas and Poinar 1988; Ting and Nissenbaum 1986) in order to avoid exacerbating the present chaotic panorama. For example, in Lower Cretaceous amber from Israel, four new species were tentatively identified as belonging to three artificial genera (Ting and Nissenbaum 1986). Detailed examination of micrographs in this study made it evident that some of them, which had been interpreted as different stages of the life cycle, particularly those relating to the new species *Peronosporites pythius*, hardly resembled fungi. These images recall vacuolated oval or ellipsoidal cells, with extruded cytoplasm in some cases. Without approximate size measurements, it is difficult to determinate which microbial group they might be included in. On the other hand, sometimes fossilized fungi have been considered as entomopathogenic because they were considered to be growing on a dead insect (Poinar and Thomas 1982, 1984; Poinar and Poinar 2005). In our opinion, it is more likely that the pictures represent a relation of saprophytism. At least, there are three certain identifications of fossilized fungi in amber; the first one corresponds to a moniliaceous fungus with septate hyphae and oblong arthroconidia similar to the extant genus *Geotrichum* that has been named *Geotrichites glaesarius* (Stubblefield et al. 1985). The second one is a fossil *Aspergillus* with excellent preservation of numerous conidiophores (Dörfelt and Schmidt 2005). Both fungi appeared on the insect surfaces, which might represent saprophytic forms that grew on dead invertebrates still exposed to the atmosphere. It is assumed that sporulation developed after embedding in the resin. The last one was a fossilized resinicolous fungus, named as *Chaenothecoptis bitterfeldensis* (Rikkinen and Poinar 2000), found in Bitterfeld amber (Miocene–Oligocene). In this study, several spore germination stages are described for the first time.

Recently, fossil evidence of carnivorous or predatory fungi has been reported from amber deposits of Archingeay/Les-Nouillers in southwestern France (Early Cretaceous). The mycelium consists of irregularly septated and branched hyphae. From some lateral hyphae rings as trapping devices have been originated which have been preserved together with their prey, small nematodes. In addition to the rings, the fungus also developed blastospores (Schmidt et al. 2007). According to the authors, secondary spores budding primarily apically from the blastospores gave rise to yeast colonies. These fossilized fungi, therefore, had a dimorphic mode of life (Schmidt et al. 2007). Later, this microfossil was named *Palaeoanellus dimorphus* and according to the authors probably represented an anamorphous of an ascomycete (Schmidt et al. 2008).

Microscopical descriptions of isolated or nonfilamentous bacteria in amber samples are very scarce. As most of them are too small in size, we cannot appreciate any taxonomically relevant trait to identify at least the bacterial taxonomical group when the amber samples are observed by optical microscopy, unfortunately the most common methodology used in this kind of studies, or even by transmission electron microscopy (Veiga-Crespo et al. 2007). However, most studies on microbiocenosis of amber noted the presence of bacteria (Poinar et al. 1993a; Schönborn et al. 1999; Breton and Tostain 2005; Ascaso et al. 2003, 2005; Veiga-Crespo et al. 2007). At present, the most complete study corresponds to a structural and ultrastructural analysis of the fossil microbial community in a xylophagous termite entrapped in Miocene amber (20 Mya). Spirochete bacteria and wood-digesting protists were observed in the intestinal tissue of the insect, although identification of genus was not possible. A spore with a decorated calcium dipicolinate coat of a Gram-positive *Arthromitus*-like bacterium and a *Hollandina*-like spirochete were identified at electron microscopic level (Wier et al. 2002).

In short, the identification of fossilized bacteria at species or even genus levels is not an easy objective, even if it is not impossible. Nevertheless, attempts using adapted electron microscopies (TEM, SEM) should be carried out to identify prokaryotic morphotypes and/or cellular architectures. This information would help to avoid confusing assignments to bacteria and/or fungi, which must be changed occasionally.

Slime moulds

They represent a complex and polyphyletic microbial group, which has been considered as Fungi in some classifications and as Protist in others. Recent molecular analyses indicated that they represent separate lineages within the protists. Although they have almost no fossil records, it must be pointed out that all reported records appear embedded in Tertiary ambers. The first confident report of a fossilized slime mould was made by Domke (1952), examining Baltic amber (Eocene), which was identified as *Stemonitis splendens*. In the last 25 years, only three additional reports of fossilized slime moulds have been published. The first of these microfossils was a large plasmodium found in Dominican amber (Eocene–Oligocene), which probably represents a fragment of an entire reticulate plasmodium (Waggoner and Poinar 1992). Some years later, Dörfelt et al. (2003) found in a piece of Baltic amber a sorocarp which was assigned to the extant genus *Arcyria* and identified as the new species, *Arcyria*

sulcata. More recently, several sorocarps preserved on conifer debris entrapped in Baltic amber have been described (Dörfelt and Schmidt 2006). This fossilized slime mould was identified as *Protophysarum balticum* sp. nov. We agree with German colleagues that slime moulds are not identifiable without the preservation of the fructification stages (Dörfelt et al. 2003). We think that in both microbial groups, filamentous fungi and slime moulds, the detailed observation of asexual sporogenous structures only make correct identification possible and, thus, the taxonomical assignment of a microfossil or a present isolate.

Viruses and other microbial obligate parasites

Besides free-living microorganisms, the research group of the prestigious paleozoologist George Poinar has described some species of protists and even potential viruses that are parasites of different insects. As is pointed out by the authors (Poinar and Poinar 2004), the frequency of finding good preserved tissues, organelles, and microorganisms in amber is extremely low. Besides, the examination of fossilized insect pathogens must be carried out in situ, through the amber as well as behind the insect exoskeleton. Under these circumstances, staining to increase contrast for a better observation is not possible and the entire cells cannot be found within the focal plane of the microscope. Due to these major limitations and the absence of both molecular and ultrastructural data, the identification of pathogens should be considered as potential or tentative, although these findings represent a promising first step.

The first report of a fossilized pathogen was a trypanosomatid (Trypanosomatidae: Kinetoplastida) associated to a blood-filled female sand fly, entrapped in Early Cretaceous Burmese amber (Upper Albian, 100–110 Mya). This protozoa was identified as *Paleoleishmania proterus* n. gen., n. sp., a new genus established as a collective fossil genus for fossil digenetic trypanososmes associated with sand flies (Poinar and Poinar 2004). Ten of the 21 examined female sand flies in Burmese (Myanmar) amber were infected with trypanosomatids, which was considered as an epidemic infection. Later, *Paleoleishmania* was also found in the gut of sand fly larvae. According to the author (Poinar 2007), this discovery supported the hypothesis that free-living trypanosomatids could have been acquired by larvae during their feeding and then transferred to adult stage before invertebrate infection. In other amber pieces from the same deposit, a haemosporidian parasite, named *Paleohaemoproteus burmacis* gen. n., sp. n. (Haemosporida: Plasmodiidae), was described (Poinar and Telford 2005) from the abdominal cavity of an extinct species of female biting midge. The oocysts and sporozoites of this parasite resembled the same developmental stages of not only the present genus *Haemoproteus* but also those of other Plasmodiidae genera (*Hepatocystis*, *Leucocytozoon*, *Hepatocystis*, etc.) vectored by insects (Poinar and Telford 2005). Finally, another haemosporidian protist has been reported from Tertiary Dominican amber, which was identified as *Plasmodium dominicana* n. sp. (Poinar 2005). This fossil is similar to the extant avian malaria species, *Plasmodium juxtanucleare*. Different cell stages, including pedunculated öocysts, sporozoites, microgametes-like, and an ookinete, were found in the body cavity of a female *Culex* mosquito. According to Poinar (2005), this finding supports the hypothesis that *Plasmodium* (or malaria) was established in America at an early date, by the mid-Tertiary.

With regard to pathogenic protists, it is quite surprising the relatively high frequency of microfossils detected and the diversity of parasitized insects. In our opinion, it would be necessary to ratify the identity of that parasite by using alternative light and electron microscopical techniques adapted to these hard substrates, such as confocal laser scanning microscopy, SEM-BSE, and LTSEM, which have proven usefulness in this kind of study (Ascaso et al. 2003, 2005). Furthermore, considering the high reported number of parasites in each insect (Poinar 2005, 2007; Poinar and Telford 2005), it might be appropriate to make attempts to apply molecular methodologies in order to corroborate the presence of these relevant pathogens.

Finally, evidences of the existence of putative pathogenic viruses of insects in at least two amber samples from distant and different deposits have been reported. In both cases, numerous polyhedra bodies were observed by optical microscopy; these structures were identified as occluded viruses (Poinar and Poinar 2005). Occluded viruses (OV) are infectious particles composed of enveloped virions embedded within a crystalline matrix of protein. They are present in the biological cycle of some insect viruses (Baculoviridae and Rotaviridae). This polyhedron presents several microns in diameter and, thus, they can be easily visualized by light microscopy (Friesen 2007). Throughout the tissues from the thorax, abdomen, coxae, head, and proboscis of a sand fly inside a nugget of Burmese amber, a number of polyhedron-shaped bodies (2.4–9.2 μm in diameter) were observed, and they were considered as putative nuclear polyhedrosis virus (NPV; Poinar and Poinar 2005). *Nucleopolyhedrovirus* is a genus of baculoviruses with large OV particle (0.15–15 μm) containing more than 20 virions. In general, baculoviruses have a relatively narrow host range, which is limited to a single insect genus or family (Friesen 2007; Bonning 2004). This considerable specificity, the large extension of viral infection throughout the insect body as well as the fact that OV are produced in the nucleus of the NPV-infected cells, raises many reasonable doubts about the real nature of

these polyhedral bodies. The second report is about the midgut tissue of a biting midge inside Burmese amber, containing numerous polyhedra bodies (2.3–5.2 μm). The authors (Poinar and Poinar 2005) suggested the presence of a cytoplasmic polyhedrosis virus (CPV) infection, based on the size, shape, and localization of these inclusion bodies. CPVs are classified as members of the genus *Cypovirus* within the family Reoviridae (Mertens et al. 2004). They are commonly isolated from Lepidoptera and occasionally from Diptera and Hymenoptera (Shapiro et al. 2005). We do not know why the polyhedral OV are assigned to these genera, which usually present cubical bodies (Shapiro et al. 2005) that are quite different to those shown in the amber micrographs. According to Poinar and Poinar (2005), the specific location of polyhedra-like bodies in the insect tissues strongly suggests that virions were also present. So, insect polyhedrosis viruses were present 100 million years ago (Table 1).

Bacteria and yeasts identified by molecular methods and microbial resuscitation

As we will discuss later, molecular identification of ancient microorganisms is currently a subject of intense controversy. Two crucial questions are posed: DNA preservation and modern microbial contamination of amber samples. The reports of Raul Cano and collaborators had a great impact,

Table 1 Relevant fossils entrapped in ambers, mainly from Cretaceous, classified in different microbial groups

Genus/species	Microbial group	Amber deposit	Reference
Cypovirus	Reovirus	Myanmar	Poinar and Poinar (2005)
Nuclear polyhedrosis virus	Baculovirus	Myanmar	Poinar and Poinar (2005)
Arthromitus/Hollandina-like	Spirochete	Dominican Republic	Wier et al. (2002)
Leptotrichites resinatus	Sheathed bacterium	Schliersee (Germany)	Schmidt and Schäfer (2005)
Amoeba	Naked amoebae	Peñacerrada (Spain)	Ascaso et al. (2005)
Hartmannella	Naked amoebae	Peñacerrada (Spain)	This paper
Centropyxis delicatula	Testate amoebae	Schliersee (Germany)	Schmidt et al. (2004)
Centropyxis hirsuta	Testate amoebae	Italian Dolomites	Schmidt et al. (2006)
Cyphoderia	Testate amoebae	Dominican Republic	Waggoner (1996a, b)
Hyalosphenia baueri	Testate amoebae	Schliersee (Germany)	Schönborn et al. (1999)
Phryganella paradoxa	Testate amoebae	Schliersee (Germany)	Schmidt et al. (2004)
Chamydomonas	Flagellata	Peñacerrada (Spain)	Martín-González et al. (2008)
Chloromonas	Flagellata	Schliersee (Germany)	Schönborn et al. (1999)
Euglena	Flagellata	Schliersee (Germany)	Schönborn et al. (1999)
Euglena	Flagellata	Peñacerrada (Spain)	Martín-González et al. (2008)
Phacus	Flagellata	Peñacerrada (Spain)	Martín-González et al. (2008)
Paleohaemoproteus burmacis	Haemosporid	Myanmar	Poinar (2007)
Paleoleishmania proterus	Trypanosomatid	Myanmar	Poinar and Poinar (2004)
Plasmodium dominicana	Haemosporid	Dominican Republic	Poinar (2005)
Bryometopus triquetus	Ciliate	Schliersee (Germany)	Schönborn et al. (1999)
Cyrtolophosis	Ciliate	Northwestern France	Waggoner (1994a–c)
Paramecium	Ciliate	Schliersee (Germany)	Poinar et al. (1993a, b)
Paramecium	Ciliate	Peñacerrada (Spain)	Ascaso et al. (2005)
Paramecium triassicum	Ciliate	Schliersee (Germany)	Schönborn et al. (1999)
Prorodon	Ciliate	Peñacerrada (Spain)	Martín-González et al. (2008)
Pseudoplatyophrya nana	Ciliate	Schliersee (Germany)	Schönborn et al. (1999)
Arcyria sulcata	Slime mould	Baltic	Dörfelt et al. (2003)
Protophysarum balticum	Slime mould	Baltic	Dörfelt and Schmidt (2006)
Stemomitis spendens	Slime mould	Baltic	Domke (1952)
Aspergillus collembolorum	Filamentous fungi	Baltic	Dörfelt and Schmidt (2005)
Chaenothecopsis bitterfeldensis	Filamentous fungi	Bitterfeld	Rikkinen and Poinar (2000)
Geotriches glaesarius	Filamentous fungi	Dominican Republic	Stubblefield et al. (1985)
Palaeoanellus dimorphus	Dimorphic fungi	Southwestern France	Schmidt et al. (2007, 2008)

but the number of skeptical researchers regarding the resuscitation of ancient bacteria has progressively increased. In 1994, Cano et al. isolated for the first time the bacterial DNA from the abdominal tissue of four extinct stingless bees, entrapped in Dominican amber. After sequencing, a 546-bp 16S rDNA gene fragment revealed a close phylogenetic relationship between these ancient sequences and the present bacterial species *Bacillus pumilus*, *Bacillus firmus*, *Bacillus subtilis*, and *Bacillus circulans*, four *Bacillus* spp. commonly isolated from bees. Later (Cano and Borucki 1995), a bacteria spore was revived, cultured, and identified after its isolation from the abdominal content of an extinct bee preserved for 20–40 Mya inside Dominican amber. The 16S rDNA profile indicated that the ancient bacterium was closely related to the current *Bacillus sphaericus*. This paper has probably been one of the most commented on and criticized by microbiologists in recent years; however, it represents a new approach in Paleomicrobiology. Skeptics have made several criticisms and pointed out various deficiencies with regard to the reproducibility of these experiments, such as the actual existence of a complex of species in *B. sphaericus*, the analysis of more and longer sequences, the absence of more exhaustive controls, and, hence, the possible existence of contamination by modern *Bacillus*, etc. (Fischman 1995; Priest 1995; Beckenbach 1995). We think that certain objections regarding a possible contamination of the original sample are raised due to certain ignorance of the fossilization mechanism by trapping in amber. In an aerobic environment and due to polymerization, the resin becomes too hard after a few weeks or some months even if the environmental conditions are unfavorable; this makes bacterial movement or contamination from outside of the nugget impossible. Furthermore, when the resin pieces have nearly solidified, insects or any organism cannot be embedded within or penetrate the resin. To avoid external modern contamination, the amber nuggets must be exposed to appropriate doses of disinfectants or UV radiation. Of course, this does not prevent possible subsequent contamination by modern microorganisms during polymerase chain reaction (PCR) manipulation. Different research groups, headed by Raul Cano, have published at least three additional studies on bacterial survival in amber in recent years. Firstly, two bacterial isolates from plants and soil inclusions in Dominican amber have been described, including biochemical, morphological, physiological, and molecular data. According to the authors, these two strains represent a new bacterial species, which has been named *Staphylococcus succinus* (Lambert et al. 1998). An attempt to study the diversity of bacterial microbiota in samples of both Dominican and Israeli ambers was made by fatty acid profiles and/or 16S rDNA gene sequencing (Greenblatt et al. 1999). Twenty-seven microbial isolates were Gram-positive bacteria: rods, cocci, and two actinobacteria. Except for strains assigned to species of the genus *Bacillus*, phenotypic (fatty acid methyl esters (FAME) content) and genotypic (16S rDNA) identifications of these microbial isolates rarely matched. Thus, for instance, no correlation was found between the two types of analysis when isolated cocci were studied. Three main reasons have been put forward to support this study: (1) inaccessibility of the amber to the environment, particularly water, (2) successful contamination precautions, and (3) significant difference between obvious contaminants (*Staphylococcus epidermidis*) and the putative isolated ancient strains. More recently, Greenblatt et al. (2004) isolated nonspore-forming cocci from a nugget of Israeli amber (120 Mya). By molecular (16S rDNA gene sequencing and FAME analysis), biochemical, and ultrastructural features, the bacterium was identified as *Micrococcus luteus*. An important point to consider is the long-term survival of this bacterium in a nutrient-poor environment (Kaprelyants and Kell 1993; Mukamolova et al. 1995). To explain it, the authors assumed that initial survival is due to bacterial growth using the succinic acid of the resin (3–8% of amber consists of this four-carbon dicarboxylic acid). When the resin polymerized to form a solid block, the microorganism might have been able to enter in dormancy, remaining viable long enough until appropriate environmental conditions for growth became available (Greenblatt et al. 2004).

Molecular studies in amber have not been restricted to just bacteria. Spanish microbiologists have amplified, from Miocene and Oligocene amber samples (Dominican and Mexican ambers), fragments of essential genes from the yeast *Saccharomyces cerevisiae* (Veiga-Crespo et al. 2004). These genes were 18S rDNA, ATP9, and PGU1, the last two encoding the subunit 9 of the ATP synthase and an endopolygalacturonase, respectively. The comparison of current genes with their ancient counterparts showed that the degree of gene sequence conservation was not the same in all cases, so nuclear genes (18S rDNA and ATP9) seem to be better conserved than the mitochondrial one (PGU1). Other consensus sequences from species of microalgae, plants, mammals, and bacteria did not produce detectable PCR products (Veiga-Crespo et al. 2004). In 2007, the same research group amplified and sequenced fragments of ancient DNA from amber Cretaceous (Burma) and Miocene (Chiapas) samples, using specific primers for the genes 18S rDNA and AGP2 (which encodes a plasma membrane carnitine carrier), which are, respectively, essential and nonessential genes for the survival of *S. cerevisiae* (Veiga-Crespo et al. 2007). After phylogenetic analysis, the authors concluded that the 18S rDNA sequence amplified from Cretaceous amber corresponds to an ancient yeast, closely related with *S. cerevisiae.* Although a high diversity of

fossil bacteria-like microorganisms were observed in these samples by transmission electron microscopy, the primers for 16S rDNA genes did not allow positive identifications at species level (Veiga-Crespo et al. 2007).

As we have stated above, in recent years, certain studies have claimed that ancient bacteria cells and their DNA can survive for millions of years within the amber. Similar claims have been voiced in other recent reports about ancient DNA amplification or old bacteria resuscitations from halites, sediments, primary salt crystals, ancient permafrost, and old ice (Bidle et al. 2007; Fish et al. 2002; Katayama et al. 2007; Miteva and Brenchley 2005; Vreeland et al. 2000). The persistence of bacterial DNA and even cultivable cells over geological time has been questioned by several authors. Four main reasons were expounded to invalidate the authenticity of these results: first, the existence of microbial contamination with recent forms (Hebsgaard et al. 2005; Willerslev and Cooper 2005), second, the absence of reproducible results by independent laboratories (Hebsgaard et al. 2005; Willerslev and Cooper 2005), third, the resemblance of ancient records to modern bacteria at both morphological and molecular levels, in disagreement with theoretical expectations (Maughan et al. 2002; Nickle et al. 2002), and finally, the integrity of ancient DNA, a labile macromolecule that is subject to diverse types of damage (Pääbo et al. 2004; Poinar 2002). According to different authors (see review by Pääbo et al. 2004), the chemical properties of DNA probably restrict the survival of any molecules to this side of a million years even in favorable environments. However, recently, evidence of DNA repair in ancient bacteria has been reported, suggesting that bacteria are alive in samples up to half a million years in age (Johnson et al. 2007). These repair pathways could explain the maintenance of DNA integrity for extended periods of time.

Conclusions

Amber is a good alternative to salt crystals, permafrost, and geological substrates for studying the morphological organization of microfossils and microbial communities from ancient times. Amber microbiocenoses are quite diverse and include bacteria, protists, and fungi. Usually, the number of records of the same genera/species embedded in the same amber piece is scarce, in contrast with those observed in present microbial cellular populations from natural habitats. It might indicate that most cells were not preserved during fossilization. Insect microbial pathogens have also been reported. Assignments of these records to current genera and species have indicated mainly freshwater or semiterrestrial ecosystems. Reports from diverse amber samples have made the existence of morphological stasis in microorganisms over geological periods evident. Amber might also be a valuable source of ancient DNA and bacteria. Although the persistence of bacterial DNA and even cultivable cells over geological time has been seriously questioned by skeptical authors, the existence of spores or other latent cellular stages (dormant, persistent forms, etc.) as well as the detection of DNA repair in ancient bacteria open up once again the real possibility of organisms surviving under these extreme situations.

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