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Amber microfossils: On the validity of species concept

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ABSTRACT

Do terrestrial micro-organisms evolve morphologically? A recent concept suggests that morphological stasis over dozens of millions of years has persisted in microbial lineages. However, it is based on a weak fossil record. Indeed, it is already difficult to define a species with extant microbes, and this task is even harder when dealing with fossil micro-organisms. Based on research on fossils in amber, we highlighted the different problems that are raised when describing a new fossil species of micro-organisms and we discuss the concept of morphological stasis.

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R É S U M É

Les micro-organismes terrestres évoluent-ils morphologiquement ? Un concept récent suggère que des stases morphologiques (plusieurs dizaines de millions d'années) existent chez différents groupes de micro-organismes. Cependant, il est basé sur un registre fossile très critiquable. En effet, il est difficile de définir une espèce à partir de micro-organismes actuels, et cette tâche devient encore plus ardue, lorsqu'il s'agit de micro-organismes fossiles. Grâce aux recherches sur l'ambre, nous avons souligné les différents problèmes qui surviennent lors de la description d'une nouvelle espèce de micro-organismes fossiles et nous avons discuté le concept de stase morphologique.

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1. Introduction

Soft-body micro-organisms (in which we can include bacteria, protists and fungi) are the most abundant

organisms on Earth, but they are often forgotten when considering biodiversity. It is even more true when speaking about palaeobiodiversity. Indeed, one learns that the first organisms that colonise Earth were micro-organisms (Schopf et al., 2010). About 3.8 billion years ago, the first cells to appear were those of bacteria, whilst eukaryotes appear at least 2 billion years later. Except for this particular period during which only micro-organisms existed, one will never (or at least only rarely) have other references to

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the fossil record of soft-body micro-organisms when discussing the Phanerozoic period. This does not mean that between the Cambrian until now micro-organisms were not present on Earth, and that they only recently appear again. This bias in our teaching of palaeontology is caused by our poor knowledge of their fossil record with relatively few known specimens. One of the famous examples comes from the Rhynie Cherts in which many very well preserved fungal remains and other microfossils have been identified and studied (Taylor et al., 2004).

The lack of information about the fossil record of soft-body micro-organisms is problematic as it tends to bias our knowledge about palaeobiodiversity (when speaking about the Jurassic or the Cretaceous with someone, the person will surely only think about dinosaurs), and it creates other problems. For example, for several decades, many studies have been done on diverse geological events known as “biological crisis”. All these studies are based on a poor fossil record. Indeed no data about soft-body micro-organisms are available to study these events, and we do not know if they affect bacteria, protists and fungi. We can easily imagine that, during a biological crisis, their abundance would have been disturbed as food webs were affected, but we ignore if several lineages disappeared. On the contrary, we can hypothesise that many decomposers benefited from these crises.

This dearth of information is now changing as much more is being learnt about the diversity of ancient micro-organisms. In the last two decades, a new kind of micropalaeontological study blossomed. It is the study of micro-organisms preserved in amber. Even if the first studies were done during the nineteenth century (Berkeley, 1848; Göppert and Berendt, 1845; Göppert and Menge, 1886), the study of amber microfossils really picked-up at the beginning of the 1990s. As it developed more and more, it provided important data about the fossil record of diverse groups of soft-body forest micro-organisms.

Here we present a summary of the discoveries made in amber with a special focus on French amber that has been recently studied in detail (Girard, 2010). Since 2003, this mid-Cretaceous amber proved to be extremely rich in microfossils (Girard, 2010). We discuss these results from a species concept perspective, as applied to fossil microbes. We also consider the concept of morphological stasis advanced by Martín-González et al. (2008).

2. Contribution of amber to the fossil record of micro-organisms

Amber is probably the mode of preservation that provides the most fossils of micro-organisms. The first studies mentioning this kind of inclusions were published in the middle of the nineteenth century (Berkeley, 1848; Göppert and Berendt, 1845; Göppert and Menge, 1886). During more than a century after, only few works were published (most of them concerning Baltic amber). However at the end of the twentieth century, studies of amber micro-organisms increased, and many publications have been published during the last two decades. These works showed that all the groups of micro-organisms that we have identified in modern environment could have been

preserved in amber (Tables 1–7). In the following paragraphs, details about all these discoveries are given, with a special focus on mid-Cretaceous amber of France.

Bacteria (Bacteria excepted Actinobacteria and Cyanobacteria: Table 1; Actinobacteria: Table 2; Cyanobacteria: Table 3) have been first mentioned in amber by Galippe (1920) when he identified some *Microzymas* that he isolated from some amber samples and that he cultivated in his laboratory (Galippe's cultivated bacteria were most probably contaminants). Few bacteria were found in amber until the nineties when studies of DNA from amber developed. At that time, dozens of bacteria had been found in various ambers (e.g. Greenblatt et al., 1999, 2004; Veiga-Crespo et al., 2004). However, most of these studies were criticised as many scientists thought that these bacteria were only contaminants (Austin et al., 1997; Hebsgaard et al., 2005; Orlando, 2005). At present, many doubts exist on the origin of these bacteria. As shown in Tables 1–3, few bacteria have been clearly identified on a morphological basis only, in part because there is insufficient morphology for visual identification.

Concerning the mid-Cretaceous amber of France, the most abundant micro-organisms correspond to prokaryotes. Rod-shaped bacteria have been preserved in many amber pieces whilst sheathed bacteria (similar to the genera *Leptotrichites* and *Sphaerotilus*) are only preserved as tiny cortices around some amber pieces. Actinobacteria (Fig. 1.1) are also very abundant and they are usually preserved as spherical colonies of 50–75 µm in diameter (Girard et al., 2009b). This shape shows that these Actinobacteria were able to grow in the resin before its solidification. The most abundant prokaryotes are Cyanobacteria, and more precisely the species *Palaeocolteronema cenomanensis* (fig. 1.2). This taxon created 1–2 cm thick cortices around some amber pieces. In these cortices, the filaments are centripetally orientated, showing that *P. cenomanensis* grew in the resin before its fossilisation. The most unexpected discovery was the presence of fossil phycocyanin pigments in some filaments of *P. cenomanensis* (Girard et al., 2009a).

Fungi (Table 4) represent the group of amber micro-organisms that have been the most intensively studied. They were the first micro-organisms found in the nineteenth century. However some of them seem to have been identified as fungi (according to the classification system of that day), whereas they were only Actinobacteria. For example the species *Penicillites curtipes* was described in 1848 (Berkeley, 1848) but the description and illustrations given by the author indicate that this inclusion is probably an Actinobacteria. The study of the amber fungi really started in the eighties and nineties. During these two decades, several authors studied fungi (Table 4) that have been preserved in amber of various origins (Baltic Sea, Dominican Republic, Mexico, USA, France, Germany...) and of various ages (Cretaceous, Eocene, Oligocene, Miocene...). These discoveries greatly enriched the fungi fossil record, providing many fossil Ascomycota and only few Basidiomycota. As for the bacteria, study of fossil DNA from amber provided few evidences of the presence of fungi in amber

Table 1

Bacteria found in amber (excepted Actinobacteria and Cyanobacteria). For more details see Girard (2010). * and (*) indicate that the specimens or some specimens have been identified thanks to DNA studies. Taxa supporting the concept of morphological stasis are underlined.

Tableau 1

Bactéries préservées dans l'ambre (à l'exception des actinomycètes et cyanobactéries). Pour plus de détails, se référer à Girard (2010). Les * et (*) indiquent que les spécimens ou certains spécimens ont été trouvés grâce à des analyses ADN. Les taxons corroborant le concept de stase morphologique sont soulignés.

Age	Outcrop	Fossils
Triassic	Italy	Bacteria indet.*
Aptian	Israel	<u>Bacillus circulans*</u>
		<u>Bacillus coagulans*</u>
		<u>Bacillus licheniformis*</u>
		<u>Bacillus megaterium*</u>
		<u>Bacillus pumilus*</u>
		<u>Bacillus sphaericus*</u>
		<u>Micrococcus agilis*</u>
		<u>Micrococcus luteus*</u>
		<u>Paenibacillus curdanolyticus*</u>
		<u>Staphylococcus aureus*</u>
		<u>Staphylococcus epidermis*</u>
		<u>Staphylococcus warneri*</u>
Albian	Spain	<u>cf. Gallionella sp.</u>
Albian/Cenomanian?	Myanmar	<u>cf. Bacillus sp.*</u>
	USA	<u>cf. Leptothrix sp.</u>
Cenomanian	France	Bacteria indet.
		Rod-shaped bacteria
		<u>cf. Leptotrichites</u>
		<u>cf. Sphaerotilus</u>
	Germany	Filamentous bacteria
		<u>Leptotrichites resinatus</u>
		Sheathed bacteria
Eocene		
Baltic sea	<i>Bacillus elektroni</i>	<u>Bacillus subtilis</u>
		<u>Longibacillus elektroni</u>
		<u>Micrococcus elektroni</u>
		<u>Spirillum elektroni</u>
		<u>Succinococcus sp.</u>
	France	Bacteria indet.*
	USA	Bacteria indet.
Oligo-Miocene	Dominican Republic	<u>cf. Arthromitus sp.</u>
		<u>cf. Bacillus sp.</u>
		<u>Bacillus coagulans*</u>
		<u>Bacillus licheniformis*</u>
		<u>Bacillus pumilus*</u>
		<u>Bacillus sphaericus*</u>
		Bacteria Spirochetes
		<u>?Micromonospora sp.*</u>
		<u>Saccharomyces cerevisiae*</u>
		<u>Staphylococcus epidermis*</u>
		<u>Staphylococcus succinus</u>
Miocene	Amazon	Eubacteria indet.
	France	Bacteria indet.*
	Mexico	<u>cf. Bacillus sp.*</u>

(Greenblatt et al., 1999), but these results are still debated.

Eukaryotes are less abundant than prokaryotes in French mid-Cretaceous amber, but they are more diversified. Fungi are the most abundant eukaryotes. However most of them are not identifiable as just fragments of the mycelium are preserved. Only few could be identified. They mainly correspond to Ascomycota such as specimen close to the genera *Metacapnodium* and *Aspergillus*, a cladosporioid fungi (Fig. 1.3) and possible Endomycetaceae (Girard, 2010). An extraordinary discovery was the one of a carnivorous fungus. A dimorphic fungus forming mycelia and

yeast stages has been identified as a carnivorous fungus (Deuteromycotina) based on the occurrence of hyphal rings as trapping devices (Schmidt et al., 2008). More recently, bracket fungi (Basidiomycota) have been reported from faecal pellets preserved in mid-Cretaceous amber of France (Schmidt et al., 2010b).

Non-photosynthetic protists from amber were not mentioned in the literature until the 1940s (Legg, 1942) when inclusions similar to the genus *Paramecium* were found in Cretaceous amber of Canada. Then protists were known in few ambers (Table 5). Recently the Triassic amber of Italy yielded a diverse assemblage of ciliates and amoebae

Table 2

Actinobacteria found in amber. For more details see Girard (2010). The * indicate specimens identified via DNA studies. Taxa supporting the concept of morphological stasis are underlined.

Tableau 2

Actinomycètes préservés dans l'ambre. Pour plus de détails, se référer à Girard (2010). Les * indiquent que les spécimens ont été trouvés grâce à des analyses ADN. Les taxons corroborant le concept de stase morphologique sont soulignés.

Age	Outcrop	Fossils
Aptian	Israel	<u>Rathayibacter rathyi*</u> <u>?Streptomyces sp.*</u>
Cenomanian	France	Actinobacteria indet.
Turonian	USA	<u>Streptosporangopsis russelli</u> <u>Palaeomonospora dominicanus</u>
Eocene	France	Actinobacteria indet.
	USA	<u>Streptomyces sp.</u>
Oligo-Miocene	Dominican Republic	<u>Nocardioformis dominicanus</u> <u>Nocardiooides albus*</u> <u>?Streptomyces sp.*</u>

(Schmidt et al., 2006). The amber that yielded the most abundant and diversified assemblage is the Cenomanian amber of Schliersee. This amber has been intensively studied as it was supposed to be Triassic in age, but a recent study proved that it is Cenomanian (Schmidt et al., 2001). Because of its supposed age, this amber has been intensively studied and at least seven genera of ciliates and five of amoebae have been identified (Poinar et al., 1993a, 1993b; Schmidt et al., 2001, 2004; Schönborn et al., 1999). Concerning the other ambers, only sporadic discoveries have been made, but fossils to several lineages of non-photosynthetic protists were discovered.

Protists in French amber mostly correspond to testate amoebae. Most of them belong to the Arcellinida (close to the genera *Arcella* (Fig. 1.4), *Hyalosphenia* (Fig. 1.5), *Centropyxis*, *Cryptodiffugia* and *Leptochlamys*) (Girard, 2010; Schmidt et al., 2010a). Only a specimen of a euglyphid has been found, close to the genus *Assulina*. Other protists are less abundant. Only few ciliates (Fig. 1.6) have been identified (specimens close to the genera *Brachonella*, *Grossglockneria*, *Astylozoon* and other Spirotrichia). Flagellates have not been clearly identified.

During the last ten years, several publications also described amber protists (Ascaso et al., 2003, 1997; Kedves and Párdutz, 2002; Martín-González et al., 2008, 2009a, 2009b; Veiga-Crespo et al., 2007). In these publications, some ovoid micro-inclusions that look like protozoans (mostly ciliates, flagellates and amoebae according to the studies) are described and illustrated. However, some characteristics of these inclusions indicate that they are probably not protists. Most of these inclusions have many delicate internal vacuoles (sometimes several dozens) that

have been interpreted as the internal vacuoles of the protists. However this abundance of vacuoles is inconsistent with the poor preservation of protists and is probably due to a phenomenon of “vacuolisation” during amber polymerisation. In addition, despite resembling “membranous vacuoles”, they do not include more robust structures such as nuclei, cytoskeleton, flagella or organelles. Similar inclusions are preserved in mid-Cretaceous French ambers in which they can be very abundant. These inclusions show important variation in size and form in a single amber piece. This characteristic is not really compatible with the trapping of a natural population of protists. Other studies are needed to really confirm or infirm the biological origin of all these inclusions, but, according to our experience, they resemble typical non-biological inclusions found in amber, inclusions that surely cannot be assigned to protists (Girard, 2010). We propose that as a precaution, these vacuolated structures should not be considered genuine fossilised protists, as they may not be of biological origin. We suggest setting these aside when reconstructing time lines based on the fossil record, until there is convincing evidence of their biological origin.

Photosynthetic protists (Table 6) were found in Baltic amber for the first time in 1848 (Berkeley, 1848) and the oldest evidence is from the Carboniferous amber of Scotland (Smith, 1896). However, since these first discoveries, only few additional specimens of probable photosynthetic species have been found, mostly in Cretaceous ambers (from Germany, France, Myanmar and Spain) (Table 6). Some of the specimens are doubtful (especially specimen similar to euglenids) as they are very similar to most of the amber micro-inclusions that have a cell shape whilst

Table 3

Cyanobacteria found in amber. For more details see Girard (2010). Taxa supporting the concept of morphological stasis are underlined.

Tableau 3

Cyanobactéries préservées dans l'ambre. Pour plus de détails, se référer à Girard (2010). Les taxons corroborant le concept de stase morphologique sont soulignés.

Age	Outcrop	Fossils
Cenomanian	France	<u>cf. Coelosphaerium sp.</u> <u>cf. Lyngbya sp.</u> <u>cf. Plectonema sp.</u> <u>Palaeocolteronema cenomanensis</u>
Eocene	Baltic sea	<u>Discophyton electroneion</u>
Miocene	Amazon	Nostocaceae indet.

Table 4

Fungi found in amber. For more details see Girard (2010). The * indicate specimens identified via DNA studies. Taxa supporting the concept of morphological stasis are underlined.

Tableau 4

Champignons préservés dans l'ambre. Pour plus de détails, se référer à Girard (2010). Les * indiquent que les spécimens ont été trouvés grâce à des analyses ADN. Les taxons corroborant le concept de stase morphologique sont soulignés.

Age	Outcrop	Fossils
Carboniferous	Scotland	<u>Leptonema tenuis</u> <u>Peronosporoides carbonifera</u> Spores indet.
Triassic	Italy	cf. <u>Ramularia</u> sp.
Aptian	Israel	Phycomycetes indet.*
Albian	Spain	Fungi indet.
Albian/Cenomanian?	Myanmar	<u>Entropezites patricii</u> Fungi indet. <u>Mycetophagites artrebor</u> <u>Palaeoagaracites antiquus</u> Ascomycota indet. <u>Metacapnodium</u> sp. cf. <u>Aspergillus</u> Cladosporioid fungus cf. Endomycetaceae Deuteromycotina Basidiomycota Moniliales indet. <u>Palaeodikaryomyces baueri</u> <u>Pithomyces</u> sp.
Cenomanian	France	<u>Archaeomarasmius leggetti</u> Zygomycete indet.
Turonian	USA	Fungi indet. Dermatiaceae indet. <u>Acromonium succineum</u> <u>Alectoria</u> sp. <u>Anthomycete</u> sp. <u>Anzia electra</u> <u>Arachnomycelium</u> sp. <u>Aspergillus</u> sp. <u>Brachycladium thomasinum</u> cf. <u>Calicium</u> sp. cf. <u>Chaenotheca</u> sp. <u>Cladosporium</u> sp. <u>Fungites</u> sp. <u>Gonatobotrys primigenia</u> <u>Graphis</u> sp. <u>Lecanora ciliaris</u> <u>Melanosphaerites</u> sp. <u>Metacapnodium succinus</u> <u>Mucorites</u> sp. <u>Oidium moniliformis</u> <u>Parmelia Physodes</u> <u>Penicillium</u> sp. <u>Penicillites curtipes</u> <u>Pezizites</u> sp. <u>Polyporus</u> sp. <u>Ramularia olbongispora</u> Schizosaccharomycetes sp. <u>Sporotrichites heterospermus</u> <u>Stigmatomyces succini</u> <u>Stilbum succinii</u> <u>Streptothrix spiralis</u> <u>Torula heteromorpha</u> <u>Tramaites</u> sp. <u>Veionella</u> sp.
Cretaceous indet.	Canada	
Paleocene-Eocene	Austria	
Eocene	Baltic sea	
Oligocene	France	cf. <u>Xylohyphites</u> sp.
	Italy	<u>Monilites</u> sp.
	Germany	<u>Chaetothecopsis bitterfeldensis</u> <u>Metacapnodium succinus</u>

Table 4 (Continued)

Age	Outcrop	Fossils
Oligo-Miocene	Dominican Republic	<i>Aspergillus</i> sp.
		<i>Aureofungus yaniguaensis</i>
Miocene	Amazon	<i>Beauveria</i> sp.
		<i>Coprinites dominicana</i>
		<i>Entomophthora</i> sp.
		Fungi indet.
		<i>Geotrichites glaesarius</i>
		cf. <i>Geotrichum</i> sp.
		<i>Leptothyrites dominicanus</i>
		<i>Leptostromites ellipticus</i>
		<i>Parmelia</i> sp.
		<i>Protomyцена electra</i>
		<i>Xylaria</i> sp.
		<i>Dicellaesporites</i> sp.
		<i>Dicellaesporites africanus</i>
		<i>Dicellaesporites inequalibis</i>
		<i>Dicellaesporites longus</i>
		<i>Dicellaesporites</i> cf. <i>obnixus</i>
		<i>Dicellaesporites</i> cf. <i>perelongatus</i>
		<i>Dictyosporites</i> sp.
		<i>Diporicellaesporites</i> sp.
		<i>Diporicellaesporites fusoides</i>
		<i>Dyadosporites</i> cf. <i>minor</i>
		<i>Frasnacritetrus</i> spp.
		<i>Hypoxylonites</i> spp.
		<i>Inapertisporites</i> spp.
		<i>Inapertisporites clarkei</i>
		<i>Kumarisporites</i> sp.
		<i>Monosporites</i> sp.
<i>Monosporites</i> cf. <i>magnus</i>		
<i>Multicellites</i> sp.		
<i>Multicellaesporites</i> sp.		
<i>Phragmothyrtes</i> sp.		
<i>Pluricellulaesporites</i> sp.		
<i>Polycellulaesporonites</i> sp.		
<i>Psiamasporites fusiformis</i>		
<i>Quilonia</i> spp.		
<i>Reduviasporites fusiformis</i>		
<i>Trilobites</i> sp.		
Mexico	<i>Asteromites mexicanus</i>	
	<i>Oligophelenchoides atrebora</i>	

they do not have a microbial origin. Some photosynthetic protists have also been preserved in mid-Cretaceous amber from France (Girard, 2010). They mostly correspond to unicellular green algae close to the genera *Myrmecia* (Girard et al., 2009b), *Quadrigula* (Fig. 1.7), *Enallax* (Girard, 2009) and *Chlorcorona*. Filamentous green algae are rare and badly preserved in mid-Cretaceous French amber. Only a specimen close to the genus *Trentepohlia* and a possible Zygnematales have been found. Few micro-inclusions have been assigned to possible badly preserved euglenids (Girard, 2010).

Lastly, few micro-inclusions have been described as *Incertae Sedis* (Table 7). These inclusions of unknown origin are surprisingly quite rare as amber can preserve many strange inclusions. However only few authors have dared to describe microfossils as *Incertae Sedis*. Mid-Cretaceous amber from France has also preserved *Incertae Sedis*. They can have a fusiform shape or be spherical. They do not exhibit the particular vacuoles of non-microbial micro-inclusions already discussed above (Girard, 2010). It is why, without any precise assignment, these micro-inclusions are called *Incertae Sedis* as they probably do not belong to well preserved micro-organisms.

3. Problem of identifying species in amber

Amber studies raise the problem of the identification of micro-organism species. Modern species of micro-organisms, from bacteria to protists, are now mostly defined on molecular and/or genetic methods (Fenchel and Finlay, 2006). Such techniques cannot be used for microfossils from amber. Several attempts to extract ancient microbial DNA from amber have been tried since the beginning of the nineties. Hamamoto and Horikoshi (1994) were the first to announce the extraction of ancient microbial DNA from amber (more precisely from Baltic amber). In the following years, many authors published on the discovery of ancient DNA from various ambers (Baltic, Dominican, Israel, Mexican, Burmese ambers) (Alharbi, 2008; Cano and Borucki, 1995; Cano et al., 1994; Greenblatt et al., 1999, 2004; Lambert et al., 1998; Veiga-Crespo et al., 2004, 2007). However all these studies have been criticized and some authors highlighted some weaknesses in the studies about ancient DNA from amber (Austin et al., 1997; Hebsgaard et al., 2005; Orlando, 2005). Priest et al. (1995) questioned a possible contamination of the samples by modern DNA. Austin et al. (1997) raised the problem of

Table 5

Non-photosynthetic protists found in amber. For more details see Girard (2010). Taxa supporting the concept of morphological stasis are underlined.

Tableau 5

Protistes non-photosynthétiques préservés dans l'ambre. Pour plus de détails, se référer à Girard (2010).

Age	Outcrop	Fossils
Triassic	Italy	cf. <u>Centropyxis hirsuta</u> ¹ Ciliates indet. cf. <u>Coleps</u> sp. ² Diffugiidae indet. ¹
Aptian	Spain	cf. <u>Colpoda</u> sp. ² cf. <u>Prorodon</u> sp. ²
Albian	Spain	cf. <u>Amoeba</u> sp. ¹ cf. <u>Paramecium</u> sp. ² Protists indet.
Albian/Cenomanian?	Myanmar	<u>Paleohaemoproteus burmavis</u> ⁴ cf. <u>Paramecium</u> sp. ²
Cenomanian	France	Amoebae indet. cf. <u>Arcella</u> sp. ¹ cf. <u>Assulina</u> sp. ¹ cf. <u>Astylozoon</u> sp. ² cf. <u>Brachonella</u> sp. ² <u>Centropyxis perforata</u> ¹ cf. <u>Cryptodiffugia</u> sp. ¹ cf. <u>Cyrtolophosis</u> sp. ¹ cf. <u>Grossglockneria</u> sp. ² cf. <u>Hyalosphenia</u> sp. ¹ <u>Leptochlamys galippe</u> ¹ Spirotrichia indet. ² cf. <u>Bryometopus triquetrus</u> ² cf. <u>Centropyxis aculeata</u> ¹ <u>Centropyxis delicatula</u> ¹ <u>Centropyxis hirsuta</u> ¹ cf. <u>Cyclopyxis eurystoma</u> ¹ cf. <u>Cyrtolophosis mucicola</u> ² <u>Hyalosphenia baurei</u> ¹ cf. <u>Mykophagophrys terricola</u> ² cf. <u>Nassula</u> sp. ² cf. <u>Paracondylostoma</u> sp. ² <u>Paramecium triassicum</u> ² <u>Phryganella acropodia</u> ¹ <u>Phryganella paradoxa</u> ¹ cf. <u>Pseudoplatyophora nana</u> ² cf. <u>Tetrahymena rostrata</u> ² cf. <u>Triassamoeba alpha</u> ¹ cf. <u>Pontigulasia</u> sp. ¹ cf. <u>Nebela</u> sp. ¹
	Germany	
	USA	
Santonian/Campanian	Hungary	Protists indet.
Cretaceous indet.	Canada	<u>Paramecium</u> sp. ²
Eocene	Baltic sea	<u>Arcyria sulcata</u> ³ <u>Protophysarum balticum</u> ³ <u>Stemonites dominicana</u> ³
	France	Ciliates indet. Protists indet.
Oligo-Miocene	Dominican republic	Cyphodermiidae indet. ¹ <u>Plasmodium dominicana</u> ⁴ Protists indet.

¹ amoebae

² ciliates

³ myxomycetes

⁴ apicomplexa.

the reproducibility of the extraction of ancient DNA from amber. They highlighted that nobody is able to reproduce the DNA sequence extracted from amber and copal. On this observation, Austin et al. (1997) argued that this lack of reproducibility is the proof that ancient DNA does not exist in amber. Parducci and Bennett (2005) commented on this argument and concluded that it is probably the main reason

for the weak development of the research on ancient DNA. This argument shows that the problem of reproducibility was not resolved in 2005. Hebsgaard et al. (2005) explained this lack of reproducibility by inadequate experimental setups for the study of ancient DNA, providing insufficient authenticity of results. Amber had been thought to be the perfect medium for DNA preservation, but Orlando (2005),

Table 6

Photosynthetic protists found in amber. For more details see Girard (2010). Taxa supporting the concept of morphological stasis are underlined.

Tableau 6

Protistes photosynthétiques préservés dans l'ambre. Pour plus de détails, se référer à Girard (2010). Les taxons corroborant le concept de stase morphologique sont soulignés.

Age	Outcrop	Fossils
Carboniferous	Scotland	<i>Nemaclada</i> sp. <i>Nemaplana</i> sp. <i>Sphairanema</i> sp.
Triassic	Italy	Chlorophyta indet. ¹ Conjugatophyta indet. ¹ <u>cf. <i>Cosmarium</i> sp.¹</u>
Albian	Spain	<u>cf. <i>Euglena</i> sp.²</u> <u>cf. <i>Chlamydomonas</i> sp.¹</u> <u>cf. <i>Phacus</i> sp.²</u>
Albian/Cenomanian? Cenomanian	Myanmar France	<i>Paleoleishmania proterus</i> ² <u>cf. <i>Chlorcorona</i> sp.¹</u> <u>cf. <i>Closterium</i> sp.¹</u> <i>Enallax napoleoni</i> ¹ Euglenophyceae indet. ² <u>cf. <i>Monas</i> sp.¹</u> <u>cf. <i>Myrmecia</i> sp.¹</u> <u>cf. <i>Quadrigula</i> sp.¹</u> Siphonale indet. ¹ <u>cf. <i>Trentepohlia</i> sp.¹</u> <u>cf. Zygnematales¹</u> <u>cf. <i>Chlamydomonas</i> sp.¹</u> <u>cf. <i>Chlorella</i> sp.¹</u> <u>cf. <i>Chloromonas</i> sp.¹</u> <u>cf. <i>Choricystis</i> sp.¹</u> <u>cf. <i>Euglena</i> sp.²</u> Green algae indet. <i>Palaeozygnema spiralis</i> ¹ <i>Dinobryon</i> sp. ³
Turonian Eocene	USA Baltic sea France	Algae indet. <i>Chaetonemopsis pseudobulbochaete</i> ? ¹ Chlorococcales indet. ¹ <u>cf. <i>Dichotomosiphon</i> sp.¹</u> <u>cf. <i>Ovoidites</i> sp.¹</u> <u>cf. <i>Trentepohlia</i> sp.¹</u> <u><i>Scenedesmus</i> sp.¹</u>
Miocene	Amazon	

¹ green algae.

² euglenids.

³ brown algae.

in a review of ancient DNA research, argued that the only way to get very old DNA is to preserve it in a dark and cold medium (such as Siberian permafrosts). Nobody studies in detail the fate of DNA in plant resin as the polymerisation processes transform resin to amber. At the moment, there is no real proof, according to us, for ancient DNA preserved in amber.

This short review of the study of ancient DNA from amber shows that many doubts exist around the results and the study of DNA from amber cannot be used as a tool for identifying fossil micro-organisms from amber. Thus there exists only one technique that can be used: optical morphological identification. Indeed, the identification of morphological structures remains the only way

Table 7

Incertae sedis found in amber. For more details see Girard (2010). Taxa supporting the concept of morphological stasis are underlined.

Tableau 7

Incertae sedis préservés dans l'ambre. Pour plus de détails, se référer à Girard (2010). Les taxons corroborant le concept de stase morphologique sont soulignés.

Age	Outcrop	Fossils
Carboniferous Albian/Cenomanian? Cenomanian	Scotland Myanmar France	<i>Carbonacarpa annandalensis</i> Pathogen micro-organisms on insects Multi-cellular inclusion Diverse <i>Incertae Sedis</i>
Oligo-Miocene Miocene	USA Dominican Republic Amazon Mexico	Rounded and ovoid inclusions Pathogen micro-organisms on insects Pseudo-lichen Pathogen micro-organisms on insects

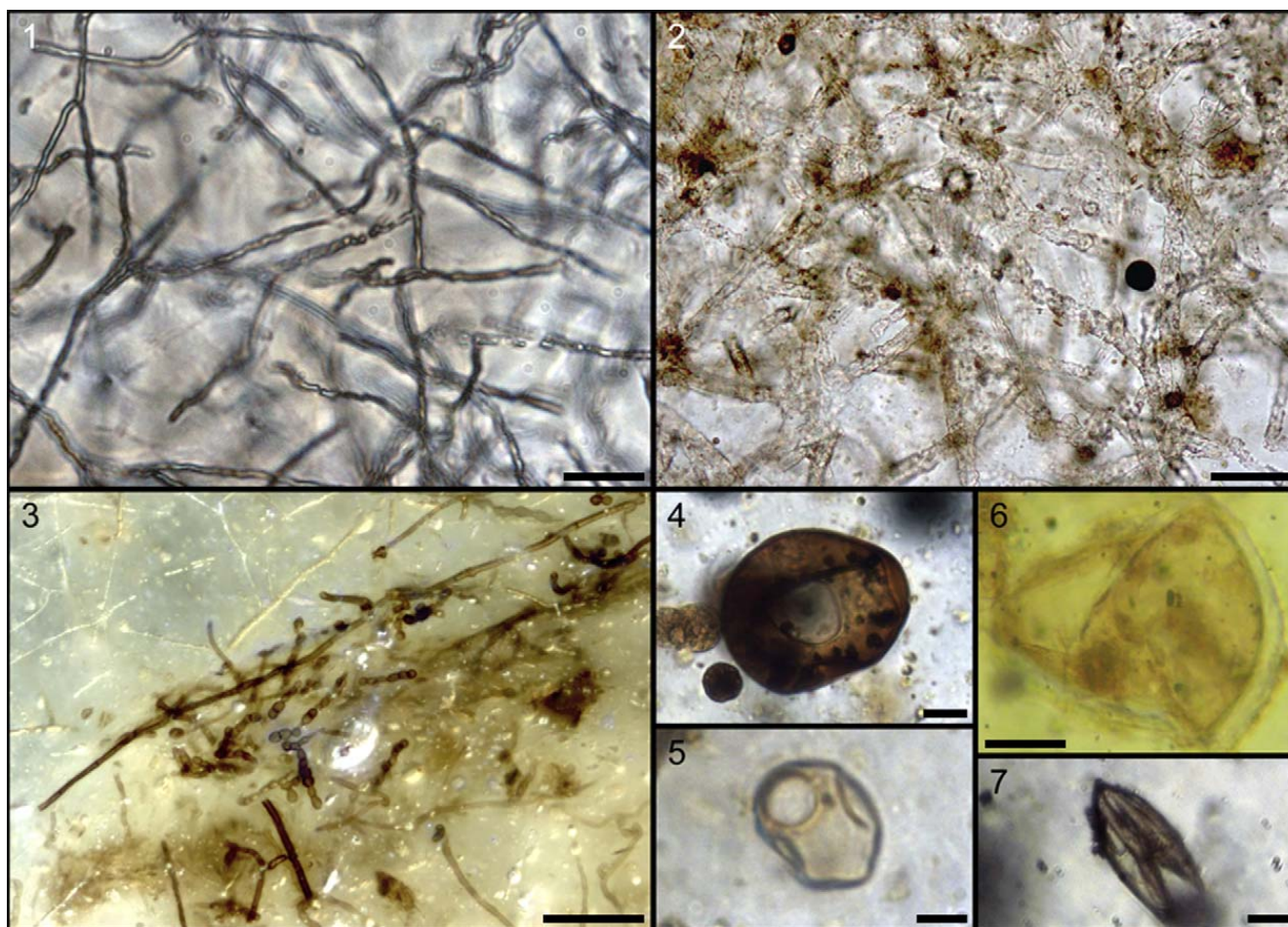


Fig. 1. Diverse micro-organisms from French mid-Cretaceous amber. 1: Actinobacteria indet. 2: *Palaeocolteronema cenomanensis*, Cyanobacteria. 3: Cladosporoid fungus. 4: cf. *Arcella* sp., amoeba. 5: cf. *Hyalosphenia* sp., amoeba. 6: Ciliate indet. 7: cf. *Quadrigula* sp., green alga. Scale bars: 5 μm (5, 7), 10 μm (1, 4), 20 μm (6) and 50 μm (2, 3).

Fig. 1. Divers micro-organismes préservés dans les ambres médio-crétacés français. 1 : Actinomycètes indéterminés. 2 : *Palaeocolteronema cenomanensis*, cyanobactérie. 3 : Champignon cladosporoïde. 4 : cf. *Arcella* sp., amibe. 5 : cf. *Hyalosphenia* sp., amibe. 6 : Cilié indéterminé. 7 : cf. *Quadrigula* sp., algue verte. Échelles : 5 μm (5, 7), 10 μm (1, 4), 20 μm (6) et 50 μm (2, 3).

to identify microfossils. However it raises issues regarding the morphological identification of micro-organisms. Regarding bacteria, it is clear that no precise affinity can be found only on morphological characteristics. Waggoner (1994b) stated that the attribution of a fossil Actinobacteria to a modern group is tentative. Such statement can be expanded to almost all groups of bacteria. Only rare cases allowed more precise identification of bacteria to genus on morphological characters (Breton and Tostain, 2005; Girard et al., 2009a; Schmidt and Schäfer, 2005). When studying bacteria from amber, one might remember that identification of bacteria is tentatively and any taxonomical attribution might be doubtful.

Regarding the eukaryotes, the problem is only a little simpler. Fungi have many diverse morphological characteristics that allow a more precise identification, especially on the identification of spores and reproductive structures. The main problem remains the fragmentation of fungal remains in amber. Most of the time, it consists only of isolated fragments of fungal hyphae. Only rare cases provide hyphae, spore and other fungal structures preserved in the same amber piece and allow precise identification (e.g. Girard, 2010; Schmidt et al., 2008, 2010a).

Regarding protists, the problem is more complex. Modern species have been traditionally identified on morphological characters. However the development of new techniques, and more precisely of genetic techniques, has provided doubts about the validity of such morphological identification. However Fenchel and Finlay (2006) argued that the identification of protists with genetic methods (such as barcoding system) is also problematic as the results greatly depends of the choice of the genes selected. Using the case of the species *Cyclidium glaucoma* (a ciliate), Fenchel and Finlay (2006) argued that morphological, physiological and ecological properties of protists are good characters to identify protist species. Such methods can thus appear perfect to identify micro-organisms from amber. However, both Adl and Gupta (2006) and Foissner (2006) provided evidence that the Fenchel and Finlay (2006) argument was simplistic, and that they had dismissed much of the existing evidence regarding the inability of morphological characters to distinguish among species of protists. Furthermore Foissner et al. (1999) showed that, depending on the resin the preservation of some protists can greatly differ from one amber to another. Many external and internal characters are quickly lost after embedding

of the micro-organisms in the resin. This is problematic for the identification of amber microfossils as most of the diagnostic features that allow the determination can be lost early in the fossilisation processes due to poor fixation. One might keep in mind this problem when studying amber microfossils. We therefore propose that species identification for fossilised micro-organisms is tentative because all tools used to identify modern species cannot be used. Only a morphotypic resemblance can be determined. This morphological identification is susceptible to error due to poor preservation of the original morphology.

4. Concept of morphological stasis

Recent discoveries of micro-organisms in various ambers (for more details, see Girard, 2010) led some authors to propose that morphological stasis exists in different microbial lineages. This concept was formally formulated by Martín-González et al. (2008) in their article entitled “Morphological stasis of protists in Lower Cretaceous amber”. These authors argued that their observation confirmed a conclusion of Fenchel and Finlay (2006), i.e. that “microbial phenotypes are ancient in term of the geological time-scale and have been maintained through stabilizing selection”.

A quick review of the literature allows one to see that the fossil record of micro-organisms in amber seems to support this concept. In the tables (Tables 1–7) of this article, the modern genera and species that have been found in amber are underlined. Schliersee amber (Cenomanian, Germany) is probably the best example. Six taxa of photosynthetic protists have been identified in this amber. Only one corresponds to a new genus (Dörfelt and Schäfer, 2000). Four green algae were attributed to the extant genera *Chlamydomonas*, *Chloromonas*, *Choricystis* and *Chlorella* and an euglenid was placed in the modern genus *Euglena* (Schönborn et al., 1999). Two fungal taxa have also been found, one belonging to a new genus, one to the extant genus *Pithomyces*. Sixteen taxa of amoebae and ciliates have also been found in Schliersee amber (Poinar et al., 1993b; Schmidt et al., 2004; Schönborn et al., 1999). This assemblage comprises only one new genus (*Triassamoeba*) and three new species (*Hyalosphenia baurei*, *Paramecium triassicum*, *Triassamoeba alpha*) whilst thirteen taxa correspond to extant species.

Schliersee amber is not unique. Most of the other ambers have preserved micro-organisms that have been attributed to modern taxa (such as the genera *Euglena*, *Paramecium*, *Centropyxis*). These discoveries seem to corroborate the concept of morphological stasis. These apparent stases can result from different factors. First many micro-organisms have broad distribution (for some a worldwide distribution) as mentioned by Fenchel and Finlay (2006). Second these authors argued protists have large population size and slow population replacement, so that phenotypes can persist for millions of years.

However, even though the concept of morphological stasis can be “sexy”, some doubt can be erected concerning its foundation. The fossil record on which it has been formulated needs to be revised. First, some amber

micro-inclusions have been described to be modern taxa of eukaryotes whilst their biological origin is still doubtful. Many of them could be non-biological inclusions only. That means many supposed fossils supporting that concept are not real fossils. Reinvestigations of all these inclusions will help to clarify this point. Second the preservation of the micro-inclusions in amber can greatly influence the identification of microfossils. For example, many testate amoebae have been found in French mid-Cretaceous amber (Girard, 2010). Most of them have been attributed to modern genera (such as *Arcella*, *Centropyxis*, *Hyalosphenia*), but their poor preservation state did not allow further identification. All the very well preserved specimens clearly show differences from modern taxa (such as the species *Centropyxis perforata* and *Leptochlamys galippe*) that differ from the modern *Centropyxis* and *Leptochlamys* by the presence of pores on their test (Schmidt et al., 2010a). Thus, with the current fossil record of eukaryotes from amber, it is difficult to corroborate or infirm the concept of morphological stasis.

What about prokaryotes? Even though the concept of morphological stasis was erected for eukaryotes, prokaryotes from amber seem also to support it. Some of the morphologically identified prokaryotes have been attributed to modern taxa (Waggoner, 1993, 1994a, 1994b, 1996). However there is little morphological diversity among prokaryotes. They are identified using molecular techniques, and their diversity is evidenced in their metabolism and DNA sequence, not by morphology. Molecular techniques can be used when dealing with amber microfossils since we are dealing with fossilised remains, and the morphological identification of amber prokaryotes is very uncertain. It is probably impossible to correctly assign most bacteria to genera based on morphological similarity. Several authors proposed that prokaryotic species cannot exist stably, due to frequent uptake of environmental DNA and due to lateral transfers of genes (Doolittle, 1999; Ochman et al., 2000; Vishwanath et al., 2004).

5. Conclusions

The fossil record of protists has been greatly improved by recent studies of microfossils. Most of the specimens have been attributed to modern genera and sometimes to modern species. The lack of DNA in fossilised amber specimens prevents identification of specimens beyond morphological similarity with extant species. The discoveries from amber support the concept of morphological stasis as formulated by Martín-González et al. (2008) although with some caution as we indicated. This concept corroborates the conclusion of Fenchel and Finlay (2006), that microbial phenotypes are geologically ancient, having been maintained through stabilising selection.

However, doubts about the real origin of several of these amber micro-organisms have been formulated (Girard, 2010; Schmidt et al., 2010a), especially regarding the vacuolated inclusions that have not biological origin according to us. It seems that many supposed protists from amber can only be non-biological protist-like inclusions mimicking protists. These possible wrongfully identified amber protists disturb our knowledge about microbe fossil record

and do not allow us to corroborate or infirm any hypothesis about the evolution of terrestrial protists.

Re-investigation of these possible protists from amber is absolutely necessary in order to clarify the fossil record of the different terrestrial protists. The concept of morphological stasis can have great repercussion in the study of amber microfossils. If it is corroborated, it can raise the problem of the definition of a fossil microbial species. Indeed, even the systematic affinities of these amber micro-inclusions are confirmed, can we really imagine that species have been maintained for several dozens of millions of years? It has been demonstrated that lateral transfers of genes are very common for microbes (especially for prokaryotes (Doolittle, 1999; Ochman et al., 2000; Vishwanath et al., 2004)). Such phenomena seem to be incompatible with the fact that species were maintained for millions of years. This problem raises, for terrestrial microbes, the problem of how to define a species, on the basis of morphology alone. Data obtained on modern taxa seems to demonstrate that morphological studies are also inappropriate for microbes. Indeed the monophyly of modern microbes could not be defined by their morphology most of the time, but resolved by molecular analyses (Simpson and Roger, 2004; Roger and Hug, 2006).

It is difficult (even impossible) to verify the monophyly of fossils species with their modern relatives. To get a better definition of a fossil microbe species, the increase of common works between molecularists and morphologists is an absolute necessity.

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