



Protist-like inclusions in amber, as evidenced by Charentes amber

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Abstract

The mid-Cretaceous amber of France contains thousands of protist-like inclusions similar in shape to some ciliates, flagellates and amoebae. The sheer abundance of these inclusions and their size variation within a single amber piece are not concordant with true fossil protists. French amber is coniferous in origin, which generally does not preserve well protists without cell walls. Thus, it would be surprising if French Cretaceous amber had preserved millions of protists. Here, we present a survey of the protist-like inclusions from French amber and attempt to elucidate their origins.

Diverse Cretaceous ambers (from Spain, Germany and Lebanon), also derived from conifer resins, contain thousands of protist-like inclusions. In contrast, Tertiary ambers and modern resins are poor in protist-like fossils. This suggests these inclusions originated from early Cretaceous plant resins, probably secreted with the resin by trees that did not survive after the Cretaceous (such as the Cheirolepidiaceae). A review of the recent literature on amber microfossils indicates several protist-like inclusions that are unlikely to have a biological origin have already been described as real fossil protists. This is problematic in that it will bias our understanding of protist evolution.

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Introduction

Amber is a fossilised tree resin known for preserving ancient organisms with exceptional fidelity (Poinar 1992, 1998). During the last two centuries, a plethora of organisms from diverse ambers have been described, including insects and arachnids (e.g., Perrichot 2005), plant and vertebrate remains (e.g., Grolle and Meister 2004; Vullo et al. 2010) and micro-organisms (Göppert and Berendt 1845; Berkeley 1848; Schönborn et al. 1999; Breton and Tostain 2005; Schmidt et al. 2006). During the 19th century and the first two-thirds

of the 20th century, amber studies were primarily focused on Eocene Baltic amber. However, beginning around 30 years ago, fossiliferous amber localities of different ages were discovered around the world. For example, microbes were recently described from Triassic amber of Italy (Schmidt et al. 2006), and diverse Cretaceous ambers recently provided data on microbes and arthropods at a time when the angiosperms were diversifying (e.g., fossils from the Lower Cretaceous amber of Spain, Delclòs et al. 2007; Peñalver et al. 2007 and from the Cenomanian amber of Germany, Poinar et al. 1993; Schönborn et al. 1999; Schmidt et al. 2001).

In the past few years, an abundance of well-preserved organisms (arthropods and micro-organisms) have been found in the mid-Cretaceous amber of France (Perrichot 2005; Schmidt et al. 2008; Girard 2009; Girard et al. 2009a,c). The arthropod fauna is dominated by forest litter organ-

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isms (Perrichot 2004; Perrichot and Girard 2009), while marine microfossils indicate the Cretaceous amber forest of SW France grew close to the sea (Girard et al. 2008, 2009c,d). Charentes amber is not a homogenous medium; on the contrary, it contains many inclusions that resemble diverse protists. Some fragments contain thousands of these protist-like inclusions. Initial observations suggested resemblance with certain modern taxa, while other characters raised doubts as to their origin. For example, is the variation in their shape and size concordant with a microbial origin? Does the concentrated abundance of such micro-inclusions support the fact they are fossilised protists? Is it likely that membranous networks and vacuoles would be preserved but not nuclei or organelles?

Here, we survey the diversity of protist-like inclusions preserved in mid-Cretaceous amber from Charentes (SW France). These fossils are compared to protist-like inclusions found in other ambers (Cretaceous one from Lebanon and Spain, Eocene Baltic amber) and modern resins (from *Araucaria* sp., *Pinus* sp. and *Shorea* sp.). The five different types of amber present in mid-Cretaceous French localities are considered.

Material and Methods

Samples

The different amber samples are from seven different amber localities of Charentes (SW France). According to the localities and the stratigraphical levels, ambers from this area have been dated as Late-Albian/Early Cenomanian by palynology (dinocysts), micropaleontology (foraminifers) and sequence stratigraphy correlations (Néraudeau et al. 2002, 2003, 2005, 2008, 2009; Perrichot et al. 2007).

Based on colour, the mid-Cretaceous outcrops of Charentes yield four different types of amber (Perrichot 2005; Girard 2010). The rarest type corresponds to translucent and yellow amber pieces. This amber is very clear and rarely contains fossils. The largest amber pieces (from a few centimetres to more than 30 centimetres in diameter) are opaque with a milky to dark brown colour and are the most abundant amber type in the localities of southwestern France. Most fossiliferous ambers are honey and red in colour. The former is recognizable because of its translucence and light-brown to honey colour. It is rich in arthropods (Perrichot 2005) and also contains numerous microfossils such as actinomycetes, fungi and various protists (Girard 2010). Red amber is characterised by a peripheral layer of cyanobacteria with a more or less translucent core of red colour (Girard et al. 2009a). This type mostly contains prokaryotic filaments but also green algae (Girard 2009) and diverse protists (Girard 2010).

A fifth amber type has been described based on its specific organisation and abundance of fossils (Perrichot 2004). It has been defined as “flattened and foliated lens-shaped structures, from 20 to 30 μm^3 , that show a gradient of transparency

ranging from a highly opaque brown side, whose surface is pockmarked by numerous pressure marks of sandstone grains, to a much more translucent yellow side, whose surface is smoother” (Perrichot 2004, p. 11). The main particularity of this amber type is the presence of dozens of arthropods and hundreds of micro-organisms in a single piece (Perrichot 2004). Most of these inclusions are typical of forest litter (Perrichot 2004; Girard et al. 2009d; Perrichot and Girard 2009).

Modern resin samples were collected on different trees in Ille-et-Vilaine (Western France, resin of *Araucaria* sp.), Vendée (Western France, resin of *Pinus* sp.), Spain (Andalusia, resin of *Araucaria* sp.), Halifax (NS, Canada, resin of *Pinus* sp.), and Thailand (Southern part of Thailand, resin of *Shoera* sp.). Amber samples from the Paleogene of Europe and the Cretaceous of Spain and Lebanon were provided by specialists (see Acknowledgements) or come from the collection of the Senckenberg Museum (Frankfurt-am-Main, Germany) that represent more than 7000 amber pieces (90% corresponding to Baltic amber).

Sample preparation

Amber is often found in environments in contact with living micro-organisms. To avoid contamination, samples were cleaned using the protocol outlined by Girard et al. (2009b). This consists of an ultrasound cleaning, H_2O_2 oxidation, followed by a HF treatment to eliminate all organic and non-organic matter found on the surface of the samples. Resin samples were not cleaned as they are easily dissolved by an oxidant such as H_2O_2 .

Fine fragments were obtained from the different amber samples and modern resin using a minute scalpel. They were mounted on slides in Canada balsam. These preparations were observed under a microscope with $\times 100$ oil immersion objective (in Rennes) or with ICT (in Halifax).

Terminology

For clarity, we provide definitions of terms used in this manuscript. “Inclusion” or “micro-inclusion” refer to any inclusions from amber, regardless of their biotic status. The terms “protist-like inclusion” and “pseudo-protist” refer to specific amber inclusions that have a shape similar to protists but several characteristics indicate that are not true fossil protists.

Results

The protist-like inclusions in French amber fall into three main groups (Fig. 1). The first type (Type A) corresponds to ovoid and spherical inclusions (Fig. 1A–D). In a single piece of amber, their size can range from a few to more than 100 μm in diameter. Some Type A are brown to dark brown, most of

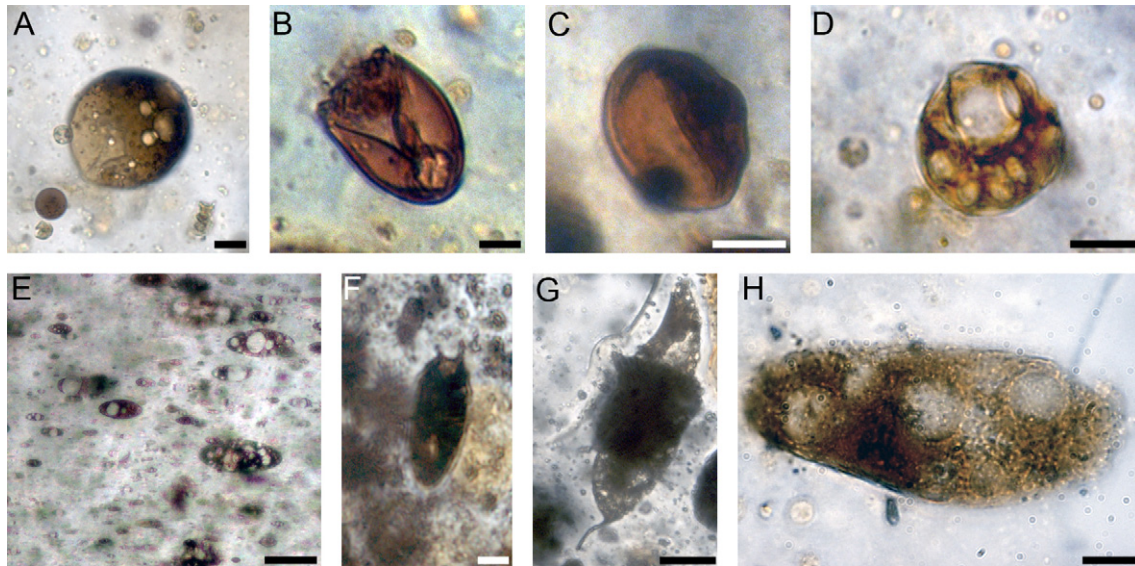


Fig. 1. The different kinds of protist-like inclusions found in mid-Cretaceous amber of Charentes. (A–C) Spherical protist-like inclusions morphologically close to amoebae of the clade Arcellinida (e.g., genus *Arcella* (A) genus *Leptoquadrula* (B) and genus *Plagiopyxis* (C)). (D) Spherical protist-like inclusions with numerous vacuoles. (E) Fusiform protist-like inclusions with numerous vacuoles. (F) Ovoid protist-like inclusions with two “horns”. (G) Euglenoid protist-like inclusions. (H) Details of a fusiform protist-like inclusions with numerous vacuoles. Scale bars: 10 μm (B, F), 20 μm (A, C, D, and H) and 30 μm (E and G).

them having a reddish colour. No preferential orientation of these inclusions has been observed. Morphologically, they look like testate amoebae, more precisely resembling extant Arcellinida (genera *Arcella*, *Microchlamys*, *Centropyxis*, etc., Fig. 1A–C). Other Type A inclusions are characterised by the presence of numerous vacuoles (Fig. 1D) and range in colour from orange to clear brown to clear red. Their size and the number of vacuoles are extremely variable. Morphologically they resemble some euglenids (such as the genus *Phacus*) or ciliates (such as the genus *Proterodon*).

The second type (B) of protist-like inclusions is ovoid to fusiform in shape (Fig. 1E–H). Their size ranges from a few micrometres to more than 1 mm in length. They possess many vacuoles (Fig. 1E and H). These protist-like inclusions are always oriented in the direction of resin flow (Fig. 1E). Certain inclusions exhibit a more globular shape but they are always slightly elongated and oriented in the direction of resin flow. Other Type B protist-like inclusions possess fine extensions at their extremities. Type B are similar in shape to some protozoans (such as the genus *Paramecium*), and those with a regular ovoid shape are very similar to some Euglenozoa (such as the genus *Phytomonas*). The more elongated specimens often exhibit a “gut” that provides them with a “euglenoid” shape.

A third type of protist-like inclusion (Type C) has been observed. This type, with no precise morphology, is rarer than the others. Type C inclusions are orange to brown in colour (sometimes they appear very dark when they are too big or too concentrated in a piece of amber) and resemble

decayed organic matter. They have never been observed to have a particular orientation.

The abundance of these three kinds of protist-like inclusions is variable and depends on the amber fragment (Table 1). Type A is primarily found in the core of red amber pieces. These protist-like inclusions are densely clustered in very dark red amber, while they are more dispersed in translucent pieces. Type A is less common in “honey” and “litter” amber and is very rare in “milky” amber. Type B is very abundant in the “milky” and dark brown amber fragments. The opacity of these amber types greatly depends of the abundance of Type B protist-like inclusions. Type B is also common in “honey” amber but relatively rare in red and “litter” ambers. Conversely, Type C is very common in “litter” amber. These inclusions are associated with diverse microfossils such as marine diatoms (Girard et al. 2008, 2009c) and actinomycetes (Girard et al. 2009d). Type C protist-like inclusions are rare in all other ambers types. We note that yellow amber is mostly devoid of inclusions but rarely contains Type A and Type C inclusions. The main particularity of yellow amber is the presence of numerous minute bubbles (less than 1 μm), which sometimes modifies the physical aspect of the amber (presence of white areas). The bubbles resemble those clouds that develop around some arthropods in amber (mostly Baltic amber), but in the yellow one, they formed without any arthropods as support.

Fig. 2 recapitulates the different protist-like inclusions found in the mid-Cretaceous amber of Charentes. Table 1 details the abundance of these protist-like inclusions in the various types of Charentes amber.

Table 1. Distribution of the three types of protist-like inclusions in the different kinds of Charentes amber.

	Yellow amber	Honey amber	Milky amber	Red amber	Litter amber
Type A	+	+++	+	+++	+
Type B	–	+	+++	+	–
Type C	+	++	+	++	+++

–: Absent; +: rare; ++: common; +++: abundant.

Discussion

Preservation of protist cells in amber

Protist-like inclusions, especially Types A and B, are very abundant in mid-Cretaceous amber from Charentes. Some pieces of amber contain more than 100 specimens per 1 cm³. The presence of these protist-like inclusions can greatly modify the aspect of the amber. Indeed, the opacity of the amber pieces appears to be directly related to the abundance of protist-like inclusions.

Other ambers (i.e., Baltic and Dominican ones) yielded no more than a few microbes in a single piece. If all the protist-like inclusions described here are real fossil protists, why would the Charentes amber have preserved such a concentration of fossil micro-organisms? That more than 75 percent of Charentes amber preserved millions or billions of protists seems unrealistic (“milky” amber represents approximately 75 percent of the amber found in the studied localities as estimated by V. Girard in 2007) is particularly troublesome because, depending on the origin of the resins, some protists (especially flagellates and ciliates) have been shown not to be well preserved (e.g., such as in resins of *Araucaria*

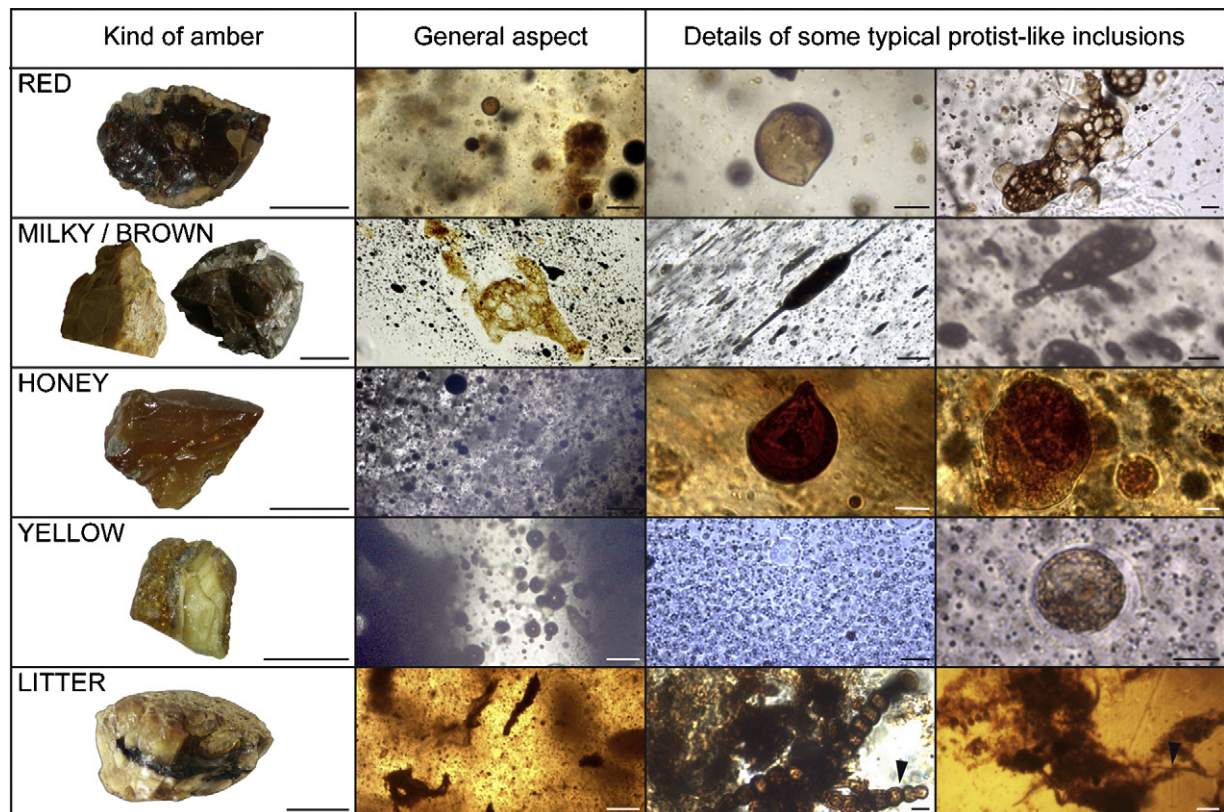


Fig. 2. The different kinds of Charentes amber and their typical protist-like inclusions. In the first column (left), an example of each kind of Charentes amber is provided. In the second column, the general microscopic aspect of these kinds of amber is illustrated. In the two last columns, details of typical protist-like inclusions of each kind of Charentes amber are given. Scale bars: 1 cm (first column), 100 μm (second column) and 10 μm (two last columns, excepted for the photo of the lower right side – 50 μm). To note that, on the two detailed photos of litter amber, you can also see a specimen of the ascomycete *Metacapnodium* (left photo, arrow) and a specimen of the diatom *Rhizosolenia* (right photo, arrow). For more details about the *Metacapnodium* specimen, see Girard et al. (2009d) and Girard (2010). To note that the *Rhizosolenia*, because of the transparency of its frustule and the fact that it is preserved within Type C protist-like inclusion, is difficult to see. For more detail, see Girard et al. (2009c).

and *Picea*; Foissner et al. 1999). The origin of the different types of Charentes amber cannot be differentiated based on their chemistry (Perrichot 2005). The fossil wood associated with Charentes amber indicates the resin was secreted by an Araucariaceae and/or a Cheirolepidiaceae (Perrichot 2005). Though nothing is known about the potential of cell preservation in Cheirolepidiaceae resins (an extinct lineage), actuotaphonomic experiments demonstrate the preservation of protists in conifer resins is imperfect and does not allow the rapid fixation or preservation of thousands of cells (Foissner et al. 1999).

Protist-like inclusions in amber and modern resin

Preparation of other ambers (Lower Cretaceous of Lebanon and Spain and Eocene amber from the Baltic sea and France, Oise region) and different modern resins (*Araucaria* from France and Spain, *Pinus* from France and *Shorea* from Thailand) illustrates that such protist-like inclusions are not specific to mid-Cretaceous French amber. Baltic and Oise ambers, as well as *Araucaria*, *Pinus* and *Shorea* resins, contain only rare protist-like inclusions (mainly similar to Type A), especially in their darker areas. Only a few protist-like inclusions are found in translucent Lebanese amber (similar to Type A), while dark Lebanese amber is dominated by Types B and C inclusions. The Lower Cretaceous amber of Spain is most similar to the mid-Cretaceous French amber. The red amber of Spain is rich in Type A protist-like inclusions and Spanish brown amber is very similar to the “milky” amber of France. Both of these Spanish ambers contain numerous Type B protist-like inclusions, always oriented in the direction of the resin flow. Brasier et al. (2009) published on Cretaceous amber from Sussex (England). Their illustrations tend to prove that Sussex amber is rich in protist-like inclusions of Type B (see Figs 6 and 7 of Brasier et al. (2009), p. 994).

Pseudo-protists from amber

Preservation of fragile organisms (such as ciliates and euglenids) is only possible in special conditions. Although amber is known to be one of the best media for organism preservation, the small differences among resins may easily cause great differences in preservation (Foissner et al. 1999). Some of the arthropods and nematodes from Charentes amber are not perfectly preserved despite the chitin cuticle (Perrichot 2005). Some specimens seem to have died before being embedded, as demonstrated by the presence of bacteria, fungi and emerging hyphae on their surface. Many other specimens are simply poorly preserved. For example, the nematodes found to date do not retain sufficient morphology for further identification (Girard 2010). The previously discussed lines of evidence suggest the resin from which the Charentes amber originated had a slow biological fixation rate and was probably not the ideal medium to preserve delicate

organisms (such as ciliates and other protists without tests or cell walls). We do not argue that there are no real protist microfossils in Charentes amber. On the contrary, convincing specimens have already been published (see Girard 2009, 2010; Girard et al. 2009d; Schmidt et al. 2010); however, we caution that it would be folly to confuse thousands of protist-like inclusions (sometimes within a single piece) for fossil specimens.

All of the protist-like inclusions possess a highly vacuolated interior, without distinguishing morphological features such as nuclei or organelles. The preservation of “vacuoles” is odd when more robust structures such as nuclei, endosymbionts or plastids, or any other organelle are not retained. Electron microscopy revealed that the preservation of membranes without artefacts requires rapid, efficient fixation in an adequately buffered medium. It is doubtful this fixation was provided by the resin, given the poor preservation of some of the micro-invertebrates. Indeed, other fossil protist inclusions described (Schönborn et al. 1999) indicate a poor fixation without ultrastructural details (such as vacuoles), but the preservation of more robust structures such as nuclei has already been highlighted (Wier et al. 2002).

The origins of these protist-like inclusions (which we will now refer to as *pseudo-protists*) must lie elsewhere. Given the above discussion and our hypothesis that they are not true micro-organisms they probably do not have a microbial origin. One explanation is that the resiniferous trees may have secreted them within the resin flows. This hypothesis accounts for why pseudo-protists are so abundant in Charentes amber and vary in size and shape. The most viscous resins may have preserved spherical protist-like inclusions (such as Type A), while the types from the more fluid resin (i.e., Type B) may have been deformed by the resin flows. Pseudo-protists were observed in modern coniferous resins, although they are not as abundant as in Charentes amber. The discrepancy in abundance among resins or ambers may be due to the botanical origin of the Charentes amber, the latter being not well defined (araucarian or cheirolepidiacean resin?). Thus ambiguity appeared as soon as the plant produces the resin. Secondly, the different processes involved in the formation of amber are not well known. Amber derives from resin by polymerisation, but the exact processes are not well parameterised. Some of these steps may have produced the pseudo-protists, but this hypothesis does not seem the most tenable. Indeed, diagenesis could have produced vacuoles when the resin progressively evolved, but the vacuoles would have a specific (probably centripetal or centrifugal) orientation in the amber piece, and the majority of pseudo-protists do not exhibit such an orientation. Most of the inclusions are orientated in the direction of resin flow, suggesting they appeared when the resin was still liquid. Moreover, the pseudo-protists possess inner structure (e.g., they can be more or less opaque or more or less vacuolar), which does not support the second hypothesis; diagenesis would primarily produce air bubbles without content or internal structure.

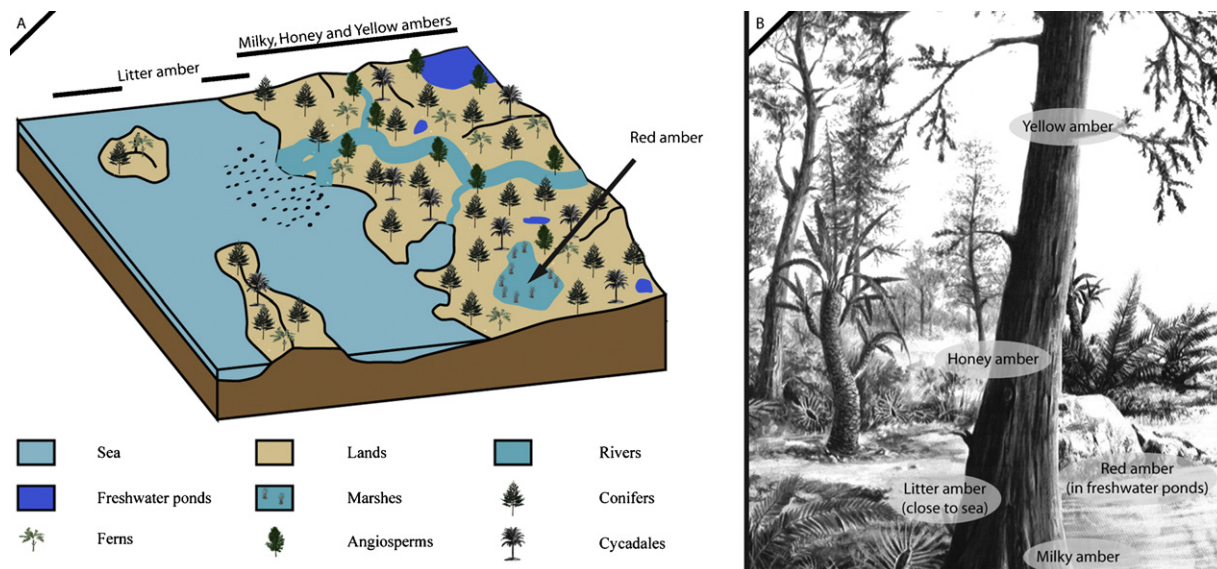


Fig. 3. Paleoenvironmental context in which the different kinds of Charentes amber and their pseudo-protists were produced. (A) Paleoenvironmental scheme showing where the different ambers of Charentes were probably produced. (B) Drawing showing the different hypothesis concerning the secretion of Charentes amber.

Abundant additional evidence also supports the first hypothesis (i.e., the resin-producing trees secreted the protist-like inclusions). In the Charentes outcrops, all the amber types are found in the same stratigraphic layer and would not have undergone a different diagenetic history. Furthermore, the study of fossil assemblages preserved in Charentes amber suggests the presence of pseudo-protists greatly depends on the environment in which the resin was produced. Yellow amber corresponds to centimetric clear and “clean” resin drops, rich in micro-bubbles and containing only a few fungal mycelia and filaments of bacteria (e.g., sheathed bacteria of the genus *Sphaerotilus* and unidentified actinomycetes; Girard 2010). The preceding lines of evidence suggest yellow amber derived from a fluid resin produced by branch or tree trunks in a relatively humid environment. “Litter” amber is the most fossiliferous. A 5 cm³ piece can contain dozens of arthropods and hundreds of microfossils including bacteria, fungi, amoebae, ciliates, etc. (Perrichot and Girard 2009). These amber pieces are unique because they also contain numerous marine microfossils such as diatoms and sponge spicules (Girard et al. 2008, 2009c). The “litter” amber is the richest in Type C protist-like inclusions, which may be due to the specific environment in which “litter” amber was produced (i.e., a forest litter in an estuarine riverbank environment). Type A protist-like inclusions are mainly found in red amber. They are associated with various freshwater microorganisms such as the cyanobacterium *Palaeocolteronema cenomanensis* (Girard et al. 2009a), diverse amoebae of the genus *Arcella* and badly preserved ciliates (Girard 2010). This association and the desiccation cracks on the amber surfaces suggest Type A protist-like inclusions are typical of resins produced in water and then dried by the sun (Girard et al. 2009a; Girard 2010). Type B inclusions are mainly

found in opaque milky to dark brown rather large amber pieces (5–30 cm) devoid of arthropod inclusions. This amber is relatively devoid of microfossils (e.g., only unbranched filaments of actinomycetes, rod-shaped bacteria and a few fungal hyphae). The large size of the resin drops (biggest amber pieces found in the outcrops, until 20–30 cm in diameter), convoluted resin flow, wood imprints in the amber pebble surface and microfossil content indicate the milky amber was produced by roots, out of soil influences (Girard 2010).

The take-away message is that the abundance and diversity of the pseudo-protists described herein point to an environmental origin. Type A protist-like inclusions may have been produced in immersed resin, Type B in resin flows secreted by roots and Type C in resin flows that fell onto forest litter. Fig. 3 recapitulates the hypotheses about the origins of the different Charentes amber types.

Proposal to discriminate pseudo-protists from real microfossils?

No unique solution exists to discriminate pseudo-protists from real microfossils in amber. When studying amber, one must be cognizant that there are probably more pseudo-protists in amber than real microfossils, at least in Cretaceous ambers. Identifying amber microbes is extremely difficult; many diagnostic features of the protists are not distinguishable when embedded in resin. Furthermore, characters are progressively lost during the fossilisation process, and the quality of preservation depends on the nature of the resin type (Foissner et al. 1999). At the moment, only those microfossils with clear diagnostic characters can be confidently identified; in the contrary case, any identification is tentative.

With this in mind, a review of the microfossil (and more precisely protists) literature reveals probable pseudo-protists that have already been published as real protists. We support the methodology of Schmidt et al. (2010), whereby the authors pointed out several doubtful amoebae published from amber, in order to progressively clean the protist fossil record (see for example Schmidt et al. 2010). We encourage this critical attitude, which may clarify the real status of diverse amber micro-inclusions, either as microfossils or pseudo-protists. Especially, our results tend to show that Cretaceous ambers are richer in pseudo-protists than more recent ambers. Thus, by prudence, most protists without cell walls or tests from Cretaceous amber should be regarded as suspect until certain safeguards have been met. We therefore recommend that for protists without cell walls or tests, authors refrain from publishing inclusions as named fossil species. For inclusions that appear to have a form similar to wall-less protists, only those with convincing additional features should be considered (for instance oral structures, flagella, extrusomes, nuclei, or other identifiable internal or external organelles). In particular, we believe it is erroneous to describe and name as fossil specimens those that appear as vacuolated bubbles without internal organelles, such as nuclei.

Conclusions

Amber is renowned for preserving numerous soft-bodied organisms (such as arthropods, fungi and bacteria). During the last couple of years, several publications detailed the discovery of protists in amber (see Girard 2010 for more detail) and doubts arose as to the true biological nature of these fossils (i.e., are they pseudo-protists or real microfossils?) (Schmidt et al. 2010). The present study highlights that many of the fossil protists described in previous works could not be fossils but pseudo-protists. The distinction is important because false identifications bias our vision of the fossil record of several groups of terrestrial protists. If pseudo-protists have been wrongfully identified as real microfossils, the various hypotheses on microbe evolutionary trends (Martín-González et al. 2008) cannot be verified by the amber record of wall-less protists as currently accepted.

At the moment it is uncertain whether many of the described protist specimen without tests or walls are of biological origin or if they are non-biological inclusions that resemble protists. In Charentes amber, rounded pseudo-protists are abundant in immersed resins, while elongated pseudo-protists are mainly present in resin secreted by roots. The abundance and diversity of pseudo-protists in a single amber piece seems to be correlated with the environmental conditions in which the resin was produced.

Further studies are needed to determine the origin of the pseudo-protists, as various questions remain unanswered. Why are pseudo-protists particularly abundant in Mesozoic ambers? Were these pseudo-protists trapped after resin secretion, were they secreted with the resin, or were they produced

during amber formation? Precise studies of these protist-like inclusions should help to better elucidate fossil plant physiology and/or paleoforests.

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