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Sensitivity of protists to preservation in plant resins for fossil formation into amber

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Summary

Several studies during the last 150 years dealt with the fossilisation of microorganisms into amber of different geological ages. Even if fossil plant resins are known to be an effective medium for preservation of some organisms, the presence of fossils of the more fragile ciliates, flagellates and amoebae in amber is still enigmatic. Previous studies showed that the botanical origin of the amber is (probably) an important parameter that governs the preservation of microorganisms into plant resins. But there are few taphonomical studies of the microbe preservation. Simple experiments in this study showed that another important parameter is the ambient temperature and the details of the first contact between the resin and the specimen, whilst humidity and light seem to not have an important role in microbe preservation. The development of such experiments in amber fossil formation should help to provide a better understanding of the environmental conditions that predominated in past resiniferous forests.

Key words: actuotaphonomy, microfossils, paleoenvironment, *Pinus* resins, protist preservation

Introduction

Since the middle of the nineteenth century, it has been shown that microfossils can be found in amber. However, between 1845 and the beginning of the 1990s, only few studies have been published on this topic (see Girard, 2010 and references therein). Over the past decade, diverse works have been published on amber microorganisms, most of them describing bacteria, fungi and protists preserved in amber (Antoine et al., 2006; Girard, 2009; Girard et al., 2009b; Poinar and Buckley, 2006; Rikkinen and Poinar, 2000, 2002; Waggoner, 1994a, 1994b, 1996). These descriptions are based on the surviving morphology and are affected by the quality of the preservation. This is of some importance because identification of fossilised protists relies on the quality of the fixation at the time of contact with the resin. Poorly preserved specimen may be deformed thus misleading their identification. As identification of fossil protists is done based on morphology, it is worth being cautious, that some

may be poorly preserved and thus not retaining their original shape. The morphology based species concept in fossil protists was considered critically in a previous discussion (Girard and Adl, 2011). We have also further noted that certain specimens of fossil protists in the literature are probably artefacts that we referred to as pseudo-protists (Girard et al., 2011). Nevertheless only few publications have dealt with trying to understand how the microorganisms become trapped and preserved in the plant resins.

Foissner and co-authors (1999) made some experiments with protists and resins. They artificially embedded three kinds of ciliates (*Paramecium aurelia* Ehrenberg, 1838, *Tetrahymena mobilis* Kahl, 1926 and *Mykophagophrys terricola* Foissner, 1995) into different plant resins (*Cycas, Araucaria* and *Picea* resins). Their experiments demonstrated that resins from different plant origin do not have the same potential of microbe preservation, or biological fixation. They concluded that *Cycas* resin has better potential than *Araucaria* or *Abies* resins. Schmidt and co-authors (2004) studied the fate of different testate amoebae embedded into diverse plant resins under stable environmental conditions, and confirmed the results obtained by Foissner and co-authors (1999) showing that the preservation of shelled microorganisms (such as testate amoebae) are easier than for naked microorganisms. Schmidt and Schäfer (2005) also carried out actuotaphonomic experiments. They tested the potential for growth of a sheathed bacterium in *Cycas* and *Pinus* resins and demonstrated it could grow in fresh resins for few days. More recently, Schmidt and Dilcher (2007) tested the preservation of aquatic organisms into *Taxodium* resin. By injuring trees, they caused resin flows into freshwater swamps. Observing the fresh resin at the microscope, they remarked on the presence of various freshwater organisms (such as green algae, diatoms, ciliates and flagellates) in the resin despite its hydrophobic properties.

Here, to better understand the processes that govern the preservation of protists in amber, we tested the potential of cell preservation in *Pinus strobus* resin under different conditions. The trapping of diverse microbes were observed under different conditions of temperature, humidity and light intensity. These experiments help to constrain the environmental parameters that lead to entrapment and preservation of protists at that first contact with resin.

Material and methods

The *Pinus strobus* resin was collected from several naturally injured trees in Halifax (Nova Scotia, Canada).The microorganisms were obtained from soil samples collected under *P. strobus* trees in the city of Halifax. Small soil samples were placed into wheat grass medium to cultivate the protists, and samples and cultures were handled according to standard procedures as described in Adl and co-authors (2007). Cultures contained a representative diversity of ciliates, flagellates, and amoebae. No attempt was made to further identify these.

Protists were concentrated by centrifugation at 500 g prior to the embedding experiments with resin. The concentrated cultures were verified at the microscope to ensure a variety of amoebae, ciliates, and flagellates. For the first set of experiments (1st method), a drop of fresh resin was suddenly placed on a drop of culture containing concentrated cells at several hundred ml^{-1}. For the other experiments (2nd method), we followed a similar protocol to that described by Foissner and co-authors (1999). A small drop of resin was placed on a slide. Then a drop of the culture medium containing concentrated cells at several hundred ml^{-1} was placed on the resin. To better locate cells in the resin, the protists contained in the drop of culture medium were coloured with diluted vital stains congo red (0.01%) and methylene blue (0.01%). The preparations were then left to dry at different temperatures: 4 °C, 14 °C, 20 °C and 37 °C. For each test, two different degrees of humidity were tested. One preparation was placed in a box at room humidity (55%) and the other was placed in a box with a higher humidity (>90%). The latter condition was achieved by placing a wet tissue paper in the box. The range of humidity and temperature chosen represent the probable range in the resiniferous forests that produced amber (Adl et al., 2011). Depending on the temperature, the humidity and the drop size, between 1 and 36 hours were required to completely evaporate the culture medium drops. Two other tests were then carried out. Drops of culture medium were placed in *Pinus strobus* resin, in one case with room humidity and in the other case with the higher humidity. These two preparations were kept at 20 °C, close to a window to test the impact of day light on microbe preservation.

For observations, the preparations were embedded with Permount (an artificial resin) and covered with a cover-slip. These preparations were then observed under a Nikon Eclipse TS100 inverted microscope with phase contrast and long distance objectives. Specimen were observed for internal structures (nucleus, vacuoles, granules, other organelles), and external features (shape, flagella, test).

Results

During the first 15 trials with the 1st method it was not possible to trap microbes into the fresh resins. The resin drop falling on the culture would completely cover the drop of culture, but the brutal contact between the resin and the culture medium completely destroyed the microbial cells.

The 2nd method allowed the preservation of microorganisms. Each preparation contained fungal hyphae and spores. They also preserved many rod-shaped and filamentous bacteria. However, despite drops of culture medium being enriched in protists by centrifugation, only few of them (less than 5%) were (mostly badly) preserved. We describe below the results of each treatment (n=5) using the 2nd method (see: Table 1 and Fig. 1).

At 4 °C, very few specimens were observed. Ciliates and flagellates lost all their internal and external characteristics. Only their general shape was still visible. Tests of amoebae were also observed, but their internal structures were not preserved.

At 14 °C, tests of amoebae were preserved. Ciliates and flagellates were more commonly preserved than at 4 °C. Moreover, their preservation was better as some internal structures (such as the nucleus) were visible. However, external structures were not always visible. In the elevated humidity experiment, ciliates were not preserved whilst few specimens of flagellates remained visible in the resin. The latter always showed some internal features (such as the nucleus), but the flagella were destroyed.

At 20 °C, some ciliates and flagellates were preserved. They were more common than at 14 °C and also better preserved. A part of the internal morphological structures have been more or less well preserved. The nucleus was clearly visible in many specimens, and most of the ciliates exhibited internal vacuoles. Some external features were preserved though poorly on some of them. On the contrary, flagellates never retained flagella. Diverse amoebae tests were observed and few of them retained internal structures such as nuclei. However, no pseudopodia were observed in any specimen. All these protists were also preserved at 20 °C when the humidity was elevated and when the preparations were placed in a better illuminated environment.

Finally, at 37 °C only few protists were observed at varying humidity. Most of them were poorly preserved, only the general shape being visible. None of the internal and external structures were retained. No naked amoebae were observed, at any temperature, in any preparation, even though they were present in the initial soil cultures.

Table 1. Experimental setup of different treatments. The symbols '—' and '+' indicate low and high experimental conditions, respectively. For humidity, symbol '—' corresponds to a humidity of ca. 55% and the symbol '+' of ca. 90%. For light, symbol '—' corresponds to darkness and symbol '+' to 'normal' light (i.e. sun light).

Experiment number	Temperature	Humidity	Light
1	4 °C	—	—
2	4 °C	+	—
3	14 °C	—	—
4	14 °C	+	—
5	20 °C	—	—
6	20 °C	+	—
7	20 °C	—	+
8	20 °C	+	+
9	37 °C	—	—
10	37 °C	+	—

Discussion

The natural preservation of very fragile organisms (such as ciliates, amoebae or flagellates) in resins requires perfect conditions. Over the past 150 years, several studies have dealt with amber microfossils (e.g. Berkeley, 1848; Waggoner, 1994a, 1994b, 1996; Rikkinen and Poinar, 2000, 2002; Poinar, 2003, 2005; Schmidt et al., 2004, 2006; Antoine et al., 2006; Schmidt and Dörfelt, 2007; Girard, 2009), but few real taphonomic studies exist, such as those by Foissner and co-authors (1999), Schmidt and co-authors (2004) and Schmidt and Dilcher (2007).

Foissner and co-authors (1999) already demonstrated that one of the main factors controlling the microbe preservation in plant resin is the botanical origin of the resin. Embedding three different species of ciliates in *Picea, Cycas* and *Auracaria* resins, they demonstrated that *Cycas* resin preserves microbes better than the two others. This particularity probably derives from the fact that *Cycas* resin is not a real resin but mucilage (Langenheim, 2003). The absence of terpenoid substances should play an important role in the preservation of microorganisms. For our experiments, we used *Pinus strobus* resin as it corresponds, according to our opinion, to one of the most common resins. Indeed Pinaceae is one of the two families (with Araucariaceae) that produces copious amount of resin (Langenheim, 2003).

Influence of temperature

Our experiments tend to show that temperature is an important parameter that controls the preservation of protists in plant resins. Depending on

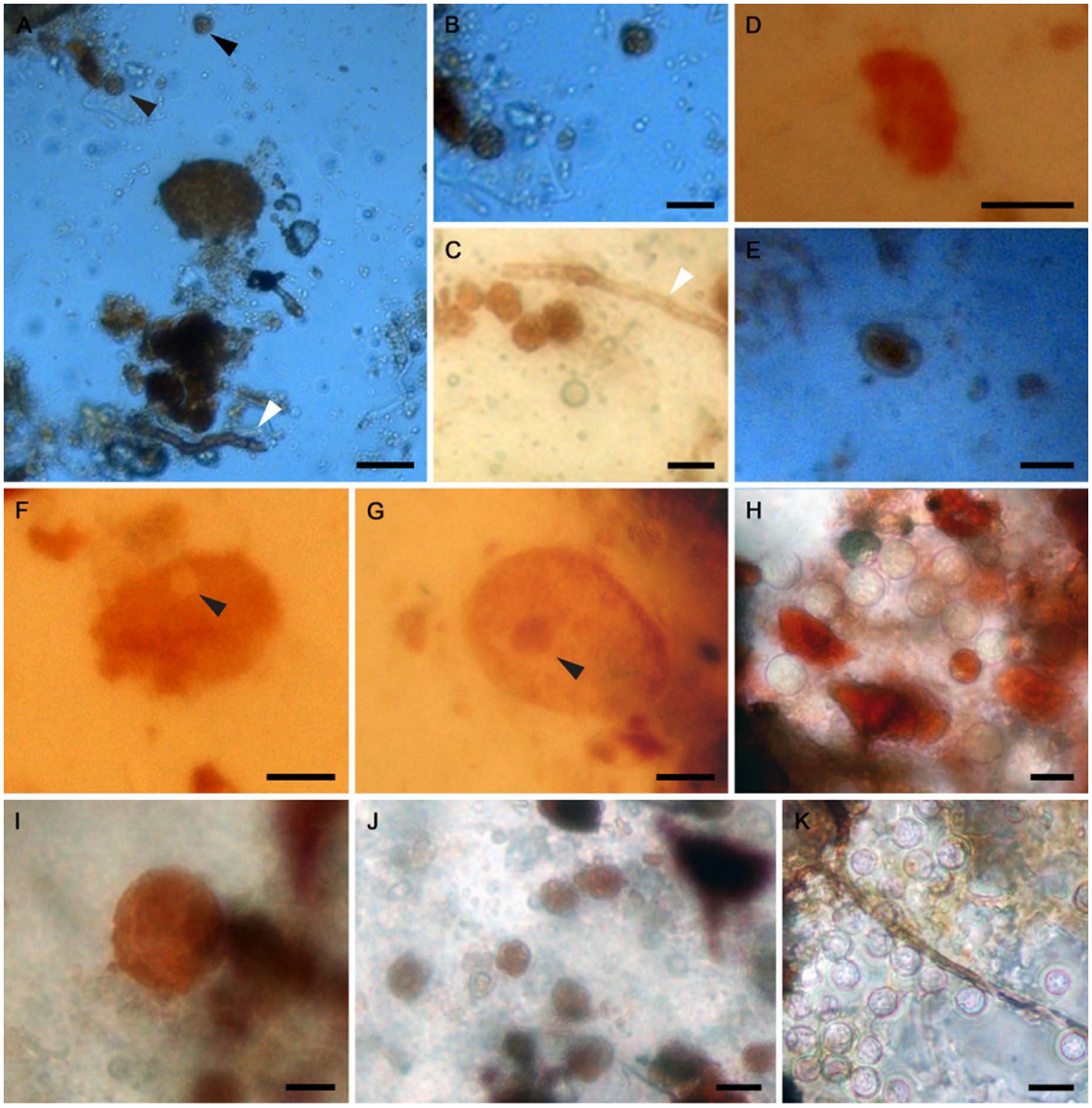

Fig. 1. State of preservation of protists embedded in *Pinus* resin with different environmental conditions. A – Several microorganisms embedded at 4 °C (low humidity). One can recognise a ciliate (in the centre, its shape changed and became more hexagonal, no internal or external features were preserved), two flagellates (black arrows) and a fragment of a fungal hypha on which a septum is visible (white arrow). B – Detail of the two flagellates shown in A. During their embedding, they became rounded. C – Fungal hyphae (white arrow) and several specimens of not-well preserved flagellates at 20 °C (low humidity). D – Not-well preserved ciliates at 20 °C (low humidity and high light intensity). Even if the specimen is not well-preserved, the shape of a cf. *Colpoda* is still recognisable, whilst at 4 °C it was not possible to identify the ciliates. E – Amoeba enclosed in a cyst at 20 °C (high humidity). F, G – Ciliate in which some internal structures (black arrows) have been preserved (20 °C and high humidity). H – Fungal spores embedded at 20 °C (high humidity). I – Ciliate embedded at 37 °C (low humidity); note that its shape is now rounded. J – Several specimens of flagellates embedded at 37 °C (low humidity). K – Fungal spores embedded at 37 °C (high humidity). Even these robust structures are badly preserved at 37 °C whilst they were very well preserved at 14 °C and 20 °C. Scale bar: 10 μm with the exception of A and E (20 μm).

the temperature, the quantity of preserved protists is not the same. Our experiments clearly showed that for high (37 °C) and low temperatures (4 °C) there were fewer preserved protists than for moderate temperatures. Concerning the lower temperature, it is possible that some substances of the resin (such as

terpenes) were progressively mixed with the water of the culture medium (water being slow to evaporate). These substances could have progressively killed the protists and dissolved their cells prior to preservation. For the higher temperature, the evaporation can be too fast, and the protists would not have had time to be entirely embedded in the resin. Cells were still exposed to the air when the drop of culture medium totally dried and a partial destruction of the cells probably occurred. This could explain the poor preservation of the protists at 37 °C.

At 14 °C, the preservation of protists was more efficient. Some robust internal structures (such as the nucleus) were preserved. The evaporation of the culture medium drop was complete after 8-10 h of experiments. The best preservation was observed at 20 °C. Both internal and external structures could be preserved. The nucleus was preserved in most of the specimens. The cells often contained vacuoles. 20 °C was the unique temperature we tested that allowed the preservation of external structures. Both evaporation and resin fluidity were optimal at this temperature and allowed a better preservation than at other temperatures. During these experiments, microorganisms stayed alive during a longer time than at other temperatures. Even after 3/4 of the evaporation time, the cells were still moving. At 4 °C, cell activity slowed down abruptly because of the cold, whilst at 37 °C it stopped quickly because of the fast evaporation of the drop of culture medium. Between 14 and 20 °C, interactions between microorganisms and oxidant agents of the atmosphere and between microorganisms and resins were not long enough to destroy cells. By staying alive and in activity longer than in the other experiments, cells were better preserved. In most cases, we observed fluid around the cells. It was probably a film of the culture medium that was trapped around the cells. This film is likely to explain the better preservation of the microbes. Indeed, in the preparations at 14 and 20 °C, the better-preserved microbes were always enclosed in these microdrops of culture medium. On the contrary, the badly-preserved specimens were those that became totally isolated from the culture medium. Our observations confirm those of Schmidt and Dilcher (2007) who noticed, observing resin flows in fresh water of a Taxodium swamp, that perfectly preserved microbes were found in small water drops engulfed in the resin flows.

Influence of humidity

Ambient humidity seems to not have a great influence on the preservation of microorganisms, at least in the range tested. Nevertheless, for a particular temperature, the preservation of microorganisms is the same at both normal and elevated humidity. However, Schmidt and Dilcher (2007) demonstrated that immersed resin flows were able to perfectly preserve tiny organisms such as algae, ciliates and amoebae. They also have been (rarely) able to observe pseudopodia of some testate amoebae, cilia of some ciliates and flagella of some algae. In our experiments, all these structures were not visible. Many factors can explain this. First, Schmidt and Dilcher (2007) studied a *Taxodium* resin whilst we used a *Pinus* resin. The small chemical differences between these two resins can provide significant variations in the effectiveness of the biological fixation as demonstrated by Foissner and co-authors (1999). Second, the observations made by Schmidt and Dilcher (2007) have been performed in natural aquatic ecosystems. It means that all the factors that govern the microbial preservation were more effective. In our experiments under terrestrial conditions, we tested a small number of parameters one by one. Important parameters of natural environments such as pH and hydrodynamics were not tested. These data can explain the differences between the two studies. Third, Schmidt and Dilcher (2007) observed that most of the microorganisms they found in *Taxodium* resin flows were in tiny water drops that were trapped inside the resin. In our experiments, we did not reproduce such encasing. The drops of culture medium were just placed at the surface of the resin, as it probably represents a more natural soil situation. Thus, microorganisms were always in contact with atmospheric oxygen, an oxidant. The poor preservation we observed may be a result of oxygen in contact with the culture drops. Being encased in the resin, as observed by Schmidt and Dilcher (2007), the drops would be protected from any oxidation and the microorganisms could be better preserved.

Influence of light

We did not observe that light had an influence on the microbial preservation and glass reduced UV penetration. Light mainly influenced the evaporation time of the drops. With light, the evaporation was 1.5 to 2 times faster than without it, probably because of the warming effect of the light. However, the preservation of microbes was not visibly affected by this factor. Meanwhile, light could have a greater effect on photosynthetic microorganisms, bacteria or fungi (Schmidt and Schäfer, 2005; Girard et al., 2009a; Rikkinen and Poinar, 2000).

Conclusions

The experiments we made provide new data on the preservation of microfossils in plant resins, by studying the initial period of contact between the resin and specimen. We conclude that smothering cells with resin (first method), by placing resin on top of the cell culture, destroys cells and nothing is preserved. However, the opposite scenario (second method), with cells in a medium slowly drying on top of some resin, provided preservation of cells. Thus, embedding of microorganisms is not a sudden event, but a process which requires a few hours of slow drying on the resin, and rapid fixation of protists in resin seems to be improbable. There is likely a role for a film of water between the cells and the resin in improving preservation. Schmidt and Dilcher (2007), observing resin flows in natural swamps, showed that acceptable preservation of microbes can be possible when the flows are produced in water. This preservation may be, considering our experiments, mainly due to the embedding of small water drops into the resin flows and the retention of water films. However, this phenomenon cannot explain the preservation of soil microorganisms in terrestrial conditions. Two other parameters tested, light and humidity, did not affect preservation. Depending on the conditions, there was variable preservation or no preservation of internal structures such as nucleus and vacuolated structures, external structures such as cell test and flagella, and shape of an organism. The best preservation conditions were executed at 14 °C and 20 °C. Under no condition was any naked amoeba preserved.

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