Further studies on fossilised ciliates (Protozoa, Ciliophora) from triassic amber

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Foissner, W., Schönborn, W., Wright, A.-D.G. & Lynn, D.H.: Further studies on fossilised ciliates (Protozoa, Ciliophora) from triassic amber
Soil Zoology in Central Europe. Tajovský, K. & Pižl, V. (eds.): 45-52. ISB AS CR, České Budějovice, 1999.
ISBN 80-902020-6-3

A recently discovered, triassic amber ("Schlierseerit") contains an abundance of excellently preserved, soft-bodied organisms, such as bacteria, fungi, algae, flagellates, ciliates, and testate amoebae. Palaeontological evidence indicated Cycadophyta as the resin source for the Schlierseerit, whereas carbon-13-nuclear magnetic resonance spectra favoured Araucariaceae. Accordingly, we investigated the ability of fresh resin from *Cycas circinalis*, *Araucaria* sp., and *Picea abies* to preserve some extant relatives (*Paramecium aurelia*, *Tetrahymena mobilis*, *Mykophagophrys terricola*) of the ciliates found in the amber. In fresh resin of *Araucaria* and *Picea*, the ciliates died within a few minutes, while they survived and preserved excellently in *Cycas* resin. Although amber formation is a complicated process, our experiments indicate the possibility that soft-bodied organisms can be preserved in certain resins. The most remarkable fossils belonged to the strictly mycophagous grossglockneriid colpodids. Today, such ciliates are invariably associated with bacterivorous species of the genus *Colpoda*, which, however, were absent in the Schlierseerit. We thus paralleled the amber investigations with a molecular estimate of the age of various colpodid lineages, using an SSr RNA clock derived from extant species. This showed that the mycophagous colpodids evolved about 240 million years ago, while *Colpoda* was slightly younger, namely, 180 million years. This perfectly matched the findings from amber, which is 220 – 230 million years old.

Keywords: Amber, colpodids, fossil ciliates, Schlierseerit, Trias.

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Introduction

Fossil records of soft-bodied protists are extremely rare (Poinar, 1992; Tappan and Loeblich, 1968). It was only recently that a whole community of microorganisms (bacteria, fungi, heterotrophic and autotrophic flagellates, ciliates, testate amoebae) was discovered in 220 – 230 million-year-old triassic amber (Poinar et al., 1994). The amber, now called "Schlierseerit", was found by Ulf-Ch. Bauer in layers of Raibler Sandstone near the village of Schliersee, Bavaria. It contained many excellently fossilised specimens, most of which corresponded to, or diverged from extant species only slightly, although some new taxa were described (Dörfelt and Schäfer, 1998; Schönborn et al., 1999). The community

Presented at the 5th Central European Workshop on Soil Zoology, České Budějovice, April 27-30, 1999

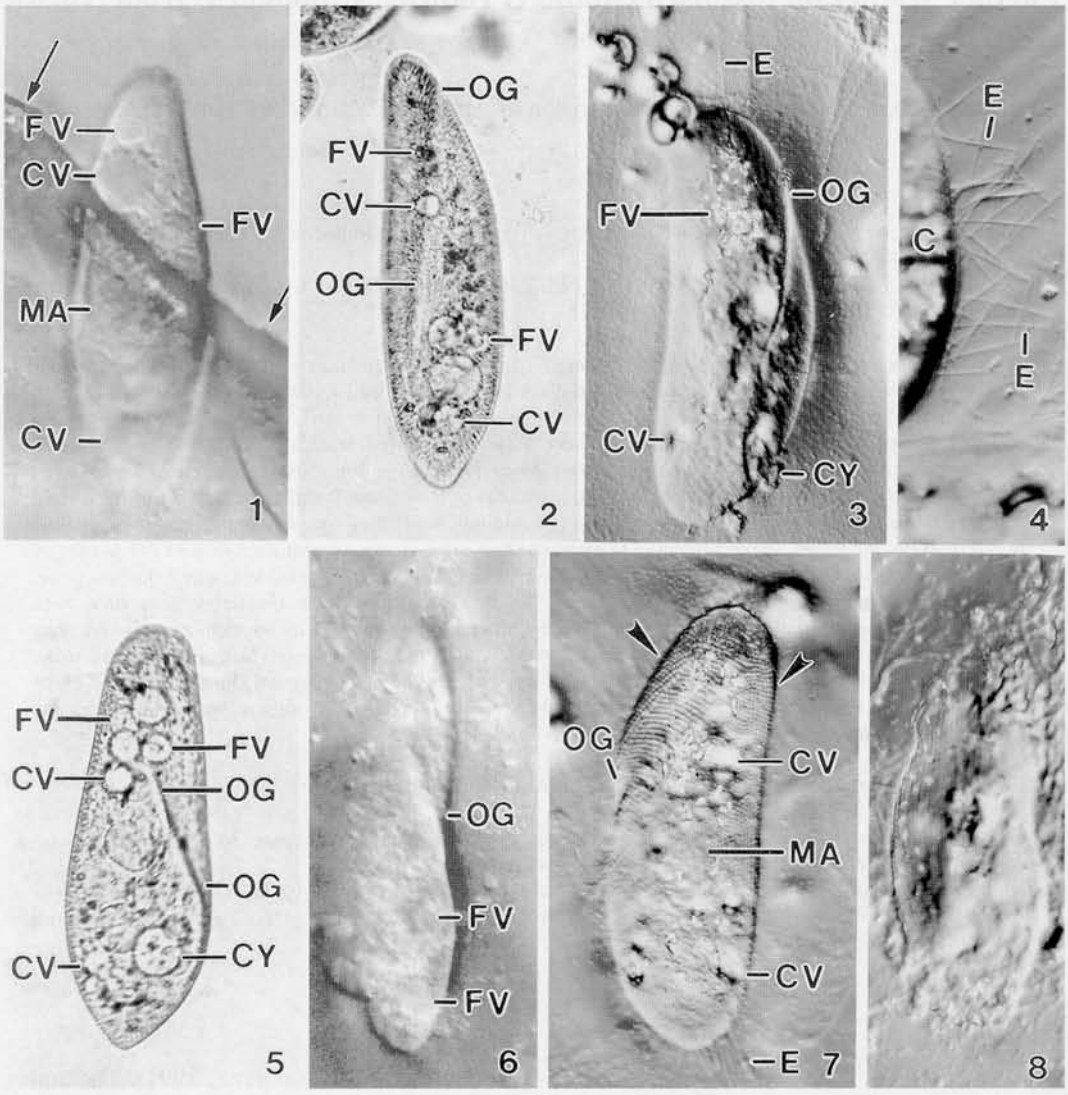

Fig. 1 – 8. Fossilised *Paramecium triassicum* from amber (1, 6) and extant *P. aurelia* from life (5) and after embedding in *Cycas* (2 – 4, 7) and *Araucaria* (8) resin. **1, 5, 6** – Dorsolateral and lateral views showing the characteristic body shape and the location of some main organelles. Note the contractile vacuoles of the fossilised specimen shown in Figure 1, which have the same location as in extant *P. aurelia* (2, 5). Arrows mark line where the amber split during preparation. **2** – Ventral view of a specimen 5 min after transference onto the resin in a drop of water. The cell is still alive but paralysed by substances diffusing from the resin into the water. **3, 4, 7** – Specimens dried on the resin surface. Body shape and some organelles, including the honey-combed cortical pattern (arrowheads), are excellently preserved. **8** – In *Araucaria* resin the cells die and disintegrate. The air-dried preparation thus hardly shows any details. 1, 3, 4, 6 – 8: differential interference contrast; 2, 5; bright field. C – ciliate, CV – contractile vacuoles, CY – cytopyge, E – extrusomes (trichocysts), FV – food vacuoles, MA – macronucleus, OG – oral groove.

structure indicated a terrestrial or semiaquatic habitat, such as leaf litter, tree-holes, or astatic ponds.

How was the excellent preservation of the organisms achieved? Poinar and Poinar (1994) suggested some sort of natural dehydration and fixation by the resin. We got the same impression because the specimens looked like those embedded in artificial resin for transmission electron microscopy. Furthermore, it is well-known and applied in several protocols (Bresslau, 1922; Foissner, 1991) that protists can be desiccated without gross destruction of some of their organelles. Accordingly, we tested this hypothesis with extant ciliates and fresh resins of *Cycas* and *Araucaria*, whose relatives were probably the resin source for the Schlierseerit (for reviews, see Poinar and Poinar, 1994; Vavra, 1996).

Up to now, the Schlierseerit was dated mainly by the geological horizon (Raibler Sandstone). However, the estimated age of 220 – 230 million years matched the age of certain ciliate lineages, which was calculated with a SSr RNA clock derived from extant species (Wright and Lynn, 1997). This surprising result will be discussed in the second part of the paper.

Materials and methods

The micrographs of the fossilised specimens are from Schönborn et al. (1999), who describe amber preparation and photography in detail. Of the ciliate species found in the amber, three extant relatives were chosen for the experiments, viz., a member of the *Paramecium aurelia*-complex, *Tetrahymena mobilis* (Kahl), and *Mykophagophrys terricola* (Foissner). As concerns the sequences used for the molecular clock, see Wright and Lynn (1997).

Paramecium aurelia highly resembles *P. triassicum* discovered in the amber (Fig. 1, 2, 5, 6), except for the size (about 150 µm vs. 50 µm). *Paramecium aurelia*, which is a very robust bacteria feeder, was cultivated in Eau de Volvic (French table water) enriched with some squashed wheat grains to stimulate bacterial growth. The culture also contained many heterotrophic flagellates, viz., *Polytomella* sp. *Tetrahymena mobilis* belongs to the *Tetrahymena pyriformis*-complex and is similar to an organism found in the amber, very likely *T. rostrata* (Fig. 9 – 12). *Tetrahymena mobilis*, which is about 50 µm long and rather fragile, is a histophagous ciliate and was thus cultivated in Eau de Volvic enriched with some meal-worm pieces. *Mykophagophrys terricola* has a size of about 30 × 20 µm and highly resembles the *Mykophagophrys terricola*-like ciliate found in the amber (Schönborn et al., 1999; Fig. 18 – 21, 25, 26). *Mykophagophrys* belongs to a group of obligate mycophagous ciliates having a minute (length 2 – 3 µm) but highly characteristic feeding tube (Fig. 17), which can be recognised in the fossilised specimens (Schönborn et al., 1999; Fig. 18, 21). It was cultivated on baker's yeast, as described by Foissner (1993) for *Pseudoplatyophrya nana*, a close relative (Fig. 17).

The resins were obtained from slightly wounded stems of the plants investigated. The *Picea abies* resin was collected from a tree in Foissner's home garden; the *Araucaria* resin was obtained from a tree in a garden on the Seychelles; and *Cycas circinalis* resin was collected from a specimen in the greenhouse at Salzburg University.

For the experiments, a small drop of resin was smeared on an ordinary microscope slide. Then, some small drops of concentrated (by mild centrifugation) ciliates were put on the resin surface with a fine-bored pipette. The drops, which remained hemispherical because the resins were rather hydrophobic, were air-dried at room temperature; evaporation required 3 – 20 min, depending on drop size, humidity, and temperature. During this time

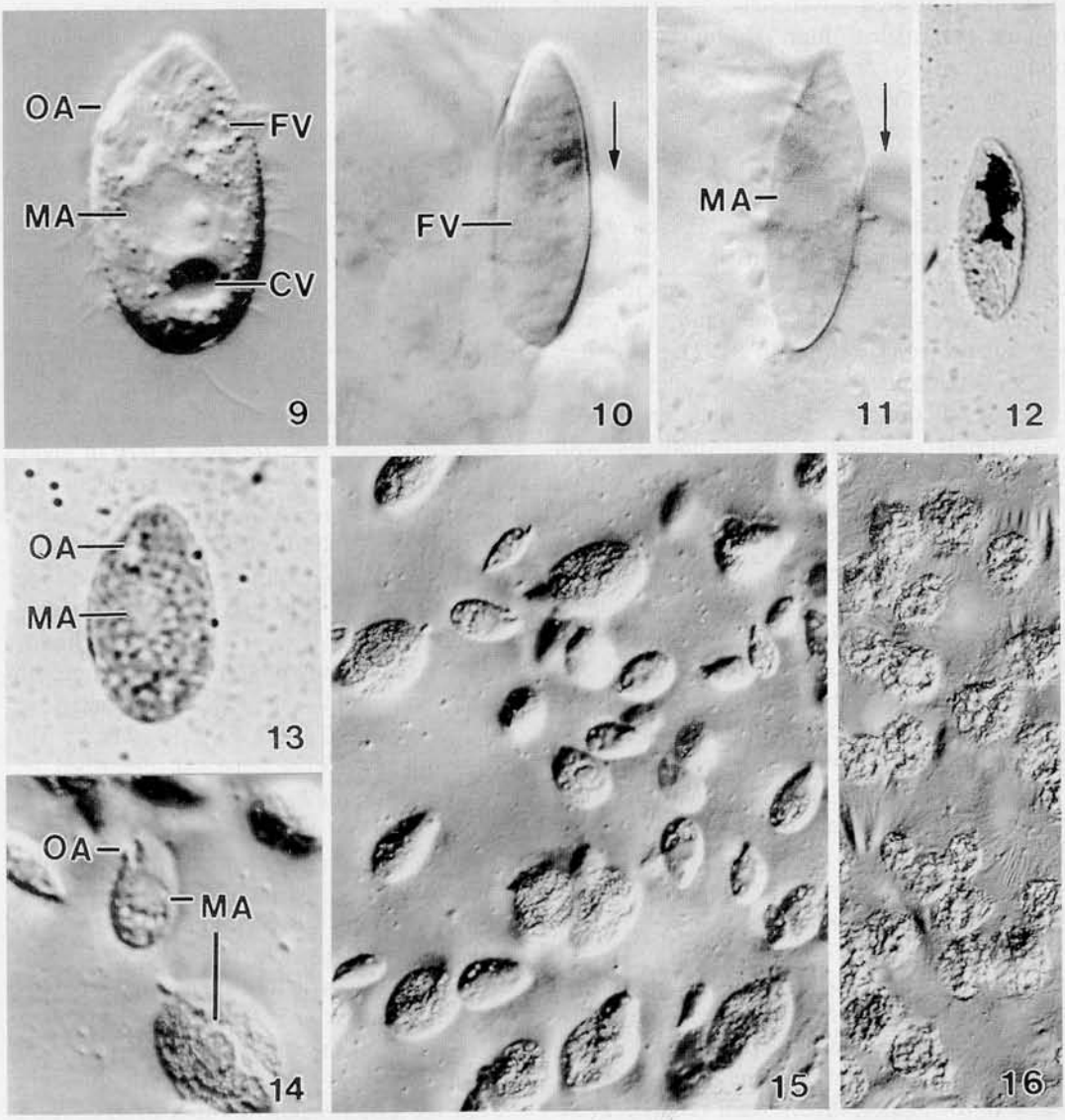

Fig. 9 – 16. Fossilised *Tetrahymena rostrata* from amber (10, 11) and extant *T. mobilis* from life (9) and after embedding in *Cycas* (12 – 15) and *Araucaria* (16) resin. **9 – 11** – Lateral views showing body shape (specimen in Figure 9 slightly flattened due to cover-glass) and some main organelles. Arrows mark line where the amber split during preparation. **12 – 15** – Specimens dried on the resin surface. Body shape and some main cell organelles, such as the oral apparatus and the macronucleus, are excellently preserved; cilia, however, are not recognisable, like in the fossil specimens (10, 11). The black inclusion in Figure 12 is air. **16** – In *Araucaria* resin the cells die and disintegrate within a few minutes. The air-dried preparation thus hardly shows any details. The resin has, however, some fixative properties because the cells do not disintegrate completely and still can be recognised in the dried preparations. 9 – 11, 14 – 16: differential interference contrast; 12, 13: bright field. CV – contractile vacuole, FV – food vacuoles, MA – macronucleus, OA – oral apparatus.

the organisms could be observed with an ordinary microscope at low magnification ($\leq \times$ 250). Finally, the dried smears were embedded in ordinary artificial resin and covered with a cover-glass. Photography was rather difficult due to the uneven resin surface.

We should mention that the experiments need some patience because the resin surface is uneven and hydrophobic, often providing only suboptimal conditions for air-drying of specimens.

Results and discussion

Resin experiments

Picea resin: The fresh resin used for the experiments was yellowish, very sticky, and had an aromatic odour. The tested organisms died and dissolved within a few minutes. Some specimens could still be recognised in the dried smears, but only the trichocysts of *Paramecium* were well-preserved.

Araucaria resin: The fresh resin was snow-white, very sticky, and without distinct odor. Within four weeks the resin became clear and almost colourless. Resin which still was supple and sticky was used for the experiments; when it was warmed to about 60°C, the resin became more fluid, giving the smears a smoother surface. Ciliates died within about 3 min, while *Polytomella* survived for up to 15 min. First, the ciliates got large blebs and then rounded up more or less distinctly. Usually, they did not dissolve completely, indicating that the resin had some sort of fixative property. Thus, many specimens still could be seen in the dried smears, although details were hardly recognisable (Fig. 8, 16). Only the trichocysts of *Paramecium* were well-preserved.

Cycas resin: The fresh resin used for the experiments was yellowish, sticky, and gelatinous. Interestingly, the resin swelled to a flabby mass when it was put into water for about 20 min; indeed, it could be dissolved in water more or less completely. Even old, very hard and dry resin showed this property, although rewetting took 24 hours. *Araucaria* and *Picea* resin did not show these properties.

When the organisms were put onto the resin, they slowed down swimming within about 5 min eventually becoming almost immobile after 5 – 10 min. At this stage, the cilia were still moving slightly and body shape was hardly altered. Thus, excellent micrographs could be taken without flash illumination (Fig. 2). About half of the specimens were still alive and possessed their natural shape when desiccation was completed, although *Mykophagophrys* was more fragile than *Paramecium* and most specimens rounded up and dissolved slightly in some experiments. The air-dried organisms were often excellently preserved, showing details such as shape, macronucleus, contractile vacuoles, food inclusions, cortical pattern, and extrusomes (Fig. 3, 7, 12 – 15, 25, 26). Indeed, they highly resembled the fossilised amber specimens (Fig. 1, 6, 10, 11, 18, 21). Extrusomes were especially well preserved both in *Paramecium* (Fig. 4) and *Mykophagophrys* (Fig. 22, 23) and looked like those stained with methyl geen-pyronin (Fig. 24). Their arrangement around the cell highly resembled the thick, peripheral rods recognisable in some fossilised specimens (Fig. 18 – 20, 25).

Obviously, the *Cycas* resin has paralysing and conservation properties for the organisms tested. Preservation could not be improved by pre-watering the resin; in such resin, preservation was usually poorer indicating that some substances were partially washed out.

Congruent fossil and gene sequence evidences about the age of colpodid ciliates

The most remarkable fossils contained in the amber belonged to a group of eight extant ciliate species, namely, the strictly mycophagous grossglockneriid colpodids.

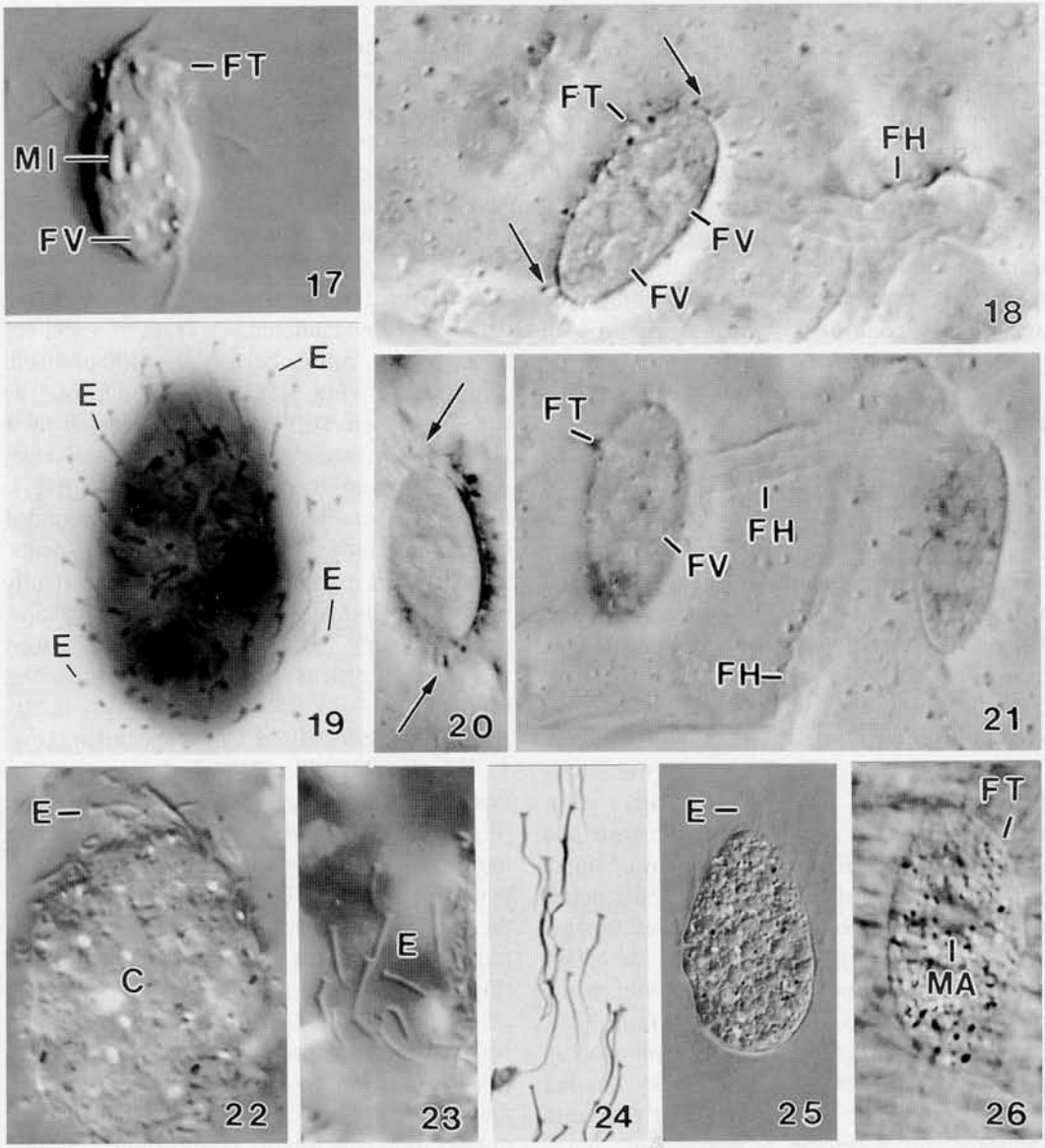

Fig. 17 – 26. Fossilised *Mykophagophrys terricola* from amber (18, 20, 21) and extant *M. terricola* after methyl green-pyronin staining (19, 24) and embedding in *Cycas* resin (22, 23, 25, 26). **17** – *Pseudoplatyophrya nana*, a relative of *Mykophagophrys*, from life. Note the highly characteristic feeding tube (FT), which is also recognisable in the fossilised specimens (18, 21). **18, 21** – Lateral view of specimens between fungal hyphae, the preferred food of mycophagous ciliates. Arrows in Figure 18 mark short, thick rods, very likely extrusomes just leaving the cell (cp. Figures 19, 20, 22, 23). **19, 20, 22, 23, 24** – The nail-shaped extrusomes (E, arrows) are well recognisable in the extant (19, 24), fossilised (20), and *Cycas* resin embedded (22, 23) specimens. **25, 26** – Specimens, packed with food vacuoles, dried on the resin surface. 17, 18, 20 – 23, 25, 26: differential interference contrast; 19, 24: bright field. C – ciliate, E – extrusomes, FH – fungal hyphae, FT – feeding tube, FV – food vacuoles, MA – macronucleus, MI – micronucleus.

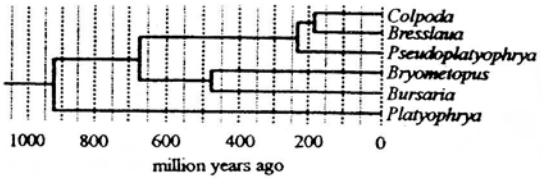

Fig. 27. The estimated time of divergence of colpodid lineages according to a SSr RNA molecular clock matches the fossil records (for details, see text).

These ciliates have a minute (length 2 – 3 µm), highly complex feeding tube, which penetrates fungal hyphae and spores and takes up their contents. Other oral structures (right and left oral ciliary field) are inconspicuous or lacking at all (buccal cavity). Thus, grossglockneriid colpodids were considered as a highly specialised, apomorphic branch of the bacterivorous and predaceous colpodids, such as *Colpoda* and *Bresslaua*, with which they share a common ancestor, as evident from morphological, ontogenetical, and sequence data (Foissner, 1993; Lynn et al., 1999). Today, the mycophagous ciliates are invariably associated with bacterivorous species of the genus *Colpoda,* which contains about 30 extant species and has rather complex oral structures (Foissner, 1993). It was thus a great surprise that we could not find *Colpoda* in the Schlierseerit.

We paralleled the amber investigations with a molecular estimate of the age of colpodids and other well-established ciliate lineages, using a SSr RNA clock derived from extant species (Wright & Lynn, 1997). This showed ciliates dating back to the Paleoproterozoic some 1980 to 2200 million years ago. The colpodids appeared about 900 million years ago and split into several lineages during the next 700 million years. The youngest lineage contained the mycophagous grossglockneriid *Pseudoplatyophrya* (Fig. 17) and the bacterivorous genus *Colpoda*, which formed a clade with the predaceous *Bresslaua*. A more detailed analysis showed that *Pseudoplaytophrya* evolved about 240 million years ago, while *Colpoda* and *Bresslaua* were slightly younger, that is, 180 million years (Fig. 27). This perfectly matched the findings from amber, which is, according to geological dating, 220 – 230 million years old. Of course, we cannot exclude that this result was pure chance. However, very recently, we discovered a new, mycophagous colpodid in mud from an ephemeral pool in South Africa (Foissner, 1999). The oral structures of this species are between those of *Pseudoplatyophrya* and *Colpoda* in appearance indicating that the congruency of the fossil and sequence data might be correct.

Acknowledgements

The authors thank Ulf-Ch. Bauer for the amber material. The technical assistance of U. Schäfer, Dr. E. Herzog, Mag. Eric Strobl, and Dr. B. Moser is acknowledged. Supported by the Austrian FWF (P-12367-BIO) and the Canadian NSERC.

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