Taiwania, **47**(4): 273-280, 2002

Ultrastructure of 100 Million Years Old Microorganisms of the Ajkaite (Upper Cretaceous Amber) from Hungary

M. Kedves[1,3] and Á. Párdutz[2]

(Manuscript received 18 June, 2002; accepted 15 November, 2002)

ABSTRACT: Ultrathin sections from Upper-Cretaceous (Santonian-Campanian) amber (Ajkaite) were investigated with transmission electron microscope (TEM). Several kinds of organic remnants were observed. In this contribution we report here the presence of three different types of morphologically intact bacteria very well preserved in amber. We could demonstrate the fine structure of the wall, the protoplasm with vacuoles, electron dense granules and flagella.This extreme form of mummification is attributed to inert dehydration and the fine structural preservation makes these ancient bacteria.

KEY WORDS: Prokaryotes, Upper Cretaceous amber, Ultrastructure.

INTRODUCTION

A wide spectrum of well preserved fossilized soft tissues from microbes to vertebrates and higher plants have been identified and studied in amber. In his monograph Walther (1911) mentioned about 33.500 arthropodous types. The fossil moss remnants from amber of different geological ages were summarized by Gyõrffy (1929). The vast majority of the observations have been made by light microscopy (Vávra, 1984; Poinar, 1992), and the first report on the fine structure of 40 million years old insect tissue was published by Poinar and Hess (1992). Light- and electron microscopic pictures of fossil angiosperm pollen grains from Eocene age were published recently (De Francheschi *et al.*, 1999, 2000; Dejax *et al.*, 2001a, b). The molecular biology methods opened new vistas in the field of paleobiology (Sykes, 1997), but the available data are rather contradictory concerning the reliability of DNA sequencing data of fossil material.

Bacteria in Baltic amber were first studied by Blunck (1929) after partially dissolving the amber. Light microscopy revealed micrococci, short and longer rods, and spiral forms, and on the basis of these data, different taxa were established. More recently several attempts have been made to revive fossil bacteria and to analyse its genetic material. These attempts have reproduced bacterial DNA sequence which are different from that of the extant species, but there is certain reservation in the literature concerning the reliability of the results obtained (Cano and Borucki 1995; Greenblatt *et al.*, 1999).

Considering the fact that the fine structural preservation of fossil material may provide important clues about the viability of DNA present in the sample, we performed TEM investigations on chronologically well defined bacteria to decide if 100 Million year old material could be used for molecular biology studies.

1. Cell Biological and Evolutionary Micropaleontological Laboratory of the University of Szeged, H-6701, P.O. Box 993, Szeged, Hungary.
2. Institute of Biophysics, Biological Research Center of the Humgarian Academy of Sciences, H-6701, P.O. Box 521, Szeged, Hungary.
3. Corresponding author.

MATERIALS AND METHODS

The occurrence of amber in the brown coal layers of Ajka was first published by Hantken (1867). Szabó (1871) established that the resinous remnants increase the caloric value of the coal. Hlasiwetz (1871) analyzed the first chemical compounds of the Ajkaite. Zechmeister (1926) ranged Ajkaite into the succinite and/or retinite. Following Rozlozsnik (1940) the Ajkaite may be ranged between the resinite and trinerite. Szádeczky-Kardoss (1953) concluded that the coal layers of Ajka originate from karstic swamp woods. The acidic water of the karstic swamps was neutralized by the limestone, and the microorganisms became very numerous. Góczán (1961) emphasized that the amber of the brown coal of Ajka may be the product of deciduous tropical woods. Recently Lantos *et al.* (1997) gave a detailed description on the stratigraphic definition of Ajkaite and clearly found it as of Upper Cretaceous origin. Based on the palynological researches the vegetation forming the coal layers may characterize in the first place by the abundance of extinct Amentiferae taxa and tropical ferns. During our first researchs amber fragments from two samples were used for TEM investigations. The microfossils of the embedding material were investigated previously (Kedves *et al.*, 2000). Based on the tissue remnants (Kedves Borbola and Priskin 2001) and the first palynological results the amber tree or the association of the vegetation forming the embedding brown coal of the two sample was completely different (Kedves and Alvarez Ramis, 2002). But the angiosperm origin seems to be the well established in both samples.

The material used in this research was collected by Mr. G. Kovács, a PhD student, from the spoil bank of the Ajka mine, No. KG-99. Small pieces of amber were placed into the gelatine capsules and some drops of propylenoxyde were added to dissolve the surface of the Ajkaite. Thereafter the samples were embedded in Araldite (Durcupan, Fluka). The ultrathin sections were made with glass knives on a Porter Blum ultramicrotome in the Cell Biological and Evolutionary Micropaleontological Laboratory. The TEM pictures were made in the EM Laboratory of the Department of Biophysics of the Biological Research Center of the Hungarian Academy of Sciences on a Tesla BS-540 (resolution 6-7 Å) and on a Zeiss EM-902 (resolution 2-3 Å) instrument. All pictures are untouched.

RESULTS

Ultrastructures of different kinds of microfossils were observed. We present here the first results concerning the fossilized prokaryots with well preserved protoplasm from the Upper Cretaceous Ajkaite. Fig. 1 illustrates the mass of microorganisms in the amber. The most important morphological and ultrastructural characteristics of these well preserved microorganisms are summarized as following.

Microorganism of type I Figs. 2-5

Small ovalid (C, D) or pyriform (B) microfossils sometimes with a short flagellum; form A, in figs. 2 and 3 are embedded in a characteristic mucilage. Size of these micro-fossils is 0.15-2.22 μm. The wall is extremely thin. Structural elements were not perceptibles. Ovaloid vacuoles (50-60 nm) and small electron dense globular units (20-30 nm) are in the protoplasm (figs. 4 & 5). These units may be the remnant of the diffuse DNA.

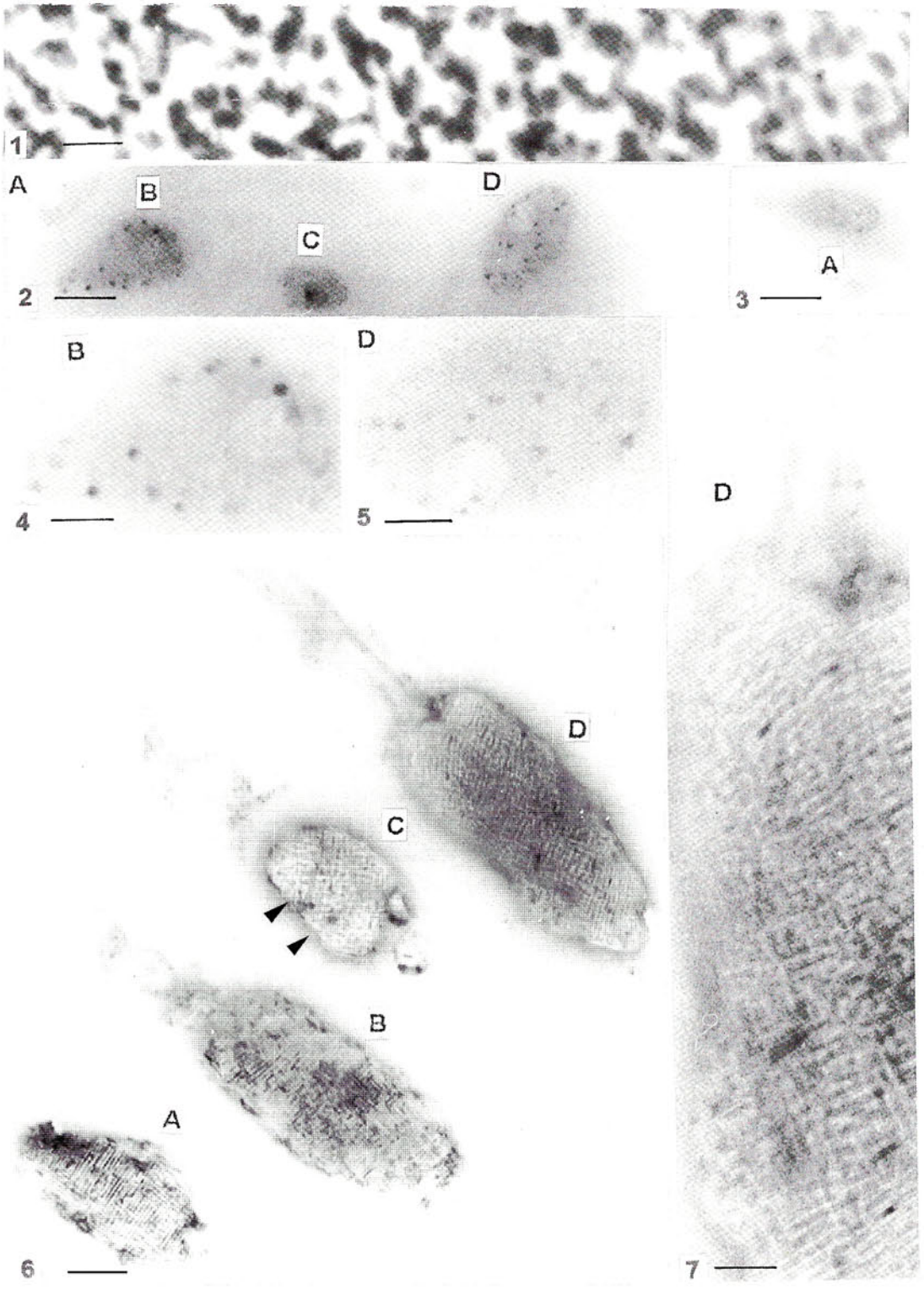


Fig. 1. TEM picture from a mass of Prokaryotes in the Ajkaite. Sample: KG-17, bar = 1 μm. Figs. 2-5. Various micro-organisms, type I, sample KG-17. Figs. 2 & 3, bar = 0.1 μm. Figs. 4 & 5, bar = 0.04 μm. Figs. 6 & 7. Various micro-organisms, type II, sample: KG-16. Fig. 6, bar = 0.1 μm. Fig. 7, bar = 0.04 μm.

Microorganism of type II. Figs. 6 &7

Microorganisms of type II are larger flagellar ovaloid microorganisms. Characteristic ultrastructural elements of the wall were observed. Striated ultrastructure is particularly well shown in specimen C and D in fig. 6. In specimen C the microstructure elements of the protoplasm are also well shown.

Microorganism of type III Figs. 8-10

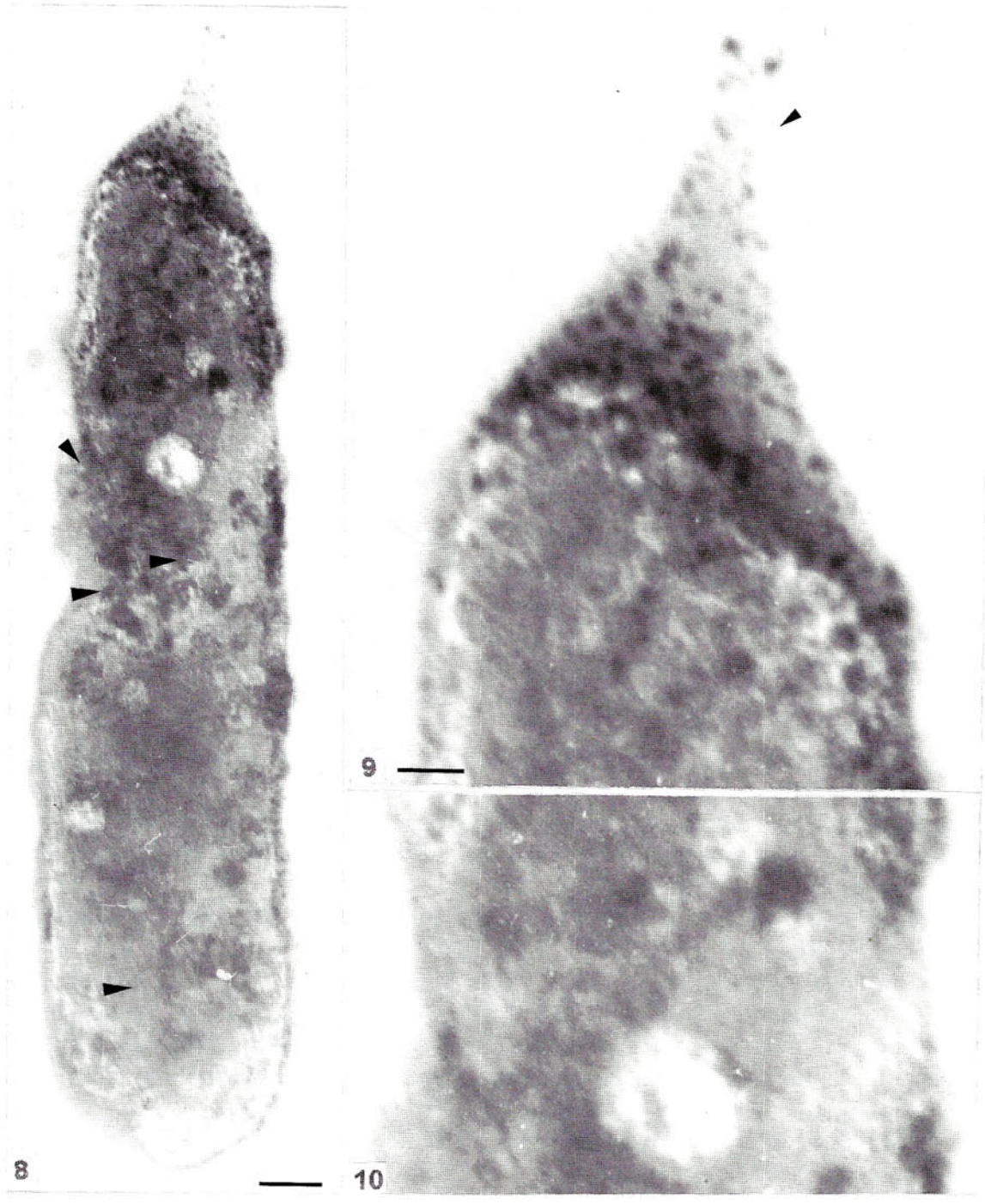
The ultrastructure of the protoplasm is well preserved in specimen illustrated in figs 8-10. This largest microorganism is elongated in form, and was in an intensive state of division. Several specimens were preserved in the amber in this way. At the upper part of this cluster of microorganism a small flagellum-like mucilagineous material (marked with arrow) was preserved with dark electron dense globular units of 15-25 nm. Well preserved fine wall ultrastructure and the organelles of the protoplasm are illustrated. Characteristic vacuoles of 0.8-1.0 μm in diameter and electron dense granular units of different size (15-20 nm) are in the protoplasm.

DISCUSSION

Concerning the methods used in the investigation of the organic fossils in the amber we have to emphasize, that it is necessary to study the ultrathin sections of the amber without dissolving the fossil resin.

The investigation of the amber is necessarily to study it by multidisciplinary concepts. A basic problem is to establish the origin of the "amber tree" (e.g.: Hillmer *et al.*, 1992; Vávra, 1991, 1993). The secondary woody fragments together with the spore- pollen assemblages of the amber containing coal or other kind of samples is useful to obtain informations for the paleovegetation and its paleoecology. The Senonian vegetation in Hungary and, generally, in Europe can be characterized with the great divergence of the extinct Amentiflorae taxa which were classified into the Stemma Normapolles by Pflug (1953). We emphasize again that based on the palynological and the woody fragment data, the two samples investigated are different (Kedves *et al.*, 2001; Kedves and Alvarez Ramis, 2002). The prokaryotes containing sample was characterized by the remarkable quantity of Pteridophyte spores, Longaxones, mostly tricolporate, and from the Brevaxones with the "Oculata Normapolles" pollen grains. This association of pollen grains was associated by different kind of secondary woody remnants: Cycadopsida, Pteridophyta or Arecales remnants-scalariform thickenings of tracheids. Gymnospermophyta, Conifero- phytina remnants-fibre tracheids, areolate bordered pits of slit-like type (*Austrotaxus, Phyllocladus, Podocarpus, Callitropsis, Dacrydium, Podocarpus*).

The origin of the Ajkaite is different, therefore the chemistry and the preservartion capacity of the samples are different. The discovery of the cytological characteristics of very old plant cells, open new opportunities in the reconstitution of the ultrastructural evolution. Regarding the ultrastructure of the fossil microorganisms and pollen grains our knowledges is in this moment in the stage of the beginning. But there are TEM data from fossil mineralized Bacteriophytes also, cf. Southam and Donald (1999). It is a hope that this branch of researches will be broadened and will obtaine several new results in the near future on different kinds of plant remnants. Finally, we emphasize the establishment of Dejax *et al.* (2001b),"some organic molecules remaining potentially preserved."



Figs. 8-10. TEM picture of microorgasnisms, type III. Sample KG-21. Fig. 8, bar = 0.1 μm. Figs. 9 & 10, bar = 0.04 μm.

ACKNOWLEDGEMENTS

The authors are very grateful to Prof. Dr. T.-C. Huang (Department of Botany, National Taiwan University, Taipei) for the translation of the abstract in Chinese language. This work was supported by Grant OTKA T 031715.

LITERATURE CITED

Blunck, G. 1929. Bakterienenschlusse im Bernstein. Centralblatt für Miner Geol. und Palaeontol. B, **11**: 554-555.

Cano, R. J. and M. K. Borucki. 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old dominician amber. Science **268**: 1060-1064.

De Francheschi, D., J. Dejax et G. De Ploeg. 1999. Pollen inclus dans l'ambre Eocène (Sparnacien du Bassin de Paris): Observation in situ, extraction et observation en microscope électronique à balayage et en microscopie photonique. XVIème Symp. APLF, Résumés des communications, p. 37, 38.

De Francheschi, D., J. Dejax et G. De Ploeg. 2000. Extraction du pollen inclus dans l'ambre (Sparnacien du Quesnoy Oise, Bassin de Paris) vers une nouvelle spécialité de la paleopalynologie. C. R. Acad. Sci. Paris **330**: 227-233.

Dejax, J., D. De Francheschi, B. Lugardon, G. De Ploëg et V. Arnold. 2001a. Le contenu cellulaire de pollen fossilisé dans l'ambre, préservé à l'état organique. XVème Symp. APLF, Vol. des résumés, p. 30.

Dejax, J., D. De Francheschi, B. Lugardon, G. De Ploëg et V. Arnold. 2001b. Le contenu cellulaire du pollen fossilisé dans l'ambre, préservé l'état organique. C. R. Acad. Sci. Paris **332**: 339-344.

Greenblatt, C. L, A. Davis, B. G. Clement, C. L. Kitts, T. Cox and R. J. Cano. 1999. Diversity of microorganisms isolated from amber. Microbial Ecol. **38**: 58-68.

Góczán, F. 1961. Die Palynologie der Senon-Bildungen des Süd-Bakony. Ann. Inst. Geol. Publ. Hung. **49**: 789-799.

Gyõrffy, I. 1929. Borostyánkövekbe ragadt mohákról és a fossilis mohák koráról. Debreceni Szemle **3**: 65-75.

Hantken, M. 1867. Az ajkai kõszéntelep geológiai viszonyai. - M.F.T. Munkálatai **3**: 98-102.

Hillmer, G., W. Weitschat and N. Vávra. 1992. Bernstein aus dem Miozän von Borneo. Naturwiss. Rdsch. **45**: 72-74.

Hlasivetz, H. 1871. Harz aus der Braunkohle von Ajka in Viszprimer Komitat. Verh. Kaiserlich- Königl. Geol. Reichanst. **1/18**: 191-192.

Kedves, M. and C. Alvarez Ramis. 2002. Types of sporomorphs from the Ajkaite containing brown coal samples from Ajka (Hungary) Types de esporomorfos determinados en muestras de lignito de las minas de Ajka (Hungria). Plant Cell Biol. Dev. **14**: 7-10.

Kedves, M., A. Borbola and K. Priskin. 2001. LM and TEM investigations on the Upper Cretaceous Ajkaite of Hungary II. Plant Cell Biol. Dev. **13**: 22-31.

Kedves, M., M. Szónoky, M. Madarász and G. Kovács. 2000. LM and TEM investigations on the Upper Cretaceous Ajkaite of Hungary I. Plant Cell Biol. and Dev. (Szeged) **12**: 8-17.

Lantos, M., M. Wagreich, Á. Siegl-Farkas, E. Bodnár and G. Császár. 1997. Integrated stratigraphic correlation of the Upper Cretaceous sequence in the borehole Bakonyjákó 528. Adv. Austro-Hungarian Geological Research, pp. 97-117.

Pflug, H. D. 1953. Zur Entstehung und Entwicklung des angiospermiden Pollens in der Erdgeschichte. Palaeontographica B, **95**: 60-171.

Poinar, G. O. Jr. (ed.). 1992. Life in Amber. Stanford University Press, pp. 69-72.

Poinar, G. O. and R. Hess. 1982. Ultrastructure of 40-million-years old insect tissue. Science **215**: 1241-1242.

Rozlozsnik, P. 1940. A csingevölgyi bányászat multja, jelene és jövõje. Vergangenheit, Gegenwart und Zukunft des Bergbaues in Csingervölgy. Ann. Hung. Geol. Inst. **3**: 1231-1245.

Southam, G. and R. Donald. 1999. A structural comparison of bacterial microfossils vs. 'nanobacteria" and nanofossils. Earth-Science Rev. **48**: 251-264.

Sykes, B. 1997. Light turning red on amber. Nature **386**: 764-765.

Szabó, J. 1871. Az ajkai kõszéntelep a Bakonyban. Földt. Közl. **7**: 124-130.

Szádeczky-Kardoss, E. 1953. Barna-és feketekõszén-fajtáink a népgazdálkodás fejlesztésének szolgálatában. - M.T.A. Müszaki Tud. Oszt. Közl. **10**: 39-56.

Vávra, N. 1984. Bernstein, die Tier und Pflanzenwelt fossiler Harze. Schriften des Vereines zur Verbreitung naturwiss. Kenntnissei. Wien **122/123**: 67-96.

Vávra, N. 1991. Chemofossils from "Baltic Amber" - a contribution to the biochemistry of the "amber tree"? In: Palaeovegetational Development in Europe, ed.: J. Kovar-Eder. pp. 423-427.

Vávra, N. 1993. Chemical Characterization of fossil Resins ("Amber") - A Critical Review of Methods, Problems and Possibilities: Determination of Mineral Species, Botanical Sources and geographical Attribution. Abh. Geol. B.-A, **49**: 147-157.

Walther, J. 1911. A föld és az élet története. K. M. Természettud. Társ. Budapest.

Zechmeister, L. 1926. Adatok az Ajkait, egy hazai fosszilis gyanta ismeretéhez. Zur Kenntnis des Ajkaites, eines fossiles Harzes aus Ungarn. Mat.- Természettud. Ért. **43**: 332-341.

匈牙利白堊紀晚期之琥珀中百萬年前的微生物之超微細構造

M. Kedves[1,3] and Á. Párdutz[2]

(收稿日期：2002 年 6 月 18 日；接受日期：2002 年 11 月 15 日)

摘　　要

本文乃利用穿透式電子顯微鏡，研究白堊紀晚期（山唐期至坎佩期）之琥珀的超薄切片，觀察到數種有機殘餘物。本文發表三種不同類型的細菌，其形態完整保存於琥珀之中；作者具體指出其細胞壁的微細構造、含液胞之原生質、高電子密度之顆粒體以及鞭毛。由於非活性脫水，致使形成此種木乃伊化的終極形式，並因這些古老細菌的微細構造保存妥當，而使其 DNA 化石成為遺傳學及親緣系統學研究之可能來源。

關鍵詞：原核生物、白堊紀晚期琥珀、微細構造。

1. Cell Biological and Evolutionary Micropaleontological Laboratory of the University of Szeged, H-6701, P.O. Box 993, Szeged, Hungary.
2. Institute of Biophysics, Biological Research Center of the Humgarian Academy of Sciences, H-6701, P.O. Box 521, Szeged, Hungary.
3. 通信作者。