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Journal of Archaeological Science 33 (2006) 1081–1087

Journal of Archaeological SCIENCE

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Ultrastructure and DNA sequence analysis of single *Concentricystis* cells from Alta Val Tiberina Holocene sediment

C. Milanesi [a], R. Vignani [a], F. Ciampolini [a], C. Faleri [a], L. Cattani [b], A. Moroni [a], S. Arrighi [a], M. Scali [a], P. Tiberi [a], E. Sensi [a], W. Wang [a], M. Cresti [a,*]

[a] *Department of Environmental Science ''G.Sarfatti'', University of Siena, P.A. Mattioli 4, 53100 Siena, Italy*
[b] *Department of Archaeology, University of Ravenna, San Vitale 28, 48110 Ravenna, Italy*

Received 1 November 2004; received in revised form 23 September 2005; accepted 21 November 2005

Abstract

We successfully combined ultrastructure and chloroplast DNA analysis for single specimens of *Concentricystis* isolated from Holocene sediment by a palynology method based on filtration, progressive sieving and percoll-gradient sedimentation to concentrate fossil cells. We compared our method with the traditional harsh chemical treatment commonly used to isolate fossil pollen and spores. With our method, the cytoplasm of *Concentricystis* was sufficiently preserved to distinguish several cell structures by light and electron microscopy. DNA analysis of *Concentricystis* involved PCR amplification using specific primers for a spacer region of the chloroplast genome. Significant homologies were found with the Angiosperm chloroplast genome by BLAST DNA search for PCR product sequences.

Keywords: Alta Val Tiberina; Holocene; Fossil sediment filtration; Light and electron microscopy; PCR; Chloroplast DNA sequence

1. Introduction

Since the early 1900s, researchers in Europe have sought to analyze and identify pollen and spores of plants found in the stratigraphic sediments of archeological sites [22] in an attempt to shed light on climatic changes and human activities in specific geographic areas during the Quaternary period. For the past 60 years, researchers in central Italy have undertaken research on biostratigraphic sequences to develop a methodological approach to determine small but significant climatic variations. They normally used a high impact acid maceration technique to extract intact fossil plant material preserved in an anaerobic environment in the sediment matrix [14]. Although this procedure is quite efficient, it does not preserve fossilized cytoplasmic components and destroys much of the information that pollen and spores could provide, as well as important ultrastructural and molecular informations.

Gorgo del Ciliegio (Fig. 1), a Holocene archeological site in the upper Tiber valley (Alta Val Tiberina) in the Apennines, was a pass to the eastern Tyrrhenian area for nomad tribes of the western Adriatic area. Preliminary stratigraphic sediment analysis was undertaken to study pollen grains and floral associations using traditional palynological chemical maceration of sediments. With this procedure, samples viewed by light microscopy revealed pollen grains, spores and many fossil specimens of *Concentricystis* [3]. These organisms, measuring 25–30 μm, have a typical spiral striation pattern on their surface and are bilaterally symmetrical. They live in freshwater marshes [15]. Researchers have long debated the evolutionary origins of these attractive organisms. Recent studies reveal their presence as far back as the Devonian era [7].

Abbreviations: C., Concentricystis; DAF, DNA amplification fingerprinting; DAPI, 4′,6-diamidino-2-phenylindole; HCl, hydrochloric acid; HF, hydrofluoric acid; LM, light microscopy; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

* Corresponding author. Tel.: +39 0577 232 895; fax: +39 0577 232 860.
E-mail address: cresti@unisi.it (M. Cresti).

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doi:10.1016/j.jas.2005.11.014

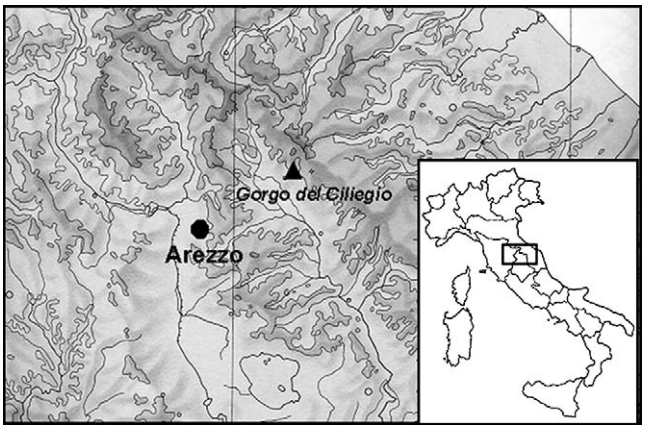

Fig. 1. Location map of the prehistoric Holocene site in Gorgo del Ciliegio, Alta Val Tiberina, 43° 35′ 25″ N, 0° 17′ 0″ W, altitude 430 m.

In this study we used both the conventional palynological technique and an alternative method involving sieving and percoll-gradient based protocols for concentrating cells [13]. *Concentricystis* were identified by DAPI staining in detritus of sediments [11]. Previous experiments demonstrated the possibility of analyzing DNA fragments out of ancient paleobotany rests [8,18]. Once selected and gathered, we examined external wall ultrastructure and analysed the fossilized cytoplasmic elements inside the wall. The cytoplasm was examined by a combination of SEM, TEM and a molecular biology technique based on PCR amplification using primers specific for chloroplast genome regions. This method helped us to formulate a hypothesis about the diagenetic history of *Concentricystis*.

2. Materials and methods

2.1. Traditional palynological treatment

The sedimentary succession material was stored at +4 °C. Dating according to pottery typology and ^{14}C was obtained by BETA Analytic Inc. 4986 S.W. 74, court Miami Florida, USA 33155. In order to analyze the percentage of palaeobotanical material and *C*. in the sediment, 10 g of different stratigraphic samples were treated by a traditional palynological method. To digest the calcium carbonate matrix, the sediment was dissolved with concentrated HCl, and to dissolve silica it was treated with HF. To remove the fluorine, the specimen was washed in water and to dissolve the humic matrix it was boiled for 10 min in 0.3 M NaOH. After rinsing in water, the small fraction containing *C*. was concentrated by heavy iodide liquid floatation [6], mounted on a clean slide with glycerine jelly, and observed with a Zeiss Axiophot 400 LM.

2.2. Isolation of C. cells

Laboratory analyses were conducted according to guidelines for excavation and conservation of microfossils for palaeogenetic studies, including methods for avoiding contamination (gloves, minimizing storage time of material in sealed plastic bags and setting up PCR under sterile conditions using a laminar flow hood). Approximately 10 g of sediment was mixed with 25 ml of sterile water. The specimen was sieved progressively with 0.30–0.008 mm filters. The particles remaining in the 0.008 mm sieve were washed repeatedly in sterile water. The material was sedimented on a percoll (Sigma) gradient (60–100%), [13] diluted with sterile water. About 5 min after sedimentation, material was collected from the bands. A Pasteur pipette was used to collect the layered material at the surface, from the 60, 80 and 100% bands and from the sediment at the bottom of the tube. The material was then placed in separate test tubes. *C*. were mostly found in the 80% layer. The material from this layer was then transferred to test tubes and washed in sterile water to remove percoll and superficial residues. To decontaminate and remove persistent organic and inorganic materials retained by *Concentricystis*, specimens were washed with 17% HCl, spun at 3000 rpm for 5 min in an Eppendorf microcentrifuge, washed with 37% HF, and spun again under the same conditions. A final thorough wash was done with water to remove acid residues. Exposure of *Concentricystis* specimens to acid did not damage the thick outer wall or the delicate cytoplasmic structures.

2.3. DAPI staining

DAPI staining was performed to locate *C*. specimens in the residues. DAPI staining for fossil materials has already been described [11]. The stained *C*. were observed in a Zeiss Axiophot fluorescence microscope under a laminar flow hood. Single *C*. specimens were collected in a sterile PCR tube for microscope observation and DNA analysis by means of a micromanipulator with a home-made sterile wood arm carrying a bristle, specifically mounted for the task.

2.4. TEM observations

Single *Concentricystis* specimens collected for TEM were transferred to a microscope cover slip for dehydration using increasing concentrations of ethanol (40, 60, 80 and 100%) at room temperature. Samples were gradually infiltrated in Spurr's [17] low viscosity resin and polymerized at 70 °C for 7 h in a chamber 0.1 mm in height, obtained by mounting two cover slips cleaned with TFL 50 dry lube (Polyscience Inc., USA). After polymerization, the slips were divided and specimens were located by light microscope to optimize sectioning. Sectioning was carried out with an LKB III ultramicrotome equipped with a glass knife. Thin sections of about 2 μm were mounted on a clean slide with glycerine jelly, and observed with a Zeiss Axiophot 400 LM. Grains that seemed to have intact fossil cytoplasm by LM were further sectioned with the ultramicrotome mounting a diamond knife. Sections of about 0.8 μm were collected on copper grids, stained for 3 min in 3% glutaraldehyde and 1% osmium tetroxide, then 2% uranyl acetate and 2% lead citrate and observed in a Philips Morgagni 268D TEM.

2.5. SEM observation

For SEM analysis a Philips XL20 SEM microscope was used. Each *C*. was placed directly on a clean standard stub and external morphology was observed under vacuum after graphitization. Sterile mQ water of 10 µl was added to each *C*. specimen in sterile 0.2 ml PCR tubes and samples were exposed to a one-step heat-shock cycle at 95 °C for 30 min. After heat-shock, samples were placed on ice until PCR mix was complete.

2.6. DNA analysis

PCR conditions were according to Suyama et al. [19], slightly modified. An MJ DNA thermal cycler was used in all amplification experiments. Primers for PCR were designed to amplify the spacer region between *rrn5* and *trnR* in chloroplast DNA. The PCR mix contained a final concentration: Taq buffer 1X (Amersham Pharmacia Biotech), 200 µM dNTP, 0.5 pmol forward primer (5′-TGGTGTCCCAGGCGTAGAG-3′) and 0.5 pmol reverse primer (5′-CGACACCGTGGTTCG-TAGC-3′), 5 U Taq DNA polymerase (Amersham Pharmacia Biotech) and sterile water up to a final volume of 100 µl. After an initial denaturing step at 94 °C for 5′, 40 PCR cycles were run consisting of 15 s at 94 °C, 15 s at 62 °C, and 25 s at 72 °C. A final extension step of 10′ at 72 °C was added. Secondary amplification was performed using 0.5 µl of the first PCR mix as template. The PCR mix was the same as described for the first amplification except for the primers, which this time were 5′-TCTACTGCGGTGACGATAC-3′ and 5′-CACG TGCTCTAATCCTCT-3′. Negative controls with no *Concentricystis* were included for the first and second amplifications. Amplification products were loaded into 1% agarose gel and run at 60 V in TAE 1X. PCR products were incorporated with Et-Br and examined with a transilluminator to check the specificity of the amplification reaction. The PCR products were cloned in *Escherichia coli* using the TOPO TA cloning kit (Invitrogen). The DNA sequencing of recombinant TOPO vectors was done according to the specifications of the user guide of the Autoread sequencing kit (Amersham Biosciences) on an ALFexpress II automated sequencer (Amersham Biosciences). The consensus sequences obtained from at least three independent experiments were analysed by BLAST search [1,12] and CLUSTAL W multiple alignment software. The consensus sequences were obtained from analysis of five different recombinant TOPO vectors harbouring the same PCR amplification fragment.

3. Results

3.1. Traditional palynology analysis

The sedimentary Holocene successions (B, C, D) analysed were nine stratigraphic layers each 20 cm thick (Fig. 2). Sample D9 from ground level was dated 3190 years BP. On the basis of geo-morphological considerations, it was determined that at this time, the Afra at a higher altitude than today and was

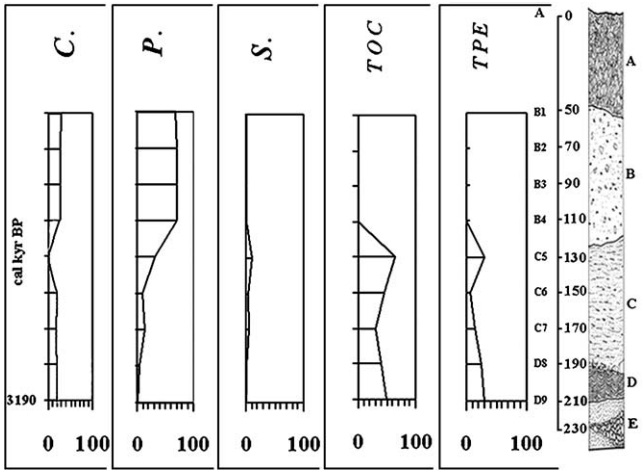

Fig. 2. Percentage of *Concentricystis* specimens (*C*.), pollen (P.), spores (S.), total organic carbon (charcoal, TOC) and total palaeobotanical elements (TPE) in stratigraphic samples. A, agricultural land; B, fluvial silt/clay sample without traces of charcoal or plant materials; C, sand/clay rich in human remains, charcoal and plant detritus; D, clay rich in human remains, charcoal and plant detritus; E, sand/pebbles without traces of human activity.

therefore close to the human settlement. Erosion created a gradient of fluvial deposits covering the archeological site. These deposits contained high concentrations of *C*. specimens and reddish clay silt as well as low concentrations of calcite crystals and microcrystals. There were higher percentages of pollen and *C*. in deposits where charcoal and palaeobotanical material were not found, indicating the end of human activity at the site. Thus strata C (three samples with evidence of human presence) and D (two samples rich in total organic charcoal and total palaeobotanic elements) were not fluvial in origin; they contained low levels of pollen and *C*. and were therefore not suitable for the analysis we carried out. In the next stratigraphic level B4, chemical palynological analysis showed much pollen, many *C*. specimens, some spores and insignificant levels of carbon and other palaeobotanical substances. This sample was therefore perfect for our molecular and microscopic characterization of *C*. Stratigraphic analysis of B generally revealed a statistically significant presence of pollen, both as number of grains and variety of plant taxa found, as well as typical associated flora. This was secondary pollen deposition caused by a fluvial deposit. The pollen indicated plants like *Picea*, *Fagus*, *Pinus*, *Fraxinus* typical of mountainous coniferous forests and diverse Mediterranean vegetation with *Pinus*, *Corylus* and *Alnus*. Herbaceous plants included *Gramineae* and *Chenopodiaceae* and *Compositae* typical of areas settled by humans, and *Polypodium* and *Verbascum* that grow in forests were found. We also found wetland flora like the *Hydrocharitaceae* and *Lemna*, in line with the concept that *C*. lived in marshy habitats. *Polygonaceae*, *Scrophularieceae* and *Valerianaceae*, typical of Mediterranean wetlands, were also found.

3.2. C. cell ultrastructure and DNA analysis

When we used the alternative method of progressive sieving and percoll-gradient sedimentation, many suitable samples

were revealed in the B4 stratification and the 80% percoll-gradient band. Fig. 3 is a light micrograph showing diffuse DAPI staining of *C*. in vitro where fossil DNA is apparent in the wall. Fig. 4 is the control of the same grain showing many details, high electron density and substantially intact cytoplasmic elements. In contrast, micrographs of the wall of *C*. treated by the conventional palynological method were not well-defined: the granule was transparent and did not appear to contain cytoplasmic components (Fig. 5). Ultrastructural observations of thin sections by LM (Fig. 6) showed cytoplasmic material in part of a *Concentricystis* granule as dark bodies of different sizes and density protected by a wall about 4 μm thick. TEM analysis of the same grain showed well preserved fossil plastid cytoplasmic material in dark bodies inside the grain (Fig. 7). Ultrastructure included irregular vesicles and traces of endoplasmic reticulum. Compact cytoplasmic aggregates were embedded in the fossilized material. SEM observations confirmed the fascinating uneven external surface of *C*. and clear, fibrous concentric striations (Fig. 8).

Four single *C*. were used for individual PCR amplifications of the spacer region of the chloroplast genome between *rrn5* and *trnR*. Each product, arbitrarily named conc 1, conc 2, conc 3 and conc 4, was analysed on agarose gel and molecular weight ranged from 318 to 347 bp. BLAST search for each product sequence confirmed the specificity of amplification for the chloroplast genome, although different orthologous sequences belonging to different Angiosperms were recognized. Namely, conc 1 significantly blasted (score > 300) to the chloroplast genome of five eudicot species (*Brassica napus*, *Spinacia olearia*, *Oenothera elata*, *Nicotiana tabacum*, *Atropa belladonna*), conc 2 (score > 200) blasted to chloroplast genome of six eudicot species (*Alnus incana*, *Panax ginseng*, *S. olearia*, *A. belladonna*, *N. tabacum*, *Castanea sativa*), conc 3 and conc 4 were significantly homologous with chloroplast genomes of five monocot species (*Triticum aestivum*, *Oryza sativa*, *Oryza nivara*, *Zea mays*, *Saccharum officinarum*). CLUSTAL W alignment revealed several gaps and indels, especially along polypurine or polypyrimidine stretches (Fig. 9) between the different sequences and this could explain the differences in BLAST results.

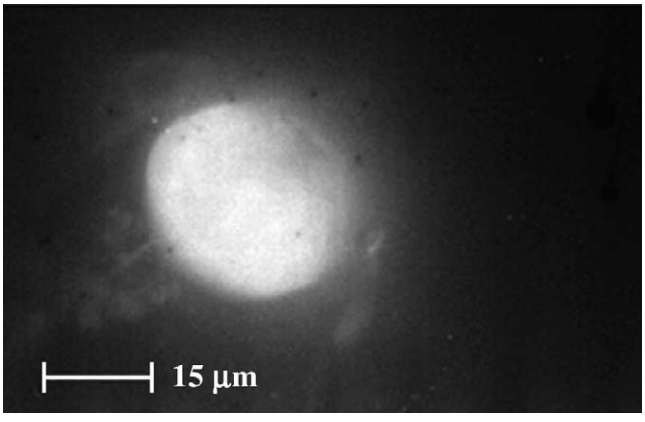

Fig. 3. Light fluorescence micrograph of *Concentricystis* after DAPI staining in vitro.

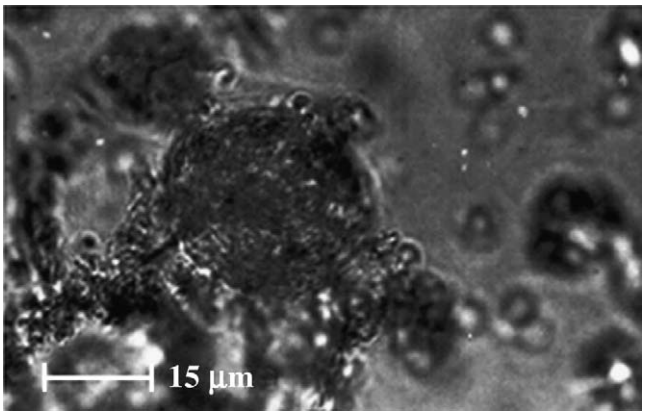

Fig. 4. Control micrograph of *Concentricystis*.

4. Discussion

The Afra has been associated with freshwater marshes and swamps in the upper Tiber valley (Alta Val Tiberina). The study site is currently at 430 m above sea-level in a gorge which forms a pass through the mountains with a small open slope inside the entrance to the gorge. The area was prone to flooding as shown by nine layers of deposited sediment. The Holocene stratifications indicated D–C were rich in human remains and charcoal, whereas sample B did not contain any charcoal or palaeobotanical materials. The B stratification was evidently colluvium. The high plant biodiversity of this stratum and the low percentage of pollen and spores in the D and C strata make it difficult to scan pollen associations, plot palynological data in a precise manner or determine palaeo-environmental conditions in distinct vegetation layers. According to classical palynological methods, *C*. were associated with wetland flora in the nine stratifications. We therefore deduced that the site was marshy and the climate was humid temperate. The soil of stratum B contained silt and clay and that of strata C and D, sand and clay. The last sediments of human origin were found in C5; all human activity suddenly ceased in the layer above, suggesting that the site was

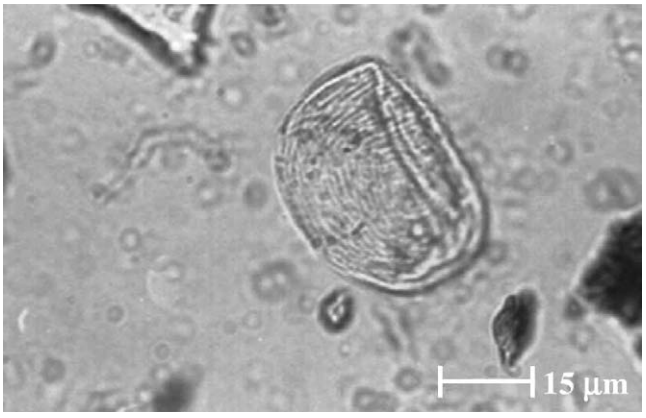

Fig. 5. Light micrograph of *Concentricystis*. The sample, processed by conventional chemical treatment, appears transparent and cytoplasmic components are not visible.

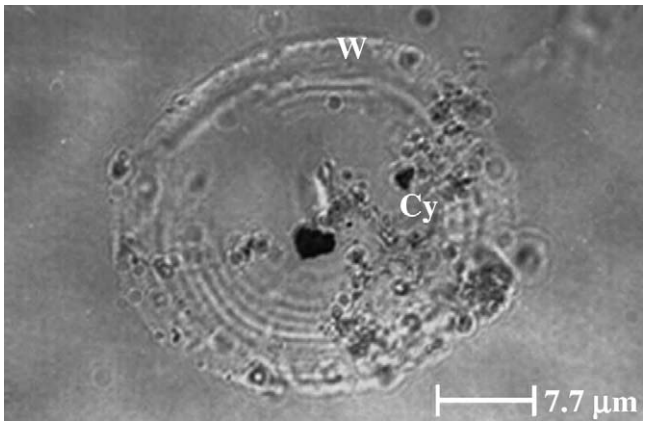

Fig. 6. Light micrograph of section of *Concentricystis*, showing cytoplasmic material (Cy) and thick wall (W).

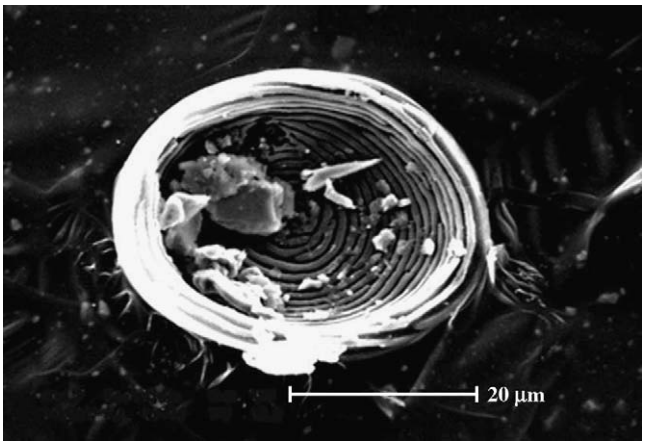

Fig. 8. SEM micrograph showing external morphology of *Concentricystis*. Resistant sediment residuals are visible on the wall.

abandoned. In B, *C*. were embedded in silt deposits, an anaerobic environment which enabled their preservation [20].

One of the main problems in the analysis of fossil DNA is the likelihood of contamination by natural exogenous factors from the micromanipulator and laboratory. Recent studies have demonstrated and we have confirmed that the mineral substrate provides formidable protection against contamination [5]. In earlier attempts, *C*. fossils were not detected by LM as they were embedded in sediment [4] and their cytoplasm therefore did not stain. The fossil particles were quickly released from the matrix by HCl and HF treatment, which also removed organic matter for subsequent molecular procedures, carried out under sterile conditions. DAPI staining confirmed the existence of fossil DNA, showing light microscope evidence of highly luminous parts of individual *C*. specimens. The diffuse nature of DAPI staining in the cells may be because fossilization disperses nuclear and plastid DNA in the cytoplasm. LM and TEM observations of ultrathin sections confirmed this dispersion.

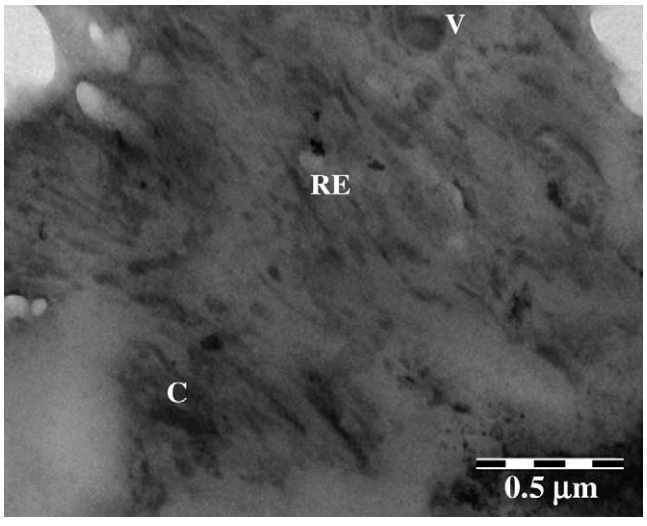

Fig. 7. Ultrastructure of fossil cytoplasm of *Concentricystis* showing vesicles (V), compact aggregates (C) and endoplasmic reticulum (RE).

The origin of *C*. has long been debated. These organisms, which have never been found alive, are known under various published names such as *Brodispora*, *Chomotriletes minor*, *Circulisporites*, *Concentricystis circulus*, *C. rubinus* and *Pseudoschizaea* [3,7,15]. They were first isolated from the Pleistocene of Israel, but they are known to exist from the Devonian. Their taxonomic collocation was exclusively morphological, on which basis the genus is reported to be very similar to genera of other probable fossil algal spores, such as *Circulisporites* [10].

The PCR primer was designed to amplify spacer regions in the chloroplast genome between *rrn5* and *trnR* of higher plants, corresponding to coding regions on both sides of eight chloroplast spacer regions of *Pinus thunbergii* and *N. tabacum* [9]. Primer choice was largely based on evidence that they are very reliable and robust molecular markers that give reliable results on residues of fossil material [19]. Our molecular data demonstrate that *C*. DNA is very similar to the chloroplast genome of Angiosperms. It seems noteworthy that all the sequences derived from *C*. DNA were significantly recognized by BLAST search in orthologous regions of different Angiosperm species. Due to the strong homology between the fossil *C*. sequence and the chloroplast genome of higher plants, we do not exclude a relation between *C*. and living algal genera of the *Zygnemataceae* [7]. This hypothesis is not new and is not incompatible with molecular data suggesting homology between fossil DNA and chloroplast DNA of higher plants, since high conservation of chloroplast DNA of higher plants and certain algae has been demonstrated [21]. Since the primers used are robust and reliable, having the capacity to amplify fossil DNA, their target is presumably highly conserved regions, less prone to degradation during aging. The fact that we obtained four similar but not identical amplification products implies the validity of the method, since amplification products from any contaminants would be identical in sequence. The observed polymorphism was to be expected for a non-coding intervening sequence and it seemed more intense in polypurine and polypyrimidine stretches, in line with previous studies on hypervariable regions [2]. The variability of non-coding chloroplast DNA regions for phylogenetic studies and population genetic analysis is well documented [16]. The

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conc3  TCTACTGCGGTGACGATACTGTAGGGGAGGTCCTGCGGCAAAATAGCTCGATGCCAGAAT 60
conc4  TCTACTGCGGTGACGATACTGTAGGGGAGGTCCTGCGGCAAAATAGCTCGATGCCAGAAT 60
conc1  TCTACTGCGGTGACGATACTGTAGGGGAGGTCCTGCGGAAAAATAGCTCGACGCCAGGAT 60
conc2  TCTACTACGGTGACGATACTGTAGGGGAGGTCCTGCGGAAAAATAGCTCGACGCCAGGAT 60
****** ****************************** ************ ***** **

conc3  GATAAAAAGCTTAACACCTCTTAATTTGACTTTTTCACTATTTTGAAATACGAAAAAGAT 120
conc4  GATAAAAAGCTTAACACCTCTTATTTT-ACTTTTTCACTATTTTGAAATACGAAAAAGAT 119
conc1  GATAAAAAGCTTAACACCTCTCATTCTTATTCCTT------TTTCAA---CGAAAAA--- 108
conc2  GATAAAA-GCTTAACACCTCTCATTCTTATTACTT------TTTCAAATTCAAAAAG--- 110
******* ************* * * * * *  **      *** **   * ****

conc3  CCAAATATAAAATGCAAAGGTCGTCTTATTCAAAACCTCAAT-----CATCACATCCCCT 175
conc4  CCAAATATAAAATGCAAAGGTCGTCTTATTAAAAACCTCAAT-----CATCACATCCCCT 174
conc1  ---TATGAAAAATCAAAAGGTCGTCTTATTCAAAACCCCAAT-----TATGACATCCCTT 160
conc2  ---AAAAATAAATGAAAAGGTCGTCTTATTCAAAACCCCAATCCAATTATGAAATCCCTT 167
*    ****  *************** ****** ****      ** * ***** *

conc3  TTCTCCCACTTCACACCTTGGAACGCGCTGTTTTTATAG------AGAGAAAGGCGCTTT 229
conc4  CTCTCCCACTTCACGCCTTGGAACGCGCTGTTTTTATAG------AGAGAAAGGCGCTTT 228
conc1  CTCTCCCACTTCACACCTCGGAACGCACCGTTCTTATAG------AGGTAAACGCGCTTT 214
conc2  CTCTCCCACTTCACACCTCGGAACTCACCGTTCTTATATTTATATAGAGAGATGCGCTTT 227
************* *** ***** * * *** *****       **  * * *******

conc3  CCCATCTTCTTAACCCGAAATG----------------A-------AGG----------- 255
conc4  CCCATCTTCTTAACCCGAAATG----------------A-------AGG----------- 254
conc1  CACATCTTCTTAACCCGAAATGAAATGGCTGGGGAGGGGAGAGGAAAGGTTCCTTTTTTT 274
conc2  CACATCTTCTTAACCGGAAACT-----GCTGGG-----GAGAGGAAAGGTTCCTTTTTTT 277
* ************* ****                          ***

conc3  --G--GTACCTCCGGGAAGATATCCAGTTGAGACTGG-TG---CCTGTAGCTCAGAGGAT 307
conc4  --G--GTACC-CCGGGAAGAGATCCAGTGGAGACGGG-TGG-GCCTGTTGCTCAGAGGAT 307
conc1  TGAGGGTACTCCCGGGAACAGATCCAGTGGAGACGGGGTGGTGCCTGTAGCTCAGAGGAT 334
conc2  T-AGGGTACTCCCGGGAACTGATCCAGTGGAGACGGGGTGTTGCCTGTAGCTCAGAGGAT 336
****  *******   ******* ***** ** **   ***** ***********

conc3  TAGAGCACGTG 318
conc4  TAGAGCACGTG 318
conc1  TAGAGCACGTG 345
conc2  TAGAGCACGTG 347
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Fig. 9. CLUSTAL W (1.82), multiple sequence alignment of amplicons consisting of chloroplast genome spacer region between *rrn5* and *trnR* from a single *Concentricystis* specimen. Several gaps and indels are visible especially in polypurine and polypyrimidine stretches. Average total similarity between sequences is 80%.

novelty of the present study is the possibility of performing PCR and microscopic characterization studies on single fossil *C*. cells, enabling hypotheses on the taxonomy of *C*. and opening new areas of palaeobotanical research.

Acknowledgements

We sincerely thank Paolo Gambassini for help with sampling at the archaeological site and Sawhney K. Vipen, University of Saskatchewan, Canada, for his kindness and critical revision of the paper.

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