

Ultrastructure and DNA Sequence Analysis of Single Fossil Sediment Cells

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Abstract — The method we have developed allows the cytoplasm of a single fossil cell to be sufficiently preserved as to be distinguished by fluorescence microscopy. Analysis of fossil DNA involved PCR amplification of the spacer region between *rrn5* and *trnR* genes in the chloroplast genome. The DNA and consensus sequences, obtained from the same PCR amplification fragment, were analysed by BLAST search and CLUSTAL W multiple alignment software.

Key words: chloroplast DNA sequence; fossil sediment filtration; light fluorescence microscopy; PCR.

INTRODUCTION

Traditional palynological chemical maceration of sediment samples to study pollen grains and floral associations (MORE *et al.* 1991) does not preserve fossilized cytoplasmic components and destroys much of the information that pollen and spores could provide, as well as important molecular information (MILANESI *et al.* 2006). We propose an alternative method involving sieving and Percoll gradient-based protocols for concentrating cells (MINAMI *et al.* 1997). Specimens were identified by DAPI staining in detritus of sediments (KAWAMURO *et al.* 1995). Fossil cytoplasm was examined by molecular biology techniques based on PCR amplification using primers specific for chloroplast genome regions.

MATERIALS AND METHODS

Sieving and Percoll gradient - Approximately 10 g of sediment was mixed with 25 ml of sterile water. The specimen was sieved progressively with 0.30- to 0.008-mm filters. The particles remaining in the 0.008-mm sieve were washed repeatedly in sterile water. By gravity the material was sedimented on a Percoll (Sigma) gradient (60-100%) diluted with sterile water (MINAMI *et al.* 1997). About 5 min after sedimentation, material was collected from the 60%, 80% and 100% bands from the sediment at the bottom of the tube. Specimens of interest

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.

DAPI staining and sample collection - DAPI staining was performed to locate fossil specimens in the residues (KAWAMURO *et al.* 1995). The stained granules were observed under a Zeiss Axiophot fluorescence microscope under a laminar flow hood. By use of a micromanipulator with a sterile wooden arm carrying a bristle specifically mounted for the task, single granules were collected in a sterile PCR tube.

DNA analysis - 10 µl sterile milliQ water was added to each single 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. Samples were placed on ice until PCR components were added. 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 (final concentrations): 1x Taq buffer (Amersham Pharmacia Biotech), 200 µM dNTP, 0.5 pmol forward primer (5'-TGGTGTCCCAG-GCGTAGAG-3'), 0.5 pmol reverse primer 5'-CGACACCGTGGTTCGTAGC-3'), 5U 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 sec at 94°C, 15 sec at 62°C, and 25 sec at 72°C. A final extension step of 10 min at 72°C was added.

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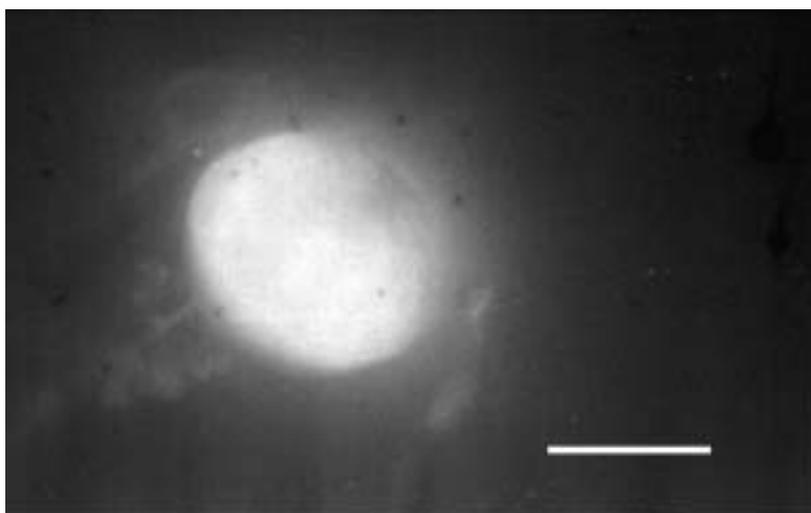


Fig. 1 — Light fluorescence micrograph of fossil single granule after DAPI staining *in vitro*. Bar = 25 μ m.

Secondary amplification was performed using 0.5 μ l 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'-CACGTGCTCTAATCCTCT-3'. Negative controls were included for the first and second amplifications. Amplification products were loaded into a 1% agarose gel and run at 60 V in TAE 1x buffer. 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 *E. 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 and CLUSTAL W multiple alignment software (ATTIMONELLI *et al.* 2002; LANAVE *et al.* 2002). The consensus sequences were obtained from analysis of five different recombinant TOPO vectors harbouring the same PCR amplification fragment.

RESULT AND DISCUSSION

Samples were mostly found in the 80% Percoll-gradient band. DAPI staining confirmed the existence of fossil DNA, with light microscopy showing highly luminous parts of individual specimens (Fig. 1). The PCR primers were de-

signed to amplify spacer regions in the chloroplast genome between the *rrn5* and *trnR* genes of higher plants. Primers used are robust and reliable, and have the capacity to amplify fossil DNA (SUYAMA *et al.* 1996). Their target is presumably a highly conserved region not particularly prone to degradation during aging. Each product was analysed on agarose gel and molecular weight ranged from 318 to 347 bp (Fig. 2). BLAST search for each product sequence confirmed the specificity

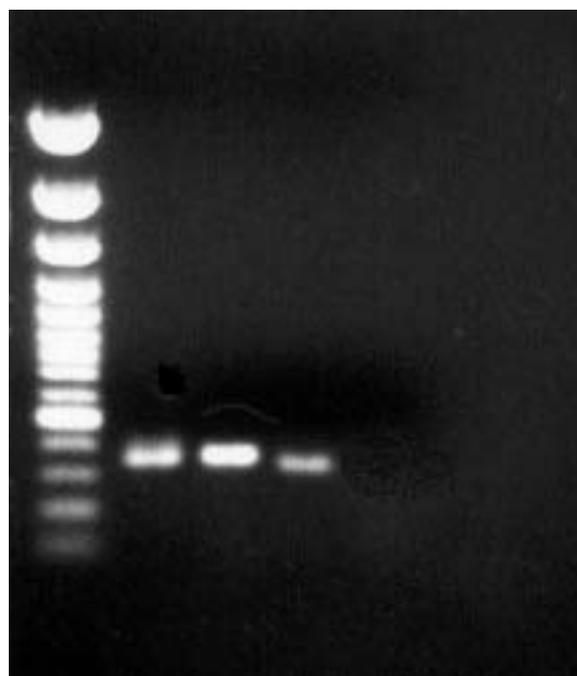


Fig. 2 — PCR products of *Concentricystis* single granule tested on a 2% agarose gel stained with ethidium bromide. Molecular weight ranged from 318 to 347 bp.

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CLUSTAL W (1.82) multiple sequence alignment
samp3 TCTACTGCGGTGACGATACTGTAGGGGAGGTCCTGCGGGCAAATAGCTCGATGCCAGGAT 60
samp1 TCTACTGCGGTGACGATACTGTAGGGGAGGTCCTGCGGGAAAATAGCTCGACGCCAGGAT 60
samp2 TCTACTACGGTGACGATACTGTAGGGGAGGTCCTGCGGGAAAATAGCTCGACGCCAGGAT 60
*****
samp3 GATAAAAAGCTTAAACACCTCTTAATTTGACTTTTTCACTATTTTGAATACGAAARAGAT 120
samp1 GATAAAAAGCTTAAACACCTCTCATTCTTATTCCTT-----TTTCAA---CGAAAR--- 108
samp2 GATAAAA-GCTTAAACACCTCTCATTCTTATTACTT-----TTTCAATTCAAAAG--- 110
*****
samp3 CCAATATAAATGCARAGGTCGTCTTATTCAAAACCTCAAT-----CATCACATCCCGCT 175
samp1 ---TATGAAJAAATCAAAAGGTCGTCTTATTCAAAACCCCAAT-----TATGACATCCCTT 180
samp2 ---AAAATAAATGAARAGGTCGTCTTATTCAAAACCCCAATCAATTATGAATCCCTT 167
*****
samp3 TTCTCCCACTTCAACACCTTGGACGCGCTGTTTTATAG-----AGGAAAGGCGCTTT 229
samp1 CTCTCCCACTTCAACACCTGGGACGCGCTTCTTATAG-----AGGTAAACGCGCTTT 214
samp2 CTCTCCCACTTCAACACCTGGGACTCAACGCTTCTTATATTTATATAGAGGATGCGCTTT 227
*****
samp3 CCCATCTTCTTAAACCCGAATG-----A-----AGG----- 255
samp1 CACATCTTCTTAAACCCGAATGAATGGCTGGGGAGGGGAGGAGGAAAGGTTCCCTTTTTT 274
samp2 CACATCTTCTTAAACCCGAATCT-----GCTGGG-----AGGAGGAAAGGTTCCCTTTTTT 277
* *****
samp3 --G--GTACTCCGGGAGATATCCAGTTGAGACTGG-TG---CCTGTAGCTCAGAGGAT 307
samp1 TGRGGTACTCCCGGACAGATCCAGTGGGACCGGGTGGTGCCTGTAGCTCAGAGGAT 334
samp2 T-AGGGTACTCCCGGACTGATCCAGTGGGACCGGGTGGTGCCTGTAGCTCAGAGGAT 336
**** *****
samp3 TAGAGCACGTG 318
samp1 TAGAGCACGTG 345
samp2 TAGAGCACGTG 347
*****

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Fig. 3 — 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%.

of amplification for the chloroplast genome, although different orthologous sequences belonging to different Angiosperms were recognized. Namely, sample 1 significantly blasted (score > 300) to the chloroplast genome of 5 eudicot species (*Brassica napus*, *Spinacia oleraria*, *Oenothera elata*, *Nicotiana tabacum*, *Atropa belladonna*), sample 2 (score > 200) blasted to the chloroplast genome of 6 eudicot species (*Alnus incana*, *Panax ginseng*, *Spinacia oleraria*, *Atropa belladonna*, *Nicotiana tabacum*, *Castanea sativa*), sample 3 showed significant homology to chloroplast genomes of 5 monocot species (*Triticum aestivum*, *Oryza sativa*, *Oryza nivara*, *Zea mays*, *Saccharum officinarum*). Our molecular data demonstrates that the amplified fossil DNA is very similar to the chloroplast genome of Angiosperms. CLUSTAL W alignment revealed several gaps and indels especially along polypurine or polypyrimidine stretches (Text-Fig. 3) between the different sequences and this could explain the differences in BLAST results.

It seems noteworthy that all the sequences derived from fossil DNA were significantly recognized by the BLAST search in orthologous regions of different Angiosperm species. The fact that we obtained three 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 (BOGANI *et al.* 2001). The variability of non-coding chloroplast DNA regions for phylogenetic studies and analysis of population genetics is well documented (SMALL *et al.* 2005).

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