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COMBINED SEM ULTRASTRUCTURE STUDIES AND PCR WITH INDIVIDUAL DIATOM CELLS

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Perforation of frustules of individual cells of the diatom *Aulacoseira skvortzowii* Edlund, Stoermer & Taylor with an extended glass needle controlled by a micro-manipulator was used to liberate the protoplast. The isolated total DNA was used in subsequent amplification with specific primers and sequencing of a fragment of the rbcL gene. Comparison of the sequence of the amplified rbcL gene fragment from *A. skvortzowii*, with those known for other plants, revealed that it is closest to the sequence of rbcL from the marine diatom *Skeletonema costatum* (12 amino acid substitutions over a fragment of 130 amino acid residues). Features of the ultra-structure of *A. skvortzowii* that are important for species identification (form and density of areolae, form of suture spine, etc.) remained intact when using this technique. The combined approach promises a breakthrough in the application of DNA sequencing to diatom research.

INTRODUCTION

As for many other organisms, the sequencing of genes of diatoms has become a valuable tool for phylogenetic analysis (e.g., Kooistra & Medlin 1996, Medlin *et al.* 1996). Existing diatom systematics relies exclusively on their morphology, especially on the diagnostic features of the siliceous frustules as revealed by SEM. Hence, along with isolating and amplifying DNA, it is always necessary to study the ultastructure of any given assemblage and make sure that all diatom cells belong to the same species. For this reason, amplification and sequencing of diatom DNA is usually done with cells taken from pure laboratory cultures. The only exception was the sequencing of a fragment of the rRNA gene of *Aulacoseira skvortzowii* Edlund, Stoermer & Taylor from Lake Baikal where DNA was extracted from *ca.* 300 cells belonging to colonies (filaments) taken from a natural population (Sherbakova *et al.* 1998). *A. skvortzowii* filaments were picked up manually under the binocular microscope. They were easy to identify because of their recognizable resting spores – no other diatoms of Lake Baikal have the same feature. However, this is not a general approach that can be done with other diatoms.

The necessity to obtain pure laboratory cultures is also a serious limitation. Up to this date, only a small fraction of the known diatom species has been successfully introduced into culture. Moreover, many diatoms change their ultrastructure in cultures, and become difficult to recognize with the keys describing species collected in the field. However, it is known that modern techniques of PCR easily permit sequencing of DNA fragments obtained from a single cell (Becker *et al.* 1996, Deliabie *et al.* 1994). In the case of diatoms, single cells can be taken from any natural population – the only problem is reliable identification of those cells from which DNA was extracted. The present paper

describes results of the application of a method that allows both amplifying and sequencing DNA from an individual diatom cell but also examining its ultrastructure by means of SEM.

MATERIALS AND METHODS

The procedure of perforating diatom cell walls was performed on an inverted microscope (Axiovert 10, Zeiss, Germany) coupled to a manual micro-manipulator (MR) from the same company. All the operations with nanoliter volumes were done with Pasteur pipettes (D = 50 μm), and glass needles (tip diameter 0.3 μm) for breaking siliceous frustules. The instruments for micro-manipulation were extended from capillaries using a micro-puller.

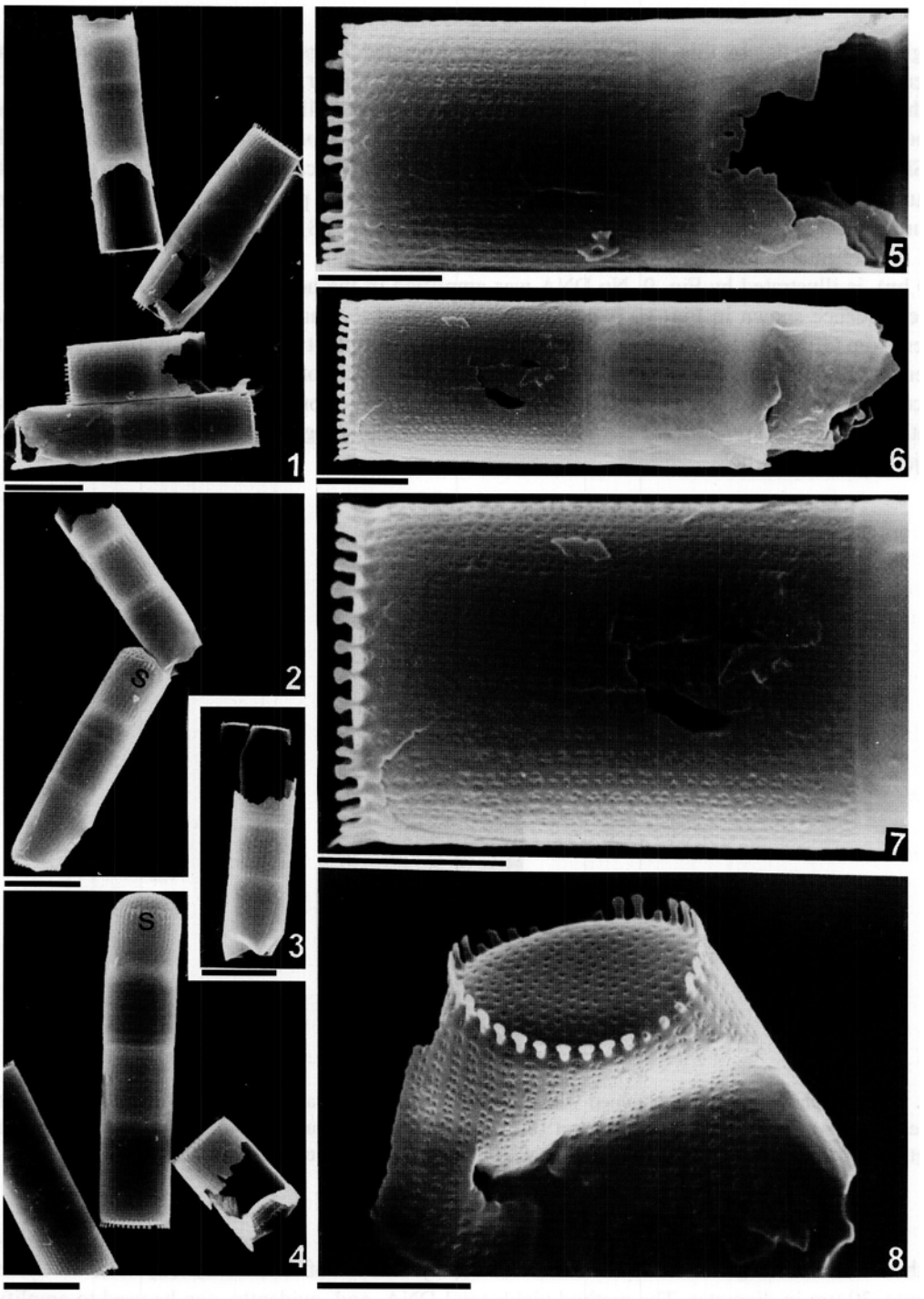
A. skvortzowii cells were taken from a laboratory culture prepared from material collected in Lake Baikal by Dr C. Gibson (Aquatic Sciences Research Division, Department of Agriculture, Northern Ireland). The cells were harvested during sporulation, fixed and subsequently stored in 80% ethanol. Fifteen cells were transferred with a Pasteur pipette onto a siliconized cover glass and placed into the center of a glued-on silicon resin ring. The glass was placed onto the stage of an inverted microscope. After evaporation of the ethanol, the mantles of the frustules were perforated near their faces by a glass needle driven by the micro-manipulator. For this purpose, the tip of the needle was moved close to the selected point on the frustule, and the manipulator base was gently knocked with a finger. By repeated moving the needle over the surface of the frustule and knocking, the mantle was broken leaving a hole. 50 nl of a proteinase K solution (0.5 mg/ml in 0.1% SDS, or 0.1% Triton X100, 10 mM Tris-HCl, pH 7.5) was placed on the cover glass. The protoplast escaped into the solution. The drop was covered with liquid paraffin, then the cover glass placed in a Petri dish, and the latter placed in an incubator. After 2 hours of incubation at 60°C, the drop was diluted with 250 nl of 0.1% Triton X100, and the solution obtained was transferred into a tube containing 15 μl of PCR buffer.

Three microlitres (one-fifth) of the DNA solution thus obtained was put into 15 μl of amplification buffer. Redundant primers complementary to regions coding for the amino acid sequences KPKLGLS and VVGKLEG of ribulose-1,5-diphosphate carboxylase were used as proposed by Hu & Tabita (1996). The primers 5'-AARCCWAARYTAGGKYTWT and 5'-TTCYTCYARYTTACCWACDAC (W= A/T, R =A/G, K = T/G, Y = C/T, D = A/G/T) served to amplify a fragment of the rbcL gene corresponding to a region of the large subunit of the enzyme. The length of the selected gene fragment was *ca.* 400 bp. The amplification was performed with AQ-polymerase and started by pre-heating the samples for 180 sec at 95°C followed by 35 cycles: 60 sec at 94°C, 60 sec at 54°C and 60 sec at 72°C with 10 minutes incubation at 72°C after the last cycle. Direct sequencing of the amplified fragment was performed by the method of Sanger (Sanger, Nicklen & Coulsen 1977) with the same primers. Both chains were sequenced resulting in an overlap of 90%.

After the solution of isolated DNA had been taken with a capillary from under the paraffin drop, the perforated frustules were left on the cover glass. They were dried in the open air, and the oil removed by washing three times with hexane. After evaporation of hexane, the preparation was treated with 30% hydrogen peroxide for 2h at 75°C, washed with water, dried and gold-plated. The perforated frustules were examined by means of a Philips 525M scanning electron microscope.

RESULTS AND DISCUSSION

Figs 1–8 illustrate the effect of breaking *A. skvortzowii* frustules with an extended glass needle, controlled by a micro-manipulator. This technique also allows separation at the sutures linking



Figs 1–8. SEM images of frustules of *A. skvortzowii* after disruption by means of micro-manipulation and subsequent removal of protoplasts for PCR. **Figs 1–5**. Fragments obtained by disruption at sutures, and between individual cell valves. **Figs 6, 7**. A perforated valve mantle. **Fig. 8**. Valve face of a perforated valve; (s) – a developing spore. Scale bars: 10 μm (Figs 1–4), 5 μm (Figs 5–8).

neighbouring cells. In this way, protoplasts are liberated (Figs 1–4). Some cells were damaged extensively (Figs 1, 3, 5), but it was also possible to make smaller holes in such way that a major part of the valve surface remained intact (Figs 6, 7) and displayed all elements of ultrastructure that are important for unequivocal identification of the species as *A. skvortzowii* (Babanazarova, Likhoshway & Sherbakov 1996, Edlund, Stoermer & Taylor 1996). In this case: diameter 8.5–9 μm, height 8–11 μm; number of areolar rows 16–18 per 10 μm; 20–25 areolae per 10 μm of row; spatulate form of suture spines; even distribution of areolae on valve face (Figs 7, 8). Spores which are shown in Figs 2 and 4 were intact. The result of amplification of DNA, a single product of the expected length (*ca.* 400 bp), is illustrated by Fig. 9. No DNA was amplified in the negative control. *A. skvortzowii* rbcL gene sequence shown in Fig. 10 is compared with that of the marine diatom *Skeletonema costatum* taken from GENBANK. Alignment of the gene fragments of the two species is unambiguous, without insertions or deletions. The number of substitutions is 46 (12%), of which 27 are in the third positions of codons. The translated DNA fragment of *A. skvortzowii* contains 12 amino acid substitutions relative to that of *Skeletonema costatum.* (Fig. 11). The placing of *A. skvortzowii* on a tree is the subject of a future article.

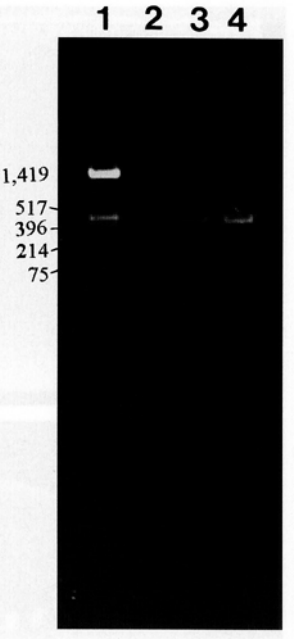


Fig. 9. Electrophoretic patterns obtained with the products of amplification of the rbcL gene of *A. skvortzowii* (lanes 3 and 4). Lane 2 is negative control (PCR without extract from *A. skvortzowii* protoplasts), lane 1 is lengths marker, PUC 18/Hinfl digest. Agarose gel (0.9%), staining with ethydium bromide.

The form and size of the diatom cell does not limit the application of this technique – the method works with a disc-like (in contrast to the cylindrical of *A. skvortzowii*) cells of *Cyclotella minuta*, 30 μm in diameter. The method yields total DNA, and, evidently, can be used to amplify any selected gene. Chloroplast and nuclear genes are sequenced for the purpose of phylogenetic reconstruction (Samigullin *et al.* 1998, Dauberg & Andersen 1997, Sorhannus *et al.* 1995). Studies of molecular evolution using diatoms presents a special interest because these plants left a unique and continuous paleontological record in both marine and lacustrine sediments.

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                .          .          .          .          .
A.skvo  ATGAAGGTTTAAAAGGCGGTTTAGATTTCTTAAAAGATGATGAGAATATA
S.cost                  T          C          G          A     T

A.skvo  AACTCACAACCATTTATGCGTTGGAGAGAGCGTTTCTTATACTGTATGGA
S.cost       T          C                A          A

A.skvo  AGGTATTAACCGTGCTTCAGCTGCAACAGGTGAAGTTAAAGGTTCTTACT
S.cost                 A          T

A.skvo  TAAACGTTACTGCTGCTACAATGGACGAAGTTATTAAACGTGCAGAGTAC
S.cost                            A     ATAC         T     T

A.skvo  GCTAAAGAAGTAGGTTCTGTAATTATCATGATCGATTTAGTTATGGGTTA
S.cost         CT  T       A CG  G T                A

A.skvo  CACAGCAATCCAAACAATTGCTATCTGGTCTCGTGAAAATGATATGATTT
S.cost     T      T   T        ATA    G          C       T A

A.skvo  TACATTTACACCGTGCAGGTAACTCAACTTATGCTCGTCAAAAAAATCAT
S.cost      C           T         T  A  C           G

A.skvo  GGTATTAACTTCCGTGTAATTTGTAAATGGATGCGT
S.cost                   T  C
```

Fig. 10. Nucleotide sequence of the amplified fragment of the chloroplast rbcL gene of *A. skvortzowii* compared to that of the marine diatom *S. costatum*.

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                .          .          .          .
A.skvo   EGLKGGLDFLKDDENINSQPEMRWRERFLNCMEGINRASAATGEVKGSYL
S.cost                                  Y

A.skvo   NITAATMEEVYKRAEYAKAVGSIVVMIDLVMGYTAIQSIAYWARENDMLL
S.cost    V     D  I        E   VII             T  I S     I

A.skvo   HLHRAGNSTYARQKNHGINFRVICKWMR
S.cost
```

Fig. 11. Aminoacid sequence of a fragment of ribulose-1, 5-biphoshate carboxylase of *A. skvortzowii* compared to that of *S. costatum*.

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