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TECHNICAL NOTE

Single-cell sequencing of dinoflagellate (Dinophyceae) nuclear ribosomal genes

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Abstract

Genetic sequences from dinoflagellates offer valuable information regarding taxonomies, phylogenies and population genetics that generally require the growth of these organisms in culture. We have developed a quick and simple method to obtain small and large subunit ribosomal gene sequences from dinoflagellates using single cells. This method, based on freeze–thaw cell lysis and a simple two-step polymerase chain reaction, provides template for sequencing in 6–8 h. We have sequenced five dinoflagellate species, including unculturable *Dinophysis* and *Ceratium* species, using fresh and frozen samples.

Keywords: dinoflagellates, *Dinophysis*, harmful algal blooms (HABs), ribosomal DNA (rDNA), single-cell

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Correct identification of species is crucial to all biological studies, and this task is particularly challenging when dealing with microorganisms. Light- and electron-microscopic identification of microorganisms is labour-intensive, requires considerable expertise and can be inaccurate due to morphological changes in the organisms caused by environmental conditions or life history. This is the case with marine dinoflagellates, which are often responsible for harmful algal blooms (HABs) that pose health threats to human and marine life, cause serious economic losses to the fishery and mariculture industries and have major environmental impacts (Hallegraeff 1995). Considerable efforts have been made over the last decade towards genetic characterization of dinoflagellate species, mostly based on nuclear ribosomal DNA (rDNA) markers, with sequence data being increasingly used in diagnostic polymerase chain reaction (PCR) and hybridization assays in HAB monitoring programmes (Scholin & Anderson 1998). A severe constraint in these efforts has been the difficulty in obtaining DNA sequences from species that do not grow in culture and are only available in small numbers from field samples. A method has been described recently to obtain sequences from single dinoflagellate cells (Marín *et al*. 2001). Independently, we have developed a rapid and simple method to obtain small subunit (SSU) and large subunit (LSU) ribosomal sequences from single dinoflagellates cells.

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Classical methods for DNA extraction from dinoflagellates require axenic clonal cultures derived from cells isolated from seawater samples. The culturing process is a lengthy and frequently unsuccessful one, as is the case with the diarrhetic toxin-producing *Dinophysis* species (Taylor *et al*. 1995). However, these limitations can be overcome by using the PCR as a 'fast-growing DNA culture'. We have used a freeze–thaw lysis and a two-step PCR method, avoiding lengthy enzymatic lysis steps (Marín *et al*. 2001), to obtain partial SSU and LSU rDNA sequences using a single dinoflagellate cell as template source. This technique could be employed to study genetic markers from seawater isolates without culturing and should be of great interest for taxonomic, phylogenetic and population genetic studies.

Dinoflagellate cultures (*Protoceratium reticulatum* strain CTCC1, *Scrippsiella trochoidea* strain CTCC14 and *Alexandrium catenella* strain CCMP1598) were obtained from Marine and Coastal Management, Cape Town. Field samples containing *Dinophysis acuminata* and *Ceratium furca* were collected with plankton nets in Lambert's Bay, South Africa. Single dinoflagellate cells either from cultures or field samples were microscopically isolated, rinsed in filtered seawater, transferred by micromanipulation into

0.2 mL thin-walled PCR tubes containing 10 μL of sterile distilled water and stored at –20 °C.

Extraction of genomic DNA from dinoflagellate cultures followed a standard phenol–chloroform procedure (Bolch *et al*. 1999). Single cells in PCR tubes were subjected to three consecutive freeze–thaw cycles to lyse the cells, alternating baths of liquid nitrogen and water at 90 °C for 20 s. An initial PCR amplification was carried out on genomic DNA from cultures and single cells using the same reaction parameters. Reaction volumes (25 μL) contained: 4 mM $MgCl_2$, 1× reaction buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl pH = 8.8; 0.01% Tween-20; Bioline), 200 μM of each dNTP, 1 μM of each primer and 1 unit of *Taq* polymerase (Bioline). Template took two forms: a single cell subjected to freeze–thaw, or 25 ng of DNA extracted from culture. Primers ss5 (5′-CTGGTTGATCCTGCCAGTAG-3′) and ss936 (5′-CCTTGGCAAATGCTTTCA/GCAG-3′) were designed from the GenBank sequence of related species *Prorocentrum micans* to amplify an approximately 940-bp fragment of the SSU. Primers D1R and D3B (Hansen *et al*. 2000) were used to amplify domains D1-D3 (1050-bp) of the LSU. Amplification reactions were run on a PCR Sprint (Hybaid) with the following thermal profile: 94 °C 2 min; 30 cycles (94 °C 30 s; 50 °C 30 s; 72 °C 90 s); 72 °C 7 min. Standard procedures were followed to minimize risk of contamination, including positive and negative PCR controls. PCR products were visualized by agarose electrophoresis and single-cell amplicons were excised. DNA from the gel slice was purified by extraction through a filter tip as follows: the gel slice was inserted into the broad end of a 200-μL filter pipette tip, from which approximately 2 cm of the narrow end had been cut off; the pipette tip was introduced into a 2-mL eppendorf tube and 50 μL of sterile distilled water added; and the eppendorf was centrifuged at 13 000 ***g*** for 1 min. We used 1–5 μL of this elution as template in a PCR re-amplification with the primers, reaction mixture and cycling parameters as described, except that the amplification cycles were now reduced to 15. Three 25 μL-reaction re-amplification replicates from each single cell were pooled and purified using a CONCERT Rapid PCR Purification System (Life Technologies) according to the manufacturer's instructions except for the final elution, which was carried out in 50 μL of sterile distilled water. Between 0.5 and 2.0 μL of purified amplicon were used in a cycle-sequencing reaction using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) and run on an ABI PRISM 377 DNA Analyser (PE Biosystems). Both strands from at least two single cells of each species were sequenced. Ribosomal DNA sequences were aligned using CLUSTALW Multiple Alignment software (Thompson *et al*. 1994).

The initial amplification reaction generally produced visible products of predicted size. When the freeze–thaw lysis step was omitted, we had limited success with unarmoured dinoflagellates, and failed to produce amplification in armoured species (results not shown). We therefore consistently used the freeze–thaw method to guarantee cell lysis. More than 70% of single cells produced a visible band of expected length. Amplicon concentration showed inter- and intraspecific variation (Fig. 1a), which is a consequence of the small amount of starting template.

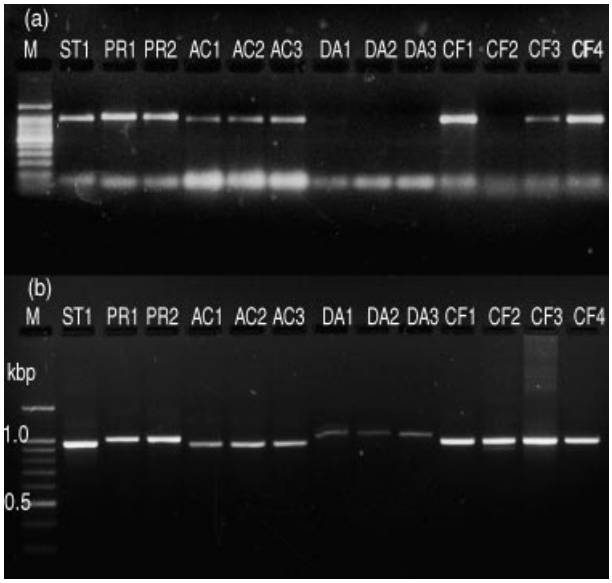

Fig. 1 Results of (a) initial amplification, and (b) re-amplification of partial small subunit (SSU) and large subunit (LSU) ribosomal DNA (rDNA) from dinoflagellates using single cells. Lanes (5 μL PCR product): M, 100-bp molecular marker; ST1, *Scrippsiella trochoidea*; PR1-PR2, *Protoceratium reticulatum*; AC1-AC3, *Alexandrium catenella*; DA1-DA3, *Dinophysis acuminata*; CF1-CF4, *Ceratium furca*. Numbers for each species denote replicate single-cell reaction for that species. PR and DA lanes are 1050-bp fragments of the LSU. ST, AC and CF lanes are 940-bp amplicons of the SSU. Negative and positive controls not shown.

Even when no product was seen after the first PCR, we excised the corresponding portion of agarose and performed the re-amplification step in the same way as the agarose excisions from visible products. Additional negative controls (an equivalent piece of agarose from a nonloaded gel section) were used to verify that no contaminants were present in the agarose. Re-amplification consistently produced visible and strong bands, even in cases when the initial amplification seemed to have failed. Variability in re-amplicon yield was greatly reduced by adjusting the amount of template between 1.0 and 5.0 μL. Figure 1(b) shows the results of re-amplification reactions. Attempts to use the initial PCR product directly as template for the re-amplification reaction produced nonspecific products, even after diluting the template.

The method described above has several advantages over the single-cell technique presented by Marín *et al*. (2001). These authors use a storage method which includes

agarose embedding, prelysis enzymatic digestion with cellulase and hemicellulase, a 48-hr incubation in proteinase K and repeated washes in TE. This represents at least three days of labour prior to the PCR step. In contrast, our method is simple, low-cost and rapid, allowing one to routinely go from single-cell to DNA sequence in one day.

We obtained sequences of approximately 800 bp from each strand using individual dinoflagellate cells as the source of template. Partial nuclear ribosomal subunit sequences for *D. acuminata*, *C. furca*, *A. catenella*, *P. reticulatum* and *S. trochoidea* have been deposited in GenBank (accession numbers AY027905–AY027909). Comparisons between sequences obtained from single cells and from source cultures (*P. reticulatum*) showed no nucleotide mismatches. Similarly, comparisons between sequences from single cells of the same species showed no variation. Sequence verification is particularly important given the highly conserved nature of ribosomal primers, the small amount of target template, and the risk of endosymbionts, parasites or environmental DNA, which could be easily amplified. Results from BLAST searches of GenBank databases confirmed that our sequences were ribosomal genes of dinoflagellates. We found minor divergences in nonconserved regions between our sequences and those available for conspecific dinoflagellates, which can be attributed to geographical variation. Where conspecifics where unavailable, we compared our sequences with those of sibling taxa.

We have described a simple, low-cost and rapid method to obtain dinoflagellate sequences from single cells, fresh or frozen. With a standard thermal cycler, template for sequencing can be prepared by this method in 6–8 h. In addition to the five species discussed in this paper, we have also sequenced two local *Alexandrium* species, *Dinophysis fortii* and *Zygabikodinium lenticulatum* (unpublished data), showing a broad applicability of this method. The main implication of the development of single-cell methods is that one can obtain sequences from species that are not easily cultured, and that these sequences can be obtained rapidly. Growing dinoflagellate cultures for sequencing purposes is no longer necessary, thus saving time and resources.

The emergence of methods to obtain DNA sequences from single dinoflagellate cells forecasts a rapid development of phylogenetic and population level studies. Dinoflagellate molecular phylogenies, to date lacking many unculturable taxa (Daugbjerg *et al*. 2000), may now be completed. A further major impact of this work is on HAB monitoring programmes, which may now be able to identify toxin-producing unculturable species such as *Dinophysis* using molecular tools.

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References

Bolch CJ, Blackburn SI, Hallegraeff GM, Vaillancourt RE (1999) Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* Graham. *Journal of Phycology*, **35**, 356–367.

Daugbjerg N, Hansen G, Larsen J, Moestrup Ø (2000) Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. *Phycologia*, **39**, 302–317.

Hallegraeff GM (1995) Harmful algal blooms: a global overview. In: *Manual on Harmful Marine Microalgae* (eds Hallegraeff GM, Anderson DM, Cembella AD), pp. 1–22. IOC-UNESCO, Paris.

Hansen G, Daugbjerg N, Henriksen P (2000) Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, comb. nov. (= *Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. *Journal of Phycology*, **36**, 394–410.

Marín I, Aguilera A, Reguera B, Abad JP (2001) Preparation of DNA suitable for PCR amplification from fresh or fixed single dinoflagellate cells. *Biotechniques*, **30**, 89–93.

Scholin CA & Anderson DM (1998) Detection and quantification of HAB species using antibody and DNA probes: progress to date and future research objectives. In: *Harmful Algae* (eds Reguera B, Blanco J, Fernandez ML, Wyatt T), pp. 253–257. Xunta de Galicia and IOC-UNESCO, Santiago de Compostela.

Taylor FJR, Fukuyo Y, Larsen J (1995) Taxonomy of harmful dinoflagellates. In: *Manual on Harmful Marine Microalgae* (eds Hallegraeff GM, Anderson DM, Cembella AD), pp. 283–317. IOC-UNESCO, Paris.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.