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Integrated Method for Single-Cell DNA Extraction, PCR Amplification, and Sequencing of Ribosomal DNA from Harmful Dinoflagellates *Cochlodinium polykrikoides* and *Alexandrium catenella*

Jang-Seu Ki,[1,2] Gi Young Jang,[2] and Myung-Soo Han[1]

[1]*Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea*
[2]*Bionex Genome Research Institute, #421 Hanyang Institute of Technology Building, Hanyang University, Seoul, 133-791, Korea*

Abstract: A simplified technique was developed for DNA sequence-based diagnosis of harmful dinoflagellate species. This protocol integrates procedures for DNA extraction and polymerase chain reaction (PCR) amplification into a single tube. DNA sequencing reactions were performed directly, using unpurified PCR products as the DNA template for subsequent sequencing reactions. PCR reactions using DNA extracted from single cells of *Cocodinium polykrikoides* and *Alexandrium catenella* successfully amplified the target ribosomal DNA regions. DNA sequencing of the unpurified PCR products showed that DNA sequences corresponded to the expected locus of ribosomal DNA regions of both *A. catenella* and *C. polykrikoides* (each zero genetic distance and 100% sequence similarity). Using the protocol described in this article, there was little DNA loss during the purification step, and the technique was found to be rapid and inexpensive. This protocol clearly resolves the taxonomic ambiguities of closely related algal species (such as *Alexandrium* and *Cochlodinium*), and it constitutes a significant breakthrough for the molecular analysis of nonculturable dinoflagellate species.

Key words: single cell, DNA extraction, Polymerase Chain reaction, ribosomal DNA sequencing.

INTRODUCTION

Harmful algal blooms (HABs) are a worldwide problem in the marine environment and have, in many cases, been linked to the presence of the toxic dinoflagellate genera *Cochlodinium* and *Alexandrium*. Traditionally species identification and monitoring of dinoflagellate populations has relied on microscopic examination of samples collected from wild populations. However, these conventional microscopic methods, based on the measurement of morphologic characters such as cell size and shape or the position of chloroplasts, are time-consuming and require considerable taxonomic experience (Godhe et al., 2001). In addition, many morphologic features are known to vary in response to changing environmental conditions, as well as during different growth stages (Sako et al., 1990), and are therefore considered unreliable for correct taxonomic identification.

The development of polymerase chain reaction (PCR) techniques has been a major step forward in resolving some

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Corresponding author: Myung-Soo Han; e-mail: hanms@hanyang.ac.kr

of the taxonomic ambiguities among the dinoflagellates, owing to their simplicity and high specificity (Godhe et al., 2001). However, PCR methods generally require the use of purified genomic DNA for the PCR template. Extraction and purification requires large quantities of cells free from contaminating organisms. This represents a special challenge for species that cannot be cultured under laboratory conditions, and in such circumstances representative material must be collected from wild populations when seasonal blooms occur (Marín et al., 2001). In addition, loss of DNA occurs in many of the procedures developed for isolation of DNA from dinoflagellate.

For these reasons Marín et al. (2001) designed a method to obtain pure DNA for PCR from small numbers of dinoflagellate cells (and even from single cells). Their protocol was used to prepare genomic DNA by a combination of physicochemical and enzymatic procedures, using cells embedded in agarose beads. Bolch (2001) also reported a PCR assay for genetic identification of dinoflagellates using DNA extracted directly from single cysts and vegetative cells. This procedure used liquid nitrogen to lyse cells. Both of these techniques were found to be useful for DNA extraction and subsequent PCR amplification from single cells without loss of DNA; however, no direct DNA sequencing, using unpurified PCR products amplified from a single cell, was tested. The 2 protocols for DNA extraction and sequence analysis described above require several steps of conventional DNA cloning of the PCR products prior to sequencing. These techniques are unsuitable for routine sequence-based identification of dinoflagellate species from natural populations owing to the time and labor required for purification.

In this report we describe an advanced technique for single-cell DNA extraction and direct PCR amplification of ribosomal DNA from the harmful dinoflagellates *Cochlodinium polykrikoides* and *Alexandrium catenella.* Because DNA sequencing can be performed directly on the PCR products, this technique is time-saving and has several potential applications for the analysis of genetic diversity within natural HAB populations.

Materials and Methods

Cultures and Strains

Clonal cultures of *Cochlodinium polykrikoides* (CCPK06) and *Alexandrium catenella* (AxCt_K01) were kindly provided by Dr. M. Chang of the Korean Ocean Research and Development Institute. The strains were maintained in f/2 medium, pH 8.2 (Guillard and Ryther, 1962), at 15°C under a light and dark regimen of 12 hour each, with an incident light intensity per second of approximately 100 μmol photons/m^2.

Single-Cell Isolation and Washing

Six drops of TE buffer were deposited on a round glass petri dish with a radius of 80 cm. One drop of culture was placed in the center of the petri dish, and individual dinoflagellate cells were isolated using a sterile micropipette under a dissecting microscope (Carl-Zeiss Ltd.). Individual dinoflagellate cells were then transferred to sterile TE buffer droplets 3 to 6 times to facilitate the removal of contaminants. Individual cells, suspended in approximately 1 μl of TE buffer, were then placed in 200-μl thin-walled PCR tubes containing a drop of mineral oil. Samples were then frozen at –20°C until analysis.

Enzymatic DNA Extraction

Proteinase K (1 μl 200 μg/ml) was then added, and the tubes were maintained at 55°C for 50 minutes with a UNO-II Thermoblock (Biometra). Samples were then incubated at 95°C for a further 10 minutes to inactivate the proteinase K and facilitate DNA denaturation. The tubes were then cooled to 4°C in preparation for PCR amplification.

PCR Amplification and DNA Sequencing

PCR primers based on conserved sequences among related dinoflagellate species were designed and synthesized. The primers, based on the small subunit (SSU) rRNA genes were as follows: AT18F01 (5′-CACCTGGTTGATCCTGCCAGTAG-3′) and AT18R02 (5′-GTTTCAGCCTTGCGACCATACTCC-3′). All of the primers used in this study were synthesized on a 10-column DNA synthesizer (PolyGen).

PCR reactions using the crude lysates extracted from single dinoflagellate cells were performed. Eight microliters of PCR master mix (0.5 U *Taq* polymerase (Promega Corp.), 0.25 mM each of all 4 dNTPs, 1 × reaction buffer, and 1.5 mM $MgCl_2$) including 10 pmol of each primer were added to the PCR tube containing approximately 2 μl of the lysate at 4°C. Thermocycling was as follows: initially 95°C for 5 minutes, followed by 40 cycles of 95°C for 20 seconds, 52°C for 30 seconds, and 72°C for 60 seconds.

After the completion of the cycles, extension was facilitated at 72°C for 6 minutes. Three microliters of PCR product was loaded onto a 1.5% agarose gel in 1 × TBE buffer (Sambrook and Russell, 1989) along with 3 μl of loading buffer. Agarose gels were stained with ethidium bromide and photographed according to standard methods (Sambrook and Russell, 1989).

DNA cycle sequencing reactions were performed directly, using PCR products as the template, without purification of the PCR-amplified fragments. The PCR products (3–6 μl) were subjected to DNA cycle sequencing using a ThermoSequenase Version 2.0 Cycle Sequencing Kit (USB) in the presence of 1.5 pmol of SAT18F01 (5′-CCAGTAGTCA TATGCTTGTC-3′) nested within the PCR primers. The sequencing primer was labeled with IRD at the 5′ end. The 4 base-specific reactions were subjected initially to 94°C for 1 minute, followed by 40 cycles consisting of 94°C for 20 seconds, 50°C for 30 seconds, and 72°C for 60 seconds in the UNO-II Thermoblock. When complete, reactions were stopped by adding 4 μl of IR2 stopper loading buffer (LiCor), and the products were heat-denatured and analyzed on a model 4200 Dual Dye Automated Sequencer according to the manufacturer's instructions.

DNA Sequence-Based Diagnosis

For sequence-based species identification, reference sequences of the SSU rRNA gene from the related species (*Alexandrium* for AxCt_K01, *Gymnodinium* and *Gyrodinium* for CCPK06), including the strains of both AxCt_K01 and CCPK06, were compiled privately. Nucleotide sequence analyzed from the test species was added to the reference sequences, and their sequences were aligned using CLUSTAL W with the default settings for gap inclusion and extension (Thompson et al., 1994). Molecular diagnosis of the unidentified dinoflagellates was performed with both genetic distance estimated by the Kimura 2-parameter model (Kimura, 1980) and similarity scores by GenDoc software between pairs of algal species from the aligned sequence data.

Results and Discussion

The preservation of HAB species is an important consideration for the extraction of DNA from single cells and subsequent PCR assays because some preservatives act as inhibitors of enzymatic reactions (Wilson, 1997). In previous studies preservatives such as formaline and Lugoil's iodine were found to interfere with DNA extraction and PCR amplification (Marín et al., 2001). The preservation reagent we used was a mixture of TE buffer and mineral oil in a PCR tube, to prevent evaporation and DNA contamination from other biological sources. The single dinoflagellate cells were stored at −20°C for several months without any degradation of DNA.

For some organisms whole cells can be used for PCR amplification, without the need for DNA isolation, since cell lysis can easily be achieved by heat. However, the cellulose shells of thecated dinoflagellates maintain their shape and resist cell lysis under the application of heat and some chemicals (e.g., sodium dodecylsulfate [SDS] and dithiothreitol [DTT]). Treatment of single cells with proteinase K, as described in this study, was found to be a rapid and effective method for the extraction of PCR-ready DNA from dinoflagellate cells.

The results of the direct PCR reaction using extracts taken from single cells are shown in Figure 1. PCR products amplified with AT18F1 and AT18R2 primers, and the lysates of both *Alexandrium catenella* and *Cochlodinium polykrikoides* yielded a fragment of the expected size of 1136 bp. The rRNA gene has been PCR-amplified more successfully than single-copy genes because the eukaryotic nuclear rDNA is tandemly organized, with copy numbers up to the order of 10^4 (Schlötterer, 1998). In previous work Bowers et al. (2000) demonstrated that the sensitivity of PCR to amplify the rRNA gene for the dinoflagellate *Pfiesteria picicida* was 0.6 cell. This suggested that the rRNA gene was a useful region for PCR amplification from single cells isolated from natural samples.

The techniques for single-cell PCR also have been used in the detection of the HIV-1 genome (Bertram, et al., 1995), and in PCR analyses of single fly (Gloor et al., 1993) and dinoflagellate cells (Bolch, 2001; Marín et al., 2001). The method used here simplifies several steps, as compared with previous protocols (Bolch, 2001; Marín et al., 2001). In addition, this technique allows direct DNA sequencing using unpurified PCR products amplified from single cells in order to avoid problems caused by the false-positive and false-negative signals possibly produced in the PCR detection.

In sequencing reactions, when a DNA band of the expected size can be seen on a gel, the concentration of DNA is greater than 50 ng/μl, since a 50-ng band of double-stranded DNA generally can be detected with ethidium bromide concentrations as low as 0.5 to 1 μg/ml. The PCR products therefore were used directly as the DNA template

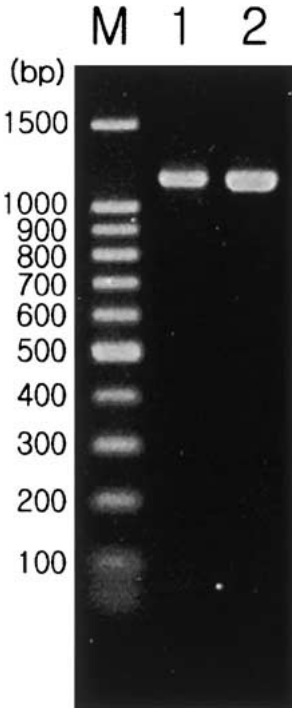

Figure 1. PCR products amplified from single-cell DNA extracts from *Cochlodinium polykrikoides* (lane 1) and *Alexandrium catenella* (lane 2) using AT18F1 and AT18R2 primers, respectively, for the ribosomal DNA region, with 100-bp ladder size marker (lane M). The size of the fragment in lanes 1 and 2 was 1136 bp.

for DNA sequencing without further check on the DNA concentration. When the band could not be visualized with ethidium bromide, or when its signal was weak, the PCR protocol was adjusted to increase the primer concentration or the number of cycles from 35 to 45. These modifications in PCR strategy efficiently improved the yield of copy DNA but also resulted in the presence of nonspecific amplified fragments. These unexpected by-products were, however, negligible for DNA sequencing reactions in the present study, possibly because of the selectivity of the nested sequencing primers and low background fluorescence in the IRD (Marziali and Aleson, 2001).

The high-quality sequence ladders of the SSU rRNA genes obtained using the SAT18F01 primer are shown in Figure 2. These sequences matched those of the preanalyzed SSU rRNA genes from the strain of AxCt_K01 (accession number AY347309) and CCPK06 (AY347309). Thus DNA sequencing of the unpurified PCR products resulted in sequences corresponding to the expected locus of rDNA of the dinoflagellate species *Alexandrium catenella* and *Cochlodinium polykrikoides.*

For sequence-based diagnosis the partial sequence of the SSU rRNA genes from the clone AxCt_K01 was added

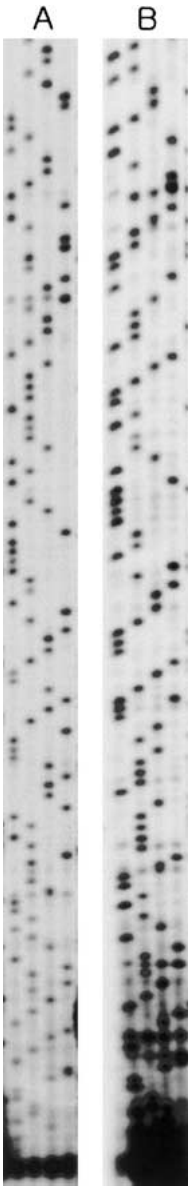

Figure 2. Data obtained by direct sequencing of unpurified PCR products from SSU rRNA genes of *Cochlodinium polykrikoides* (**A**) and *Alexandrium catenaella* (**B**). Sequences show over 100 bp from the primer. The 4 lanes in each panel are in the order A-T-G-C.

to the reference sequences, which each consisted of about 600 bp of the partial SSU rDNAs, and the compiled sequences were aligned using CLUSTAL W software. The partial sequence of the SSU rRNA genes from the clone AxCt_K01 was zero genetic distance and 100% sequence similarity with a strain of YSC9811, obtained from GenBank (accession number AB088334). The results therefore showed that the strain of AxCt_K01 was correctly identified as the toxic dinoflagellate *Alexandrium catenella*. In like manner the partial sequence of the SSU rRNA gene from

Table 1. Reference Species and Their Sequences for Sequence-Based Species Diagnosis of Clone AxCt_K01 and CCPK06[a]

Reference species	GenBank access. no.	Strain name	Isolation locale[b]	Genetic distance	Similarity score (%)
				Between AxCt_K01	
A. catenella	AY347308	AxCt_K01	South Korea/Nanpo	**0.000**	**100**
A. catenella	AB088334	YSC9811	South Korea/Yosu	**0.000**	**100**
A. tamarense	AB088295	HAT4	Japan/?	0.058	94
A. tamiyavanichi	AB088318	TAMI22012	Japan/Harimanada	0.069	92
A. cohorticula	AF113935	ACMS01	?	0.073	92
A. affine	AB088282	AFF37	Japan/Harimanada	0.075	92
A. minutum	U27499	7	?	0.093	90
A. insuetum	AB088298	AI104	Japan/?	0.102	89
A. ostenfeldii	U27500	?	?	0.106	89
A. fraterculus	AB088290	DPW9709	South Korea/?	0.111	89
A. margaelefii	U27498	?	?	0.117	88
A. pseudogonyaulax	AB088302	APSN	Japan/?	0.130	87
				Between CCPK06	
C. polykrikoides	AY347390	CCPK06	South Korea/Sarang-do	**0.000**	**100**
Gym. sanguineum	U41085	CCMP1321	?	0.054	94
Gyr. galatheanum	AF274262	CCCM 555	?	0.058	94
Gym. mikimotoi	AF022195	MUCC098	?	0.059	94
Gyr. aureolum	AF172713	KT-77D	?	0.062	93
Gym. breve	AF172714	CCMP718	?	0.065	93
Gyr. dorsum	AF274261	UTEX LB 2334	?	0.071	93
Gyr. uncatenum	AF274263	CCCM533	?	0.071	93
Gym. beii	U37365	?	?	0.078	92
Gym. simplex	U41086	CCMP419	?	0.084	91

[a]The nucleotide sequences consisted of about 600 bp of the SSU rDNA from the related species, including the unidentified species. Molecular diagnosis was performed using both genetic distance and similarity score between the aligned sequences. Boldface indicates matched species.

the clone CCPK06 was identical to that of the SSU rDNA from GenBank (*Cochlodinium polykrikoides*; AY347309), and the homology between them was found to be 100% sequence similarity and zero genetic distance (Table 1). The results suggest that direct DNA sequencing using the unpurified PCR products from single cells is useful for sequence-based genetic identification and clearly resolves the taxonomic ambiguities of closely related algal species (such as *Alexandrium* and *Cochlodinium*).

The new approach described here has been applied for the species diagnosis of single cells isolated from field samples. The HAB samples were collected from the coastal waters of Korea for the genera *Alexandrium, Gymnodinium, Gyrodinium*, and *Cocnlodinium*, and at Juam reservoir in Korea for a freshwater dinoflagellate, *Peridinium*, when seasonal blooms took place. PCR reactions successfully amplified the target rDNA regions, using primer sets

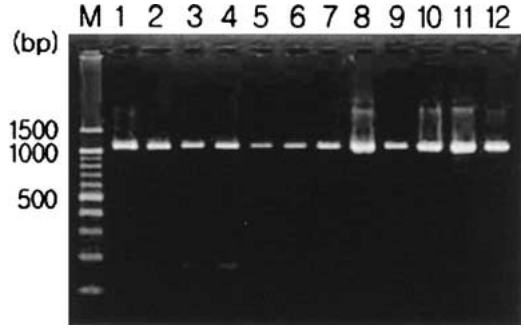

Figure 3. PCR products amplified from DNA extracts of single cells isolated from field samples using AT18F1 and AT18R2 primers for the ribosomal DNA region. Lanes 1–6, unidentified armoured dinoflagellate (genus *Alexandrium*); lanes 7–11, unidentified unarmoured dinoflagellate (genera *Cochlodinium* and *Gymnodinium*); lane 12, unidentified freshwater dinoflagellate (genus *Peridinium*); lane M, 100-bp ladder size marker. The size of the fragments was around 1100 bp.

Table 2. Sequence-Based Species, Diagnosis of Single Cells Isolated from Field Samples

Lane	Isolation locale (in Korea)	Matched sequence (GenBank access. no.)	Similarity score (%)	Genetic distance	Seq.-based diagnosis
1	Masan Bay	AB088295	100	0.000	***A. tamarense***
2	Nanpo, Jinhae Bay	AY347308	100	0.000	***A. catenella***
3	Nanpo, Jinhae Bay	AY347308	99.5	0.005	***A. catenella***
4	Jangmok	AY347308	99.5	0.005	***A. catenella***
5	Masan Bay	AB088295	100	0.000	***A. tamarense***
6	Chilchon-do	AB088295	100	0.000	***A. tamarense***
7	Hakdong	AY347390	100	0.000	***C. polykrikoides***
8	Narodo	AY347390	100	0.000	***C. polykrikoides***
9	Nanpo, Jinhae Bay	AF022193	100	0.000	***Gym. catenatum***
10	Jindong	AF022197	100	0.000	***Gym. impudicum***
11	Jangmok	U41085	99.2	0.005	***Gym. sanguineum***
12	Juam Reservoir	AF231805	98.3	0.017	***P. bipes***[b]

[a]Sequencing reactions were carried out using the PCR products (Figure 3), and about 600 bp of the SSU rDNA was sequenced. The nucleotide sequence was searched by BLAST, and a sequence having the highest sequence similarity score was selected as the matched sequence. Boldface represents the identified species name as judged by sequence similarity or genetic distance. All the samples, except *Peridinium* species, were identified by phenotypic analysis.
[b]Represents the morphologically unidentified species.

(AT18F1 and AT18R2 primers) and DNA extracts of single cells (Figure 3). For sequence-based diagnosis of the cells, the nucleotide sequences analyzed from the PCR products were compared with the reference sequences of SSU rDNA from the marine dinoflagellates, including a freshwater *Peridinium*. The dinoflagellate cells at the genus level were identified to the species level, as judged by sequence similarity (Table 2). A relatively high degree of sequence homology (>99.2%) between the revealed and the matched sequence was recorded for *A. tamarense, A. catenella, C. polykrikoides, G. catenatum*, and *G. sanguineum*, respectively. The genetic variations, which might be caused by the large geographic separation of the same species, were significantly lower at the intraspecies level than at the interspecies level (see Table 2), and the values did not affect the identification of *Alexandrium* and *Gymnodinium* cells collected from the coastal area of Korea. All the cells were identified phenotypically, and the results were concordant with the sequence-based species identification.

Mainly on the basis of sequence data, the cell from Juam Reservoir was identified as *P. bipes*, as judged by 98.3% sequence similarity with a strain of *P. bipes* (accession number AF231805). The cell, however, had not been identified phenotypically as *P. bipes*, since the genus *Peridinium* contained at least 220 species, and their thecal formula and plate shape, which were used as taxonomic characters, were quite difficult to observe under the light microscope. Furthermore, the data available in public resources to use as reference sequences for each *Peridinium* were not sufficient to discriminate their taxonomy.

As we described above the sequence-based diagnostic method is dependent on sequence comparison with the prerevealed sequence for species identification. The method therefore could be applied to identify several species previously known to be different, but it, also was applied to identify an unpresented dinoflagellate or HAB species from a studied area when nucleotide sequences of the same genomic regions (e.g., 18S rDNA sequence) had been reported in public resources. To date the nucleotide sequences from the HAB species, except for several *Alexandrium* and *Gymnodinium* species, have not been reported sufficiently. For the wide application of this method, further studies are needed to determine the nucleotide sequences of the rRNA and other genes of more samples collected from local or different geographic regions. In the present study we demonstrated the applicability of sequence-based diagnosis from single dinoflagellate cells, including nonculturable species.

The method described here is a rapid and inexpensive way to the isolate and preserve dinoflagellate DNA. This protocol constitutes a significant breakthrough in the application of PCR techniques to many nonculturable dinoflagellates isolated from natural samples. In addition, it is suitable for molecular diagnosis (e.g., sequence-based typ-

ing and the detection of infectious disease) and large-scale DNA sequencing of the same genomic regions from related species for molecular evolution studies.

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References

Bertram, S., Hufert, F.T., Neumann-Haefelin, D., and von Laer, D. (1995). Detection of DNA in single cells using an automated cell deposition unit and PCR. *Biotechniques* 19:616–620.

Bolch, C.J.S. (2001). PCR protocols for genetic identification of dinoflagellates directly from single cysts and plankton. *Phycologia* 40:162–167.

Bowers, H.A., Tengs, T., Glasgow, H.B, Burkholder, J.M., Rublee, P.A., and Oldach, D.W. (2000). Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. *Appl Environ Microbiol* 66:4641–4648.

Godhe, A., Otta, S.K., Rehnstam-Holm, A.S., Karunasagar, I., and Karunasagar, I. (2001). Polymerase chain reaction (PCR) based detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in field samples from Southwest India. *Mar Biotechnol* 3:152–162.

Guillard, R.R.L., and Ryther, J.H. (1962). Studies of marine planktonic diatoms, 1: *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can J Microbiol* 8:229–239.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120.

Marín, I., Aguilera, A., Reguera, B., and Abad, J.P. (2001). Preparation of DNA suitable for PCR amplification from fresh or fixed single dinoflagellate cells. *Biotechniques* 30:88–93.

Sako, Y., Kim, C.H., Ninomiya, H., Adachi, M., and Ishida, Y. (1990). Isozyme and cross analysis of mating populations in the *Alexandrium catenella/tamarense* species complex. In: *Toxic Phytoplankton Blooms in the Sea*, Granili, E., Sundstrom, B., Eldler, L., and Anderson, D.M. (eds.). Amsterdam, Netherlands: Elsevier, pp 95–102.

Sambrook, J., and Russell, D.W. (1989). Molecular Cloning: A Laboratory Manual. New York, N.Y.: Cold Spring Harbor Press, pp 54–517.

Schlötterer, C. (1998). Ribosomal DNA probes and primers. In: *Molecular Tools for Screening Biodiversity*, Karp, A., Isaac, P.G., and Ingram, D.S. (eds.). London, U.K.: Chapman & Hall, pp 267–276.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.

Wilson, L.G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* 63:3741–3751.