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# Polymerase Chain Reaction in Detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in Field Samples from Southwest India

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**Abstract:** Polymerase chain reaction (PCR) primers were constructed for the detection of two toxic dinoflagellate species, *Gymnodinium mikimotoi* and *Alexandrium minutum.* The primers amplified a product of expected size from cultured cells of *G. mikimotoi* and *A. minutum.* The species-specific primers targeting *G. mikimotoi* did not yield any product with a wide range of other cultured algae used as negative controls. Primers designed for *A. minutum* were species-group-specific since it PCR yielded a product from the closely related species *A. ostenfeldii* and *A. andersonii,* but not from other species of this genus tested. The confirmation of PCR products was performed by digestion of the products with restriction enzymes. Sensitivity analyses of the primers on DNA template from cultured cells was positive by PCR at a DNA template concentration of $1.5 \times 10^{-4}$ ng/µl (0.3 cells/L) for *A. minutum,* and at a DNA concentration of $2.5 \times 10^{-2}$ ng/µl (697 cells/L) for *G. mikimotoi.* The PCR method for detection of *G. mikimotoi* and *A. minutum* was applied on field samples collected with a plankton net. *Gymnodinium mikimotoi* could be detected in 11 field samples by microscopy, and all these field samples were positive by PCR. The cell counts of *G. mikimotoi* in simultaneously collected water samples ranged from 306 to 2077/L. *Alexandrium minutum* could be detected by microscopy in 3 different field samples. The cell counts in water samples collected at the same time as the net samples ranged from 115 to 1115 cells/L. *Alexandrium minutum* was detected by PCR in these field samples, with the exception of the sample displaying the lowest cell count (115 cells/L). Plankton samples that were negative by microscopy for any of the two target species were also negative by PCR. All the PCR products from field samples were confirmed by restriction enzyme digestion. The application of PCR-based detection of harmful algal bloom species for aquaculture and monitoring purposes in natural field samples is discussed.

**Key words:** PCR, Dinophyceae, *Gymnodinium mikimotoi, Alexandrium minutum,* natural samples, Arabian Sea.

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## Introduction

Harmful algae are a concern all over the world (Hallegraeff, 1995). In India there have been problems due to paralytic shellfish poisoning (Karunasagar et al., 1984; Karunasagar and Karunasagar, 1998), diarrhetic shellfish poisoning (Karunasagar et al., 1989), and fish kills (Subramanyan, 1954; Karunasagar and Karunasagar, 1992). Monitoring coastal water for the presence of harmful algae would be essential to access the potential for bloom formation. Normally this type of monitoring involves microscopic examination of plankton. This is time-consuming and requires consdierable taxonomic experience, as the identification is based on morphological characters. In addition, the species of interest often occurs as a minor component in a mixed plankton community. An adequate method for rapid and specific identification with high sensitivity would therefore be of great interest.

Worldwide *Gymnodinium mikimotoi* Miyake & Kominami ex Oda has been reported to be involved in fish kills (e.g., Karunasagar and Karunasagar, 1992; Nakamura et al., 1996; Gentien, 1998). Microscopic identification based on morphological features of *G. mikimotoi,* as well as for other species belonging to the order of Gymnodiales, is obstructed by the difficulty of preserving naked dinoflagellates. Characteristic details of the species are often distorted when cells are fixed (Takayama et al., 1998). Furthermore, taxonomic problems have been associated with *G. mikimotoi* because morphological features vary in response to changes in environmental conditions of growth stages (Partensky et al., 1988).

Members of the paralytic shellfish toxin (PST)–producing genus *Alexandrium* Halim have identical plate tabulations (Taylor and Fukuyo, 1998). *Alexandrium minutum* Halim is distinguished from other species belonging to the same genus by minute details of the apical tabulation (Taylor et al., 1995). This means that morphological identification of *A. minutum* requires high taxonomic skills and a well-trained eye. During recent years, there has been considerable interest in using antibody and DNA-based methods to detect potentially toxic algae (Anderson, 1995; Scholin and Anderson, 1998). Studies have involved development of antibodies against cell-surface-associated antigens or toxins, and nucleic acid probes for ribosomal RNA and DNA or spacer regions that separate genes within the rDNA operon. Methods with excellent specificity and sensitivity have been developed for a wide range of harmful algal species. A few papers report successful detection of algae from field samples by these methods (e.g., Scholin et al., 2000), but the majority of these techniques have so far been tested only on cultured cells and not on natural populations (Vrieling and Anderson, 1996; Scholin and Anderson, 1998).

Methods based on polymerase chain reaction (PCR) are gaining popularity for detection of pathogens within the biomedical discipline owing to its simplicity and high specificity. Relatively few papers, however, are published describing successful attempts to identify and detect microalgae by PCR. PCR-based detection of dinoflagellates from field samples has been reported for *Lingulodinium polyedrum* (Stein) Dodge (Rollo et al., 1995) and *Dinophysis acuminata* Claparède & Lachmann (Puel et al., 1998). Haley et al. (1999) described PCR-based detection of *Alexandrium tamarense* Lebour from water samples spiked with cultured cells, and detection of 4 species of cultured strains from the genus *Alexandrium* using PCR technique and genus-specific primers was reported by Penna and Magnani (1999).

In this article we describe PCR-based detection of *G. mikimotoi* and *A. minutum* and the protocol that has been applied for detection of these organisms in field samples.

## Materials and Methods

### Primer Design

The small subunit (SSU) rDNA sequences of *Gymnodinium mikimotoi* (EMBL accession number AF022195) and *Alexandrium minutum* (EMBL accession number AMU27499) were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and compared with sequences of closely related species using BLAST. Primers for detection of the two species were designed using the software OLIGO.EXE structure 3.4 (Wojciech Rychlik Boreland Int. The primer sequences and their binding sites (i.e., he 5′-end of the primer corresponding to base number in the GenBank sequences) and the target sites of the primers on rDNA sequences from closely related species are as indicated in Figure 1. The specificity of the primers for their target species was checked against EMBL and GenBank databases using EMBL FASTA server.

### Collection of Field Samples

Plankton was collected approximately once a week, during the period November 1998 to March 1999. The samples were nonquantitative and collected with a 10-µm plankton

| Species | Primer sequence | Number of bp amplified | Primer 5' binding site |
|---|---|---|---|
| *G. mikimotoi* | 5'TGC ATC AGC TGG CGA TAG ATC (forward) | 1433 | 276 |
| | 5'CAG GAA CTG AAC ACT GCG GCA (reverse) | | 1709 |
| *A. minutum* | 5'CTC TGC TTG GAA TTT TGG TGA (forward) | 1147 | 230 |
| | 5'GCC CAC ATA ACC GGA ATT ACG (reverse) | | 1377 |

| | *G. mikimotoi* (forward) | *G. mikimotoi* (reverse) |
|---|---|---|
| | 5'TGC ATC AGC TGG CGA TAG ATC | 5'CAG GAA CTG AAC ACT GCG GCA |
| *Gymnodinium mikimotoi* | TGC ATC AGC TGG CGA TAG ATC | GTC CTT GAC TTG TGA CGC CGT |
| *Pentapharsodinium tyrrhenicum* | GGC ATC AGC TGG CGA TGA ATC | GTC CTT GAC TTG TGA CGA CGT |
| *Heterocapsa triquetra* | GGC ATC CGC TGG CGA TGA ATC | GCA CTG G–C TTG TGA CGA CGT |
| *Cachonina hallii* | AGC ATC -GC TGG CGA TGA ATC | GCA CTG G–C TTG TGA CGA CGT |
| *Prorocentrum micans* | GGC ATC CGC TGG CGA TGA ATC | GTC CTT GAC TTG TGA CGA CGT |
| *Gymnodinium catenatum* | GGC ATC AGC TGG CGA TGA ATC | GCC CTA GGT TTG TAA CGA CGT |
| *Gyrodinium impudicum* | GGC CTC GCC TAG CGA TGG ACT | GTCGCAT GGC TTG TAA CGG CGT |

| | *A. minutum* (forward) | *A. minutum* (reverse) |
|---|---|---|
| | 5'CTC TGC TTG GAA TTT TGG TGA | 5'GCC CAC ATA ACC GGA ATT ACG |
| *Alexandrium minutum* | CTC TGC TTG GAA TTT TGG TGA | CGG GTG TAT TGG CCT TAA TGC |
| *A. ostenfeldii* | CTC TGC TTG GAA CCT TGG TGA | CGG GTG TAT TGG CTT TAA TGC |
| *A. margalefii* | CTC TGC TTG GAA TTT TGG TGA | CGG GTG TAT TAG CTT TRA TGC |
| *A. tamarense* | CTC TGC TTG GAT ACT TGATTGA | CGG GTG TAT TAG CTT TAA TGT |
| *A. fundyense* | CTA TGC TTG GAC ACT TGAATGA | CGG GTG TAT TAG CTT TAA TGT |
| *Pentapharsodinium tyrrhenicum* | CTC TGC CTG GTC TTG TGG TGA | CGG GTG CAT TGG CTC CAA TGC |
| *Gymnodinium mikimotoi* | CTC TGC CTG GTC TCG TGG TGA | CGG GTG CAT TGG CTT CAA TGC |

**Figure 1.** Sequences of primers designed for the detection of *Gymnodinium mikimotoi* and *Alexandrium minutum.* Lengths of amplified fragments and the 5′ end of the primer corresponding to base number in GenBank sequences are indicated. The designed primers are aligned with the most closely related species according to BLAST.

net (Hydrobios; upper diameter, 15 cm) directly from the shore at Someshwar and Totham Beach, or from a boat in the Nethravathi River and a few nautical miles offshore Mangalore (Figure 2, Table 1). The plankton net was kept in streaming water and dragged behind the boat at a depth of 1 to 2 m for approximately 10 minutes. Simultaneously surface water was collected at the sampling sites in plastic containers and fixed in Lugol's iodine solution (Willén, 1962) for later enumeration in cases in which the PCR reactions showed positive results.

Live net samples were examined in bright-field and phase-contrast illumination at magnifications of ×100, ×400, and ×1000, using a Leitz Laborlux S microscope, and qualitative observations of species present in the samples were recorded (Table 2). The following sources were used to identify phytoplankton to species or genus level: Dodge (1982), Thomsen (1992), and Tomas (1997). Enumeration of cells was performed using 50–52-ml Utermöhl-type sedimentation chambers (Utermöhl, 1958). Numbers of cells per liter of the amplified species were counted at magnification ×200 using an inverted Leitz DM IL microscope.

## DNA Extraction

Cells from cultures (Table 3) were collected by mild centrifugation (3000 *g*) for 5 minutes. The pellet was resuspended in 420 μl of autoclaved milli-Q water (Millipore Corp., Molsheim, France). Thereafter 20 μl of 250 mM ethylenediaminetetraacetate (EDTA, pH 8.0), 50 μl of 10% sodium dodecylsulfate (SDS), 5 μl of 1 M Tris-HCl (pH 7.5), and 5 μl of 1 M NaCl was added. DNA was extracted with buffered phenol–chloroform–isoamyl alcohol (24:24:1). The mixture was vortexed for about a minute and centrifuged at 15,000 *g* for 3 minutes. The upper aqueous phase was extracted twice with phenol–chloroform–isoamyl alcohol, and DNA was precipitated at −20°C with one-tenth volume of 3 M Na-acetate (ph 4.5), and 2 volumes of absolute EtOH for a minimum of 1 hour (Ausubel et al., 1987). DNA was pelleted by centrifugation at 15,000 *g* for 30 minutes. The pellet was washed with cold 70% EtOH and centrifuged again at the same speed, and the new pellet was dried for 5 minutes in 49°C. Before the DNA was used in the PCR reaction, it was resuspended in 25 to 50 μl of sterile milli-Q water. Concentration and purity of DNA were checked in a spectrophotometer at the wavelengths 260 and 280 nm. DNA samples were stored at −20°C.

In order to obtain DNA from field samples, cells were collected by mild centrifugation and lysed as described for cultured cells. After adding milli-Q water, EDTA, SDS, Tris-HCl and NaCl, the mixture was kept at 49°C for 1 to 2 hours. DNA was thereafter purified using Clean Genei Kit (Bangalore Genei Pvt. Ltd., Bangalore, India) following the protocol of the producers.

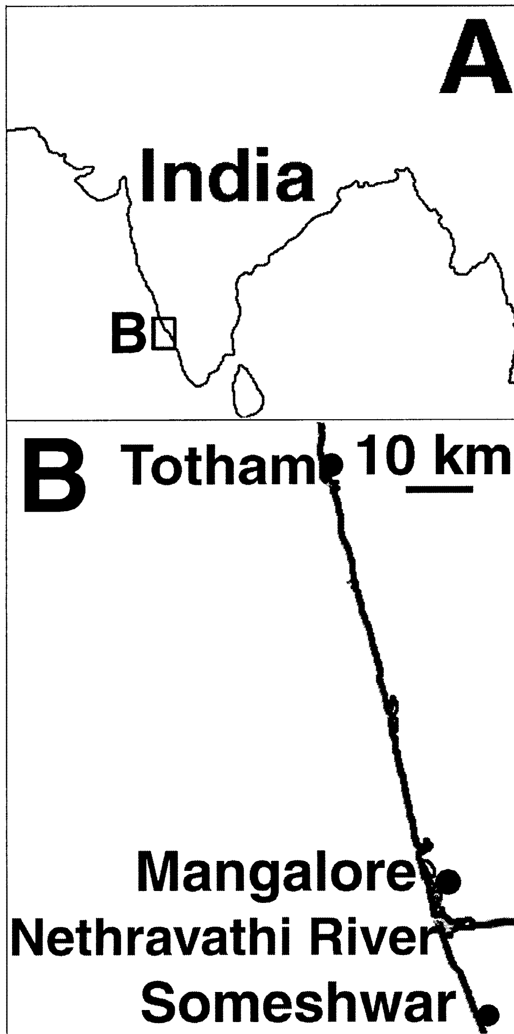


**Figure 2.** **A:** India. Coastal S.W. Karnataka State inset. **B:** Coastal S.W. Karnataka, showing the locations where plankton samples were collected.

**Table 1.** Details of Natural Plankton Sample Collection

| Sample no. | Date | Location (depth, m) |
|---|---|---|
| 1 | 11/19/99 | Nethravathi estuary (5) |
| 2 | 12/26/99 | Offshore Mangalore (10) |
| 3 | 1/5/99 | Someshwar Beach (4) |
| 4 | 1/14/99 | Offshore Mangalore (10) |
| 5 | 1/14/99 | Offshore Mangalore (35) |
| 6 | 1/27/99 | Offshore Mangalore (10) |
| 7 | 2/8/99 | Totham Beach (4) |
| 8 | 2/11/99 | Offshore Mangalore (20) |
| 9 | 2/18/99 | Offshore Mangalore (20) |
| 10 | 2/25/99 | Offshore Mangalore (10) |
| 11 | 2/25/99 | Offshore Mangalore (20) |
| 12 | 3/4/99 | Offshore Mangalore (10) |
| 13 | 3/4/99 | Offshore Mangalore (20) |

## Polymerase Chain Reaction Amplifications

All DNA from cultures was amplified directly with primers designed for the target species. DNA from field samples was amplified first with SSU rDNA eukaryotic-specific primers (Medlin et al., 1988), and the obtained products were thereafter used as template DNA for the next reaction with species-specific or species-group-specific primers—i.e., nested PCR.

The PCR reactions were run in total volumes of 50 µl each, consisting of *Taq* buffer with 1.5 mM $MgCl_2$, 0.5 µg of each primer, 200 µM of each deoxynucleotide triphosphate, 0.5 to 2 µl of template DNA, 3 U of *Taq* polymerase (Bangalore Genei Pvt. Ltd.), and sterile milli-Q water. Amplification with SSU rDNA eukaryotic-specific primers was carried out in a thermal cycler (Techne Genius) as follows: initially 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C 1 minute, annealing at 45°C 1 minutes, and extension at 72°C 1 minute. After the cycles, extension was completed at 72°C for 7 minutes. The protocol for amplification using species-specific or species-group-specific primers was initially 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C 1 minute, annealing at 57°C 1 minute, and extension at 72°C 1 minute. After the cycles, extension was completed at 72°C for 5 minutes.

The PCR product was cooled to 28°C and 20 µl of the product was loaded together with 5 µl of dye buffer onto 0.8% agarose gel in 0.5 × TBE buffer (Ausubel et al., 1987). Ethidium-bromide-stained gels were studied under UV transillumination.

## Sensitivity Analyses with Cultured Strains

Exponentially growing cells of cultured *G. mikimotoi* (K-0286) and *A. minutum* (CCMP113) were used to quantitatively investigate the sensitivity of the PCR using the species-specific or species-group-specific primers. Ten milliliters of each monoclonal culture was fixed in Lugol's iodine solution and poured into an Utermöhl-type sedimentation chamber. The cell count of the cultures was determined in an inverted Leitz DM IL microscope. Fifty milliliters of each culture was used for DNA extraction. The extraction was performed as described for cultured cells above. The quantity and purity of the DNA from the cultured cells was checked by the spectrophotomeric method, and the DNA

**Table 2.** Qualitative Record of Dinoflagellate Species and Other Phytoplankton Taxa Present in the Net Samples*

| Taxa/Sample no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dinophyceae | | | | | | | | | | | | | |
| *Alexandrium minutum* | | | | x | x | | | | | x | | | |
| *Alexandrium* sp. | | | | | | | | | x | | | | |
| *Ceratium furca* | x | x | x | x | x | | x | x | xxx | x | x | x | x |
| *Ceratium fusus* | | x | x | x | x | | x | x | x | x | x | x | x |
| *Ceratium horridum* | | | x | | x | | | x | | | x | | x |
| *Ceratium triops* | | x | x | | | | x | x | x | x | x | x | x |
| *Cochlodinium polykrikoides* | | xxx | | | | | | | | xx | x | x | x |
| *Cochlodinium* sp. | | | | | x | | | | x | | | x | |
| *Dinophysis* cf. *acuminata* | | | | x | | | | | | | | | |
| *Dinophysis caudata* | | x | x | x | x | | x | x | x | x | x | x | |
| *Dinophysis rotundata* | | x | | | | | | x | x | x | | | |
| *Diplopsalis* sp. | | x | x | x | x | x | | x | x | x | x | x | x |
| *Gonyaulax polygramma* | | x | xx | x | x | | x | x | x | | | x | |
| *Gonyaulax spinifera* | | | | | | | | | | x | x | x | |
| *Gonyaulax* cf. *verior* | | | | | | | | | | | | x | |
| *Gymnodinium* cf. *breve* | | | | x | | | | | | | | | |
| *Gymnodinium mikimotoi* | | x | x | x | x | | x | x | x | x | x | x | x |
| *Gymnodinium sanguineum* | | | x | x | x | | | x | x | x | | x | x |
| *Gyrodinium* cf. *instriatum* | | x | xx | | | | | | x | x | | | |
| *Gyrodinium* sp. 1 | | | x | | | x | | x | | | | | |
| *Gyrodinium* sp. 2 | | | | | x | | | | x | | | | |
| *Katodinium* sp. | | | | x | | | | x | | | | | |
| *Ornithocercus magnificus* | | | | x | | | x | | | | | | |
| *Phalacroma rapa* | | | | | | | | x | | | | | |
| *Pheopolykrikos hartmanii* | | | | | | | | | | | | | x |
| *Polykrikos kofodii* | | | | x | | | | | | | | | |
| *Prorocentrum compressum* | | x | | x | x | | | x | | | | | |
| *Prorocentrum gracile* | | | x | x | x | x | x | | x | x | x | x | x |
| *Prorocentrum* cf. *mexicanum* | | | | | | | | | | | | | x |
| *Prorocentrum micans* | | x | x | x | x | | x | x | x | | x | | x |
| *Protoceratium reticulatum* | | | | x | | | | x | | | | | |
| *Protoperidinium conicum* | | | x | x | | | | | | | | | |
| *Protoperidinium depressum* | | x | x | x | | | x | x | x | | x | x | x |
| *Protoperidinium divergens* | | | | | x | | | | | | | | |
| *Protoperidinium oblongum* | | | x | | x | | x | | x | x | x | x | |
| *Protoperidinium* cf. *pellucidum* | | | x | | | | | | x | x | | x | x |
| *Protoperidinium* sp. | | | | x | | x | | x | | | | | x |
| *Pyrophacus steinii* | | | | x | | | | | | | | x | |
| *Scrippsiella* sp. | | | x | x | x | | x | | | | | | x |
| Bacillariophyceae | xxx | xx | xx | xxx | xx | xx | xx | xx | xx | xx | xx | xxx | xx |
| Euglenophyceae | x | | | | | | | | | | | | |
| Prymnesiophyceae | x | | | | | | | | | | | | |
| Cyanophyceae | xx | | x | | | | x | x | xxx | xxx | x | x | |
| Dictyochophyceae | | | x | | | | x | x | x | | | x | |
| Choanoflagellida | x | | | | | | | | | | | | |

*An x indicates present; xx, common; xxx, abundant.

**Table 3.** Algal Cultures Used as Positive and Negative Controls

| Species | Culture location | Strain number |
|---|---|---|
| *Alexandrium minutum* | Provasoli Guillard Center for Marine Microalgae (CCMP) | CCMP113 |
| *Gymnodinium mikimotoi* | Göteborg Unviersity Marine Algal Culture Collection (GUMACC) | 36 |
| *G. mikimotoi* | Scandinavian Culture Center for Algae and Protozoa (SCCAP) | K-0286 |
| *Alexandrium andersonii* Balech | Dept. Phycology University of Copenhagen | JL24 |
| *A.* cfr. *fundyense* Balech | SCCAP | K-0271 |
| *A. ostenfeldii* (Paulsen) Balech & Tangen | SCCAP | K-0287 |
| *A. tamarense* (Lebour) Balech | SCCAP | K-0055 |
| *A. tamarense* | Kalmar Algal Collection (KAC) | KAC2 |
| *Gymnodinium catenatum* Graham | CSIRO Marine Research | PTL01-4 |
| *G. chlorophorum* | SCCAP | K-0539 |
| *G.* cfr. *placidum* | SCCAP | K-0308 |
| *G. sanguineum* Hirasaka | SCCAP | K-0405 |
| *Gyrodinium impudicum* | Dept. Phycology University of Copenhagen | JL41 |
| *Prorocentrum micans* Ehrenberg | GUMACC | 35 |
| *Heterocapsa triquetra* (Ehrenberg) Stein | GUMACC | 43 |
| *Amphidinium carterae* Hulburt | GUMACC | 13 |
| *Prymnesium parvum* N. Carter | GUMACC | 37 |
| *Rhodomonas* sp. Karsten | GUMACC | 9 |
| *Dunaliella tertiolecta* Butcher | GUMACC | 5 |
| *Tetraselmis* sp. Stein | GUMACC | 19 |
| *Phaeodactylum tricornutum* Bohlin | GUMACC | 2 |
| *Porphyridium cruentum* (Gray) Nägeli | GUMACC | 25 |

was thereafter serially diluted in the range 10 to $10^6$. Amplification conditions with direct PCR using species-specific or species-group-specific primers and the stepwise diluted DNA extracted from the cultures as template were as described for cultures above.

## Confirmation of PCR Products by Restriction Enzyme Digestion

The expected restriction enzyme patterns of the PCR products were acquired by using the software program CLONE 4.0 (Educational and Scientific Software). The restriction enzymes chosen for the analyses of the PCR products and the expected lengths of fragments are displayed in Table 4. The uniqueness of the lengths of the fragments obtained after restriction was checked using the software program DNA STRIDER 1.2. (CEA, France). The sequences of the SSU rDNA gene of the cultures used as negative controls (listed in Table 3) and the 20 most closely related species according to BLAST were investigated in the same manner.

**Table 4.** Restriction Enzymes Used for the Confirmation of PCR Products

| Species | Enzyme | Expected length of fragments (bp) |
|---|---|---|
| *G. mikimotoi* | *Eco*31I | 1055,378 |
| | *Mun*I | 1165,268 |
| | *Xba*I | 865,568 |
| *A. minutum* | *Bcl*I | 736,411 |
| | *Bss*HII | 944,203 |
| | *Mun*I | 833,314 |
| | *Mbo*II | 838,309 |

Amplified species-specific or species-group-specific

PCR products from field samples and cultures were cleaned with Clean Genei kit (Bangalore Genei Pvt. Ltd.). Twenty microliters of each purified PCR product was digested with restriction enzymes in 30-μl reactions for 1 to 2 hours as directed by the manufacturer. The digested products were thereafter mixed with 5 μl of dye buffer and loaded onto 0.8% agarose gel in 0.5 × TBE buffer. Ethidium-bromide-stained gels were studied under UV transillumination.

## Results

The primers designed in this study amplified DNA fragments of expected sizes from cultured cells of *Gymnodinium mikimotoi* (Figure 3, A, lane 1) and *Alexandrium minutum* (Figure 3, B, lane 1). The species-specific primers for *G. mikimotoi* did not yield any product with a wide range of unialgal cultures representing several different taxonomic classes (Table 3). Primers designed to be specific for *A. minutum* (Figure 1) yielded a PCR product with the closely related species *A. ostenfeldii* and *A. andersonii* but not with the more distant species *A. tamarense* and *A.* cfr. *fundyense* within the same genus.

As shown in Figure 3, C and D, *G. mikimotoi* PCR products yielded two fragments of 865 and 568 bp, and of 1165 and 268 bp after digestion with restriction enzyme, *Xba*I and *Mun*I, respectively. The *A. minutum* PCR products digested with *Bcl*I yielded fragments of the expected sizes, 736 and 411 bp (Figure 3, E). When PCR products from *A. minutum* were digested with *Mun*I and *Bss*HII, fragments of 833 and 314 bp, and 944 and 203 bp, respectively, were obtained (Figure 3, F). The restriction maps of the SSU rDNA gene of the species used as negative controls (Table 3) and the closest related species of *G. mikimotoi* and *A. minutum* did not yield similar restriction patterns for the enzymes listed in Table 4, as tested with data from GenBank and EMBL.

Sensitivity analyses of the direct species-specific PCR on cultured cells of *G. mikimotoi* (K-0286) showed a visible band after gel electrophoresis at a minimum concentration of $2.6 \times 10^{-2}$ ng/μl template DNA (Figure 3, G). This DNA concentration corresponded to a cell concentration of 697 cells per liter. A positive result of direct PCR with a visible band after electrophoresis using cultured *A. minutum* (CCMP113) was obtained at a DNA concentration of $1.5 \times 10^{-4}$ ng/μl (Figure 3, H). This DNA concentration corresponded to a cell count of 0.3 cells per liter.

Results in Table 5 show the application of the PCR method for detection of *G. mikimotoi* and *A. minutum* in field samples. The former could be detected by microscopy in 11 field samples, and these samples were also positive by PCR. Typical results are illustrated in Figure 3, A. The water samples collected at the same time as the net hauls displayed a range of 306 to 2077 *G. mikimotoi* cells per liter (Table 5). *A. minutum* was encountered by microscopy in net hauls on 3 separate occasions (Table 2), but could only be detected by PCR assay in 2 of these samples (Table 5, Figure 3, B). The water samples collected at the same time as the net hauls for quantitative enumeration of the number of cells present in the water ranged from 115 to 1115 *A. minutum* cells per liter. The field sample, which had a corresponding count of 115 *A. minutum* cells per liter in the quantitative water sample did not yield any PCR product (Table 5). The PCR products of all field samples were confirmed by restriction mapping, and typical results are presented in Figure 3, C–F. Plankton samples, which were negative by microscopy for any of the target species, were first amplified with eukaryotic-specific primers to demonstrate the ability of the DNA extract. Thereafter this DNA was amplified with species-specific and species-group-specific primers. None of the field samples, negative by microscopic examination, were positive by PCR using the designed primers (Table 5).

PCR using species-specific or species-group-specific primers yielded bright bands when template DNA originated from cultured cells. With field samples, however, direct PCR often yielded very faint bands. Therefore nested PCR was performed on all field samples. Eukaryotic-specific primers were used in the first PCR. The PCR product obtained was then used as template in the second reaction with species-specific or species-group-specific primers. These nested reactions yielded unambiguous results, and the results of the restriction enzyme digestion confirmed that the products obtained are specific to the dinoflagellates of interest.

All field samples examined in this study displayed high species diversity with respect of phytoplankton, and there was no dominance of any of the two target species, *G. mikimotoi* or *A. minutum,* at any time. Cells of *A. ostenfeldii* and *A. andersonii,* species closely related to *A. minutum,* were not encountered in the field samples (Table 2).

## Discussion

Results of this study show that PCR would be very useful for the detection of *Gymnodinium mikimotoi* and *Alexandrium*

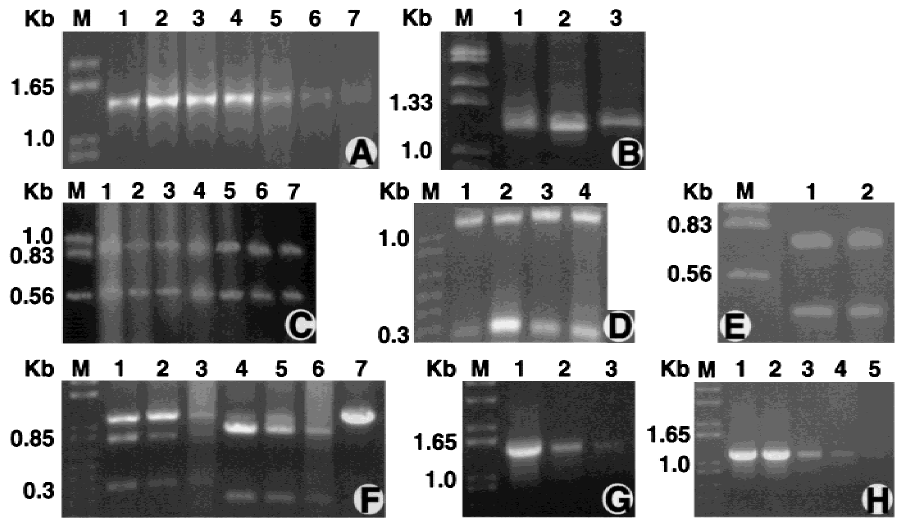


**Figure 3.** **A:** PCR products using species-specific SSU rDNA primers and template DNA from cultured cells of *G. mikimotoi* (direct PCR) and field samples (nested PCR). The expected length of fragment is 1433 bp. M indicates molecular marker (1 kb plus DNA ladder). Lane 1, cultured *G. mikimotoi* (K-0286); lane 2, field sample 4; lane 3, field sample 5; lane 4, field sample 7; lane 5, field sample 10; lane 6, field sample 11; lane 7, field sample 12. **B:** PCR products using species-group-specific SSU rDNA primers and template DNA from cultured cells fo *A. minutum* (direct PCR) and field samples (nested PCR). The expected length of fragment is 1147 bp. M indicates molecular marker (λ DNA/*Eco*RI + *Hin*dIII double digest). Lane 1, cultured *A. minutum* (CCMP113); lane 2, field sample 4; lane 3, field sample 5. **C:** Restriction patterns after digestion with *Xba*I, on PCR products using species-specific SSU rDNA primers and template DNA from cultured *G. mikimotoi* (36) and field samples containing *G. mikimotoi.* The expected lengths of fragments were 865 and 568 bp. M indicates molecular marker (λ DNA/*Eco*RI + *Hin*dIII double digest) Lane 1, digested PCR product of cultured *G. mikimotoi*; lane 2, field sample 3; lane 3, field sample 7; lane 4, field sample 10; lane 5, field sample 11; lane 6, field sample 12; lane 7, field sample 13. **D:** Restriction patterns after digestion with *Mun*I, on PCR products using species-specific SSU rDNA primers and template DNA from cultured *G. mikimotoi* (36) and field samples containing *G. mikimotoi.* The expected lengths of fragments were 1165 and 268 bp. M indicates molecular marker (1-kb plus DNA ladder). Lane 1, digested PCR product of cultured *G. mikimotoi*; lane 2, field sample 2; lane 3, field sample 4; lane 4, field sample 10. **E:** Restriction patterns after digestion with *Bc*I on PCR products, using species-group-specific SSU rDNA primers and template DNA from cultured *A. minutum* (CCMP113) and field samples containing *A. minutum.* The expected lengths of fragments are 736 and 411 bp. M indicates molecular marker (λ DNA/*Eco*RI + *Hin*dIII double digest). Lane 1, digested PCR product of cultured *A. minutum*; lane 2, digested PCR product of field sample 5. **F:** Restriction patterns after digestion with *Mun*I (expected lengths of fragments 833 and 314 bp) and *Bss*HII (expected lengths of fragments, 944 and 20 bp) on PCR products, using species-group-specific SSU rDNA primers and template DNA from cultured *A. minutum* (CCMP113) and field samples containing *A. minutum.* Undegraded PCR product is present at 1147 bp in all lanes. M indicates molecular marker (1-kb plus DNA ladder). Lane 1, *Mun*I-digested PCR product of cultured *A. minutum*; lane 2, *Mun*I-digested PCR product of field sample 4; lane 3, *Mun*I-digested PCR product of field sample 5; lane 4, *Bss*HII-digested PCR product of cultured *A. minutum*; lane 5, *Bss*HII-digested PCR product of field sample 4; lane 6, *Bss*HII-digested PCR product of field sample 5; lane 7, undegraded PCR product *A. minutum* (CCMP113). **G:** Sensitivity of detection of *G. mikimotoi* DNA template, extracted from cultured cells (K-0286). The extracted DNA was serially diluted. M indicates molecular marker (1-kb plus DNA ladder). Lane 1, $1.3 \times 10^{2}$ ng/μl; lane 2, 1.3 ng/μl; lane 3, $2.6 \times 10^{-2}$ ng/μl. **H:** Sensitivity of detection of *A. minutum* DNA template, extracted from cultured cells (CCMP113). The extracted DNA was serially diluted. M indicates molecular marker (1-kb plus DNA ladder). Lane 1, $1.5 \times 10^{2}$ ng/μl; lane 2, 1.5 ng/μl; lane 3, $1.5 \times 10^{-2}$ ng/μl; lane 4, $1.5 \times 10^{-3}$ ng/μl; lane 5, $1.5 \times 10^{-4}$ ng/μl.

*minutum* in field samples. This is an important finding as previous records of successful identification of harmful microalgae by molecular methods in natural field samples are rare.

The extracted DNA from field samples used as template DNA in the PCR reactions came exclusively from net hauls, which is a nonquantitative method of plankton sampling. Hence, the values of cells per liter of the target species displayed in Table 5 only function as an indicator that the species of interest can be detected in rather low concentrations in their environment, and moreover as a nondominating member in a natural plankton community (Table 2). No estimates have been done to exactly calculate how many *G. mikimotoi* or *A. minutum* cells were present in the field samples from which template DNA was extracted.

On 11 separate occasions, *G. mikimotoi* was present in the water in relatively low numbers (Table 5). The template DNA, from which the species-specific reactions gave a clear

**Table 5.** Details of Amplified Field Samples

| Sample no. | Species | Positive by species-specific PCR | Positive by microscopy | Cell count of amplified species (cells/L) in simultaneously collected water samples |
|---|---|---|---|---|
| 2 | *G. mikimotoi* | Yes | Yes | 700 |
| 3 | *G. mikimotoi* | Yes | Yes | 600 |
| 4 | *G. mikimotoi* | Yes | Yes | 900 |
| 5 | *G. mikimotoi* | Yes | Yes | 577 |
| 7 | *G. mikimotoi* | Yes | Yes | 596 |
| 8 | *G. mikimotoi* | Yes | Yes | 306 |
| 9 | *G. mikimotoi* | Yes | Yes | 327 |
| 10 | *G. mikimotoi* | Yes | Yes | 827 |
| 11 | *G. mikimotoi* | Yes | Yes | 1173 |
| 12 | *G. mikimotoi* | Yes | Yes | 885 |
| 13 | *G. mikimotoi* | Yes | Yes | 2077 |
| 4 | *A. minutum* | Yes | Yes | 750 |
| 5 | *A. minutum* | Yes | Yes | 1115 |
| 10 | *A. minutum* | No | Yes | 115 |
| 1 | Negative control | No | No | |
| 6 | Negative control | No | No | |

positive signal by PCR for the presence of *G. mikimotoi,* comprised a very heterogeneous phytoplankton community (Table 2). On 3 different occasions *A. minutum* was present in the water at concentrations ranging from 115 to 1115 cells per liter. *A. minutum* was detected by microscopy in all samples, but the PCR reaction only detected *A. minutum* in 2 of these (Table 5). The reason sample 10 (Tables 1 and 5) did not yield any positive signal by PCR, though it was positive by microscopy, could be that the low cell number (115 cells/L) is the limit of detection for this method in natural samples, given the experiment setup described in "Materials and Methods." It could also be explained by a nonrepresentative net haul, or a failure in the procedure of extracting DNA from that particular sample. The character of the plankton community with respect to species composition and dominance in sample 10 was unusual due to a bloom of *Trichodesmium* sp. (Table 2). However, *G. mikimotoi* was detected from the same sample both by microscopy and by PCR, but the density of *G. mikimotoi* cells was higher, 827 cells/L (Table 5).

A quantitative approach has been made with cultured cells of the target species: *A. minutum* could be detected at a DNA template concentration of $1.5 \times 10^{-4}$ ng/μl, while *G. mikimotoi* could be detected at a DNA template concentration of $2.5 \times 10^{-2}$ ng/μl (Figure 3, G–H). The DNA concentrations correspond to cell concentrations of 0.3 and 697 cultured cells per liter. Puel et al. (1998) found that *Dinophysis acuminata* could be detected from natural samples by species-specific PCR at a concentration of 30 cells/L. Presence of *Alexandrium tamarense* could be confirmed by PCR in filtered seawater samples spiked with cultured cells at a DNA template concentration of $2 \times 10^{-4}$ ng/μl (Haley et al., 1999). Genus-specific primers together with $^{32}$P-labeled probes for *Alexandrium* sp. could detect cultured *A. lusitanicum* Balech at a DNA template concentration of 1.5 fg, corresponding to 100 cells (Penna and Magnami, 1999). These results together with our sensitivity analysis of the primers targeted for *A. minutum,* which gave a positive PCR signal at a cell concentration of 0.3 cells per liter, suggest that PCR is a very sensitive method for detecting marine microalgae. Our result in the sensitivity analysis *G. mikimotoi,* where 697 cells per liter was required to obtain a positive result, was less satisfactory. We suggest that the difference in this sensitivity analyses between the two species could be due to differences in quality of the DNA, although the same method of extraction has been used. A possible explanation is that the DNA extraction protocol used (Ausubel et al., 1987) is better optimized for the genus *Alexandrium* than for *Gymnodinium.*

We also want to stress that the DNA template concen-

trations of *G. mikimotoi* and *A. minutum* needed to obtain a positive PCR product presented here were obtained from unialgal cultures. There is no evidence that these figures would be true in natural field samples when there is a mixed population of species and possibly interference from substances such as humic acids or salts that could inhibit polymerase activity. Natural communities of algae also exhibit differential growth phases, as opposed to unialgal cultures, which could further affect the PCR reaction.

Although primers for the detection of *A. minutum* were designed for species-specific detection (Figure 1), they also amplified DNA from cultured cells of *A. ostenfeldii* and *A. andersonii.* Several phylogenetic studies of *Alexandrium* have reported two different species complexes within this genus based on rDNA genetic information: *A. tamarense/fundyense/catenella/excavtum/affine* and *A. minutum/ostenfeldii/andersonii/margalefii* (e.g., Scholin and Anderson 1994, 1996; Walsh et al., 1998). Our primers were able to distinguish between the two groups, but because of the cross reaction with cultured cells of *A. ostenfeldii* and *A. andersonii,* these could not be denoted as species-specific for *A. minutum* but rather species-group-specific.

Besides being sensitive and specific for identifying and confirming the presence of harmful algae, the PCR-based method is rapid. Thus this technique could be helpful in monitoring programs and serve as an early warning system for the presence of harmful algal species in low concentrations. Our results suggest that for such monitoring programs, it would be better to perform a nested PCR—i.e., amplifying with eukaryotic-specific primers first and then amplifying the species-specific region. Sensitivity of nested PCR has been demonstrated to be at least 10,000 times higher than that of direct PCR under identical conditions (Miserez et al., 1997). Direct PCR of field samples in this study often resulted in very faint bands or no bands at all on UV transillumination, thus yielding apparent false negatives. This was probably due to the presence of DNA from other species and the low concentration of the target species. Cultures of single species, in contrast, were readily amplified by direct PCR.

The restriction patterns of the PCR product obtained from the field samples were as expected, which confirms that the reactions were specific (Figure 3, C–F). These results together with the findings that no other species generated similar restriction maps with the chosen enzymes, indicate the exitence of species- or species-group-specific regions in the SSU rDNA of *G. mikimotoi* and *A. minutum.* It is also noteworthy that in all field samples, there was a heterogeneous population of phytoplankton (Table 2). The observation that *G. mikimotoi* and *A. minutum* could be detected by PCR in such samples shows that the reactions are specific and sensitive.

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