

# Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay

TORSTEIN TENGS,\* HOLLY A. BOWERS,\* ANDREW P. ZIMAN,\* DIANE K. STOECKER† and DAVID W. OLDACH\*

\*Institute of Human Virology, University of Maryland at Baltimore, 725 West Lombard Street, Baltimore, MD 21201, USA,

†University of Maryland Center for Environmental Science, Horn Point Laboratory, PO Box 775, Cambridge, MD 21613, USA

## Abstract

Nuclear and chloroplast-encoded small subunit ribosomal DNA sequences were obtained from several strains of the toxic dinoflagellate *Gymnodinium galatheanum*. Phylogenetic analyses and comparison of sequences indicate that the chloroplast sequences show a higher degree of sequence divergence than the nuclear homologue. The chloroplast sequences were chosen as targets for the development of a 5′–3′ exonuclease assay for detection of the organism. The assay has a very high degree of specificity and has been used to screen environmental water samples from a fish farm where the presence of this dinoflagellate species has previously been associated with fish kills. Various hypotheses for the derived nature of the chloroplast sequences are discussed, as well as what is known about the toxicity of the species.

**Keywords:** dinoflagellates, *Gyrodinium galatheanum*, PCR, plastid, small subunit ribosomal RNA, taqman

Received 15 June 2000; revision received 9 October 2000; accepted 9 October 2000

## Introduction

Algal blooms, including those of harmful species, sometimes visibly discolour surface waters and are then referred to as 'red tides'. Even at densities that do not discolour water, blooms can be harmful; due to high biomass, blooms can decrease water quality, primarily due to increased biological O<sub>2</sub> demand, and can sometimes cause die-off of aquatic life. Some harmful algae produce physical or chemical irritants, and some produce toxins, all of which can have negative impacts on marine life (Hallegraeff 1993; Smayda 1997). Exposure to algal toxins in water, aerosols or seafood can also impact marine mammal and human health (Chang *et al.* 1997; Grattan *et al.* 1998; Harvell *et al.* 1999; Scholin *et al.* 2000).

The dinoflagellate species *Gymnodinium galatheanum* (Syn. *Gyrodinium galatheanum*) is widespread in marine and estuarine waters and has been linked to fish kill events in Europe, Africa and North America (Nielsen & Strømngren 1991; Nielsen 1993, 1996; Aneer & Löfgren 1996; Freeman & Pienaar 1999; K. A. Steidinger, personal communication;

D. E. Terlizzi, personal communication; see also <http://www.fmri.usf.edu/ecohab/galateanum.htm>). Although this species can be difficult to identify in field samples, no molecular detection assay had been developed for it.

*G. galatheanum* is photosynthetic and, therefore, has plastids (a more general term for chloroplasts). Plastids originated, in evolutionary terms, through the engulfment of a cyanobacterium (-like) cell by a eukaryote (-like) cell, and have a genome derived from the original cyanobacterium genome (for review, see Delwiche & Palmer 1997). All plastid genomes investigated to date encode small subunit (SSU) ribosomal RNA (rRNA). Because of the cyanobacterial origin of this gene, the sequence is quite distinct from its nuclear homologue (nuclear SSU rRNA), and SSU rDNA has been sequenced from a broad range of plant and protist plastids. This sequence information has been used primarily to investigate various hypotheses on the endosymbiotic origin of plastids in protists with different pigmentation patterns (Helmchen *et al.* 1995; Medlin *et al.* 1995; Nelissen *et al.* 1995; Tengs *et al.* 2000). In the case of *G. galatheanum* (and other dinoflagellates with 19′hexanoyloxy-fucoanthin as their main carotenoid), their plastid SSU rDNA sequences have recently been demonstrated to be most closely related to those of haptophytes (sometimes called prymnesiophytes),

Correspondence: David W. Oldach. Fax: 410 706 1992; E-mail: oldach@umbi.umd.edu

a distantly related group of protists, and phylogenetic analyses indicate a tertiary endosymbiotic origin of these plastids (Tengs *et al.* 2000).

Many of today's molecular detection assays are based on nuclear ribosomal genes. These genes (especially nuclear SSU rRNA) have been sequenced from a broad range of organisms, and they are present in tandem arrays in high copy numbers. Probes and primers are generally designed from an alignment where multiple sequences are compared to ensure that the assay has the correct selectivity. Some assays rely solely on polymerase chain reaction (PCR) primer-design to obtain the appropriate specificity and sensitivity, whereas others rely on probe hybridization to DNA and/or RNA (for examples, see Cangelosi *et al.* 1997; Worden *et al.* 2000). Other methods combine probe hybridization with PCR, and one way of combining these steps is through the use of the 5'-3' exonuclease assay in a 'real time' PCR amplification (Holland *et al.* 1991; Oldach 1999). This assay, marketed commercially as the TaqMan™ assay, is PCR-based and relies on the hybridization of a labelled probe to the PCR amplicon during the ongoing reaction (i.e. in real time). The probe contains two fluorescent dyes, of which one is an efficient 'quencher' through resonance energy transfer of the other. When the probe is intact, the quencher reduces the signal emitted from the excited dye when stimulated by light of the proper wavelength. The assay is performed using a DNA polymerase that has 5'-3' exonuclease activity, resulting in cleavage of annealed probes during each round of DNA synthesis. As a result of oligonucleotide (probe) cleavage, the excitation dye is no longer coupled to its quencher and it is thus free to emit light that can be detected by a real time PCR instrument. The result is a quantitative, fast and sensitive assay where specificity is enhanced by the utilization of both primer and probe targets.

A problem often encountered when designing probes and primers for a specific locus in a particular genome (species) is poor taxon sampling from related organisms. Nuclear SSU rDNA sequences are often the only gene sequence data available from a reasonable number of close relatives, and sometimes this gene does not allow for an assay with desired selectivity. Many harmful organisms are photosynthetic, and plastid genomes are well characterized from an expanding number of species (for insights, see Delwiche & Palmer 1997; Martin *et al.* 1998). By designing probes and primers to detect plastid SSU rRNA genes instead of nuclear SSU rRNA, the assays can be made very specific for numerous reasons. First, some plastid genes tend to have an elevated evolutionary rate, generating more sequence variation between related taxa than seen when comparing nuclear SSU sequences (Tengs *et al.* 2000). Second, in the case of photosynthetic dinoflagellates like *G. galatheanum*, cross-hybridization with nonphotosynthesizing lookalikes (such as members of the heterotrophic dinoflagellates considered *Pfiesteria*-like dinoflagellates —

although *Pfiesteria piscicida* has been shown to sometimes retain cryptophyte plastids as kleptochloroplasts, Lewitus *et al.* 1999) may be eliminated. Third, cross-hybridization with relatives containing plastids of a different endosymbiotic origin (like the 19'hexanoxyloxy-fucoanthin-containing dinoflagellates vs. the peridinin-containing dinoflagellates, Tengs *et al.* 2000) is virtually eliminated.

With these considerations in mind, and after initial examination of nuclear SSU rDNA sequence alignments revealed that this locus would not contain sequence variation sufficient to distinguish between *G. galatheanum* and closely related dinoflagellates, we selected the *G. galatheanum* plastid SSU rRNA gene as a target for development of a real time PCR detection assay. Plastid SSU rDNA sequences were generated from five different isolates of *G. galatheanum*, and probes and primers were designed to permit detection of these sequences in a TaqMan™ assay. The sensitivity of the assay was assessed by analysing serial dilutions of pure *G. galatheanum* cells. The specificity of the assay was confirmed through testing of panels of other dinoflagellates and algae species with similar pigmentation. Finally, the assay was performance-tested through testing of environmental water samples from sites where the presence of *G. galatheanum* had been confirmed using traditional methodologies.

## Materials and methods

### Cultures and DNA isolation

DNA was isolated from five different cultures of *Gymnodinium galatheanum*. Cultures CCMP 415 and CCMP 416 (both isolated at the same time at 63° N, 10° E, North Atlantic Ocean, Norway) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), and Dr Aishao Li isolated the two other strains; one from the Chesapeake Bay, Maryland (isolate 'GE'), and another from a commercial fish farm, Hyrock Farm, located in Princess Anne on the eastern shore of Maryland on the Manokin River, a subestuary of Chesapeake Bay (isolate 'GE-2'). DNA from *G. galatheanum* Braarud (isolated from the Oslofjord, Norway) was obtained as described elsewhere (Tengs *et al.* 2000). DNA was also extracted from all other available dinoflagellate strains with plastids similar to *G. galatheanum* (and some other selected dinoflagellate species) and multiple strains of haptophytes. In addition, DNA was extracted from 29 water samples (approximately 50 mL, fixed in 1% Lugol's, SIGMA) collected from estuarine aquaculture ponds at Hyrock Farm and from a dilution series of cells from the GE-2 isolate (25 000, 2500, 250, 25 and 2.5 cells/mL).

All extractions were done by filtering samples onto a 5-µm filter paper, and using a DNeasy plant mini kit (QIAGEN) according to the manufacturer's recommendations (total elution volume for DNA = 200 µL).

### PCR and sequencing

Amplification of full-length nuclear SSU rDNA was performed using universal primers designed with reference to an alignment comprised of 173 SSU rDNA sequences (forward Gen18S primer TCCTGCCAGTAGTCATATGC, reverse Gen18S primer TGATCCTTCTGCAGGTTCACTAC, all primer and probe sequences are written in 5'–3' direction). An alignment containing 72 plastid and cyanobacterial SSU rDNA sequences was also made. Both alignments were made using sequences downloaded from GenBank and the software Pileup [GCG (Genetics Computer Group), The Wisconsin Package Version 8.1-UNIX, Deveraux *et al.* (1984)]. The plastid sequence from a Norwegian isolate of *G. galatheanum* Braarud (GenBank Accession no. AF172716) was also included in the plastid/cyanobacterial SSU rDNA alignment. With reference to the plastid/cyanobacterial SSU rDNA alignment, two primers were designed to specifically amplify plastid SSU rDNA from *G. galatheanum* (forward G.gal\_plastid primer GATGAATACTCAGAATCGGAAAAG and reverse G.gal\_plastid primer GCACCACCTATTTCTACTTACC) when used in combination with two general primers flanking the gene (forward Gen16S CTGGCTCAGGATGAACGCT and reverse Gen16S GAGGTGATCCAGCCGCA). All amplifications were carried out using [Mg<sup>2+</sup>] 3 mM and *Taq* DNA polymerase (GIBCO) in a 50- $\mu$ L reaction.

An aliquot of the products from all of the amplification reactions was run on a 1.5% agarose gel, and the remaining PCR products were purified (QIAquick PCR purification kit, QIAGEN, used according to the manufacturer's recommendations) for direct sequencing when a clean product of the correct size was observed.

Sequencing reactions was carried out using a BigDye sequencing kit (Perkin Elmer), and the products were interpreted using an ABI 377 automatic sequencing machine (Perkin Elmer).

### Phylogenetic analyses

To ensure that the sequences obtained were correct, BLAST searches were done using 'Advanced BLAST' (Altschul *et al.* 1997). Our novel sequences [Accession nos (plastid and nuclear SSU rDNA): CCMP 415; AF272048 and AF272045, CCMP 416; AF272052 and AF272049, GE isolate; AF272047 and AF272046, GE-2 isolate; AF272051 and AF272050] were also added to the matrices using the program SeqPup (Gilbert 1996) in order to perform simple phylogenetic analyses. Ambiguously aligned areas were excluded from the two matrices and selected sequences were used in distance (Minimum Evolution, ME) analyses (Swofford 2000).

Maximum likelihood value for proportion of invariable sites (pinvar) was initially measured from neighbour-joining (NJ)/Kimura 2-parameter (K2P) topologies using

the two matrices. These values were in turn used to calculate Tamura and Nei (TrN) distances for the nuclear SSU rDNA sequences and LogDet distances for the plastid sequences to be used in a ME analyses. For the analysis of the nuclear sequences, 1859 characters and 39 taxa were used, whereas 1487 characters and 33 taxa were used for the plastid analysis. Heuristic searches were carried out with 25 random addition of the sequences and tree-bisection-reconnection (TBR) branch swap on both data sets, and finally bootstrap analyses were performed (100 replicates and the same method as the initial analysis).

All phylogenetic analyses were carried out using PAUP\* (Swofford 2000), and alignments together with strain references for the sequences used are available upon request (see also Tengs *et al.* 2000 and Oldach *et al.* 2000 for strain information).

### *G. galatheanum* specific real time PCR assay

With reference to the plastid/cyanobacterial SSU rDNA alignment, a set of two *G. galatheanum* specific primers (forward G.gal\_Taq Primer: CGCAACCCTTGTTTGGTCAG, reverse G.gal\_Taq primer: AGTAAGCGGCTCTTTGCTTAAACC) and a probe were designed (G.gal\_TaqProbe: TGAGAAATCGGAGGAAGGTAAGGATGACG) using the software Primer Express (Test version, Perkin Elmer). The probes had 5' ends labelled with 5-carboxyfluorescein (5-FAM – used as dye) and 3' labelled with 5-carboxy-tetramethylrhodamine (5-TAMRA – used as quencher) and were synthesized by OPERON Technologies, Inc. Total size of the expected amplicon was 149 bp. The real time PCRs were performed using 10  $\mu$ L capillary tubes in a LightCycler™ (Wittwer *et al.* 1997) with [Mg<sup>2+</sup>] 4 mM and *Taq* DNA polymerase (GIBCO) in a 7- $\mu$ L reaction. The primer concentration used was 0.2  $\mu$ M and the probe concentration was 3  $\mu$ M. Cycling parameters used were 50 cycles at 95 °C denaturation (0 s) and 55 °C annealing (20 s) with no elongation step.

Aliquots (10 mL) of the dilution series of *G. galatheanum* cells from the GE-2 strain were assayed to test sensitivity of the assay, and the water sample from fish ponds at Hyrock Farm were screened to test the applicability of the assay when working with Lugol's fixed environmental samples. DNA isolates from the other strains and species were also screened to ensure that there was no nonspecific cross-hybridization of primers/probes, and all of the *G. galatheanum* strains were screened as positive controls (Table 1).

## Results

### Nuclear SSU rDNA sequences

The nuclear SSU rDNA sequences derived from *Gymnodinium galatheanum* cultures CCMP 415 and CCMP 416 were

Species	Strain reference	Assay
<i>Gymnodinium galatheanum</i> F	CCMP 415	+
<i>G. galatheanum</i> F	CCMP 416	+
<i>G. galatheanum</i> F	Hyrock Farms, GE-2 (Dr Aishao Li)	+
<i>G. galatheanum</i> F	Chesapeake Bay, GE (Dr Aishao Li)	+
<i>G. galatheanum</i> F	Braarud	+
<i>G. breve</i> F	Wilson	-
<i>G. breve</i> F	Davis	-
<i>G. breve</i> F	Piney Island	-
<i>Gyrodinium aureolum</i> F	Hulburt	-
<i>G. mikimotoi</i> F	CCMP 429	-
<i>G. mikimotoi</i> F	CCMP 430	-
<i>IsochrYSIS galbana</i> H	Horn Point Environmental Laboratory	-
<i>Prymnesium</i> cf. <i>patelliferum</i> H	Dr Bente Edvardsen, University of Oslo	-
<i>Chrysochromulina polylepis</i> H	Dr Bente Edvardsen, University of Oslo	-
<i>Emiliania huxleyi</i> H	Dr Bente Edvardsen, University of Oslo	-
<i>Pavlova gyrans</i> H	Butcher	-
<i>Coolia monotis</i> H	CCMP 305	-
<i>Gambierdiscus toxicus</i> P	CCMP 1600	-
<i>Lingulodinium polyedrum</i> P	CCMP 421	-
<i>Heterocapsa triquetra</i> P	CCMP 449	-
<i>G. sanguineum</i> P	CCMP 417	-
<i>Alexandrium tamarense</i> P	CCMP 116	-
<i>Pfiesteria piscicida</i> N	Florida Marine Research Institute	-

**Table 1** Specificity of the *Gymnodinium galatheanum* assay when tested against various dinoflagellate and haptophyte species

F, 19'hexanoxyloxy-fucoxanthin-containing dinoflagellates; H, Haptophytes; P, Peridinin-containing dinoflagellates; N, Non-photosynthetic dinoflagellate species [although *P. piscicida* has been shown to sometimes retain cryptophyte plastids (Lewitus *et al.* 1999)].

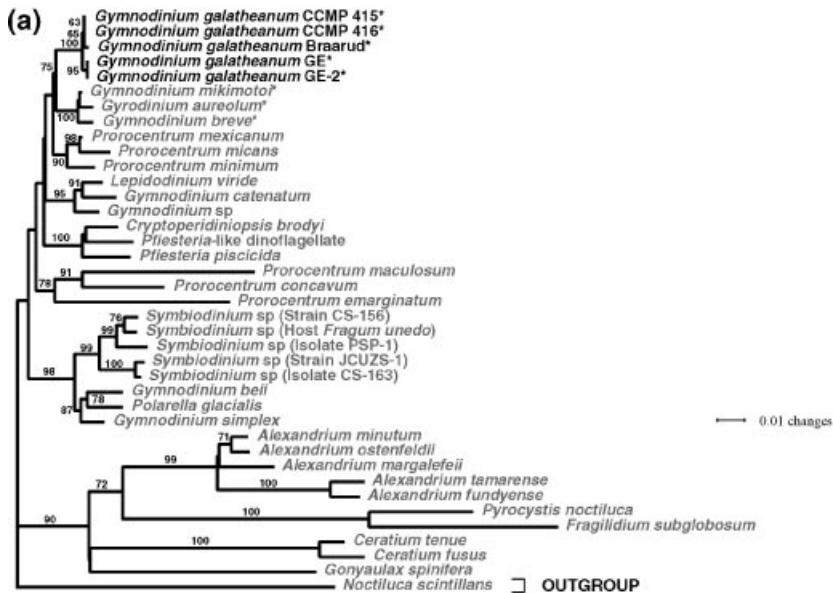
identical to the sequence from the Braarud strain in Norway except for some ambiguities seen in the Braarud sequence (Accession no. AF172712). These sequences differed by only 2 bp from that derived from the two North America strains (GE-2 and GE), which were identical to each other (Fig. 1a). The 2 bp difference seen between these sequences constitutes a set of covarying characters that maintains the structure of the encoded rRNA when mapped onto a secondary structure map of the molecule (Maidak *et al.* 1999; Fig. 2).

The phylogenetic analysis was performed using *Noctiluca scintillans* as an outgroup and included only dinoflagellate SSU rDNA sequences (Fig. 1a). *N. scintillans* has been shown to belong to a primitive lineage of dinoflagellates, and can thus be used to root tree topologies showing dinoflagellate phylogeny (Saunders *et al.* 1997). The 19'hexanoxyloxy-fucoxanthin-containing dinoflagellates grouped together with moderate support (75%) and the internal placement of this group within the dinoflagellates remains unresolved from this analysis (Fig. 1a). The phylum dinoflagellata has been shown to belong within a group of protists called Alveolata, which includes ciliates and apicomplexans (for review, see Cavalier-Smith 1993).

#### Plastid SSU rDNA sequences

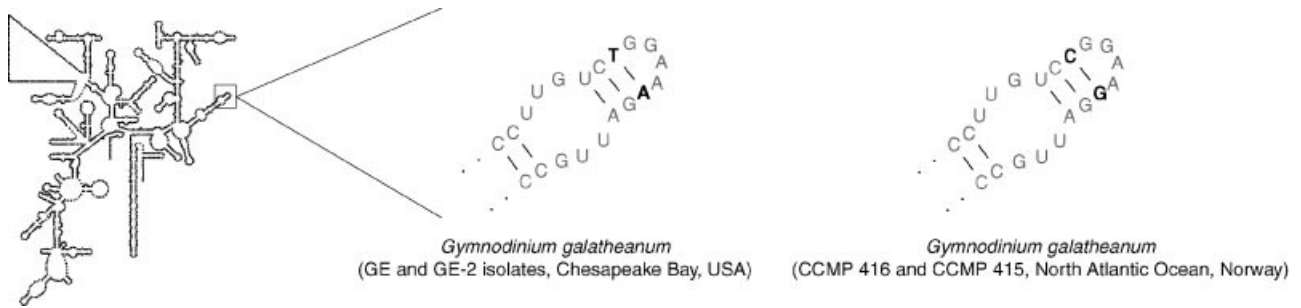
Although the examined *G. galatheanum* nuclear SSU rDNA sequences showed a very high degree of sequence similarity when compared with each other, the plastid SSU sequences demonstrated greater sequence variation. They showed the same pattern (Norwegian isolates CCMP 415/416 identical to each other and close to the Braarud sequence, and the two North America strains GE/GE-2 identical to each other), but the distances between the sequences was much larger (Fig. 1b). When comparing CCMP 415/416 with GE-2/GE, the sequences were different in a total of 57 sites (counting both insertions/deletions and nucleotide differences using the same alignment as for the phylogenetic analysis).

Upon inspection of the plastid/cyanobacteria SSU rDNA alignment, several insertions unique for the *G. galatheanum* sequences were observed. Although the sequences appeared derived when compared with the rest of the haptophyte/19'hexanoxyloxy-fucoxanthin-containing dinoflagellate sequences, the *G. galatheanum* strains shared a great number of synapomorphic characters and grouped together with high bootstrap support (100%) in the phylogenetic analysis (Fig. 1b). The phylogenetic analysis was done using plastid



**Fig. 1** Distance trees (Minimum Evolution) showing phylogenetic relationship between SSU rDNA sequences. Vertical bars connecting CCMP 415/416 and *Gymnodinium galatheanum* GE-2/GE isolates indicate sequence identity in both the nuclear and plastid sequences. Bootstrap values above 60% are indicated. (a) Nuclear SSU rDNA (18S) topology showing phylogenetic relationships between various dinoflagellates. Tamura and Nei (TrN) distances were used with random addition of the sequences, tree-bisection-reconnection (TBR) branch swap and pinvar set to 0.60. The same topology was found 25 out of 25 times using heuristic searches. (b) Alignment of plastid SSU rDNA (16S) sequences analysed using random addition of the sequences, TBR branch swap and LogDet distances (pinvar set to 0.49). The same tree topology was found 25 out of 25 times using heuristic searches. Accession numbers and strain references for the sequences are as listed in Tengs *et al.* 2000. Scale bars indicate inferred number of substitutions per site using LogDet and TrN distances, respectively. \*Dinoflagellate species with 19'hexanoyloxy-fucoanthin as main carotenoid.

Note: although sequences CCMP 415/416 are identical (like *G. galatheanum* GE-2/GE), they will not necessarily group together with 100% bootstrap support. The reason for this is that when bootstrapping a data set to generate pseudoreplicates, and subsequently analysing these data sets, distances of zero cause branches to collapse, creating polytomies. So, for instance, if two sequences are identical and close to another distinct sequence, polytomies can occur because the bootstrapping will not always sample the characters that distinguishes the sequences.



**Fig. 2** Secondary structure of nuclear encoded *Babesia bigemina* SSU rRNA (closest relative of dinoflagellates where secondary structure information is available, Maidak *et al.* 1999) indicating that the 2 bp difference seen between CCMP 415/416 and *Gymnodinium galatheanum* GE-2/GE isolates corresponds to two coevolving nucleotides that conserves the structure of the encoded rRNA.

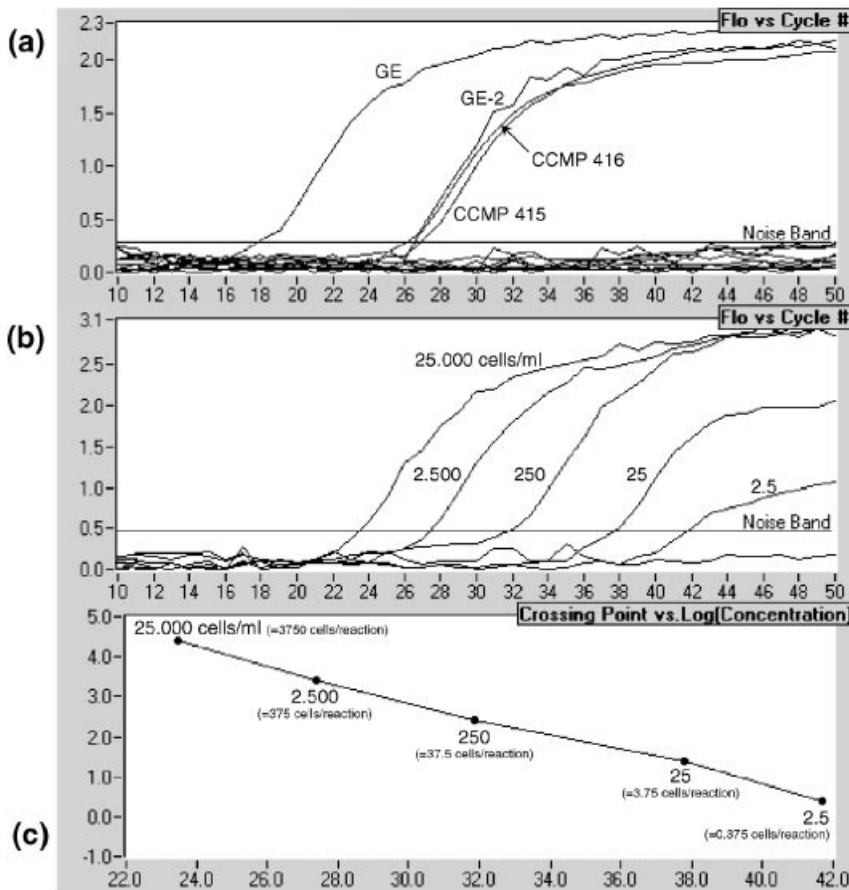


Fig. 3 Output from the LightCycler™ when performing *Gymnodinium galatheanum* assays. (a) Result from screening of multiple *G. galatheanum* strains and DNA from other dinoflagellates and haptophytes. The negative samples (signal below noise band) are: *G. breve*, *G. aureolum*, *G. mikimotoi*, *Isochrysis galbana*, *Chrysochromulina polylepis*, *Emiliania huxleyi*, *Alexandrium tamarense* and *Pfiesteria piscicida* (Table 1). (b) Serial dilution of *G. galatheanum* cells (strain isolated from Hyrock Fish Farm, 10 mL filtered) is assayed. (c) Cycle number where the fluorescence level rises above background noise plotted against the concentration of cells used.

SSU rDNA sequences from a broad range of taxa and glaucophyte sequences were used as a root (Martin *et al.* 1998). There was strong bootstrap support (99%) for grouping the plastids of haptophytes together with those of the 19'-hexanoyloxy-fucoxanthin-containing dinoflagellates, but low support (46%, data not shown) for monophyletic origin of these dinoflagellate plastids.

#### Real time PCR detection of *G. galatheanum*

The derived nature of the plastid sequences permitted design of PCR primers and probe predicted to have high specificity for the *G. galatheanum* sequences. The assay detected all five *G. galatheanum* strains examined, and did not cross react with any of the closely related species, nor did it detect DNA from organisms with similar plastid types (Table 1).

When tested on the serial dilution of *G. galatheanum* cells in sterile seawater medium, the real time PCR assay had a lower detection level of approximately 2.5 cells/mL when 10 mL water samples were used (Fig. 3b,c). Because DNA was eluted in 200  $\mu$ L of elution buffer, and 3  $\mu$ L of DNA

was used to perform the assay, this is the equivalent of 0.375 cells/reaction. This level of sensitivity of the assay was confirmed by capillary isolating *G. galatheanum* cells and successfully detecting individual cells (data not shown). By plotting the cycle number where the fluorescence level rises above background noise (noise band is set automatically by the LightCycler™ software to be 10% of the net fluorescence signal from the assay) against the cell concentrations, the sum of squared residuals divided by number of points is 0.098, indicating a high degree of linearity for the assay (Fig. 3c).

Multiple of the water samples from the fish farm gave positive result when screened (data not shown), consistent with morphological identification of *G. galatheanum* from the same sites using microscopy techniques.

#### Discussion

##### *Ecology, toxicity and morphology of Gymnodinium galatheanum*

*Gymnodinium galatheanum* was transferred to *Gyrodinium galatheanum* based on the amount of cingulum displacement

by Taylor (1992) but in the literature it is still often referred to as *Gymnodinium galatheanum*. In phylogenetic analyses based on nuclear-encoded large subunit (LSU) rDNA (domains D1–D3) it groups with *Gymnodinium breve* and *Gymnodinium mikimotoi* (Syn. *Gyrodinium aureolum*, *Gyrodinium nagasakiense*) (Hansen *et al.* 2000; see also Tengs *et al.* 2000). *G. breve* (syn. *Ptychodiscus breve*), *G. galatheanum* and *G. mikimotoi* are all characterized by unusual photosynthetic pigments for dinoflagellates, including presence of chlorophyll  $c_3$  and lack of peridinin as the major light-harvesting carotenoid (Johnsen & Sakshaug 1993). All three dinoflagellate species are considered toxic and have been implicated in fish kills or have been associated with other harmful effects in marine ecosystems (Hallegraeff 1993; Steidinger & Tangen 1997; see also <http://www.fmri.usf.edu/ecohab/fhab.htm>).

*G. galatheanum* is widespread in temperate coastal and estuarine waters and has been reported from Africa, Europe and North America (Braarud 1957; Aneer & Löfgren 1996; Nielsen 1996; Freeman & Pienaar 1999; Li *et al.* 2000). A similar or perhaps the same species has been observed in the mid-east (Abboud-Abi *et al.* 2000). Although it can grow as a strict photoautotroph, using inorganic nutrients and light as a source of carbon and energy, growth of *G. galatheanum* is stimulated by ingestion of algal prey, and thus it is a facultative mixotroph (Li *et al.* 1999). Harmful effects on other marine organisms have only been experimentally observed at high densities ( $\geq 10^5$  cells  $\text{ml}^{-1}$ ) of *G. galatheanum* (Nielsen & Strömgen 1991; Nielsen 1993), but the mechanism of toxicity for this species is not yet characterized.

Within the last 5 years, *G. galatheanum* has been identified from the south-eastern coast of the USA, although it is likely that the species was present previously, but misidentified. However, it has recently been implicated in fish kills in estuarine aquaculture ponds in Texas, South Carolina and Maryland (K. A. Steidinger, personal communication; Terlizzi *et al.* 2000; D. E. Terlizzi, personal communication). *G. galatheanum* has also been identified from a historic fish kill area in Florida (K. A. Steidinger, personal communication). At Hyrock Farms in Maryland, fish kills associated with *G. galatheanum* blooms occurred in 1996, 1997 and 1999 and resulted in the loss of 20 000, 8000 and 5000 hybrid striped bass, respectively (Terlizzi *et al.* 2000; D. E. Terlizzi, personal communication). Economic losses in estuarine aquaculture due to decreased growth and mortality of fish caused by *G. galatheanum* can be considerable, and thus are an important management concern in some areas (Terlizzi *et al.* 2000). Fish ponds are highly eutrophic habitats with altered food web structure, and it seems possible that changes induced by anthropogenic activities, including eutrophication, may stimulate or permit dense blooms of this species. *G. galatheanum* may, thus, be expected to become more of a problem in coastal waters in the future.

Although *G. galatheanum* is a common species, it can be

difficult to identify in field samples because of its small size,  $< 20 \mu\text{m}$ , and lack of distinctive armour. In the past, it was probably often over-looked or misidentified in samples examined using light microscopy to identify and count dinoflagellate cells. In investigations using high performance liquid chromatography (HPLC) to detect the presence of dinoflagellates based on the presence of peridinin, it would not have been detectable because of its unusual pigment composition. Thus, a molecular detection assay is particularly appropriate for this species.

#### *SSU rDNA sequences and detection assay*

When comparing the nuclear SSU rDNA sequences derived from the different *G. galatheanum* strains, very little sequence variation was observed (see Fig. 1a). The fact that the GE-2 isolate was identical to the GE isolate (both nuclear and plastid SSU rDNA sequences) was not surprising because water from the Chesapeake Bay is used in the ponds. But, when comparing plastid SSU rDNA sequences from the different sites (North America and Norway), genetic polymorphism is observed. Several explanations can be hypothesized for this sequence diversity, which may reflect a higher evolutionary rate in plastid vs. nuclear SSU rDNA sequences. It may be that DNA repair mechanisms in plastids are deficient in comparison with those seen in eukaryote nuclei (as has been described for DNA repair in mitochondrial genomes, see Croteau *et al.* 1999). Another explanation could be that the (hypothesized) high evolutionary rate seen in plastids is strictly a product of population genetics, where plastids (within a cell) behave like small populations of organisms. The plastids divide by binary fission, so in the absence of exchange of genetic material occurring between plastids, mutations may be more readily fixed leading to faster accumulation (than that observed in organisms with haploid and diploid life stages). A more general explanation for the derived nature of some plastid genomes could be that this solely reflects the changes in selection pressure a symbiont goes through when adopting to a new environment (evolving from being an endosymbiont to becoming a stable organelle). This phenomenon has been observed for fungi adapting to a mutualistic lifestyle (Lutzoni & Pagel 1997).

Although the *G. galatheanum* plastid SSU rDNA sequences from the different sites were distinct, we considered it unlikely that this reflected multiple independent endosymbiotic events. The plastids of *G. galatheanum* are probably the result of myzocytosis or phagocytosis with multiple subsequent gene-transfers (Tengs *et al.* 2000), and this may, in an evolutionary perspective, be considered a complex process. Although conceivable that multiple such events have occurred independently, the most parsimonious explanation is a monophyletic origin of all 19'hexanoyloxy-fucoxanthin-containing dinoflagellate plastids.

The great majority of photosynthetic dinoflagellates have plastids containing peridinin as their main carotenoid. A plastid SSU rDNA sequence has been reported from the peridinin-containing dinoflagellate *Heterocapsa triquetra* (Zhang *et al.* 1999), but this sequence is extremely derived when compared to other plastid SSU rDNA sequences (Tengs *et al.* 2000), and was virtually impossible to include in our plastid/cyanobacteria SSU rDNA alignment in any meaningful way. It is, therefore, highly unlikely that an assay designed to detect plastids of a different pigmentation pattern will cross react with any peridinin-containing species.

Probes and primers were designed to detect all the different strains of *G. galatheanum*. Care was also taken to design the primer/probe system to target a region of the gene with a mutation rate as low as possible while still retaining a high degree of specificity for this species. However, it is not inconceivable that there are strains of *G. galatheanum* that this assay might not detect without some modification.

The LightCycler™ is designed so that the amount of amplified DNA can be measured quantitatively – as the number of amplicons increases, the fluorescence-level increases accordingly. By using the cycle number were the amplification gives a signal above a certain threshold as a direct indicator for how many copies of the target were present before the PCR, this assay avoids some of the problems encountered when trying to do a quantitative amplification without a real time PCR machine. Most amplification reactions reach a plateau-level after a certain number of cycles, and this is not necessarily an accurate indication of template amount initially added to the reaction (Morrison & Gannon 1994). For the *G. galatheanum* assay described herein, several factors will affect the quantitative performance of the screening process, including integrity of the isolated DNA and DNA extraction efficiency. Some dinoflagellates have also been shown to have DNA content that varies dependent not only on the life-stage of the cells, but also on population growth-phase (Rizzo 1987). Quantification assays targeting plastid DNA sequences may be affected in this way (i.e. the difference in cycle number at which signal rises above background in Fig. 3a might not be solely due to differences in cell number), although we believe that the variation is probably mostly due to changes in nuclear DNA content. This detection assay can also be modified into a standard PCR-based method. The PCR amplicons from the *G. galatheanum* real time PCR were visualized using ethidium bromide stained 0.8% agarose gels, and all of the samples that were positive gave strong bands of the correct size (149 bp, data not shown).

In summary, we have developed a rapid and highly specific real time PCR assay for detection of a harmful algal blooms (HAB) species of economic significance,

*G. galatheanum*. The assay should provide a useful tool for further studies of the ecology of this organism and its impacts on aquaculture and natural aquatic systems. Due to their derived nature, plastid genomes represent useful targets for molecular detection assays, and may provide unique insights into some evolutionary events underlying eukaryotic diversity. The derived nature of plastid sequences should also provide useful information in studies of population genetics and 'strain' or subspecies characterization among dinoflagellates (and other species) of interest.

## Acknowledgements

The authors would like to thank Aishao Li, Dan Terlizzi, Bente Edvardsen, Kamran Shalchian-Tabrizi and Karen Steidinger for providing culture material and algal DNA so that this assay could be developed. T. Tengs thanks Dr Kjetill S. Jakobsen for mentorship of preliminary work leading up to this project.

## References

- Abboud-Abi Saab M, Chedid S, Therese-Kassab M (2000) Relationships between toxic phytoplankton and environmental factors in fishing harbors in Lebanese waters. *Abstract submitted for the ninth conference on Harmful Algal Blooms*. Hobart Tasmania.
- Altschul SF, Madden TL, Schaffer AA *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.
- Aneer GL, Löfgren S (1996) *ALGAL BLOOM – Some Questions and Answers*. Report published by the Information Office for the Baltic Proper at the County Administrative Board of Stockholm (Web-page address: <http://www.ab.lst.se/infobalt/TEXT/QUEST1.HTM#CONTENTS>).
- Braarud T (1957) A red water organism from Walvis Bay (*Gymnodinium galatheanum* n. sp.). *Galathea Report*, **1**, 137–138.
- Cangelosi GA, Hamlin AM, Marin R 3rd *et al.* (1997) Detection of stable pre-rRNA in toxigenic *Pseudo-nitzschia* species. *Applied Environmental Microbiology*, **63**, 4859–4865.
- Cavalier-Smith T (1993) Kingdom protozoa and its 18 phyla. *Microbiological Reviews*, **57**, 953–994.
- Chang FH, Anderson DM, Kulis DM *et al.* (1997) Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. *Toxicon*, **35**, 393–409.
- Croteau DL, Stierum RH, Bohr VA (1999) Mitochondrial DNA repair pathways. *Mutation Research*, **434**, 137–148.
- Delwiche CF, Palmer JD (1997) The origin of plastids and their spread via secondary symbiosis. *Plant Systematics and Evolution*, **11** (Suppl.), 53–86.
- Deveraux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research*, **12**, 387–395.
- Freeman AJ, Pienaar RN (1999) *Gymnodinium cf. galatheanum*: a toxic marine dinoflagellate from Namibia. *Presentation given at the 38th annual Microscopy Society of Southern Africa (MSSA) meeting*. University of the Free State (UFS), South Africa.
- Gilbert D (1996) *SeqPup, Version 0.6. A biological sequence editor and analysis program*. <ftp://iubio.indiana.edu/molbio/seqpup/>
- Grattan LM, Oldach D, Perl TM *et al.* (1998) Learning and memory difficulties after environmental exposure to waterways containing

- toxin-producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. *Lancet*, **352**, 532–539.
- Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. *Phycologia*, **32**, 79–99.
- 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.
- Harvell CD, Kim K, Burkholder JM *et al.* (1999) Emerging marine diseases — climate links and anthropogenic factors. *Science*, **285**, 1505–1510.
- Helmchen TA, Bhattacharya D, Melkonian M (1995) Analyses of ribosomal RNA sequences from glaucocystophyte cyanelles provide new insights into the evolutionary relationships of plastids. *Journal of Molecular Evolution*, **2**, 203–210.
- Holland PM, Abramson RD, Watson R *et al.* (1991) Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences of the USA*, **88**, 7276–7280.
- Johnsen G, Sakshaug E (1993) Bio-optical characteristics and photoadaptive responses in the toxic and bloom-forming dinoflagellates *Gyrodinium aureolum*, *Gymnodinium galatheanum*, and two strains of *Prorocentrum minimum*. *Journal of Phycology*, **29**, 627–642.
- Lewitus A, Glasgow HB, Burkholder JM (1999) Kleptoplastidy in the toxic dinoflagellate *Pfiesteria piscicida* (Dinophyceae). *Journal of Phycology*, **35**, 303–312.
- Li A, Stoecker DK, Adolf JE (1999) Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. *Aquatic Microbial Ecology*, **19**, 163–176.
- Li A, Stoecker DK, Coats DW (2000) Mixotrophy in *Gyrodinium galatheanum* (Dinophyceae): Grazing responses to light intensity and inorganic nutrients. *Journal of Phycology*, **36**, 33–45.
- Lutzoni F, Pagel M (1997) Accelerated evolution as a consequence of transitions to mutualism. *Proceedings of the National Academy of Sciences of the USA*, **94**, 11422–11427.
- Maidak BL, Cole JR, Parker CT *et al.* (1999) A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Research*, **27**, 171–173.
- Martin W, Stoebe B, Goremykin V *et al.* (1998) Gene transfer to the nucleus and the evolution of chloroplasts. *Nature*, **393**, 162–165.
- Medlin LK, Cooper A, Hill C *et al.* (1995) Phylogenetic position of the Chromista plastids based on small subunit rRNA coding regions. *Current Genetics*, **28**, 560–565.
- Morrison C, Gannon F (1994) The impact of the PCR plateau phase on quantitative PCR. *Biochimica et Biophysica Acta*, **1219**, 493–498.
- Nelissen B, Van de Peer Y, Wilmotte A *et al.* (1995) An early origin of plastids within the cyanobacterial divergence is suggested by evolutionary trees based on complete 16S sequences. *Molecular Biology and Evolution*, **12**, 1166–1173.
- Nielsen MV (1993) Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. *Marine Ecology Progress Series*, **95**, 273–277.
- Nielsen MV (1996) Chemical composition of the toxic dinoflagellate *Gymnodinium galatheanum* in relation to irradiance, temperature and salinity. *Marine Ecology Progress Series*, **136**, 205–211.
- Nielsen MV, Strømgren T (1991) Shell growth response (*Mytilus edulis*) exposed to toxic microalgae. *Marine Biology*, **108**, 263–267.
- Oldach D (1999) 'Real-time' polymerase chain reaction. *Gastroenterology*, **116**, 763–764.
- Oldach DW, Delwiche CF, Jakobsen KS *et al.* (2000) Heteroduplex mobility assay-guided sequence discovery: elucidation of the small subunit (18S) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal culture and environmental sample DNA pools. *Proceedings of the National Academy of Sciences of the USA*, **97**, 4303–4308.
- Rizzo PJ (1987) The biochemistry of the dinoflagellate nucleus. In: *The Biology of Dinoflagellates* (ed. Taylor FJR), pp. 143–174. Blackwell scientific publications, Boston.
- Saunders GW, Hill DRA, Sexton JP *et al.* (1997) Small-subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypothesis with molecular systematic methods. *Plant Systematics and Evolution*, **11** (Suppl.), 237–239.
- Scholin CA, Gulland F, Doucette GJ *et al.* (2000) Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature*, **403**, 80–84.
- Smayda TE (1997) What is a bloom? A Commentary. *Limnology and Oceanography*, **42**, 1132–1136.
- Steidinger KA, Tangen K (1997) Dinoflagellates. In: *Identifying Marine Phytoplankton* (ed. Tomas CR), pp. 387–584. Academic Press, San Diego.
- Swofford DL (2000) PAUP\*. *Phylogenetic Analysis Using Parsimony (\*and Other Methods)*, Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Taylor FJR (1992) The taxonomy of harmful marine phytoplankton. *Giornale Botanico Italiano*, **126**, 209–219.
- Tengs T, Dahlberg OJ, Shalchian-Tabrizi K *et al.* (2000) Phylogenetic analyses indicate that the 19'hexanoyloxy-fucoanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Molecular Biology and Evolution*, **17**, 718–729.
- Terlizzi DE, Stoecker D, Glibert PM (2000) *Gyrodinium galatheanum*: a threat to estuarine aquaculture waters. In: *Responsible Aquaculture in the New Millennium. Abstracts of Contributions Presented at the International Conference AQUA 2000, Nice, France, May 2–6, 2000* (eds. Flos R, Cressell L), pp. 700. European Aquaculture Society Special Publication 28, Ostende, Belgium.
- Wittwer CT, Ririe KM, Andrew RV *et al.* (1997) The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques*, **22**, 176–181.
- Worden AZ, Chisholm SW, Binder BJ (2000) *In situ* hybridization of *Prochlorococcus* and *Synechococcus* (marine cyanobacteria) spp. with rRNA-targeted peptide. *Applied Environmental Microbiology*, **66**, 284–289.
- Zhang Z, Green BR, Cavalier-Smith T (1999) Single gene circles in dinoflagellate chloroplast genomes. *Nature*, **400**, 155–159.

---

T. Tengs is a graduate student in molecular systematics and dinoflagellate phylogeny. His work on the dinoflagellates was initiated at the University of Oslo under the guidance of K. Jakobsen, and the present work was performed at the Institute of Human Virology, Baltimore in the laboratory of D. Oldach. Dr Oldach is investigating both clinical (human health effects) and molecular ecology of *Pfiesteria piscicida* and other toxicity associated dinoflagellates. H. Bowers is a research associate in this Laboratory. A. Ziman worked as a summer student on this project and D. Stoecker studies estuarine aquatic ecology. This work was supported by ECOHAB (Ecology and Oceanography of Harmful Algal Blooms) Grant Number R827084 (administered by the U.S. Environmental Protection Agency).

---