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# Molecular studies of *Dinophysis* (Dinophyceae) species from Sweden and North America

ANN-SOFI REHNSTAM-HOLM[1]*, ANNA GODHE[2] AND DONALD M. ANDERSON[1]

[1]*Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*
[2]*Marine Botany, Göteborg University, Box 461, SE-405 30 Göteborg, Sweden*

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Diarrhoeic shellfish poisoning has increasingly become a problem throughout the world. Because the causative organisms *Dinophysis* spp. cannot be cultured in the laboratory, new approaches are needed to obtain ecological and physiological information. In this study, *D. acuminata, D. norvegica* and *D. acuta* were collected directly from field samples and used in polymerase chain reactions. The D1–D2 region of the large-subunit ribosomal RNA gene was amplified, cloned and sequenced. Sequence analyses showed that *D. acuminata* and *D. norvegica* were nearly identical (> 99%), and that *D. acuminata* showed an intraspecies variation of 0.8%. The *D. acuta* sequence was 98.7% similar to that of *D. acuminata.* The slight differences between *D. norvegica* and *D. acuminata* suggest that they may have evolved into separate species rather recently. Phylogenetic analyses show that species within the Dinophysiales order should be included in the 'GPP complex', a lineage associated with a diverse array of taxa within the orders Gymnodiniales, Prorocentrales and Peridiniales. The Prorocentrales and Dinophysiales would be sister groups within the GPP complex. Amplification of Swedish *D. acuminata* isolates always resulted in a single LSU rDNA fragment. In contrast, amplification of the North American *D. acuminata* always produced two distinct fragments. The longer (735 bp) fragment showed 99.3–100% homology among all sequenced clones of different *D. acuminata* field isolates. The shorter gene fragment had a 70 bp deletion, but it was otherwise highly homologous to the larger gene fragment. This fragment is possibly a pseudogene and might be an important genetic marker. A variable region that is suitable as a target for a probe to identify *Dinophysis* was also identified. *Dinophysis* specificity was confirmed for the probe, in that hybridization to cultured representatives of dinoflagellates and environmental samples containing mixed phytoplankton assemblages resulted in specific labelling of *D. acuminata, D. norvegica* and *D. acuta,* but not other dinoflagellates. No labelling of *D. rotundata* was observed.

## INTRODUCTION

Blooms of toxic or harmful algae, now collectively termed as harmful algal blooms (HABs), have become increasingly prevalent in coastal waters over the past several decades. The number of toxic blooms, known toxins and toxic species, the economic losses and the type and number of resources affected have all increased (Anderson 1989; Hallegraeff 1993). One fundamental question is whether this expansion is directly related to human activities, such as eutrophication (e.g. Smayda 1990), or whether it is a trend linked to climate change, increased numbers of observers, improved toxin detection methods, or other factors (Anderson 1989). To answer these questions, accurate estimates are needed of the abundance and distribution of individual species or strains in regions where toxic blooms occur, as well as information on the genetic relatedness of HAB populations to other populations of the same species that occur in different regions.

A few dozen phytoplankton species have the capacity to produce toxins that can be accumulated through the food web (Hallegraeff 1993). Consumption of shellfish that have accumulated these algal toxins during feeding causes a variety of poisoning syndromes in humans. In Europe, diarrhoeic shellfish poisoning (DSP) has become one of the predominant threats in this regard. A peculiarity about this syndrome is that the causative organisms *Dinophysis* spp. are present in most coastal temperate waters throughout the world, yet many countries have no history of DSP outbreaks (Hallegraeff *et al.* 1995). Another related observation is that there may be a difference in toxicity between geographically isolated clones and different blooms of the diarrhoeic shellfish toxin (DST)-producing algae within the same water mass. For example, regular autumn blooms of toxic *D. acuminata* Claparède & Lachmann in the Scandinavian waters are interspersed with non-toxic blooms (Rehnstam-Holm *et al.* 1999; Dahl & Johannesson 2001). One of the major reasons for our general lack of knowledge regarding the basic biology and genetics of the *Dinophysis* spp. is that, thus far, they have proven to be unculturable.

Because of the DSP problem, *Dinophysis* spp. are of great interest and importance in phytoplankton-monitoring programmes in many parts of the world. A crucial problem in all such programmes is the rapid and accurate identification and enumeration of the plankton species, because this becomes increasingly difficult with high-density sampling and long-term monitoring if time-consuming microscopical analysis of discrete samples is employed. Species identification can also be problematic. For example, with *Dinophysis* there is confusion regarding the *D. acuminata–D. sacculus* Stein complex (Bravo *et al.* 1995; Zingone *et al.* 1998). Classical morphology-based taxonomy reveals a high number of morphotypes

* Corresponding author (ann-sofi.holm@microbio.gu.se). Present addresses: Clinical Bacteriology, Göteborg University, Guldhedsgatan 10A, SE-413 46 Göteborg, Sweden, and Department of Mathematics and Natural Sciences, Kristianstad University, SE-291 88, Kristianstad, Sweden.

**Table 1.** Isolation details for *Dinophysis* species.[1]

| Species | Clone number | Accession number | Type of isolation | Number of cells | PCR material |
|---|---|---|---|---|---|
| *D. acuminata* | US131 | AF414683 | micropipette | 1 | whole cell |
| *D. acuminata* | US138 | AF414684 | micropipette | 1 | whole cell |
| *D. acuminata* | US152 | AF414685 | micropipette | 25 | whole cells |
| *D. acuminata* | SW204 | AF414680 | micropipette | 1 | whole cell |
| *D. acuminata* | SW205 | AF414681 | flow cytometer | ND | DNA |
| *D. acuminata* | SW208 | AF414682 | flow cytometer | ND | DNA |
| *D. norvegica* | SW1 | AF414686 | micropipette | 1 | whole cell |
| *D. norvegica* | SW20 | AF414687 | micropipette | 1 | whole cell |
| *D. norvegica* | SW209 | AF414688 | micropipette | 10 | whole cells |
| *D. acuta* | SW13 | AF414689 | micropipette | 10 | whole cells |
| *D. acuta* | SW45 | AF414690 | flow cytometer | ND | DNA |
| *D. acuta* | SW206 | AF414691 | micropipette | 5 | whole cells |

[1] ND, not determined; PCR, polymerase chain reaction.

within this complex. In Europe, *D. acuminata* has traditionally been regarded as a northern species and *D. sacculus* as the southern European species. However, borderline morphotypes are commonly recorded, especially from the French Atlantic coast (Bravo *et al.* 1995). The ultimate solution to these problems is to determine the genetic differences between the species, and this approach has been strongly recommended by researchers within the field (Bravo *et al.* 1995).

The identification and classification of dinoflagellates have in the past been focused mainly on the morphological studies of vegetative cells and cysts. Recently, however, the accumulation of sequence data from genes like those coding for ribosomal RNA (rRNA) has led to new classification schemes and phylogenetic inferences. Furthermore, these data have formed the basis for the design of species-specific 'molecular probes' (e.g. Scholin *et al.* 1994a; Anderson 1995; Scholin & Anderson 1998). Both small-subunit (SSU) and large-subunit (LSU) rDNAs have the advantage of being highly conserved and also highly variable within the same molecule (Olsen *et al.* 1986; Sogin *et al.* 1986; Field *et al.* 1988). Conserved stretches of the molecule provide excellent areas for correct alignment to rDNA sequences from other organisms, and provide targets for universal or kingdom-specific sequencing primers. Moreover, these areas can be used for phylogenetic comparisons between distantly related taxa. Conversely, highly divergent areas of the molecule are suitable for phylogenetic studies of closely related species and can be used as target sequences for species-specific identification probes. A rapidly growing number of published sequences for phytoplankton, especially for toxic or harmful species, are available for comparative analysis (Scholin & Anderson 1998).

The LSU rDNA has become the dominant choice for the phylogenetic analysis of dinoflagellates (Daugbjerg *et al.* 2001). The complete nucleotide sequence of the LSU rRNA gene is known for the dinoflagellate *Prorocentrum micans* Ehrenberg and contains a total of 12 divergent domains named D1 to D12 (Lenaers *et al.* 1989). LSU rRNA dinoflagellate phylogeny has been studied mainly on the basis of comparisons using domains D1–D3 and D8–D9 (Lenaers *et al.* 1991; Zardoya *et al.* 1995). Species of *Dinophysis* have until now been excluded from these analyses, however, because of a lack of success in cultivating them, which has made sequence information difficult to obtain.

Here, we present molecular data from Swedish isolates of the potentially toxin-producing species *D. acuminata, D. acuta* Ehrenberg and *D. norvegica* Claparède & Lachmann. The LSU rDNA sequence information was derived from the D1–D2 region. A nontoxic isolate of *D. acuminata* from the North American east coast was also included in this study to determine if there is any genetic difference between toxic and nontoxic clones from widely different geographical origins. We also developed an rDNA-based probe for the identification of *Dinophysis* spp.

## MATERIAL AND METHODS

### Sampling

Field samples containing *Dinophysis* spp. were collected in the Skagerrak, near the mouth of Gullmar Fjord and close to Kristineberg Marine Research Station, Sweden, and from Perch Pond, Falmouth, MA (Table 1). Live dinoflagellate cells (*D. acuminata, D. acuta, D. norvegica*) were isolated into separate 1.5 ml microcentrifuge tubes (Eppendorf). Individual cells were isolated from a concentrated plankton sample, using a micropipette, and transferred through several drops of sterile seawater before finally being transferred into the Eppendorf tube. Some tubes contained single cells, whereas others contained up to 25 cells that were combined to form a single sample. Cells were also isolated using the sorting feature of a Becton Dickinson FACS-SCAN flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). *Dinophysis* species were separated from each other and from co-occurring phytoplankton on the basis of size (forward angle light scatter) and phycoerythrin (orange) fluorescence. When a distinct population of cells could be resolved and gated on the basis of these parameters, the sorting feature of the instrument was activated and the cells were collected in a vial. The purity of the sorted cells and the identification of the *Dinophysis* species were then confirmed microscopically. The cells were either suspended in microfuge tubes containing 500 µl of DNA–ethylenediamine tetraacetic acid (EDTA) buffer (10 mM Tris–Base, pH 8.0; 100 mM EDTA; 1% sodium dodecyl sulfate) or directly added to the polymerase chain reaction (PCR) reaction mixture (see subsequently).

### DNA extraction, sequencing and phylogenetic analysis

Algal cells collected in DNA–EDTA buffer were boiled for 2 min, and the crude DNA was collected by spin filtration

through a Microcon-10 filter device (Amicon). The complete D1–D2 region of the LSU rRNA was amplified using primers designed from *Prorocentrum micans,* previously described by Scholin *et al.* (1994b). The PCR reactions were performed either with 1 μl of crude DNA or on algal cells (1–25 cells) directly added to 20-μl reaction mixtures containing 2 μl PCR buffer, 2 μl deoxynucleoside triphosphate mix (1 mM each of the nucleotides dATP, dCTP, dTTP and dGTP), 1 μl $MgCl_2$ (25 mM), 1 μl (1 μM) each of the 28S rRNA–specific primers D1R (forward) and D2C (reverse) (Scholin *et al.* 1994b) and 2 U of *Taq* polymerase (Promega). All amplifications were achieved by using a Perkin–Elmer 2400 Thermal Cycler. After an initial denaturation step of 5 min at 94°C, repetitive rounds of 20 s at 94°C, 1 min at 50°C and 1 min at 72°C were performed for 35 cycles. The programme was ended with an extension of 5 min at 72°C. Five microlitres of the PCR product was analysed by low-melting agarose electrophoresis, and bands of predicted length (~ 700 bp) were cut from the gel. The gel slices were melted, and 1 μl of this agarose–PCR product was immediately used in a new round of amplification, using the same programme until the amount of the amplification product was estimated to be at least 100 ng.

Ligation and transformation were performed using the Original T/A Cloning Kit from Invitrogen BV (Netherlands). Ligation was performed directly with 3 μl of the agarose–PCR product (*c.* 1–10 ng), 1 μl of 10× ligation buffer, 2 μl (25 ng $\mu l^{-1}$) precut pCR 2.1 vector, 1 μl T4 ligase and ultrapure, deionized water to a final volume of 10 μl. The ligation reaction was incubated at 16°C for 18 h. Competent *Escherichia coli* INVaF′ cells were used in the transformation process, according to the kit instructions. If applicable, at least 10 successfully transformed white *E. coli* colonies were picked for further analysis. Isolated colonies were grown overnight at 37°C in 3 ml Luria Broth medium containing 50 μg $ml^{-1}$ of ampicillin. Plasmids were isolated using the Wizard miniprep kit (Promega). Purified plasmids were analysed by *Eco*RI restriction enzyme digestion to verify correct inserts. Nucleotide information was obtained using M13 primers (M13 forward-20 and M13 reverse-40) and the SequiTherm EXEL TM Long-Read DNA sequencing kit LC (Epicenter Technologies) for automated sequencing. Reaction products were run on a LI-COR R Sequencer. The sequences are deposited at GenBank, accession numbers AF414680–AF414691.

Each clone was sequenced at least five times in each direction (often > 10 times), and 5–25 clones were sequenced, depending on the species. The cloned material was derived from 1–25 individually picked cells on each occasion, or in three cases, from collections (by flow cytometry) of several hundred cells (see Table 1).

## Phylogenetic analysis

The sequences of the D1–D2 fragments (positions 53–424 in the *P. micans* sequence: Lenaers *et al.* 1989) were aligned with representative members within the class Dinophyceae (orders from Gymnodiniales, Suessiales, Gonyaulacales, Peridiniales and Prorocentrales, according to the taxonomic system of Fensome *et al.* 1993), using the Ribosomal RNA Database Program (Maidak *et al.* 1997). The alignments were then edited manually. The ciliate *Tetrahymena pyriformis* Furgason was used as the outgroup species. Maximum parsimony (MP), maximum likelihood (ML) and distance analyses were achieved using the heuristic search in PAUP 4.0* (Swofford 1990). Bootstrap analysis was performed in 800 replicates, using a 50% majority-rule consensus. In the parsimony analyses, all variable nucleotide characters were equally weighted and alignment gaps were treated as a fifth base. The most parsimonious trees were sought by random (50 replicates) sequential additions of taxa. The settings for ML analysis were according to the Hasegawa–Kisumo–Yano model (Swofford 1990). A starting tree was obtained via stepwise addition. The distance analysis started with a tree obtained via neighbour joining.

## Probe design and *in situ* hybridization

The alignments revealed a variable region suitable as a target for an identification probe at the genus level. The target sequence is located within the region between variable domains D1 and D2 (*P. micans* numbering 305–327). A presumably *Dinophysis*-specific oligonucleotide, 5′-TGAAGTTAAMCA-ATGGYTTGGG-3′, labelled with either biotin or fluorescein, was designed using Probe Assistant 1.0.0 (Chaotic Software, Saratoga, CA), synthesized and subsequently used for *in situ* hybridization experiments with preserved algal cells. The specificity for the probe was checked against EMBL and GenBank databases using the EMBL FAST server. The probe showed ~ 73% homology to the target region in the closely related species *P. micans* and *P. minimum* and only ~ 64% homology to the more distantly related dinoflagellate *Alexandrium tamarense* (Lebour) Balech. A biotinylated probe, previously designed by Distel *et al.* (1991) to specifically detect a shipworm bacterial symbiont, was used as a negative control. A fluorescein- or biotin-labelled universal probe, targeted towards the conserved SSU rRNA region 502–519 (Field *et al.* 1988), was used as a positive control.

Hybridization was performed on formaldehyde-methanol–preserved cells, as previously described by Anderson *et al.* (1999). Generally, the cells were preserved with 2% formaldehyde (final concentration) for 5 min on ice, pelleted by centrifugation (1000 × *g*) and resuspended in 1 ml of sterile seawater, after which 10 ml of ice cold 100% methanol was added. The samples were stored at −20°C, if not analysed immediately. Appropriate aliquots (50–500 μl) of cells were withdrawn for hybridization experiments, placed into 0.5 ml microfuge tubes and centrifuged at 1500 × *g* for 5 min. The pelleted cells were resuspended into 50 μl of hybridization buffer consisting of 10 μl 25× SET (3.75 M NaCl, 25 mM EDTA, 500 mM Tris–HCl), 0.5 μl 10% Tergitol NP-40, 0.5 μl Poly(A) (10 ng $\mu l^{-1}$), 5 μl formamide and 34 μl MQ-water. Prehybridization took place at 45°C for 30 min, after which 5 μl (50 ng $\mu l^{-1}$) of the appropriate fluorescein- or biotin-labelled probe was added. The cells were hybridized overnight in the dark, pelleted by centrifugation at 2000 × *g* for 5 min and washed once in 50 μl 0.2× SET for 10–30 min at 50°C. The cells were thereafter either pelleted and resuspended into a 5× SET–glycerol solution for direct observation or diluted into washing buffer for amplification of the hybridization signal. This was performed using the Tyramide signal amplification (TSA-direct) kit from NEN™ Life Science Products (Boston, MA), according to the protocol of the producer with minor modifications (Roth *et al.* 1996). Hybridized cells were

washed three times in 100 µl washing buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.05% TWEEN 20) and thereafter diluted into 50 µl blocking buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking reagent) at room temperature for 30 min. Subsequently, the cells were pelleted and resuspended into 100 µl of a 1 : 100 dilution of Streptavidin–horseradish peroxidase (HRP) solution (included in the kit), incubated at room temperature for 4.5 h and washed for 3 × 5 min in washing buffer, with agitation. Fluorophore tyramide working solution (300 µl) was added, and the cells were incubated for 10 min, washed for 3 × 5 min in washing buffer with agitation and mounted in a 1 : 4 glycerol–washing buffer mixture. The amplified hybridization signal was directly visualized by fluorescence microscopy on a Zeiss Axioskop Microscope fitted with a fluorescein bandpass (excitation 465–495 nm, emission 615–645 nm) filter set and a 50 W mercury vapour light source.

## RESULTS

### Phylogenetic analysis

Sequence patterns were investigated within variable domains D1 and D2 and the corresponding conserved flanking regions (position 57–391 in *P. micans*). Both regions have comparable length with respect to all dinoflagellate sequences to date, which allowed easy alignment. MP analysis generated four nearly identical trees, all with a length of 805 steps, a consistency index of 0.617, a rescaled consistency index of 0.491 and a retention index of 0.746. The ML analysis (4008 steps) and the distance method (score of 1.874) generated single identical trees (Fig. 1).

The LSU rDNA data showed a division of the Dinophyceae into three groups: (1) the so-called 'GPP complex' *sensu stricto* (Saunders *et al.* 1997), which includes taxa within the orders Gymnodiniales, Prorocentrales and Peridiniales; (2) the *Symbiodinium* complex; and (3) the Gonyaulacales. Bootstrap analysis supported this pattern. The trees confirmed that the Dinophysiales are closely related to the Prorocentrales, except for the benthic species *P. lima* (Ehrenberg) J.D. Dodge. The phylogenetic position of *P. lima* has been much debated recently. Some argue that *Prorocentrum* should be split into two genera, *Prorocentrum* and *Exuviaella,* and that *P. lima* should be classified into *Exuviaella* (McLachlan *et al.* 1997). Species of *Exuviaella* are defined to be relatively large marine prorocentroids producing DST and having a primarily benthic habitat. Because of these uncertainties, *P. lima* was excluded from our analysis.

The analyses also showed that *D. acuminata* and *D. norvegica* are nearly identical in their D1–D2 region, with only one base difference unique for *D. norvegica* (G exchanged with C at position 497; see Fig. 2). The *D. acuta* sequence showed a similarity value of 97% compared with *D. acuminata.*

### Biogeographical comparisons

Amplification of the Swedish *D. acuminata* isolates always resulted in a single fragment of a specific length. Successive cloning and sequencing of this LSU rDNA fragment showed > 99% homology (Fig. 2). In contrast, amplification of the

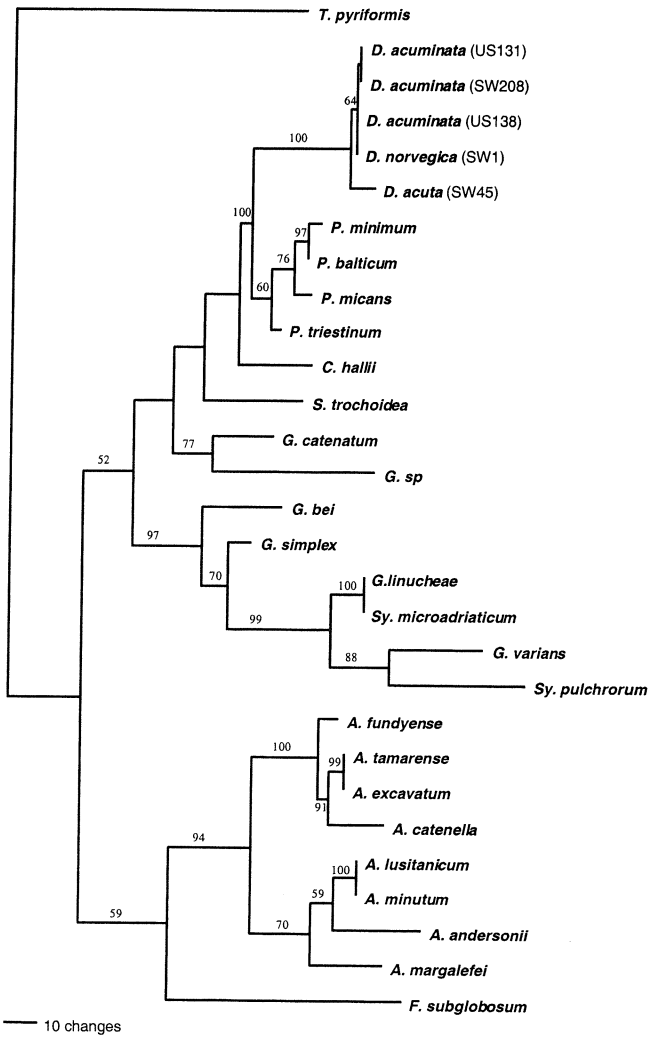



**Fig. 1.** The most parsimonious tree derived from an alignment of the D1 and partial D2 regions together with their surrounding conserved flanking regions. The ciliate *T. pyriformis* was used as the outgroup. Numbers indicate the percentage of the frequency of a particular branch (50% majority-rule) after 500 replicate samplings using bootstrap (Swofford 1990). *A = Alexandrium, C = Cachonina, D = Dinophysis, F = Fragilidium, G = Gymnodinium, P = Prorocentrum, S = Scrippsiella, Sy = Symbiodinium, T = Tetrahymena.*

D1–D2 region from the North American *D. acuminata* (single or pooled cells) always produced two distinct fragments. Cloning and sequencing revealed that the North American *D. acuminata* genome contained two classes of the LSU rRNA genes (Fig. 2). The longer 735-bp fragment (clone 131) showed 98.9–99.9% homology with the other American and Swedish field isolates. The shorter gene fragment (clone 138) had a large 70-bp deletion close to the DIR2 priming site, but was otherwise highly homologous to the larger gene fragment (similarity value of 97%, Fig. 2).

### *Dinophysis*-specific probes and signal amplification

Highly variable regions within the D1–D2 domains of LSU rRNA genes constitute ideal targets for identification probes at the species and strain levels (Anderson 1995; Scholin & Anderson 1998). Alignment of *Dinophysis* sequences from closely related species revealed a variable region, suitable as

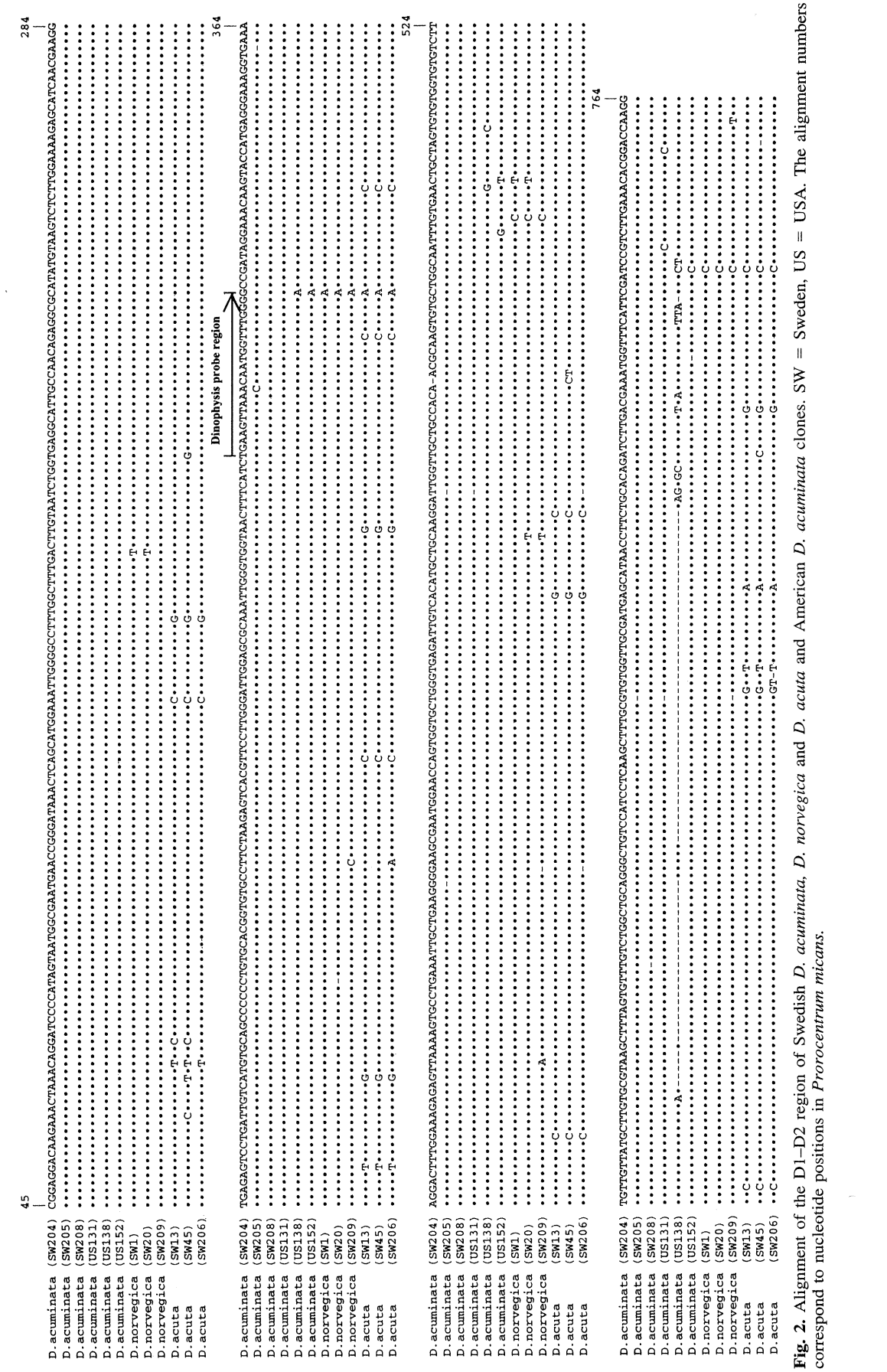



**Fig. 2.** Alignment of the D1–D2 region of Swedish *D. acuminata, D. norvegica* and *D. acuta* and American *D. acuminata* clones. SW = Sweden, US = USA. The alignment numbers correspond to nucleotide positions in *Prorocentrum micans.*

a target for a genus-specific identification probe (Fig. 2). Given the nearly identical LSU rDNA sequences for *D. norvegica* and *D. acuminata* over the regions examined, it was not possible to design a species-specific probe. Using fluorescent *in situ* hybridization, the genus probe was tested against formaldehyde-methanol–preserved cultures of *P. micans* (CCMP21) and *P. minimum* (Pavillard) Schiller (CCMP1329), both obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME; *A. catenella* (Whedon & Kofoid) Balech (ACQH01, obtained from S. Hall); *P. lima* (P1V); *Gymnodinium catenatum* Graham (GCURFII), maintained at D.M. Anderson's laboratory at the Woods Hole Oceanographic Institution, and several natural plankton samples containing mixed species of dinoflagellates, such as *Ceratium furca* (Ehrenberg) Claparède & Lachmann, *Protoperidinium divergens* (Ehrenberg) Balech and *Heterocapsa triquetra* (Ehrenberg) Stein. No cross-reactivity was observed with any of these species (data not shown). *Dinophysis* specificity of the probe was confirmed by hybridization to field samples containing mixed species of dinoflagellates, which resulted in the labelling only of *Dinophysis* spp. (*D. acuminata, D. norvegica* and *D. acuta:* Fig. 3). However, the hybridization signal from the fluorescein-labelled probe was masked by strong autofluorescence of the pigments within the *Dinophysis* chloroplasts. This is commonly observed with marine organisms (Lange *et al.* 1996; Lim *et al.* 1996). In *Alexandrium* and other dinoflagellates, chlorophyll autofluorescence during probing is typically removed by fixation in fomaldehyde and then methanol (e.g. Anderson *et al.* 1999). With *Dinophysis,* phycoerythrin remained within the cell after this procedure; so the autofluorescence persisted.

To circumvent this problem, we adapted a method for the amplification of the hybridization signal. TSA is a powerful amplification system based on a patented catalytic reaction. The method uses HRP to catalyse the deposition of fluorescent tyramide around the hybridized region. This leads to a 1000-fold enhancement in signal strength. Using this method with our *Dinophysis* probe, a strong signal was obtained from the hybridized probe (Figs 3, 5, 9). However, on some occasions, relatively high background fluorescence was observed (Figs 5, 9). This could not be reduced by prolonged incubation of the blocking agent or by an additional blocking reaction with unlabelled streptavidin, which only resulted in a reduction of the signal intensity. Treatment of the sample with 3% $H_2O_2$ before hybridization, as recommended by the manufacturer, did not significantly reduce the background. Nevertheless, however disturbing it was to us, the nonspecific background signal was always significantly lower than the specific signal, so that the positive cells could be easily identified (Figs 5, 9).

## DISCUSSION

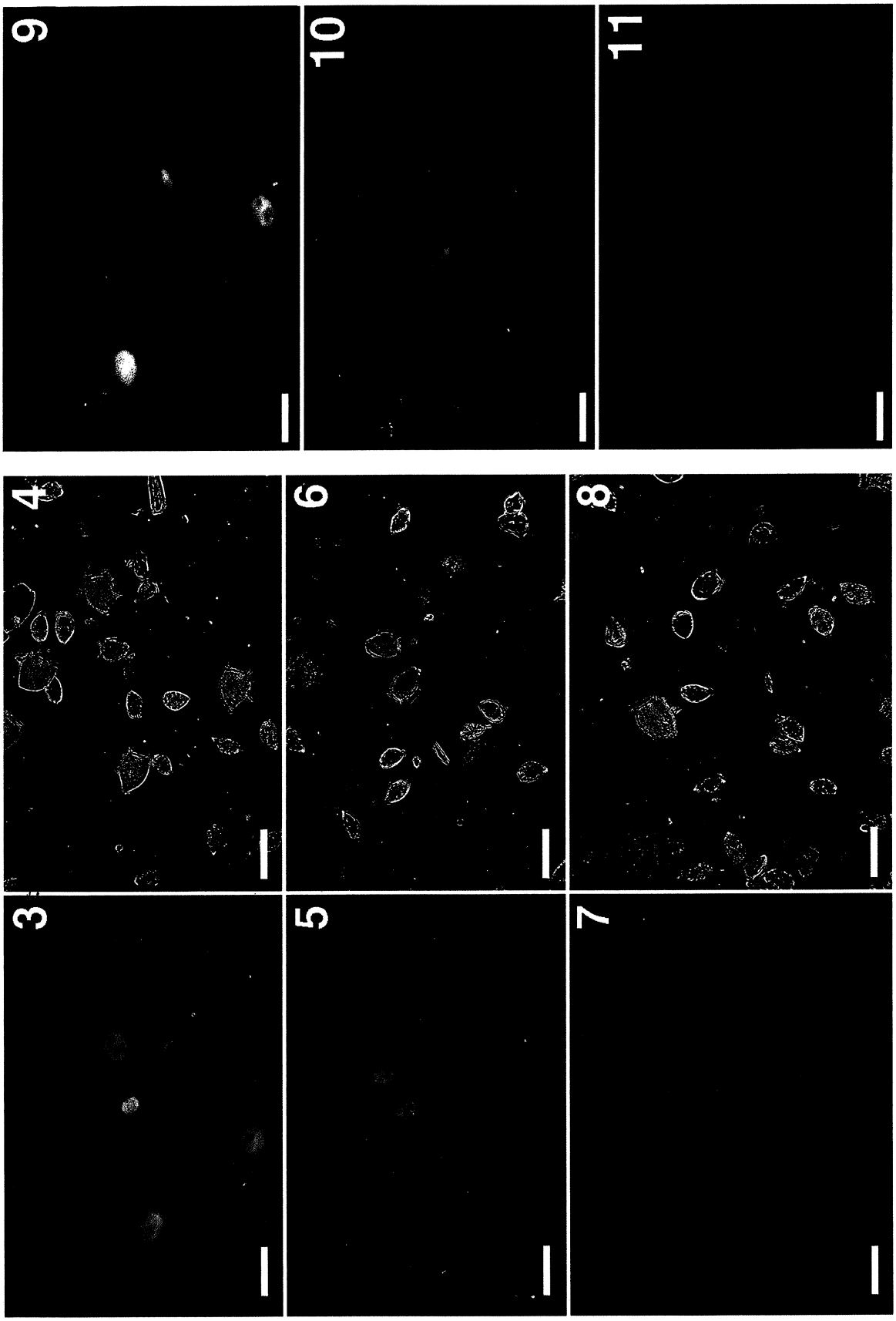
### Phylogeny

Dinoflagellates and their phylogeny have attracted considerable interest recently, not only because of their ecological and economical impact as common HAB species but also because they represent an ancestral group of organisms with unique features. Traditionally, they are regarded as algae, but modern molecular phylogenetic studies place them between the ciliates and apicomplexans (Van de Peer & De Wachter 1997). Several investigators have inferred molecular dinoflagellate phylogenies from comparisons of SSU rRNA or LSU rRNA gene sequences (Lenaers *et al.* 1991; Zardoya *et al.* 1995; Saunders *et al.* 1997; Daugbjerg *et al.* 2001). These studies have suggested that evolutionary theories based on the morphology and evolution of thecal plates (increasing numbers, decreasing numbers or fragmentation) should be reconsidered. In the excellent review by Saunders *et al.* (1997), a large number of dinoflagellate SSU rDNA sequences were compared in the context of traditional (morphological) classification. They concluded that four distinctive lineages can be recognized in the Dinophyceae: the *Amphidinium* line, the Gonyaulacales, the *Symbiodinium* group and the GPP complex. The GPP complex is a recently derived lineage associated with a large diversity of taxa within the orders Gymnodiniales, Prorocentrales and Peridiniales. Our analysis of the LSU rDNA D1–D2 region distinctly shows that members within the order Dinophysiales should be included in this diverse group. The phylogenetic analyses clearly indicate, as had been predicted previously from morphology and toxin composition profiles (Lenaers *et al.* 1991; Saunders *et al.* 1997), that the Prorocentrales and Dinophysiales are sister groups within the GPP complex.

All three *Dinophysis* species analysed can be identified rather easily on the basis of their morphological characters. We were therefore surprised to find that *D. acuminata* and *D. novegica* differed by only one difference in 735 bp in the D1–D2 region of LSU rDNA, whereas there were up to six differences between different isolates of the same species (99.2–100% similarity). By analysing SSU rDNA and the first internal transcribed spacer (ITS1), a similar pattern has recently been discovered by B. Edvardsen *et al.* (unpublished observations). They noted that the three photosynthetic species, *D. acuminata, D. norvegica* and *D. acuta,* were very similar with respect to the SSU rRNA gene (five to eight differences out of 1802 bases). Moreover, ITS1 was identical within each species and differed by less than 10 bases among species. These findings indicate that the photosynthetic *Dinophysis* species may have evolved rather recently.

### Pseudogenes

Toxic *D. acuminata* isolates from Sweden appeared to contain a single gene for LSU rRNA. Nontoxic American *D. acuminata,* however, contained two distinctive classes of LSU rRNA genes in roughly equal amounts. An explanation of this result may be a mixture of at least two different *D. acuminata* genotypes being pooled before PCR amplification. Because our sequences were sometimes obtained from a mixture of 5–25 cells that had been isolated from field populations and pooled in a single vial, it is quite possible that morphologically similar, but genetically different, strains were present in each of our samples. However, comparison between cloned LSU rDNA fragments from repeated microscopical isolation of single or several cells of the North American *D. acuminata* revealed very low variance (98.9–100% similarity). The major difference between the two gene classes was always a large deletion of 70 bp. This suggests two alternative explanations – the shorter PCR product is generated from a nonfunctional




**Figs 3–8.** Fluorescence *in situ* hybridization and signal amplification field samples. Figs 3 and 5 show samples hybridized with the *Dinophysis* probe; in Fig. 7 the sample was hybridized with the negative control probe (shipworm probe: Distel *et al.* 1991). Scale bars = 50 µm. (Figs 3, 5, 7: Epifluorescent microscope images; Figs 4, 6, 8: the corresponding bright field micrographs.)

**Fig. 3.** Field sample with *D. acuminata* and *D. norvegica.*

**Fig. 5.** Field sample with *D. acuminata.*

**Fig. 7.** Field sample with both *D. acuminata* and *D. norvegica.*

**Figs 9–11.** Epifluorescence micrographs of *in situ* hybridized field samples. The film exposure time was 6 s in all cases. Scale bars = 50 µm.

**Fig. 9.** Field sample containing *D. acuminata* and *D. norvegica* hybridized with the biotinylated *Dinophysis* genus probe and signal amplified using Tyramide signal amplification.

**Fig. 10.** The same sample hybridized with an fluorescein isothiocyanate–labelled universal probe with no signal amplification (Medlin *et al.* 1988).

**Fig. 11.** Negative control using the Tyramide signal amplification system without the probe.

**Table 2.** Taxa and the respective accession numbers for the LSU rRNA gene sequences used in this study.[1]

| Species | Accession number |
|---|---|
| *Prorocentrum micans* | M14649 |
| *P. minimum* | L38637 |
| *P. balticum* | AF042816 |
| *P. triestinum* | AF042815 |
| *Cachonina hallii* | AF033867 |
| *Scrippsiella trochoidea* | AF206705 |
| *Gymnodinium catenatum* | AF375857 |
| *Gymnodinium* sp. | U94907 |
| *G. beii* | AF06900 |
| *G. simplex* | AF060901 |
| *G. linucheae* | AF060893 |
| *G. varians* | AF060899 |
| *Symbiodinium microadriaticum* | AF060896 |
| *S. pulchrorum* | AF060892 |
| *Alexandrium fundyense* | U44928 |
| *A. tamarense* | AF033534 |
| *A. excavatum* | AY056824 |
| *A. catenella* | AY056823 |
| *A. lusitanicum* | L38624 |
| *A. minutum* | L38625 |
| *A. andersonii* | U44937 |
| *A. margalefii* | AF033531 |
| *Fragilidium subglobosum* | AF033868 |
| *Tetrahymena pyriformis* | X54004 |

[1] LSU rRNA, large subunit ribosomal RNA.

gene (pseudogene) or it is produced during PCR amplification, if the template forms a loop structure at that point. The latter explanation is highly unlikely. If the deletion were a PCR artefact, this should have resulted in unequal amounts of each of the two products in repetitive PCR reactions and not the roughly 1 : 1 ratio that was observed on every occasion. However, that this ratio truly reflects the abundance of the two classes of LSU rRNA within North American *D. acuminata* remains to be demonstrated.

The secondary structures of the divergent domains in LSU rRNA are well conserved. Studies have shown that if these expansion segments are deleted or replaced with unrelated sequences of equal length, cells cease to grow (Engberg *et al.* 1990; Sweeny *et al.* 1994). Conversely, if a specific divergent domain in one species is replaced with the homologous divergent domain from another species, growth is restored. This leads to the conclusion that there is considerable selective pressure to preserve the secondary structure of divergent domains, especially of D2 and D8. Whether this is because of the function of the region itself, or because only particular secondary structures are tolerated within otherwise functionally important regions, has yet to be proven; but whatever the explanation, the high selection pressure suggests to us that the shorter PCR fragment that was detected in the North American *D. acuminata* is a pseudogene. Pseudogenes have previously been shown to exist in *Alexandrium.* Scholin *et al.* (1993) described two distinct SSU rRNA genes in *A. fundyense,* termed A and B. The B gene was unexpressed, as shown by reverse transcriptase sequencing, supporting the conclusion that the B gene was a pseudogene unique to the *A. fundyense* clone. Yeung *et al.* (1996) obtained two distinct PCR-generated LSU rDNA products from a toxic isolate of *A. catenella.* The shorter fragment had an 87 bp deletion and a 6 bp difference, compared with the longer gene fragment. We observed a similar pattern in the North American *D. acuminata,* with a 70 bp deletion and a 33 bp difference, compared with the longer fragment. In future, the presence or absence of the 70 bp section could be used as a genetic marker to distinguish among different isolates of *D. acuminata.* It would be very convenient if that marker was also specific for nontoxic strains of that species. Additional isolates from other regions must be analysed before either of these possibilities can be accepted. As always, the lack of cultures of the *Dinophysis* species makes this more difficult than it might otherwise be.

### Biogeographical and population comparisons

In this study, we sequenced and cloned one to a few *D. acuminata, D. acuta* and *D. norvegica* cells isolated directly from the environment. This provided a useful picture of the sequence variants (ribotypes) existing in the same water mass during a single bloom. Comparison between cloned LSU rDNA fragments from different micropipette-isolated *D. acuminata* revealed small differences between isolates from the same geographical origin (similarity values > 99%). Scholin *et al.* (1994b) have been able to show a tight coupling between the ribotype and the geographical source in cultures of species within the *A. tamarense* complex, but these were separate cultures derived from multiple locations in a large region and were not isolated from the same water samples. In our case, the sequences of replicate isolates of *D. acuminata* (Swedish or North American), *D. norvegica* or *D. acuta* cells taken directly from environmental samples gave a unique picture of the sequence variations existing in the same water mass during a single bloom. These variations were low for all studied species (similarity values 98.7–100%: Fig. 2).

Scholin *et al.* (1994b) found that several morphospecies of *Alexandrium* (*A. tamarense, A. fundyense* and *A. catenella*) from the same geographical region (e.g. North America) were genetically identical (in LSU rDNA), whereas isolates of a single species from geographically separated regions showed numerous sequence differences. Ribotypes were thus consistent with the geographical origin, not the morphospecies. In our study of *Dinophysis* spp. we demonstrated virtually no significant sequence difference between the North American and Swedish *D. acuminata* isolates (similarity values 99.2–99.9%). However, our finding of a pseudogene associated only with the North American *D. acuminata* clone supports the theory that the genetic difference among morphologically identical isolates of *Dinophysis* may be correlated with the geographical origin.

### Application of identification probes

Several types of nucleic acid techniques are now used for the identification of microscopic species in clinical or environmental samples. One of the most commonly used methods is the comparative sequencing of the rDNA. This serves as a basis for phylogenetic analysis as well as for the design of rDNA-targeted oligonucleotide probes. Identification requires hybridization between the probe and its target rDNA in extracts or in preserved, intact cells. Detection is then achieved using radioactivity, fluorescence, chemiluminescence or colorimetric methods. Most of the effort in oligonucleotide detection of algal species has been based on laboratory cultures,