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Multiple simultaneous detection of Harmful Algal Blooms (HABs) through a high throughput bead array technology, with potential use in phytoplankton community analysis

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Abstract

As an alternative to traditional, morphology-based methods, molecular techniques can provide detection of multiple species within the HAB community and, more widely, the phytoplankton community in a rapid, accurate and simultaneous qualitative analysis. These methods require detailed knowledge of the molecular diversity within taxa in order to design efficient specific primers and specific probes able to avoid cross-reaction with non-target sequences. Isolates from Florida coastal communities were sequence-analyzed and compared with the GenBank database. Almost 44% of the genotypes obtained did not match any sequence in GenBank, showing the existence of a large and still unexplored biodiversity among taxa. Based on these results and on the GenBank database, we designed 14 species-specific probes and 4 sets of specific primers. Multiple simultaneous detection was achieved with a bead array method based on the use of a flow cytometer and color-coded microspheres, which are conjugated to the developed probes. Following a parallel double PCR amplification, which employed universal primers in a singleplex reaction and a set of species-specific primers in multiplex, detection was performed in a cost effective and highly specific analysis. This multi-format assay, which required less than 4 h to complete from sample collection, can be expanded according to need. Up to 100 different species can be identified simultaneously in a single sample, which allows for additional use of this method in community analyses extended to all phytoplankton species. Our initial field trials, which were based on the 14 species-specific probes, showed the co-existence and dominance of two or more species of *Karenia* during toxic blooms in Florida waters.

Keywords

Bead array; Harmful Algal Bloom; Luminex; Simultaneous molecular detection

1. Introduction

The spatial and temporal distribution of total phytoplankton biomass is better documented and understood than the distribution of individual phytoplankton species because of the greater ease of measuring total biomass. Total phytoplankton biomass is usually estimated by the concentration of chlorophyll a, which can be easily extracted and measured fluorometrically

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or spectrophotometrically. While not as quantitatively accurate, chlorophyll spatial and temporal patterns can also be examined using satellite or airborne remote sensing, or using towed or moored instruments that detect the in vivo fluorescence of chlorophyll. By contrast, the identification and quantification of individual species remains difficult, time consuming, tedious, and requires a great deal of expertise and experience. In general, individual cells must be examined to distinguish species by their morphological characteristics.

Light microscopy is often not adequate for discerning some of the small differences between species and electron microscopy must be used, increasing the expense and time needed. Often, even a morphologically defined “species” is inadequate because of the existence of sibling species. An understanding of the population dynamics of a species is dependent upon the correct identification of the genetic species, which is often not the “morphological species”. The correct identification of species is particularly acute in the study of toxic Harmful Algal Blooms, as only individual genetic species produce the toxins that present an increasing hazard, throughout the world, to the health of humans and various organisms in the ecosystem (Smayda, 1990; Hallegraeff, 1993; Hackett et al., 2004). To satisfy these requirements, a genetic analysis is needed. An understanding of the factors that lead to Harmful Algal Blooms requires information of the spatial and temporal distribution of the species before they increase to high concentrations. Furthermore, samples must be taken over large areas and frequently over long time periods. Rapid and inexpensive analysis is therefore an important factor.

Because a rather high percentage of toxic Harmful Algal Blooms are caused by dinoflagellates, we directed most of our effort on distinguishing different species of dinoflagellates. For field studies, we focused on *Karenia brevis*, the toxic dinoflagellate that causes most of the toxic Harmful Algal Blooms along the west coast of Florida.

Several alternative detection methods based on molecular assays have been developed in the last decade. These methods comprised RFLP (Restriction Fragment Length Polymorhism) (Adachi et al., 1994), HMA (Heteroduplex Mobility Assay) (Oldach et al., 2000), Real-time PCR (Bowers et al., 2000; Tengs et al., 2001; Gray et al., 2003; Galluzzi et al., 2004; Hosoi-Tanabe and Sako, 2005), FISH (Fluorescent In Situ Hybridization) (Miller and Scholin, 1996; John et al., 2005; Takahashi et al., 2005), NASBA (Nucleic Acid-based Sequence Amplification) (Casper et al., 2004, 2007; Patterson et al., 2005), SHA (Sandwich Hybridization Assay) (Scholin et al., 1996; Tyrrell et al., 2002) and NPA-SH Sandwich Hybridization Assay Nuclease Protection (Cai et al., 2004). More recently, detection methods for some of the most common toxic dinoflagellates were developed based on bead (Ellison and Burton, 2005) or nanoparticle (Galluzzi et al., 2006) array technology.

All molecular-based methods require the support of detailed phylogenetic data. Unfortunately, phytoplankton phylogeny is poorly resolved and in particular the phylogeny of dinoflagellate species. In the late 1800s and early 1900s four genera of dinoflagellates (*Gymnodinium, Peridinium, Amphidinium* and *Prorocentrum*) were described based on morphological characteristics. In the 1960s a major revision was carried out by Balech that resulted in the inclusion of several new genera (Balech, 1974, 1977, 1989). The morphology was based mainly on the pattern of the thecal plates (composed of celluose or other polysaccharides, whose role is to harden the cell wall) and their number. For this reason, the taxonomy of athecate dinoflagellates (naked dinoflagellates) progressed slowly.

Based on ultrastructural studies, rDNA large subunit (LSU) sequence data and previous (Saunders et al., 1997) small subunit (SSU) data, Daugbjerg et al. (2000) divided the genus *Gymnodinium* into 4 genera. Additional phylogenetic studies based on the LSU rDNA have been conducted on a diversity of genera and species, many of which were newly described

(Hirashita et al., 2000; Guillou et al., 2002; De Salas et al., 2003, 2004a,b; Murray et al., 2005; Haywood et al., 2004; Botes et al., 2003).

Other genes have also been studied: the encoded form II Rubisco gene, chloroplast 23S, SSU rRNA, mitochondrial cytochrome *B*, Internal Transcribed Spacer regions (ITS) (Zhang and Lin, 2003; Pochon et al., 2006; Litaker et al., 1999; Lin et al., 2006a,^{b}; Zhang et al., 2005; Yoshida et al., 2003; D'Onofrio et al., 1999; Gottschling et al., 2005; Montresor et al., 2003). Further phylogenetic analyses on dinoflagellates are needed, however, to clarify the relationship between molecular and morphology-based taxonomy and to provide taxonomic support to molecular detection methods.

The goals of the present study included: (1) assessment, through sequencing data, of HAB community diversity in west Florida waters, (2) development of a rapid, sensitive, reliable and cost-effective procedure to allow simultaneous detection of toxin-producing species in a multi-format assay and to provide, at the same time, high specificity, (3) application of this procedure in preliminary field trials.

Goal 1 was achieved with sequence analysis of isolates from Florida waters and related cultures from the CCMP laboratory, to compare with sequences in the GenBank database whereas (2) and (3) employed Luminex Xmap technology based on a flow cytometer that analyzes multiple color-coded fluorescent microspheres (beads) coupled with specific sequences. These sequences represent probes that were designed based on sequence analysis of individual species. The 5.6 μm polystyrene carboxylated beads are internally dyed with two different fluorophores (red and infrared) in a variety of combinations generating up to 100 different microsphere sets with specific spectral addresses. These unique microspheres are covalently coupled to probes that are designed to be complimentary to target DNA sequences. The covalent linkage occurs between the carboxyl group of the microsphere and the C12 modification of the probe. Target DNA is biotinylated, denatured and hybridized to the microsphere-probe set under stringent conditions. A reporter molecule, such as streptavidin-R-phycoerythrin, is added to detect hybridization.

The microsphere-probe sets are interrogated individually in a fast flowing fluid as they pass by two separate laser beams in the Luminex 100 analyzer. A 635 nm classification laser excites the 2 fluorophores contained inside the microspheres, allowing the classification of each coupled bead based on its spectral address. A 532 nm reporter laser excites the reporter fluorochrome (streptavidin-R-phycoerythrin) on the surface, permitting quantification of the hybridized biotinylated amplicon (captured DNA). The fluorescent signals are converted into intensity units by a digital signal processor.

The multi-format structure of this assay, which permits qualitative detection of 100 species at one time, allows for additional, simultaneous analysis of phytoplankton community diversity. The method could provide, in this way, a more precise understanding of the biodiversity that can be found in Florida waters before, during and after a toxic bloom.

2. Materials and methods

2.1. Strains studied

Isolates from Florida waters and voucher specimens were studied in comparison for our Florida waters community analysis. Twenty-three strains of voucher specimens (Table 1) from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton, from the Algae Collection at the University of Texas and from the Algae Collection at Florida International University were cultured. Seawater for the cultures was collected from the northern Tongue of the Ocean, Bahamas. The seawater and individual nutrient stock solutions were all autoclaved separately in Teflon bottles to make BWM media (Brand, 1986). The cultures were maintained at 21-23 °C and a light intensity of 59 uEinst m^{-2} s^{-1} provided by cool-white fluorescent bulbs on a 14:10 photoperiod. Twenty-nine cultures (Table 2) were isolated from Florida waters, several of which were deposited at CCMP (the detailed isolation method for these cultures will be described in a future publication).

For our field studies surface seawater samples were collected in Florida waters from Pine Island Sound in July 2006 and off Charlotte Harbor in October 2006. The latter samples were taken during a Florida Monitoring and Event Response for Harmful Algal Blooms (MERHAB) Cruise. Specific locations for both cruises and microscopic counts for the MERHAB Cruise are reported in Tables 7 and 8.

2.1.1. DNA extraction—

The steps in our methods are synthesized in Table 3. DNA extraction for the 29 cultures in Table 2 was achieved with the Bio101 FastDNA kit (Qbiogene) and the FastPrep instrument (Qbiogene) according to the manufacturer's instructions. DNA extraction for voucher specimens and environmental samples was carried out according to the following procedure. Two hundred microliters of each sample were filtered through SUPOR 800 filters (0.8 μM pore, 25 mm diameter). All filters were frozen at −80 °C. One tenth of each filter (about 2.5 mm) was punched out with a hole-punch, placed in a microtube containing 100 μl of water and boiled for5 min at 100 °C. Genomic DNA concentration was quantified with a Nano Drop ND-100 spectrophotometer using absorbance of 260 nm. Due to the PCR inhibition showed by several single culture samples, the procedure was modified for these samples, which were spun for 10 min at 13,000 rpm in a microcentrifuge (Galaxy 16DH, VWR). The pellet was then re-dissolved in 100 ml of seawater and filtered. The purpose of this wash step was to eliminate possible inhibitors, which were present in single culture samples due to the cultural media or to substances produced by the cultures themselves at some stage of their growth.

2.1.2. Sequence analysis—

A PCR amplification followed the extraction. The PCR mixture was comprised of 5 μl of buffer (containing 15 mM Mg_2Cl_2), 10 pmol per each primer, 1.5 μl dNTPs (10 mM) and 1.5 units of DyNAzyme polymerase (New England Biolabs). Template amount was based on the genomic DNA concentration. Final DNA concentration varied from 10 to 40 ng. Sterile water was added to bring the mixture to the final volume (50 μl). Primers D1R and D2C (Scholin et al., 1994) were synthesized by Sigma Genosys (Table 4).

The PCR program consisted of one cycle of denaturation at 95 °C for 5 min followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 50 °C and 1 min extension at 70 °C. The final extension at 72 °C was for 8 min. The program was run in a PTC-200 Thermocycler (MJ Research). Samples were checked by electrophoresis on a 2% agarose gel and purified (Qiagene, QIAquick PCR Purification Kit). Sequencing was performed on an ABI 3730 sequencer (Applied Biosystems, CA, USA). The sequences obtained were analyzed with Seqman 5.51 (DNASTAR for MacOSx), visually corrected, analyzed through GenBank BLAST and aligned in Megalign (DNASTAR for MacOSx) with related sequences available in GenBank. Sequences obtained were deposited into the GenBank database, GenBank accession numbers for all the strains sequenced can be found in Tables 1 and 2. Phylogenetic analysis employed PAUP 4.0b 10 with neighbor joining analysis.

2.1.3. Luminex analysis

2.1.3.1. Region of study and primer design: The region elected for primer and probe design was the D1D2 domain of the large subunit rRNA. The choice was based on the relevant presence of D1D2 sequences in the GenBank database and on the variability of the region, which permits inter-specific discrimination for the species of interest (Daugbjerg et al., 2000; Hirashita et al., 2000; Guillou et al., 2002; De Salas et al., 2003, 2004a,b; Murray et al., 2005; Haywood et al., 2004). Data present in GenBank showed also intraspecific variability for some species (*Scrippsiella trochoidea* AY628427, AF042819 and AF260393).

Universal primers D1R and D2C (Table 4) were used to generate a "universal" amplicon (length range from 550 to 650 bp according to the species). In addition four "specific" amplicons (Table 5) were produced in a multiplex PCR by specific primers, which were also designed in the D1D2 domain LSU rDNA. The 4 sets of specific primers were designed to enhance amplification of species less represented in environmental samples. The species were *Prorocentrum minimum, Prorocentrum rhatymum, Prorocentrum micans, Prorocentrum mexicanum, S. trochoidea* and *Protoceratium reticulatum*. The amplicon lengths were, respectively, 165 bp (minimum), 290 bp (rhatmicmex), 440 bp (Scripp) and 550 bp (Protoce) long (Table 5). Primer sequences, species amplified and related information are in Table 5. Universal and specific PCR products were both biotinylated (all reverse primers had a biotin moiety) and hybridized with the probes in Table 6, which were previously conjugated to carboxylated microspheres.

2.1.3.2. Probe design: Fourteen probes were designed based on species sequences (Table 6). Sequences were obtained from one or more strains for each species and compared with related GenBank sequences in sequence alignments obtained with Megalign Program (DNAStar).

Specific parameters of the probe sequences were analyzed with the Programs Oligo (Mol. Biol. Ins.) and Mac Vector (Oxford Molec.) to assess probe functionality. The chosen probes had lengths ranging from 20 to 25 bp, with a G/C ratio between 43.4 and 61.9%. Secondary structure was avoided, when possible, and was never more than moderate, according to Sigma-Genosys DNA calculations (http://www.sigma-genosys.com/calc/DNACalc.asp). Following Luminex Corporation's suggestions (Dunbar, S.A., personal communication), shorter probes (20-21 bp) were favored for species with a low number of mismatches in the probe sequence in order to ensure adequate sequence discrimination. When the number of mismatches and the consequent specificity were higher, slightly longer probes (23-24) were used to achieve larger signals. Also higher melting temperatures were favored to obtain stricter specificity. Temperatures ranged from 62 to 72.8 °C with a ΔG (free energy of reaction) from −6.6 to −10.1. The number of mismatches with the closest species was at least two. The mismatches were localized in the center of the probe (Dunbar, 2006) in order to obtain a maximum destabilizing effect. All the probe sequences were analyzed through GenBank BLAST for specificity.

The probes were synthesized by IDT Technologies with a 5′ Amino Modifier C12 that allows for covalent binding to the carboxyl groups on the microsphere's surface. The probes were initially designed for amplicons generated between Universal primers D1R and D2C. The binding site within the amplicon was in the second half of the sequence, near the 3′ end, to avoid possible interference of the secondary structure. This position was maintained when the specific primers were used, designing the additional primers around the existing probes.

All the probes were species specific with the exception of *S. trochoidea* probe, which is a cluster probe. Due to the existence of 3 different GenBank sequences for this species and to the high number of different strains found in the environment, the probe was designed to include voucher CCMP 1735 and isolates FIU 16, FIU 26 and FIU 30.

2.1.3.3. Coupling reaction: The coupling reaction followed a carbodiimide method (Fulton et al., 1997) with slight modifications (Diaz and Fell, 2004). Multianalyte carboxylated microspheres were purchased from MiraiBio (Alameda, CA, USA).

The beads (2.5×10^6 in number) were re-suspended by vortex and sonication for 20 s and centrifuged at 13,000 rpm for 2.5 min. The re-suspension of the pellet was in 50 μl of 0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid; Sigma], pH 4.5. Variable amounts of the probe and 2.5 μl of a 10mg/ml solution of EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride; Pierce] were added. After vortexing and incubating the beads for 30 min in the dark, at room temperature, 2.5 μl of the EDC solution were added again, followed by a second incubation (room temperature for 30 min in the dark). Two wash steps in 500 μl of 0.02% Tween 20 and in 500 μl of 0.1% SDS, respectively, were carried out with a microcentrifugation at 13,000 rpm for 2 min to pellet the beads after each wash. Finally the beads were resuspended in 50 μl of TE, pH 8, to a final concentration of 5×10^4 beads/μl. The amount of probe in the coupling reactions was optimized for each microshere set and ranged from 0.2 to 0.35 nmol (Table 6). Conjugated microspheres were stored in the dark at 5 °C. Following Luminex recommended procedures, three of the most represented probes (430 brevis, 530 mikimotoi and Scripps; Table 6) were assessed in a coupling verification test where biotinylated oligonucleotides, which represented the reverse and complement of each probe, were used in place of biotinylated amplicons.

2.1.3.4. PCR amplifications prior hybridization: Prior to hybridization two parallel amplifications were performed: a singleplex with universal primers (Table 4) and a multiplex with specific primers (Table 5). Separate amplifications were required due to the production of random amplicons when universal and specific primers were used in a combined reaction.

Both PCR mixtures contained 7 μl of buffer (15 mM Mg_2Cl_2), 2.5 μl dNTPs (10 mM) and 2.5 units of polymerase. In the 4 primer sets multiplex, however, primer concentration was 7.5 pmol per each primer, in contrast with 20 pmol per each primer utilized in the singleplex. Template amount was based on the genomic DNA concentration. Final DNA concentration varied from 10 to 40 ng. Sterile water was added to bring the mixture to final volume (50 μl). All the reverse primers had a biotin moiety at the 5′ end to allow labeling of the biotinylated oligonucleotides through a reporter molecule (streptavidin-R-phycoerythrin).

The program consisted of one cycle of denaturation at 95 °C for 10 min followed by 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 50 °C and 1 min extension at 69 °C. The final extension at 69 °C was for 30 min. Samples were checked by electrophoresis on a 2% agarose gel, but no purification step was carried out.

2.1.3.5. Hybridization assay: The hybridization assay was performed in 3 M TMAC (tetramethyl ammonium chloride) solution (50 mM Tris, pH 8.0/4 mM EDTA, pH 8.0/0.1% Sarkosyl). This salt stabilizes ATs pairs sensibility to melting temperature, so that probes with different base composition can work uniformly under the same hybridization conditions.

Duplicate samples containing 10 μl of biotinylated amplicon were diluted in 7 μl of 1 × TE buffer (pH 8) and 33 μl of 1.5 × TMAC solution containing a bead mixture of circa 5000 microspheres for each set of probes. The reaction mixture was incubated for 10 min at 95 °C with a PTC-200 Thermocycler (MJ Research). This step was followed by a 40 min incubation at 55 °C. The beads were pelleted by centrifugation at 2250 rpm for 3 min. Incubation at 55 °C for 5 min followed careful removal of the supernatant. Subsequently 300 ng streptavidin-R-phycoerythrin (Invitrogen) from a 1 mg/ml stock solution were diluted in 75 μl of 1 × TMAC and added to each sample. The amplicons were then labeled for 5 min at 55 °C. After a second centrifugation (2250 rpm for 3 min), the supernatant was removed and 75 μl of 1 × TMAC were again added to wash unbound label.

2.1.3.6. Detection analysis: The samples were run on a Luminex 100 analyzer where 100 microspheres of each set were analyzed. The median fluorescence intensity (MFI) values were calculated by Luminex 1.7 proprietary software (on a PC interfacing the Luminex analyzer) as a direct statistical average of each bead analyzed in a sample. A blank (no target DNA) and a set of positive and negative controls were included in the assay. All samples were run in duplicates. The signal obtained from a blank sample was subtracted from the MFI value. A response was considered positive when the corrected MFI value (MFI value after subtraction of the blank value) was at least twice the blank value (Diaz and Fell, 2005).

2.1.3.6.1. Probe singleplex: Each probe was initially tested in singleplex experiments against the appropriate target (specific DNA from a cultured organism) to assess probe design and sensitivity. Probes that did not show a positive signal with the appropriate target (approximately 7.1% of all the designed probes) were modified by adding a few nucleotides on each end or replaced when modifications would drastically change the probe structure. Serial dilutions of genomic DNA and of biotinylated amplicons and serial dilutions of single cultures before filtration were used to determine probe sensitivity.

2.1.3.6.2. Probe multiplex: Probes with a positive evaluation in the singleplex test were assessed in multiplex experiments for possible cross-reactivity. All 14 probes were tested against samples containing individual target DNAs. Probes that showed cross-reactivity (positive response for non specific target) were altered or replaced (approximately 7.1% of all the designed probes).

2.1.3.6.3. Probe and target multiplex: As a final step, probes were tested against amplicons that contained more than one target DNA. These amplicons were first obtained from PCR where DNA templates, extracted from different species, were added to the PCR mix. Subsequently, different cultures were used to spike seawater samples. The resulting mixture was filtered, extracted, amplified and Luminex-tested.

2.1.4. Field studies—Surface seawater samples were collected in Florida waters from Pine Island Sound in July 2006 and off Charlotte Harbor in October 2006. The latter samples were taken during a Florida Monitoring and Event Response for Harmful Algal Blooms (MERHAB) Cruise. Specific locations for both cruises and microscopic counts for the MERHAB Cruises are reported in Tables 7 and 8. Microscopic counts for samples from Pine Island Sound were for *Karenia* sp. and ranged from 0 cell/ml at station 4 to 95 cell/ml at station 10. The amount of biotinylated amplicon in the hybridization assay was increased to 17 μl when field samples were analyzed.

3. Results

3.1. HAB community diversity

Based on culture sequences we constructed a phylogenetic tree that comprised specimen vouchers (from CCMP, from the University of Texas and from Florida International University), strains isolated in Florida waters and GenBank sequences of related species. *Chattonella subsalsa* and *Heterosigma akashiwo*, two HAB raphidophytes found in Florida waters, were used as the outgroup. The goal was to provide a phylogenetic estimate of the ecological diversity among taxa, which is an essential step for probe design. Therefore, fourteen different dinoflagellate genera (*Akashiwo*, *Alexandrium*, *Amphidinium*, *Cachonina*, *Ceratium*, *Fragilidium*, *Gonyaulax*, *Gymnodinium*, *Heterocapsa*, *Karenia*, *Karlodinium*, *Prorocentrum*, *Protoceratium* and *Scrippsiella*) were analyzed.

Due to the large size, the phylogenetic tree was depicted in Fig. 1 as a stick tree, divided into clusters, which were represented in subsequent figures (Figs. 2–5). In addition to cluster names, single strain names of sequences, which were unrelated to any of the clusters, can be found in

Fig. 1. Cultures from CCMP, from the University of Texas and from Florida International University were reported as listed in those collections (the names *Katodinium rotondatum* and *Lyngulodinium polyedra* were replaced with their updated synonyms *Heterocapsa rotundata* and *Gonyaulax polyedra*) (see Table 1). Isolates from Florida waters appear as FIU (Florida International University) followed by the isolation numbers (see Table 2). GenBank accession numbers for all the strains sequenced can be found in Tables 1 and 2.

Without taking in account the three voucher specimens that lack a D1D2 GenBank submission (*Heterocapsa pygmaea*, *Ceratium longipes* and *Prorocentrum cassubicum*), only 65% of the remaining cultures had their collection label confirmed. For all the other voucher cultures, our sequences aligned with a species different from the collection label, or did not find any match in the GenBank database. This lack of confirmation for voucher cultures can possibly be explained by misidentification or mislabeling. A third hypothesis could be that some cultures in collection represent a mixture of similar species and only one of them was successfully extracted or amplified.

The “unique” sequences in the tree, which did not match any GenBank submission, comprised 43.6% of the total number of different genotypes, demonstrating the existence of a large and still unexplored biodiversity among taxa. Further analyses in different genes should evaluate the possibility that these sequences represent species already described, but not yet sequenced in the D1D2 region, such as species in the genera *Scrippsiella* (Gottschling et al., 2005) and *Heterocapsa* (Yoshida et al., 2003). Sequences that differed by only a few bp (one to four) from GenBank submissions were not taken into account as separate species, with the assumption that the mismatches were due to differences in sequencing procedures.

3.1.1. Unarmored dinoflagellates—Among the unarmored or naked dinoflagellates (species lacking thecal plates) we analyzed the following genera: *Karenia, Karlodnium, Gymnodinium, Akashiwo* and *Amphidinium*. The genera *Karenia* and *Karlodinium* were created after review of the genus *Gymnodinium* (Daugbjerg et al., 2000) and they both are robust monophyletic clusters as shown in Fig. 1. The two genera appear to cluster together, although with low bootstrap values (Fig. 2, bootstrap values lower than 50% are not reported). In recent years, several new species of *Karenia* were described, such as *Karenia asterichroma* (De Salas et al., 2004a), *Karenia umbella* (De Salas et al., 2004b), *Karenia papilionacea, Karenia bidigitata* and *Karenia selliformis* (Haywood et al., 2004), *Karenia cristata* and *Karenia bicuneiformis* (Botes et al., 2003). The only available cultures are, however,*K. brevis* and *Karenia mikimotoi*. In the *Karenia* cluster three strains listed by CCMP as *brevis*, FIU 45 and *K. mikimotoi* CCMP 429 confirmed the respective GenBank sequences. In the *Karlodinium* cluster, isolate FIU 41 is identical to *Karlodinium micrum* AY245692. One *Gymnodinium* species (*Gymnodinium pulchellum*) appears strictly related to the *Karlodinium* cluster (98% bootstrap; Fig. 2). The closeness of *Karenia* species, *Karlodinium* species and *G. pulchellum* was morphologically confirmed by the presence of a common ultra-structural character, an intercingular tubular structure of the cingulum (Haywood et al., 2004). In contrast, the remaining *Gymnodinium* species appear in a cluster with high bootstrap value (98%; Fig. 2), but also in three unrelated branches (*Gymnodinium corii, G. falcatum* and *Gymnodinium* sp.), which include FIU 52, a perfect match for *G. corii* AF318226, and FIU 57, a close match for GenBank U94907 *Gymnodinium* sp. The *Akashiwo* genus (Fig. 2) contains only one species, *Akashiwo sanguinea*. Isolate FIU 34 is one bp different from one of the two GenBank sequences submitted for this species (DQ779987) and more than ten bp different from the other (AF2603397).

The genus *Amphidinium* (Fig. 3) is a compact cluster, which reaches a bootstrap value of 100% without inclusion of the species *Amphidinium incoloratum*, 58% with inclusion. The species *A. semilunatum*, however, stands separate with no apparent relation to this group (Fig. 1). Our sequence for *Amphidinium operculatum* CCMP 1344 (Fig. 3) did not match any sequence in GenBank and clustered between *Amphidinium eilatiensis* and *Amphidinium carterae* (100% bootstrap value), almost identical to isolate FIU 2. In contrast, *A. operculatum* CCMP 120, identical to isolate FIU 14, matched almost perfectly (one mismatch) the GenBank sequence of a different species, AY460587 *Amphidinium gibbosum* (Fig. 3). Also CCMP 1342, labeled as *Amphidinium massartii*, did not match AAY455670 *A. massartii* and showed a sequence identical to AAF260381 *Amphidinium klebsii* (Fig. 3). Strain FIU 32 aligned in a clade with *A. klebsii* and *A. massartii* (82% bootstrap value). *A. carterae* CCMP 1314 confirmed GenBank sequence AF260380 for the same species.

3.1.2. Armored dinoflagellates—The armored dinoflagellate genera examined were: *Coolia, Cachonina, Heterocapsa, Protoceratium, Fragilidium, Alexandrium, Ceratium, Gonyaulax, Scrippsiella* and *Prorocentrum*. The genus *Coolia* (Fig. 3), contains two species identical in their D1D2 sequence (*Coolia monotis* and *Coolia malayense*). Isolate FIU 39 is strictly related to *Coolia monotis/Coolia malayense* (100%), but represents a different species. *Cachonina hallii* clusters within the genus *Heterocapsa* (Fig. 3). Bootstrap value for the whole group is 86%. CCMP 1734 *H. rotundata* (Fig. 3), almost a match for isolate FIU 5, is considerably distant from *H. rotundata* GenBank AF260400. CCMP 448 *Heterocapsa triquetra* is a close match for *H. triquetra* GenBank AF260401, while isolate FIU 11 is identical to the other submission for the same species, EF613355. Isolates FIU 10, FIU 31 and FIU 12R did not align with any GenBank sequence, showing a high biodiversity in this cluster. *H. pygmaea* UTEX 2421 also clustered within the genus. GenBank sequences X61735 and X61736 for *H. pygmaea* included only the D1 domain and were not used in our tree.

In the *Protoceratium* cluster (Fig. 3), CCMP 1721 *P. reticulatum* (Fig. 3) differed considerably from GenBank sequence AY027907 *P. reticulatum*, which was a perfect match for isolate FIU 20. The genus *Protoceratium*, as well as *Fragilidium, Alexandrium, Ceratium* and *Gonyaulax*, belong to the Gonyaulacales, a morphologically diverse order that lacks a unique morphological character common to all the taxa. The only noticeable relationship among these genera is between *Alexandrium* and *Fragilidium* with a 68% bootstrap value (Figs. 1 and 4). The two genera also share the same plate pattern (Fensome et al., 1993). In the *Fragilidium* cluster (Fig. 4) FIU 49, which is related to *Fragilidium subglobosum* AF033868 (bootstrap100%), represents a different species in this monospecific genus. *Alexandrium* is a well-supported group (bootstrap 99%) that contains *Ceratium furca*, a species related to *Alexandrium tropicale* (Fig. 4). Our sequence of CCMP 1493 *Alexandrium tamarense* was a perfect match for AB088229 *Alexandrium catenella*, one of the species in the *A. tamarense* species complex (John et al., 2005). Four other *Ceratium* species cluster with 100% bootstrap support (Fig. 4) including *C. longipes* CCMP 1770. (There are no D1D2 sequence in GenBank for this species.) *Gonyaulax* (Fig. 4) is a split genus with five well-supported species in a clade and the species *G. polyedra* standing separately (Fig. 1). Our sequence for CCMP 1738 G. polyedra (Fig. 1) was related, but not identical to AF377994 (100%).

Species belonging to *Scrippsiella* (Fig. 4), a cyst forming genus, group in a well supported cluster (bootstrap 98%). *S. trochoidea* (Fig. 4) is a particular case, with three different GenBank sequences (AY628427, AF042819 and AF260393). Our sequence analysis for CCMP 1735, labeled as *S. trochoidea*, generated a sequence different from any of the three GenBank submissions and from any other sequence in the *Scrippsiella* cluster. In addition, sequence analysis of Florida waters isolates showed one strain with an identical sequence to *S. trochoidea* AY628427 and four strains with a different sequence from any other species. Intraspecific diversity in the ITS regions was also reported for this species (Montresor et al., 2003).

A peculiar situation is reflected in the genus *Prorocentrum* (Fig. 5). According to previous LSU (Zardoya et al., 1995) and SSU (Grzebyc and Sako, 1998) analyses, *Prorocentrum* species are divided in two distinct clusters: one comprising planktonic and bentho-planktonic species (*P. mexicanum, micans, minimum*), the other including strictly benthic species (*P. lima, hoffmannianum*). Although this dichotomy appears in our phylogenetic representation (see Prorocentrum 1 and Prorocentrum 2; Fig. 5), the situation is complicated by the presence of a third cluster (Prorocentrum 3; Fig. 5) including additional species such as *P. cassubicum, Prorocentrum levis, Prorocentrum foveolatum, Prorocentrum playfar-iii* and *Prorocentrum concavum*. Furthermore, three species (*Prorocentrum emarginatum, Prorocentrum borbonicum* and *Prorocentrum compressum*) appear to be unrelated to any of the three groups (Fig. 1). Taxonomy in this genus has not, however, progressed uniformly since the first morphological description of the species. The AlgaeBase database reports 48 species for the genus *Prorocentrum*, several of which were described based on morphology (Faust, 1990, 1993a,b,1994,1997); the GenBank database contains, however, sequences for only 25 different species, two of which undescribed (*P. donggang* and *P. tainan*). Also, identical D1D2 sequences were deposited for different species (*P. minimum/Prorocentrum balticum, Prorocentrum donghiaense/Prorocentrum dentatum, Prorocentrum hoffmannianum/ Prorocentrum belizeanum*).

Among collection vouchers, FIU PL *Prorocentrum lima* was identical to GenBank sequence L38634 *P. lima*, while *P. lima* CCMP 1368 (Fig. 5, Prorocentrum 2), which was 19 bp different, represented a different species. CCMP vouchers for *P. hoffmannianum, P. mexicanum, P. micans* and *P. minimum* differed only a few bp (one to four) from the corresponding GenBank sequences (Fig. 5, Prorocentrum 1 and 2). *P. cassubicum* UTEX 1596 (from the University of Texas algae collection), species not present in the GenBank database, strictly clustered with *P. foveolatum* AY259173 in the clade Prorocentrum 3.

Among environmental isolates, FIU 21 was identical to *P. minimum* AF042813, FIU 9 differed by one bp from *Prorocentrum rhathymum* AY259167 and FIU 40 differed by three bp from *P. hoffmannianum*, AY259171. FIU 22 clusters with *P. minimum/balticum* and *Prorocentrum donghaiense/dentatum* (100 boostrap value), FIU 23 clusters with *P. micans* and *Prorocentrum gracile* (100) and FIU 25 aligns very closely to *P. rhathymum* (89), but they all represent different species.

Lastly, in the outgroup (Fig. 1), the raphidophyte species *C. subsalsa* matched perfectly with FIU 42. Isolate FIU 51 differed by one bp from the other raphidophyte species, *H. akashiwo*.

3.2. Luminex results

3.2.1. Lab samples

3.2.1.1. Probe singleplex: Universal primers and species-specific sets of primers (Tables 4 and 5) produced amplicons that gave a Luminex positive response when tested against a single appropriate target DNA. Each of the 14 probes designed showed sensitivity to target DNA with MFI corrected values ranging from 420 (*Scrippsiella*) to 1300 (*P. hoffmannianum*). Similar wide-ranged results are not unusual in Luminex assays (Diaz and Fell, 2005). Several theories (Graves, 1999;Southern et al., 1999;Inacio et al., 2003;Peplies et al., 2003;Diaz and Fell, 2005) have been elaborated to explain their variability based mainly on the probe itself (base composition, base stacking, steric hindrance, kinetics, hairpin structure, position of the binding site) or on possible secondary structure in the denatured target DNA, but the mechanisms have not been clarified. Differences were also found in the intensity of the signal when probes were tested against different strains of the same target (Fig. 6). This kind of heterogeneity is probably due to differences in DNA quality and copy numbers among strains. Serial dilutions of genomic DNA and amplicons, as well as serial dilution of single cultures before filtration, also showed differences in signal response. In Fig. 7 are reported the results for culture dilutions of *P. micans*.

3.2.1.2. Probe multiplex: Multiplexing of the probes was conducted by testing all 14 probes against single species samples (Fig. 8). A slight decrease in the signal, compared to the singleplex results, occurred for the majority of the probes. Heterogeneity among probes can be noticed in the same trend shown in probe singleplex results (*Scrippsiella* 200/*P. hoffmannianum* 950).

3.2.1.3. Probe and target multiplex: Initially PCR products generated by Universal primers were used in Luminex tests on probe and target multiplexing. Use of universal primers allows for a simple and less expensive procedure, avoiding multiple-primers-PCRs and parallel amplifications.

A first stage of multi-probe-multi-target tests were performed adding DNA from different species in the PCR mix. In experiments with uniform amounts of DNA added to the PCR mix, the Luminex responses were consistently positive (Fig. 9). In spite of employing equal amounts of DNA, however, the difference in MFI values, for the two species contained in each sample, varied greatly from 50 (*H. akashiwo* 1150, *P. reticulatum* 1200) up to 850 MFI (*H. akashiwo* 1100, *P. mexicanum* 250). Also, the MFI absolute values for each species differed noticeably, in some cases, when compared to the MFI values for the same species tested singularly (*K. mikimotoi* 300 Fig. 9 and 650 Fig. 8). The difference always consisted in a reduced signal for the multi target samples. PCR mixtures with non-uniform amounts of DNA produced, instead, inconsistent Luminex results where species with smaller amount of target were generally “overwhelmed” by the most represented species and often went undetected (data not shown).

The second stage, in the multi-probe-multi-target testing, was performed spiking a water sample with multiple cultures, followed by filtration and DNA extraction. Luminex results with these samples were also variable. In this case, the inconsistency occurred even when the number of filtered cells per species was approximately identical. Differences in the extracted DNA quality and concentration for the different cultures, even within the same extraction sample, were probably responsible for this situation.

To improve and stabilize Luminex response in multi-probe-multi-target testing, a multiple-primers-PCR, which employed specific sets of primers, was added to the universal primers singleplex. Performing amplifications with specific primers increased PCR yield and specificity, for the species less represented in the sample, and allowed their detection in Luminex analyses. Specific primer sets (Table 5) were designed for a selected group of species prevalent in Florida waters, which included species numerically less represented in the environment, during HABs, and species unsuitable for multiplex reactions due to their sequence structure. Five different species (*P. minimum, P. rhathymum, P. micans, P. mexicanum* and *P. reticulatum*) and all the strains belonging to the *S. trochoidea* cluster were detected using the four sets of primers (Table 5). Forward and reverse specific sets generated different size amplicons, a feature that allowed a pre-Luminex PCR validation by a minigel electrophoresis (Fig. 10) where different size bands marked the presence of the four different amplicons. As previously reported, specific amplicons were used in Luminex tests with the same probes designed for universal amplicons. Hybridization can be improved choosing a probe-binding site close to the 3′ end of the amplicon sequence, to minimize the potential formation of secondary structures between the formed duplex and the biotinylated end. For this reason, the design of specific primers took into account the position of the binding site that was maintained as in the amplicons generated with universal primers or improved (moved closer to the 3′ end). The four sets of specific primers gave positive Luminex results when tested in DNA mix with random amount of DNA, as well as in cell mix with a random number of cells (Fig. 11).

3.2.2. Field samples—In our initial field trials, environmental samples were collected in Florida waters from Pine Island Sound in July 2006 and off Charlotte Harbor in October 2006. The latter samples were taken during a Florida Monitoring and Event Response for Harmful Algal Blooms (MERHAB) Cruise. Surface seawater samples were analyzed with the Luminex system (Figs. 12 and 13). The results showed the co-existence and dominance of two or more species of *Karenia* during toxic blooms in Florida waters. Four out of the nine *Karenia* species described are presently reported and regularly counted in Florida waters: *K. brevis, K. mikimotoi, K. papilionacea* and *K. selliformis*. In the present study we were able to design species-specific probes for the only two species available in culture: *K. brevis* and *K. mikimotoi*.

Microscopic counts for *Karenia* sp. were carried out on samples from Pine Island Sound and ranged from 0 cell/ml at station 4– 95 cells/ml at station 10. As shown in Fig. 12, a positive signal for *K. brevis* was obtained with the Luminex assay for all the samples analyzed, including station 4. A positive signal for *K. mikimotoi* was also shown for stations 5 and 9. For the October MERHAB Cruise we have counts at four stations for three different species of *Karenia: K. brevis, K. mikimotoi* and *K. papilionacea* (Table 8). Unidentified cells were recorded as *Karenia* sp. *K. brevis* was the most abundant species in both assays (Fig. 13 and Table 8). It is interesting to note, however, that, while in the microscopic counts the second biggest group is *Karenia* sp., the Luminex results reported a positive signal for *K. mikimotoi* at all stations (Fig. 12), even at station 44 where no cells of *K. mikimotoi* could be distinguished in the counts (Table 8). A possible explanation might be that cells of *K. mikimotoi*, even though present at all stations, could not be distinguished through microscopy due to their position on the slide. Design of additional species-specific probes (and potentially primers), when more *Karenia* species will be available in culture, might also help in solving this problem.

4. Discussion

Our first goal (assessment of HAB community diversity) was accomplished through sequence analysis of specimen vouchers and natural isolates. Label identification was confirmed for the majority of the voucher cultures. However, 35% of the voucher sequences aligned with a species different from the label, or did not find any match in the GenBank database. Misidentification, mislabeling or presence of a mixed culture might be responsible for this situation. The most interesting result was that 43.6% of our total sequence analysis (total number of different genotypes from specimen vouchers and natural isolates) showed a “unique” sequence, which did not match any GenBank submission. These results demonstrate the existence of a large and still unexplored phytoplankton biodiversity. Further analyses in different genes should be considered, however, for these cultures, to establish whether or not they may constitute new species.

Goals 2 and 3 (respectively early detection of HAB species and preliminary field application of the procedures) were realized through a qualitative bead array method based on the use of a flow cytometer (Luminex 100) and color-coded microspheres. For the bead array fourteen probes were designed, based on sequence analysis, for 14 microalgal species and four sets of species specific (or group specific) primers were employed, in addition to the universal primers, to enhance the presence of species of low abundance. The design of these specific primers was the key to success for species detection in analysis of samples, such as environmental samples, that contain multiple species in different concentrations.

Two parallel amplifications, followed by hybridization and Luminex analysis, allowed rapid identification of at least nine species of microalgae independently from their abundance. The singleplex PCR with Universal primers was performed for the detection of *K. brevis*, *K. mikimotoi* and *H. akashiwo*. A parallel multiplex PCR with sets of specific primers was carried out to amplify *P. minimum*, *P. rhathymum*, *P. micans*, *P. mexicanum*, *P. reticulatum* and *S. trochoidea*. The need for two separate PCR reactions is due to the possible production of random PCR amplicons if universal and specific primers, designed in the same gene, are present in the same amplification.

The two parallel amplicons obtained from single and multiplex PCR can be mixed in a single sample before hybridization and Luminex analysis, with consequent reduction in costs and time. If an enhanced signal is required, however, singleplex and multiplex PCR products can be kept separate and can be hybridized and analyzed as two different samples, increasing concentration and consequently signal response. Our LSU sequence analysis showed also sufficient inter-species variability to allow the design of additional species-specific primers for the majority of the species.

K. brevis and *K. mikimotoi*, did not show sufficient LSU variability to allow species-specific primer design without compromising probe closeness to the reverse primer. Increasing the distance of the probe site from the biotinylated end of the amplicon was avoided since it implied an increased risk of secondary structure formation between binding site and biotinylated end with consequent signal reduction. The regular abundance of the two species in any Florida HAB, however, provides high DNA yield and amplicon production. *H. akashiwo* does not require specific primers, since it could be detected in universal-primers-generated-amplicons in combination with any other species and with a constantly high MFI value (see Fig. 9, more than 900 MFI).

In recent years several new species of *Karenia* were described in different geographical locations: *K. asterichroma* (De Salas et al., 2004a), *K. umbella* (De Salas et al., 2004b), *K. papilionacea*, *K. bidigitata* and *K. selliformis* (Haywood et al., 2004), *K. cristata* and *K. bicuneiformis* (Botes et al., 2003) and *K. brevisulcata* (Chang, 1999). Unfortunately, the present lack of availability for *Karenia* cultures other than *K. mikimotoi* and *K. brevis* does not permit design of additional species-specific probes (and potentially primers) for the genus. A sequence for these species could be obtained cloning environmental samples and compared with the existing GenBank sequences. Lack of the specific culture, however, would still prevent proper testing of the probe design.

Future efforts should concentrate on an accurate assessment of the genetic variability in LSU and/or other genes of the new *Karenia* species, in particular *K. papilionacea* and *K. selliformis*, which are regularly reported in Florida waters in addition to *K. brevis* and *K. mikimotoi*. The acquisition of sequences to compare to the ones presently available in the GenBank database, would enable us to expand and integrate our present method. A different gene, which might offer larger variability and material for higher specificity, might be considered in probe design for the whole genus *Karenia*. This possibility could eliminate universal primers and replace the parallel amplifications with a single multiplex containing the present four LSU sets of primers and new sets of *Karenia* species (or group) specific primers. The use of a single PCR reaction would result in considerable reduction of time and costs.

Finally, the multi-format structure of this assay, which permits detection of 100 species at one time, could be advantageously used for additional, simultaneous analysis of phytoplankton community diversity. The method could provide more precise information of the biodiversity before, during and after a toxic bloom, which would contribute to a more comprehensive evaluation of the HAB phenomenon.

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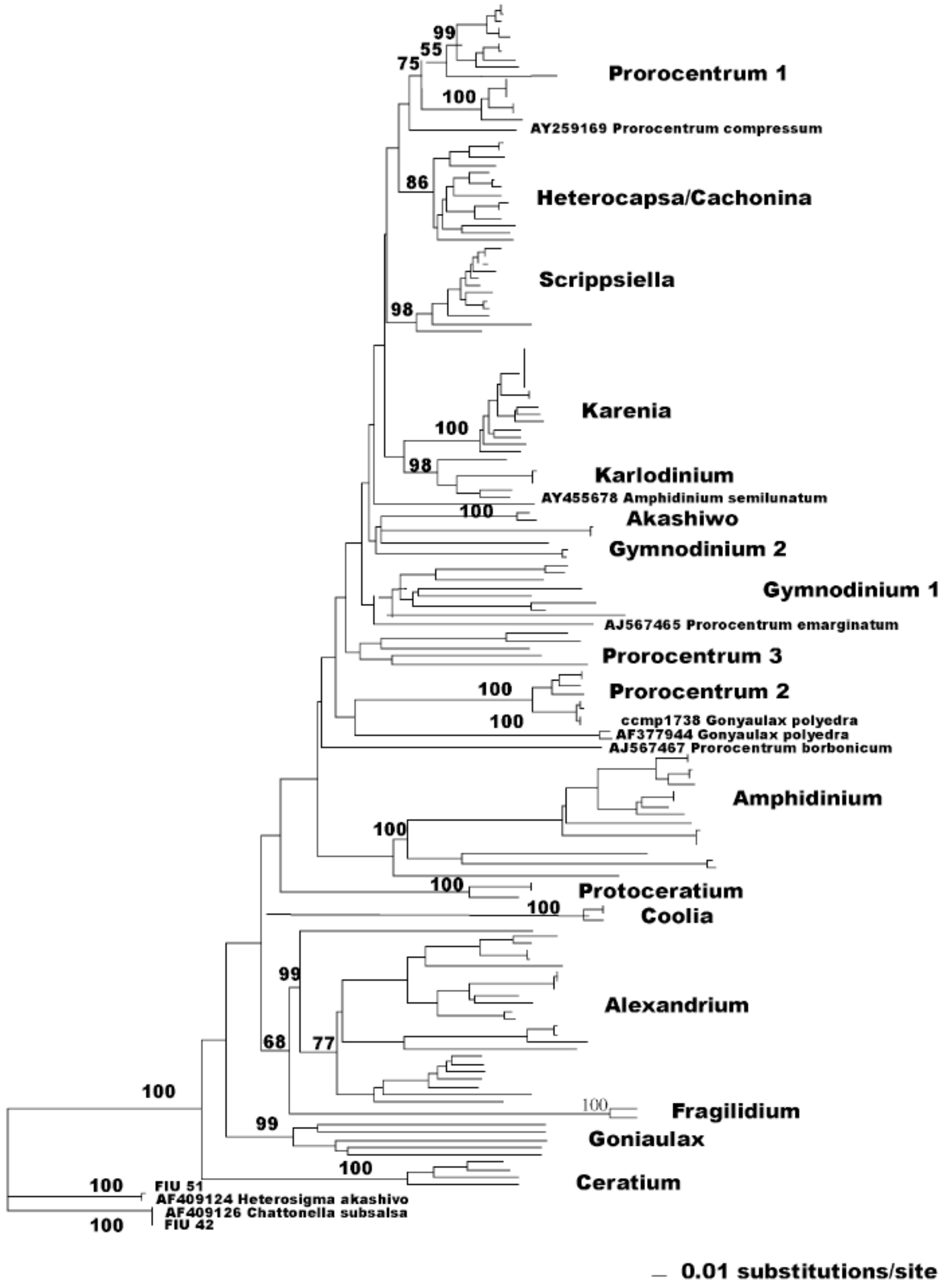
99
55
75
Prorocentrum 1
100
AY259169 Prorocentrum compressum
86
Heterocapsa/Cachonina
Scrippsiella
98
100
Karenia
98
Karlodinium
AY455678 Amphidinium semilunatum
100
Akashiwo
Gymnodinium 2
Gymnodinium 1
AJ567465 Prorocentrum emarginatum
Prorocentrum 3
100
Prorocentrum 2
100
ccmp1738 Gonyaulax polyedra
AF377944 Gonyaulax polyedra
AJ567467 Prorocentrum borbonicum
Amphidinium
100
100
Protoceratium
100
Coolia
99
Alexandrium
68
77
100
100
Fragilidium
99
Goniaulax
100
Ceratium
100
FIU 51
AF409124 Heterosigma akashivo
AF409126 Chattonella subsalsa
100
FIU 42
0.01 substitutions/site

Fig. 1.

LSU D1D2 rRNA phylogenetic stick tree comprising CCMP specimen vouchers, FL waters isolates and related GenBank sequences. The tree was constructed with neighbor joining analysis. Bootstrap values are reported on branches when higher than 50%. Clades are shown to indicate their orientation within lineages (see Figs. 2–5).

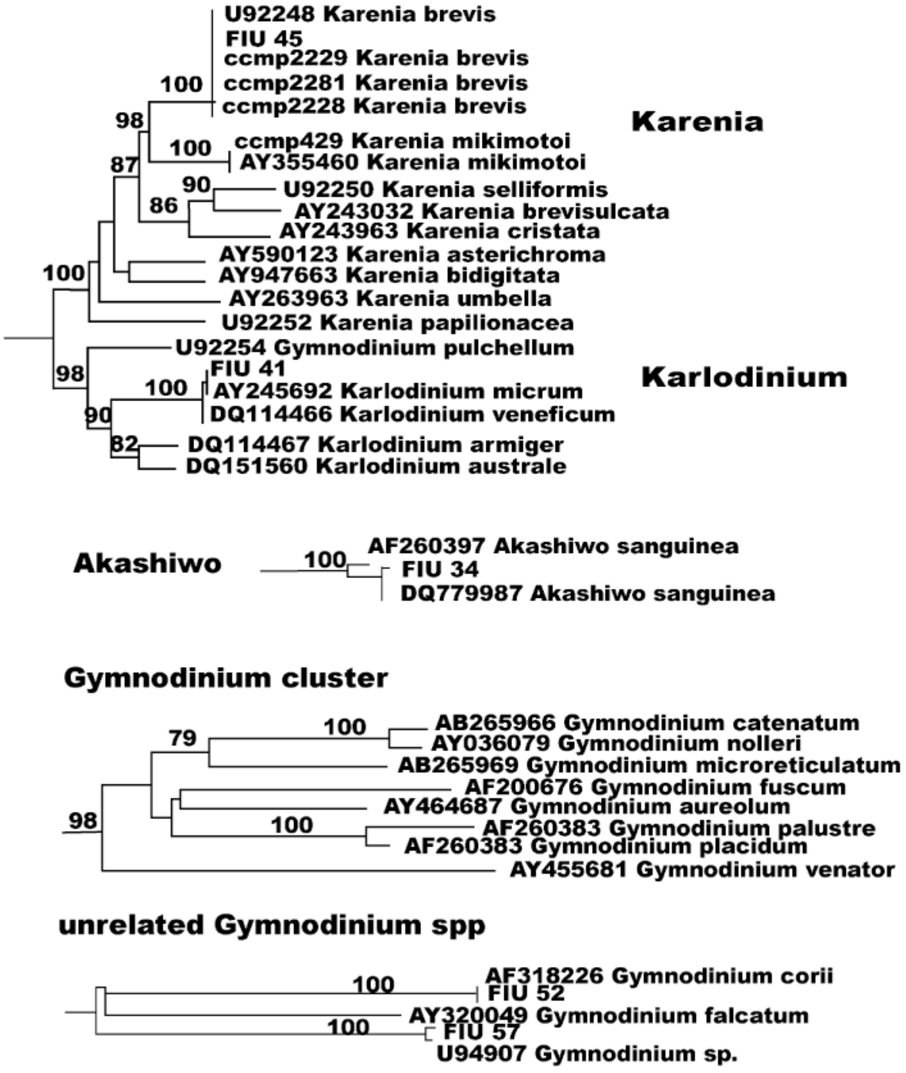
U92248 Karenia brevis
FIU 45
ccmp2229 Karenia brevis
ccmp2281 Karenia brevis
ccmp2228 Karenia brevis
100
98
Karenia
ccmp429 Karenia mikimotoi
AY355460 Karenia mikimotoi
100
87
90
U92250 Karenia selliformis
AY243032 Karenia brevisulcata
86
AY243963 Karenia cristata
AY590123 Karenia asterichroma
AY947663 Karenia bidigitata
100
AY263963 Karenia umbella
U92252 Karenia papilionacea
U92254 Gymnodinium pulchellum
98
FIU 41
Karlodinium
100
AY245692 Karlodinium micrum
90
DQ114466 Karlodinium veneficum
82
DQ114467 Karlodinium armiger
DQ151560 Karlodinium australe
Akashiwo
100
AF260397 Akashiwo sanguinea
FIU 34
DQ779987 Akashiwo sanguinea
Gymnodinium cluster
100
AB265966 Gymnodinium catenatum
79
AY036079 Gymnodinium nolleri
AB265969 Gymnodinium microreticulatum
AF200676 Gymnodinium fuscum
AY464687 Gymnodinium aureolum
98
100
AF260383 Gymnodinium palustre
AF260383 Gymnodinium placidum
AY455681 Gymnodinium venator
unrelated Gymnodinium spp
100
AF318226 Gymnodinium corii
FIU 52
AY320049 Gymnodinium falcatum
100
FIU 57
U94907 Gymnodinium sp.

Fig. 2.

Karenia, *Karlodinium*, *Akashiwo* and *Gymnodinium* clusters and unrelated *Gymodinium* species as from Fig. 1.

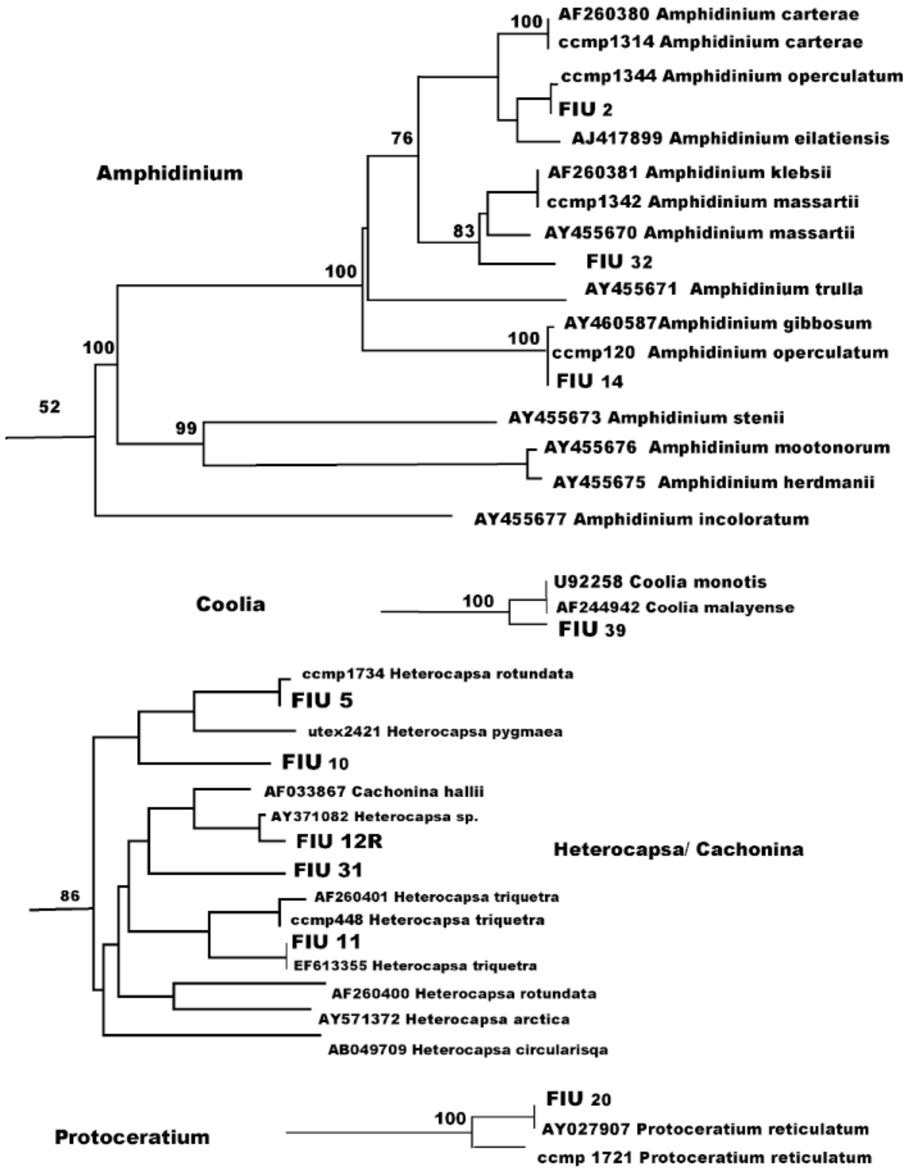
Amphidinium
100
AF260380 Amphidinium carterae
ccmp1314 Amphidinium carterae
ccmp1344 Amphidinium operculatum
FIU 2
76
AJ417899 Amphidinium eilatiensis
AF260381 Amphidinium klebsii
ccmp1342 Amphidinium massartii
83
AY455670 Amphidinium massartii
FIU 32
100
AY455671 Amphidinium trulla
100
AY460587Amphidinium gibbosum
ccmp120 Amphidinium operculatum
FIU 14
100
52
AY455673 Amphidinium stenii
99
AY455676 Amphidinium mootonorum
AY455675 Amphidinium herdmanii
AY455677 Amphidinium incoloratum
Coolia
100
U92258 Coolia monotis
AF244942 Coolia malayense
FIU 39
ccmp1734 Heterocapsa rotundata
FIU 5
utex2421 Heterocapsa pygmaea
FIU 10
AF033867 Cachonina hallii
AY371082 Heterocapsa sp.
FIU 12R
Heterocapsa/ Cachonina
FIU 31
86
AF260401 Heterocapsa triquetra
ccmp448 Heterocapsa triquetra
FIU 11
EF613355 Heterocapsa triquetra
AF260400 Heterocapsa rotundata
AY571372 Heterocapsa arctica
AB049709 Heterocapsa circularisqa
Protoceratium
100
FIU 20
AY027907 Protoceratium reticulatum
ccmp 1721 Protoceratium reticulatum

Fig. 3.

Amphidinium, Coolia, Heterocapsa/Cachonina and *Protoceratium* clusters as from Fig. 1.

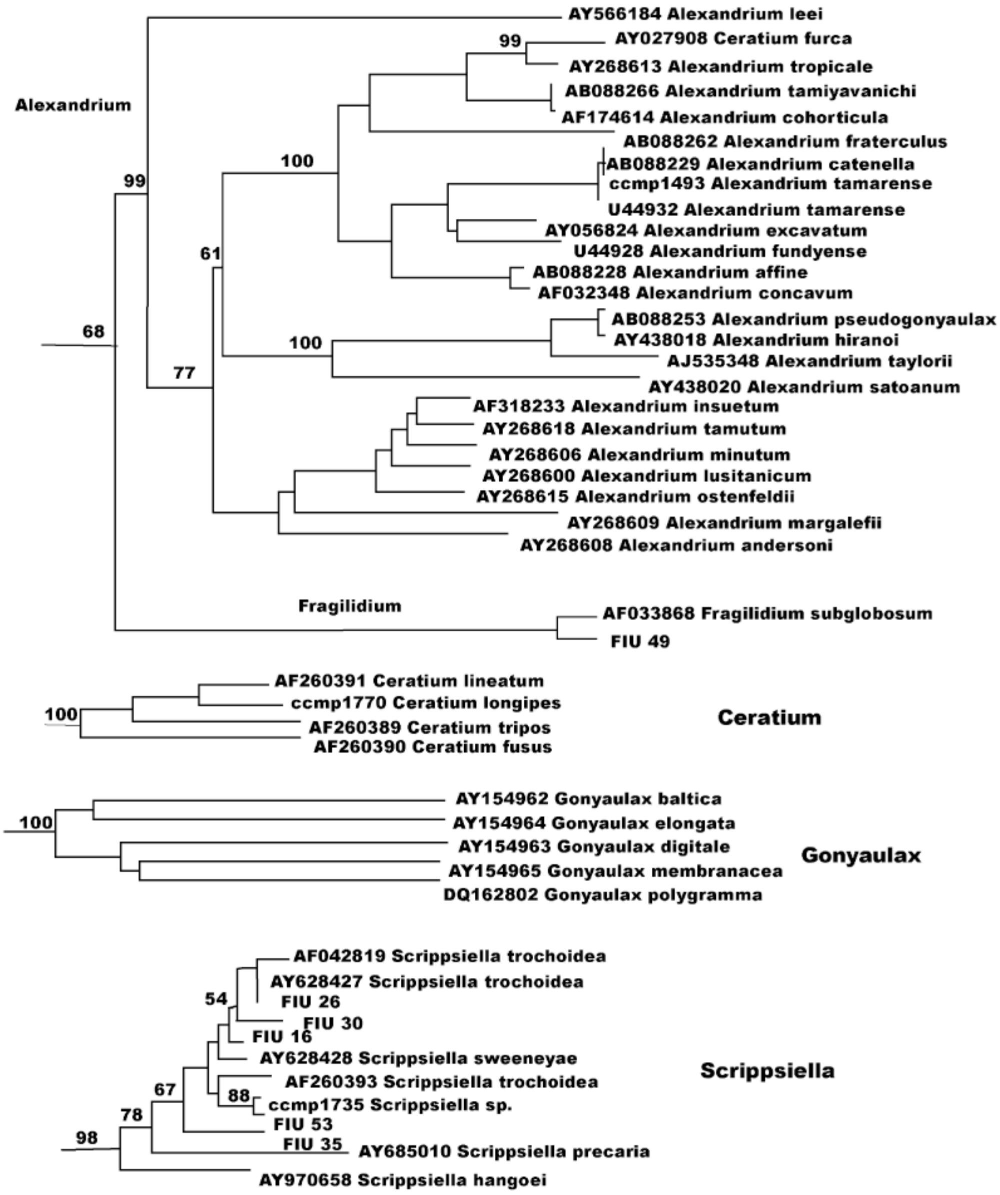
AY566184 Alexandrium leei
99
AY027908 Ceratium furca
AY268613 Alexandrium tropicale
AB088266 Alexandrium tamiyavanichi
AF174614 Alexandrium cohorticula
Alexandrium
AB088262 Alexandrium fraterculus
100
AB088229 Alexandrium catenella
ccmp1493 Alexandrium tamarense
99
U44932 Alexandrium tamarense
AY056824 Alexandrium excavatum
61
U44928 Alexandrium fundyense
AB088228 Alexandrium affine
AF032348 Alexandrium concavum
68
AB088253 Alexandrium pseudogonyaulax
100
AY438018 Alexandrium hiranoi
AJ535348 Alexandrium taylorii
77
AY438020 Alexandrium satoanum
AF318233 Alexandrium insuetum
AY268618 Alexandrium tamutum
AY268606 Alexandrium minutum
AY268600 Alexandrium lusitanicum
AY268615 Alexandrium ostenfeldii
AY268609 Alexandrium margalefii
AY268608 Alexandrium andersoni
Fragilidium
AF033868 Fragilidium subglobosum
FIU 49
AF260391 Ceratium lineatum
ccmp1770 Ceratium longipes
100
AF260389 Ceratium tripos
AF260390 Ceratium fusus
Ceratium
AY154962 Gonyaulax baltica
100
AY154964 Gonyaulax elongata
AY154963 Gonyaulax digitale
Gonyaulax
AY154965 Gonyaulax membranacea
DQ162802 Gonyaulax polygramma
AF042819 Scrippsiella trochoidea
AY628427 Scrippsiella trochoidea
54
FIU 26
FIU 30
FIU 16
AY628428 Scrippsiella sweeneyae
Scrippsiella
AF260393 Scrippsiella trochoidea
67
88
ccmp1735 Scrippsiella sp.
78
FIU 53
98
FIU 35
AY685010 Scrippsiella precaria
AY970658 Scrippsiella hangoei

Fig. 4.

Alexandrium, Fragilidium, Ceratium, Gonyaulax and *Scrippsiella* clusters as from Fig. 1.

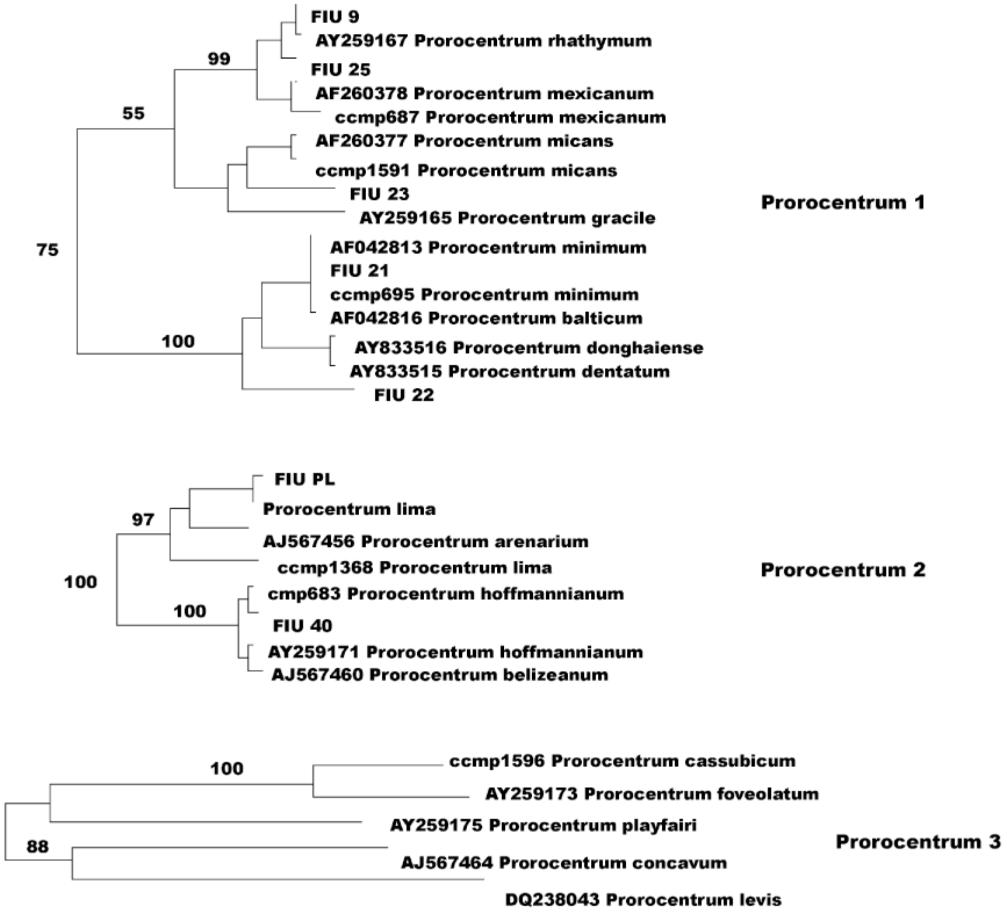
FIU 9
AY259167 Prorocentrum rhathymum
99
FIU 25
55
AF260378 Prorocentrum mexicanum
ccmp687 Prorocentrum mexicanum
AF260377 Prorocentrum micans
ccmp1591 Prorocentrum micans
FIU 23
Prorocentrum 1
AY259165 Prorocentrum gracile
75
AF042813 Prorocentrum minimum
FIU 21
ccmp695 Prorocentrum minimum
AF042816 Prorocentrum balticum
100
AY833516 Prorocentrum donghaiense
AY833515 Prorocentrum dentatum
FIU 22
FIU PL
Prorocentrum lima
97
AJ567456 Prorocentrum arenarium
100
ccmp1368 Prorocentrum lima
Prorocentrum 2
cmp683 Prorocentrum hoffmannianum
100
FIU 40
AY259171 Prorocentrum hoffmannianum
AJ567460 Prorocentrum belizeanum
ccmp1596 Prorocentrum cassubicum
100
AY259173 Prorocentrum foveolatum
AY259175 Prorocentrum playfairi
88
Prorocentrum 3
AJ567464 Prorocentrum concavum
DQ238043 Prorocentrum levis

Fig. 5.

Prorocentrum 1, 2 and 3 clusters as from Fig. 1.

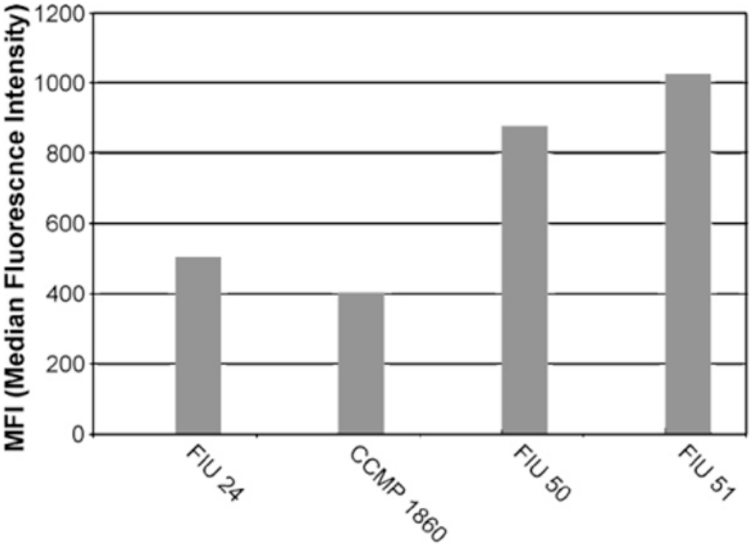

Fig. 6.

Probe singleplex with different strains of the same target (*Heterosigma akashiwo*).

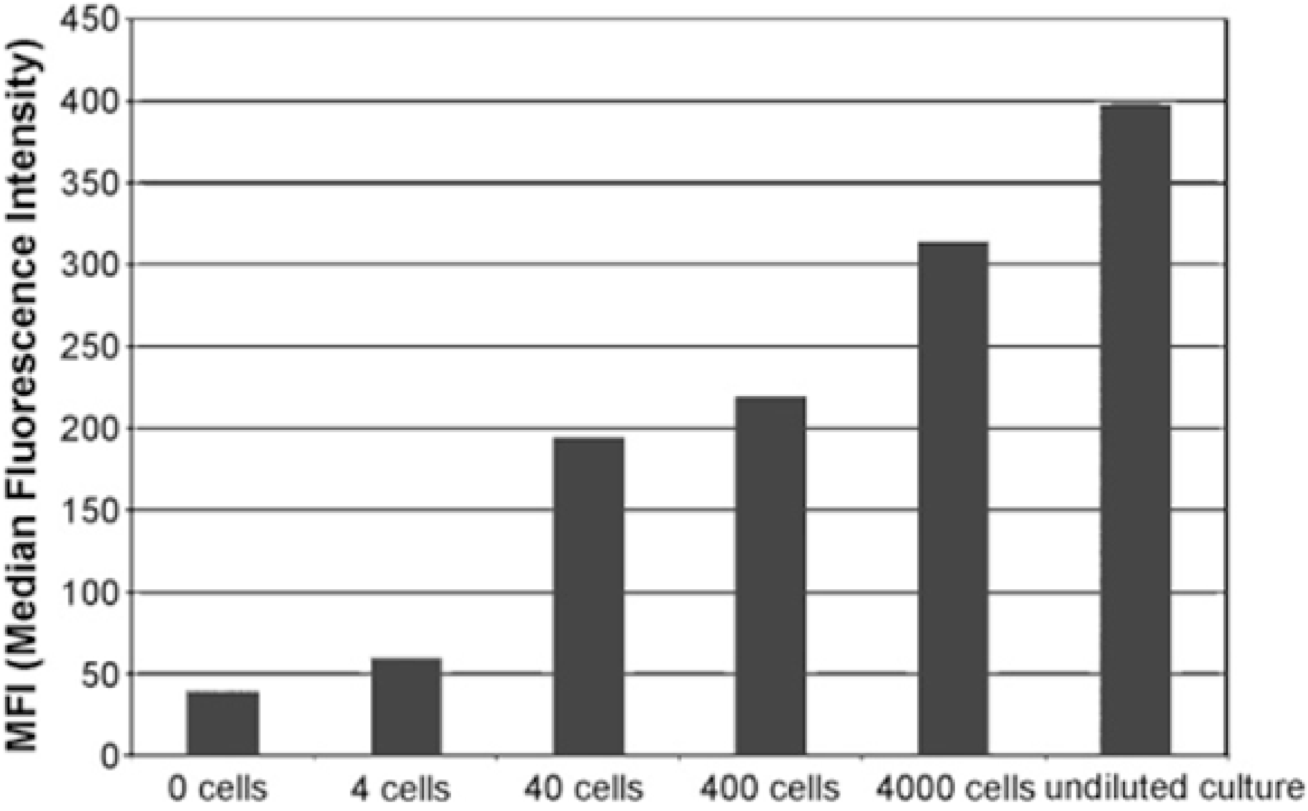

Fig. 7.
Probe singleplex with culture dilutions of the same target (*Prorocentrum micans*).

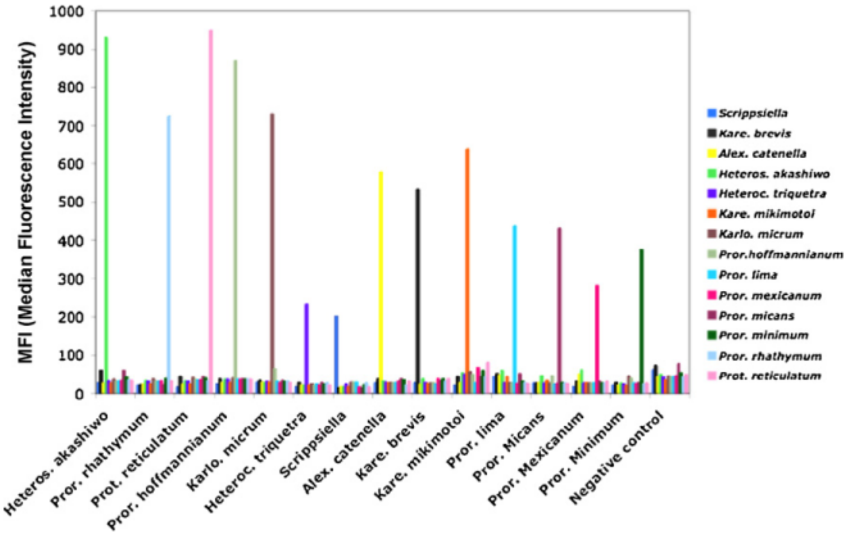

Fig. 8.

Probe multiplex with 14 probes against single-species samples.

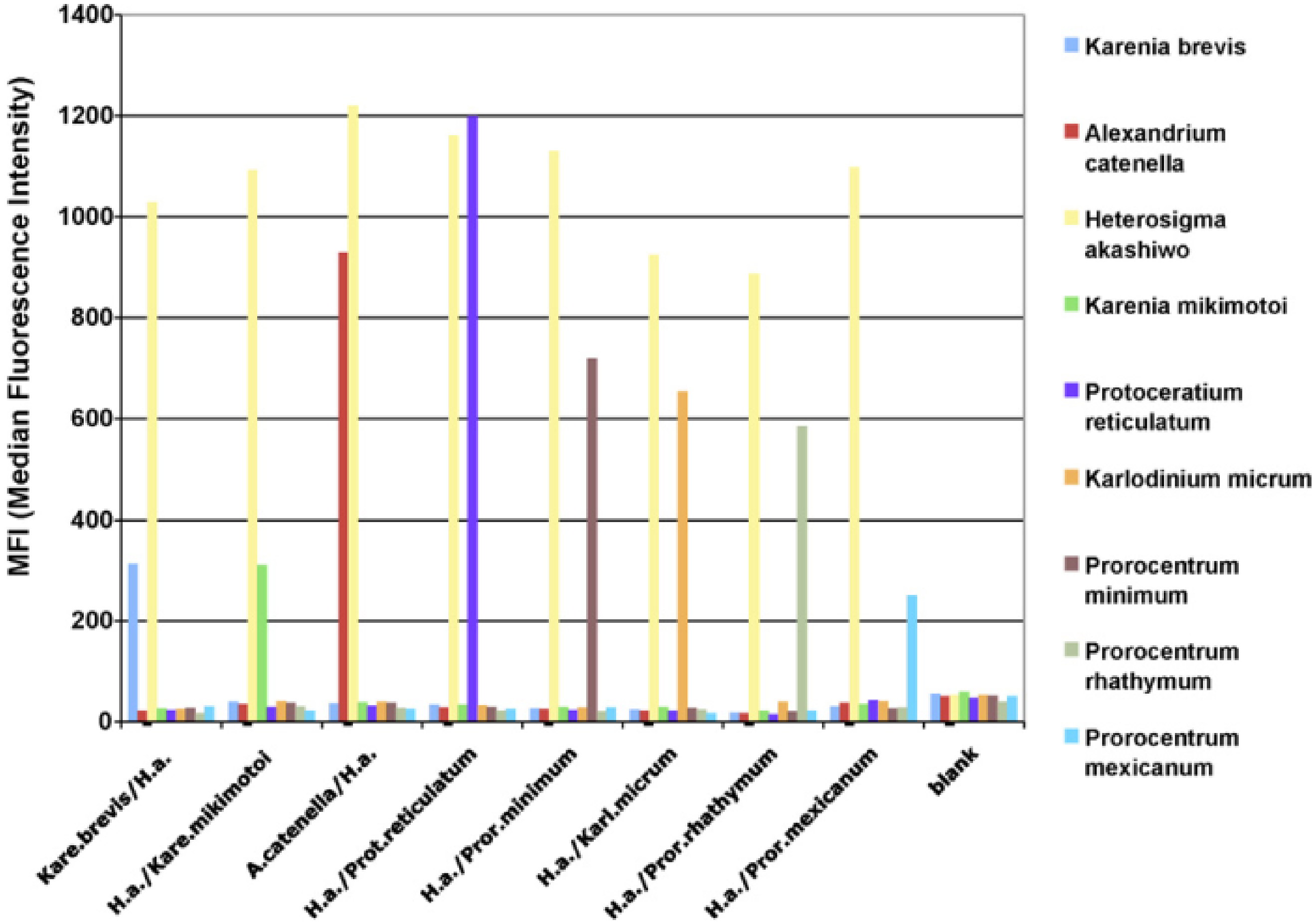

Fig. 9.

Probe and target multiplex with 9 probes and 2-species samples containing uniform DNA amounts for each species.

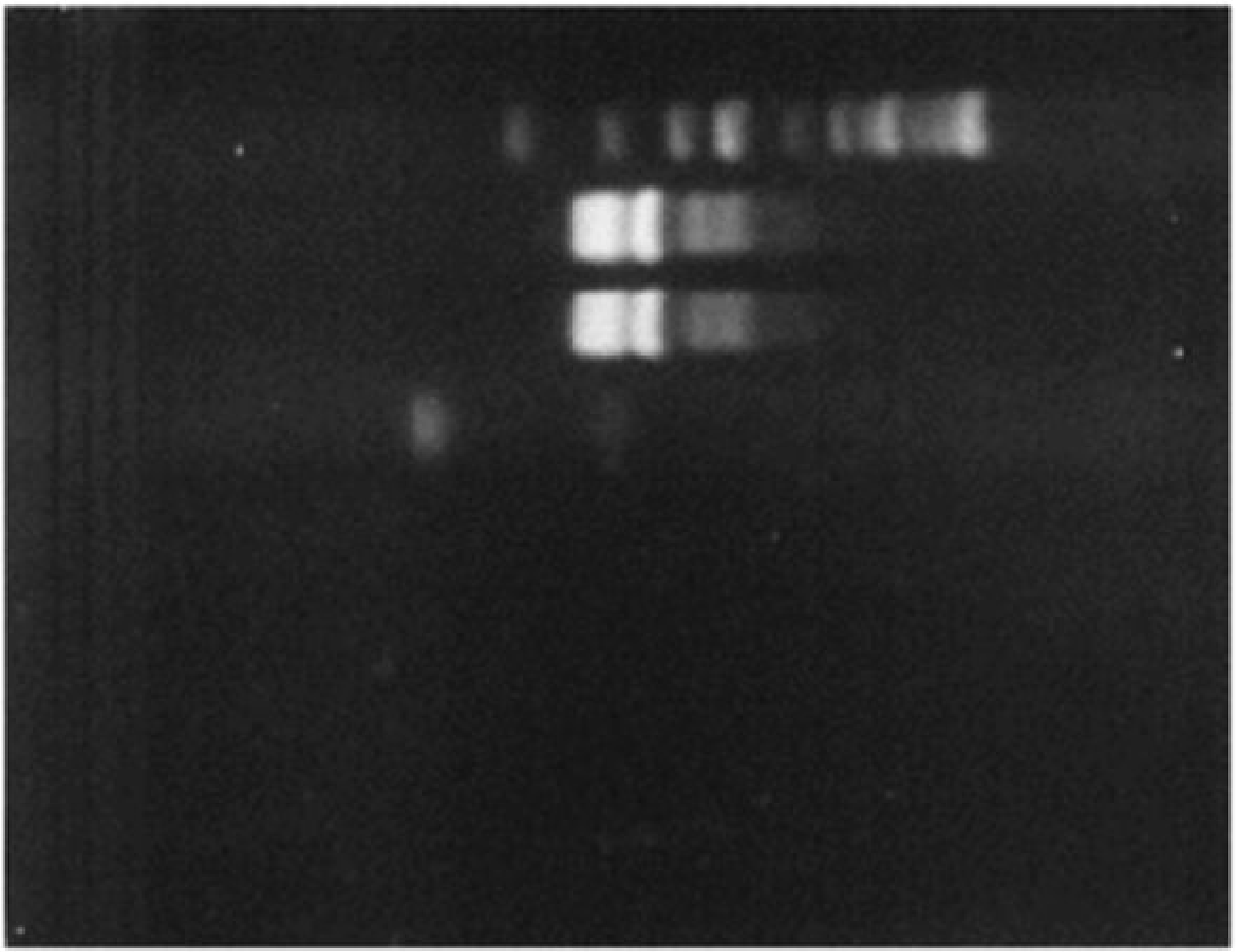

Fig. 10.

Minigel electrophoresis showing different size amplicons generated by specific primers sets.

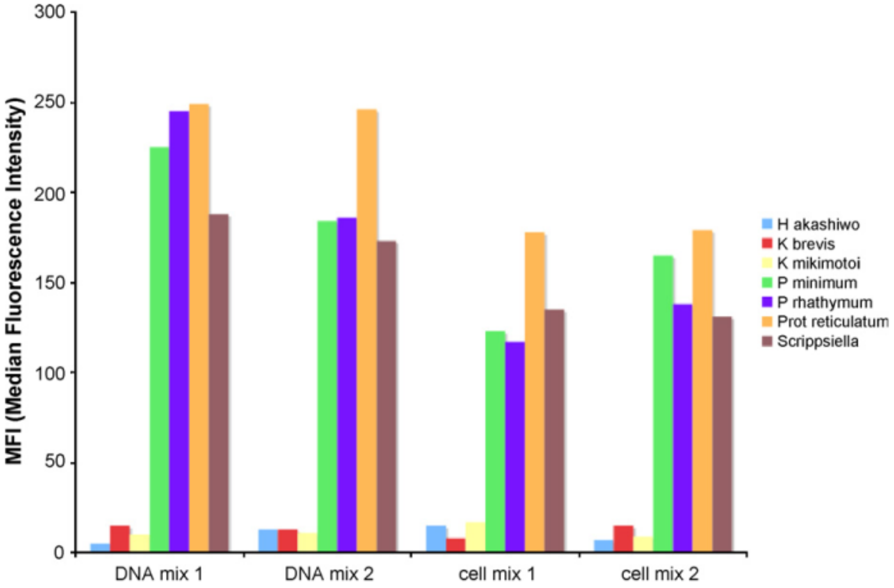

Fig. 11.
Probe and target multiplex with 7 probes and 4-species amplicons generated with species specific primers. The first two samples were originated from a PCR DNA mix with random amount of DNA for each of the 4 species. For the second two samples a cell mix containing a random number of cells for each species was extracted and amplified.

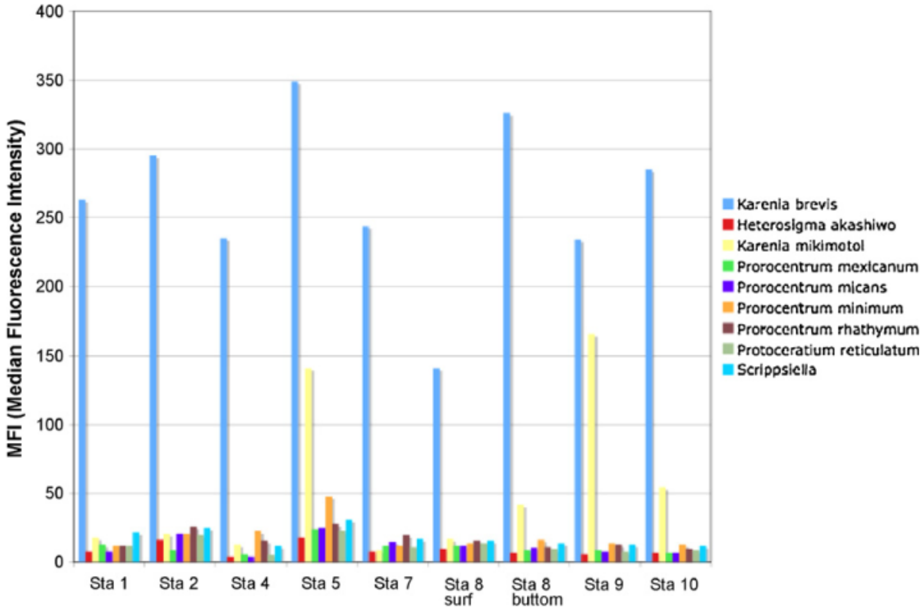

Fig. 12.

Probe multiplex on environmental samples collected in Florida waters from Pine Island Sound in July 2006.

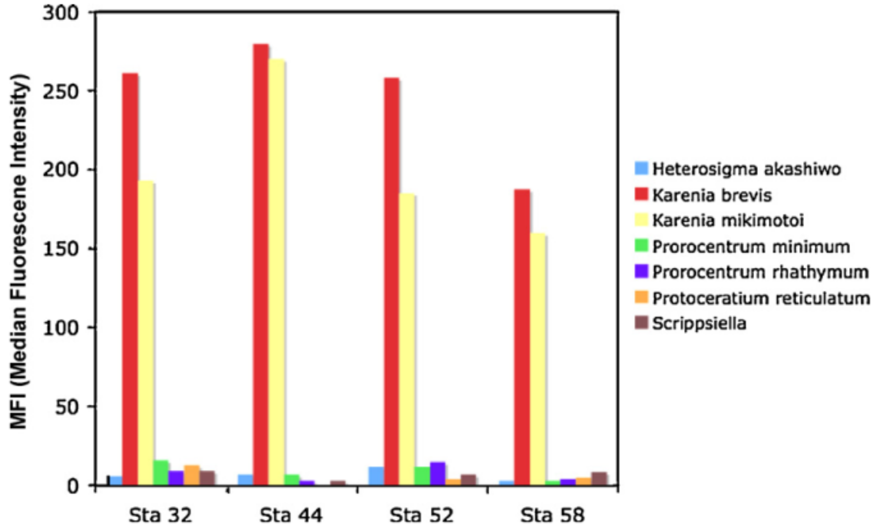

Fig. 13.

Probe multiplex on environmental samples collected off Charlotte Harbor in October 2006 (MERHAB Cruise).

Table 1

List of voucher specimens studied

Collection label	Collection number	Sequencing identification	GenBank accession number
Alexandrium tamarense/catenella	CCMP 1493	Identical to *Alexandrium catenella* AB088229	EU165300
Amphidinium carterae	CCMP 1314	Identical to *Amphidinium carterae* AF260380	EU165301
Amphidinium massartii	CCMP 1342	Identical to *Amphidinium klebsii* AF260381	EU165302
Amphidinium operculatum	CCMP 120	I bp different from *Amphidinium gibbosum* AY460567	EU165303
Amphidinium operculatum	CCMP 1344	Related to *Amphidinium carterae* and *Amphidinium eilatiensis*	EU165304
Ceratium longipes	CCMP 1770	Related to *Ceratium lineatum*	EU165305
Heterocapsa pygmeae	UTEX 2421	Clusters in the *Heterocapsa* group	EU165306
Heterocapsa triquetra	CCMP 448	4 bp different from *Heterocapsa triquetra* AF2604401	EU165307
Karenia brevis	CCMP 2228	Identical to *Karenia brevis* U92248	EU165308
Karenia brevis	CCMP 2229	Identical to *Karenia brevis* U92248	EU165309
Karenia brevis	CCMP 2281	Identical to *Karenia brevis* U92248	EU165310
Karenia mikimotoi	CCMP 429	Identical to *Karenia mikimotoi* AY355460	EU165311
Katodinium rotundatum (Heterocapsa rotundata)	CCMP 1734	Clusters in the *Heterocapsa* group, far from *Heterocapsa rotundata* AF260400	EU165312
Lingulodinium polyedra (Gonyaulax polyedra)	CCMP 1738	8 bp different from *Gonyaulax polyedra* AF377944	EU165313
Prorocentrum cassubicum	UTEX 1596	Related to *Prorocentrum foveolatum* AY259173	EU165314
Prorocentrum hoffmannianum	CCMP 683	2 bp different from *Prorocentrum hoffmannianum* AY259171	EU165315
Prorocentrum lima	FIU PL	Identical to *Prorocentrum lima* L38634	EU165316
Prorocentrum lima	CCMP 1368	19 bp different from *Prorocentrum lima* L38634	EU165317
Prorocentrum mexicanum	CCMP 687	3 bp different from *Prorocentrum mexicanum* AF260378	EU165318
Prorocentrum micans	CCMP 1591	1 bp different from *Prorocentrum micans* AF260377	EU165319
Prorocentrum minimum	CCMP 695	Identical to *Prorocentrum minimum* AF042813	EU165320

Collection label	Collection number	Sequencing identification	GenBank accession number
Protoceratium reticulatum	CCMP 1721	More than 30 bp from *Protoceratium reticulatum* AY027907	EU165321
Scrippsiella sp.	CCMP 1735	Clusters in the *Scrippsiella* group	EU165322

CCMP, Provasoli-Guillard Center for the Culture of Marine Phytoplankton; UTEX, Culture Collection of Algae, University of Texas; FIU, Culture Collection of Algae, Florida International University.

Table 2

List of Florida water isolates studied

FIU number/CCMP number	Sequencing identification	GenBank accession number	Number of identical strains
FIU 2	Related to *Amphidinium carterae* and *Amphidinium eilatiensis*	EU165276	
FIU 5/CCMP 2770	Clusters in the *Heterocapsa/ Cachonina* group	EU165271	3
FIU 9	1 bp different from *Prorocentrum rhathymum* AY259167	EU165279	3
FIU 10	Clusters in the *Heterocapsa/ Cachonina* group	EU165272	
FIU 11	Clusters in the *Heterocapsa/ Cachonina* group	EU165273	2
FIU 12R	5 bp different from *Heterocapsa* sp. AY371082	EU165274	
FIU 14/CCMP 2819	1 bp different from *Amphidinium gibbosum* AY460587	EU165277	2
FIU 16/CCMP 2797	6 bp different from *Scrippsiella trochoidea* AY628427	EU165285	2
FIU 20/CCMP 2776	Identical to *Protoceratium reticulatum* AY027907	EU165290	2
FIU 21/CCMP 2812	Identical to *Prorocentrum minimum* AF042813	EU165280	2
FIU 22	Related to *Prorocentrum donghiaense, Prorocentrum dentatum, Prorocentrum minimum* and *Prorocentrum balticum*	EU165281	
FIU 23/CCMP 2772	Related to *Prorocentrum micans* and *Prorocentrum gracile*	EU165282	1
FIU 25	6 bp different from *Prorocentrum rhathymum* AY259167	EU165283	
FIU 26	Identical to *Scrippsiella trochoidea* AY628427	EU165286	2
FIU 30/CCMP 2810	Clusters in the *Scrippsiella* group	EU165287	
FIU 31	Clusters in the *Heterocapsa/ Cachonina* group	EU165275	
FIU 32/CCMP 2774	Related to *Amphidinium massartii* and *Amphidinium klebsii*	EU165278	1
FIU 34/CCMP 2796	More than 10 bp different from *Akashiwo sanguinea* AF2603397	EU165292	1
FIU 35/CCMP 2810	Clusters in the *Scrippsiella* group	EU165288	
FIU 39	Related to *Coolia monotis* U92258/*Coolia malayense* AF244942	EU165293	

FIU number/CCMP number	Sequencing identification	GenBank accession number	Number of identical strains
FIU 40/CCMP 2804	3 bp different from *Prorocentrum hoffmannianum* AY259171	EU165284	
FIU 41/CCMP 2778	Identical to *Karlodinium micrum* AY245692	EU165294	
FIU 42/CCMP 2793	Identical to *Chattonella subsalsa* AF409126	EU165295	4
FIU 45/CCMP 2820	Identical to *Karenia brevis* U92248	EU165296	
FIU 49	Related to *Fragilidium subglobosum* AF033868	EU165297	
FIU 51/CCMP 2817	1 bp different from *Heterosigma akashiwo* AF409124	EU165291	2
FIU 52	Identical to *Gymnodinium corii* AF318226	EU165298	
FIU 53/CCMP 2775	Clusters in the *Scrippsiella* group	EU165289	
FIU 57	3 bp different from *Gymnodinium* sp. U94907	EU165299	

FIU, Culture Collection of Algae, Florida International University; CCMP, Provasoli-Guillard Center for the Culture of Marine Phytoplankton.

Table 3

List of steps carried out in our procedure

DNA extraction (2.1.1)	
Sequencing analysis (2.1.2)	PCR amplification with universal primers (no biotin moiety added) followed by electrophoresis verification, purification and determination of partial LSU rDNA sequences
Luminex analysis (2.1.3)	Primer design based on sequencing results (2.1.3.1)
	Probe design based on sequencing results (2.1.3.2)
	Coupling reaction (2.1.3.3)
	PCR amplifications: singleplex with universal primers (biotinylated reverse) and multiplex with 4 sets of specific primers (biotinylated reverse) (2.1.3.4)
	Hybridization assay (2.1.3.5)
	Detection analysis (probe singleplex, probe multiplex, probe and target multiplex) (2.1.3.6)
Field studies (2.1.4)	

Table 4

List of universal primers used

Primer name	**Primer sequence; primer length**	**Melting *T*; G/C%; Sec. struct.**
D1R F	ACCGCTGAATTTAAGCATA; 19bp	57.4 °C; 36.84%; weak
D2C R	CCTTGGTCCGTGTTTCAAGA; 20bp	65 °C; 50%; moderate

Table 5

List of specific primers designed

Primer name; spp. amplified	**Primer sequence; primer length**	**Melting *T*; G/C %; Sec. struct.**
Scripp F; all spp. in the *Scrippsiella* complex	GAATTGTAGTCTGGAGATGTACCAC; 25 bp	61.5 °C; 44%; weak
Scripp R	BioCTGAAGTTTTCCCCAGTTGC; 20 bp	63.5 °C; 50%; weak
Protoce F; *Protoceratium reticulatum*	ATGGCGAATGAAGAAGGAGT; 20 bp	62.5 °C; 45%; none
Protoce R	BioGATCCACAAGCTAAATCTGAACCT; 24 bp	63.3 °C; 41.67%; very weak
Minimum F; *Prorocentrum minimum*	GGGTCATGGTAGCTCGTCTA; 20 bp	61.7 °C; 55%; very weak
Minimum R	BioCGTCTTTGTGTCAGGGAAAT; 20 bp	61.5 °C; 45%; none
Rhatmicmex F; *Prorocentrum rhathymum, micans* and *mexicanum*	CTTGGCGAGATTGTCATACG; 20bp	63.2 °C; 50%; none
Rhatmicmex R	BioCGTCTATGTGTCAGGGAAGC; 20 bp	62.3 °C; 55%; none

Table 6

List of Luminex probes designed

Species name; probe name; probe amount	**Probe sequence; probe length**	**Melting *T*; G/C%; secondary structcure; Δ*G***
Heterosigma akashiwo; (HA1); 0.35 nm	GTTCCGCGGGAAATGTTCAGTG; 22 bp	71.4 °C; 54.5%; moderate; −6.7
Karlodinium micrum; (KM2); 0.35 nm	AGCATGACGCGCACTTTGTTTCTC; 24 bp	72 °C; 50%; none; −6.7
Karenia brevis; (430 brevis); 0.35 nm	TGTTGTCTAAGGTGATAGCTTGC; 23 bp	62 °C; 43.4%; very weak; −8.5
Scrippsiella trochoidea; (Scripps); 0.35 nm	AGTAAGTCTTGAACAGGACATGCG; 24 bp	65.2 °C; 45.8%; weak; −10.1
Karenia mikimotoi; (530 mikimotoi); 0.35 nm	CGCTTCCGAGTGACTGAATG; 20 bp	65.8 °C; 55%; very weak; −6.9
Heterocapsa triquetra; (HT7bis); 0.35 nm	GCATTGTGTCTTCACACCCCTG; 22 bp	68.8 °C; 54.5%; moderate; −9.7
Alexandrium catenella, *Alexandrium tamarense*; (AC8); 0.35 nm	GGGTTTTGGCTGCAAGTGCAA; 21 bp	71.1 °C; 52.3%; weak; −8.8
Prorocentrum micans; (Pmic); 0.2 nm	AGTGCTGCTTGATCCTGTGTTG; 22 bp	66.5 °C; 50%; none; −7
Prorocentrum minimum; (PI3); 0.35 nm	GCAGTGTCCTTGGCATTCTGGA; 22 bp	70.5 °C; 54.5%; weak; −8.2
Prorocentrum lima; (Plim); 0.2 nm	GACGAATATGTCTGGGTGCATC; 22 bp	65.3 C; 50%; weak; −8.1
Prorocentrum hoffmannianum; (Phoff); 0.2 nm	ACGAAGGTGCCTGGTCGCAT; 20 bp	71.2 C; 60%; weak; −10.1
Prorocentrum mexicanum; (PE4); 0.35 nm	GTCTTCGGGTGGGCGAATGTG; 21 bp	72.8 C; 61.9%; moderate; −6.6
Prorocentrum rhathymum; (PH5); 0.35 nm	GTCTTCGGGCGGGTGAATGTG; 21 bp	72.8 C; 61.9%; very weak; −6.6
Protoceratium reticulatum; (PR6); 0.35 nm	GTGAATGCGCAATGTTGCTTG; 21 bp	72.8 C; 61.9%; very weak; −6.6

Table 7

List of collection sites

Station	Time (EDT)	Latitude	Longitude
July 27, 2006			
Stn 1	06:05	26°27.227′N	82°00.241′W
Stn 2	07:40	26°28.623′N	82°05.179′W
Stn 4	08:40	26°33.614′N	82°10.437′W
Stn 5	09:02	26°36.554′N	82°12.108′W
Stn 7	10:35	26°41.592′N	82°13.995′W
Stn 8	11:00	26°44.394′N	82°14.960′W
Stn 9	12:17	26°46.150′N	82°18.498′W
Stn 10	12:36	26°44.968′N	82°17.591′W
October 9–13, 2006 MERHAB Cruise			
Stn 32	19:13	26°44.422′N	82°20.421′W
Stn 44	07:20	26°40.706′N	82°20.330′W
Stn 52	18:26	26°42.821′N	82°20.747′W
Stn 58	06:15	26°41.492′N	82°19.481′W

Table 8

Microscopic counts for the 9–13, October 2006 MERHAB Cruise

	K. brevis	**K. mikimotoi**	**K. papilionacea**	**Karenia *sp.***
Station 32	57	2	3	32
Station 44	79		2	46
Station 52	251	4	3	158
Station 58	59	1	1	34

Numbers refer to cells per ml.