

IDENTIFICATION OF *PFIESTERIA PISCICIDA* (DINOPHYCEAE) AND
PFIESTERIA-LIKE ORGANISMS USING INTERNAL TRANSCRIBED
SPACER-SPECIFIC PCR ASSAYS¹

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The putative harmful algal bloom dinoflagellate, *Pfiesteria piscicida* (Steidinger et Burkholder), frequently co-occurs with other morphologically similar species collectively known as *Pfiesteria*-like organisms (PLOs). This study specifically evaluated whether unique sequences in the internal transcribed spacer (ITS) regions, ITS1 and ITS2, could be used to develop PCR assays capable of detecting PLOs in natural assemblages. ITS regions were selected because they are more variable than the flanking small subunit or large subunit rRNA genes and more likely to contain species-specific sequences. Sequencing of the ITS regions revealed unique oligonucleotide primer binding sites for *Pfiesteria piscicida*, *Pfiesteria shumwayae* (Glasgow et Burkholder), Florida “Lucy” species, two cryptoperidiniopsis species, “H/V14” and “PLO21,” and the estuarine mixotroph, *Karodinium micrum* (Leadbetter et Dodge). These PCR assays had a minimum sensitivity of 100 cells in a 100-mL sample (1 cell·mL⁻¹) and were successfully used to detect PLOs in the St. Johns River system in Florida, USA. DNA purification and aspects of PCR assay development, PCR optimization, PCR assay controls, and collection of field samples are discussed.

Key index words: diagnostics; evolution; *Karodinium micrum*; PCR; *Pfiesteria piscicida*; *Pfiesteria shumwayae*; *Pfiesteria*-like organisms; ribosomal genes

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit; PLOs, *Pfiesteria*-like organisms; rDNA,

the genomic DNA genes that code for rRNA; SSU, small subunit

In most organisms, the internal transcribed spacer (ITS) regions diverge significantly during speciation, allowing even closely related species to be unambiguously identified (Bargues et al. 2000, Dungan et al. 2002, Rauscher et al. 2002). Indeed, ITS sequence data have been used for species differentiation in groups as diverse as dinoflagellates (Hudson and Adlard 1996, Connell 2001), fungi (Lu et al. 2002, Luo and Mitchell 2002), plants (Baldwin 1992, Baum et al. 1998), coelenterates (Van Oppen et al. 2002), rotifers (Gomez et al. 2002), platyhelminths (Gonzalez et al. 2002, Jousson and Bartoli 2002), insects (Rafferty et al. 2002), and amphibians (Furlong and Maden 1983). In contrast, the small subunit (SSU), 5.8S, and large subunit (LSU) genes, which flank the ITS regions, diverge more slowly during speciation and are more conserved between species (Bargues et al. 2000). Therefore, the probability of finding unique sequences suitable for developing species-specific PCR assays is higher in the ITS region than in the SSU, 5.8S, or LSU genes. This article describes the development, optimization, and use of ITS-based PCR assays to detect *Pfiesteria piscicida* (Steidinger et Burkholder) and five other morphologically similar dinoflagellates in natural phytoplankton assemblages.

We developed these PCR detection methods because the putatively toxic dinoflagellate, *P. piscicida*, co-occurs with numerous other small dinoflagellates that are morphologically indistinguishable using LM techniques. Currently, the only reliable way to morphologically identify *P. piscicida* in environmental samples is

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by using SEM procedures that are both laborious and expensive and not well suited for rapid sample processing (Steidinger et al. 1996, Truby 1997). The inability to distinguish toxic from nontoxic species has potential consequences for adequately protecting public and environmental health. Also, the presence of multiple look-alike species in an assemblage makes understanding the ecology and abundance of any single species difficult. Species-specific PCR assays offer a cost-effective solution for detecting and quantifying harmful algae in phytoplankton assemblages (Ruble et al. 1999, Bowers et al. 2000, Oldach et al. 2000, Guillou et al. 2002) and for elucidating the ecological role of morphologically indistinguishable or “cryptic” species.

MATERIALS AND METHODS

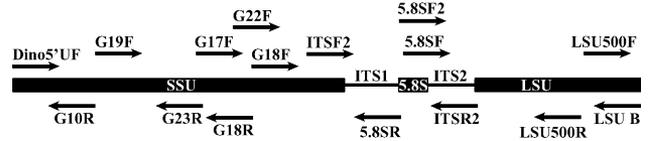
Cultures and growth conditions. The species investigated in this study included *Pfiesteria piscicida*, *P. shumwayae* Glasgow et Burkholder, Florida “Lucy” species, two cryptoperidiniopsoid sp., “PLO21” and “H/V14,” and *Karlodinium micrum* Leadbeater et Dodge (formerly *Gyrodinium/Gymnodinium galatheanum*). The “cryptoperidiniopsoid” and the “Lucy” species have not yet been formally described. However, the Kofoidian plate series characteristics of “cryptoperidiniopsoid” and “Lucy” species are given in Steidinger et al. (2001).

Pfiesteria piscicida, *P. shumwayae*, Florida “Lucy” species, and cryptoperidiniopsoid species “H/V14” and “PLO21” are heterotrophs and were cultured in sterile Gulf Stream water diluted to 15 psu, 23 ± 1° C, 14:10-h light:dark cycle, approximately 50 μmol photons·m⁻²·s⁻¹. *Karlodinium micrum* is a mixotrophic species and was cultured in F/40 at 15–20 psu, 23 ± 1° C, 14:10-h light:dark cycle, approximately 50 μmol photons·m⁻²·s⁻¹. All species were fed a small amount of the prey species *Rhodomonas* sp. (CCMP767) every 2–3 days. Cultures were transferred to new flasks with fresh media every 8–12 days. The autotrophic species, *Karenia brevis* Hansen and Moestrup (= *Gymnodinium breve*), was used during the PCR assay development as a negative control. This species was cultured in F/2 (Guillard and Ryther 1962) or K media (Keller et al. 1987) at 23 ± 1° C, 14:10-h light:dark cycle, approximately 100 μmol photons·m⁻²·s⁻¹, 30 psu. The prey species *Rhodomonas* CCMP767 was grown in F/2-Si, 1-L glass or polycarbonate bottles, 30 psu, 25 ± 0.2° C, 14:10-h light:dark cycle, approximately 80 μmol photons·m⁻²·s⁻¹.

DNA extraction, PCR amplification, and DNA sequencing procedures. Approximately 50 mL of each heterotrophic culture (approximately 1 × 10³ cell·mL⁻¹) were concentrated by filtration onto a 47-mm, 3-μm pore size Nucleopore polycarbonate filter (Whatman, Clifton, NJ, USA). These heterotrophs were allowed to graze down the *Rhodomonas* before harvesting. As controls, 50 mL of log phase *K. brevis* and the *Rhodomonas* strain CCMP767 were similarly filtered onto a 3-μm Nucleopore filter. DNA was extracted from the filters using a DNeasy Tissue Kit following the

TABLE 1. Primers used to amplify and sequence dinoflagellate SSU, ITS1, 5.8S, ITS2, and the first approximately 900 bp of the LSU.

Primer name	Sequence (5'–3')
Forward primers	
Dino5'UF	CAACCTGGTGATCCTGCCAGT
G19F	CATCTAAGGAAGGCAGCAGG
G17F	ATACCGTCTAGTCTTAACC
G22F	TGGTGGAGTGATTGTCTGG
G18F	CAATAACAGGTCTGTGATGC
ITSF2	TACGTCCTGCCCTTTGTAC
5.8SF	CATTGTGAATTGCAGAATTCC
5.8SF2	GTCTCGGCTCGAACAACGATG
LSU500F	GCAAACAAGTACCATGAGGG
Reverse primers	
G10R	CCGGCGCTGCTGGCACCAGAC
G23R	TTCAGCCTTGGCACCATAC
G18R	GCATCACAGACCTGTTATTG
5.8SR	CATCGTTGTTCCGAGCCGAGAC
ITSR2	TCCCTGTTTCATTGCCATTAC
LSU500R	CCCTCATGGTACTTGTGTTGC
LSU B	ACGAACGATTTGCACGCTCAG



manufacturer’s protocol (Qiagen, Valencia, CA, USA). Filters that were not immediately processed were stored at –80° C.

The SSU, ITS1, 5.8S, ITS2, and first 66 bp of the LSU were amplified using the Dino5'UF and ITSR2 primers (Table 1). The amplification reaction mixtures contained 20 mM Tris-HCl, pH 8.4, 3 mM MgCl₂, 50 mM KCl, 25 pmol of each primer, 200 μM of dNTPs, 0.5 units Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Rockville, MD, USA), and 20 ng of genomic DNA in a total volume of 50 μL. The DNA was amplified in a Robocycler (Stratagene, La Jolla, CA, USA) using the following cycling conditions: 2 min at 95° C followed by 35× (30 s at 95° C, 45 s at 60° C, 2.5 min at 72° C) with a final extension of 7 min at 72° C. The primers 5.8SF and LSU-B were used to amplify the region containing the last approximately 100 bp of the 5.8S gene through the first approximately 900 bp of the LSU. The amplification mixture was a 50-μL volume identical to that described above. These reactions were amplified using an initial denaturation of 2 min at 95° C followed by 30× (30 s at 95° C, 40 s at 63° C, 1.25 min at 72° C) with a final extension of 5 min at 72° C. The Dino5'UF and LSU-B primers were capable of amplifying the entire SSU–5' LSU region. However, this primer pair also amplified the *Rhodomonas* CCMP767 DNA equally well. This made it impossible to obtain a single PCR product spanning the entire SSU–LSU gene region that was not contaminated with *Rhodomonas* DNA.

TABLE 2. Species-specific PCR primer sequences. All primers except DinoDUF1 are reverse primers.

Dinoflagellate species	Primer name	Sequences (5'–3')	Size (bp)	10× Buffer
Universal dinoflagellate	DinoDUF1	GTCCCTGCCCTTTGTACA		
<i>Pfiesteria piscicida</i>	PpiDR1	AAATTCACGGTGATGATGAGATTA	212	Invitrogen mix
<i>Karlodinium micrum</i>	KmDR1	AATAGCGATAGCTTCGCAG	323	Invitrogen mix
<i>Pfiesteria shumwayae</i>	PshDR1B	TCCGACAGAGTCCGATGC	257	Optibuffer #4
Florida sp. “Lucy”	FluDR1B	CACACTCCGAGCAAAGAAG	394	Optibuffer #1
Cryptoperidiniopsoid sp. “H/V14”	CbrDR1B	GGCGACGAGAACGTTAAG	272	Optibuffer #10
Cryptoperidiniopsoid sp. “PLO21”	Csp21DR1C	TAATCAAGCTTCCCGAAGG	404	Optibuffer #4

Each reverse primer was used in conjunction with the universal dinoflagellate forward primer. The expected PCR product sizes and optimal 10× buffers are listed for each reverse primer.

A 5- μ L aliquot of each PCR reaction was checked for the presence of a specific amplification product by agarose gel electrophoresis (1% 40 mM Tris-acetate, 1 mM EDTA, pH 8.0 (TAE), 50 V) and ethidium bromide staining. PCR reactions containing specific products were cleaned using the QIAquick PCR purification kit (Qiagen), quantified spectrophotometrically, and sequenced on an ABI377 DNA sequencer using the Deoxy Terminator Cycle sequencing kit (Applied Biosystems – ABI, Foster City, CA, USA). Sequencing was performed following the manufacturer's instructions and using approximately 10 ng of DNA for each 100 bp of template DNA and 25 pmols of primer. DNA templates were sequenced completely in both directions using the primers listed in Table 1. The resulting SSU to 5' LSU sequence for each species was assembled using the Vector NTI program (Informax Inc., Bethesda, MD, USA).

Species-specific PCR assay development. The SSU–5' LSU sequences obtained in this study and other related dinoflagellate sequences available from GenBank were aligned using the CLUSTAL-W algorithm (Thompson et al. 1994) included in the MacVector 7.0 software package (Oxford Molecular Ltd., Oxford, UK). These alignments were used to identify unique ITS sequences and to develop species-specific PCR assays.

Each PCR assay used the conserved forward primer, Dino DUF1, located approximately 150 bp from the 3' end of the SSU (Table 2). Species-specific reverse primer sites in the ITS regions were selected for evaluation (Innis et al. 1999, McPherson et al. 2000). All primer pairs were designed to anneal at 56° C (Table 2). Each PCR reaction contained 25 pmol of each primer, 200 μ M of dNTPs, 0.5 units Platinum *Taq* DNA polymerase (Invitrogen), and approximately 20 ng of genomic DNA in a total volume of 50 μ L. The pH, Tris-HCl, MgCl₂, and KCl concentrations were optimized with a 12 buffer optimization kit (Opti-Prime, Stratagene) (Table 3). Amplifications were carried out in a Robocycler with the following profile: 2 min at 95° C followed by 35 \times (20 s at 95° C, 30 s at 56° C, 45 s at 72° C) with a final extension of 5 min at 72° C. Aliquots (5 μ L) from each amplification were separated on 3% 3:1 Nusieve (BioWhittaker, Walkersville, MD, USA) TAE agarose gels run at 50 V. The size of the PCR products was verified using either a 100- or 123-bp molecular weight ladder (Promega, Madison, WI, USA and Roche, Basel, Switzerland, respectively). After optimization, the primer pairs were tested for possible cross-reactivity with a panel of DNAs, including the other species for which assays were being developed, *K. brevis*, and the *Rhodomonas* sp. used as food for the heterotrophic dinoflagellates. The *Rhodomonas* controls were particularly important because residual DNA from these food species contaminated each of the heterotrophic DNA preparations.

Determining minimal limits of detection for each assay. Water samples were collected from the Neuse River estuary, North Carolina, USA. These samples came from a region of the estuary where the salinity was approximately 15 psu, the same salinity as used to culture each dinoflagellate species. Cells from *P. piscicida* and five PLO species were added to individual Neuse River water samples such that a series of cell concentrations ranging from 1 to 50 cells·mL⁻¹ in a final volume of 100 mL were generated. These spiked samples were gently filtered (<10 cm Hg) through a 47-mm, 3- μ m Nucleopore polycarbonate filter and extracted using the Mo Bio UltraClean soil DNA extraction kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). Blank cell

TABLE 3. Opti-Prime PCR buffers from Stratagene used for optimizing the species-specific PCR assays (Lanes 1–12).

Lane	Buffer no.	pH	[Tris-HCl(mM)]	[MgCl ₂ (mM)]	[KCl(mM)]
1	1	8.3	10	1.5	25
2	2	8.3	10	1.5	75
3	3	8.3	10	3.5	25
4	4	8.3	10	3.5	75
5	5	8.8	10	1.5	25
6	6	8.8	10	1.5	75
7	7	8.8	10	3.5	25
8	8	8.8	10	3.5	75
9	9	9.2	10	1.5	25
10	10	9.2	10	1.5	75
11	11	9.2	10	3.5	25
12	12	9.2	10	3.5	75
13	LT ^a	8.4	20	3.0	50

The Opti-Prime buffers systematically alter pH and the MgCl₂ and KCl concentrations of the PCR reaction mixes. This facilitates rapid PCR optimization. Lane 13, Life Technologies (^aLT) buffer supplied with Platinum *Taq* DNA polymerase.

addition treatments were included to identify contaminating target cells already present in the Neuse River water. Precise *Pfiesteria* or PLO DNA concentrations could not be measured spectrophotometrically due to the presence of other contaminating DNAs from microorganisms in the Neuse River water. We therefore used a 2- μ L volume of extracted DNA per PCR reaction. DNAs from these samples were amplified using the species-specific primers and the reaction conditions described above. The limits of detection were determined using agarose gel electrophoresis and ethidium bromide staining.

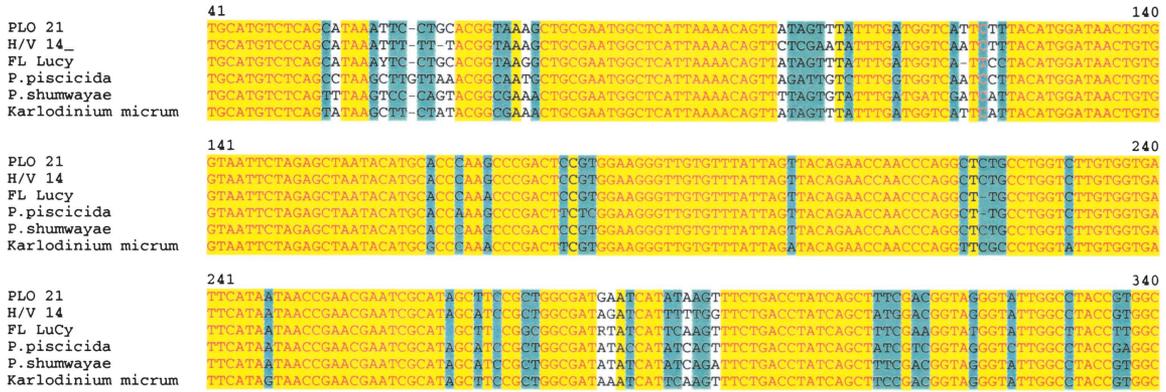
Evaluating DNA carry-over during sample filtration. To test for residual DNA contamination of the filtration units, 100 mL of cultured *P. piscicida* or *P. shumwayae* (100–1000 cells·mL⁻¹) was filtered onto 47-mm, 3- μ m Nucleopore membranes using polyphenylsulfone magnetic filter funnels (Pall Gelman Corp., Ann Arbor, MI, USA), commonly used in field sampling. The filter was removed and genomic DNA was extracted as described above. The genomic DNA was PCR amplified either with the *P. piscicida* primers, PCOLSUF160 and PpLSU450R (Vogelbein et al. 2002), or the *P. shumwayae* primers, PFBSSU paired with either 16S-like primer B (Vogelbein et al. 2001) or PFBITSR201L (Vogelbein et al. 2002). After filtration of the cultured cells, the filter funnel apparatus was rinsed three times with sterile distilled water and autoclaved. A sterile 47-mm, 3- μ m Nucleopore membrane was placed into the autoclaved apparatus, and 100 mL of sterile distilled water was filtered through the funnel. Any residual genomic DNA was extracted from the filter and PCR amplified.

Field trials of species-specific PCR assays. To field test the species-specific PCR assays, samples were collected from seven stations along the length of the St. Johns River estuary, Florida, USA (18 July 2000 to 25 July 2002; see Table 4 for station locations). Surface and bottom water samples were collected at each station (depth permitting) with a discrete water sampler. To remove larger zooplankton, approximately 10 L was passed through a

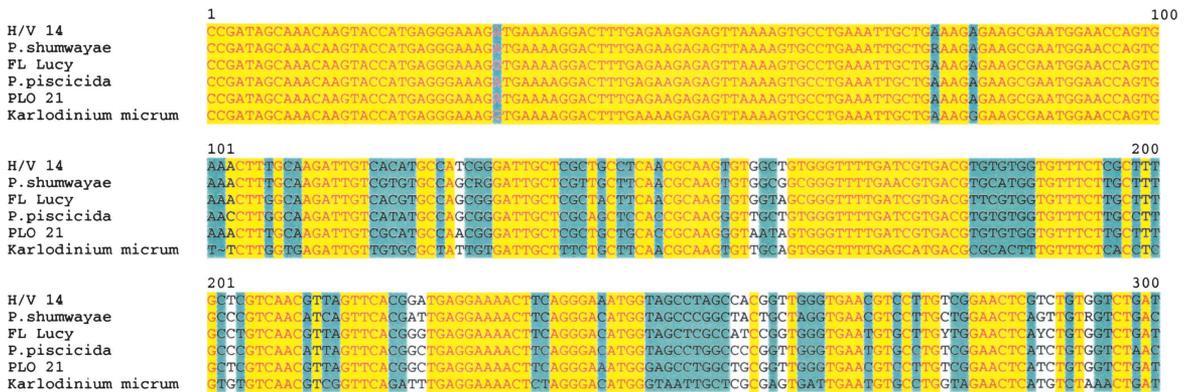
TABLE 4. St. Johns River (SJR) sampling stations and station descriptions.

Station	Latitude	Longitude	Station description
F1	30° 26.166	81° 30.537	Clapboard Creek N of Pelotes Is. ~4 km N of SR105
F2	20° 22.729	81° 32.324	Mill Cove entrance ~1000m S of Channel Marker 43
F3	30° 22.987	81° 33.685	SJR 1/4 mi. W of Dames Point Bridge, 30 m off S shore
F4	30° 23.475	81° 39.311	Trout River 0.8 km W of US 17 off S shore, near North Shore Park
F5	30° 21.574	81° 37.181	SJR off N end of Talleyrand Bulkhead, across river from Jacksonville University
F6	30° 16.611	81° 42.697	Ortega River between US 17 & SR 211, off E end Ortega River Marine docks
F7	30° 9.018	81° 41.894	Doctors Inlet, 100 m on E side of US 17

A. Most Variable SSU Sequence



B. Most Variable LSU Sequence



c. ITS 1 Sequence Alignment

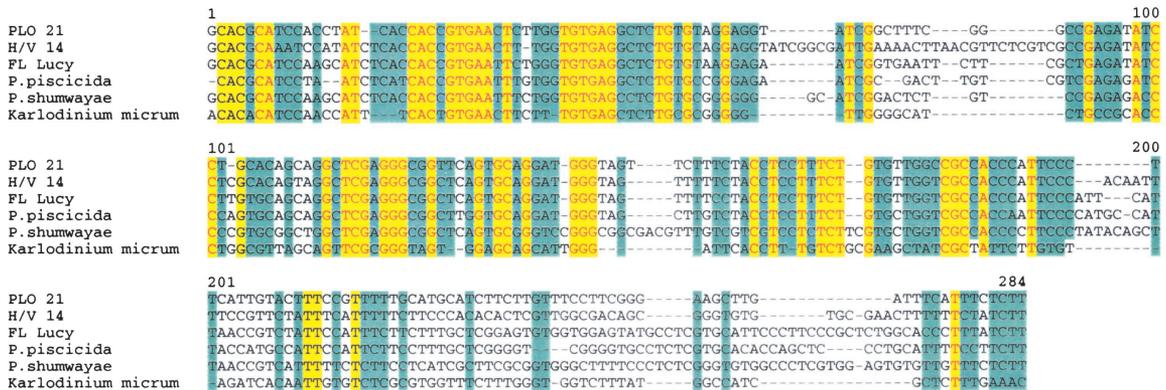


FIG. 1. (A) Sequence alignment showing the most variable 300 bp of the SSU gene for *Pfiesteria piscicida*, *P. shumwayae*, Florida “Lucy” sp., cryptoperidiniopsoid sp. “H/V14,” cryptoperidiniopsoid sp. “PLO21,” and *Karlodinium micrum*. (B) The most variable 300-bp region of the LSU gene for the same six species. It should be noted that this 300-bp section was selected from an approximately 900-bp fragment at the 5’ end of the LSU gene. This region contained several of the more variable domains found in the LSU gene. (C) Sequence alignment of the ITS1 region for the same six species demonstrating the greater sequence variability in ITS1 than in the LSU and SSU genes. Yellow regions indicate homologous sequence shared by all six species. Blue regions indicate sequence where two base pairs are common among the six species. White regions indicate three to four nucleotide differences or significant gaps among the six species. Contiguous variable regions are desirable for designing species-specific primers. GenBank accession numbers for the SSU and LSU sequences were as follows: *P. piscicida*, AY245693; *P. shumwayae*, AY245694; Florida “Lucy,” AY245689; cryptoperidiniopsoid sp. “H/V14,” AY245690; cryptoperidiniopsoid sp. “PLO21,” AY245691; and *Karlodinium micrum*, AY245692. ITS sequences are as follows: *P. piscicida*, AF352333–AF352337; *P. shumwayae*, AF352338–AF352345; Florida “Lucy,” AF352346–AF352348; cryptoperidiniopsoid sp. “H/V14,” AF352349–AF352351; cryptoperidiniopsoid sp. “PLO21,” AF352355–AF352358; and *Karlodinium micrum*, AF352365–AF352367.

153- μm mesh into a plastic carboy. Samples from each station were put in a cooler with ambient water and transported back to the laboratory. Approximately 500 mL of surface and bottom water from each sampling station were measured with a clean graduated cylinder and then poured into a beaker and gently mixed. The pooled sample was next poured through a 30- μm mesh to remove larger plankton and debris. Disposable 100-mL analytical test filter funnels (Nalgene Nunc International, Rochester, NY, USA) were assembled with 47-mm, 3- μm polycarbonate membrane filters. A 100-mL aliquot from each station was vacuum filtered at <10 cm Hg. After filtration, the filter was stored in a cryovial and frozen in liquid N_2 . The samples were stored at -80°C pending PCR assay analysis.

PCR amplification controls for field trials. To assess potential DNA contamination and PCR inhibitors in the extracted field samples, each PCR assay included a positive control, a negative DNA control, two blank extraction controls, and three spiked DNA controls. The positive control contained a known amount target DNA in the PCR reaction mixture and ensured that the PCR reagents were properly assembled and that the DNA *Taq* polymerase was functional. The negative control consisted of adding 1 \times reaction buffer to a subset of reaction mixes to identify contaminated reagents or cross-contamination between samples. The blank extraction controls were incorporated during the DNA extractions of the St. Johns River samples to test for potential DNA contamination during the extraction process. The spiked controls consisted of adding target DNA to a subset of the St. Johns River samples to identify DNA polymerase inhibitors, such as carbohydrate and phenolic (humic) compounds, that are difficult to eliminate during the extraction process (Tebbe and Vahjen 1993, Kreader 1996).

RESULTS AND DISCUSSION

Unique DNA sequences in the ITS regions of *P. piscicida* and five PLO species were used as a basis for species-specific PCR assays. In this study, diagnostic PCR primer pairs were successfully designed to target *P. piscicida*, *P. shumwayae*, cryptoperidiniopsis spp. "H/V14" and "PLO21," Florida "Lucy" sp., and *K. micrum*. The assays were capable of detecting these species in natural assemblages at concentrations of 100 cells per 100 mL sample (1 cell·mL⁻¹).

Sequence alignments showed that the ITS regions were considerably more variable than the surrounding SSU and LSU regions and contained more variable sites suitable for developing species-specific PCR assays (Fig. 1). This does not mean that highly accurate species-specific PCR assays cannot be developed from SSU or LSU sequences. Indeed, SSU-based PCR assays have been successfully developed for *P. piscicida* (Ruble et al. 1999, Bowers et al. 2000, Oldach et al. 2000). Those assays have comparable sensitivities with the assays developed in this study. However, more sequencing is generally required to locate species-specific primer binding sites in the SSU and LSU. The lower divergence rate between species in the SSU and LSU regions also means a greater statistical probability of homologous sequences occurring in related species (Fig. 1). In addition, the ITS sequences are relatively short compared with the SSU and LSU genes, making them more cost effective to sequence. The result is a higher number of unique primer binding sites per base pair sequenced.

Direct DNA sequencing did not reveal any ambiguities in the ITS regions resulting from deletion or substitution events. These data indicated that direct se-

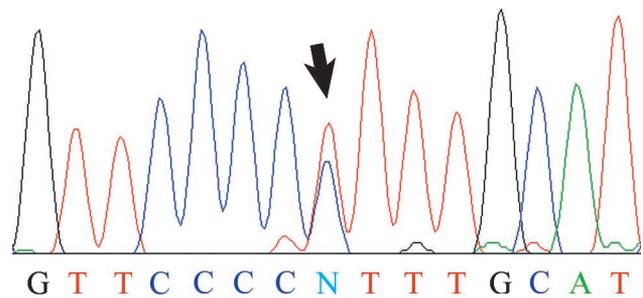


FIG. 2. A sequencing chromatogram of a polymorphism in the ITS2 region of *Pfiesteria shumwayae* (arrow). Sequencing of individually cloned ITS regions from the same single cell isolate has shown that these polymorphisms are authentic.

quencing of dinoflagellate ITS PCR products is feasible. However, polymorphisms at specific base locations were apparent in the sequencing chromatograms (Fig. 2). These polymorphisms were a consequence of the ITS regions being present at multiple locations in the genome and a subset of the ITS copies having acquired mutations. When polymorphisms did occur, one of the peaks was generally dominant. This indicated that a majority of the ITS copies within the genome were identical. To ensure the specificity of the PCR assays, each potential primer binding site was carefully examined to ensure that there were no obvious polymorphisms present at that site. Those primer binding sites containing any polymorphisms were rejected.

We used a conserved forward primer in a fixed location for each assay that we developed. This made it possible to select reverse primers to target either the ITS1 or ITS2 regions such that each species-specific amplification product had a unique length. Each primer pair was tested for cross-reactive PCR amplification using a battery of genomic DNAs from other PLO species, *Rhodomonas* spp., and *K. brevis* to establish their specificity (Fig. 3A). Reverse primers that consistently gave species-specific PCR amplification products (Fig. 3B) when used in combination with the universal SSU forward primer are listed in Table 2. Because DNA polymerases and primer binding are sensitive to pH, Mg^{2+} , and K^+ concentrations, each PCR assay was optimized to determine the best reaction conditions for that assay (Fig. 3C and Table 3). Suboptimal PCR reaction conditions often resulted in nonspecific amplification products and were common in field-extracted DNA samples where Mg^{2+} concentrations and pH differences were sufficient to alter optimal PCR conditions. When nonspecific amplification occurred, we systematically altered the Mg^{2+} or pH to produce specific PCR products, as shown in Figure 3C.

Each PCR assay in this study had a minimum sensitivity level of at least 100 cells in a 100-mL water sample (1 cell·mL⁻¹) (Fig. 4). However, it should be noted that though the ITS regions are excellent for developing DNA based assays, the ITS transcripts do not always make good candidates for developing RNA-based *in situ*

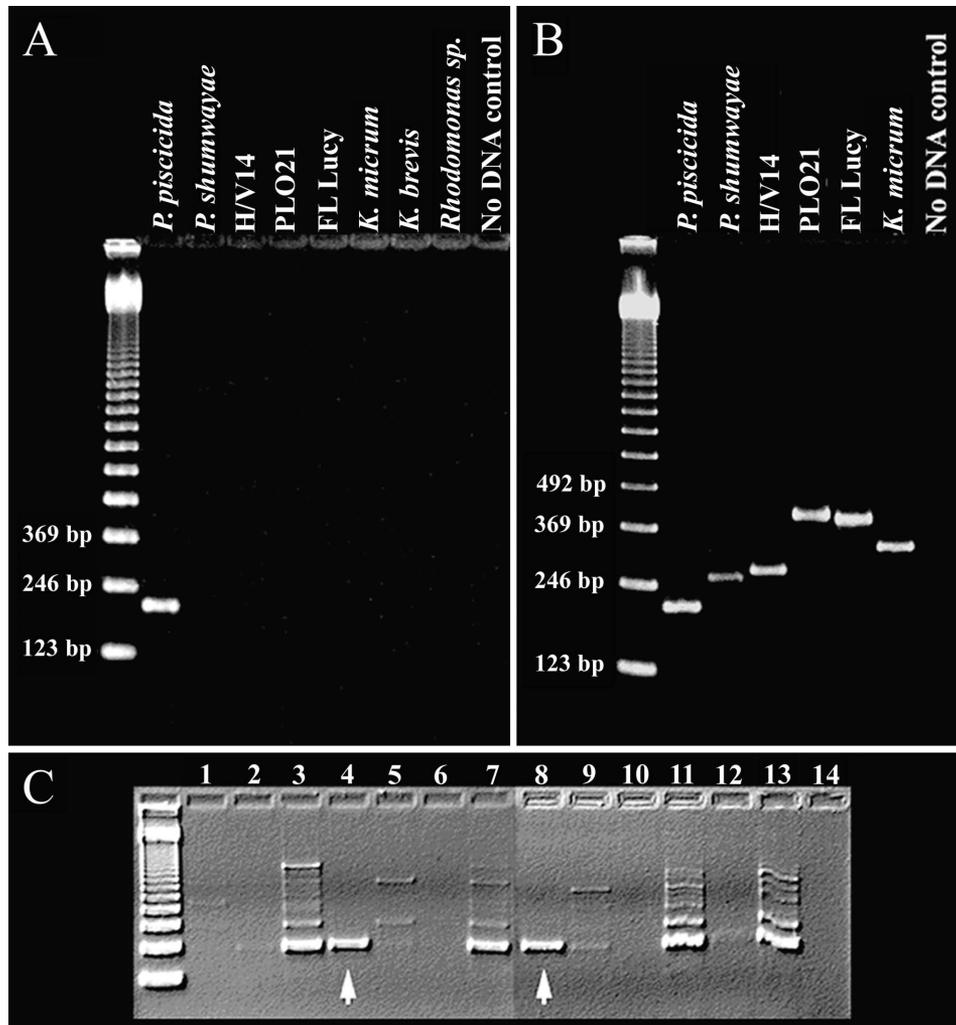


FIG. 3. (A) A *Pfiesteria piscicida* specific PCR assay using genomic DNA from *P. piscicida*, five PLOs, *Karenia brevis*, and *Rhodomonas* CCMP767. DNA from each species of PLO was used to test each species-specific PCR primer set for potential cross-reactivity. (B) The characteristic PCR product sizes generated by each species-specific assay are shown. The optimal PCR buffer conditions used for each assay are listed in Table 2. (C) A typical PCR optimization using *P. shumwayae* DNA showing the changes in fidelity of primer set DinoDUF1/PshDR1B (Table 2). Lanes 1–12 correspond to Stratagene Opti-Prime buffers 1–12 (Table 3). The Tris HCl, pH, MgCl₂, and KCl concentrations for each buffer are listed in Table 3. Lane 13 used the Invitrogen Life Technologies buffer (Table 3). Lane 14, negative DNA control. Arrows indicate reactions that generated a single species-specific PCR product. A 123 bp ladder was used for A, B, and C.

hybridization probes or RT-PCR and sandwich hybridization assays. Many ITS RNA transcripts are degraded immediately after transcription and are not found at high levels in the cell (Cangelosi et al. 1997, Allmang et al. 1999, 2000, Anderson et al. 1999).

Laboratory experiments designed to simulate field-sampling conditions and test for DNA contamination between sampling sites were performed. Repetitive use of a filtration manifold, as is typically used to collect chl *a* samples, resulted in cross-contamination due to carry over of DNA and cells. Rinsing the filtration units with sterile deionized water did not prevent the contamination from occurring. Similarly, autoclaving failed to destroy DNA that was adhered to the walls of the filtration unit. *Pfiesteria piscicida* and *P. shumwayae* DNA extracted from a filtered culture was

successfully PCR amplified as expected. However, subsequent amplification of DNA extracted from sterile distilled water using an autoclaved filtration unit through which *P. shumwayae* cells had previously passed also yielded a faint PCR product of the expected size for *P. shumwayae*. These results indicated that for each sample, disposable filtration units and forceps should be used to minimize the likelihood of cross-contamination during the handling of field-collected samples.

Environmental samples were collected periodically from the St. Johns River, Florida, between 18 July 2000 and 25 July 2002. A small subset of samples (26 and 28 March 2001) was assayed to validate the use of the PLO species-specific primers with field-collected water samples. On these dates, *K. micrum*, *P. piscicida*, and cryptoperidiniopsoid sp. “H/V14” were widely distrib-

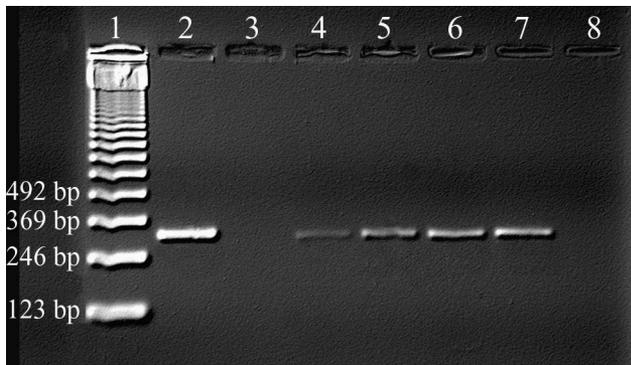


FIG. 4. PCR limits of detection assay using *Karlodinium micrum* cells diluted in 15 psu Neuse River water to simulate field-collected samples. Final concentrations ranged 0 to 50 cells·mL⁻¹. Water samples of 100 mL were filtered and extracted for total DNA. All species-specific primer sets were capable of detecting 100 cells in 100 mL (1 cell·mL⁻¹). Lanes 1–8: 1, 123-bp ladder; 2, positive control; 3, 0 cells·mL⁻¹; 4, 1 cell·mL⁻¹; 5, 5 cells·mL⁻¹; 6, 10 cells·mL⁻¹; 7, 50 cells·mL⁻¹; 8, negative DNA control.

uted in the river system, whereas *P. shumwayae* was not detected at all (Fig. 5). Blank extraction controls were negative, and spiked DNA controls demonstrated that the extracted DNA lacked significant inhibitors. The assays are currently being used to assess the environmental factors associated with the distribution and abundance of these PLO species in the St. Johns River.

This study showed that species-specific PCR assays based on unique ITS sequences could be developed for dinoflagellate species. Targeting the ITS was advantageous because, though relatively short and economical to sequence, this region contained a significant number of species-specific primer binding sites. ITS sequence data reduced the time and money needed to develop new species-specific PCR assays relative to sequencing SSU or LSU regions. As reported for other dinoflagellate species (Adachi et al. 1994, 1995, 1997, Hudson and Adlard 1996, Baillie et al. 2000), the ITS region of PLO dinoflagellates enabled us to distinguish closely related species. Sequencing the ITS region therefore provided important taxon information at the same time it allowed identification of unique primer binding sites for species-specific PCR assay development. This taxonomic information may prove useful, particularly in uncharacterized dinoflagellate groups. ITS sequence data will also likely prove useful for developing species-specific PCR assays in other algal groups.

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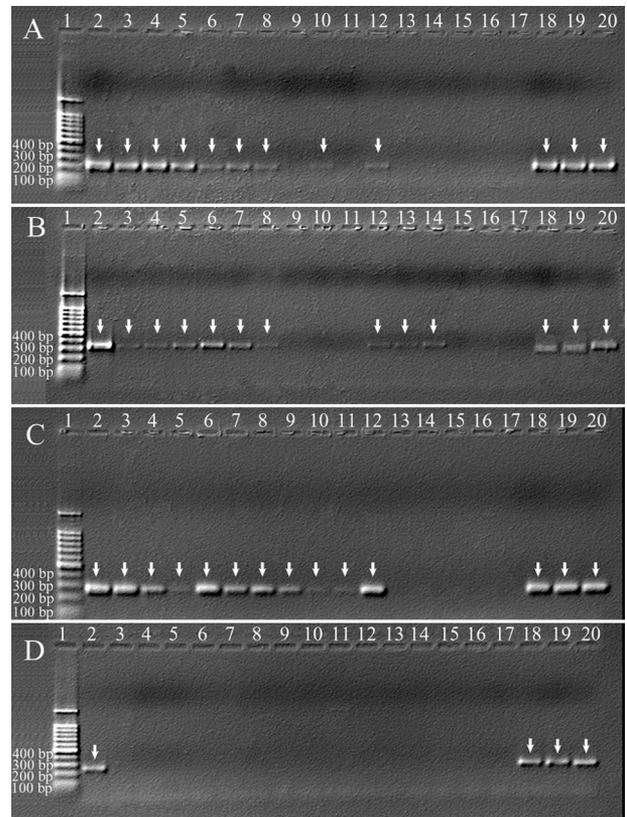


FIG. 5. Validation of species-specific PCR assays using environmental samples. Representative samples from seven stations along the St. Johns River, Florida, USA, were extracted and assayed. Arrows indicate positive PCR products. (A) *Pfiesteria piscicida* specific primers. (B) *Karlodinium micrum* specific primers. (C) cryptoperidiniopsisoid sp. "H/V14" specific primers. (D) *Pfiesteria shumwayae* specific primers; this species was not detected in the St. Johns River samples. For all gel images (A–D): Lanes: 1, 100-bp ladder; 2, positive control; 3–14, St. Johns River samples; 15, negative DNA control; 16–17, blank DNA extractions; 18–20, spiked DNA controls.

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