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GENETIC VARIATION AMONG STRAINS OF *PSEUDOPFIESTERIA SHUMWAYAE* AND *PFIESTERIA PISCICIDA* (DINOPHYCEAE)[1]

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The putatively toxic dinoflagellates *Pseudopfiesteria shumwayae* (Glasgow et J. M. Burkh.) Litaker, Steid., P. L. Mason, Shields et P. A. Tester and *Pfiesteria piscicida* Steid. et J. M. Burkh. have been implicated in massive fish kills and of having negative impacts on human health along the mid-Atlantic seaboard of the USA. Considerable debate still remains as to the mechanisms responsible for fish mortality (toxicity vs. micropredation) caused by these dinoflagellates. Genetic differences among these cultures have not been adequately investigated and may account for or correlate with phenotypic variability among strains within each species. Genetic variation among strains of *Ps. shumwayae* and *P. piscicida* was examined by PCR–RFLP analysis using cultures obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), as well as those from our own and other colleagues' collection efforts. Examination of restriction digest banding profiles for 22 strains of *Ps. shumwayae* revealed the presence of 10 polymorphic restriction endonuclease sites within the first and second internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene of the rDNA complex, and the cytochrome oxidase subunit I (COI) gene. Three compound genotypes were represented within the 22 *Ps. shumwayae* strains. Conversely, PCR–RFLP examination of 14 strains of *P. piscicida* at the same ITS1, 5.8S, and ITS2 regions revealed only one variable restriction endonuclease site, located in the ITS1 region. In addition, a dinoflagellate culture listed as *P. piscicida* (CCMP 1928) and analyzed as part of this study was identified as closely related to *Luciella masanensis* P. L. Mason, H. J. Jeong, Litaker, Reece et Steid.

Key index words: **COI; genetic variation; PCR–RFLP; *Pfiesteria piscicida*; *Pseudopfiesteria shumwayae*; ribosomal genes**

Abbreviations: **BLAST, basic local alignment search tool; bp, base pair; CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; COI, cytochrome oxidase subunit I; CYTB, cytochrome b; DGGE, denaturing gradient gel electrophoresis; *Exo* I, Exonuclease I; ITS, internal transcribed spacer; NCBI, National Center for Biotechnology Information; PLDs, *Pfiesteria*–like dinoflagellates; SAP, shrimp alkaline phosphatase; SNP, single nucleotide polymorphism; VIMS, Virginia Institute of Marine Science**

P. shumwayae and *P. piscicida* are estuarine dinoflagellates that have been associated with fish-kill events and presumptive impacts on human health within several estuarine systems along the eastern seaboard of the United States (Burkholder et al. 1992, 1995, Glasgow et al. 1995, Steidinger et al. 1996, Burkholder and Glasgow 1997, Grattan et al. 1998). Their distribution appears to be worldwide (Jakobsen et al. 2002, Rhodes et al. 2002, 2006, Rublee et al. 2005, Park et al. 2007a,b). The field of research on *Ps. shumwayae* and *P. piscicida* has been beset by considerable debate on two fundamental properties of these organisms, the mechanism(s) responsible for fish mortality (dinoflagellate toxicity vs. micropredation) (Burkholder et al. 1992, Burkholder and Glasgow 1997, Burkholder 1999, Vogelbein et al. 2002, Lovko et al. 2003) and the capacity for stage transformations during the life cycle (Burkholder and Glasgow 1997, 2002, Litaker et al. 2002a,b, Peglar et al. 2004). Although these organisms have been documented and discussed at length in the literature, strain variability in *Ps. shumwayae* and *P. piscicida* has rarely been considered.

A diverse capacity for toxin expression (Tox-A, Tox-B, and noninducible) has been reported, and toxigenicity in some strains is suggested to be regulated by nutritional status of the culture (Burkholder et al. 2001a,b). However, the definition for toxicity remains ambiguous, and many strains have not been tested adequately. In addition, variations in micropredation behavior among species and strains of *Ps. shumwayae* and *P. piscicida* have been observed (Lovko et al. 2003). It is evident from a review of the literature that several research groups have conducted experiments with different strains of *Ps. shumwayae* and *P. piscicida* and have reported different modes of pathogenicity; this highlights the

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need for genetic characterization of these strains to interpret and compare experimental results more accurately.

Strain variation is a common feature in many algal species (Scholin and Anderson 1996, Bolch et al. 1999), especially with regard to toxin expression (Cembella et al. 2002, Wang et al. 2005) and genetic variation (Bolch et al. 1999, Evans et al. 2004, Gomez and Gonzalez 2004, Martínez et al. 2006). Scholin et al. (1994) and Higman et al. (2001) reported that toxic and nontoxic species of *Pseudonitzschia* and strains of *Alexandrium tamarense* were distinguishable by analysis of rDNA sequences. Nucleotide polymorphisms in the ITS regions (and 5.8S rDNA genes) of both *Ps. shumwayae* and *P. piscicida* have been recorded previously (Litaker et al. 2003, 2007). In contrast, Tengs et al. (2003) observed no differences in the ITS1 and ITS2 regions, and the 5.8S gene, among isolates of *P. piscicida*, while Marshall et al. (2006) reported finding no differences in the 5.8S rDNA gene between several strains of *Ps. shumwayae*, and a 1 bp difference between isolates of *P. piscicida*.

In the current study, we describe the analysis of 22 strains of *Ps. shumwayae* and 14 strains of *P. piscicida* by PCR–RFLP analysis of the ITS1, 5.8S gene, and ITS2 rDNA regions, and the cytochrome oxidase subunit I (COI) gene. Our objective was to develop a simple method to genotype *Ps. shumwayae* and *P. piscicida* isolates and examine whether genetic differences exist between geographically separate isolates.

MATERIALS AND METHODS

Cultures. Clonal cultures of *Ps. shumwayae* and *P. piscicida* were obtained from the CCMP and through our own and other colleagues' sampling efforts. The cells were cultured in sterile Gulf Stream water diluted to 15 psu, at 23°C under a 14:10 light:dark (L:D) cycle. All species were fed small amounts of *Rhodomonas* sp. (CCMP 767) every 2–3 d. Prior to filtering the cells for DNA extraction, the dinoflagellates were allowed to graze down the *Rhodomonas* to minimize the DNA obtained from the food source and maximize capture of *Ps. shumwayae* and *P. piscicida* cells.

DNA extraction. Aliquots of 20 mL of each culture ($\sim 2 \times 10^3$ cells $\cdot$ mL^{-1}) were filtered onto a 3 μm Nucleopore (Costar, Cambridge, MA, USA) filter to concentrate the cells. As a control for PCR amplifications, 15 mL of *Rhodomonas* sp. (CCMP 767) was also filtered (as above). Genomic DNA was extracted from the filters using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Purified DNA was eluted into 50 μL of the supplied extraction buffer, quantified using a Hoefer DyNA Quant 200 fluorometer (Pharmacia Biotech Inc., Piscataway, NJ, USA) and stored at −20°C.

PCR amplification. Several sets of primers were designed (Table 1) based on DNA sequences available in our laboratory database and in GenBank to target the rDNA regions (ITS1/5.8S/ITS2), the COI gene, and other regions of *Ps. shumwayae* and *P. piscicida* genomes, such as the cytochrome b (CYTB) gene of *P. piscicida* (see Results and Discussion). The amplification reactions contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 2.5 μM of each primer, 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA, USA), 0.4 mg $\cdot$ mL^{-1} BSA, and 30–50 ng genomic DNA in a total volume of 20 μL. The following cycling conditions were used for each locus: an initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 30 s at the annealing temperature (varied with primers used, see Table 1), 72°C for 90 s, and a final extension step of 72°C for 5 min. To check for uniform amplification across all samples, 5 μL aliquots of the amplified products were visualized by agarose gel electrophoresis (2% w/v), stained with ethidium bromide, and viewed under a UV light source. Amplification products selected for sequence analysis were excised from the agarose gels using a sterile scalpel blade and purified using a Qiaquick Gel Extraction Kit (Qiagen).

Sequencing of amplification products. Amplification products from a number of primary PCRs were cloned and sequenced to confirm that the correct target was amplified. Cloning and sequencing were performed according to methods described previously (Small et al. 2007). Briefly, purified PCR products were cloned into the plasmid pCR®4-TOPO® (Invitrogen, Carlsbad, CA, USA) and transformed into competent *Escherichia coli* using a TOPO TA Cloning® Kit (Invitrogen) following the manufacturer's protocols. Transformed bacterial colonies were screened for inserts using a PCR-based screening reaction using M13 primers (M13F 5′-GTAAAACGACGGCCAG-3′ and M13R 5′-CAGGAAACAGCTATGAC-3′). Aliquots of 3 μL of all PCR products were analyzed on an agarose gel as

TABLE 1. *Pseudopfiesteria shumwayae* and *Pfiesteria piscicida* primer sequences, annealing temperatures, and restriction endonucleases from informative regions.

Species	Region	Primer name	Primer sequence 5′–3′	Annealing temp. (°C)	PCR product size (bp)	Polymorphic restriction enzyme sites
Ps. shumwayae	ITS1	PsRFLPITS1F1	ACGCATCCAACCATCTCACCAC	60	316	*Nci* I, *Hpy*CH4 IV,
		PsRFLPITS1R1	GCTGTGCCCTTCATCGTTGTTC			*Bmg* BI, *Bst* UI, *Bsl* I
	5.8 S	PsRFLP5.8SF1	CGTGGAGTGTGTTGTTTCTC	68	314	*Bst* UI
		PsRFLP5.8SR1	CACCACCCCGAAAGAGAGGA			
	ITS2	PsRFLPITS2F2	CTTTCTCTGAGCCTGTCTCGGAGGATCTG	66	306	*Bsl* I, *Bsm* I
		PsRFLPITS2R2	GTCTTGGGGGCTGAGAGGCGAGCTCTC			
		PsRFLPITS2F3	GAACAACGATGAAGGGCACAGC	66	311	*Sph* I
		PsRFLPITS2R3	AATGCGTCAAAGGCTCCAACCC			
	CO1	PsRFLPCox1F1	GCTCTTCTTTTTAGTAATGCCTGG	65	816	*Dra* I
		PsRFLPCox1R1	TTGAGCAGTAACAATGCCG			
P. piscicida	ITS1	PpRFLPITS1F1	ACGCATCCTAATCTCATCACCG	60	374	*Nla* IV
		PpRFLPITS1R1	CCCGAAACCGCATTTTACG			
	ITS2	PpRFLPITS2F1	CGTAAAATGCGGTTTCG	60	365	*Bbs* I (CCMP 1928)
		PpRFLPITS2R1	TTAGTTTCTTTTCCTCCGC			

described above, and products from clones containing the correct insert size were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo* I) (Amersham Biosciences, Piscataway, NJ, USA) prior to sequencing. PCR fragments were bidirectionally sequenced using the Big Dye Terminator kit (Applied Biosystems) with M13 sequencing primers and one-eighth the recommended concentration of Big Dye. Aliquots of 10 μL of each sequencing reaction product were electrophoretically separated on an ABI 3100 Genetic Analyzer (Applied Biosystems), and sequences were imported into Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) for trimming of vector sequences. Consensus sequences were aligned using the CLUSTAL-W program in the MacVector sequence analysis package (MacVector Inc., Cary, NC, USA).

RFLP analysis. Following PCR, aliquots of 2.5 to 5 μL of the amplified products were screened with up to 30 restriction endonucleases to identify enzymes that demonstrated intraspecific polymorphisms for each region. Digested products were visualized by agarose gel electrophoresis (2% w/v), stained with ethidium bromide, and viewed under a UV light source. Gel images were recorded using an Alpha Innotech (Alpha Innotech Corporation, San Leandro, CA, USA) gel documentation system. Fragment lengths were compared with each other and with a size standard (50 bp ladder; Sigma Inc., St. Louis, MO, USA) to assess polymorphisms. Several of the clonal cultures were found to have two different ITS region RFLP profiles, which is consistent with previous observations of polymorphisms among multiple copies of the rRNA gene complex (Litaker et al. 2003). Therefore, the ITS and 5.8S gene genotypes were given two letter designations (i.e., AA, AB, or BB). Amplifications and digestions were carried out in triplicate to ensure reproducibility of the results.

Estimation of genetic relatedness. Composite genotypes for each strain were assigned based on the RFLP patterns as visualized by agarose gel electrophoresis. Genetic relationships among the 22 strains of *Ps. shumwayae* were assessed by neighbor-joining analysis based on the total number of pairwise character differences using PAUP (Swofford 2000).

Confirmation of culture identity. Species-specific PCR assays for *P. piscicida* were performed according to the methods of Vogelbein et al. (2001) to confirm culture identity. In addition, the 3′ end of the ITS1 region, the 5.8S rRNA gene, and the ITS2 region from CCMP 1928 (listed as *P. piscicida*) were amplified and sequenced to confirm culture identity (see Results and Discussion). A 20 mL culture of CCMP 1928 (direct from CCMP, without our own subculturing efforts) was filtered onto a 3 μm Nucleopore polycarbonate filter, and DNA extracted as described above. The amplification reactions contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.5 mM of each dNTP, 2.5 μM of each primer (PpRFLPITS1F1 and PpRFLPITS2R1, see Table 1), 0.2 U · μL^{-1} *Taq* polymerase, 0.4 mg · mL^{-1} BSA, and 1 μL of DNA in a total volume of 20 μL. Amplifications were performed with an initial denaturation of 94°C for 4 min, followed by 40 cycles at 94°C for 1 min, 58.5°C for 1 min, 72°C for 90 s, with a final elongation at 72°C for 5 min. Following amplification, 15 μL of PCR product was analyzed by agarose gel electrophoresis (2%), stained with ethidium bromide, and visualized under UV light. The ~800 bp fragment was excised from the agarose gel using a sterile scalpel blade and purified using a Qiaquick gel extraction kit (Qiagen). Cloning and sequencing were performed according to the methods described above. Six clones were sequenced, and the data deposited in GenBank under the following accession numbers (GQ478043–GQ478048). Sequence similarity searches of the National Center for Biotechnology Information (NCBI) database were carried out using the basic local alignment search tool (BLAST) (Altschul et al. 1990).

RESULTS

Ps. shumwayae. Polymorphisms in the ITS1 and ITS2 regions and 5.8S gene of *Ps. shumwayae* were identified in silico (i.e., virtually using the sequence analysis software MacVector) based on sequences available in GenBank and the Virginia Institute of Marine Science (VIMS) database (Litaker et al. 2003). These three regions were successfully amplified from the 22 strains, and the polymorphisms confirmed experimentally by RFLP analysis (see Fig. 1, A–B, for examples). A polymorphism in the *Ps. shumwayae* COI gene was also identified (Fig. 1C). Genotypes were assigned to each of the strains based on banding patterns (Table 2). Three compound genotypes were detected within the 22 strains of *Ps. shumwayae*, and neighbor-joining analysis was conducted to examine the relatedness of the three genotypes (Fig. 3). Endonuclease digestion profiles of the strains CCMP 2359 and 2360, both isolated from the Neuse River in North Carolina, were identical (genotype 1) and were similar to two other strains (genotype 2); one that had been isolated from the Tasman Bay, New Zealand (CCMP 2360), and another from a commercial fish tank in Gloucester Point, Virginia (VIMS VA1) (7/10 identical restriction digest profiles). The remaining strains of *Ps. shumwayae* from the Pamlico River in North Carolina (Ps-VIMS 1047-1066, NOGA 3-24, PATS B, and CCMP 2089, $n = 17$) and a single isolate from Chincoteague Bay in Maryland (CCMP 2359) all had the same composite genotype (genotype 3) and were distinct from strains represented by genotypes 1 (1/10 identical restriction digest profiles) and genotype 2 (0/10 identical restriction digest profiles).

P. piscicida. The sequence of *P. piscicida* strains was conserved over the ITS1 and ITS2, and 5.8S rDNA regions; however, a single polymorphic site in the ITS1 was identified and confirmed by RFLP analysis (Fig. 1D and Table 3). No COI gene sequence variation was observed among *P. piscicida* strains. Attempts were made to amplify the mitochondrial CYTB gene in the *P. piscicida* strains using previously published primers (PPCOB3F and PPCOB3R) and amplification conditions (Zhang and Lin 2002). The primers repeatedly failed to produce any amplification product when using DNA templates from CCMP 2354. Because these primers did not amplify all *P. piscicida* strains, RFLP analysis of the CYTB gene region was not investigated further.

PCR–RFLP results for *P. piscicida* CCMP 1928 targeting the ITS1 and ITS2 regions indicated that this strain was subtly different from the other *P. piscicida* cultures examined. Differences were observed in the length of the PCR amplication product and the RFLP banding patterns (Fig. 2). Differences were also observed by PCR–RFLP for the 5.8S rRNA gene and the COI gene (data not shown). To

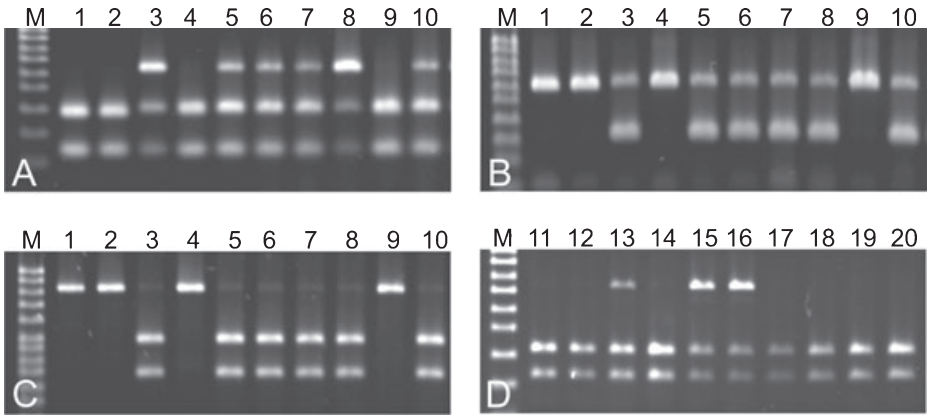

FIG. 1. PCR–RFLP analysis of *Pseudopfiesteria shumwayae* (A, B, C) and *Pfiesteria piscicida* (D). (A) ITS1 region digested with the restriction endonuclease *Nci* I. (B) ITS2 region digested with the restriction endonuclease *Sph* I. (C) COI gene digested with the restriction endonuclease *Dra* I. (D) ITS1 region digested with the restriction endonuclease *Nla* IV. Lane 1, CCMP 2357; lane 2, CCMP 2358; lane 3, CCMP 2359; lane 4, CCMP 2360; lane 5, NOGA 3; lane 6, NOGA 9; lane 7, NOGA 17; lane 8, NOGA 24; lane 9, VIMS VA1; lane 10, PATS B; lane 11, CCMP 1830; lane 12, CCMP 1831; lane 13, CCMP 1834; lane 14, CCMP 1901; lane 15, CCMP 1921; lane 16, CCMP 2091; lane 17, CCMP 2354; lane 18, CCMP 2361; lane 19, CCMP 2362; lane 20, CCMP 2363. Lane M, 50 bp molecular weight marker. ITS, internal transcribed spacer.

TABLE 2. *Pseudopfiesteria shumwayae* genotypes resulting from PCR–RFLP analysis of genomic DNA amplified from 22 clonal cultures.

Sample name	Geographic source	ITS1A *Nci* I	ITS1B *Hpy* CH4 IV	ITS1C *Bmg* BI	ITS1D *Bst* UI	ITS1E *Bsl* I	5.8S *Bst* UI	ITS2A *Bsl* I	ITS2B *Bsm* I	ITS2C *Sph* I	CO1 *Dra* I
CCMP 2357	Neuse River, NC	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA
CCMP 2358	Neuse River, NC	BB	BB	AB	BB	BB	AA	BB	AA	AA	AA
CCMP 2359	Chincoteague Bay, MD	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
CCMP 2360	Tasman Bay, N.Z.	BB	BB	AB	BB	AB	AA	AB	AB	AA	AA
VIMS 1047	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1048	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
CCMP 2089	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1057	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1058	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1060	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1061	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1062	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1063	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1064	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1065	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS 1066	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
NOGA 3	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
NOGA 9	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
NOGA 17	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
NOGA 24	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
PATS B	Pamlico River, NC	AB	AB	AA	AB	AA	AB	AA	AA	AB	BB
VIMS VA1	Gloucester Point, VA	BB	BB	AB	BB	AB	AA	AB	AB	AA	AA

validate our results, CCMP 1928 was reordered, and DNA was extracted directly from the isolate without additional culturing. PCR–RFLP results for the ITS1 and ITS2 regions were again different from the other *P. piscicida* cultures, confirming the previous findings. Sequencing and BLAST analysis of an rDNA region PCR fragment encompassing the 3′ end of the ITS1, the 5.8S gene, and the ITS2 from this dinoflagellate revealed a 99% similarity to *L. masanensis* ribotype 1 (Mason et al. 2007).

DISCUSSION

This study represents the first detailed genetic characterization of the differences among strains of *Ps. shumwayae* and *P. piscicida* isolated from different geographic locations. Sequence divergence between the 14 strains of *P. piscicida* was limited, with only a single variable restriction endonuclease site identified. In comparison, 10 polymorphic restriction endonuclease sites were identified in the 22 strains of *Ps. shumwayae* analyzed, giving rise to three

TABLE 3. *Pfiesteria piscicida* genotypes resulting from PCR–RFLP analysis of genomic DNA amplified from 14 clonal cultures.

Sample name	Geographic source	ITS1 *Nla* IV
CCMP 1830	Chicamacomico River, MD	BB
CCMP 1831	Chicamacomico River, MD	BB
CCMP 1834	Pokomoke River, MD	AB
CCMP 1901	Chicamacomico River, MD	BB
CCMP 1902	Chicamacomico River, MD	BB
CCMP 1921	Chicamacomico River, MD	AB
CCMP 2091	Raleigh, NC	AB
CCMP 2354	Chincoteague Bay, MD	BB
CCMP 2361	Newport River NC	BB
CCMP 2362	Neuse River, NC	BB
CCMP 2363	Calibogue Sound, SC	BB
CCMP 2423	Neuse River, NC	BB
VIMS NOGA 02	Raleigh, NC	AB
VIMS P11	Gloucester Point, VA	BB

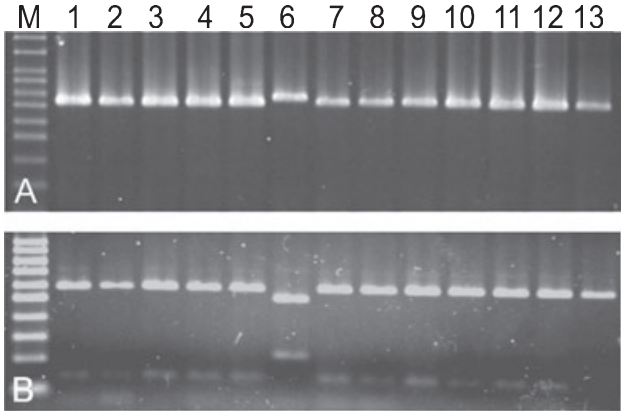

FIG. 2. PCR and RFLP analysis of *Pfiesteria piscicida* and CCMP 1928. (A) PCR of ITS1 region. (B) RFLP of the ITS2 region. Lane 1, CCMP 1830; lane 2, CCMP 1831; lane 3, CCMP 1834; lane 4, CCMP 1901; lane 5, CCMP 1921; lane 6, CCMP 1928; lane 7, CCMP 2091; lane 8, CCMP 2354; lane 9, CCMP 2361; lane 10, CCMP 2362; lane 11, CCMP 2363; lane 12, CCMP 2423; lane 13, VIMS-P11. Lane M, 50 bp molecular weight marker. Note the different length of amplification product (A) and RFLP banding pattern (B) in lane 6 (CCMP 1928).

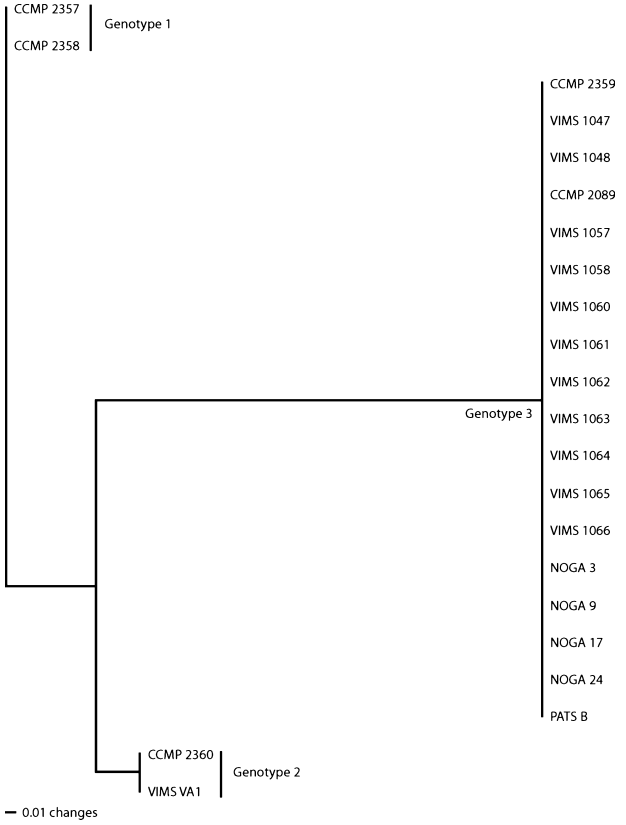

FIG. 3. Neighbor-joining analysis illustrating the genetic relationships among the 22 strains of *Pseudopfiesteria shumwayae* analyzed.

compound genotypes demonstrating structure in the geographic distribution of the strains. The primers used in this study amplified the correct target in all strains tested and, in combination with RFLP analysis will be a useful resource to investigate *Ps. shumwayae* and *P. piscicida* genotypes in field and laboratory samples. In addition, a *P. piscicida* culture (CCMP 1928) analyzed as part of this study was identified as a *Luciella* sp., most likely *L. masanensis.*

The ITS1 and ITS2 regions that exist in between the SSU, 5.8S, and LSU rRNA genes are suitable targets for DNA-based species/strain identifications. Though transcribed, these spacer regions do not code for functional gene products; therefore, they may diverge considerably during speciation. These regions have previously been used as targets for species-specific diagnostic assays for *Ps. shumwayae* and *P. piscicida,* and *Pfiesteria*–like dinoflagellates (PLDs) (Litaker et al. 2003) and have recently been used in the identification of a wide range of dinoflagellates (Litaker et al. 2007). Coyne et al. (2001) reported finding distinct ''strains'' of *P. piscicida* in water and sediment samples using denaturing gradient gel electrophoresis (DGGE) analysis of a 311 bp fragment of the SSU gene; however, it remains unclear whether this variation (1 or 2 bp over 311 bp) was due to single nucleotide polymorphisms (SNPs) in the rDNA repeat regions or from nonspecific amplifications via the use of a general eukaryotic forward primer. In contrast, Tengs et al. (2003) reported finding no differences in the DNA sequences of the SSU and 5.8S genes, nor in the ITS1 and ITS2 regions of 21 isolates of *P. piscicida* from North Carolina and Maryland, including purported isolates varying in toxicity (i.e., Tox-A, Tox-B, and noninducible). In addition, Marshall et al. (2006) reported finding limited intraspecific genetic variability between 10 strains of *P. piscicida* (1 bp difference in the 5.8S rRNA gene); however, they also reported finding no differences between nine *Ps. shumwayae* strains at this same locus, including several CCMP strains analyzed in the current study. The present study confirmed previous findings of the conserved nature of the rDNA

regions in *P. piscicida* (Tengs et al. 2003, Park et al. 2007a,b) and also indicated sequence conservation of the COI gene; however, a polymorphic site in the ITS1 region was identified and confirmed by RFLP analysis. Restriction digest profiles of strains of *P. piscicida* showed no significant structure in geographic distribution and indicated that although a RFLP was identified, the rRNA locus of *P. piscicida* is highly conserved and that other targets will be required to assist in distinguishing among genetic strains.

In contrast to *P. piscicida*, nine RFLPs were identified within the ITS1-5.8S-ITS2 regions of the 22 strains of *Ps. shumwayae* analyzed (Table 2). In combination with the RFLP identified in the COI gene, this resulted in the identification of three genotypes (1, 2, and 3, see Fig. 3) represented within the strains analyzed. The genetic distance analysis indicated that the Neuse River (genotype 1) and Gloucester Point/Tazman Bay strains (genotype 2) were similar, while the Pamlico River/Chincoteague Bay strains (genotype 3) were very different from both the Gloucester Point/Tazman Bay genotype and the Neuse River genotype. Species of *Ps. shumwayae* and *P. piscicida* are thought to have a global distribution, and in addition to being present in waters of several states along the East Coast of the USA, they have been identified in northern Europe (Jakobsen et al. 2002), in New Zealand (Rhodes et al. 2002, 2006), and more recently from ballast water originating from Indonesia (Park et al. 2007a), and in the Antarctic (Park et al. 2007b). It is surprising that given the close proximity of the Pamlico and Neuse rivers to one another in North Carolina (both flow into the Pamlico estuary), two considerably different genotypes were found exclusively in samples from either location. The concept of different populations occupying a similar geographic location is not new, as Kim et al. (2004) reported finding morphologically similar, yet genetically distinct, populations of *Peridinium limbatum* in neighboring lakes. In this example, however, the lakes sampled were completely separated, which is not the case for the Neuse and Pamlico rivers. Bolch et al. (1999) also observed clustering of *Gymnodinium catenatum* isolates into estuarine groups. The discovery of different *Ps. shumwayae* strains occupying geographically proximate areas suggests that genetic exchange is limited between these areas, and that the different genotypes may represent distinct populations. Bolch et al. (1999) suggested that for *G. catenatum*, blooms may arise within estuaries from benthic cysts, undergo cyst formation, and resettle in the same areas, effectively isolating estuarine blooms from those of neighboring estuaries. We believe that such a scenario may explain what is happening for *Ps. shumwayae* in the North Carolina estuaries; however, further analysis of additional samples from multiple locations is required to definitively address this discovery.

It is also surprising that the *Ps. shumwayae* strain isolated from a commercial fish tank for sheephead minnows (*Cyprinodon variegates*) in Gloucester Point, Virginia (VIMS VA1, isolated in 2001), and the strain from Tasman Bay, New Zealand (CCMP 2360, also isolated in 2001), had identical RFLP profiles. It is unlikely that the two strains evolved to have identical genomes (as assessed by PCR–RFLP) and therefore may represent an example of the global transport of *Ps. shumwayae* and *P. piscicida* by ballast water, as has been suggested by Doblin et al. (2004) and Park et al. (2007a). This finding may also be the case for why a strain of *Ps. shumwayae* from Chincoteague Bay, Maryland (CCMP 2359), was identical to all of the Pamlico River strains of *Ps. shumwayae* from North Carolina. Further support for this speculative hypothesis comes from Drake et al. (2007) who document substantial numbers of commercial vessels arriving at the Port of Hampton Roads (encompassing terminals in the lower Chesapeake Bay cities of Norfolk, Newport News, Portsmouth, and Chesapeake, Virginia) and suggest that a high level of microorganisms is present in ballast water discharged, greatly increasing the chance for unintentional transport of exotic organisms to and from the Chesapeake Bay. However, the sheephead fish tank in Gloucester Point was situated entirely indoors, in isolation from external water sources. Alternative scenarios include the unintentional transport of *Ps. shumwayae* cells with experimental fish or shellfish, or cross-contamination when held in scientific laboratories. Despite the circumstantial evidence for dispersal routes, the probable source population of this strain remains unclear. Further efforts are underway to identify genetic differences in *Ps. shumwayae* strains by additional molecular analyses, and these may shed new light on the geographic distribution and origin of the different strains.

Some *Ps. shumwayae* strains were polymorphic at several of the rDNA region restriction sites. Both *Ps. shumwayae* and *P. piscicida* are known to have multiple copies of the rDNA region in their genomes with a small subset acquiring mutations (Litaker et al. 2003, 2007). The variations observed in the *Ps. shumwayae* rRNA gene complex are likely explained by SNPs in repeated copies of these regions, resulting in a common ITS region sequence in addition to less frequent variant copies. This explanation is supported by the finding that in the presumably single-copy mitochondrial COI gene sequence, only a single allele was observed (Fig. 1D and Table 2). Alternatively, the ploidy of the cultures could be diploid, and at the polymorphic sites, the tandemly repeated copies of rRNA gene complex could represent one variant, while on the other chromosome, there could be multiple copies of the other variant. However, current evidence suggests a haplontic life cycle for *Ps. shumwayae* and *P. piscicida* (Litaker et al. 2002a, Parrow and Burkholder 2003,

2004). Interestingly, genotype 1 (from the Neuse River) was found to be monomorphic for 8/9 rDNA region polymorphic sites, whereas genotype 2 (from Tasman Bay/Gloucester Point) was monomorphic at 5/9 rDNA region polymorphic sites, and genotype 3 (from the Pamlico River/Chincoteague Bay) was monomorphic at 4/9 rDNA polymorphic sites. The reasons for this remain unclear, but it does indicate that there is a dominant ITS rRNA (ITS1-5.8S-ITS2) ''type'' present in the Neuse River samples.

The purported ''noninducible'' strains of *Ps. shumwayae* that are publicly available via the CCMP (2327, 2358, 2359, and 2360) had different composite genotypes. Conversely, strains with the same composite genotype (CCMP 2359 and 2089) are reported as having different toxicity profiles (noninducible and Tox-B, respectively) (Burkholder et al. 2005). RFLP analysis of the rDNA regions and others (COI/CYTB) is not expected to discriminate between toxic and nontoxic strains, as the molecular machinery responsible for toxin production, if it exists, is unlikely to be linked with these gene profiles. The future characterization of *Ps. shumwayae* strains by PCR–RFLP would allow for ''genotypic anchoring'' of strains that may allow for more accurate comparison of research results. It would have been interesting to analyze so-called Tox-A strains by PCR-RFLP; however, these were not, and are still not, publicly available.

Significantly, we were unable to amplify a partial fragment of the mitochondrial CYTB gene from all strains of *P. piscicida* using the diagnostic primers PPCOB3F and PPCOB3R (Zhang and Lin 2002), and our primers designed to amplify a smaller fragment (data not shown). Both sets of primers failed to produce any amplification product using DNA from Pp-MD7 (CCMP 2354). The reasons for this remain unclear, as the above isolate produced intense single-reaction products from species-specific PCR to confirm culture identity and from all other *P. piscicida* primers developed in this study. There may be polymorphisms in the CYTB primer binding sites, but this is unlikely given that the same isolates failed to amplify using either of the CYTB-targeted primer sets. Regardless of the mechanism, this finding may indicate that Pp-MD7 (CCMP 2354) is not a ''true'' *P. piscicida* isolate and may be another PLD, or that the *P. piscicida* diagnostic assay of Zhang and Lin (2002) is not inclusive for all strains, and it may cross-react with *L. masanensis* (see below). Further molecular and EM studies are needed to address this discovery.

During the course of this study, it was also discovered that a *P. piscicida* culture (CCMP 1928) had subtly different PCR amplicon lengths and RFLP digestion profiles when compared to the other strains of *P. piscicida*. Zhang and Lin (2002) had previously detected CCMP 1928 using both SSU and CYTB-targeted primers designed to detect *P. piscicida*; however, subsequent species-specific PCR tests carried out in this study confirmed that this culture as we obtained it from CCMP was not a species of *P. piscicida*, and analysis of an rDNA fragment encompassing the ITS1-5.8S-ITS2 regions indicated that this dinoflagellate culture was closely related (99% similarity) to *L. masanensis* ribotype 1 (Mason et al. 2007). Further support for this misidentification comes from Sullivan and Andersen (2001) who noted that CCMP1928 had a noticeably higher salinity tolerance than six other *P. piscicida* isolates. *L. masanensis* resembles *P. piscicida* in size and shape and would be easily misidentified using standard LM techniques. We recommend that future *Ps. shumwayae* and *P. piscicida* spp. and PLD submissions to CCMP are accompanied by EM and molecular analysis to confirm identification.

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Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–10.

Bolch, C. J. S., Blackburn, S. J., Hallegraeff, G. M. & Vaillancourt, R. E. 1999. Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol.* 35:356–67.

Burkholder, J. M. 1999. The lurking perils of *Pfiesteria*. *Sci. Am.* 281:42–9.

Burkholder, J. M. & Glasgow Jr., H. B. 1997. *Pfiesteria piscicida* and other toxic *Pfiesteria*-like dinoflagellates: behaviour, impacts, and environmental controls. *Limnol. Oceanogr.* 42:1052–75.

Burkholder, J. M. & Glasgow, H. B. 2002. The life cycle and toxicity of *Pfiesteria piscicida* revisited. *J. Phycol.* 38:1261–7.

Burkholder, J. M., Glasgow, H. B. & Deamer-Melia, N. J. 2001b. Overview and present status of the toxic *Pfiesteria* complex (Dinophyceae). *Phycologia* 40:186–214.

Burkholder, J. M., Glasgow, H. B., Deamer-Melia, N. J., Springer, J., Parrow, M. W., Zhang, C. & Cancellieri, P. J. 2001a. Species of the toxic *Pfiesteria* complex, and the importance of functional type in data interpretation. *Environ. Health Perspect.* 109:667–79.

Burkholder, J. M., Glasgow Jr., H. B. & Hobbs, C. W. 1995. Distribution and environ-mental conditions for fish kills linked to a toxic ambush-predator dinoflagellate. *Mar. Ecol. Prog. Ser.* 124:43–61.

Burkholder, J. M., Gordon, A. S., Moeller, P. D., Law, J. M., Coyne, K. J., Lewitus, A. J., Ramsdell, J. S., et al. 2005. Demonstration of toxicity to fish and to mammalian cells by *Pfiesteria* species: comparison of assay methods and strains. *Proc. Natl. Acad. Sci. U. S. A.* 102:3471–6.

Burkholder, J. M., Noga, E. J., Hobs, C. W., Glasgow Jr., H. B. & Smith, S. A. 1992. New phantom dinoflagellate is the causative agent of major estuarine fish kills. *Nature* 358:407–10.

Cembella, A. D., Quilliama, M. A., Lewisa, N. I., Baudera, A. G., Dell'Aversanoa, C., Thomasa, K., Jellett, J. & Cusack, R. R. 2002. The toxigenic marine dinoflagellate *Alexandrium tamarense* as the probable cause of mortality of caged salmon in Nova Scotia. *Harmful Algae* 1:313–25.

Coyne, K. J., Hutchins, D. A., Hare, C. E. & Cary, S. C. 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. *Aquat. Microb. Ecol.* 24:275–85.

Doblin, M. A., Drake, L. A., Coyne, K. J., Rublee, P. A. & Dobbs, F. C. 2004. *Pfiesteria* species identified in ships' ballast water and residuals: a possible vector for introductions to coastal areas. *In* Steidinger, K. A., Landsberg, J. H., Tomas, C. R. & Vargo, G. A. [Eds.] *Harmful Algae 2002.* Fl. Fish and Wildlife Cons. Comm., Fl. Inst. Oceanogr., and Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg, Florida, pp. 317–9.

Drake, L. A., Doblin, M. A. & Dobbs, F. C. 2007. Potential microbial bioinvasions via ships' ballast water, sediment, and biofilm. *Mar. Pollut. Bull.* 55:333–41.

Evans, K. M., Bates, S. S., Medlin, L. K. & Hayes, P. K. 2004. Microsatellite marker development and genetic variation in the toxic marine diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae). *J. Phycol.* 40:911–20.

Glasgow Jr., H. B., Burkholder, J. M., Schmechel, D. E., Tester, P. A. & Rublee, P. A. 1995. Insidious effects of a toxic estuarine dinoflagellate on fish survival and human health. *J. Toxicol. Environ. Health* 46:501–22.

Gomez, P. I. & Gonzalez, M. A. 2004. Genetic variation among seven strains of *Dunaliella salina* (Chlorophyta) with industrial potential, based on RAPD banding patterns and on nuclear ITS rDNA sequences. *Aquaculture* 233:149–62.

Grattan, L. M., Oldach, D., Perl, T. M., Lowitt, M. H., Matuszak, D. L., Dickson, C., Parrott, C., et al. 1998. Learning and memory difficulties after environmental exposure to waterways containing toxin producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. *Lancet* 352:532–9.

Higman, W. H., Stone, D. M. & Lewis, J. M. 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. *Phycologia* 40:256–62.

Jakobsen, K. S., Tengs, T., Vatne, A., Bowers, H. A., Oldach, D. W., Burkholder, J. M., Glasgow, H. B., Rublee, P. A. & Klaveness, D. 2002. Discovery of the toxic dinoflagellate, *Pfiesteria*, from northern European waters. *Proc. R. Soc. Lond. B* 269:211–4.

Kim, E., Wilcox, L., Graham, L. & Graham, J. 2004. Genetically distinct populations of the dinoflagellate *Peridinium limbatum* in neighbouring northern Wisconsin lakes. *Microb. Ecol.* 48:521–7.

Litaker, R. W., Vandersea, M. W., Kibler, S. R., Madden, V. J., Noga, E. J. & Tester, P. A. 2002a. Life cycle of the heterotrophic dinoflagellate *Pfiesteria piscicida* (Dinophyceae). *J. Phycol.* 38:442–63.

Litaker, R. W., Vandersea, M. W., Kibler, S. R., Madden, V. J., Noga, E. J. & Tester, P. A. 2002b. Reply to comment on the life cycle and toxicity of *Pfiesteria piscicida* revisited. *J. Phycol.* 38:1268–72.

Litaker, R. W., Vandersea, M. W., Kibler, S. R., Reece, K. S., Stokes, N. A., Steidinger, K. A., Millie, D. F., Bendis, B. J., Pigg, R. J. & Tester, P. A. 2003. Identification of *Pfiesteria piscicida* (Dinophycae) and *Pfiesteria*-like organisms using internal transcribed spacer-specific PCR assays *J. Phycol.* 39:754–61.

Litaker, R. W., Vandersea, M. W., Reece, K. S., Stokes, N. A., Yonish, B. A., Kibler, S. R., Black, M. N. D., Steidinger, K. A., Lutzoni, F. M. & Tester, P. A. 2007. Recognizing dinoflagellate species using ITS rDNA sequences. *J. Phycol.* 43:344–55.

Lovko, V. J., Vogelbein, W. K., Shields, J. D., Haas, L. W. & Reece, K. S. 2003. A new larval fish bioassay for testing the pathogenicity of *Pfiesteria* spp. (Dinophyceae). *J. Phycol.* 39:600–9.

Marshall, H. G., Hargraves, P. E., Burkholder, J. M., Parrow, M. W., Elbrachter, M., Allen, E. H., Knowlton, V. M., et al. 2006. Taxonomy of *Pfiesteria* (Dinophyceae). *Harmful Algae* 5:481–96.

Martínez, R., Añíbarro, C. & Fernández, S. 2006. Genetic variability among *Alexandrium tamarense* and *Alexandrium minutum* strains studied by RAPD banding pattern analysis. *Harmful Algae* 5:599–607.

Mason, P. L., Litaker, R. W., Jeong, H. J., Ha, J. H., Reece, K. S., Vogelbein, W. K., Stokes, N. A., et al. 2007. Description of a new genus of *Pfiesteria*-like dinoflagellate, *Luciella* gen. nov. (Dinophyceae), including two new species: *Luciella masanensis* sp. nov. and *Luciella atlantis* sp. nov. *J. Phycol.* 43:799–810.

Park, T. G., Bell, E. M., Pearce, I., Rublee, P. A., Bolch, C. J. S. & Hallegraeff, G. M. 2007b. Detection of a novel ecotype of *Pfiesteria piscicida* (Dinophyceae) in an Antarctic saline lake by real-time PCR. *Polar Biol.* 30:843–8.

Park, T. G., Bolch, C. J. S. & Hallegraeff, G. M. 2007a. Morphological and molecular genetic characterization of *Cryptoperidiniopsis brodyi* (Dinophyceae) from Australian-wide isolates. *Harmful Algae* 6:718–33.

Parrow, M. W. & Burkholder, J. M. 2003. Reproduction and sexuality in *Pfiesteria shumwayae* (Dinophyceae). *J. Phycol.* 39:697–711.

Parrow, M. W. & Burkholder, J. M. 2004. The sexual life cycles of *Pfiesteria piscicida* and cryptoperidiniopsoids (Dinophyceae). *J. Phycol.* 40:664–73.

Peglar, M. T., Nerad, T. A., Anderson, O. R. & Gillevet, P. M. 2004. Identification of amoebae implicated in the life cycle of *Pfiesteria* and *Pfiesteria*-like dinoflagellates. *J. Eukaryot. Microbiol.* 51:542–52.

Rhodes, L. L., Adamson, J. E., Rublee, P. A. & Schaefer, E. F. 2006. Geographic distribution of *Pfiesteria* spp. (Pfiesteriaceae) in Tasman Bay and Canterbury, New Zealand (2002–2003). *N. Z. J. Mar. Freshw. Res.* 40:211–20.

Rhodes, L. L., Burkholder, J. M., Glasgow, H., Rublee, P. A., Allen, C. & Adamson, J. E. 2002. *Pfiesteria shumwayae* (Pfiesteriaceae) in New Zealand. *N. Z. J. Mar. Freshw. Res.* 36:621–30.

Rublee, P. A., Remington, D. L., Schaefer, E. F. & Marshall, M. M. 2005. Detection of the dinozoans *Pfiesteria piscicida* and *P. shumwayae*: a review of detection methods and geographic distribution. *J. Eukaryot. Microbiol.* 52:83–9.

Scholin, C. A. & Anderson, D. M. 1996. LSU rDNA-based RFLP assays for discriminating species and strains of *Alexandrium* (Dinophyceae). *J. Phycol.* 32:1022–35.

Scholin, C. A., Villac, M. C., Buck, K. R., Krupp, J. M., Powers, D. A., Fryxell, G. A. & Chavez, F. P. 1994. Ribosomal DNA sequences discriminate among toxic and non-toxic *Pseudonitzschia* species. *Nat. Toxins* 2:152–65.

Small, H. J., Shields, J. D., Moss, J. A. & Reece, K. S. 2007. Conservation in the first internal transcribed spacer region (ITS1) in *Hematodinium* species infecting crustacean hosts found in the United Kingdom and Newfoundland. *Dis. Aquat. Org.* 75:251–8.

Steidinger, K. A., Burkholder, J. A., Glasgow, H. B., Truby, E. W., Garrett, J. K., Noga, E. J. & Smith, S. A. 1996. *Pfiesteria piscicda* gen. et. sp. nov. (Pfiesteriaceae, fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. *J. Phycol.* 32:157–64.

Sullivan, B. E. & Andersen, R. A. 2001. Salinity tolerances of 62 strains of *Pfiesteria* and *Pfiesteria*-like heterotrophic dinoflagellate (Dinophyceae). *Phycol. Res.* 49:207–14.

Swofford, D. L. 2000. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4.0b4a. Sinauer Associates, Sunderland, Massachusetts.

Tengs, T., Bowers, H. A., Glasgow, H. B., Burkholder, J. M. & Oldach, D. W. 2003. Identical ribosomal DNA sequence data from *Pfiesteria piscicida* (Dinophyceae) isolates with different toxicity phenotypes. *Environ. Res.* 93:88–91.

Vogelbein, W. K., Lovko, V. J., Shields, J. D., Reece, K. S., Mason, P. L., Haas, L. W. & Walker, C. C. 2002. *Pfiesteria shumwayae* kills fish by micropredation not exotoxin secretion. *Nature* 418:67–9.

Vogelbein, W. K., Shields, J. D., Haas, L. W., Reece, K. S. & Zwerner, D. E. 2001. Skin ulcers in estuarine fishes: a comparative pathological evaluation of wild and laboratory-exposed fish. *Environ. Health Perspect.* 109:687–93.

Wang, D. Z., Jiang, T. J. & Hsieh, D. P. H. 2005. Toxin composition variations in cultures of *Alexandrium* species isolated from the coastal waters of southern China. *Harmful Algae* 4:109–21.

Zhang, H. & Lin, S. 2002. Detection and quantification of *Pfiesteria piscicida* by using the mitochondrial cytochrome *b* gene. *Appl. Environ. Microbiol.* 68:989–94.