Detection of the Dinozoans Pfiesteria piscicida and P. shumwayae: A Review of Detection Methods and Geographic Distribution

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Abstract:
Molecular methods, including conventional PCR, real-time PCR, denaturing gradient gel electrophoresis, fluorescent fragment detection PCR, and fluorescent in situ hybridization, have all been developed for use in identifying and studying the distribution of the toxic dinoflagellates *Pfiesteria* piscicida and *P. shumwayae*. Application of the methods has demonstrated a worldwide distribution of both species and provided insight into their environmental tolerance range and temporal changes in distribution. Genetic variability among geographic locations generally appears low in rDNA genes, and detection of the organisms in ballast water is consistent with rapid dispersal or high gene flow among populations, but additional sequence data are needed to verify this hypothesis. The rapid development and application of these tools serves as a model for study of other microbial taxa and provides a basis for future development of tools that can simultaneously detect multiple targets.
Key Words: Dinoflagellates, harmful algae, molecular methods.

Article:

SINCE it was first discovered in 1988, *Pfiesteria piscicida*, and its sister species, *P. shumwayae*, have been the focus of in-tense research and frequent controversy (c.f. Burkholder and Glasgow 2002; Burkholder et al. 2005; Samet et al. 2001). The controversial aspects have arisen at least in part because *Pfiesteria* is an “unconventional” organism, difficult to identify and culture, with a complex life cycle, complex nutrition, and complex interactions with other organisms, including fish and mammals (c.f. Burkholder, Glasgow, and Deamer-Melia 2001a; Grattan et al. 1998; Levin et al. 2003). Additionally, because of its response to anthropogenically introduced nutrients and its potential impacts on marine resources and human health, it has maintained a high public as well as scientific visibility.

Studies in the past decade suggest that *Pfiesteria* is a cosmopolitan organism with a worldwide distribution (Jakobsen et al. 2002; Rhodes et al. 2002, unpubl. data; Rublee et al. 1999, 2001, 2002, 2004). If this is the case, then one might ask why was *Pfiesteria* only recognized in the late 1980s, despite a long history of biological research in coastal areas? The answer lies primarily in the fact that *Pfiesteria* spp. are small cryptic estuarine dinoflagellates that look very much like many other estuarine dinoflagellate species, and secondarily in the ephemeral nature of its occurrence in estuarine systems. Indeed, the discovery of these organisms has stimulated much research directed toward the understanding of other small, potentially harmful dinoflagellates in coastal areas, and the discovery of other, as yet incompletely identified, estuarine species (c.f. Litaker, Scholin, and Vasta 1999; Parrow and Burkholder 2003).

Initial discovery and subsequent detection of *P. piscicida* relied on microscopic and culturing techniques (Burkholder, Glasgow, and Hobbs 1995). Definitive identification relied on SEM observation of the thecal plate structure (Glasgow et al. 2001a; Steidinger et al. 1996, 2001). For identification of *Pfiesteria* in environmental samples, a three-step process started with light microscope observation of a water sample. If a sufficient

population of cells (≥ 300 cells/liter) that looked and behaved like *Pfiesteria* (*Pfiesteria*-like organisms or PLOs) was observed, then a sample of water and/or sediment was introduced into a bioassay chamber with fish prey under a specific set of conditions (Burkholder et al. 2001b). If fish died within 3 wk, then the third step in the identification, examination of thecal plate structure using SEM, was undertaken. Initially, only cultures that manifested fish death in the bioassays were carried through the entire process and ultimately identified as *Pfiesteria* if their plate structure matched.

Light microscope and histochemical methods have been used to look for the presence of *Pfiesteria* cells at the sites of fish lesions and in fish guts in laboratory experiments (Burkholder, Glasgow, and Hobbs 1995; Vogelbein et al. 2001). These methods demonstrated that individual *Pfiesteria* cells are associated with physical attack and histopathologies of affected larval finfish, but the methods are not species specific and therefore cannot be applied in environmental samples where dinoflagellates with similar morphologies may be common.

Since the "gold standard" identification method was labor intensive and took weeks for identification, alternative procedures (Table 1) which bypassed the bioassay stage were desirable— especially in light of the potential health risks associated with exposure to Pfiesteria. The first of these consisted of detection of long (≈1,500–1,700 bp; Rublee et al. 1999) or short (≈200 bp; Oldach et al. 2000) fragments of SSU rRNA genes amplified by PCR. The PCR primers were developed in collaborative studies based on sequence determination from bona fide *P. piscicida* cultures (GenBank Accession number AF077055). These approaches were validated on genomic DNA extracted from cultures, whole water samples associated with fish kills in Maryland in 1997, and genomic DNA isolated from a fish kill in North Carolina in 1998, as well as archived samples from historical fish kills. These methods reduced the analytical time to 1–2 d, dependent on the DNA extraction method used prior to PCR amplification. This was soon followed by development of real-time PCR protocols (Bowers et al. 2000), which reduced the turn around time of sample analysis even more. When combined with commercial DNA extraction protocols (Qiagen, or PureGene), the time for analysis of water samples could be as little as 2–3 h from the time a sample arrived at the analytical laboratory. The real-time PCR protocol is highly sensitive because of the use of species-specific primers, fluorescent probes, and the amplification of short fragments. This approach has become a method of choice for screening large numbers of samples. An additional advantage of real-time PCR is that it can be at least semi-quantitative (c.f. Bowers et al. 2000).

Table 1. *Pfiesteria* spp. detection methods.

Method	Target	Sensitivity/Throughput	Uses	References
Bioassay/SEM	Whole cell	High/Low	Detect in sed/water	Burkholder, Glasgow, and
	Toxin	High/Low	Detection of toxicity	Deamer-Melia (2001a);
				Burkholder et al. (2001b, c)
"Direct" PCR	SSU rDNA	Moderate-High/High	Detect in sed/water	Rublee et al. (1999)
	SSU rDNA			Oldach et al. (2000)
	rDNA-NTS			Saito et al. (2002)
	cytb			Zhang and Lin (2002)
	rDNA-ITS			Litaker et al. (2003)
HMA assay	SSU rDNA	High/Moderate	Test clonal cultures	Oldach et al. (2000)
FISH	SSU rDNA	High/Moderate	Visualize whole cells	Allen (2000); Kempton (1999)
	SSU rDNA			Glasgow et al. (2001b)
	SSU rDNA			Vogelbein et al. (2001)
	SSU rDNA			Litaker et al. (2002)
Real-time PCR	SSU rDNA	High/High (semi-quantiative)	Detect in sed/water	Bowers et al. (2000)
DGGE	SSU rDNA	High/Moderate (Determines strain variation)	Detect in sed/water	Coyne et al. (2001)
PCR-FFD	SSU rDNA	Very high/high	Detect in sed/water	Coyne et al. (2001)

Molecular assays of environmental samples of dinoflagellates are potentially very difficult, because there are several potential sources of error. First, rapid collection and stabilization of cellular components (e.g. nucleases) are essential to prevent degradation of the target molecules. A second challenge lies in the efficiency of extraction and purification of the nucleic acid from the field sample. Many dinoflagellates, including *Pfiesteria* spp., have varied life stages including amoeboid, vegetative, and cyst forms (Burkholder and Glasgow 2002; Burkholder et al. (2001 a, c; 2005) Pfiester and Popovský 1979). Thus, extraction buffers may not be equally efficient at lysing different forms of the target species. Further, if the extraction and subsequent purification of target molecules is incomplete, impurities may interfere with PCR amplification. Finally, there may be biases in

the PCR reaction itself. Overlying each of these procedural concerns is the fact that the genome size and therefore the amount of DNA in dinoflagellates can be quite variable, especially if maintained in long-term culture (c.f. Holt and Pfiester 1982; Parrow and Burkholder 2002).

All molecular assays utilize target nucleic acid derived from cultures or clones as a positive control in reactions in order to as-sure that the reagents have not degraded. If the quantity of the positive control is known, the strength of that signal provides an approximate comparison for the strength of the signal in the environmental sample. Beyond this, several strategies have been used to estimate the quantity (or number of targets) in the starting material. The simplest approaches use known quantities of the target material as spiked internal standards (e.g. Coyne et al. 2001) or in a dilution series for comparison with the strength of signal in the sample (e.g. Bowers et al. 2000). If such standards are added as purified target DNA during the sample extraction protocol or only in the reaction assay, then it generally gives a good indication of the signal strength in the reaction, but does not adequately account for extraction efficiency. Even if cultured whole cells are used as an internal standard by addition to the raw sample, extraction efficiency may not be adequately assessed since cultured whole cells may not represent the mix of morphological forms present in the environmental sample and since the genome size of the cultured cells may not be equivalent to that of the naturally occurring population (c.f. Parrow and Burkholder 2002). An additional approach has been competitive PCR (e.g. Saito et al. 2002). In this method, a plasmid is added to the PCR reaction which has identical primer sites as the target (i.e. a competitor in the reaction) but which generates an amplicon of a different size than that of the target. Assaying the relative amount of the two products produced over a range of proportional additions of the competitor allows estimation of the sensitivity of the amplification and a quantitative estimation of the number of targets in the starting material.

Since the initial development of PCR primers to the SSU rDNA target, a number of authors have developed primers to other parts of the rDNA gene. These include primers directed at NTS (Saito et al. 2002) and ITS (Litaker, Scholin, and Vasta 1999) regions. Additionally, Zhang and Lin (2002) developed PCR primers to the cytochrome b gene. These developments, especially the latter are most welcome, as they provide longer sequences and a second gene that can be exploited for phylogenetic comparisons.

Oldach et al. (2000) also utilized a heteroduplex mobility assay (HMA) for detection of *Pfiesteria* species. This method relies on duplicate PCR reactions of a known “driver” genome and the unknown sample to be tested using primers at a broad taxonomic level. In practice, Oldach et al. (2000) used primers that amplified all (or nearly all) dinoflagellates, and the driver genome was *Gymnodinium sanguineum* DNA. After the initial PCR reaction, reaction products are mixed in a 1 : 1 ratio, are denatured by warming the DNA, and then the mixture is cooled to allow strands to reanneal. Reannealed DNA fragment can be of three types: driver–driver, sample–sample, or driver–sample. If the driver sequence and the sample sequence are identical, then each type of reannealed fragment is actually a homoduplex, and when run out on an acrylamide gel they all migrate at the same rate forming a single band. If the driver and sample are different, then the double-stranded fragments (two homoduplexes and one hetroduplex) will migrate at different rates and a characteristic “signature” will show up on the gel. This approach is ideal for determination of the purity of cultures (c.f. Fig. 3 in Oldach et al. 2000) and has been especially useful in validating the purity and identity of cultures in collections, such as the CCMP. It can also be used for detection of the characteristic signature in field samples, but since the number of additional heteroduplex and homoduplex bands increases arithmetically with each additional source of DNA (within the taxon amplified by the primers) it rapidly becomes difficult to sort out or even see the signature bands in complex samples.

Fluorescent in situ hybridization has also been used as a means of detecting *Pfiesteria* species in both cultures and environmental samples. Kempton (1999) and Allen (2000) used fluorescein-conjugated PCR probes to assess cultures and environmental samples. They found the procedure to be sensitive, but that all steps in the process—fixation, membrane permeabilization, and hybridization—had to be carefully monitored for good results, and that it did not seem amenable to rapidly screen environmental samples. Burkholder et al. (2001a) used different alexafluor dyes in conjunction with PCR probes to discriminate between the two *Pfiesteria*

species and a related cryptoperidiniopsoid dinozoan. An as yet unrealized goal of this work was to be able to isolate cells from natural samples by fluorescent cell sorting, but the complex mixture of organic and inorganic materials in natural sample interferes with the specificity of binding only to target cells. Recently, Litaker et al. (2002) have developed PNA-based fluorescent probes, which have high sensitivity and specificity. The advantage of FISH approaches is the direct observation of target cells, but significant drawbacks are that it is labor intensive, requires careful attention to hybridization conditions to minimize non-specific binding, and can be extremely difficult to apply in environmental samples.

Coyne et al. (2001) adapted denaturing gradient gel electrophoresis (DGGE) for detection of *P. piscicida* in natural estuarine samples. In this approach, short taxon-specific fragments are amplified from genomic DNA extracted from natural samples, and the products separated by electrophoresis on a gel, which finely discriminates between nearly identical fragments due to the urea concentration gradient in the gel. Utilizing this method, Coyne et al. (2001) not only found *P. piscicida* widely distributed throughout Delaware and Maryland estuaries, but also found at least five different variants of the 311-bp amplified region of the small subunit rDNA (Genbank Accession numbers AF363585– AF363589). They attributed this to allelic variation, but it is not clear whether this variation is among strains or lies within the genome of individuals organisms, or both, since there are multiple copies of the SSU in the genome.

Fluorescent fragment PCR (FFG-PCR) was also developed for *Pfiesteria* detection by Coyne et al. (2001). This method is very sensitive, and it indicated a very high incidence of *Pfiesteria* in mid-Atlantic coastal waters during 1999 (up to 90% positive hits in samples tested during the first year of application). Frequency of occurrence in subsequent years has been lower, but still indicative of widespread endemic *Pfiesteria* populations.

Several alternative detection methods have been proposed, but have not yet reached fruition. These include an electrochemical approach (Litaker et al. 2001), which although promising, has not developed sufficient sensitivity. The use of antibodies and lectins as cell surface markers (c.f. Scholin et al. 2003) has also been suggested during a workshop on *Pfiesteria* detection in 1999 (Litaker et al. 1999), but it appears that the successful development and ease of using PCR approaches discouraged development of these approaches.

APPLICATION OF METHODS

The detection methods described have been useful as part of risk assessments related to fish kill events, and as a research tool to determine distribution patterns of *Pfiesteria* species. The primary purpose for development of the molecular methods was to aid in determining if *Pfiesteria* species were causative agents of fish kills and if there was a risk of human exposure. By 1997–1999, the potential for human health impact had been realized (Glasgow et al. 1995; Grattan et al. 1998), but definitive methods for identifying *Pfiesteria* as the causative agent of an event had not been established. Indeed, until a specific field test for *Pfiesteria* toxin is available, absolute determination of *Pfiesteria* as a causative agent remains problematic. Currently, the approach is a conservative application of Henle–Koch postulates (Burkholder et al. 200 1c), which rely as much on the absence of other causative factors as they do on the presence of toxic forms of *Pfiesteria*. Nevertheless, screening of fish lesion/kill events, including historical events, has suggested that *Pfiesteria* species have been active players in fish lesion or fish kill events in coastal waters (Burkholder et al. 1995; Glasgow et al. 2001b). In this regard, molecular tools have been particularly advantageous since even under “bloom” conditions, *Pfiesteria* may constitute only 10% of the total phytoplankton numbers.

Regional US East Coast distribution. The geographic distribution of *Pfiesteria* species, initially conducted by conventional PCR, has predominantly relied on the real-time PCR approach since its introduction (Bowers et al. 2000). Prior to the advent of molecular methods, the geographic range of *Pfiesteria* species had been established from the Delaware inland bays on the U.S. East Coast to Mobile Bay, Alabama on the U.S. Gulf Coast (Burkholder and Glasgow 1997). Subsequently, the range has been extended southward to the southern tip of Texas, and northward to Long Island, New York (Rublee et al. 1999; Villareal, Simons, and Rublee 2004). We have also detected *Pfiesteria* in samples from Rhode Island and Mississippi (data not shown).

Some information has emerged on the regional distribution of *Pfiesteria* species and responses to meteorological forcing events. For example, through early summer of 1999 low rainfall resulted in drought conditions through much of the eastern United States. During August of that year, a fish kill was observed in the Tuckahoe River in southern New Jersey, at a location where the water was normally fresh, but had become brackish because of salt water intrusion as a result of the low flow conditions. Microscopic observation of water samples revealed approximately 60 *Pfiesteria*-like cells/mL, not enough to trigger further evaluation by bioassays (Burkholder, J. NCSU and Ruppel, B. NJ Dept. Env. Protection, pers. commun.). The kill event eventually subsided, but a decision was made to test water and sediment samples from the river using PCR. However, on the day before sampling was scheduled, and some weeks after the end of the fish kill, the remnants of hurricane Floyd dropped several inches of rain in the area and normal flow was reestablished. Despite this return to normal, sediment samples taken in October, 1 km above, and 1 km below the fish kill site tested positive for *P. piscicida*. These results are not sufficient to confirm that *P. piscicida* was the causative agent of the fish kill, but they do confirm its presence in the river.

Three hurricanes passed over North Carolina in 1999, which caused significant flooding and both sediment deposition and scouring of the coastal estuaries (Burkholder et al. 2004). It appeared that *Pfiesteria* populations along with those of other organisms were displaced down the estuaries as a result of this flushing with fresh water. The evidence for this displacement was two-fold: first, no fish kill events linked to *Pfiesteria* have been recorded since 1998, and second, the frequency of positive tests for *Pfiesteria* in coastal NC was low following the storm events (although this was also the period in which the molecular methods were first used extensively). Numbers of samples positive for *Pfiesteria* in NC estuaries appeared to increase during 2002 and it appeared that populations were reestablishing (Fig. 1), but since then additional storm activity likely has reversed that process (Burkholder et al. 2004). The abundance of *Pfiesteria* species was also limited in South Carolina estuaries following the same period of high storm activity (Lewitus et al. 2002).

Global distribution. During 2001, we requested field samples from international colleagues. One of the first positive samples was from Trondheim, Norway, in waters previously considered too cold for *Pfiesteria* species. Fortuitously, Jakobsen et al. (2002) had simultaneously isolated and identified both *P. piscicida* and *P. shumwayae* in sediment samples from Oslofjord, clearly establishing that these organisms were not restricted to warm waters as previously thought. Additional samples have now established that *Pfiesteria* is cosmopolitan, since it has been detected in at least one location on every continent (Rublee et al. 2004). The most unusual sample to have recorded a positive hit has been from Ace Lake, an isolated saline lake in Antarctica, although efforts to confirm the presence of *Pfiesteria* by amplification and sequencing of SSU rDNA fragments from that sample have not been successful.

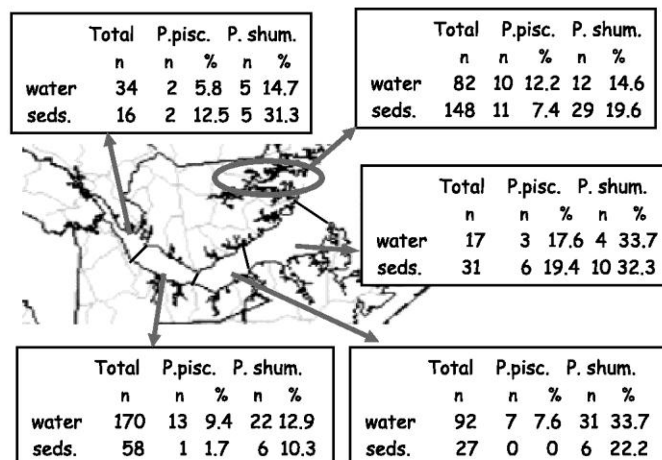

Fig. 1. Results of assays for *Pfeisteria* species in the Neuse River, NC during 2002–2003. Prior to hurricanes in 1999, highest abundance and activity of *Pfiesteria* was in regions II and III.

The most extensive studies of distribution to date outside of the U.S. have been conducted by Rhodes et al. (2002; in prep.) in New Zealand. They have documented the presence of both species of *Pfiesteria* in multiple locations in both the North and South Islands. Despite the widespread presence of *Pfiesteria* species in New

Zealand, it was thought that the risk of fish lesion or kill events there was generally low because of high tidal amplitudes and limited estuarine development, including low nutrient conditions and the absence of large populations of schooling fish. They did suggest, however, that coastal, lagoonal lakes might be locations of *Pfiesteria* abundance and at higher risk, especially since they have detected the presence of the organisms in such lakes.

Table **2.** Sources of sequences used.

Isolate/Sample ID	Date	Location of collection	Genbank entry	Length	References
Pfiesteria piscicida					
NCSU B-125-4 (12/9)	12/96	Beaufort Pt., NC, USA	AF330600	2779	Tengs et al. (2003)
NCSU B-125-PAC	12/96	Beaufort Pt., NC, USA	AF330601	2779	Tengs et al. (2003)
NCSU 125-4[a]	9/96	Beaufort Pt., NC, USA	AF330602	2779	Tengs et al. (2003)
NCSU 125-4 (12/2)	12/96	Beaufort Pt., NC, USA	AF330603	2779	Tengs et al. (2003)
NCSU 125-4 1997	12/96	Beaufort Pt., NC, USA	AF330604	2779	Tengs et al. (2003)
NCSU 125-4	12/96	Beaufort Pt., NC, USA	AF330606	2779	Tengs et al. (2003)
NCSU 113-2	9/97	Neuse River, NC, USA	AF077055	1800	Oldach et al. (2000)
NCSU 113-2	9/97	Neuse River, NC, USA	AF330611	2779	Tengs et al. (2003)
NCSU B-113-3	9/97	Neuse River, NC, USA	AF330605	2779	Tengs et al. (2003)
NCSU B-89B	7/98	Neuse River, NC, USA	AF330607	2779	Tengs et al. (2003)
NCSU 114-1-5	9/97	Neuse River, NC, USA	AF330612	2779	Tengs et al. (2003)
NCSU 113-4	9/97	Neuse River, NC, USA	AF330616	2779	Tengs et al. (2003)
NCSU 113-3-B	9/97	Neuse River, NC, USA	AF330617	2779	Tengs et al. (2003)
Noga-P		Pamlico River, NC, USA	AY245693	3361	Litaker et al. (2003)
NCSU B-98T	9/97	Chicamacomico River, MD, USA	AF330609	2779	Tengs et al. (2003)
NCSU 97-2 1997	9/97	Chicamacomico River, MD, USA	AF330613	2779	Tengs et al. (2003)
NCSU 97-1	9/97	Chicamacomico River, MD, USA	AF330610	2779	Tengs et al. (2003)
NCSU 98-3 1997	9/97	Chicamacomico River, MD, USA	AF330614	2779	Tengs et al. (2003)
NCSU 98-A	9/97	Chicamacomico River, MD, USA	AF330615	2779	Tengs et al. (2003)
Florida DEP	9/97	Chicamacomico River, MD, USA	AF330618	2779	Tengs et al. (2003)
CCMP 1830	1/98	Chicamacomico River, MD, USA	AF330619	2779	Tengs et al. (2003)
CCMP 1831	1/98	Chicamacomico River, MD, USA	AF330620	2779	Tengs et al. (2003)
MMRCC#981020BR01C	9/97	Chicomococomico R, MD, USA	AF149793	1785	Litaker et al. (2003)
NCSU 102-1	8/97	Pocomoke River, MD, USA	AF330608	2779	Tengs et al. (2003)
CCMP 1834	1/98	Pocomoke River, MD, USA	AY121846	1794	Peglar, Nerad, & Gillevet (2001)
—	?	Atlantic Coast USA	AY112746	6434	Saito et al. (2002)
NY (four samples)[b]	2003	Long Island, NY, USA		460	Rublee, unpubl.
CAW (four samples)[b]	2003	Lake Ellesmere, New Zealand		450	Rhodes et al., unpubl.
—	2000	Oslofjord, Norway	AY033488	1788	Jakobsen et al. (2002)
Pfiesteria shumwayae					
NCSUSp. B 112456	6/97	Beaufort Pt. NC, USA	AF218805	1800	Oldach et al. (2000)
Noga-S	?	Pamlico River, NC, USA	AY245694	3434	Litaker et al. (2003)
Clone N-1	1991	Pamlico River, NC, USA	AF080098	1785	Litaker et al. (1999)
652-T	2000	NZ		550	Rhodes et al. (2002)
Norway	2000	Oslofjord		246	Rublee, unpubl.
CAW (six samples)	2003	Lake Ellesmere, NZ		407	Rhodes et al., unpubl.
Crypotoperidiniopsds					
''Shephard's Crook''			AY251291	1744	Tengs & Seaborn, unpubl.
CCMP1828 ''Crypt. brodyii''	9/97	Kings Creek, MD, USA	AF080097	1783	Litaker et al. (1999)
''crypto-C''	?	?	AY251292	1744	Tengs & Seaborn, unpubl.
CCMP1873 ''P-like''	11/98	Willington River, GA, USA	AY033487	1787	Tengs & Seaborn, unpubl.
P-like clone POC-8	?	?	AY121856	1792	Peglar, Nerad, & Gillevet (2001)
Lucy-8	?	?	AY251290	1742	Tengs & Seaborn, unpubl.
Lucy	?	Indian R., FL	AY245689	3347	Litaker et al. (2003)
Crypto H/V14	?	Neuse River, NC	AY245690	3400	Litaker et al. (2003)
Crypto PLO21	?	St. Lucie R., FL	AY245691	3327	Litaker et al. (2003)
Karlodinium micrum					
Pimo5JulC4	?	St. John's R., FL	AY245692	3348	Litaker et al. (2003)
Vorticella campanula					
Vorticella campanula	?	?	AF335518	1733	Miao, Yu, & Shen (2001)

[a]Actively toxic culture.
[b]Sequences generated by direct sequencing using forward and reverse primers.

SEQUENCE ANALYSES

An advantage of PCR-based approaches for detection is that the amplified fragments can also be used in sequence analysis, either by generating clones from amplified fragments or by direct sequencing. Knowing the sequence allows insight into additional questions of scientific interest: how much variability is there among isolates across geographic or temporal ranges? What is the phylogenetic position of the target organism? If it is widely distributed, can a source location be identified? The available collection of sequence data for *Pfiesteria*

and *Pfiesteria*-like species is limited (65 entries in Genbank), but is enough to at least begin to answer interesting questions. For example, Tengs et al. (2003) examined a 3000-bp fragment of the rDNA (including 18S, ITS1, 5.8S, ITS2, NTS, and partial LSU regions) of 24 *P. piscicida* clones from four geographic locations to search for any sequence differences between toxic and non-toxic strains. They found that all sequences were identical.

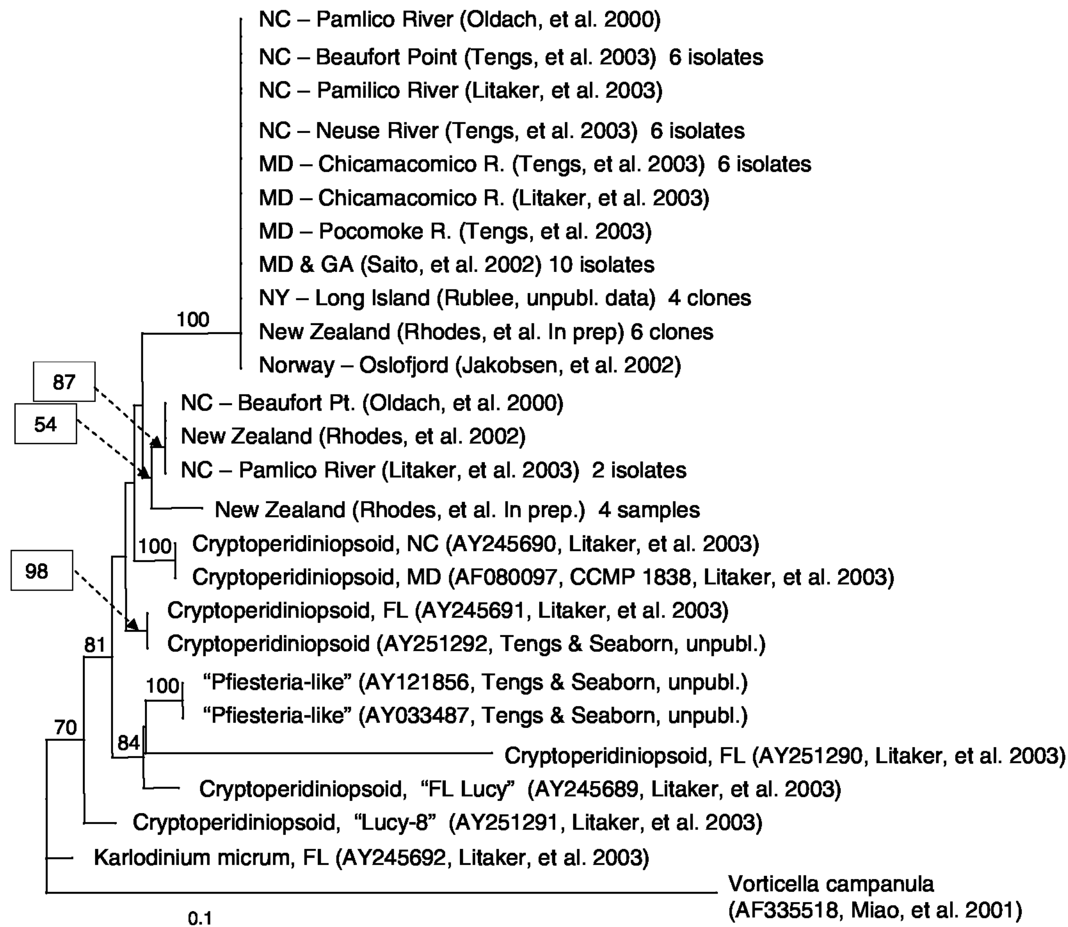

Fig. 2. Neighbor-joining tree based on shared 409-bp sequence from the small subunit rDNA of *Pfiesteria* and ''*Pfiesteria*-like'' (including cryptoperidiniopsoid) sequences. Bootstrap percentages are shown for nodes with greater than 50 support. An additional 142-bp sequence generated by *Pfiesteria shumwayae* specific primer amplification from a field sample from Oslofjord in Norway (c.f. Jakobsen et al. 2002) is not included in the phylogram, but the sequence was identical over its range to three *P. shumwayae* sequences shown. Most entries in the phylogram consist of longer ribosomal sequences (≈1,800–3,700 bp, including SSU, ITS1, 5.8S, ITS2, and partial LSU). Phylograms generated with entries that have longer sequences do not change relationships among *Pfiesteria* species, although different topologies are generated among the ''*Pfiesteria*-like'' and cryptoperidiniopsopid entries. *Vorticella campanula* was added as an outgroup to root the tree; *Karlodinium micrum* was added as an additional outgroup to remianing sequences. See Table 2 for complete listing of entries.

A comparison of sequence data available in GenBank suggests that variability among *Pfiesteria* isolates including those from Norway and New Zealand is very low. This includes comparisons among isolates from which fairly long sequences of the ribosomal genes (SSU, ITS 1, 5.8S, ITS2, and partial LSU), or NTS genes are available (Litaker, Scholin, and Vasta 2003; Saito et al. 2002; Tengs et al. 2003). Differences in sequences were never greater than might be expected from base calling error in the sequencing process (1 or 2 base pairs per sequence). Thus, for both *Pfiesteria* spp. there was little variation across a large geographic range. This might suggest widespread recent dispersal, which would be supported by recent analyses of ballast water in large ships, in which both *Pfiesteria* species have been detected by PCR (Doblin et al. 2004; Drake et al. in press). This mechanism of dispersal has previously been suggested for other dinoflagellate species (c.f. Bolch et al. 1998), but the lack of variation in the rDNA of *Pfiesteria* species precludes any suggestion of source location. Ribosomal DNA is relatively conserved because of functional constraints, even though it has non-transcribed regions; so it is possible the sequence analysis of other genes may show enough variability to suggest dispersal patterns.

Recently, we have sequenced SSU rDNA fragments of up to 540 bp from *Pfiesteria* samples collected in 2003

for *P. piscicida* in New Zealand and New York, USA, and for *P. shumwayae* samples from New Zealand (direct sequencing using forward and reverse primers on reaction products of at least four different PCR reactions). We found no sequence variation across the SSU rDNA gene in *P. piscicida* in these samples, but the *P. shumwayae* sequences from four samples in New Zealand were significantly different (13 differences over a 407-bp region in the SSU rDNA). Interestingly, all these New Zealand samples came from a lagoonal lake in New Zealand that is generally isolated from marine waters. Further, we were not successful in our attempts to amplify additional fragments for sequencing using PCR primers that are routinely used on other *Pfiesteria* isolates. This may suggest that there is either a significant *P. shumwayae* variant or possibly a third *Pfiesteria* species. A phylogram based on a 407-bp common region among all *Pfiesteria* and "*Pfiesteria*-like" Genbank entries illustrates this relationship, along with the uncertain position of many of the related small estuarine dinoflagellates that have been sequenced in related work (Table 2 and Fig. 2). When comparisons are made among longer sequences (when available), it does not substantially change the topology of the tree.

CONCLUSIONS

Although much has been learned about the distribution of *Pfiesteria* and PLOs during the last decade, there is still much to learn. Continued assays of field samples over a range of temporal and spatial scales, including assessments of sequence variability for both rDNA and other genes should help resolve questions of variability and dispersal. If the assays are widely used, they can also provide insight into risks associated with *Pfiesteria* populations in countries other than the United States, a concern that remains unresolved. Despite a lack of any indication of such problems, it may yet be important in locations with extensive estuarine-dependent fisheries or aquaculture. An additional question is: will we see a resurgence of *Pfiesteria* activity once environmental conditions and populations are reestablished at sites that formerly exhibited high activity? We suspect the answer will be yes, but a general expectation of an increased number of storms along the eastern coast of the U.S. may preclude such reestablishment far beyond the foreseeable future.

We have not addressed issues of *Pfiesteria* activity in this paper or the methods to address them. Development of activity methods such as toxicity bioassays (c.f. Burkholder et al. 2001c, 2005), toxin assays (Fairey et al. 1999; Kimm-Brinson et al. 2001; Melo et al. 2001; Moeller et al. 2001), and assays of gene expression (e.g. SAGE, Coyne et al. 2001, 2005) will be instrumental in understanding the role of these dinoflagellates. Determining the structure of the *Pfiesteria* toxin is also essential for definitive determination of whether *Pfiesteria* is the causative agent in various fish kill or lesion events, and to define the health risks associated with exposure.

Finally, we anticipate that the methods described here as well as other developments will lead to further discoveries and identification of dinozoans, an important component of both marine and freshwater aquatic ecosystems.

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