Detection of a novel ecotype of Pfiesteria piscicida (Dinophyceae) in an Antarctic saline lake by real-time PCR

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Abstract:

The heterotrophic dinoflagellate *Pfiesteria piscicida* was detected in Ace Lake in the Vestfold Hills, eastern Antarctica by using real-time PCR based on 18S rDNA sequences. Antarctic water samples collected in 2004 were tested by species-specific real-time PCR assays for the identification of *P. piscicida* and *P. shumwayae*. Positive results were shown with *P. piscicida*-specific real-time PCR, and PCR products were examined by sequence analysis for confirmation. A phylogenetic tree made from partial 18S rDNA sequences showed that the Antarctic clone clustered with *P. piscicida*. This result suggests that *P. piscicida* is present in the extreme conditions of an Antarctic saline lake which has not contained fish for thousands of years.

Article:

Introduction

Pfiesteria piscicida and *P. shumwayae* (PS) are hetero-trophic dinoflagellates linked to numerous fish kills in the US mid-Atlantic estuaries (Burkholder et al. 2001). *P. piscicida* can reproduce sexually in cultures maintained with algae or fish (Parrow et al. 2002; Parrow and Burkholder 2004), although sexual reproduction in *P. shumwayae* has so far only been demonstrated in the presence of finfish or shellfish. Translucent zoospores of PS are often difficult to discern and are morphologically indistinguishable using light microscopy (Steidinger et al. 2001). Thecal plate tabulation can be visualized by electron microscopy but this process requires cultivation of cells and is time consuming (Mason et al. 2003).

Real-time PCR has been shown to be a powerful tool for rapid identification of PS in environmental samples (Bowers et al. 2000; Zhang and Lin 2005). A global distribution of PS has been suggested by using PCR-based detection methods (Rublee et al. 2004). PS have been detected under a wide range of temperature and salinity in coastal areas (Rublee et al. 2004), and a wide salinity tolerance of *P. piscicida* and *Pfiesteria*-like dinoflagellates have also been reported in culture experiments (Sullivan and Anderson 2001).

The Vestfold Hills in eastern Antarctica cover an area of 410 km^2 and host over 150 lakes of which about 20% are saline (Pickard et al. 1986) and 30 are meromictic (Gibson and Burton 1996). The Vestfold Hills and many of its lakes were formed by isostatic rebound after the last major glaciation, but more recently models have been developed relating sea level changes to ice sheet history (Zwart et al. 1998). A review of a range of published palaeolimnological data indicates that eustatic sea level changes occurred more rapidly than isostatic rebound (Hodgson et al. 2004). There is good evidence of marine incursions and successive periods of meromixis in the sedimentary history of Ace Lake (Roberts and McMinn 1999; Cromer et al. 2005). Ace Lake was isolated from the marine environment 8,000 to 10,000 years ago and has been a stable saline meromictic lake system for the last 4,000 years, with a maximum depth of 25 m and an area of ca. 0.2 km^2 (Gibson and Burton 1996; Laybourn-Parry et al. 2002). Water temperatures in the mixolimnion can reach 5°C when the ice cover is lost

during summer (January to February) (Bell and Laybourn-Parry 1999). When ice cover is largely retained in summer, the temperatures remain between -1°C and 1°C, and salinity is approximately 20‰ (Bell and Laybourn-Parry 1999; Laybourn-Parry et al. 2002). The trophic structure of Ace Lake is dominated by benthic micro-organisms, many of which are of marine origin (Franzmann and Dobson 1993; Perriss et al. 1995). The microbial plankton is dominated by bacteria and a few phytoflagellate species including the prasinophyte, *Pyramimonas gelidicola*, and the cryptophyte, cf. *Geminigera cryophila*. Only one zooplankton species, Paralabidocera antarctica, inhabits the lake and there are no fish (Laybourn-Parry 1997; Bell and Laybourn-Parry 1999). *P. piscicida* has been previously detected in Ace Lake by PCR-based assays (Rublee et al. 2004) but this has not been confirmed by sequence analysis.

In this study, Antarctic water samples were analyzed by real-time PCR assays using *Pfiesteria*-specific primers. Positive PCR products were isolated from the water samples, sequenced, and examined for their phylogenetic affinities.

Materials and methods

Selection of species-specific real-time PCR assays for detection of P. piscicida and P. shumwayae

Pfiesteria shumwayae-specific real-time PCR using SYBR Green I (Zhang and Lin 2005) and *P. piscicida*-specific real-time PCR using fluorigenic 5' nuclease (TaqMan) chemistry (Bowers et al. 2000) were chosen for detection of P. *shumwayae* and *P. piscicida*, respectively. The *P. shumwayae*-specific primer set and the *P. piscicida*-specific primer–probe set were previously designed from mitochondrial cytochrome b gene (cob) (Zhang and Lin 2005) and from SSU rDNA (Bowers et al. 2000), respectively (Table 1). Specificity of these assays has been tested against other related species and has been confirmed by sequence analysis of PCR amplicons from natural waters (Bowers et al. 2000; Zhang and Lin 2005; Rhodes et al. 2006). Prior to use of both real-time PCR assays for Antarctic water samples, specificity was also tested with Australian genotypes of *Cryptoperidiniopsis brodyi* (CBWA12, CBSA4, CBHU1, CBDE1, and CBDE10) because these sequences were not available when these assays were developed.

Species-specific real-time PCR assays on Antarctic water samples

The water samples employed were collected from Ace Lake on 1st July and 11th November 2004. Ace Lake, a saline lake, is located at 68°28.4'S, 78°11.1'E in the Vestfold Hills area of eastern Antarctica. It is 10 km from the ice cap and 150 m from the ocean. A motorized ice-drill with a 25 cm diameter auger (Jiffy, USA) was used to drill through the ice and access the water at the deepest point of the lake. Samples were collected at 2 m intervals from 0 to 12 m using a 2.5 l polycarbonate Kemmerer bottle. Two hundred milliliters of samples from depths 0, 2, 4, 6, 8, 10, 12 m were concentrated by filtration onto a 25 mm glass fiber filters. The filters were stored in CTAB buffer at room temperature until DNA extraction. Genomic DNA of the samples were extracted with a CTAB extraction protocol (Scholin et al. 2003). To check for PCR quality DNA, amplification of the extracted DNAs were performed with an internal positive control (DNA extracts from *C. brodyi*) in conventional PCR using primer ITSF2 and 5.8SR (Table 2) (Litaker et al. 2003). PCR products were loaded to gel electrophoresis in 2% agarose gels and visualized by ethidium bromide staining.

Table 1 The real-time PCR primer and probe sequences used for detection of *P. piscicida* and *P. shumwayae*

Dinoflagellate	Primer and probe	Sequence (5′ → 3′)	Reference
P. piscicida	107	CAGTTAGATTGTCTTTGGTGGTCAA	Bowers et al. (2000)
	320	TGATAGGTCAGAAAGTGATATGGTA	
	Probe	FAM-CATGCACCAAAGCCCGACTTCTCG-TAMRA	
P. shumwayae	PSMTF2	TGACTTTCTAACTTCTAACTTCTTTACATC	Zhang and Lin (2005)
	PSCOBR1	AACACCATCCATAGAATATTTCTCTCATG	

Pfiesteria piscicida-specific real-time PCR assay was performed with 2 µl DNA from each Antarctic sample, 10 ~l of platinum quantitative PCR supermix-UDG (Invitrogen, Australia Pty), forward and reverse primers each at a final concentration of 0.2 µM, fluorogenic probe at a final concentration of 0.15 µM, PCR grade water to a

final volume of 20 µl. For *P. shumwayae*-specific real-time assay, the amplification mixture contained 10 µl of platinum SYBR Green qPCR supermix-UDG (Invitrogen, Australia Pty), 0.4 µl of each primer at a concentration of 10 µM, 7.2 µl of PCR grade water, and 2 µl of DNA sample. Thermal cycling conditions consisted of 50°C for 2 min and 95°C for 2 min followed by 45 cycles (50 cycles for SYBR Green) of 95°C for 10 s and 60°C for 45 s (40 s for SYBR Green). The real-time PCR was performed with the Rotor-Gene RG-3000A (Corbett research) detection instrument. Positive PCR products with SYBR Green real-time PCR were further checked by agarose gel electrophoresis to confirm the amplicon size.

Sequencing environmental clones

For species verification of real-time PCR amplicons from Antarctic samples, positive PCR products of the target species were examined by agarose gel electrophoresis, and the amplicons on agarose gel were amplified by the 'band stab' approach (Bolch 2001). The band was stabbed with a stainless steel syringe needle and the needle was dipped into a conventional PCR reaction mixture. *P. piscicida*-specific primers (for-ward: 107 and reverse: 320) were used for the PCR amplification. The reaction mixture contained 35.6 µl of PCR grade water, 10 µl of buffer, 8 µl of DNTPs, 12 µl of $MgCl_2$, 10 µl of BSA, 10 µl of each primer (10 µM), 0.6 µl of Taq polymerase, and 2 µl of template DNA. PCR amplification was carried out in a DNA thermocycler with an initial denaturation step for 2 min at 94°C followed by 39 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 60°C, and 2 min at 72°C. Following the PCR assay, the amplified products were purified using a Qiaquick PCR purification kit (Qiagen, Hildon, Germany), ligated into the pGEM-T vector system (Promega, Madison, WI, USA), and transformed into *Escherichia coli* cells following the manufacturer's instructions (Promega, Madison, WI, USA). Plasmid DNAs were isolated and purified using DNA purification system (Promega, Madison, WI, USA). The plasmids containing target DNA were sequenced with pUC/M13 primers (Pro-mega, Madison, WI, USA) using Beckman-Coulter DNA sequencing chemistry on a CEQ2000 capillary electrophoresis sequencer.

Phylogenetic analysis of partial sequences

Partial SSU rDNA sequences were aligned with sequences of *Pfiesteria* and other closely related organisms available on GenBank using ClustalX (Thompson et al. 1997). The dinoflagellate Shepherd's Crook was chosen as an outgroup taxon, and the final alignment was manually adjusted. Bayesian analysis (likelihood) was carried out using MrBayes (Huelsenbeck and Ronquist 2001). Nucleotides were treated as independent unordered characters of equal weight and gaps were interpreted as missing. The parameters of Bayesian analysis were set to 150,000 generations, four by four nucleotide substitution, general time-reversible model with gamma-distributed among-site rate variation (GTR + G + I), and sampling once every 100 trees; parameter values of GTR + G + I model were estimated during the Bayesian analysis.

Results

Cultures

Antarctic water samples that showed positive signals in *P. piscicida*-specific real-time PCR assays were observed under light microscopy, but no zoospores or cysts of *P. piscicida* were visible. After additions of *Pyramimonas* sp., *Rhodomonas salina*, copepods, and salmon tissue into water samples from Ace Lake, algal prey species swam actively, but no germination of *Pfiesteria* cysts or feeding behavior was observed in the water samples.

Table 2 Conventional PCR primers for confirmation of PCR quality template DNA

Primer name	Sequence (5′ → 3′)	Reference
ITSF2	TACGTCCCTGCCCTTTGTAC	Litaker et al. (2003)
5.8SR	CATCGTTGTTCGAGCCGAGAC	

Detection of P. piscicida from Antarctica by real-time PCR assays

The sample sets included 14 water samples from Ace Lake in Antarctica. Water samples collected from 2 m (1st July 2004), 4 m, and 10 m (11th November 2004) gave a positive response to the *P. piscicida*-specific real-

time PCR assays (Fig. 1). In contrast, no samples gave positive responses to *P. shumwayae*-specific real-time PCR assay. Additional verification of the specificity of the real-time PCR assays in the environmental samples was conducted by sequence analyses of positive PCR amplicons. The SSU rDNA sequence data from the PCR positives (GenBank accession no: DQ991383) were compared to the *P. piscicida* sequence, which showed that the sequences of the PCR positives were identical to those of *P. piscicida*. A phylogenetic tree made from partial SSU rDNA sequences (211-bp) showed that environmental clones clustered with *P. piscicida* (Fig. 2). When compared to a phylogram based on full sequences of SSU rDNA, these phylogenetic results were not significantly different (not shown). These results strongly imply the presence of *P. piscicida* in Antarctica.

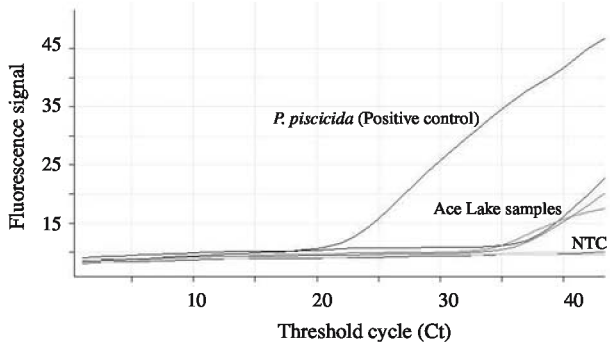

Fig. 1 Detection of *P. piscicida* from Ace Lake samples using SSU rDNA-based real-time PCR. Fluorescent signals were observed from 2 m (1st July 2004), 4 m, and 10 m (11th November 2004) water samples. *NTC* no-template control

Discussion

Detection of Pfiesteria species in Ace Lake by real-time PCR assays

Growth of the heterotrophic *P. piscicida* varies in relation to a variety factors including prey types and concentrations (Burkholder et al. 2001; Lin et al. 2004). *P. piscicida* has been shown to grow on a broad range of microbial taxa, and the trophic structure of the plankton in Ace Lake is dominated by bacteria, algae, and protozoans (Bell and Laybourn-Perry 2003). *P. piscicida* has also been known to be able to function as a phototroph by acquiring chloroplasts from algal prey (Lewitus et al. 1999a, b). Chloroplasts retained inside a food vacuole of *P. piscicida* were photosynthetically active for a week (Lewitus et al. 1999a). The ability of direct nutrient acquisition (N-labeled nitrate, ammonium, glutamate, and urea) by kleptoplastidic *P. piscicida* has been shown by Lewitus et al. (1999b) suggesting that kleptoplastidy might be a survival mechanism during unfavorable (prey-depleted) conditions. The nutritional versatility of *P. piscicida* might enhance survival in the extreme Ace Lake ecosystem where temperatures are low, light is limiting, particularly in the winter months, and ice cover prevails for the majority of the year. Mixotrophic phytoplankton are prevalent in Antarctic lakes and mixotrophy is considered to be advantageous in allowing phytoplankton to sustain population numbers either heterotrophically or photosynthetically (Bell and Laybourn-Parry 1999, 2003). For example, the prasinophyte *P. gelidicola*, considered to be a phototrophic species in temperate regions, was observed to ingest and grow on bacteria in Ace Lake (Bell and Laybourn-Parry 1999, 2003) and cells can also take up dissolved organic substrates to supplement survival (E.M. Bell, personal observation). The uptake of dissolved organic carbon of different molecular weights by *Pyramimonas* species has been documented in Highway Lake that is close to Ace Lake in the Vestfold Hills (Laybourn-Parry et al. 2002). The Ace Lake population of the cryptophyte *G. cryophila* is also mixotrophic and a strong dependence on mixotrophy has been documented for cryptophytes in Dry Valley Antarctic lakes even during mid-summer (Roberts and Laybourn-Parry 1999; Marshall and Laybourn-Parry 2002; Laybourn-Parry et al. 2005).

Methodological considerations

Pfiesteria piscicida-specific real-time PCR which tests specificity of the assay against closely related dinoflagellates was developed by Bowers et al. (2000). This assay has been used for detection of *P. piscicida* in field samples (Rublee et al. 2004, 2006; Lin et al. 2006; Bowers et al. 2006). Environmental water samples could contain *Pfiesteria*-like dinoflagellates that are not yet described and these may cross react with the species-specific PCR primers leading to false conclusions (Bowers et al. 2000). Therefore, assay specificity was further confirmed by sequence analysis of positive real-time PCR amplicons. The sequence analyses showed

that the environmental samples had sequences identical to *P. piscicida* providing confirmation of the specificity of the real-time PCR assay. Although the partial SSU-based phylogram clearly clustered the Ace Lake clone with *P. piscicida* from the US and Indonesia, it is possible that the phylogram shown in Fig. 2 may not resolve the phylogeny of *Pfiesteria* and related species because of short sequences (211-bp) and the conservative nature of SSU rDNA. However, the detection of *P. piscicida* from Ace Lake indicates that this species has both a wide geographical distribution (from tropical and temperate regions to Antarctica) and a broad tolerance to environmental variables. Ongoing efforts are needed to try and culture this fascinating and enigmatic dinoflagellate from Ace Lake.

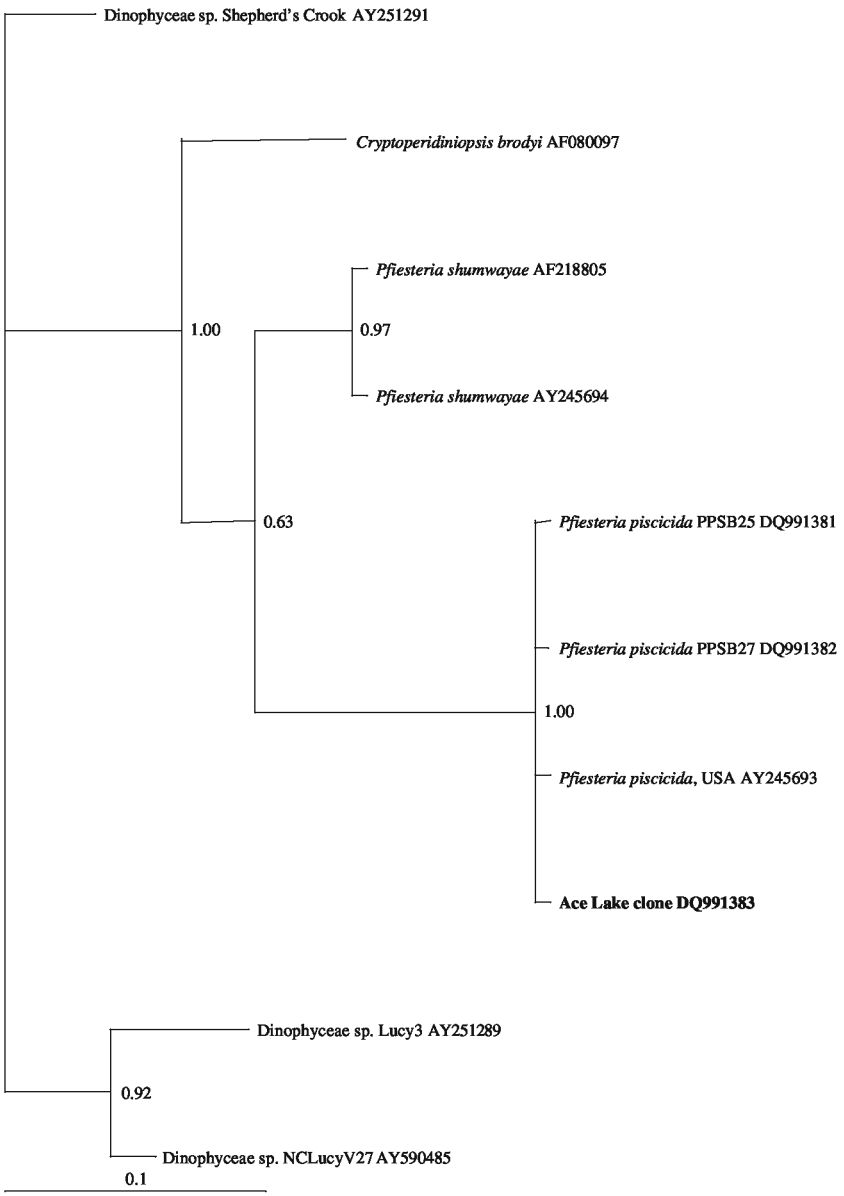

Fig. 2 Phylogram based on Bayesian analysis of partial SSU rDNA sequences (211-bp) from Ace Lake clone and closely related organisms. Ace Lake clone was sampled from Ace Lake, Antarctica on 11th November 2004. This clone was detected by *P. piscicida*-specific real-time PCR and was sequenced. *Numbers* adjacent to each node represent probabilities derived from 150,000 generations using a general time-reversible evolution model with gamma-distributed (= 1.705) among-site rate variation. Strains sequenced in the present study are shown in *bold*. Consistency index (*CI*) = 0.936, rescaled consistency index (*RC*) = 0.881, retention index (*RI*) = 0.941, and homoplasy index (*HI*) = 0.0637

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