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Discovery of the toxic dinoflagellate *Pfiesteria* in northern European waters

Kjetill S. Jakobsen[1], Torstein Tengs[2], Andreas Vatne[1], Holly A. Bowers[2], David W. Oldach[2], JoAnn M. Burkholder[3], Howard B. Glasgow Jr[3], Parke A. Rublee[4] and Dag Klaveness[1*]

[1]*Department of Biology, University of Oslo, PO Box 1066 Blindern, 0316 Oslo, Norway*
[2]*University of Maryland School of Medicine and Institute of Human Virology, Room 552, Medical Biotechnology Centre, 725 West Lombard Street, Baltimore, MD 21201, USA*
[3]*Center for Applied Aquatic Ecology, North Carolina State University, Suite 104, 620 Hutton Street, Raleigh, NC 27606-7510, USA*
[4]*Department of Biology, University of North Carolina, Greensboro, NC 27402, USA*

Several dinoflagellate strains of the genus *Pfiesteria* were isolated by culturing techniques from sediment samples taken in the Oslofjord region of Norway. *Pfiesteria piscicida*, well known as a fish killer from the Atlantic coast of America, was identified by genetic methods and light microscopy. The related species *Pfiesteria shumwayae* was attracted from the sediment by the presence of fish, and has proved toxic. This present survey demonstrates the wide distribution of these potentially harmful species, but so far they have not been connected with fish kills in Europe.

Keywords: *Pfiesteria*; Europe; toxic algae; environmental polymerase chain reaction

1. INTRODUCTION

The discovery of the estuarine, ambush-predator dinoflagellate *Pfiesteria piscicida* Steidinger & Burkholder (Burkholder *et al.* 1992; Steidinger *et al.* 1996) as a causative agent of major estuarine fish kills, and then a second toxic species, *Pfiesteria shumwayae* Glasgow & Burkholder (Glasgow *et al.* 2001*a*), have stimulated research on toxic dinoflagellates. Despite emerging knowledge about conditions leading to toxic *Pfiesteria* outbreaks, their deleterious environmental impacts (Burkholder & Glasgow 1997; Burkholder *et al.* 2001*a*), and their effects on laboratory animals and exposed humans (Glasgow *et al.* 1995; Levin *et al.* 1997, 1999; Grattan *et al.* 1998; Oldach *et al.* 1999; Kimm-Brinson *et al.* 2001; Schmechel & Koltai 2001), the geographical distribution of these organisms had been documented only in the US Atlantic and Gulf Coasts. Here we report, we believe for the first time, toxic *Pfiesteria* from European waters.

2. METHODS AND RESULTS

As an initial strategy in searching for *Pfiesteria* along the eastern Atlantic Coast (Europe), we used enrichment and capillary isolation methods to establish clonal cultures of phagotrophic dinoflagellates from estuarine sites in the Oslofjord region of Norway (59–60° N, 10–11° E). By using a dinoflagellate-specific 18S rRNA primer pair upon single cells (Oldach *et al.* 2000; Tengs *et al.* 2000), and subsequent direct sequencing of multiple polymerase chain reaction (PCR) products (a 142 bp region spanning the 3′-end of the gene), several of the clonal cultures grown with *Rhodomonas* algal prey (strains 105, 106, 123 and 132) gave sequences identical to that reported for *P. piscicida* (GenBank accession no. AF077055). These clonal cultures came from a locality at the mouth of the River Sandvikselva (59° 53′ N, 10° 32′ E, Sandviksbukta), at a depth of 13 m. The normal yearly temperature amplitude for the bottom water in this area is 5–20 °C, and the salinity (PSU) range is 5–28. Sequencing the entire 18S gene (GenBank, accession no. AY033488) from these cultures revealed that they were identical, and three single nucleotide differences between the original *P. piscicida* (a toxic clonal culture from the laboratory of J.M.B. and H.B.G.) and the Norwegian isolates were identified.

DNA extracted directly from sediment samples from the Sandvikselva site was also tested using 5′–3′ exonuclease assays specific for *P. shumwayae* and *P. piscicida* (Bowers *et al.* 2000). Both assays gave positive signals from multiple samples, indicating the presence of benthic cysts or cells of both species.

The clonal cultures of *P. piscicida* and *P. shumwayae* obtained from sediments were zoospores, with occasional thin-walled temporary cysts. Fluorescence light microscopy and scanning electron microscopy (SEM) revealed that the thecal-plate tabulations were in agreement with those of *P. piscicida* (Burkholder *et al.* 1992) and *P. shumwayae* (Steidinger *et al.* 1996; Burkholder *et al.* 2001*a*) (figure 1; additional detail in the sulcal area (zoospore, ventral view) was revealed by electronic enhancement (figure 1*a*)).

As is shown in the phylogenetic tree (figure 2) constructed from full-length 18S rDNA sequences, *P. piscicida* and the Norwegian isolate form a tight cluster, whereas the closely related *P. shumwayae* (a toxic clonal culture from the laboratory of J.M.B. and H.B.G. (Glasgow *et al.* 2001*a*)) is clearly the most divergent. The *P. shumwayae* isolate from the Sandviks-bukta sediment is identical to

* Author for correspondence (dag.klaveness@bio.uio.no).

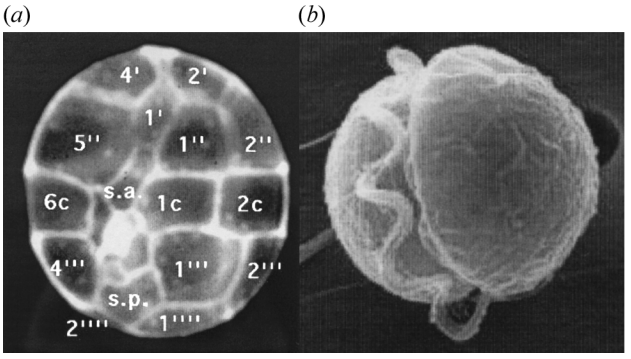

Figure 1. (*a*) Fluorescence light micrograph of a cell from the clonal culture no. 132 from Sandviks-bukta, showing the ventral aspect of a compressed zoospore of *Pfiesteria piscicida* (note five precingular plates as 5", whereas *P. shumwayae* has six precingular plates); stained by Fluorescent Brightener 28 (Sigma), with the signal electronically enhanced. Scale bar, 1μm. (*b*) Scanning electron micrograph of a toxic zoospore of *P. shumwayae* in suture-swollen preparation, induced from the sediment (Sandvika site) during exposure to live fish. Scale bar, 1μm.

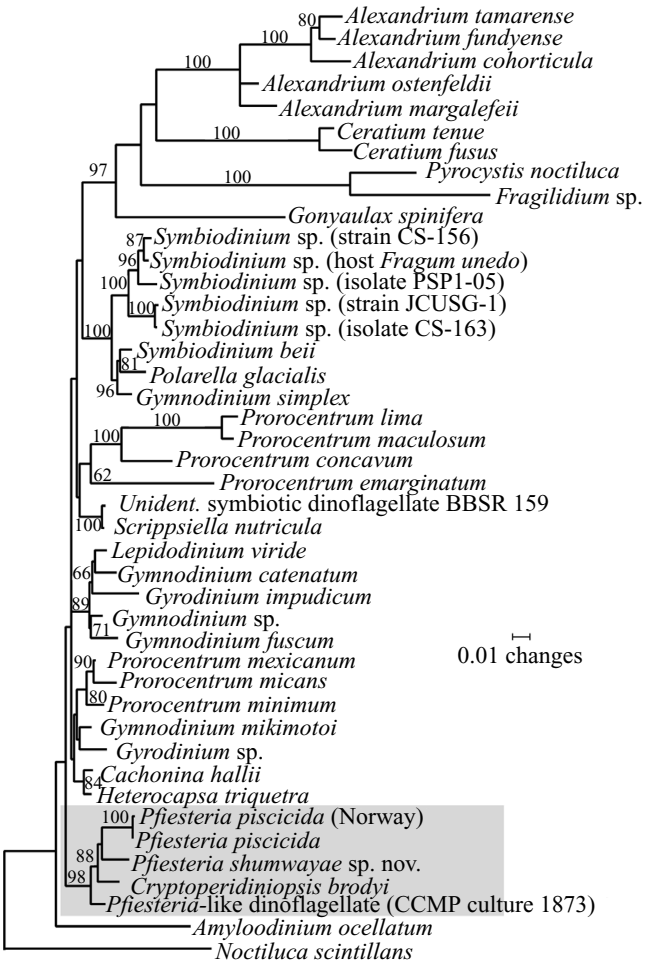

Figure 2. Minimum-evolution/maximum-likelihood-distance phylogenetic tree generated using the entire 18S rRNA sequence. The analysis is based on 1851 characters from an alignment of 43 species. All parameters (base frequencies, general time-reversible (GTR) substitution matrices, γ correction (four categories) and proportion of invariable sites) were optimized simultaneously from a neighbour-joining/Kimura 2 parameter topology. *Noctiluca scintillans* is used as an outgroup. The same tree was found 25 times in 25 heuristic searches with random addition of sequences and tree bisection and reconnection branch swap. The numbers at the nodes indicate bootstrap values over 60% (100 replicates).

the American isolate referred to above in the entire 18S region. Between *P. piscicida* and *P. shumwayae*, there are 58 nucleotide sites including both indels and point mutations. The phylogenetic tree also supports the monophyletic clade of the *Pfiesteria* group, which includes the cryptoperidiniopsoid species reported in recent phylogenetic work (Litaker *et al.* 1999; Bowers *et al.* 2000; Oldach *et al.* 2000; Tengs *et al.* 2000).

As *Pfiesteria* spp. have benign as well as toxic strains (Marshall *et al.* 2000; Burkholder *et al.* 2001*a–c*; Glasgow *et al.* 2001*a*,*b*; Parrow *et al.* 2001), it was essential to confirm whether the European isolates were toxic. Sediment samples from the mouth of the River Sandvikselva, a location at which *P. shumwayae* had been detected, were tested for ichthyotoxic *Pfiesteria* using standardized fish bioassays that follow Henle–Kochs' postulates (Evans 1976; Harden 1992), modified for toxic rather than infectious agents, as developed and refined by Burkholder *et al.* (1995, 2001*a–c*), Burkholder & Glasgow (1997), Marshall *et al.* (2000) and Samet *et al.* (2001). Dinoflagellate populations isolated and cloned from fish-killing assays (800–1200 zoospores ml^{-1}) were confirmed as *P. shumwayae* using PCR and SEM (suture-swollen cells (Burkholder *et al.* 2001*a*; Glasgow *et al.* 2001*a*)). Cloned *P. shumwayae* was retested (five clones per sample, each in separate bioassays) with fish and confirmed as ichthyotoxic (fish death in less than 4 h, versus sustained healthy control fish that had been treated similarly but without exposure to clonal *P. shumwayae*). Control fish bioassays had been maintained for comparison in both sets of test bioassays, in which fish were maintained similarly but without exposure to the natural samples or to cloned *P. shumwayae*, and all control fish remained healthy. In a final step, the dinoflagellates from the second set of fish bioassays with clonal *P. shumwayae* cultures were reisolated and reconfirmed as *P. shumwayae* using PCR and SEM, and cross-confirmed by the laboratories of P.A.R. and D.W.O. (Burkholder *et al.* 2001*a*,*c*).

From the samples tested, toxic *P. shumwayae* strains were detected in the presence of live fish. *Pfiesteria* spp. thrive as non-toxic stages, without active toxin production, except when they detect sufficient fresh materials from live fish to stimulate toxicity (Burkholder *et al.* 1997, 2001*a*; Glasgow *et al.* 2001*a–c*). Our samples were taken in the absence of diseased fish or fish kills. Therefore, the toxic strains of *P. shumwayae* that were detected in the standardized fish bioassays are not believed to have been in an actively toxic mode for fish when they were collected (Burkholder *et al.* 2001*a*; Glasgow *et al.* 2001*b*).

3. DISCUSSION

The data presented here are, to our knowledge, the first demonstration of *Pfiesteria* strains from the eastern Atlantic. Our results, implicating a trans-Atlantic distribution of the *Pfiesteria* complex, raise the question how old are these species on both sides of the Atlantic? The DNA

sequences being identical, or almost identical, does not allow us to conclude whether it has been recently introduced at one of the two continents or if the distribution reflects a continuous distribution (i.e. a genetic homogeneous distribution due to extensive and ongoing gene flow) of *Pfiesteria*. However, a scenario in which the trans-Atlantic distribution of the species complex was created by a single and ancient event is highly unlikely, judging from the very similar rDNA sequences. Toxic *Pfiesteria* thrives in estuarine waters affected by pollution from nutrient over-enrichment (Burkholder *et al.* 1997, 2001*a*,*b*; Lewitus *et al.* 1999; Magnien *et al.* 2000; Glasgow *et al.* 2001*b*,*c*). No reports have, as yet, identified *Pfiesteria* as a cause of unexplained fish deaths in European waters; therefore, the populations discovered here, may so far have exhibited low levels of toxicity that have not affected fauna within their immediate microenvironment at a level that can be detected by humans.

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