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The Systematic Position of the Parasitoid Marine Dinoflagellate *Paulsenella vonstoschii* (Dinophyceae) Inferred from Nuclear-Encoded Small Subunit Ribosomal DNA

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Members of the genus *Paulsenella* Chatton are marine phagotrophic dinoflagellates that specifically attack marine diatoms. In this phylogenetic study, we show that *Paulsenella* groups with *Amyloodinium ocellatum* (Brown) Brown et Hovasse, *Pfiesteria piscicida* Steidinger et Burkholder (Dinophyceae), *Pfiesteria shumwayae* Glasgow et Burkholder, and the cryptoperidiniopsoids, all members of the order Peridinales. In the phylogenetic tree, *Paulsenella* diverged after *Amyloodinium ocellatum* but prior to *Pfiesteria* and the cryptoperidiniopsoids. This suggests that *Paulsenella* also belongs to the order Peridinales and its earlier description as gymnodinioid and athecate has to be revised.

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Key words: dinoflagellate; feeding behaviour; *Paulsenella vonstoschii*; small subunit (SSU) rRNA.

Introduction

Heterotrophic dinoflagellates are a morphologically, phylogenetically, and functionally diverse group of protists. A remarkable variety of feeding mechanisms is found among them, including engulfing food cells, external digestion of food cells by means of a membranous veil (pallium feeding), or sucking out cell contents with an extensible feeding tube (myzocytosis) (Elbrächter 1991). In the field, many planktonic diatoms are

attacked and consumed by host-specific nano-protists of various taxonomic groups, which can cause high mortality rates among phytoplankton populations (Drebes et al. 1996; Kühn et al. 1996; Schnepf et al. 2000; Tillmann et al. 1999). Until now two dinoflagellate genera are known that are host-specific for diatoms. *Gyrodinium undulans* Hulbert feeds only on one diatom species and

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Abbreviations: BI, Bayesian inference; PP, posterior probabilities; PPC, *Paulsenella/Pfiesteria/cryptoperidiniopsoids*

(under laboratory conditions) on zooplankton eggs (Drebes and Schnepf 1998). The genus *Paulsenella* includes three species (*Paulsenella chaetoceratis* (Paulsen) Chatton, *P. kornmannii* Drebes and Schnepf, and *P. vonstoschii* Drebes and Schnepf) that specifically attack diatoms and take up cell contents by myzocytosis (Drebes and Schnepf 1988). *Paulsenella vonstoschii* has been observed to almost eliminate a diatom population (Drebes and Schnepf 1988). The life cycle of *Paulsenella* has been described in detail by Drebes and Schnepf (1982, 1988) but its taxonomic position has been unclear. The result of this phylogenetic study shows that *Paulsenella* is included in the *Pfiesteria*/*Amyloodinium* clade (Litaker et al. 1999) where it diverges prior to *Pfiesteria*. This is supported by the feeding behaviour and life cycle of *Paulsenella*, which is similar to that of *Pfiesteria* and the cryptoperidiniopsoids, the latter being an unclassified group of estuarine phagotrophic dinoflagellates (Steidinger et al. 2001).

Results

In our phylogenetic tree, *Paulsenella* diverges after *Amyloodinium ocellatum* and is sister to *Pfiesteria piscicida*, *P. shumwayae*, and cryptoperidiniopsoid sp. (Figure 1). This sister relationship is fairly robust with support values ranging from 93–92% posterior probabilities (PP) with the three replicate BI runs and 85% NJ bootstrap support. The support for the next higher clade containing all of these species plus *A. ocellatum* is even higher with 98% on each replicate run; however, bootstrap analysis places *A. ocellatum* with a basal polytomy separating all the major dinoflagellate clades. The short branch lengths separating the basal divergences can be seen in the branch length BI tree mirroring the consensus tree in Figure 1. *Paulsenella* is not closely related to other heterotrophic and parasitic dinoflagellates in our tree. The absolute number of base pair differences in the taxa in the clade to which *Paulsenella* belongs ranges from 26 to 37, which represents a

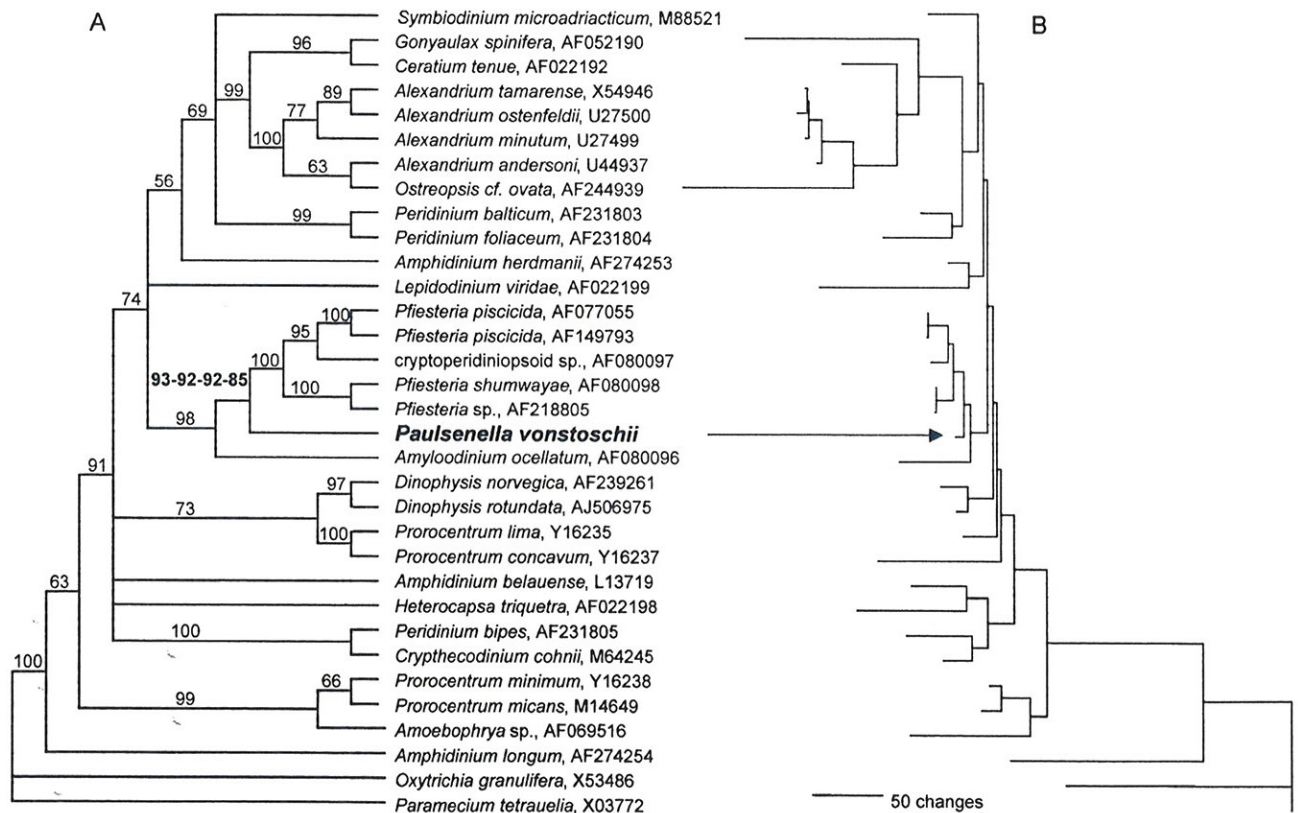
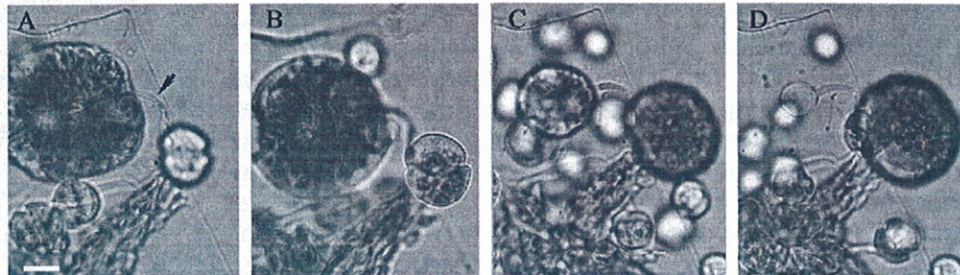


Figure 1. Phylogenetic reconstruction of the dinoflagellates showing the position of *Paulsenella* using Bayesian inference methods. **A.** Consensus tree of 1500 trees. Posterior probabilities are shown on each node. At the *Paulsenella* node, both the three replicate BI posterior probabilities and the NJ bootstrap value are presented. **B.** Branch length tree from the BI analysis inverted horizontally to match the labels of the consensus tree.

Table 1. Summary of the absolute number of base pair differences and pairwise differences between species in the clade to which *Paulsenella* belongs based on SSU rDNA.

	1	2	3	4	5	6	7
1 <i>Amphidinium longum</i>	—	0.041	0.041	0.037	0.039	0.038	0.030
2 <i>Pfiesteria piscicida</i> AF022199	50	—	0.001	0.026	0.026	0.023	0.028
3 <i>Pfiesteria piscicida</i> AF149793	50	1	—	0.025	0.026	0.024	0.029
4 <i>cryptoperidiniopsis</i> sp.	45	31	30	—	0.001	0.022	0.024
5 <i>Pfiesteria shumwayae</i>	47	32	31	1	—	0.023	0.025
6 <i>Pfiesteria</i> sp.	46	28	29	27	28	—	0.021
7 <i>Paulsenella vonstoschii</i>	37	34	35	29	30	26	—

**Figure 2.** Light micrographs of *Paulsenella vonstoschii* feeding on *Mediopyxis* Kühn, Hargraves & Medlin (time series approximately 1 h). **A.** Vegetative cell feeding with feeding tube (arrow) on plasmolysed diatom protoplast. Scale bar, 10 μ m. **B.** Cells with ingested chloroplasts contained in a single food vacuole in epicone. **C.** Cell increases in size; the infected diatom apparently attracts starved vegetative cells. **D.** Feeding tube is still attached to the diatom protoplast emptied of all cell contents. The enlarged dinoflagellate has lost its typical morphology and the dinokaryon is not discernible.

difference of 2 to 3% (Table 1). *Paulsenella* is extremely closely related to *Pfiesteria* but ranges from 4 to 19% difference to other members of the tree. The close relationship between these taxa can be seen by the branch-length BI tree mirrored to the consensus tree.

All *Paulsenella*/*Pfiesteria*/cryptoperidiniopsoids (PPC) and *A. ocellatum* potentially cause mortality in their hosts/prey (i.e., fish or microalgae) by feeding. Starved *P. vonstoschii* vegetative cells showed a rapid chemosensory response to *Helicotheca tamesis* and readily infected all host cells. *Belleriochea malleus* did not appear to induce a feeding response, but nonetheless a few cells were eventually infected. In the new centric diatom genus, *Mediopyxis* (Kühn et al. in press), some cells induced a feeding response by *Paulsenella*, leading to the aggregation of dinoflagellates, whereas other cells did not. Still only few vegetative cells succeeded to feed on the host protoplast (Figure 2 A–D). Ingestion of host cells, however, was far less successful than in *H. tamesis*; penetrating the frustule was presumably the largest problem.

Discussion

Our phylogenetic analyses have clearly placed *Paulsenella* into a dinoflagellate clade that is united by their mode of feeding. Dinospores are attracted to prey by chemosensory clues, feed myzocytotically by means of an extensible feeding tube, and can swell significantly during food uptake. The basal part of the *Paulsenella* peduncle is ensheathed by an extracytoplasmic tube, which is presumably cellulosic (Schnepf and Elbrächter 1992); this feature has not been investigated for *Pfiesteria*/cryptoperidiniopsoids. Whereas the host range of *Paulsenella* is restricted to either one diatom genus or closely related species, *Pfiesteria* and cryptoperidiniopsoid vegetative cells have a broader range of target organisms, feeding on various microalgae, bacteria, microfauna, and fish tissue (Burkholder et al. 2001; Glasgow et al. 2001; Lewitus et al. 1999; Seaborn et al. 1999; Vogelbein et al. 2002).

These taxa are also united by features of their life cycle (Parrow and Burkholder 2003b). All PPC described thus far have motile (flagellate) and

nonmotile (cyst) phases during their life histories (Drebes and Schnepf 1988; Litaker et al. 2002; Parrow and Burkholder 2003a,b). In the sexual reproduction cycle, two isogamous or anisogamous gametes fuse and form a triflagellated planozygote, with two longitudinal flagella and one transversal flagellum that engages in feeding activity (Parrow and Burkholder 2003a,b). Encystment of planozygotes leads to the formation of a zygotic cyst. Zygotic cysts exhibit nuclear cyclosis, a conspicuous phenomenon generally associated with meiosis in dinoflagellates (Elbrächter 2003), but see Litaker et al. (2002). Planozygotes in *Pfiesteria* (*P. piscicida*) and cryptoperidiniopsoids can also form non-reproductive temporary cysts (Parrow and Burkholder 2004). Nuclear and cytoplasmic division of the protoplast produce two secondary cysts, each of which releases two flagellated vegetative cells, thus producing four offspring in *Paulsenella* (Drebes and Schnepf 1988) and two offspring in *Pfiesteria* and cryptoperidiniopsoids (Parrow and Burkholder 2004). Deviations might be present in *Paulsenella* in which nuclear cyclosis was reported in both the vegetative stage and planozygotes, neither of which was reported to have two longitudinal flagella.

During asexual reproduction, the encystment of the vegetative stage leads to the formation of primary reproductive cysts. Sequential divisions, the number of which depend on the size of the vegetative stage (Drebes and Schnepf 1988 for *Paulsenella* and Parrow and Burkholder 2003b for *Pfiesteria*), lead to secondary or even tertiary cysts; in the case of the latter, eventually releasing eight offspring. Several types of cysts have been described in most PPC but were addressed by different terms: (i) zygotic cysts showing nuclear cyclosis (primary cyst, hypnozygotes, meiocysts, temporary cyst); (ii) division cysts used for asexual reproduction releasing 2 vegetative cells (reproductive cyst, mitotic cyst, zoosporangia, gametangia, primary, and secondary cysts, etc.); (iii) temporary cysts releasing one vegetative cell (non-reproductive, thin-walled); and (iv) resting cysts with thickened cell walls.

In recent literature, the exotoxin secretion of *Pfiesteria* has been the subject of debate. Vogelbein et al. (2002) showed that *P. shumwayae* vegetative cells kill fish by myzocytosis and not by toxin production; however, Burkholder et al. (2005) have recently reported it to be toxic. Likewise, no toxin production has been observed for the cryptoperidiniopsoids (Burkholder et al. 2005). It has to be noted that all life phases of *Paulsenella* spp., which is ancestral to the other PPC, are non-

toxic. This indicates that toxin production, if present, is likely to have evolved later.

Finally, some assumptions must be made for the tabulation pattern of *Paulsenella*. The Kofoidan tabulation pattern of *A. ocellatum* and *Pfiesteria piscicida* has been determined to be typical of the Peridiniales (Fensome et al. 1999; Landsberg et al. 1994). Studies by Drebes and Schnepf (1988), based solely on light microscopical observations, classified *Paulsenella* vegetative cells to be gymnodinioid and athecate. The plate tabulation of *Paulsenella* has not yet been investigated, but because *Amyloodinium*, the basal divergence in the clade to which *Paulsenella* belongs, is peridinioid and not gymnodinioid and because the sister group to *Paulsenella*, *Pfiesteria*, is also peridinioid, it follows that *Paulsenella* must also be peridinioid and not gymnodinioid. True gymnodinioid taxa are very distantly related to *Paulsenella*. Thus, given the position *Paulsenella* in our phylogenetic tree, it is very likely that this genus should be placed in the order Peridiniales. This entire group (PPC) cannot be considered gymnodinioid because two of its members have been determined to be peridinioid.

Methods

Culture and phylogenetic analysis: Several strains of *Paulsenella vonstoschii* were isolated in August 2003 from plankton samples collected with a 20 or 80 µm mesh plankton net from the Wadden Sea off List/Sylt (North Sea). Cultures were established by isolating *Helicotheca tamesis* (Shrubsole) Ricard infected with *Paulsenella vonstoschii* with a mouth pipette. Dinoflagellates were maintained in culture with *Helicotheca tamesis* in plastic Petri dishes in modified f/2 medium at 15 °C on a 16:8 light : dark cycle. When most diatoms were infected, a few µl of infected cultures were transferred into new host cultures (various approaches to cryopreserve *Paulsenella vonstoschii* were not successful). For host range studies, cells of *Bellerochea malleus* Brightwell and *Mediopyxis* (member of the Lithodesmiales forming short colonies) were added to vegetative cell cultures. Dinoflagellates were used directly for DNA extraction. PCR amplification and sequencing was performed with minor modifications according to the protocol used by Kühn et al. (2004). For the phylogenetic analysis, an 18S data set was downloaded from an ARB software database (<http://www.arb-home.de>) to which published and unpublished dinoflagellate sequences were added to represent a range of

dinoflagellates that would permit the accurate placement of the *Paulsenella* sequence. We selected at least one representative of each order of dinoflagellates for our data set. The data set contained 33 taxa and 1300 nucleotides, which were selected, based on a filter determined by the presence of each nucleotide selected in at least 50% of all taxa. This data set was analysed using Bayesian inference (Huelsenbeck et al. 2001). The analysis was done using MrBayes V. 3.0 (<http://morphbank.ebc.uu.se/mrbayes/>). We ran the Bayesian search three separate times using the general time reversal GTR model with an undefined gamma distribution, during 3,000,000 generations, and saving every 1000th tree. We discarded the first 1500 trees; the remaining 1500, all with higher PP, were used to construct a consensus tree onto which 'credibility values' for each clade are shown, which represent the percentage of those 1500 trees having the corresponding clades. A branch length tree from this analysis with a high log likelihood score mirrors the consensus tree so that distances between taxa can be seen. We also performed a bootstrap analysis of 1000 replicates using a minimum evolution analysis with maximum likelihood distances within PAUP* (Swofford 2002). The bootstrap values for *Paulsenella* are placed on the BI tree. A table of absolute base differences and pairwise distances for the closest relatives of *Paulsenella* was calculated from the minimum evolution analysis.

P. vonstoschii can be obtained from Stefanie Kühn, University of Bremen, and the alignment will be made available upon request by Linda Medlin.

Nucleotide Accession Numbers: The *Paulsenella* SSU rDNA sequence has the accession number AJ968729.

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