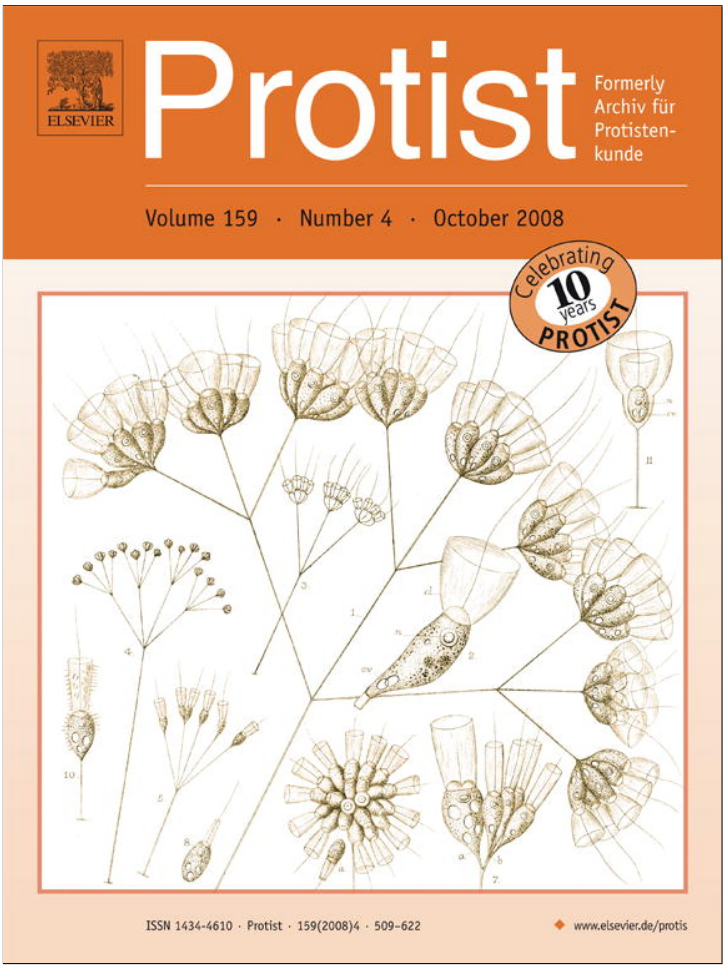

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ORIGINAL PAPER

Description, Host-specificity, and Strain Selectivity of the Dinoflagellate Parasite *Parvilucifera sinerae* sp. nov. (Perkinsozoa)

Rosa Isabel Figueroa[1,2], Esther Garcés[1,2], Ramon Massana, and Jordi Camp

Institut de Ciències del Mar (ICM-CSIC), Pg. Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain

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A new species of parasite, *Parvilucifera sinerae* sp. nov., isolated from a bloom of the toxic dinoflagellate *Alexandrium minutum* in the harbor of Arenys de Mar (Mediterranean Sea, Spain), is described. This species is morphologically, behaviourally, and genetically (18S rDNA sequence) different from *Parvilucifera infectans*, until now the only species of the genus *Parvilucifera* to be genetically analyzed. Sequence analysis of the 18S ribosomal DNA supported *P. sinerae* as a new species placed within the Perkinsozoa and close to *P. infectans*. Data on the seasonal occurrence of *P. sinerae*, its infective rates in natural and laboratory cultures, and intra-species strain-specific resistance are presented. Life-cycle studies in field samples showed that the dinoflagellate resting zygote (resting cyst) was resistant to infection, but the mobile zygote (planozygote) or pellicle stage (temporary cyst) became infected. The effects of light and salinity levels on the growth of *P. sinerae* were examined, and the results showed that low salinity levels promote both sporangial germination and higher rates of infection. Our findings on this newly described parasite point to a complex host—parasite interaction and provide valuable information that leads to a reconsideration of the biological strategy to control dinoflagellate blooms by means of intentional parasitic infections.

Key words: dinoflagellates; *Parvilucifera*; parasites; perkinsids; toxic phytoplankton.

Introduction

Within less than a year, three publications (Delgado 1999; Erard Le Denn et al. 2000; Norén et al. 1999) reported the existence of apparently very similar parasitic flagellates. The reported organisms had a shared outer morphology during the immobile parasite stage and the ability to kill dinoflagellates, a well-known group of microalgae that are the causative agents of harmful algal blooms (HABs) in many coastal waters.

The genus of parasites, *Parvilucifera*, was defined by Norén et al. (1999), whose findings suggested that *Colpodella* and *Perkinsus*, together with *Parvilucifera*, represent a distinct group that combines the characteristics of dinoflagellates and apicomplexans. These authors proposed considering this new group, the Perkinsozoa, as a taxon on the same level as the Dinoflagellata, Apicomplexa, and Ciliophora, thus adding a fourth phylum to the alveolates. Nevertheless, the existence of the phylum needs to be

e-mail figueroa@icm.csic.es (R.I. Figueroa), esther@icm.csic.es (E. Garcés).
[1]Corresponding authors.
[2]The authors contributed equally to this work.

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clarified. Perkinsids were long considered apicomplexan-like flagellates because they posses an apical complex reminiscent of those in apicomplexans and colpodellids (see Leander and Keeling 2004 for revision). However, molecular phylogenies strongly indicate that dinoflagellates and apicomplexans are more closely related to each other than to ciliates, and that *Perkinsus* is more closely related to dinoflagellates than to apicomplexans (Fukuda and Endoh 2008; Shalchian-Tabrizi et al. 2006). Additionally, recent molecular surveys from the marine picoplankton have also recovered two additional independent alveolate groups, closer to dinoflagellates, that are mostly undescribed and of unknown morphology (López-García et al. 2001).

The first *Parvilucifera* species known, *P. infectans*, was originally isolated from the dinoflagellate genus *Dinophysis* on the west coast of Sweden (Norén et al. 1999). The *Parvilucifera*-like parasite described by Delgado (1999) was isolated from a red tide of *Alexandrium catenella* in the Mediterranean Sea, although the description did not include genetic analyses to establish the parasite's identity.

Parvilucifera has also subsequently been identified in both North America and Australia (Moestrup and Norén 2003), and a new species, *Parvilucifera prorocentri*, was recently described based on morphological features (Leander and Hoppenrath 2008). However, the morphology of this species differs remarkably from that of *P. infectans*, e.g., with respect to the nature of the sporangial envelope surface and the existence of a germ tube, which is a feature of *Perkinsus* sporangia. In the absence of genetic analysis, the position of this species in the genus remains to be determined. Studies have shown that, although *Parvilucifera infectans* infects several species of dinoflagellates, its main target is *Alexandrium* spp., a very important genus that is responsible for paralytic shellfish poisoning (Delgado 1999; Norén et al. 1999). By contrast, *P. prorocentri* is known to infect only *Prorocentrum fukuyoi* (Leander and Hoppenrath 2008).

The infectivity and growth parameters of parasites are known to be influenced by environmental conditions (Van Donk and Bruning 1995). For example, light and phosphorous levels modulate infection of the diatom *Asterionella formosa* by the cytrid *Rhizophydium planktonicum*, through control of the production and generation time of new zoospores (Bruning 1991a, 1991b). These results underline the relevance of studying infectivity and the effects of physical factors in order to understand and forecast the development of parasitic outbreaks. Studies of this kind make even more sense if the parasite is to be used to control the blooming of a second species — a strategy that has been proposed to counter dinoflagellate proliferations (Anderson 1997).

In April 2007, live samples obtained from a bloom of *Alexandrium minutum* in the harbour of Arenys de Mar (Mediterranean Sea, Spain) were studied in our laboratory. After only 5 days, the *Alexandrium minutum* population was exterminated by a parasitic infection, whereas the population of *Prorocentrum triestinum*, initially present at a very low concentration, grew and eventually replaced *A. minutum* as the main representative population. Our observations suggested that the "culprit species" of parasite differed from *P. infectans*, which is known to infect the genus *Prorocentrum* (Delgado 1999). This hypothesis was the starting point of the present study, which had two main objectives: first, to identify and describe the parasite, and second, to understand its seasonal occurrence, host specificity, and the environmental factors controlling its virulence. To achieve these goals, the morphological characteristics of the parasite were examined by light microscopy and scanning electron microscopy (SEM), while its genetic identification was based on the nucleotide sequence of its 18S ribosomal gene. Additionally, field and laboratory samples of dinoflagellates were infected under different environmental conditions. Our results indicate that a morphologically and genetically new *Parvilucifera* species (*Parvilucifera sinerae* sp. nov.) inhabits the Mediterranean coasts of Spain, and that the infection by this new parasite is specific to different species and strains of dinoflagellates.

Results

Preliminary Observations and Parasite Bloom Dynamics

The presence of a *Parvilucifera*-like parasite was observed during a red tide of *Alexandrium minutum* in 2006. The parasite was selective within the phytoplanktonic community in that species accompanying *A. minutum* were either not or only slightly infected. During the four months of sampling (December 2005 until April 2006), *A. minutum* densities increased from 10^2 to 10^5 cells L^{-1} during the developmental phase from January to February 2006. The maintenance

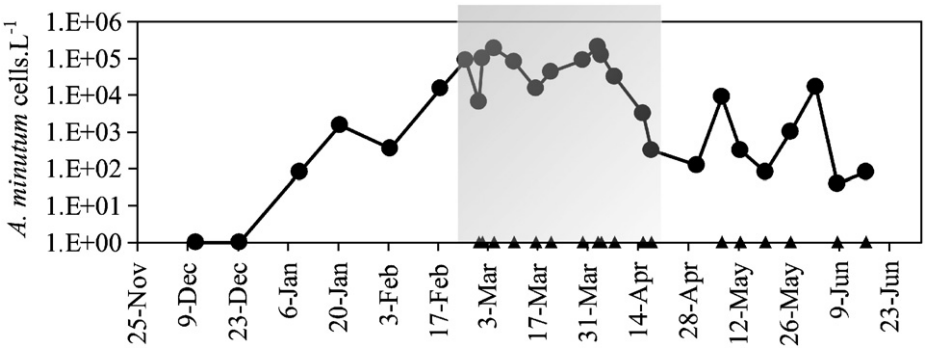


Figure 1. Temporal fluctuations in the cell density of *Alexandrium minutum* (cells L^{-1} ●) in the surface waters of the Arenys de Mar harbor in 2005—2006. ▲ Field samples incubated in the laboratory. Shadowed area: positive samples infected by *Parvilucifera* (presence of mature sporangia).

phase of the dinoflagellate bloom occurred in March to early April 2006. The decline phase of the bloom, during early May, was marked by a sharp decrease in cell numbers (Fig. 1). The parasate *Parvilucifera* sp. was identified in 67% of the samples collected by inspecting and was coincident with high cell densities of *A. minutum* (10^5 cells L^{-1}) during March and early April 2006. Infected *A. minutum* cells were immobile and nuclear staining revealed a diffuse nuclear area. Dark sporangia partially conserved the theca of *A. minutum*. Unfixed water samples showed that the sporangia released small flagellated cells.

The other main dinoflagellates accompanying *A. minutum* during the bloom, such as *Dinophysis sacculus*, *Scrippsiella* sp., and *Prorocentrum micans*, did not appear to be infected by the parasite. *Scrippsiella* sp. peaked at the same time as *A. minutum*, reaching a density of 30,000 cells L^{-1}, whereas the maximum densities of *D. sacculus* and *P. micans* were only 1000 and 5600 cells L^{-1}, respectively.

Isolation of *Parvilucifera sinerae* sp. nov. and Rates of Infection of Vegetative and Sexual Stages of *A. minutum* in Natural Samples

On April 3, 2007, sexuality was induced in a subsample of *Alexandrium minutum* consisting of cells isolated by filtration of a natural-bloom sample, obtained as part of a field study of sexual reproduction in *A. minutum*. After 2 days, $4.3 \pm 1.8\%$ of the population was killed by the infection, and $96.9 \pm 2.2\%$ of the population was killed after only 5 days. Infection began 2 days after sexual induction, but this did not prevent gamete fusion (Fig. 2a) or planozygote formation. The presence of planozygotes was confirmed after nuclear staining, based on the occurrence of nuclear morphologies present only during syngamy (Figueroa et al. 2006) (Fig. 2b). The planozygotes were counted by flow cytometry following the method of Figueroa et al. (2007), and a concentration of 20.9 ± 5.7 planozygotes mL^{-1} was determined. However, the final yield was only 4.4 ± 0.5 resting cysts mL^{-1}, which pointed to active planozygote infection. By contrast, resting cysts were resistant to parasite infection.

Several sporangia (parasite immobile stages) were isolated from this experiment and a parasite culture was subsequently established.

Morphology of the Flagellated Zooid and Sporangium of *Alexandrium minutum* by SEM

The free-living stage of *Parvilucifera sinerae* sp. nov. is a flagellated zooid with an elongated body, about 2.7 μm long by 2.2 μm wide, that is refractile and characterized by the presence of two flagella (Fig. 3a). The posterior flagellum is bare, about 1.5 μm long, and progressively thinner towards the end (Fig. 3b, c). The anterior flagellum exhibits hairs over its full length and it is about 7 μm long (Fig. 3d). Late stages of infection were easily recognized because the parasite caused the dinoflagellate body to become round (Fig. 4a), killed the host, and provoked detachment of the dinoflagellate thecal plates (Fig. 4b) so that a sporangium eventually formed (Fig. 4c). The

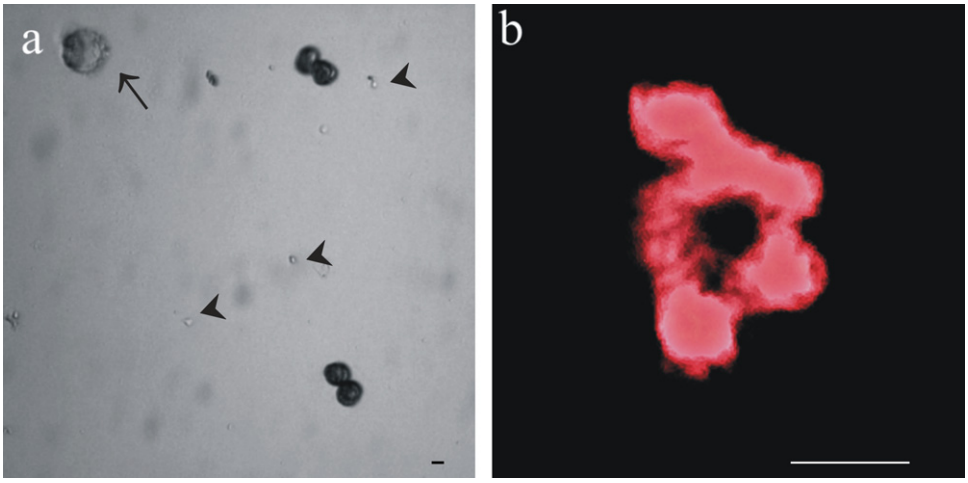


Figure 2. **a**. Fusing gamete pairs of *Alexandrium minutum* in an infected natural population in which there is active sporangial germination (arrow) and swimming zooids (arrowheads). **b**. Zygote formation was confirmed by the presence of cells with nuclear morphologies that are present only during syngamy. Scale bars: 10 μm in **a,** and 5 μm in **b**.

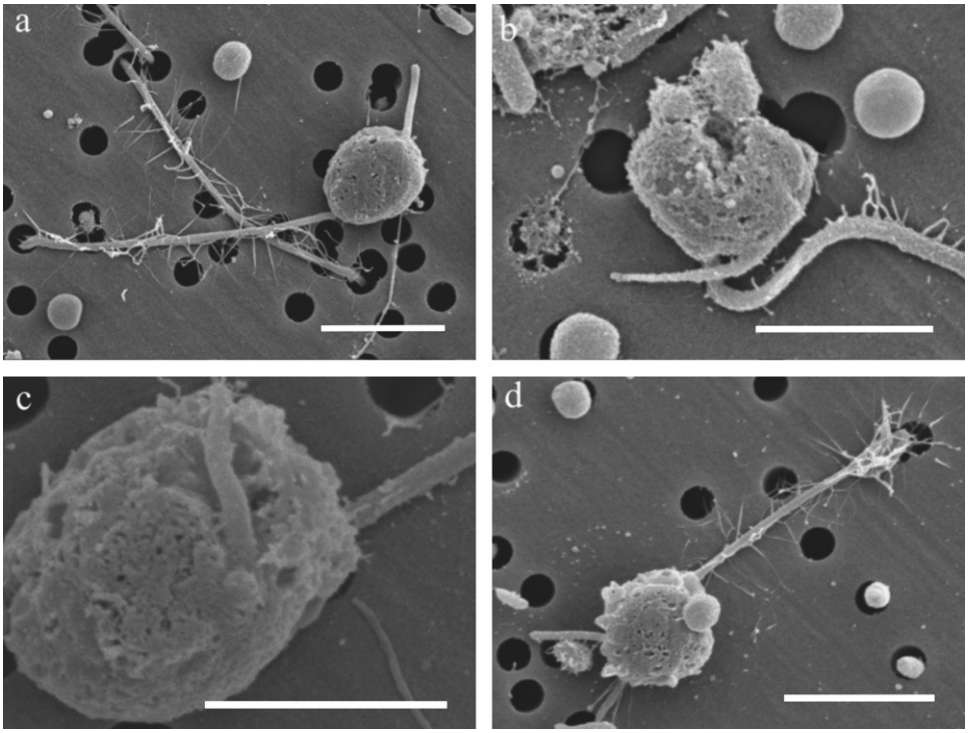


Figure 3. Morphology of the zooid stage of *Parvilucifera sinerae* as seen with SEM. Scale bars: 3 μm in **a** and **d**, 2 μm in **b** and **c**.

mature sporangium (13.4—44.9 μm, depending on the host) is characterized by the presence of an outer net of star-shaped filaments (Fig. 4d). Zooids escape from the mature sporangium through 4—5 apertures in the wall (Fig. 4e). Each aperture is covered by an operculum, which was open in empty sporangia (Fig. 4f).

The longevity of the zooid once it had emerged from the host was about 10 min when placed in a drop of 50 μL.

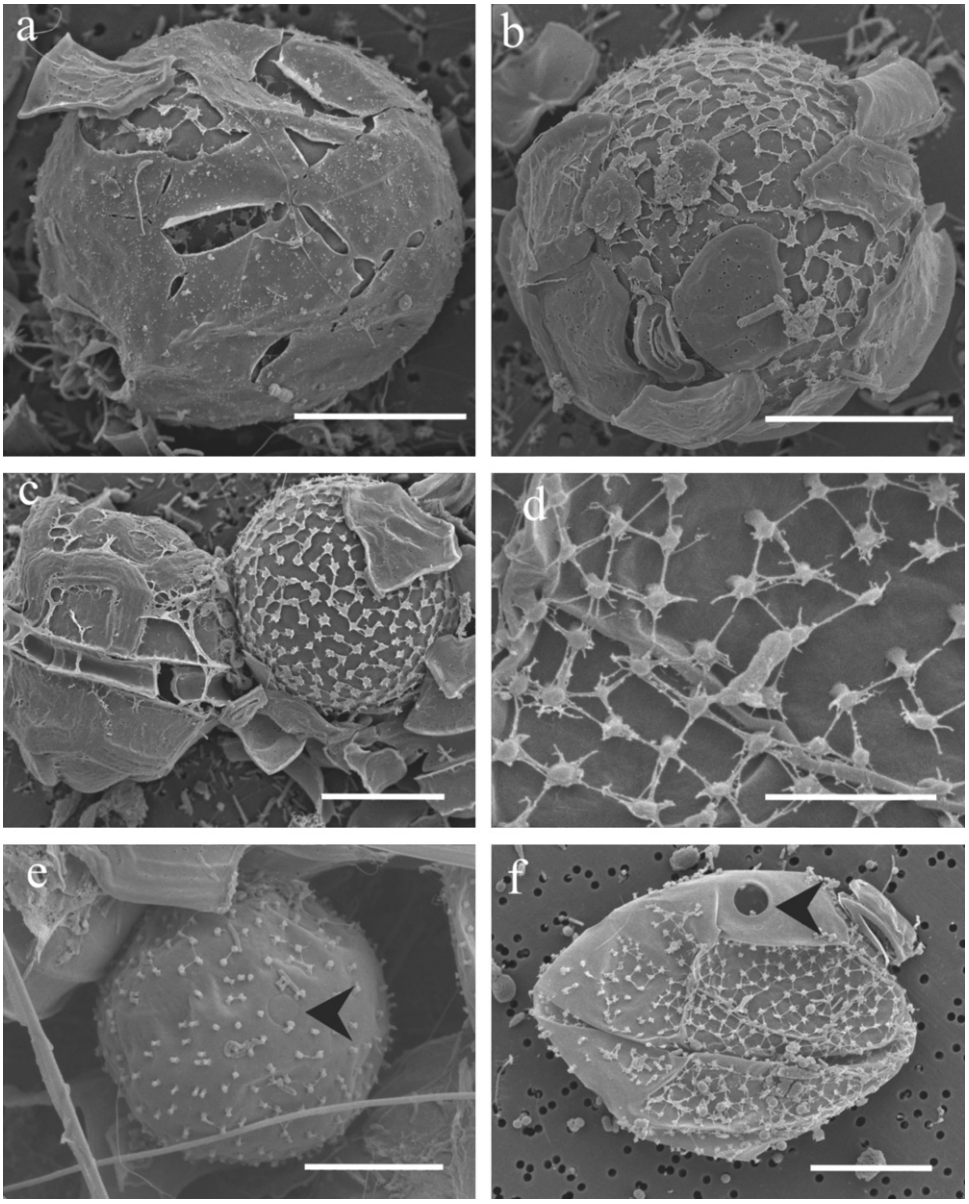


Figure 4. Sporangium morphology of *Parvilucifera sinerae* as seen with SEM. **a**. Sporangium still covered by most of the *Alexandrium minutum* cell thecal plates. **b**. Sporangium almost free of thecal plates. **c**. Sporangium and non-infected *Alexandrium minutum* cell. **d**. Detail of the sporangium reticulation pattern. **e**. Closed operculum (arrow head). **f**. Empty sporangium showing opened operculum (arrow head). Scale bars: 10 μm except in **d,** which is 3 μm.

Infection Studies with *Parvilucifera sinerae* sp. nov.

Table 1 lists the species tested and indicates their sensitivity or resistance to infection. *Heterosigma akashiwo*, *Prorocentrum micans*, *P. triestinum*, *Alexandrium taylorii*, *Gyrodinium impudicum*, and *Scrippsiella trochoidea* were resistant to infection, whereas *Alexandrium catenella*, *A. peruvianum*, *A. ostenfeldii*, and *Gymnodinium catenatum* were infected. However, although the previously explained field experiment confirmed 100% infection of *A. minutum*, geographically distant strains of *Alexandrium minutum* and *Protoceratium reticulatum* strains could not be infected (Table 1). The times and levels of infection were dependent

Table 1. Infectivity of *Parvilucifera infectans* and *Parvilucifera sinerae* in marine dinoflagellates, *n*(cultures infected) > 2.

	Strain	History of the cultures		Infecting species	
		Year of isolation	Location	*P. infectans*	*P. sinerae*[a]
Athecate host species					
Akashiwo sanguinea	CCMP1321	1997	USA	Yes	**No**
Gymnodinium catenatum	VGO 12V	1993	Vigo (Spain)	Yes	Yes
	VGO 743	2004	Argelia		
Gymnodinium impudicum	ICMB 209	1996	Barcelona (Spain)	—	No
Gymnodinium nolleri	922I	1998	Kattegat (Denmark)	—	Yes
Karlodinium veneficum	Karlo 5	2000	Alfacs (Spain)	—	No
Karenia brevis	CCMP 718 (Wilson clone)	1960	Florida (USA)	—	Yes
Thecate host species					
Alexandrium andersonii	ICMB 222	2007	Alfacs (Spain)	Yes	Yes
Alexadrium catenella	Cat G01	2006	Tarragona (Spain)	Yes	Yes
Alexandrium insuetum	ICMB 218	2006	Arenys del Mar (Spain)	—	Yes
Alexandrium margalefii	ICMB 220	2007	Alfacs (Spain)	—	Yes
Alexandrium minutum	VGO 651	2003	Port Saint Hubert		**No**[b]
	VGO 663	2003	(Brittany)	Yes	**Yes**
	VGO A7	2005	Baiona (Spain)		Yes
	Natural sample	2007	Arenys del Mar (Spain)		Yes
Alexandrium ostenfeldii	AT01	2001	Tvärminne (Baltic Sea)	Yes	Yes
Alexandrium peruvianum	VGO 10C	2002	Palamós (Spain)	Yes	Yes
	VGO 11C	2002			
Alexandrium taylorii	CSIC-AV8	1995	La Fosca (Spain)	No	No
Kryptoperidinium foliaceum	VGO B9	2005	Baiona (Spain)	No	No
Protoceratium reticulatum	VGO 758	2003	Els Alfacs (Spain)	—	**Yes**[b]
	CCMP 1721	1994	Florida (USA)		No
Prorocentrum micans	Natural sample	2007	Arenys del Mar (Spain)	Yes	No
Prorocentrum triestinum	Natural sample	2007	Arenys del Mar (Spain)	—	No
Scrippsiella trochoidea	ICMB 213	1988	Plymouth (USA)	Yes	**No**
	Natural sample	2007	Arenys del Mar (Spain)		
Diatoms					
Thalassiosira weissflogii	CCAP 1085	1999	UK	—	**No**

[a]Data of *P. infectans* are based on Delgado (1999) and the review of Park et al. (2004).
[b]Strain specificity. Differences between both species are highlighted in bold.

on the species studied. Sensitive *Alexandrium* strains were infected the fastest, with the population becoming extinct in ⩽6 days. By contrast, *Karenia brevis* and *Gymnodinium catenatum* were infected relatively slowly and the cultures were still alive up to 2 weeks after infection (Table 2).

Table 2. Times for host extinction of *Parvilucifera sinerae* in dinoflagellate cultures ($n = 2$).

	Strain	Time for host extinction (<10 cell mL^{-1})
Athecate host species		
Gymnodinium catenatum	VGO 12V	>15 days
Karenia brevis	CCMP 718	>15 days
Thecate host species		
Alexandrium andersonii	ICMB 222	6 days
Alexadrium catenella	Cat G01	5 days
Alexandrium insuetum	ICMB 218	5 days
Alexandrium margalefii	ICMB 220	3—4 days
Alexandrium minutum	VGO 651	Resistant
	VGO 663	5 days
	VGO A7	5 days
Alexandrium ostenfeldii	VGO517	5 days
	AT01	5 days
Alexandrium peruvianum	VGO 10C	6 days
	VGO 11C	5 days
Protoceratium reticulatum	VGO 758	6 days
	CCMP 1721	Resistant
	CCMP 404	5—6 days

Morphologically very similar species behaved very differently. For example, the related species *Gymnodinium nolleri* and *Gymnodinium catenatum* had different parasite sensitivities, because *G. nolleri* was infected faster than *G. catenatum*, which was also infected to a lesser extent. Moreover, *G. impudicum*, easily confused with *G. catenatum* because of their morphological similarity, was resistant to infection (Table 1).

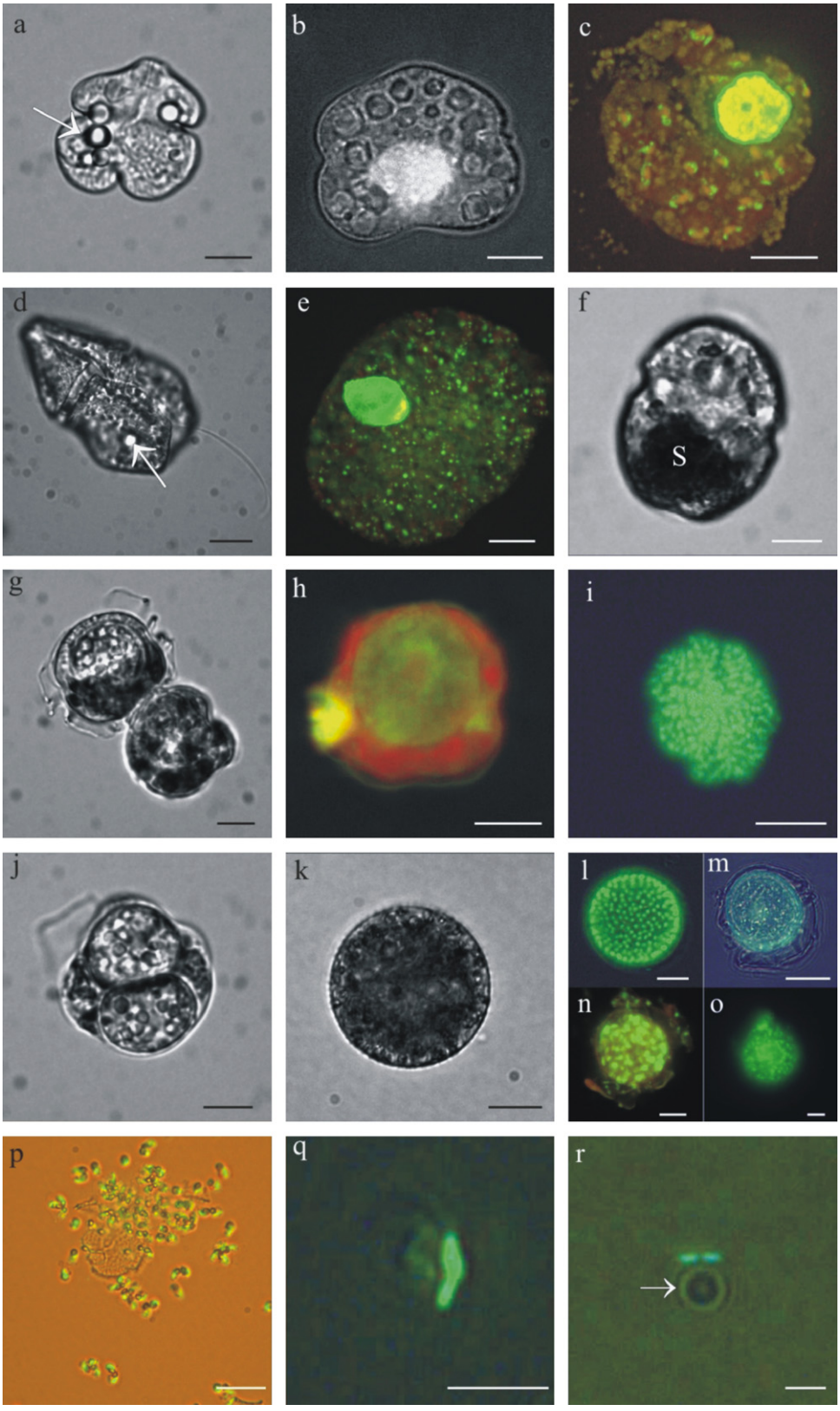
The diatoms tested, *Thalassiosira weissflogii* and *Stephanodiscus* sp. were not infected.

Direct microscopic examination of living or preserved cells allowed mature infections to be identified based on the presence of very dark and spherical sporangia. Early stages of infection were recognizable using nuclear staining, which revealed two distinct infection pathways that depended on the host species:

(i) *Cytoplasmic infection*: In athecate species, such as *Karenia brevis* (Fig. 5a—c) and *Gymnodinium catenatum* (Fig. 5d—f), early stages of infection were distinguishable by the presence of vacuole-like structures in the cytoplasm (Fig. 5a, b, d). These vacuoles contained the zooid stage, because the zooid nuclei became apparent after nuclear staining (Fig. 5c, e). At late stages of infection, the sporangium accounted for only part of the cell volume (Fig. 5f), in contrast to thecate species, as discussed below. Cytoplasmic infections were also observed in *Gymnodinium nolleri*.

Because the nucleus of the host was intact in slightly vacuolated cells, the dinoflagellates were viable for a longer period of time, so they could swim and even divide until the concentration of zooids became too high, which eventually provoked cell death and sporangium formation.

(ii) *Nuclear infection*: In thecate species, such as *Alexandrium catenella*, infection was first detectable by the appearance of a dark area in the antapex (Fig. 5g, h), a morphology related to chloroplast degradation and infection of the U-shaped nucleus (Fig. 5i). This process was usually accompanied by detachment of the dinoflagellate theca (Figs 5g and 4). In general, a host cell gave rise to a single sporangium, although the formation of two sporangia per cell was sometimes observed (Fig. 5j). Later, the sporangium matured and became darker. This final morphology was quite similar at the light-microscopy level in all species tested (Fig. 5k). The nuclei of numerous zooids per sporangium were observed after SYBR green staining, although the number and pattern distribution of the zooids within each of the sporangia varied among the host species studied (Fig. 5l—o). After a brief



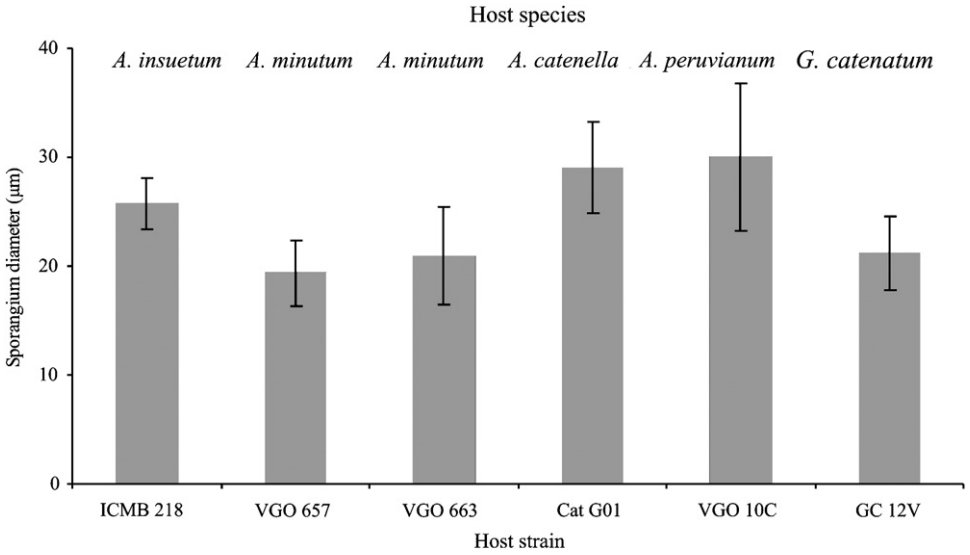


Figure 6. Sporangial size ($n = 30$) in different species and strains. Values are means $\pm$ SD.

maturation period (24—48 h), the sporangium liberated the zooids, which were identifiable by their refractile bodies (Fig. 5p, r). Frequently, the nucleus of the zooid was elongated (Fig. 5q), and two nuclei per zooid, perhaps indicating mitosis, were observed on a few occasions (Fig. 5r). The number of zooids liberated per sporangium depended on the latter's size and ranged from 50 to 500. It generally took 5—10 min for most zooids to leave a sporangium. After the zooids were released, a residual body was seen inside the sporangium.

In our study, sporangium size was directly related to the particular species and strain (Fig. 6). There was a positive relationship between the size of the species and that of the sporangia in the thecate species, with smaller species (*A. minutum* 13—25 μm length, 13—22 μm width) associated with smaller sporangia. However, this was not the case for athecate species: *G. catenatum* was the largest species tested (38—53 μm length, 33—45 μm width) but its sporangia were similar in size to those of *A. minutum* (Fig. 6).

Effects of Salinity and Light Levels on Infection Rates and Sporangia Germination

Experiments to examine the effects of salinity and light conditions on infection rates showed that while light intensity did not significantly affect the infection rate, lower salinity levels did, low salinity levels promoting higher infections rates (Infected cells = 450+225Salinity, $F = 8{,}33$; $P < 0.05$).

Based on the rates of infection of *Alexandrium* cultures, the time needed by the sporangia to germinate was determined to be 24—48 h. To establish the minimum time for sporangial germination and test whether mobile stages emitted a signal that induced this process, thirty-six sporangia were isolated in 1 L medium containing different salinities and following different isolation

Figure 5. Stages of *Parvilucifera sinerae* and infection of different host species. Examples of cytoplasmic infection in *Karenia brevis* (**a—c**) and *Gymnodinium catenatum* (**d, e, f, n; g, h, i**). The arrows indicate vacuole-like structures and S means sporangium. The various stages of nuclear infection in *Alexandrium catenella* (**g, h, i**). Formation of two sporangia inside an *Alexandrium minutum* cell (**j**). Mature sporangium (**k**). Sporangial morphology in different host species after sybr staining: (**l**) *Protoceratium reticulatum*; (**m**) *Alexandrium minutum*; (**n**) *Gymnodinium catenatum*, and (**o**) *Alexandrium insuetum.* Sporangium liberating zooids (**p**). Elongated parasite nucleus after sybr staining (**q**). Presumably parasite mitosis with sybr staining and refractile body (arrow) (**r**). Scale bars: 10 μm unless in q and r in which are 1 μm.

conditions. This approach resulted in the following observations: (i) The lack of a mobile dinoflagellate culture delayed the time needed for the sporangia to germinate from around 2 to about 6 days (data not shown); (ii) the presence of a non-infective life stage of a dinoflagellate (resting stage) did not affect sporangial germination, because germination proceeded within a period of time similar to that needed in the absence of the resting stage; (iii) salinity levels affected significantly both the germination time and the ability of the sporangia to germinate (ANOVA at $P<0.05$). At a salinity of 31, the time needed for germination was 5.6 ± 1.8 days whereas at a salinity of 20 only 3.3 ± 1.7 days were needed; likewise, the percentage of the population that germinated increased from 58% to 96%.

Phylogenetic Analyses

Complete SSU rDNA sequences were obtained from a group of approximately 50 individually isolated sporangia. The sequence consisted of 1780 characters, with no ambiguous position found. The sequence alignment is available upon request. The parasite strain isolated from the Arenys de Mar harbor grouped together with *P. infectans*, the only sequenced member of the genus *Parvilucifera*. The difference among the two

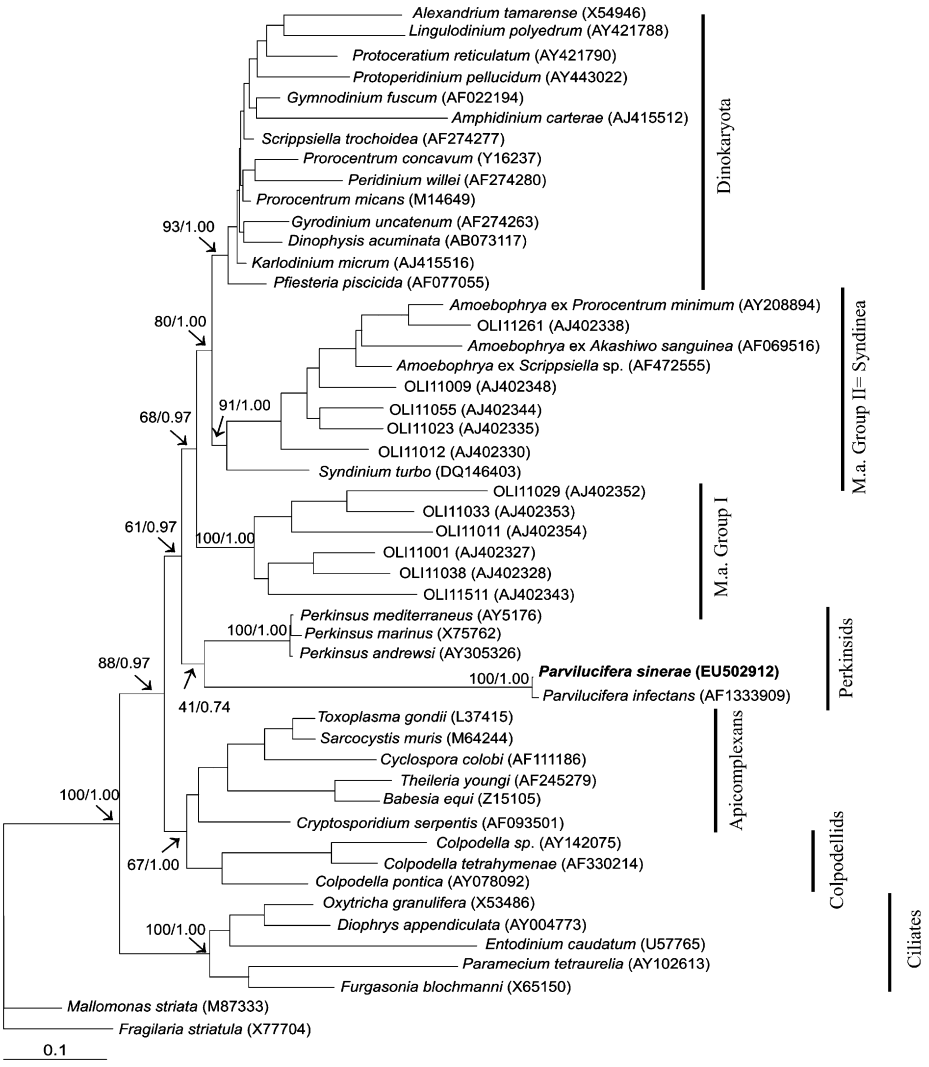


Figure 7. Small subunit rRNA tree based on the full length of the gene (1660 positions), inferred with Bayesian inferences applying the GTR+G+I model. Maximum-Likelihood analysis following 1000 boostrap resampling. Maximum-Likelihood Boostrap Values (left), Bayesian Posterior Probabilities (right).

species was 1.2% (20 different position in 1713 bp compared) (Fig. 7). There was a low support (BVs < 50%) and moderate Posterior Probabilities (PP = 0.74) for a clade comprised of *Parvilucifera* and *Perkinsus* species (including *P. andrewsi, P. mediterraneus and P. marinus*). The Perkinsozoa (*Parvilucifera* and *Perkinsus*) also grouped with moderate support in the ML analyses with a cluster comprised of marine alveolates Groups I and II and Dinokaryota (BVs < 70%). However, Bayesian Posterior Probabilities were high for this clade (PP = 0.97).

Discussion

Eukaryotic parasites have long been considered potential agents for controlling the noxious and/or toxic episodes caused by dinoflagellate blooms in marine environments (Coats and Bockstahler 1994; Coats et al. 1996; Nishitani et al. 1985; Salomon et al. 2003). In the present study, morphological, behavioural and molecular data provided the basis for the first description of *Parvilucifera sinerae*, a new species of dinoflagellate parasite belonging to the genus *Parvilucifera*.

Morphological Characteristics

Until now, *Parvilucifera infectans* was the only species of the genus that had been genetically characterized. *P. infectans* infects a number of different dinoflagellate species, including several species of *Alexandrium*, *Prorocentrum*, and *Dinophysis* (Delgado 1999; Norén et al. 1999). Morphologically, *Parvilucifera sinerae* is very similar to *P. infectans* with respect to its mobile zooid stage, which, by light and negative staining (TEM), was shown to have a large refractile body and two dissimilar flagella (one long, one short). The nucleus is oval, although it becomes U-shaped during mitosis. The morphology of the sporangium is also similar in relation to the presence of a surface reticulation pattern, which however, looks different (Moestrup, personal communication). Recently, another new species of *Parvilucifera* was reported (Leander and Hoppenrath 2008); however, its position within the genus is not clear and the morphology of the sporangium, of only 5—15 μm and consisting of spherical bodies and amorphous spaces, significantly differs from that of either *P. infectans* or *P. sinerae*.

When compared to zooids of other groups, *P. sinerae* zooids are smaller than those of *Sydindinium* (Marine Alveolate Group II), which survive longer (up to 2 days in filtered seawater) (Skovgaard et al. 2005). Few details are known for comparison to *Duboscquella* (Marine Alveolate Group I), whose zooids have similar size and shape. Other characteristics, such as the morphology of the flagella, have not yet been studied (Harada et al. 2007).

Infection Pathways

After penetrating the host, *P. sinerae* grows and divides to yield 50—500 flagellated cells, thereby transforming the degraded host cell into a sporangium. The number of zooids per sporangium and the morphology and size of the sporangium depend on the host species. After 1—2 days, the sporangium releases the swimming zooids, which infect a new host. From infection until the formation of new sporangia takes 1—5 days, depending on the host species. However, in contrast to what has been reported for *P. infectans*, *P. sinerae* did not significantly infect *Scrippsiella trochoidea* and *Akashiwo sanguinea*, even when field and laboratory specimens were tested.

P. sinerae infection proceeds by two different mechanisms. In athecate species, infection is first noted by the formation of vacuole-like structures within the host cytoplasm. These presumably function as a primary defense mechanism against infection. In fact, athecate species are infected more slowly than thecate species, in which nuclear infection is the faster pathway. For example, *Alexandrium* and *Protoceratium* were infected relatively quickly, such that the population was totally extinct in about 6 days. To our knowledge, neither the early stages of infection nor zooid proliferation patterns have been described for *P. infectans*.

Factors Influencing Maturation Times and Germinability

Temperature, host availability, and salinity have been suggested to explain the geographical distribution of *P. infectans* (Johansson et al. 2006). Regarding the behaviour and physiology of *P. sinerae*, we observed the following:

(i) Laboratory studies showed that the maturation times of individually isolated sporangia were longer than those of sporangia remaining in culture, suggesting that host chemical signals promote sporangial germination. Moreover, field studies corroborated the existence of a

host-density-dependent factor, because the occurrence of *P. sinerae* coincided with seasonal peaks in the density of *A. minutum*. A similar observation was reported by Johansson et al. (2006) for the spatial distribution of *P. infectans* on the Swedish coast. These authors proposed that the parasite needed a threshold host abundance to successfully proliferate, given the short survival time of the infective zooid outside the host (Delgado 1999). However, this general pattern has not been described for other protist parasites of dinoflagellates. For example, the infection pathways of *Amoebophyra* are mainly related to environmental factors, not to biological ones, because a low phosphorous concentration rather than high densities of *A. catenella* correlate with *Amoebophyra ceratii* infection (Nishitani et al. 1985).

(ii) Decreasing salinity levels increased sporangial germinability and *P. sinerae* infection rates. These observations were consistent with those of Delgado (1999), who reported that the exit of *Parvilucifera* sp. zooids from cysts was enhanced by the addition of freshwater. This finding is in contrast to the observations of Johansson et al. (2006), who noted that *P. infectans* was only found in the most saline coastal regions of Sweden. However, it must be considered that salinities in the Mediterranean Sea are considerably higher. Rapid sporangial germination may not be synonymous with an optimal survival strategy, because there must be an adequate coincidence of time and space regarding sporangial germination and host availability to assure the survival of parasite offspring. Other factors, such us temperature and light, may also affect sporangial germination, given that sporangia can be activated by shifting cysts from storage in a refrigerator (darkness and low temperature) to room conditions (Erard Le Denn et al. 2000; Norén et al. 1999).

Life-Cycle Stages Sensitive to Infection

Dinoflagellates have been reported to form pellicle (also called temporary) cysts when exposed to filtrates of infected cultures of *Parvilucifera*. For example, Toth et al. 2004 reported that, upon infection by *P. infectans*, *Alexandrium ostenfeldii* formed pellicle cysts resistant to the parasite. However, results from the species tested in this study indicate that this is a short-lived strategy and that these cells also soon become infected. Different stages of the dinoflagellate life cycle, from vegetative cells to sexual forms, are characterized by distinct features. Although sexual cysts are resistant, swimming planozygotes are vulnerable to infection. This implies that the success of a sexual strategy to avoid infection involves a trade-off between the expense of sexuality and environmental and host-specific factors such as parasite density or the time needed for a planozygote to encyst.

In addition to the specificity of its infection of dinoflagellate species, *P. sinerae* showed strain specificity at the intra-species level. This was the case for *A. minutum* and *P. reticulatum*, although this characteristic might be common place in other species as well, given that relatively few strains have been tested. The *A. minutum* resistant strain is geographically distant from the other *A. mintum* strains. An in-depth study of this resistant effect is ongoing in our laboratory.

Molecular Identification

The 18S rRNA sequence analyses supported that the parasite isolated in Arenys de Mar harbour was a species belonging to the *Parvilucifera* genus, but different from *P. infectans. P. sinerae* clusters with *P. infectans* with similar distances to the different *Perkinsus* species. Although *Parvilucifera* and *Perkinsus* have been suggested to form a new taxon, the Perkinsozoa, (Norén et al. 1999), they grouped with low support in the ML and Bayesian analyses. Johansson et al. (2006) proposed the detection of *P. infectans* through the use of primer pairs. However, our results suggest that there are multiple species target probe within the descriptions of *P. infectans*, as was found for the endoparasitic dinoflagellate *Amoebophyra ceratii* on the basis of 18S rRNA sequence analyses (Janson et al. 2000). Because the above-mentioned primers also cover *P. sinerae* , we argue that they are genus- instead of species-specific. As for *A. ceratii*, there may well be a cluster of species within *Parvilucifera*, each of which is capable of infecting only a narrow range of dinoflagellate species.

Ecological Perspectives

While parasitic protozoa may play a role in limiting the proliferation of their hosts, this possibility has been more or less unexplored at the level of the ecosystem (Brugerolle 2002). Given that blooms of toxin-producing species are very

harmful to fishery and aquaculture, a biological control system making use of parasites has been proposed (Anderson 1997). Specifically, *Amoebophyra* sp. (Taylor 1968) and *Parvilucifera* (Delgado 1999; Norén et al. 1999) were examined for their potential application in the biocontrol of toxic dinoflagellates such as *Dinophysis* and *Alexandrium*. However, the strain resistances shown by *Amoebophrya* (Janson et al. 2000) and *P. sinerae* pose difficulties in the successful development of a dinoflagellate-bloom control strategy through intentional parasite infection and point to the necessity for further study of this approach, also in *P. infectans*.

P. sinerae has a significant impact on dinoflagellate populations under laboratory conditions. In culture (closed flasks), the parasite is capable of removing a significant fraction of the dinoflagellate biomass within a short time. Although estimates of host mortality following infections of natural dinoflagellate populations by this parasite were not obtained, infection by this parasite of natural *A. minutum* populations did not bring about a decrease or an end to the bloom. It can thus be expected that the effective use of *P. sinerae* in controlling harmful algal blooms in natural environments will depend on prior knowledge of the ecology of *Parvilucifera*, both in natural occurrences and with respect to its exploitation as a biological control agent. Specifically, the behaviour of the parasite and of the sensitive host at various cell densities, the stages of the parasite population's life cycle, the most prevalent locations of the blooms, and the hydrodynamics of those sites (renovation of the water, currents) all remain to be determined.

Formal Description

Parvilucifera sinerae Figueroa et Garcés sp.nov. (Figs 3—5).

Type illustration: (Figs 3—4), from material originally collected at the harbor of Arenys de Mar. on the Spanish Mediterranean Coast in April 2007.

Diagnosis: alveolate endoparasite in dinoflagellates. Motile cells with two unequal flagella arising from the anterior part of the cell. Sporangium formed inside the host and covered by small spicules. Each sporangium produces numerous swarmers. There are two routes of host infection, either nuclear in thecate host or cytoplasmatic in athecate species. Strain specificity in target hosts, showing local adaptation to Mediterranean strains are as follows: type host: *Alexandrium* (strain specificity, not *A. taylori*), *Karenia, Gymnodinium catenatum* and *nolleri, Protoceratium reticulatum* (strain specificity). *Akashiwo sanguinea* and *Scrippsiella trochoidea* not significantly infected.

Fixed specimens are deposited with the identification numbers 20070525 A1, 20070525 A2, 20070525 B1 and 20070525 B2 in the Electronic Microscopy Laboratory of the previously cited public institution (contact address: Institut de Ciènces del Mar, Passeig maritim de la Barceloneta 37-39, E08003 Barcelona, Spain).

Etymology: sinerae (Latin) referring to the nick name of the locality, Arenys de Mar, by the Catalan writer Salvador Espriu.

Methods

Field sampling strategy: Initiation of the dinoflagellate bloom was evaluated on the basis of *Alexandrium minutum* cell numbers, determined during routine monitoring of a specific site in the harbor of Arenys de Mar (Mediterranean Sea, Spain; sampled 1—2 times per week). When cell densities increased (early March 2006, cell densities $>10^4$ cells L^{-1}), a fresh and living sample of the bloom was collected. Four to seven liters of the sample were stored in a 5- to 8-L PLC square-shaped bottle and transported back to the laboratory. Samples were pre-filtered through a 60-μm mesh to remove major debris and grazers and then incubated in a culture chamber for 20—24 days at 15 °C with a light:dark (L:D) cycle of 10:14 h and irradiance conditions of 120 μmol photon m^{-2} s^{-1} (Gyrolux fluorescence tubes, Sylvania, Germany). After the incubation period, the entire sample volume was filtered through a 10-μm mesh. Subsamples were examined under a Leica-Leitz DM-IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and micrographs were taken with a ProgRes C10 Plus digital camera (JENOPTIK Laser, Optik, Systeme GmbH, Germany).

Additional samples (150 mL) obtained from the routine monitoring station were immediately fixed with Lugol's iodine solution for microscopic analyses of the dinoflagellate assemblage. The general procedure for identifying and quantifying the target species involved sedimentation (24 h) of a subsample in 50-mL settling chambers and then counting the cells occurring in an appropriate area (depending on cell density) following the method of Throndsen (1995) and using an inverted Leica microscope. Dinoflagellates were identified according to the criteria of Steidinger and Tangen (1997).

Flow cytometric analyses and nuclear staining: The number of planozygotes formed in the sexual out- and self-crosses was estimated by flow cytometry according to the method of Figueroa et al. (2007). Briefly, the method takes advantage of the light-induced synchronisation of cell division in *Alexandrium* in order to calculate zygote formation. Since *A. minutum* cells arrest in the G1 phase of the cell cycle after 48 h of darkness, the number of diploids (2*n*) formed in a synchronised sexual cross sampled immediately after the end of the dark period are zygotes and not dividing cells containing double the amount of DNA.

Synchronization: For cell synchronization, the sexual crosses of the *A. minutum* strains was performed in duplicate

flask bottles of 50 mL (Iwaki, Tokio, Japan) with the same conditions described in the main manuscript. The cultures were synchronized as follows: After the completion of a 24 h 12L:12D cycle, the incubators lights were turned off for a period of 48 h. Samples for DNA and cell concentration were taken from duplicates once the cycle was restored ($t0\,h = 0\,h$ of light after synchronization or $t8\,h = 8\,h$ of light after synchronization).

Analyses: A 20 mL aliquot of culture was fixed with 1% of paraformaldehyde for 10 min and washed in PBS pH 7 (Sigma-Aldrich, St. Louis, USA) ($1200g \times 10$ min). The pellet was resuspended in 5 mL of cold methanol and stored for 12 h at 4 °C to extract chlorophyll. The cells were then washed twice in PBS (pH 7) and the pellet was resuspended in a staining solution (PBS, 3 μg mL^{-1} propidium iodide and 1.1 μg mL^{-1} RNaseA) for at least 2 h before analysis. We used a Becton and Dickinson FACScalibur bench machine with a laser emitting at 488 nm. Samples were run at low speed (approx. 18 μL min^{-1}) and data were acquired in linear and log mode until around 10,000 events had been recorded. We added 10 μL per sample of a 10^6 mL^{-1} solution of yellow-green 0.92 μm Polysciences latex beads as an internal standard. Fluorescence emission of propidium iodide was detected at 617 nm. ModFit LT (Verity software House) was used to compute peak numbers, coefficients of variation (CVs), means and standard deviations for the population DNA fluorescence distributions.

Establishment of a *P. sinerae* sp. nov. culture: Four liters of seawater from an almost monospecific bloom of *Alexandrium minutum* that pervaded the harbor of Arenys de Mar (Mediterranean Sea, Spain) in April 2007 were filtered through a 60-μm plankton net. The filtrate was recovered in a 15-μm plankton net in order to collect the size fraction characteristic of the dinoflagellate community. These concentrated *A. minutum* cells were inoculated to a final concentration of 300 cells mL^{-1} into 2-L Nalgene flasks filled with L1 medium with no phosphates added to induce sexuality, following the protocol described by Figueroa et al. (2007). After 5 days, several *P. sinerae* sporangia were individually lifted, washed in several drops of filtered seawater, and used to infect a clonal culture of *Alexandrium minutum* (VGO A7). The established culture was maintained by inoculation with fresh *A. minutum* every 5 days at 20 °C and 90 μmol photons m^{-2} s^{-1} with a L:D cycle of 12:12 h. The strain of *Parvilucifera sinerae* ICMB 851 was deposited in the culture collection of the institut de Ciènces del Mar (ICM) of Barcelona (contact address: Institut de Ciènces del Mar, Passeig maritim de la Barceloneta 37-39, E08003 Barcelona, Spain). Unfortunately, this strain is no longer available because it died after 3 months in culture. However, new clones, most probably *P. sinerae* but pending molecular identification, are currently being isolated in our laboratory.

Scanning electron microscopy: Natural and cultured samples of the parasite strain were fixed for 2 h in 2% OsO_4 (Sigma-Aldrich, St. Louis, MO, USA), diluted in seawater (salinity 36), or fixed for 2 h in 2% glutaraldehyde (Sigma-Aldrich). Cells were subsequently washed with distilled water (2 h), filtered onto a Nuclepore filter (8-μm and 0.8-μm pore size; Whatman, Maidstone, UK), and dehydrated in a graded ethanol series (25%, 50%, 70%, 99%) for 15 min per step, followed by three 15-min rinses in 100% ethanol. Samples were critical-point dried in liquid CO_2 using a BAL-TEC CPD 030 critical-point drying apparatus. Filters were subsequently glued to SEM stubs with colloidal silver, sputter-coated with gold palladium, and examined with a Hitachi S-3500N (Nissei Sangyo Co. Ltd., Tokyo, Japan) scanning electron microscope operating at 5 kV.

Infection studies: To infect the cultures, newly formed parasite sporangia were individually isolated and washed in several drops of seawater before infection. The strains were classified as sensitive (*S*) or resistant (*R*) to infection based on the infection capability of a parasite inoculum of 10 sporangia mL^{-1} added to 1 mL of an exponentially growing dinoflagellate culture (3000—6000 cells mL^{-1}). The sporangia formed in the infected cultures were counted over the entire plate at least once a day for 10 days. Putative resistant (*R*) cultures were infected twice and with replicates to confirm resistance.

To test the effect of different light and salinity levels on *P. sinerae* infectivity, a natural-bloom sample of *Alexandrium minutum* was induced to sexuality following the method of Figueroa et al. (2007). The description of the percentage of healthy cells after inoculation of the parasite was obtained by using a first-order orthogonal design with a quadruple replication in the centre of the experimental domain (Box et al. 1989). The independent variables used to define a first-order orthogonal design were: salinity (*S*) and irradiance (*I*) with the following ranges.

The selected range for salinity was 38—28. alinities were prepared by diluting seawater at salinity 31 and 28 by de-ionized water (MilliQ, Millipore).

The experimental range of the irradiance selected was 120—80 μmol photon m^{-2} s^{-1}. Irradiance was provided by cool-white fluorescent lamps located in the frontal wall. Initial irradiance was 120 μmol photon m^{-2} s^{-1} and the different irradiances (100 and 80 μmol photon m^{-2} s^{-1}) were obtained by situating the bottles in different position to archive different percentage intensities. Irradiance as photosynthetic active radiation (PAR) was measured in the position of the bottles with a QSL quantum sensor (LI-185B, Li-Cor Inc.).

Statisca 6.0 program (StatSoft. Inc. 2001) was used to obtain an empirical equation to describe the % of healthy cells after parasite infection as a function of salinity and irradiance and to test the statistical significance of the coefficients.

Thirty-six sporangia of *P. sineare* were individually isolated, washed in several drops of seawater, and transferred to sterile polystyrene Petri dishes (Iwaki, Japan, 6.4-mm diameter) filled with 300 μL of L1 medium at two different salinities (20 and 31) and at a salinity of 31 combined with a resting cyst of *Alexandrium peruvianum*. The sporangia were incubated at 20 °C and 90 μmol photons m^{-2} s^{-1} with a L:D cycle of 12:12 h. The isolated cells were monitored every 24 h for 10 days.

DNA extraction: Several sporangia (40—50) were individually isolated from an infected culture of *Alexandrium minutum*. Each sporangium was washed in several drops of filtered seawater and manually crushed with the tip of a pipette. This suspension was lysed by several rounds of freezing in liquid nitrogen and thawing.

PCR: Small subunit (SSU) rRNA genes were PCR-amplified with the eukaryotic primers EukA and EukB (Medlin et al. 1988). The 50-μL PCR mixture contained 5 μL of DNA extract as template, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 1.25 units of *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA), and the PCR buffer supplied with the enzyme. PCRs were run in an automated thermocycler (MJ Mini cycler, Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: an initial denaturing step at 94 °C for 3 min, 30 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 1 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min.

Sequencing: Amplified PCR products were purified with a PCR purification kit (Qiagen, Venlo, The Netherlands) and sequenced with a Big Dye Terminator Cycle Sequencing kit (v.3) in an ABI PRISM model 3100 (V3.3) automated sequencer (Applied Biosystems, Foster City, CA, USA). The PCR primers and the internal eukaryote-specific primers 516r (Amann et al. 1990), 528f (Elwood et al. 1985), and 1209f (Giovannoni et al. 1988) were used to sequence the complete SSU rRNA gene. Sequences were aligned and assembled using ChromasPro software (Technelysium Pty Ltd, Tewantin, Australia).

Phylogenetic analysis: The complete SSU rDNA sequence obtained, as described above, was aligned using ClustalW 1.82 (Thompson et al. 1994) with a selection of alveolate sequences from databases: 14 dinoflagellates, 6 marine alveolates of Group I, 9 marine alveolates of Group II, 3 perkinsids, 6 apicomplexans, 3 colpodellids, and 5 ciliates. Highly variable regions of the alignment were located and removed using Gblocks (Castresana 2000) with parameters optimized for rDNA alignments (minimum length of a block: 5; gaps allowed in half positions). Maximum-likelihood analysis was carried out in GARLI (serial version, v0.942; Zwickl 2006) with 1000 bootstrap (Felsenstein 1985) pseudoreplicates. All parameters were used in default options, except for the number of generations that the program should run with no significant improvements in the scoring of the topology, which was set to 5000. Phylogenetic analyses with GARLI were run under the GTR+G+I model, because the covarion model was not implemented. Phylogenetic analyses with GARLI were run at the University of Oslo Bioportal (http://www.bioportal.uio.no/). Bayesian analysis was carried out with MrBayes v3.0B (Huelsenbeck and Ronquist 2001) using the GTR+G+I model. Bayesian posterior probabilities were computed by running four chains for 2,000,000 generations using the program default model parameters. Trees were sampled every 100 generations and 1000 trees were discarded as "burn-in" upon examination of the log likelihood curve of the sampled trees, so that only trees in the stationary phase of the chain were considered.

Nucleotide sequence accession numbers: The newly determined sequence was deposited in the GenBank database under the accession number EU502912.

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