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Life cycle and molecular phylogeny of the dinoflagellates *Chytriodinium* and *Dissodinium*, ectoparasites of copepod eggs

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Abstract

The dinoflagellates *Chytriodinium affine*, *C. roseum* and *Dissodinium pseudolunula* are ectoparasites of crustacean eggs. Here, we present new observations regarding their life cycle based on coastal plankton samples and incubations and analyze their molecular phylogeny using the small subunit ribosomal RNA gene (SSU rDNA) as a marker. In contrast to the typical stages already documented for its life cycle, we observed that *D. pseudolunula* dinospores may exceptionally differentiate inside a globular cyst. Despite its parasitic life style, the cysts and dinospores of *D. pseudolunula* contain chlorophyll *a*. We obtained the first SSU rDNA sequences for the genera *Chytriodinium* (the type *C. roseum* and *C. affine*) and *Dissodinium* (*D. pseudolunula*). Classical taxonomical schemes have ascribed these genera to the order Blastodiniales. However, our SSU rDNA-based phylogenetic analysis shows that these ectoparasites form a clade in the *Gymnodinium* sensu stricto group, unarmored dinokaryotic dinoflagellates of the order Gymnodiniales. They branch in a subgroup composed of warnowiids, polykrikoids, the type of *Gymnodinium*, *G. fuscum* and *G. aureolum*. Although *Chytriodinium* and *Dissodinium* appear to be relatives based on SSU rDNA phylogeny, feeding and host specificity, their life cycles are substantially different. Based on these data we consider that the type of life cycle is a poor criterion for classification at the family level. We suggest that the morphology of the infective cell is probably the most reliable phenotypic characteristic to determine the systematic position of parasitic dinoflagellates.

Keywords: Alveolata; Blastodiniales; *Gymnodinium*; Parasitic dinophyceae; SSU rDNA

Introduction

Copepods are the most abundant metazoans in the sea and represent a key trophic link in pelagic food webs (Mauchline 1998). Numerous parasites have been shown to influence the mortality and fecundity of the copepod populations (Théodoridès 1989). The lipid-rich copepod eggs are the target of several specialized parasites. In particular, the dinoflagellates *Chytriodinium* Chatton and *Dissodinium* Klebs in Pascher (= *Diplodinium* Klebs) have dinospores able to infest planktonic crustacean eggs, absorb the host content and form one or two successive cysts that produce new dinospores. The cysts of *Dissodinium pseudolunula*

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Swift ex Elbrächter et Drebes, often reported as *Gymnodinium lunula* Schütt, *Dissodinium lunula* (Schütt) Pascher or *Pyrocystis lunula* (Schütt) Schütt, are known from earlier plankton studies (Claparède and Lachmann 1858). *Dissodinium pseudolunula* has often been confused with the superficially similar free-living thecate dinoflagellate *Pyrocystis lunula*, as both genera form primary and secondary cysts of somewhat similar size and shape during certain stages of their life cycles (Elbrächter and Drebes 1978). The genus *Dissodinium* contains two species: *D. pseudocalani* (Gönnert) Drebes (= *Sporodinium pseudocalani*) and *D. pseudolunula* that were first considered as endoparasites. Initially it was understood that the dinospore was able to penetrate the egg and consequently the primary cyst was confused with the egg membrane (Dogiel 1906; Gönnert 1936). However, subsequent work (Drebes 1969, 1978) demonstrated that both *Dissodinium* species were indeed ectoparasites.

Dogiel (1906) investigated the life cycle of *D. pseudolunula* (as *Gymnodinium lunula*) in the Mediterranean Sea, in addition to three other dinoflagellates known to parasitize crustacean eggs. Since their dinospores resembled those of *Gymnodinium*, he classified them into the genus *Gymnodinium* Stein as *G. affine* Dogiel, *G. parasiticum* Dogiel and *G. roseum* Dogiel. Chatton (1912, 1920) reinterpreted Dogiel's observations, considering that these species were ectoparasites and multiplied by palinsporogenesis (unequal products of reproduction). Chatton transferred the three Dogiel's new species into the genus *Chytriodinium* Chatton. Later, Cachon and Cachon (1968) demonstrated that reproduction took place by palintomic multiplication (repeated binary fission, without an intermediate stage of nutrition and growth, leading to the formation of identical products of reproduction). More recently, *Schizochytriodinium calani* Elbrächter and *Syltodinium listii* Drebes were added to the list of dinoflagellate ectoparasites of copepod eggs from the Arctic Ocean and North Sea, respectively (Elbrächter 1988; Drebes 1988).

In earlier taxonomic schemes, *Dissodinium pseudolunula* (as *Pyrocystis*) and *Chytriodinium* were placed in the orders Dinococcales Pascher and Blastodiniales Chatton, respectively. Other authors placed *Dissodinium* together with *Pyrocystis*, a free-living photosynthetic thecate dinoflagellate, in the order Pyrocystales Apstein (Chrétiennot-Dinet et al. 1993; Gómez 2005). However, most authors have placed *Dissodinium* and *Chytriodinium* in the order Blastodiniales (Drebes 1969, 1978; Taylor 1987), more specifically within the family Chytriodiniaceae Cachon et Cachon, or under its own order, Chytriodiniales Loeblich III (Loeblich III 1982; Cachon et al. 1969; Cachon and Cachon 1987; Taylor 1987). Fensome et al. (1993) placed *Dissodinium* and *Cachonella* Rose et Cachon in the family Cachonellaceae P.C. Silva within the Blastodiniales and *Chytriodinium* as incertae sedis in that same order.

Consequently, based on morphological data, the systematic position of *Chytriodinium* and *Dissodinium* remains ambiguous. Recent molecular phylogeny studies have demonstrated that the Blastodiniales are polyphyletic, with several species branching among non-parasite dinokaryotic dinoflagellates (Kühn and Medlin 2005; Skovgaard et al. 2007). Phylogenetic analyses using partial large subunit ribosomal DNA (LSU rDNA) sequences, placed *D. pseudolunula* within the *Gymnodinium* sensu stricto group with the unarmored dinoflagellates of the order Gymnodiniales Lemmermann (Kim et al. 2008).

No records of the genus *Chytriodinium* have been reported within the last 40 years of published observations (Cachon and Cachon 1968). New observations of the life cycle of *Dissodinium pseudolunula* have not been reported since the work published by Elbrächter and Drebes (1978) in the North Sea. Here we illustrate, for the first time, photographic records of the life cycle of these organisms in the Mediterranean Sea. In addition, we present the first phylogenetic analyses based on SSU rDNA sequences of the genera *Chytriodinium* and *Dissodinium* from single-cell specimens collected from the western Mediterranean, the type locality of both species of *Chytriodinium*. Finally, in the light of molecular data of closely related species, we discuss the applicability of the type of life cycle (series of cysts), host, feeding, and general morphology of the trophont or infective dinospores for the classification of parasitic dinoflagellates.

Materials and methods

Sampling and isolation

From October 2007 to September 2008, seawater samples were collected from the pier at Station Marine d'Endoume, Marseille (43°16′48″N, 5°20′57″E, bottom depth 3 m). A strainer with netting apertures of 20, 40 or 60-μm was used to collect the organisms. Between 10 and 100 liters were filtered depending on the concentration of particulate matter. In addition, we also studied samples collected during several monitoring research cruises to the SOMLIT (Service d'Observation en Milieu LITtoral) station in the Bay of Marseille (43°14′30″N, 05°17′30″E, bottom depth 60 m). Seawater samples were collected with a 12-l Niskin bottle at 40 and 55 m depth and filtered as described above. The plankton concentrate was scanned in settling chambers at 100 × magnification with a Nikon Eclipse TE200 inverted microscope. Cells were photographed alive at 200 × or 400 × magnification with a Nikon Coolpix E995 digital camera. In order to test the occurrence of chlorophyll *a*, live specimens were observed under blue-light with an

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inverted epifluorescence microscope (Nikon Eclipse TE2000). For single-cell PCR, the primary cyst of *D. pseudolunula* or the trophont of *C. affine* (attached to the copepod egg) were isolated with a micropipette and transferred to separate Utermöhl chambers containing filtered and sterilized seawater. After the formation of cysts containing mature dinospores (24-48 h after), a fine capillary micropipette was used to transfer the samples to a second Utermöhl chamber and they were washed several times in serial drops of 0.2-μm filtered and sterilized seawater. Finally, the complete cyst with mature dinospores was picked up and deposited into a 1.5 ml Eppendorf tube containing several drops of 100% ethanol. For *Chytriodinium roseum*, we observed only two chains of sporocytes that was immediately isolated for single-cell PCR. The samples were maintained at ambient temperature in darkness until molecular analysis.

PCR amplification of small subunit rRNA genes (SSU rDNAs) and sequencing

Ethanol-fixed cysts were centrifuged for 5 minutes at 3,000 rpm. Ethanol was removed by evaporation in a vacuum desiccator and the specimens re-suspended directly in 50 μl of Ex TaKaRa (TaKaRa, distributed by Lonza Cia., Levallois-Perret, France) PCR reaction mix containing 10 to 20 pmol of the eukaryotic-specific SSU rDNA primers EK-42F (5′-CTCAARGAY-TAAGCCATGCA-3′) and EK-1520R (5′-CYGCAG GTTCACCTAC-3′). PCR reactions were performed under the following conditions: 2 min denaturation at 94 °C; 10 cycles of 'touch-down' PCR (denaturation at 94 °C for 15 s; a 30-s annealing step at decreasing temperature from 65 down to 55 °C −1 °C decrease with each cycle-, extension at 72 °C for 2 min); 20 additional cycles with 55 °C of annealing temperature; and a final elongation step of 7 min at 72 °C. A nested PCR reaction was then carried out using 2 to 5 μl of the first PCR reaction in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the eukaryotic-specific primers EK-82F (5′-GAAACTGCGAA;TGGCTC-3′) and EK-1498R (5′-CACCTACGGAAACCTTGT-TA-3′) and similar PCR conditions as above except for an increase in the total number of cycles from 30 to 35. A third, semi-nested PCR was carried out under similar conditions using the dinoflagellate specific primer DIN464F (5′-TAACAATACAGGGCATC-CAT-3′) and 0.3 to 3 μl of the second PCR reaction as template. Amplicons of the expected size were then sequenced bidirectionally using primers DIN464F and EK-1498R (Cogenics, Meylan, France), yielding sequences of ~1,200 bp. The sequences were deposited in GenBank with accession numbers FJ473378-FJ473380 and FJ663049.

Phylogenetic analyses

The new sequences were aligned to a large multiple sequence alignment containing 1100 publicly available complete or nearly complete (>1,300 bp) dinoflagellate SSU rDNA sequences using the profile alignment option of MUSCLE 3.7 (Edgar 2004). The resulting alignment was manually inspected using the program ED of the MUST package (Philippe 1993). Ambiguously aligned regions and gaps were excluded in phylogenetic analyses. Preliminary phylogenetic trees with all sequences were constructed using the Neighbour Joining (NJ) method (Saitou and Nei 1987) implemented in the MUST package (Philippe 1993). These trees allowed identifying the closest relatives of our sequences, which were selected together with a sample of other dinoflagellate species to carry out more computationally-intensive Maximum Likelihood (ML) and Bayesian Inference (BI) analyses with a data set of 1,202 sites. ML analyses were conducted with the program TREE-FINDER (Jobb et al. 2004) by applying a GTR + Γ + I model of nucleotide substitution, taking into account a proportion of invariable sites, and a Γ-shaped distribution of substitution rates with four rate categories. BI analyses were carried out with the program PHYLO-BAYES through the application of a GTR + CAT Bayesian mixture model (Lartillot and Philippe 2004).

Results

Dissodinium pseudolunula

The primary (spherical) and secondary (lunate, crescent-shaped) cysts of *D. pseudolunula* were a common component of the spring phytoplankton assemblage in the coastal NW Mediterranean Sea. The first cysts appeared in early February, coinciding with the development of the spring diatom bloom and the presence of large size opaque copepod eggs. The last cysts were observed in early June. The different stages of the life cycle of *Dissodinium pseudolunula* are illustrated in the figures (Figs 1–17). We omitted the detailed description of our observations in the Mediterranean Sea because they coincided to those already documented in the North Sea (Elbrächter and Drebes 1978). However, we exceptionally observed that the dinospores began to differentiate before the secondary cysts were released from the primary cyst. In fact, we observed two globular cysts containing dinospores in the natural samples (Figs 11, 12). In order to verify their identity, we carried out PCR analysis of each of these globular cysts with dinospores. The SSU rDNA sequences were identical to that of the dinospores found in the lunate cyst of *D. pseudolunula*, showing that the dinospores

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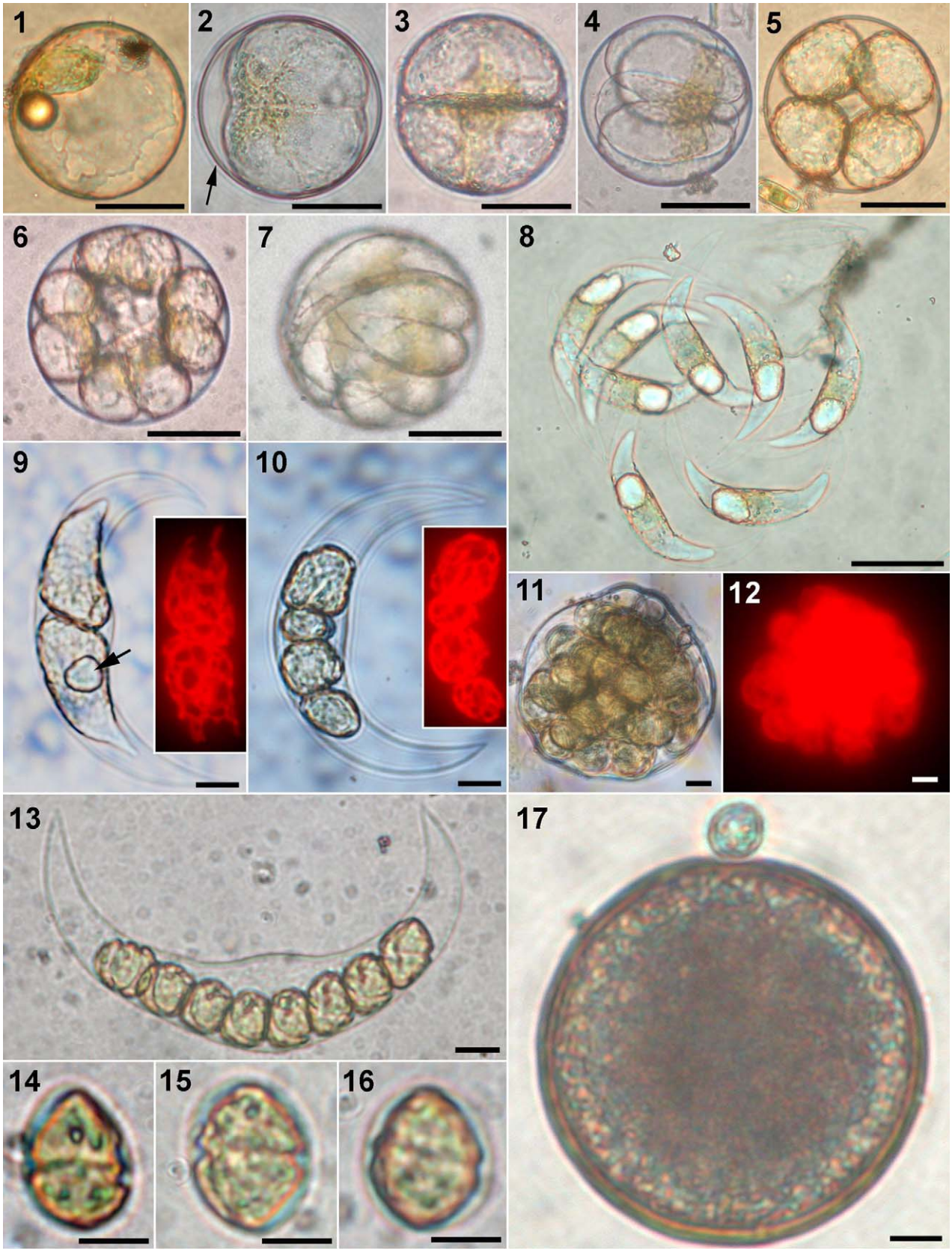

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may indeed occasionally differentiate before the rupture of the primary cyst wall.

The fast-swimming dinospores of *D. pseudolunula* could not be easily distinguished from other surrounding gymnodinioid cells in the live plankton samples. We carried out short-term laboratory incubations with isolated primary cysts in order to investigate the life cycle and the morphology and behavior of the dinospores. A recently detached primary cyst required two days for the formation of the secondary cysts and the dinospores. These incubations often resulted in aberrant secondary cysts with anomalous shapes and a lower number of dinospores when compared to natural samples. The released dinospores swam actively and after several minutes, they became non-motile and encysted inside a hyaline membrane (Figs 14–16). No division of the dinospores was observed. We added fresh copepod eggs to test whether re-infections could occur. However, the dinospores did not infect them, so that infections were only observed in natural samples (Fig. 17).

As the secondary cysts and the dinospores showed a yellow-green pigmentation, we looked for the presence of chlorophyll *a* using epifluorescence microscopy. Instead of typical globular plastid accumulations, the chlorophyll *a* showed a reticulate distribution in the periphery of the cell (Figs 9, 10). The dinospores were placed in cultures under different light intensities in a nutrient-rich (f/2) medium that has been successfully used with other photosynthetic unarmored dinoflagellates. However, the dinospores did not proliferate under these conditions.

Chytriodinium affine

Chytriodinium affine (Dogiel) Chatton was observed attached to small or medium-sized copepod eggs from June to September. The dinospore attached to the egg surface from the hyposome, it transformed into a spherical trophont stage and grew to a size of 50 to 80 μm. Whereas the infective dinospore of *D. pseudolunula* detached as a primary cyst as soon as the egg contents were absorbed (less than 1 hour) and subsequently started to divide, the trophont of *C. affine* remained attached to the copepod egg until the dinospores were mature. Multi-infection was a common feature in *Chytriodinium* (Figs 18, 19), although no more than two trophonts developed successfully from a single host (Figs 22, 26, 27). The dinospore was attached to the host by means of a feeding tube, enlarged at its base. An ampulla with one large trophic vacuole formed at the end of the peduncular disk that gradually absorbed the egg cytoplasm (Figs 20–22, 24–25). These structures are here named following the terminology by Cachon and Cachon (1968). The trophont maintained a spherical shape during this process (Figs 20–26). It continued feeding while the dinospores formed by palintomic sporogenesis. The chain of dinospores coiled itself inside of a fine hyaline membrane (Figs 28, 29). When all the dinospores were formed, the cyst containing the chain of dinospores detached from the empty egg, leaving the trophont membrane (Fig. 30). After the rupture of the membrane, the chain of dinospores gradually decomposed by releasing single cells that began to swim and dispersed (Fig. 31). The colorless dinospores (9 μm long) showed a hemispheric episome. The dinospores divided synchronously again (Fig. 32). In contrast to *D. pseudolunula*, the dinospores of *C. affine* were able to infect fresh copepod eggs immediately after the last binary division. Each cycle, from the infection of the host to the liberation of dinospores, was completed in ~24 hours and provided up to 200 dinospores from a trophont.

Chytriodinium roseum

One specimen ascribed to the genus *Chytriodinium* type species, *C. roseum*, was found in samples from the NW Mediterranean. It was identified from a sample collected in the Bay of Marseille on June 24th 2008 at 55 m depth (Figs 33–35). Due to the paucity of individuals, we decided to isolate it immediately for single-cell PCR instead of trying a temporal incubation that might have resulted in loss of the specimen. At the observed stage of development, the chain was not fully developed and about half of the host content was already consumed. The host membrane began to collapse in the area where the trophont was attached (Fig. 35). The growing parasite formed a chain with two ellipsoidal lobules with a round junction. The proximal lobule (70 μm long), attached to the egg, showed transversal constrictions or septa in the distal extreme. In the proximal extreme, the trophont showed a large structure that appeared to be a vacuole. The distal lobule showed three transversal septa that divided it into four sections (Fig. 34). We ascribed this chain of

Figs 1–17. Light micrographs of different stages of the life cycle of *Dissodinium pseudolunula* collected off Marseille, France, in spring 2008. **1**. Primary cyst recently detached from the copepod egg. **2-7**. Sequence of cell division inside of the primary cyst. The arrow in the Fig. 2 points to the rigid cellulosic wall. **9-10, 13**. Binary division of the protoplasm to form the dinospores. The arrow in the Fig. 9 points to the tentative residual vacuole. The insets in the Figs 9, 10 showed the chloroplasts illuminated with blue light under epifluorescence microscopy. **11-12**. Exceptional differentiation of the dinospores inside of a globular cyst. This cyst was used for PCR amplification. **14-16**. Free dinospores, note the hyaline capsule. **17**. Dinospore infecting a copepod egg. Scale bars: 50 μm in the Figs 1–8 and 10 μm in the Figs 9–17.

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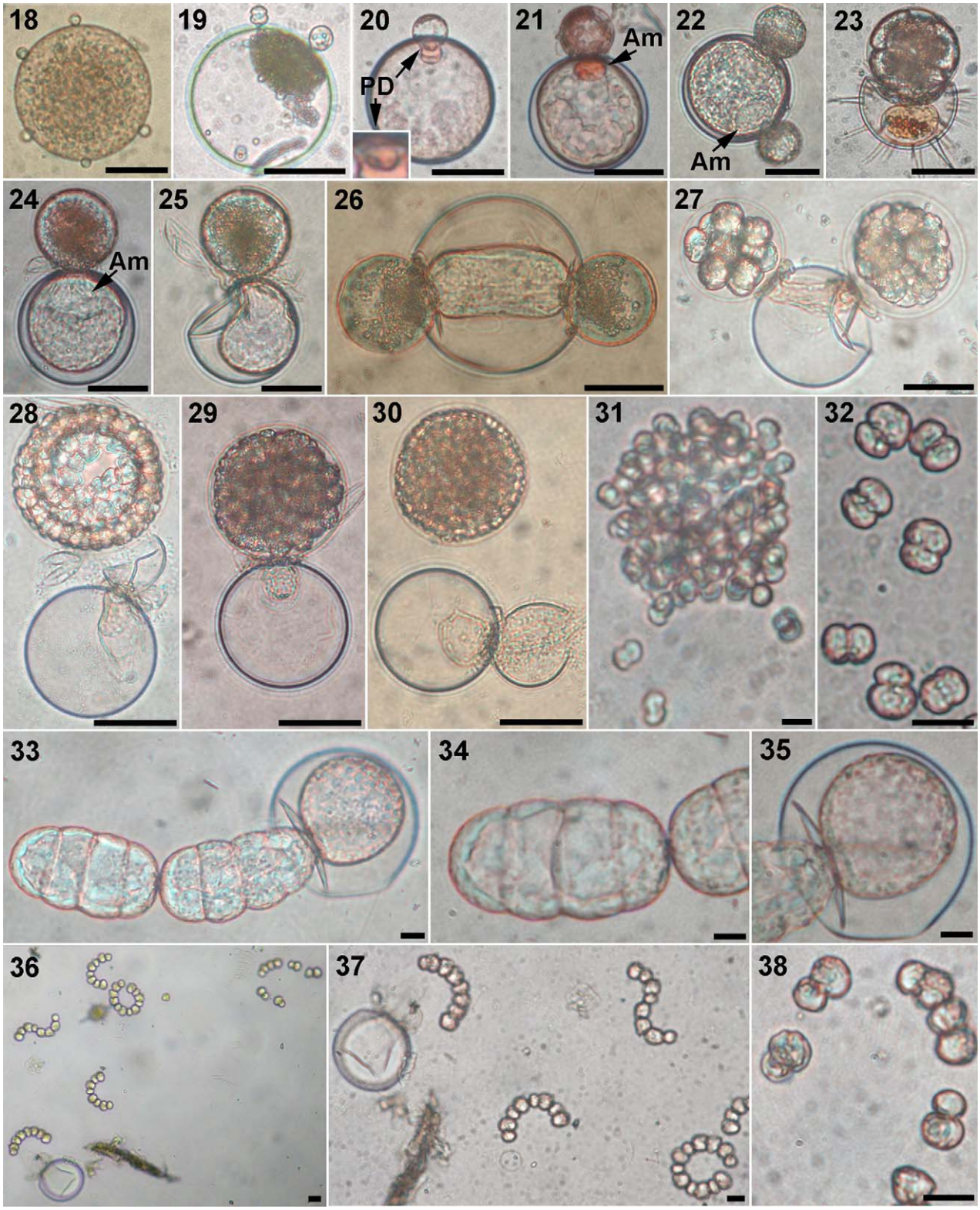

Figs 18–35. Light micrographs of the life cycle of *Chytriodinium affine* (Figs 18–32) and two groups of sporocytes of *C. roseum* (Figs 33–35) collected off Marseille, France, in summer 2008; **18-19** Copepod eggs multi-infected; **20-25** Development of the trophont; Peduncular disk (**PD**), Ampulla (**Am**). **25** Note the collapse of the copepod egg membrane; **26-27** Host infected by two trophonts; **27** Note the different degree of differentiation between the two parasites; **28** Chain of sporocytes detached from the host; **29-32** The sporocytes detached from the host, leaving behind the primary cyst; **31** The chain fragmented and the dinospore began to disperse; **32** Dinospores under binary division; **33-35** Chain of sporocytes of *Chytriodinium roseum* used for PCR analysis; **36-38**. Other individuals of *C. roseum* dissociated into several chains. Scale bar of 50 μm in Figs 18–30 and of 10 μm in Figs 31–38.

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dinospores to the species *C. roseum* in accordance with the illustration by Cachon and Cachon (1968, plate II, i–j). In *C. affine,* the numerous dinospores developed in a coiled chain inside of a hyaline spherical membrane that was absent in the chain of *C. roseum*.

Another specimen of *C. roseum* was retrieved from a surface sample collected in the Bay of Marseille on September 2nd 2008 (Figs 36–38). The sporocytes formed six chains. The closer chains to the empty egg were formed of eight sporocytes. One chain was formed of 12 sporocytes and other chain showed some sporocytes (11-14 μm in diameter) were dissociated and under binary division (Fig. 38).

Molecular phylogeny

Preliminary phylogenetic analyses of *D. pseudolunula*, *C. affine* and *C. roseum* sequences were included in a large dinoflagellate SSU rDNA sequence alignment containing more than 1100 sequences. The preliminary analysis indicated that these species branched close to representatives of the Gymnodiniales (data not shown). The phylogeny was further investigated by applying Maximum Likelihood (ML) and Bayesian Inference (BI) methods upon a more restricted taxonomic sampling, including 67 taxa representing different Gymnodiniales and the main dinoflagellate orders (Peridiniales, Dinophysiales, Prorocentrales, Gonyaulacales, Suessiales), with Syndiniales as outgroup taxa. The two species of *Chytriodinium* were closely related (97% sequence identity), and formed a moderately supported group together with the sequence of *Dissodinium pseudolunula* (ML bootstrap proportion, BP, of 76%, and BI posterior probability of 0.82). This low support could partly be due to the different evolutionary rates of the two *Chytriodinium* sequences. In fact, *C. affine* showed a branch twice longer than *C. roseum*, which could induce tree reconstruction problems, in particular long branch attraction artifacts. In agreement with this idea, the removal of the *C. affine* sequence resulted in a tree where *D. pseudolunula* and *C. roseum* formed a group with better support (BP of 88% and PP of 0.96, data not shown).

The clade formed by *Chytriodinium* and *Dissodinium* branched with the *Gymnodinium* sensu stricto group (Fig. 39). This group appeared split in two major subgroups. The first one was composed of *Gymnodinium* species available from phototrophically growing cultures, colonial species related to *Gymnodinium catenatum* Graham (*G. microreticulatum* Bolch, Negri et Hallegraeff, *G. nolleri* Ellegaard et Moestrup, *G. impudicum* (Fraga et Bravo) G. Hansen et Moestrup) and the unicellular *G. dorsalisulcum* Murray, de Salas et Hallegraeff. *Lepidodinium viride* Watanabe, Suda, Inouye, Sawaguchi et Chihara/*Lepidodinium chlorophorum* (Elbrächter et Schnepf) G. Hansen, Botes et de Salas also branched in this subgroup, although with a low bootstrap support (Fig. 39). The other subgroup included both apochlorotic and chloroplast-containing genera with a high diversity of ultrastructural features and trophic behaviors. Among the species forming this cluster, only the freshwater type of *Gymnodinium*, *G. fuscum* Stein and *G. aureolum* (Hulburt) G. Hansen have been maintained growing phototrophically in stable cultures. *Gymnodinium fuscum* branched at a basal position in the clade of pseudocolonial species of *Polykrikos* Bütschli. The phagotrophic warnowiids represented by the genera *Erythropsidinium* Hertwig and *Warnowia* Lindemann, and the type of *Pheopolykrikos* Chatton, the chloroplast-containing *Pheopolykrikos beauchampii* Chatton, formed a weakly supported clade. These species branched relatively close to *G. fuscum*/*Polykrikos*, although with a low bootstrap support (54%). At a basal position appeared a clade formed by *Gymnodinium aureolum* and another unidentified *Gymnodinium* species. The *Chytriodinium* clade appeared in the most basal position of this subgroup of the *Gymnodinium* s.s. group. The inclusion of these new sequences reinforced the separation of the *Gymnodinium* type and other congeneric species related to *G. catenatum* (Fig. 39).

Discussion

Dissodinium pseudolunula is widely distributed in marine neritic habitats. In contrast, the records of *Chytriodinium* are scarce, mainly restricted to the western Mediterranean Sea (Dogiel 1906; Cachon and Cachon 1968) and the subtropical Atlantic Ocean (Elbrächter 1988). Drebes and Elbrächter carried out intensive studies on the parasitic dinoflagellates in the North Sea, but no record of *Chytriodinium* was reported. *Chytriodinium* appears to have a clear warm-water distribution.

Our molecular phylogeny study suggests that *Chytriodinium* and *Dissodinium* derived from a common ancestor. The type of feeding and host appear to be similar for these ectoparasitic dinoflagellates but, apparently, they do not compete for the same resources since their peaks in abundance are temporally decoupled in the western Mediterranean Sea. *Chytriodinium affine* is able to rapidly respond to the increase in host availability during the summer with the formation of the infective dinospores occurring in 24 hours. In contrast, *D. pseudolunula* requires at least two days from infection to the liberation of the new dinospores and probably some additional time for the dinospores to be infective since, according to our observations, recently released dinospores appeared unable to infect new hosts. This

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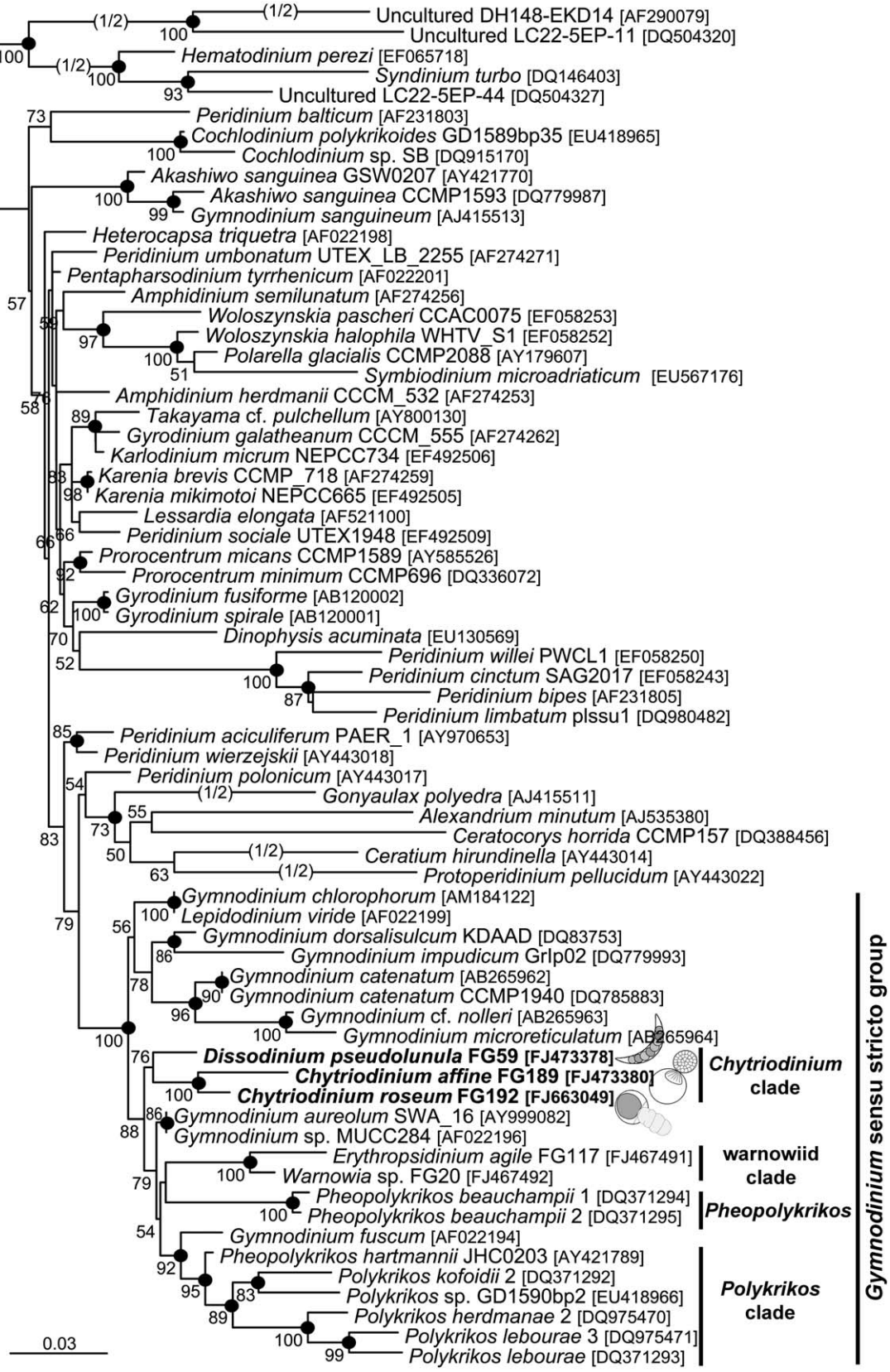

Fig. 39. Maximum likelihood phylogenetic tree of dinoflagellate SSU rDNA sequences, based on 1,202 aligned positions. Names in bold represent sequences obtained in this study. Numbers at the nodes are bootstrap proportions (values under 50% are omitted). Nodes supported by posterior probability values >0.90 in Bayesian Inference analyses are indicated by black circles. Several branches leading to fast-evolving species have been shortened to one third (indicated by 1/3). Accession numbers are provided between brackets. The scale bar represents the number of substitutions for a unit branch length.

agrees with the results obtained by Drebes (1984) that suggested that the dinospores of *D. pseudolunula* may need a post-maturation time before they become able to infect a host. Drebes (1984) also found that these dinospores could survive for up to four weeks. In contrast, our incubation experiments suggest that the *C. affine* dinospores disappeared in a few hours.

The dinospores of *C. affine* were colorless and we did not observe any cyst formation, while the dinospores of *D. pseudolunula* contained chlorophyll *a* and were encysted. Stoecker (1999) reported that phototrophy in *D. pseudolunula* might be a mechanism to increase survival during dispersal. However, it is uncertain whether the chlorophyll *a* is photosynthetically functional and able to maintain the dinospores until a suitable host is available. Chloroplasts are well developed and appear functional in other parasitic dinoflagellates such as *Protoodinium* Hovasse, *Piscinoodinium* Lom and *Crepidoodinium* Lom et Lawler and some species of *Blastodinium* Chatton (Cachon and Cachon 1987). The other species of *Dissodinium*, *D. pseudocalani*, lack chloroplasts (Drebes 1969). Since dinoflagellates show a strong tendency to lose and replace plastids (Saldarriaga et al. 2001), the fact that a parasitic dinoflagellate continues to synthesize chlorophyll *a* suggests a function related to the survival of the dinospores. The ultrastructure of the putative chloroplasts of *D. pseudolunula* needs to be investigated.

The warnowiids and heterotrophic polykrikoids branched in the SSU rDNA phylogenies within the *Gymnodinium* s.s. group, whose members are endowed with a high diversity of chloroplasts (Daugbjerg et al. 2000; Hansen et al. 2007; Hoppenrath and Leander 2007b). The *Chytriodinium* clade branched in the subgroup of type *G. fuscum*. In the LSU rDNA phylogenies, *G. fuscum* appeared as sister of the heterotrophic *Gymnodinium venator* Flø Jørgensen et Murray, previously known under the genus *Amphidinium* as *A. pellucidum* C. Herdman (Flø Jørgensen et al. 2004). The species of *Lepidodinium* and *Gymnodinium aureolum* are able to grow phototrophically in cultures. These species have a peduncle, whose function is speculated to be a feeding structure (Hansen 2001; Hansen et al. 2007). In our SSU rDNA tree, the clade *Lepidodinium* and *G. aureolum*/*Gymnodinium* sp. appeared in different subgroups of *Gymnodinium* s.s. group, although these clades have a very low bootstrap support. *Gymnodinium aureolum* appeared relatively close to the *Chytriodinium* clade and it is uncertain whether its peduncle emerging from the sulcal region might be related to the peduncular disk issued from the hyposome in the *Chytriodinium* clade.

The SSU rDNA phylogeny would support the reclassification of *Chytriodinium roseum* and *C. affine* under the genus *Gymnodinium*, as Dogiel (1906) originally described it. This is also the case for *D. pseudolunula*. The name *Gymnodinium lunula* has been largely used for life stages of both, *D. pseudolunula* and *Pyrocystis lunula*. To avoid confusion, a new combination, *Gymnodinium pseudolunula*, may be proposed. However, before proposing the transfer of *Chytriodinium* and *Dissodinium* species into *Gymnodinium*, we must be sure that the species currently ascribed to *Gymnodinium* form a unique clade. The addition of new sequences in the *Gymnodinium* s.s. group is revealing that the freshwater *Gymnodinium* type is separated from the marine representatives (*G. catenatum* and related species) (Hoppenrath and Leander 2007a, b; Kim et al. 2008; Gómez et al. 2009). Hence, a further split of the current *Gymnodinium* s.s. into separate genera cannot be discarded and, therefore, we prefer at present to conserve *C. roseum*, *C. affine* and *D. pseudolunula* under their current nomenclature. At any rate, since the SSU rDNA phylogeny showed that *Chytriodinium* and *Dissodinium* formed a clade, it would be possible to group all these species under the genus *Chytriodinium*, which has the priority versus *Dissodinium*.

The type of life cycle has been used traditionally for the classification of parasitic dinoflagellates into families. Fensome et al. (1993, p. 175) placed *Dissodinium* and *Cachonella* into the Cachonellaceae according to the presence of at least two vegetative cyst stages. However, in this study we observed that the *Dissodinium* dinospores can occasionally be formed inside a globular cyst (Figs 11, 12), indicating that *Dissodinium* has the capacity to modify its life cycle. Therefore, the type of life cycle is not an appropriate characteristic for the classification of the parasitic dinoflagellates. A similar argument may apply also to the mode of feeding as a general diagnostic feature. The genus *Paulsenella* Chatton was initially included within the Chytriodiniaceae (Cachon et al. 1969; Loeblich III 1982; Elbrächter 1988), although the SSU rDNA phylogeny indicated later an affiliation among the thecate dinoflagellates of the order Peridiniales (Kühn and Medlin 2005). *Paulsenella* and its relatives, *Pfiesteria* and the cryptoperidiniopsoids, form a clade that is united by their mode of feeding, myzocytotically by means of an extensible feeding tube. Consequently, Kühn and Medlin (2005) suggested the mode of feeding as a character useful for the classification of parasitic dinoflagellates, but this assumption is not valid for *Chytriodinium* and *Dissodinium* because, although they share the type of feeding, their closest relatives include numerous photosynthetic species.

Although, in general, trophonts easily lose the dinoflagellate characters and the morphology of unrelated species may resemble by convergence (Cachon and Cachon 1987), in the case of *Chytriodinium* and *Dissodinium*, the type of dinospore appeared informative and supported their systematic affiliation. The members

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of the *Gymnodinium* s.s. group possess a loop-shaped apical groove running anticlockwise (Daugbjerg et al. 2000). Due to the low resolution of the inverted light microscopy, we were unable to confirm the occurrence of this type of apical groove in *C. affine* and *D. pseudolunula*. Unfortunately, the swimming infective dinospores are delicate, have a short life and, as such, cannot be easily distinguished from other plankton cells. For example, the dinospores of *Blastodinium* have been recently demonstrated to be thecate, while they were considered gymnodinioid in previous studies (Skovgaard et al. 2007). *Paulsenella* has been placed between the thecate dinoflagellates, but despite the studies in cultures, there is no evidence of thecal plates in any life stage (Kühn and Medlin 2005). It appears that the diagnostic features present in the dinospores that may reveal their systematic position are only visible during discrete periods of their life cycle. Therefore, molecular techniques are not only complementary to classical taxonomy, but they appear to be the only alternative to resolve the phylogeny of parasitic dinoflagellate species in cases where the diagnostic morphological characteristics cannot be observed.

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