

**Dinoflagellates *Amyloodinium* and *Ichthyodinium* (Dinophyceae),
parasites of marine fishes in the South Atlantic Ocean**

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ABSTRACT: The morphology and molecular phylogeny of the parasitic dinoflagellates *Ichthyodinium chabelardi* and *Amyloodinium ocellatum* was investigated off Brazil (South Atlantic Ocean). This is the first record of *Ichthyodinium* and the first molecular data of both parasites from the southern hemisphere. *Ichthyodinium chabelardi* infected the yolk of eggs of feral populations of Argentine anchovy (*Engraulis anchoita*; Engraulidae) and Brazilian sardinella (*Sardinella brasiliensis*; Clupeidae) in different seasons. The SSU rRNA and ITS gene sequences were identical and confirmed *Ichthyodinium* as a host generalist. The new sequences clustered with the type species *I. chabelardi* from the North Atlantic and environmental sequences from the Pacific Ocean. A second species from the western Pacific remains undescribed. *Amyloodinium ocellatum* was isolated from the gills of a cultured cobia fish (*Rachycentron canadum*) after causing mortality. The SSU rRNA gene sequence of the Brazilian isolate was almost identical to those from the northern hemisphere. This suggests a single species with a widespread distribution, although it is uncertain whether the species has a natural pantropical distribution or is the result of artificial distribution due to the human-induced fish transport.

KEYWORDS: fish parasite · yolk egg parasite · amyloodiniosis · marine velvet disease · ichthyoplankton infestation · Dinoflagellata

INTRODUCTION

The sustainability of global fisheries requires precise assessments of resource abundance in space and time, and the factors that impact fish mortality. Parasitism is one important factor often neglected in fisheries assessments (Timi and Mackenzie 2015). In particular, mariculture (fish farming) is an expanding alternative to natural fisheries, but the maintenance of large numbers of fish confined in a small area create conditions that lead to an increase in the risk of parasite infection (Shinn et al. 2014).

This study specifically examined three commercially important South American fish species, the Brazilian sardinella, the Argentine anchovy and cobia to determine if they were susceptible to parasitism by either *Ichthyodinium* or *Amyloodinium* species. The Brazilian sardinella, *Sardinella brasiliensis* (Clupeidae), is a pelagic fish known from the continental shelves between Florida and Argentina with catches that reached 100,000 tons in 2013 (FAO FishStat). This species constitutes Brazil's most important fishery resource, but since the 1970s catches varied greatly (Cergole & Dias Neto 2011). The Argentine anchovy, *Engraulis anchoita* (Engraulidae) is an endemic species of the continental shelves of Brazil, Uruguay and Argentina. This species constitutes an emerging fish resource in Brazil (Carvalho & Castello, 2013). The cobia (*Rachycentron canadum*) has aroused great interest in the mariculture sector as an emerging species. The production started in Asia and spread rapidly to other continents because of their positive characteristics such as rapid weight gain (Liao et al. 2004).

The syndinean dinoflagellate *Ichthyodinium chabelardi* was first described in eggs of the sardine (*Sardina pilchardus*) in the Mediterranean Sea (Hollande & Cachon 1952), and further reported infecting other fishes important for human consumption in the European Atlantic (Pedersen & Køie 1994, Meneses et al. 2003, Skovgaard et al. 2009, 2010, Sørensen et al. 2014), and the Asian Pacific (Yuasa et al. 2007, Mori et al.

2007, Shadrin et al. 2010). The parasite consumes the vitelline vesicle of the embryo, growing and dividing until the vesicle finally bursts, killing the embryo and releasing the dinospores. Studies revealed that *Ichthyodinium chabelardi* might infect to up 90% of the fish eggs (Meneses et al. 2003) and possibly lead to the decrease of some stocks, such as the sardine fisheries (Gestal et al. 2006). In molecular phylogenies, the sequences of *Ichthyodinium* cluster within the basal dinoflagellates of the Marine Alveolate Group I that include *Euduboscquella*, a parasitoid of ciliates, as the closest known relative (Skovgaard et al. 2009, 2010).

The dinokaryotic dinoflagellate *Amyloodinium ocellatum* is a nonspecific, marine fish ectoparasite that causes extensive economic losses in the aquaculture and aquarium industries (Kuperman & Matey 1999). This obligate parasite is responsible for amyloodiniosis or ‘marine velvet disease’, one of the most serious diseases of almost every species of marine fishes of temperate and warm waters (Noga & Levy 1995). The pathogenicity is associated with attachment to the fish tissue (gills and skin) using rhizoids that embed deep in epithelial cells causing hyperplasia, inflammation, hemorrhage and necrosis. *Amyloodinium ocellatum* can cause devastating disease and mortality because it is able to reproduce quickly when fish are crowded, especially in closed systems. The life cycle comprises an infective stage (dinospore) that attaches to the host tissue, forming the feeding stage (trophont), which damages the host. After feeding, the trophont detaches from the host, falls off the host, and forms the reproductive stage (tomont), which divides to form up to 256 infective dinospores (Brown & Hovasse 1946, Noga & Levy 2006). *Amyloodinium ocellatum* is responsible of the mortality of cobia farmed in cages in Brazil (Moreira et al. 2013), but molecular data are restricted to isolates from the northern hemisphere (Litaker et al. 1999, Levy et al. 2007a, Litaker et al. 2007, Picón-Camacho et al. 2013).

MATERIALS AND METHODS

Collection of *Ichthyodinium*

Within the context of a study of the parasitism of marine zooplankton, samples were collected by using a plankton net (80 µm mesh size) in the surface waters of the South Atlantic Ocean off Ubatuba (23° 32' 20.15" S; 45° 5' 58.94" W, 15 m depth) from December 2013 to December 2015. Aliquots of plankton tows were examined in glass dishes with a dissection microscope. Infected eggs were isolated with a micropipette and placed in settling chambers. Images were recorded on an inverted microscope Olympus IX73 (Olympus Inc., Tokyo, Japan) using a digital camera (Cyber-shot DSC-W300; Sony, Tokyo, Japan) mounted on the microscope's eyepiece. For molecular analysis, infected eggs in the last stage of the infection (before the dinospore release), were washed several times with 0.2 µm filtered and autoclaved sterilized seawater. The chorion was pierced with a pointed glass pipette, and the parasite cells were removed with a fine capillary and deposited in a 0.2 mL Eppendorf tube filled with absolute ethanol.

Collection of *Amyloodinium*

Individuals of cultured cobia presented erratic swimming and gasping at the water surface in the tanks of the mariculture experimental facilities of the marine station of the Oceanographic Institute of the University of São Paulo at Ubatuba, Brazil. Gills of recently dead individuals were examined with a dissecting microscope. Tomonts were isolated with a micropipette and washed several times in glass dishes filled with 0.2 µm filtered seawater. The tomonts were placed in 6-well tissue culture plates with 0.2 µm filtered seawater in an incubator at 23°C. The developmental stages were recorded with an inverted microscope as reported above. After two days, each tomont produced 64128

cells. At this stage, before formation of the swimmers, the tomites were isolated for molecular analyses. The cells were washed several times in 0.2 µm filtered and sterilized seawater and deposited in a 0.2 mL Eppendorf tube filled with absolute ethanol. Other tomites were allowed to complete development until the formation of the infective dinospores.

PCR amplification and sequencing

The samples were kept at room temperature and in darkness until the molecular analyses could be performed. Prior to DNA extraction, the 0.2-mL Eppendorf tubes containing *Ichthyodinium* and *Amyloodinium* were opened and ethanol was allowed to evaporate overnight on the benchtop in a covered container. Cells were resuspended in 50 µL extraction buffer (final concentrations 1 mg mL⁻¹ bovine serum albumin, 10 mM Tris pH 7.4, 100 mM KCl, 1 mM EDTA, 50% glycerol). A negative extraction control was 50 µL of extraction buffer in a sterile 0.25 mL microcentrifuge tube. The tubes were frozen at -80 °C for 20 min followed by rapid warming to room temperature for 20 min. Two µL of the extracted product was used as DNA template for polymerase chain reaction (PCR).

To amplify SSU rDNA fragments, nested PCR amplifications were performed. In the first round, the primers EukA and EukB (Table 1) were used in a reaction with GoTaq polymerase (Promega, Madison, WI, USA). For this initial reaction, the following thermocycler program was: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 2 min; final extension at 72 °C for 7 min. In the nested round of PCR, 1 µL of the first PCR product was used as DNA template and the primer pairs EukA/892R, 570F/1200R

and 892/EukB2 were used (Table 1). Conditions for the second round of PCR were the same as the first, except that the cycling extension time was shortened to 1 min.

For the Internal Transcribed Spacer amplification (ITS) using the 18S primer 1200F and the 28S primer NL1fR (Table 1), the thermocycler program was: initial denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 2 min; final extension at 72 °C for 7 min. The nested amplification with ITS primer ITS1 and 28S primer CTB6R yielded an ~1200 bp product and was: initial denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min; final extension at 72 °C for 7 min. All PCR products were cleaned up using the MinElute PCR Purification Kit (Qiagen, Germantown, MD, USA) and directly sequenced at GeneWiz (South Plainfield, NJ, USA). Chromatograms were checked and assembled using Sequencher v 5.4.6 (Gene Codes, Ann Arbor, MI, USA), and the contig exported as a FASTA file.

Table 1. Primers used in this study.

Primer name	Sequences (5'-3')	Use	References
EukA	AACCTGGTTGATCCTGCCAG T	Initial amplification, nested PCR, sequencing	Modified from Medlin <i>et al.</i> (1988)
EukB	GATCCTTCTGCAGGTTACCC TAC	Initial amplification	Modified from Medlin <i>et al.</i> (1988)
570F	GCTATTGGAGCTGGAATTA	nested PCR,	Weekers <i>et al.</i> (1994)

	C	sequencing	
892F	CCAAGAATTCACCTCTGAC	nested PCR, sequencing	Elwood <i>et al.</i> (1985)
892R	GTCAGAGGTGAAATTCTTG G	nested PCR, sequencing	Modified from Elwood <i>et al.</i> (1985)
1200R	GGGCATCACAGACCTG	nested PCR, sequencing	Weekers <i>et al.</i> (1994)
1200F	CAGGTCTGTGATGCCC	Initial amplification	Weekers <i>et al.</i> (1994)
EukB2	GATCCTKCTGCAGGTTACCC TA	Initial amplification	Modified from Medlin <i>et al.</i> (1988)
ITS1	TCCGTAGGTGAACCTGCGG	nested PCR, sequencing	https://nature.berkeley.edu/brunslab/tour/pimers.html
NL1fR	TAGATGAAATTTACCACCC A	Initial amplification	https://nature.berkeley.edu/brunslab/tour/pimers.html
CTB6R	CCTCCGCTTACTTATATGC	nested PCR, sequencing	https://nature.berkeley.edu/brunslab/tour/pimers.html

Phylogenetic analyses

An alignment was made using ClustalW (Larkin *et al.* 2007) in MEGA7 (Kumar *et al.* 2016) with the new sequences and ones of *Ichthyodinium* and *Amyloodinium* available in GenBank, plus a number of environmental sequences with highest similarity detected through a BLAST search. The sequences *Ichthyodinium* sp. ex *Anguilla anguilla* (972 bp, KF731662), *Ichthyodinium* sp. PS01 ex *Gadus morhua* (955 bp, FJ940898), *Ichthyodinium* sp. PBT ex *Thunnus orientalis* (573 bp, AB488441) were excluded because they were short and they were identical to longer sequences already

included in the alignment. Two short environmental sequences from the Caribbean Sea (GU823763, 1196 bp; GU824976, 1387 bp) that branched within the group of the European sequences of *Ichthyodinium* were also excluded. The alignment further included species of *Euduboscquella* spp., syndineans (*Amoebophrya*, *Hematodinium*, *Syndinium*), perkinsids (*Perkinsus*, *Parvilucifera*), several dinokaryotic dinoflagellates including all the available sequences of *Amyloodinium*, and the apicomplexan *Toxoplasma* for rooting the phylogenetic tree. The evolutionary history was inferred by using the RAxML method (Stamatakis et al. 2008) with default settings and allowing the program to terminate bootstrapping (Pattengale et al. 2010) at the Cipres Portal (Miller et al. 2010). The sequences were deposited in DDBJ/EMBL/GenBank under accession numbers MH248943, MH248956, MH249038, MH571751 and MH590044.

RESULTS

Observations of *Ichthyodinium*

Five fish eggs with different infection development stages were observed in August 2015 (Fig. 1A–E). The eggs were ellipsoidal in shape, a distinctive characteristic of the family Engraulidae. Based on the shape and size (~300 µm long, ~100 µm wide), they were readily identifiable as eggs of the Argentine anchovy (*Engraulis anchoita*), the most common and abundant pelagic fish species in the region. The developmental stages showed growth of the parasite while the embryo was still alive. The earlier stages of infection visible under light microscopy were recognizable as single spherical structures 10–20 µm in diameter (Fig. 1A). The parasite consumed the contents of the yolk sac, resulting in darker parasite masses and death of the embryo (Fig. 1B). The parasite masses divided to produce hundreds of spherical spores. At this stage, an infected egg was isolated for molecular analysis.

Several spherical eggs were observed in May 2005. They showed a large perivitelline space, and a segmented yolk sac with an oil globule that is a characteristic of the members of Clupeidae (Fig. 1F–I). This morphological character and the size corresponded to the Brazilian sardinella (*Sardinella brasiliensis*) another common and abundant pelagic fish in southern Brazil. Some eggs were not apparently infected, while other showed early stages of infection with *Ichthyodinium* that were recognizable as one or more spherical, almost transparent structures inside the yolk sac of embryos. At later developmental stages, yolk sacs became successively more filled with dark parasite cell masses and the lipid droplet has disintegrated (Fig. 1E–F). At this stage, the egg was pierced and hundreds of spherical spores were sucked out for molecular analysis. Several eggs were incubated in filtered seawater to complete the life cycle and to release the dinospores. Unfortunately, antibiotics were not available and the eggs became infected by bacteria and fungi.

We obtained SSU rRNA gene and ITS sequences of *Ichthyodinium* from an infected egg of *Sardinella brasiliensis* isolated on March 20, 2015, and from an infected egg of *Engraulis anchoita* isolated on August 5, 2015. The SSU and ITS sequences of the two Brazilian isolates of *Ichthyodinium* were identical (100%). This confirms that the same species is able to infect different hosts in different seasons. In the SSU phylogeny, all the available sequences of *Ichthyodinium* and several environmental clones branched as a sister group of species of *Euduboscquella* with strong support (BP, 100%) within the Marine Alveolate Group I (Fig. 2). The Brazilian isolates clustered with the sequences of *Ichthyodinium chabelardi* from Europe and several environmental sequences from the Pacific Ocean. This was also observed in the ITS phylogeny (Fig. S1). *Ichthyodinium chabelardi* was first described infecting eggs of the European sardine in the coast of Algeria (SW Mediterranean Sea). Sequences from the

Mediterranean Sea are not available, but they are four SSU rDNA sequences of isolates infecting the eggs of the European sardine in Portuguese waters (FJ440623–26, FJ440627). We consider that these sequences are representative of the 'true' *Ichthyodinium chabelardi*. The environmental sequences of KP404655 and KP404710 from Vietnam and our sequences of *Ichthyodinium* have a similarity of 99% (they only differed in two nucleotides). Three sequences of *Ichthyodinium* infecting fishes from Asia clustered together in a lineage with a longer branch. Two environmental sequences from the East Pacific (KJ757231, KJ757687) and one sequence from the Sargasso Sea (EF172830) were basal to the clade of *Ichthyodinium* with strong support (100%) (Fig. 2).

Observations of *Amyloodinium ocellatum*

The gills of recently dead cultured cobia showed a few trophonts still attached, while most of the trophonts were already transformed into tomons that easily detached (Fig. 3A–C). The trophonts were pear-shaped with a peduncle with motile rhizoids (Fig. 3D–F). The trophont became ellipsoid and darker, and developed a hyaline outer layer as it transformed to a tomont (Fig. 3G–H). A red granule was visible that calls to mind a stigma or eyespot (Fig. 3G). The first phase of multiplication showed synchronic divisions and the tomons remained enclosed in a hyaline outer layer (Fig. 3I–P). After two days at 23 °C, tomons of 64- and 128-cell stage were observed. At this point, they were isolated for molecular analysis. A few tomons were allowed to complete the life cycle and the next day swimming dinospores were observed (Fig. 3Q–T). The dinospores (~12–14 µm wide) showed a pronounced anteroposterior flattening. The cingulum was deeply impressed, and the episome was shorter than the hypotheca. There was a red/orange stigma in the ventral side (Fig. 3Q–R), but it was not easy to determinate whether the stigma was in the episome or hyposome. *Amyloodinium*

showed a triphasic life cycle with a parasitic feeding trophont, encysted reproductive tomont and free-swimming infective dinospore (Fig. 3).

We obtained the SSU rRNA gene sequence of *Amyloodinium* infecting the gills of cobia. The sequence of 915 base pairs only differed in one nucleotide (99.9% similarity) from that of *Amyloodinium ocellatum* isolated from a clown fish in the Gulf of Mexico (accession number AF080096). All the available sequences of *Amyloodinium* clustered together with strong support (100%) within the dinokaryotic dinoflagellates. There were no environmental sequences among the top blast hits (Fig. 2).

DISCUSSION

We provide the first molecular data of *Amyloodinium* and *Ichthyodinium* from the southern hemisphere. The new sequences were similar to those from the northern hemisphere, and support *Amyloodinium ocellatum* and *Ichthyodinium chabelardi* as widespread species (Fig. 2). *Amyloodinium ocellatum* infects cobia, and *I. chabelardi* is reported for the first time infecting the fish genera *Engraulis* and *Sardinella*. This confirms these fish parasites as host generalists.

Amyloodinium belongs to a clade of relatively well-known dinoflagellates that includes the ectoparasites *Paulsenella* and *Tintinnophagus*, and the “*Pfiesteria* group”, containing *Pfiesteria piscicida*, *Pseudopfiesteria shumwayae*, *Luciella* spp., and *Cryptoperidiniopsis brodyi* (Fig. 2, Litaker et al. 1999, Levy et al. 2007b). When compared to its relatives, the swarmer of *Amyloodinium* showed a cell compression that is uncommon among the planktonic dinoflagellates (Fig. 3R–T, Landsburg et al. 1994). Tentatively, the cell compression could facilitate the adhesion to the host surface. The life cycle of *Amyloodinium* includes a benthic stage that may be required to fulfill its life cycle in the open ocean, but to date environmental *Amyloodinium* sequences have

not been reported from open ocean surveys. Although *Amyloodinium* SSU rDNA sequences are very divergent from their closest relatives, there is very little sequence difference between the different isolates. This supports the hypotheses that they are able to infect a wide range of hosts, and that they are globally distributed. Specifically, the movement of fishes associated with aquaculture and aquarium activities may facilitate the dispersal of *Amyloodinium*.

Up to date, *Ichthyodinium* has been only reported as a parasite in the vitellus of fish eggs, the so-called ichthyoplankton. There are no observations infecting other organisms. The environmental molecular surveys reveal that *Ichthyodinium* is present in warm or temperate open ocean waters, with tentative undescribed species (Fig. 2). Edgcomb et al. (2011) attributed about 2.5% of total protistan sequences from an open ocean site in the Caribbean Sea to *Ichthyodinium*. The sequences of *Ichthyodinium* from Brazil clustered with environmental sequences (KJ757231, KJ757697) retrieved from the tropical eastern Pacific Ocean at 2500 m depth (Fig. 2). This site, ~200 m above the seafloor, is a mid-oceanic ridge with deep-sea hydrothermal vents with endemic fishes. The Asian isolates (Yuasa et al. 2007, Mori et al. 2007, Shadrin et al. 2010) and these environmental sequences suggest undescribed species of *Ichthyodinium*. This study reports the first observation of *Ichthyodinium* from the south hemisphere, and its role on the fishery stocks remains understudied.

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Supporting material

Fig. S1. rDNA Internal Transcribed Spacer (ITS) phylogenetic tree of *Ichthyodinium*.

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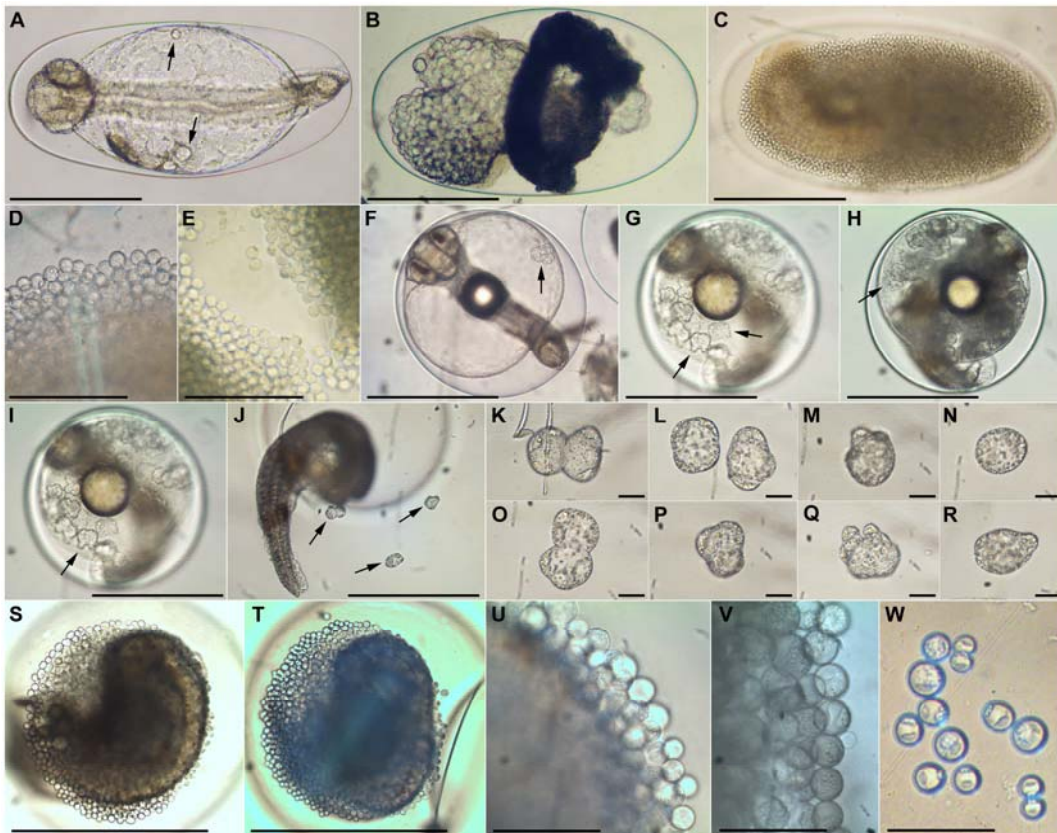
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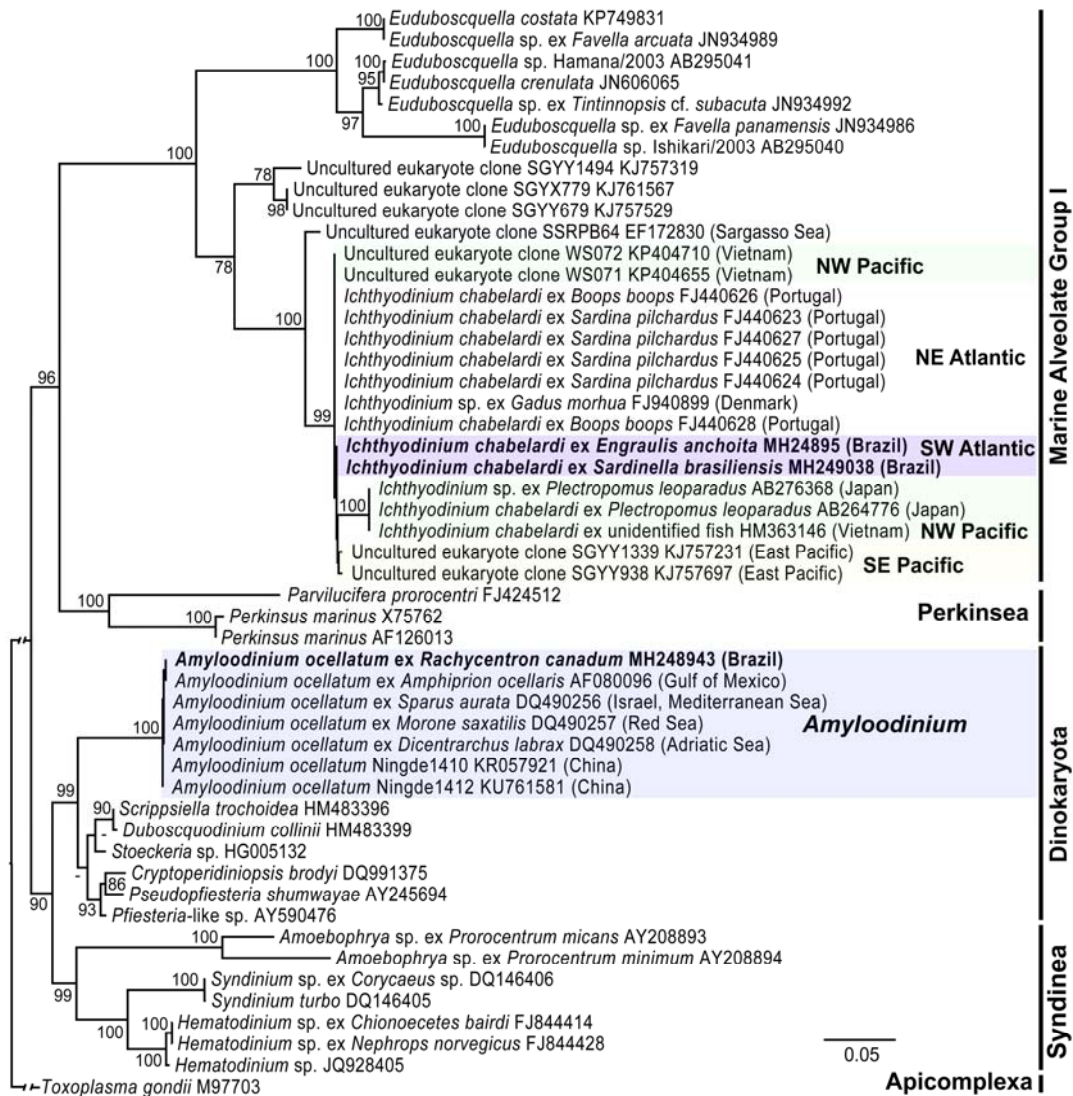


Fig. 2. SSU rDNA phylogenetic tree of new sequences of *Ichthyodinium* and *Amyloodinium* and other alveolates. Newly sequenced species are shown in bold type. Numbers after each taxon name are GenBank accession numbers. The bootstrap values ≥ 70 are noted to the left of internodes. The apicomplexan *Toxoplasma gondii* was used as outgroup. Scale bar = 0.05 substitutions per site.

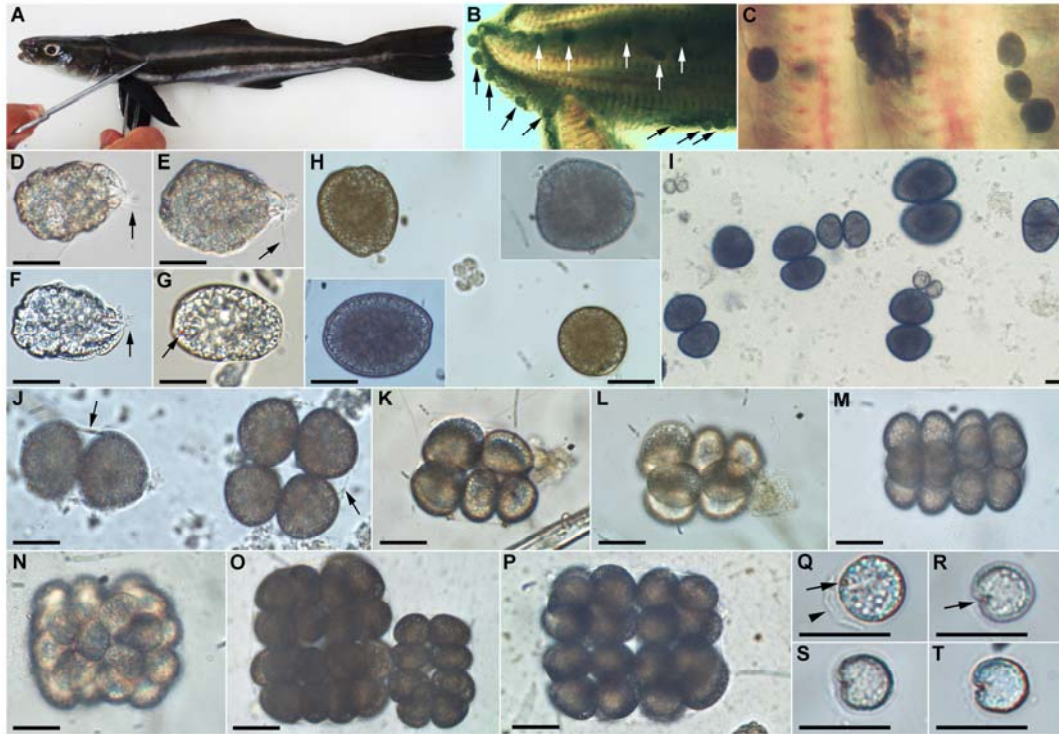


Fig. 3. Light micrographs of the life stages of *Amyloodinium ocellatum* from Ubatuba, Brazil. (A) Infected cobia (*Rachycentron canadum*). (B–C) Trophonts (feeding stage) on gill tissue. (B) The arrows point the parasites. (C) Trophonts on gill tissue. (D–F) Trophonts recently detached from the gills. The arrow points the rhizoid-like complex. (G–P) Tomonts (reproductive stage). (G) The arrow points to a red spot. (H) Tomont before the first division. (I) First phase of multiplication with synchronic tomont division (palintomic sporogenesis). (J) Tomont (2- and 4-cell stages). The arrow points the cell membrane. (K–L) Tomont (8-cell stage). (M–N) Tomont (16-cell stage). (O–P) Tomont (32-cell stage). (Q–T) Infective stages (swarmers). (Q) The arrowhead points a detached hyaline layer. (Q–R) The arrows point a red spot. Scale bar = 20 μm .

Supporting material

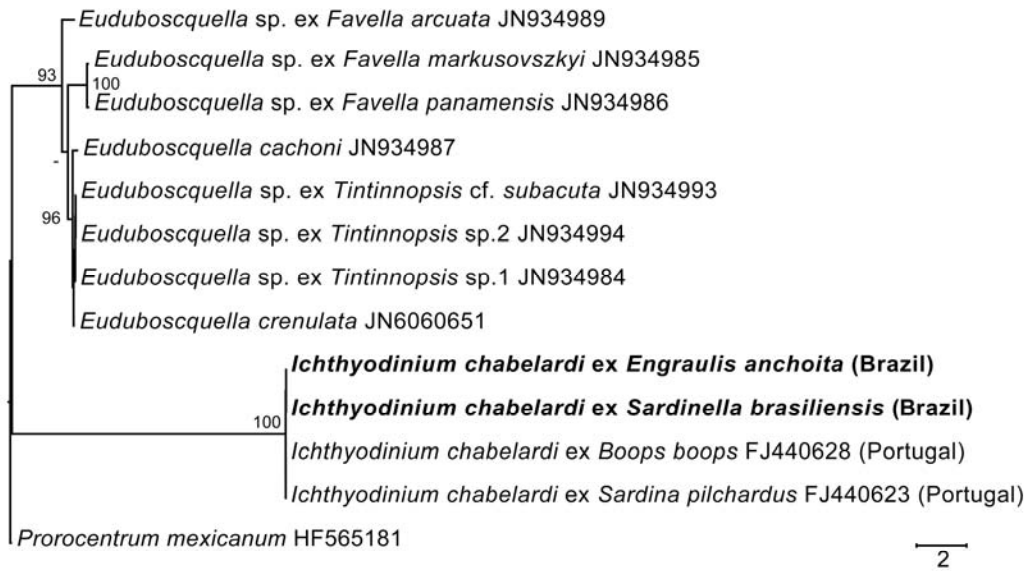


Fig. S1. rDNA Internal Transcribed Spacer (ITS) phylogenetic tree of *Ichthyodinium* and euduboscquelloid alveolates (Marine Alveolate Group I). Newly sequenced species are shown in bold type. Numbers after each taxon name are GenBank accession numbers. The bootstrap values ≥ 70 are noted to the left of internodes. The dinokaryotic dinoflagellate *Prorocentrum mexicanum* was used as outgroup. Scale bar = 2 substitutions per site.

Fig. S1. rDNA Internal Transcribed Spacer (ITS) phylogenetic tree of *Ichthyodinium*.