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Genetic diversity of Amoebophryidae (Syndiniales) during *Alexandrium catenella/tamarense* (Dinophyceae) blooms in the Thau lagoon (Mediterranean Sea, France)

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Abstract

During toxic spring and fall blooms produced by the dinoflagellate *Alexandrium* in the Thau lagoon (Mediterranean Sea), we monitored the presence of Amoebophryidae (Syndiniales), a group of parasites virulent toward a wide range of dinoflagellate hosts. A PCR-biased approach unveiled the presence of at least 10 different parasitic groups during *Alexandrium* proliferation. However, fluorescent in situ hybridization failed to reveal parasitic infection inside *Alexandrium* cells in field populations. In contrast, several co-occurring, less abundant thecate dinoflagellate species were infected by Amoebophryidae, showing up to 10 % of infected cells. We concluded that *Alexandrium* populations were not infected by these local parasites, at least during our survey. In order to check this resistance capacity on a more global scale, we cross-infected several *Alexandrium* strains isolated from the Thau lagoon with one strain of the parasite *Amoebophrya* sp. originating from Salt Pond, MA, USA. All of these hosts were strongly infected by the North American parasite, leading to the conclusion that blooming *Alexandrium* in the Thau lagoon were not particularly resistant to this kind of parasite. These results provide additional evidence that dinoflagellates may become invasive when they successfully escaped their natural enemies in time and/or space (the "enemy release" hypothesis).

Keywords: Dinoflagellate; Parasite; Invasive species

1. Introduction

Many species of *Alexandrium* (Dinophyceae) produce red tides potentially toxic to humans. These species synthesize potent paralytic neurotoxins that can rapidly accumulate in bivalve tissues during algal proliferation. In the last two decades, *Alexandrium* toxic bloom events have considerably increased, with many species suddenly emerging in regions previously free from such algae (Maguer et al., 2004; Penna et al., 2005; Vila et al., 2001). Along the French coast, the first toxic *Alexandrium minutum* red tide was officially recorded in July 1985 by the REPHY network from the bay of Vilaine in southern Brittany (the IFREMER phytoplankton monitoring survey, http://www.ifremer.fr/envlit/surveillance/phytoplancton_phycotoxines). This species then rapidly extended towards northeastern Brittany (Chambouvet et al., 2008). After this first outbreak, toxic blooms of the microalgae were detected almost every year in several estuaries in Brittany. In particular, this species is now well established in the Penzé estuary and was detected with remarkable regularity every June for almost 10 years (Fig. 1). Such regularity is quite atypical for phytoplanktonic blooms and may be partially explained by exceptionally favorable environmental conditions, such as marked eutrophication of this river and better retention of *A. minutum* resting cysts in the sediment. Because of this regularity, the Penzé estuary rapidly became a reference study site to understand optimal environmental conditions for bloom induction (Maguer et al., 2004). However, since 2003, although *A. minutum* is still observed every June, its proliferation now appears to have stabilized at low and non-toxic concentrations.

A recent study identified a parasitoid, *Amoebophrya ceratii* Cachon (Amoebophryidae), as one of the probable causative agent possibly regulating proliferation (Chambouvet et al., 2008; Montagnes et al., 2008). Amoebophryidae (also called marine

alveolate group II) belongs to Syndiniales (Alveolata) and is a widespread group of marine parasites (Guillou et al., 2008). Based upon SSU rRNA gene phylogenies, Amoebophryidae was found to include at least 44 separated clusters, most of them exclusively known by environmental sequences (Guillou et al., 2008). Species boundaries are not clearly delineated and *A. ceratii* is now preferentially referred to as a complex species (Coats et al., 1996). This group of parasitoids is able to infect most, if not all, dinoflagellate species (Cachon, 1964). Infections observed in the Penzé estuary were mostly host-specific, with a single parasitic clade preferentially infecting a single dinoflagellate host species year after year (Chambouvet et al., 2008). This host specialization also provides an explanation for the extensive genetic diversity of this group of parasitoids. Infection begins by the entrance of a small infective biflagellate cell, called the dinospore, into the host (Cachon, 1964). The parasitoid completes its maturation in 2 to 3 days (Coats and Bockstahler, 1994; Coats and Park, 2002) and leaves its host as a long worm-shaped filament of cells known as the vermiform stage (Cachon, 1964). Within a few hours, each constitutive cell of this vermiform is freed to produce hundreds of new infective dinospores, leading to rapid control of the host population (Cachon, 1964; Chambouvet et al., 2008; Coats and Bockstahler, 1994; Coats and Park, 2002).

Both in situ observations (Chambouvet et al., 2008) and in silico modelling (Montagnes et al., 2008) suggested that this parasitoid is an efficient controlling factor, potentially able to prevent toxic bloom proliferation. Without the parasitoid, red tides may occur in the field due to the absence of this natural biological control (Chambouvet et al., 2008).

The presence of a novel invasive species of *Alexandrium* along the French coasts offered us the possibility of testing this hypothesis. The recent invasion by *A. catenella* of the western basin of the Mediterranean Sea has been extensively reported over the last decade.

This species was first detected along the Catalan coasts (Margalef and Estrada, 1987) and expected to have expanded along the northwest Mediterranean coasts (Vila et al., 2001). This species was first observed in 1995 in the Thau lagoon. Since 1998, significant algal blooms and PSP events are recurrent in this ecosystem (Abadie et al., 1999). Since that year, this toxic species has bloomed regularly during the spring and fall in the Thau lagoon, with toxin contamination in bivalves frequently exceeding 80 µg.eq.STX.hg^{-1} (Fig. 1). Two isolates of this invader were identified as belonging to group IV, closely related to Japanese strains (Lilly et al., 2007; Lilly et al., 2002). Consequently, it was assumed that *A. catenella* may have recently been introduced into the Thau lagoon from an Asiatic area. Microsatellite markers used with 61 Mediterranean and 23 Japanese strains demonstrated that these *A. catenella* strains represented separate populations (Masseret et al., 2009). Thus, the exact origin of *A. catenella* proliferating in the Thau lagoon remains to be determined. Intergenic ribosomal regions (ITS1-5.8S rDNA-ITS2) obtained from more than 500 monoclonal strains established during the spring and fall of 2007 revealed the presence of a second ribotype belonging to group III (Genovesi et al., 2010).

Since their introduction, no natural pathogen able to infect these *Alexandrium* populations has been reported from the Thau lagoon. In the present work, we sought to determine whether Amoebophryidae could be detected in the Thau lagoon during these *Alexandrium* blooms, and whether these parasitoids were able to infect such newly toxic invasive species during spring and fall 2007.

2. Materials and methods

2.1. Abundance of dinoflagellates

The Thau lagoon is located on the French Mediterranean coast (43°24 N-3°36 E, Fig. 2) and covers 75 km^2 with a mean depth of 4.5 m (10 m maximum, Fig. 2). This zone is one of the most important oyster productions in Europe. Since 1998, recurrent blooms of the toxic dinoflagellate *A. catenella* have periodically prevented these economic activities. This ecosystem is actively surveyed weekly by the program 'Réseau de Surveillance de Phytoplancton et des Phycotoxines' (REPHY) managed by the Ifremer (http://www.ifremer.fr). Abundant toxic microalgae observed by the REPHY are released annually and publically available at the following site: http://www.ifremer.fr/envlit/.

Every year, A. *catenella*/*tamarense* blooms are initiated at Angle Creek (Fig. 2). This semi-enclosed area constitutes one of the important areas where *Alexandrium* resting cysts accumulate in the sediment, and is thus the most intensively surveyed site for future bloom initiations (Genovesi, 2006). During the spring and fall of 2007, 8 different stations were sampled weekly. Seven of them were located inside Angle Creek (A00, A3, A5, B3, B5, C3 and C5), and one, station A9, was located just in front of Angle Creek (Fig. 2).

Dinoflagellate concentrations were assessed at all stations. For this purpose, seawater was collected between 50 cm and 1 m in depth using a pumping system. One sample (100 ml) was directly fixed on board using formaldehyde (2 % final concentration). For counts, these samples were decanted for several days and supernatant was removed in order to reduce the sample to a final volume of 2-10 ml (exact volumes were considered). Many subsamples of the discarded volume were examined by microscopy and were systematically free of cells. For final quantification, the totality of one or more subsamples of 50 µl (collected after

homogenization of the sample by at least 15 complete reversals of the sample) was counted in a microtank of 300 µl using an inverted microscope (Axiovert Zeiss).

To ensure the quality of counts at low densities (< 100 cells l^{-1}), 20 to 30 l were systematically concentrated on board through a 20 µm net sieve into a final volume of about 30 ml (exact volumes were considered and samples were concentrated ± 1000 times). This sample was then fixed using formaldehyde (2% final concentration). In the laboratory, 50 to 100 µl of this concentrate were collected and counted as previously explained. Counts obtained using these two methodologies (untreated and concentrated samples) were compared on 10 samples based upon a Wilcoxon rank test. No significant difference was detected (effective pairing: rs Spearman = 0.9876 ; $p<0.0001$).

2.2. Genetic diversity and sequence analyses

Only one station per date was selected for genetic analyses of eukaryotes. Because *Alexandrium* spp. are known to occur as a patchy mode, densities of *Alexandrium* spp. were rapidly checked on board. For this purpose, 100 µl of the sample concentrated and fixed on board were mounted on a glass slide with a cover slide and densities roughly assessed using a conventional microscope (Axiolab, Zeiss). When the concentration was considered sufficient, 10 l of untreated seawater were stored on board and rapidly brought to the laboratory (usually within 2 h). In the laboratory, 4 to 6 l of this sample were filtered for genetic diversity for 30 min using a peristaltic pump through a 47-mm-diameter polycarbonate filter with a porosity of 3 µm (Osmonic poretics) in filter housing connected in series to a 0.2 µm pore size Sterivex unit (Millipore). Filters were submerged in a lysis buffer (EDTA 40 mM, Tris-HCl 50 mM, saccharose 0.75 M) and kept at -80 °C until DNA extraction.

DNA was extracted using a CTAB (cetyltrimethyl ammonium bromide) protocol, as described by Doyle et al. (1987) with some modifications. Sterivex filters were incubated in 3 % (w/v) CTAB solution (0.1 M Tris-HCl pH=8.0, 3 % CTAB (Sigma), 1.4 M NaCl, 0.2 % β-mercapto-ethanol (Sigma), 20 mM EDTA pH=7.2) preheated to 60 °C for 2 h. Nucleic acids were extracted twice with an equal volume of chloroform-isoamyl alcohol (96:4). After two centrifugations for 15 min at 11,000 g (4 °C), the aqueous phase was transferred to a clean tube and two volumes of cold isopropanol were added and incubated at -20 °C overnight to precipitate the nucleic acids. The nucleic acids were thus recovered by centrifugation (11,000 g for 10 min at 4 °C). Tubes were drained by gravity and the pellet was dried. Then, pellets were re-suspended in Milli-Q sterile water prior to storage at -20 °C.

The genetic diversity of eukaryotes was first assessed on the larger size fraction using the general eukaryotic set of primers 328f-329r amplifying the whole SSU rDNA gene (Moon-van der Staay et al., 2000). Amoebophryidae genetic diversity was then assessed by a biased-PCR approach using the ALV01 probe as forward primer in complement to the 329r reverse primer, with a final amplicon length of 1040 bp (Chambouvet et al., 2008).

Amplifications were performed using the GoTaq® Flexi PCR kit (50 µl final volume) as described by Promega (1/5 5X Green GoTaq® Flexi Buffer, 4 mM $MgCl_2$, 10 pmol of each primer, 100 µM final concentration dNTPs and 2.5 U of GoTaq® Flexipolymerase). Thirty-five cycles were used, each consisting of a 30 s denaturation step at 95 °C, a 1 min annealing step at 55 °C and a 3 min extension step at 72 °C. After the final cycle, the reaction mixture was incubated at 72 °C for 10 min. PCR amplification was checked on 1 % agarose gel. Amplified PCR products were cloned into the TOPO® TA cloning vector (Invitrogene) and inserted into competent One-Shot® TOPO 10 F' *E. coli*. Plasmids were purified with the "plasmid Miniprep96" kit (Millipore). Sequencing was done using the Applied Biosystems Sequencing kit version 3.1 and an ABI PRISM model 377 (version 3.3) automated sequencer

with plasmid primers M13F and M13R at the Ouest-Genopole platform of the Station Biologique de Roscoff (France). Sequences were deposited in GenBank (accession numbers HQ337025 to HQ337055).

For parasite diversity, around 50 clones per genetic library were sequenced. The KeyDNAtools website (available online http://keydnatools.com) was used to identify putative chimera and provide rapid evaluation of sequence taxonomy (Guillou et al., 2008). One representative clone per clade and per genetic library was selected to be entirely sequenced. Selected sequences were aligned with other related published sequences, mostly from the Penzé estuary (Chambouvet et al., 2008), using Clustal W2 software (Larkin et al., 2007). The best nucleotide substitution model was determined using JModeltest 0.1.1 (Posada, 2008) and a transitional model with six free parameters and unequal base frequencies (TIM2+I+G) was selected using both Akaike and Bayesian information criterion, with the following parameters: Lset base=(0.2496 0.1906 0.2678 0.2919) nst=6 rmat=(1.8426 3.6963 1.8426 1.0000 6.6534 1.0000) rates=gamma shape=0.4720 ncat=4 pinvar=0.2600 (-lnL = 5685.9704). Settings given by the jModeltest were used to perform neighbor joining (NJ), maximum likelihood (ML) and Bayesian analyses. NJ and maximum parsimony (MP) were performed using PAUP 4.0b10 (Swofford, 2002). Bootstrap values for NJ and MP were estimated from 1,000 replicates. For MP, the number of rearrangements was limited to 5,000 for each bootstrap replicate. In addition, we used Bayesian reconstruction for analysis of complete sequences using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), starting with a random tree run for 2,000,000 generations in four chains, with a burn-in of 5,000 generations in order to ensure the analysis was based on a stable chain. Data remaining after discarding burn-in samples were used to generate a majority-rule consensus tree where the percentage of samples recovering any particular clade of the consensus tree represented the clade's posterior probability (Huelsenbeck and Ronquist, 2001).

2.3. Fluorescent in situ hybridization

For fluorescent in situ hybridizations, 2 l were concentrated on board through a 20 µm net sieve into a final volume of 180 ml. This sample was fixed on board with 1 % (final concentration) of paraformaldehyde (Sigma). In the laboratory, the sample was filtered by gravity through 10 µm polycarbonate filters (Osmonic poretics) and dehydrated using ethanol series (50, 80, and 100 %, 3 min each). Filters were then stored at -80 °C until use.

Parasites belonging to Amoebophryidae were detected inside their hosts using the modified CARD-FISH protocol, as explained by Chambouvet et al. (2008). Briefly, hybridizations were carried out at 42 °C for at least 15 h and then two successive washing steps at 46 °C for 20 min were necessary to remove background. In this study, we used the ALV01 oligonucleotide probe (5'-GCC TGC CGT GAA CAC TCT -3'), which had previously been successfully tested on parasites of *A. minutum* in the Penzé estuary (Chambouvet et al., 2008). At the end of the hybridization procedure, cells were counterstained with propidium iodide (final concentration 10 µg ml^{-1}) to visualize nuclei, and calcofluor (fluorescent Brightener 28, Sigma, 1% final concentration) to stain dinoflagellate theca. Photographs were taken using an epifluorescence microscope BX51 (Olympus optical CO, Tokyo, Japan) equipped with a mercury light source. Field samples collected from the Penzé estuary (Brittany, France), where parasites belonging to Amoebophryidae are known to occur, were used as positive control. For prevalences (percentage of infected hosts), a minimum of 50 cells were counted. This information is only available for thecate dinoflagellates, as naked dinoflagellates could not be identified after the FISH procedure.

2.4. Infection experiments

A strain of *Amoebophrya ceratii* was isolated from water sampled at Salt Pond, MA, in 2003 during a bloom of *Alexandrium tamarense* (unknown genetic clade). This strain belongs to Amoebophryidae clade 2, as suggested by its SSU rRNA gene sequence (Fig. 4, sequence HQ337038). Cultures of this parasite are currently maintained using *A. tamarense* as host (belonging to group I of the *tamarensis* complex species), following methods explained by Coats and Park (2002). These strains are maintained at 20 °C on a 14:10 light: dark cycle at 70-80 µmol $m^{-2}.s^{-1}$ in F/2 medium based on autoclaved seawater (salinity of 30 psu) (Guillard and Ryther, 1962). Different *Alexandrium* strains were isolated over the years from the Thau lagoon (Table 1), all of which belong to group IV of the *tamarensis* complex species (Masseret et al., 2009). These strains were sent to the Smithsonian Environmental Research Center and acclimated using the same culture conditions as for the parasitic strain. After their acclimation, once the cultures reached sufficient density, 2 ml aliquots were placed in 24-well plates. Freshly-produced parasites were collected by gravity filtration through a 12 µm nucleopore filter to remove the remaining host. To each well, a small volume of parasites was added. After 4 days, wells were observed under an Axioscope inverted, fluorescence microscope (450 to 490 nm excitation; 520 nm barrier filter). Positive infections were determined by the presence of the green fluorescence of the parasite and the presence of the beehive structure. In order to ensure that infections could be propagated for several generations, 1 ml samples of previous incubations were used as inoculants for another series of infections in a new well containing an uninfected host.

3. Results

3.1. Abundance and genetic diversity of Alexandrium *spp. in 2007*

Alexandrium spp., independently assessed by REPHY data and by Genovesi et al. (2010), occurred at similar periods of the year (Fig. 3). During spring, blooms extended over one month, from the middle of May to the middle of June. This period was longer in the fall and extended from the middle of September to early November. During the spring, the six samples collected each week from April 24 to June 7 largely covered the blooming period. In the fall, this survey was stopped after October 15 for material reasons, and only 3 dates (September 20 and October 11 and 15) were sampled. However, those three dates covered the maximal densities of *Alexandrium* spp. observed during the fall. Concentrations of *Alexandrium* spp. in our samples ranged from 20 cells l^{-1} on April 26 to 52,400 cells l^{-1} on June 7 during the spring. Densities were higher in the fall, with more than 1.2 10^5 cells l^{-1} observed on September 20 and October 15. Other accompanying dinoflagellate species were present, such as *Peridinium quinquecorne*, *Scrippsiella* sp., *Prorocentrum micans*, *Gymnodinium* sp., *Protoperidinium* sp., *Gonyaulax* sp. and *Dinophysis sp.*, with concentrations never exceeding 6.4 10^3 cells l^{-1} (Table 2). *Scrippsiella trochoidea* was only detected during the spring.

3.2. Genetic diversity

Genetic diversity using general eukaryotic primers was performed on a sample collected during the spring bloom (May 24, 2007). Out of 82 clones, 19 belonged to metazoa (15 Crustacea, 2 Mollusca, and 2 Polychaeta), two clones were affiliated with Thraustochytriidae (Labyrinthulida), 5 were diatoms and one clone was a Cryptophyceae. The remaining sequences belonged to dinoflagellates (55 clones). Among these, 40 clones were affiliated with the *Alexandrium catenella/tamarense* species complex. Phylogenetic

analyses revealed that 36 clones belonged to group IV of the *tamarensis* complex species, closely related to sequence AJ535392 (John et al., 2003), whilst 3 clones belong to group III of the *tamarensis* complex species, closely related to sequence AJ535391 (John et al., 2003).

In order to reveal the genetic diversity of Syndiniales, not detected using general eukaryotic primer amplifications, biased-PCR towards Amoebophryidae was performed on three different dates in the spring (April 26, May 24, and June 7 2007) and one date in the fall (October 15, 2007, Table 2). Among 125 total clones retrieved, 105 belonged to Syndiniales group II (Amoebophryidae). The rest of the sequences were affiliated with polychaetes (3 clones), Cryptophyceae (9 clones) and ciliates (8 clones). All together, 10 clades out of the 44 already described within Amoebophryidae were found in the Thau lagoon (Fig. 4). Clade distribution was very different between the two seasons, with only two clades (clade 1 and 4) retrieved during both the spring and fall periods, having very similar sequences: 95-98% identity between sequences obtained from the two seasons in clade 1 and 99% identity in clade 4. Clades 1 and 2 were the dominant ribotypes in terms of number of clones retrieved during the spring, while clade 8 was the most frequent ribotype found in October. Finally, clades 2, 14, 23, 28 and 44 were only retrieved in the spring (although 3 genetic libraries were made from samples taken in this period) and clades, 8, 10, 11 and 12 were only retrieved in fall. A new clade compared to the published work by Guillou et al. (2008) was detected, including a sequence retrieved from the Thau lagoon in June and two sequences retrieved from the Penzé estuary. Environmental sequences belonging to clades 1, 2 and 4 were closely related to sequences obtained from isolated cultures of *Amoebophrya* spp. infecting a wide range of dinoflagellate species.

3.3. Detection of Amoebophryidae by CARD FISH

All samples collected in the presence of *Alexandrium* spp. (6 dates over the spring bloom and 3 dates over the fall bloom) were checked for the presence of Amoebophryidae. Infected hosts targeted by the specific Syndiniales group II probe were observed in all samples. Each time, different maturation stages of the parasite were observed within different hosts (Fig. 5). Only dinoflagellates were observed to be infected (see examples of *Prorocentrum micans*, *Protoperidinium* sp., *Scrippsiella trochoidea* on Fig. 5), with prevalences up to 11.5 % in *Prorocentrum micans* on May 24 and 13% in *Peridinium quinquecorne* on June 7 (Table 2). However, *Alexandrium* spp. never bound the probe whatever the date considered. Absence of infection was further confirmed by the shape of the *Alexandrium* nucleus, stained by propidium iodide, which always presented a typical U-shape, without deformation or supernumerary nuclei.

3.4. Cross-infection

Cross-infection was performed using the *Amoebophrya* ex *A. tamarense* (GOM) strain isolated from the Salt Pond and four strains of *A. catenella* isolated from the Thau lagoon between 1998 and 2003. In all cases, the *Amoebophrya* strain was able to successfully infect each host culture and infections were maintained over many generations for over two months.

4. Discussion

Alexandrium spp. blooms were usually initiated in the Angle Creek, where the highest concentrations are frequently observed, and were able to extend into other areas of the lagoon when favorable hydrodynamic conditions appeared. In 2007, *Alexandrium* spp. were the most abundant dinoflagellates during the two sampling periods, with occurrences extending over more than a month during the fall. Genetic analysis performed in the spring confirmed the presence of at least two distinct genetic ribotypes within the *A. catenella/tamarense* species complex belonging to groups III and IV, as previously reported by Genovesi et al. (2010). No blooming cells observed after the FISH technique appeared infected by Amoebophryidae, although the ALV01 probe used does not cover the entire genetic diversity of this group. In fact, no probe is specific for the entire Amoebophryidae group, which is highly genetically diverse. The screening of this probe on database published by Guillou et al. (2008) revealed that it is still the best consensus probe, targeting 33 clades out of the 44 described within Syndiniales group II (clades 7, 18, 19, 20, 22, 29, 34, 35, 36, 37 and 40 have at least one mismatch with the ALV01 sequence motif). This probe matches the 11 complete sequences obtained from cultivated *Amoebophrya* deposited in GenBank. No other potential parasitic infections, including viral infection, were detected based on the shape of the nucleus and cell morphology in general.

This absence of infection was not caused by the incapacity of *Amoebophrya* spp. to proliferate in the Thau lagoon. On the contrary, the Syndiniales group II was particularly genetically diverse during *Alexandrium* blooms, with almost a quarter of the worldwide repertory of clades detected in only four samples. Most importantly, co-occurring dinoflagellate species examined during the study, although less abundant by two orders of magnitude than *Alexandrium* spp., were infected by Amoebophryidae. This fact, combined with coverage of the monitored periods, led us to believe that the absence of a signal by the

FISH technique observed in *Alexandrium* populations hardly resulted from the variability of the infection rate in time and space. These results strongly suggest that the dominant *Alexandrium* populations occurring in the Thau lagoon in spring and fall 2007 were apparently not infected by all Amoebophryidae retrieved from the genetic libraries (the 10 clades observed) during the monitored period. This absence of detected Amoebophryidae parasites capable of infecting *Alexandrium* spp. could help to explain the long bloom periods in the Thau lagoon (> 1 month), which might eventually result in toxic events since 1998. These results led us to suggest that species boundaries are not easy to cross, at least in the field.

Cross-infections in culture demonstrated that several toxic strains of *A. catenella* isolated from the Thau lagoon between 1998 and 2003 were not particularly resistant to Amoebophryidae, as they could be experimentally infected by Amoebophryidae with an *Alexandrium*-infecting strain of *Amoebophrya* from the Salt Pond (USA). This *Amoebophrya* sp. maintained in a host strain belonging to group I of the *tamarensis* complex species is thus also able to infect strains belonging to group IV. However, it is well known that cross-infections in the laboratory often provide more permissive conditions than environmental conditions (Poulin and Keeney, 2007). The question is open as to whether or not the Amoebophryidae infecting other dinoflagellates could have the capacity to adapt to *Alexandrium* spp. in the Thau lagoon. The lack of evidence for clear co-evolution between *Amoebophrya* strains and their dinoflagellate hosts clearly indicated the capacity of such parasitoids to jump from one host to another (Guillou et al., 2008). Primary host changes are not uncommon in parasitic interactions. We cite the example of fish parasites in the genus *Gyrodactylus*, which have jumped between different host families. Such events often lead to parasite speciation. However, these mechanisms require long-term cohabitation between a parasite and the novel host species (Zietaria and Lumme, 2002).

The arrival of a species in a new ecosystem may result in interruption of interactions between the host and its pathogens, hence allowing proliferation of the host released from this biological control. This phenomenon was previously described for plants, in which the commonly accepted mechanism of invasion is introduced by the "enemy release hypothesis" (ERH) (Keane and Crawley, 2002). This hypothesis had previously been proposed to explain the recent increase in frequency and occurrence of toxic dinoflagellate blooms (Chambouvet et al., 2008; Salomon et al., 2003). Empirically, it appears that blooms of *A. minutum* occurring from the 1990s on in the Penzé estuary likely came under control of at least one *Amoebophrya* parasite after 2001. Thus, more than 10 years were apparently necessary for biological controlling factors to efficiently reduce *A. minutum* proliferation. In the Thau lagoon, *Alexandrium* spp. appeared for the first time in 1998, more than 10 years ago, suggesting that the emergence of this type of biological control may require a long period of time. The use of Amoebophryidae as a biological control might be very tempting to reduce such a period. We know that strains of *A. catenella* isolated from the Thau lagoon are sensitive to a parasitic strain isolated from the eastern coast of North America. However, the use of biological control against pests has often been found to have worse effects than the pests themselves. Major problems indeed occurred when introduced biological controls lacked specificity and suddenly proliferated by attacking autochthonous hosts or preys. For example, the use of *Harmonia axyridis*, a voracious coccinellid beetle native to Asia, which was introduced in North America and Europe to control aphids and scale insects, has now become a dangerous invader that threatens the endemic species (Koch, 2003).

In conclusion, the Thau lagoon offers a unique opportunity for investigating the process allowing Amoebophryidae parasites to adapt to a newly arrived potential host species in a semi-enclosed ecosystem. This study was a first attempt to evaluate the Amoebophryiade infectious reservoir present in the Thau lagoon, with a list (probably non-exhaustive) of ten

parasitoid clades that must be considered potential candidates for further adaptation to the invasive *Alexandrium*. As *Alexandrium* spp. are present throughout the Mediterranean Sea, it would be very interesting to investigate whether these species are infected in other areas and to compare them using Amoebophryidae genetic clades.

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Fig. 1. Occurrence of *Alexandrium spp.* in the Penzé estuary (North Brittany, France, upper part) and the Thau lagoon (south of France, last part) over the last two decades (1987 to 2007). Stars represent toxicity of bivalves with > 80 µg.eq.STX.hg^{-1}. For the Thau lagoon, cell densities in light and dark gray correspond to blooms occurring during spring and fall, respectively. Data provided by the French phytoplankton monitoring network REPHY (Ifremer, http://wwz.ifremer.fr/envlit/surveillance/phytoplancton_phycotoxines).

Fig. 2. Sampling area in the Angle Creek of the Thau lagoon represented on different geographic scales. The Penzé estuary (see figure 1) is also indicated in the largest scale. The IFREMER sampling point and stations sampled during this study are shown in the bottom panel.

Fig. 3. Occurrences of *Alexandrium* spp. in the Thau lagoon during 2007. *Alexandrium* spp. abundances were obtained from two independent sources (maximal abundances published by Genovesi et al. 2010 and the REPHY, Ifremer, http://wwz.ifremer.fr/envlit/surveillance/phytoplancton_phycotoxines). Spring and fall periods monitored in the frame of this study are indicated by dashed lines.

Fig. 4. Phylogenetic analyses of environmental sequences belonging to Amoebophryidae (Syndiniales group II) detected in the Thau lagoon during spring and fall blooms. Bayesian phylogeny based on analysis of 82 full-length sequences 740 pb in length (including outgroup). Environmental sequences from the Thau lagoon are in bold. Neighbor-joining and maximum parsimony bootstrap values associated with the Bayesian posterior probability higher than 70 % are shown at the principal node on the phylogenetic tree. The scale bar corresponds to 0.1 % sequence divergence.

Fig. 5. Detection of parasitic Amoebophryidae by FISH within different dinoflagellates during the monitoring survey. 1) Infected cell of *Prorocentrum micans* from October 11, 2007. 2) Infected cell of *Protoperidinium* sp. from October 11, 2007. 3) Infected cell of *Scrippsiella trochoidea* from April 26, 2007. 4) Non-infected cells of *Alexandrium* spp. from October 11, 2007. Red fluorescence shows nuclei of cells stained by propidium iodide. Blue fluorescence shows cellulosic theca of dinoflagellate cells revealed by calcofluor. Green fluorescence shows the general oligonucleotide probe ALV01 targeting small subunit ribosomal RNA (SSU rRNA) of the parasitoid by FISH. Scale bars = 10 µm.

Table 1. List of strains used in this study

Taxonomy	Strain name	Origin	Year of isolation	Isolation procedure	Reference
Hosts					
A. catenella group IV of the *tamarensis* complex species	ACT1	Thau lagoon	2002	Cyst germination, monoclonal	Laabir et al. 2002
	ACT2	Thau lagoon	2002	Cyst germination, monoclonal	Laabir et al.2002
	ACT3	Thau lagoon	2003	Vegetative cell, monoclonal	Laabir et al. 2007
	ATTL01	Thau lagoon	1998	Vegetative cell, monoclonal	Lilly et al. 2002
Parasitoid					
Amoebophrya sp.	GOM	Salt Pond, MA (USA)	2003	*A. tamarense* as primary host	This study

Table 2. Host densities and infections by Amoebophryidae during spring and fall *Alexandrium* spp. blooms. Dinoflagellate concentrations are in cells l^{-1}. Prevalences in thecate dinoflagellates (percentage of infected host counted from at least 50 different observations) were assessed by FISH for *Alexandrium* spp. abundances superior to 30,000 cells l^{-1}. Prevalences are provided in parenthesis after concentration of hosts. When fewer than 50 host cells were counted, the eventual presence of infections was indicated by a +. The variance in data depended on the cell concentration, and the CI (95 %) was estimated at 51.2 % for a concentration of 300 cells l^{-1}, less than 20.3 % for 3,000 cells l^{-1}, and less than 6.2 % for 30,000 cells l^{-1}.

Date	*Alexandrium spp.*	*Peridinium quinquecorne*	*Prorocentrum micans*	*Dinophysis* sp.	*Scrippsiella trochoidea*	*Gymnodinium* sp.	*Protoperidinium* sp.	*Gonyaulax* sp.	Other dinoflagellates	Samples sites
Spring bloom										
26 April 2007 [a]	20	0	0	570	6400	15	218	1244	22,680	A3
11 May 2007	5400	420	850	110	760	133	493	209	1920	C5
16 May 2007	7200	110	340	70	250	417	59	355	450	C5
24 May 2007 [a]	31,500	870	620 (11.5 %)	0	2480 (9 %)	0	248	0	3723 (3 % [b])	A3

31 May 2007	290	30	40	0	0	51	13	0	523	A00
07 June 2007 [a]	52,400	280 (13 %)	50 (1.2 %)	90	870 (6.5 %)	426	124 (+)	53 (+)	674	C5
Fall bloom										
20 September 2007	123,000	160 (+)	410	20	0	119	99	607	1910	B3
11 October 2007	75,300	1100 (+)	1490 (1.8 %)	0	0	398	498	2488	600	B3
15 October 2007[a]	168,400	230	580 (+)	230	0	466	2213	7222	1747	B3

[a] Dates selected to assess *Amoebophrya* spp. genetic diversity.

[b] Prevalence based on small unidentified thecate dinoflagellates

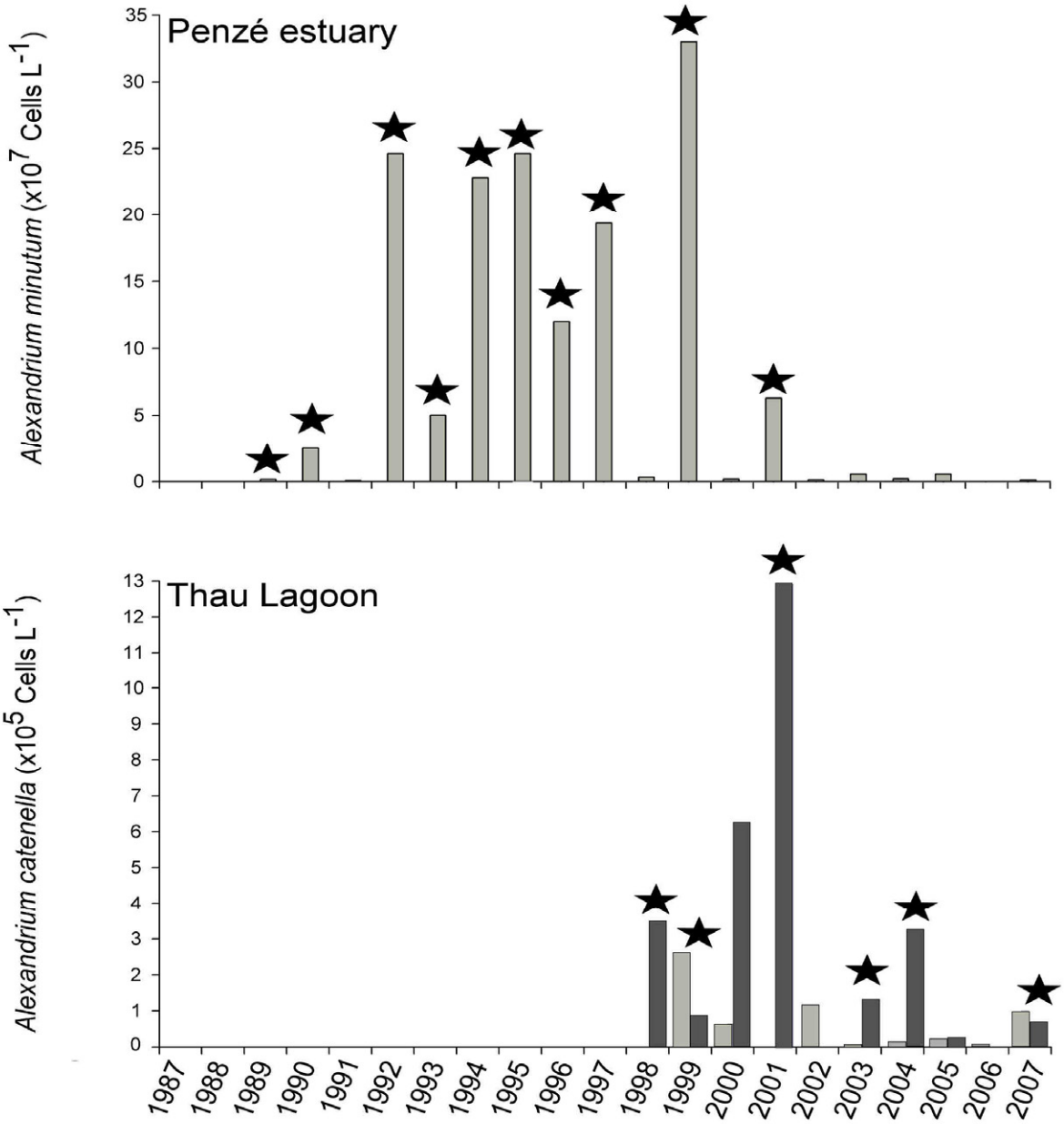
Penzé estuary
Alexandrium minutum (x10^7 Cells L^-1)
0
5
10
15
20
25
30
35
Thau Lagoon
Alexandrium catenella (x10^5 Cells L^-1)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007

Figure 1

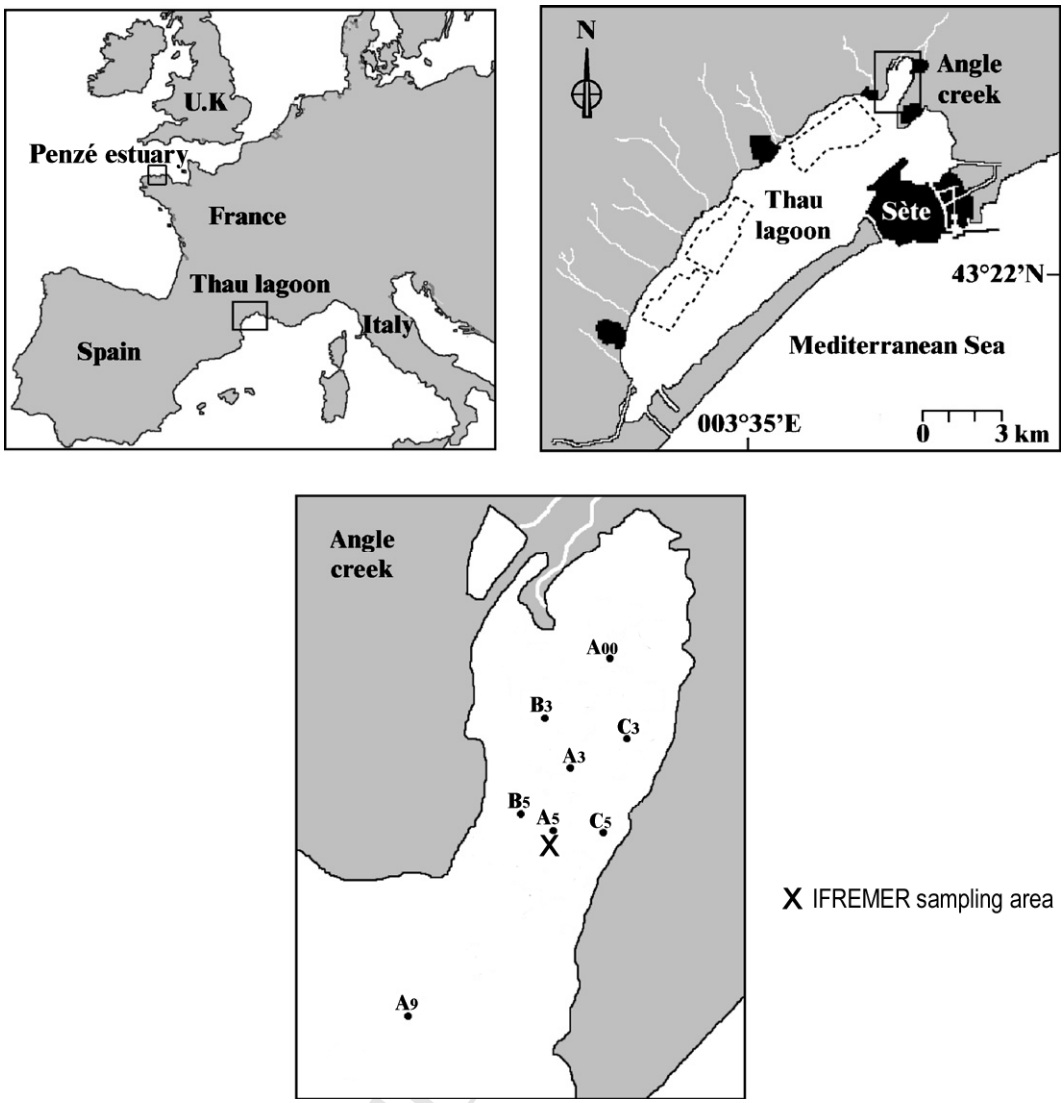

Figure 2

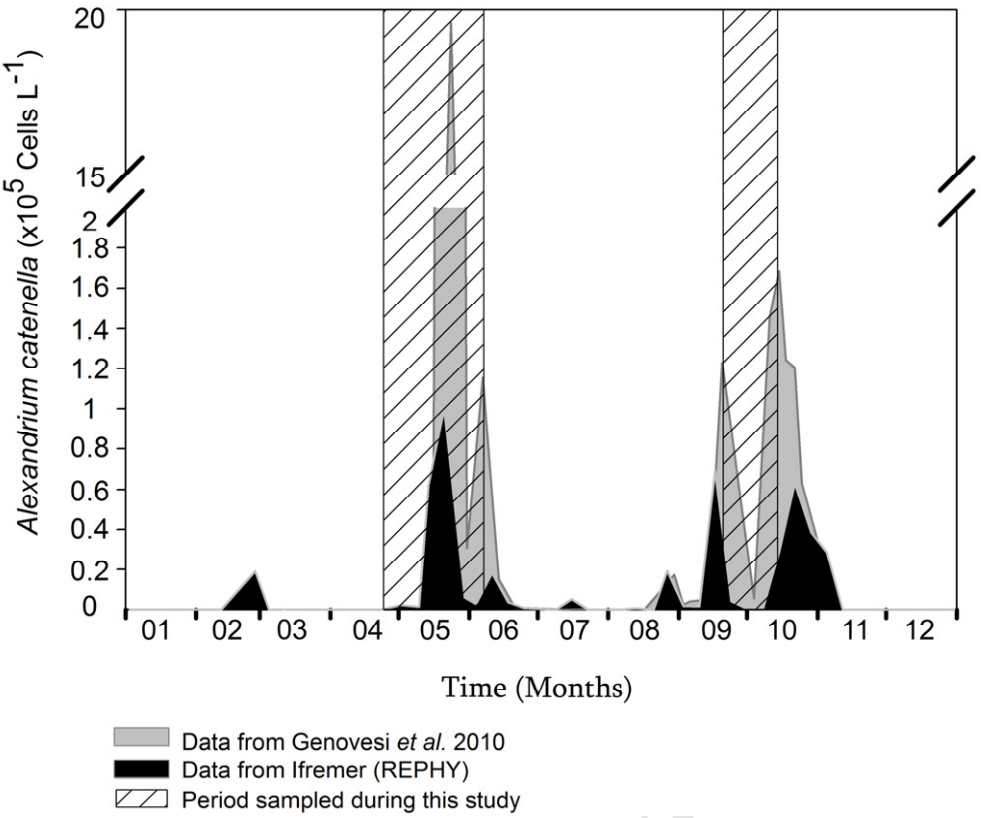

Figure 3

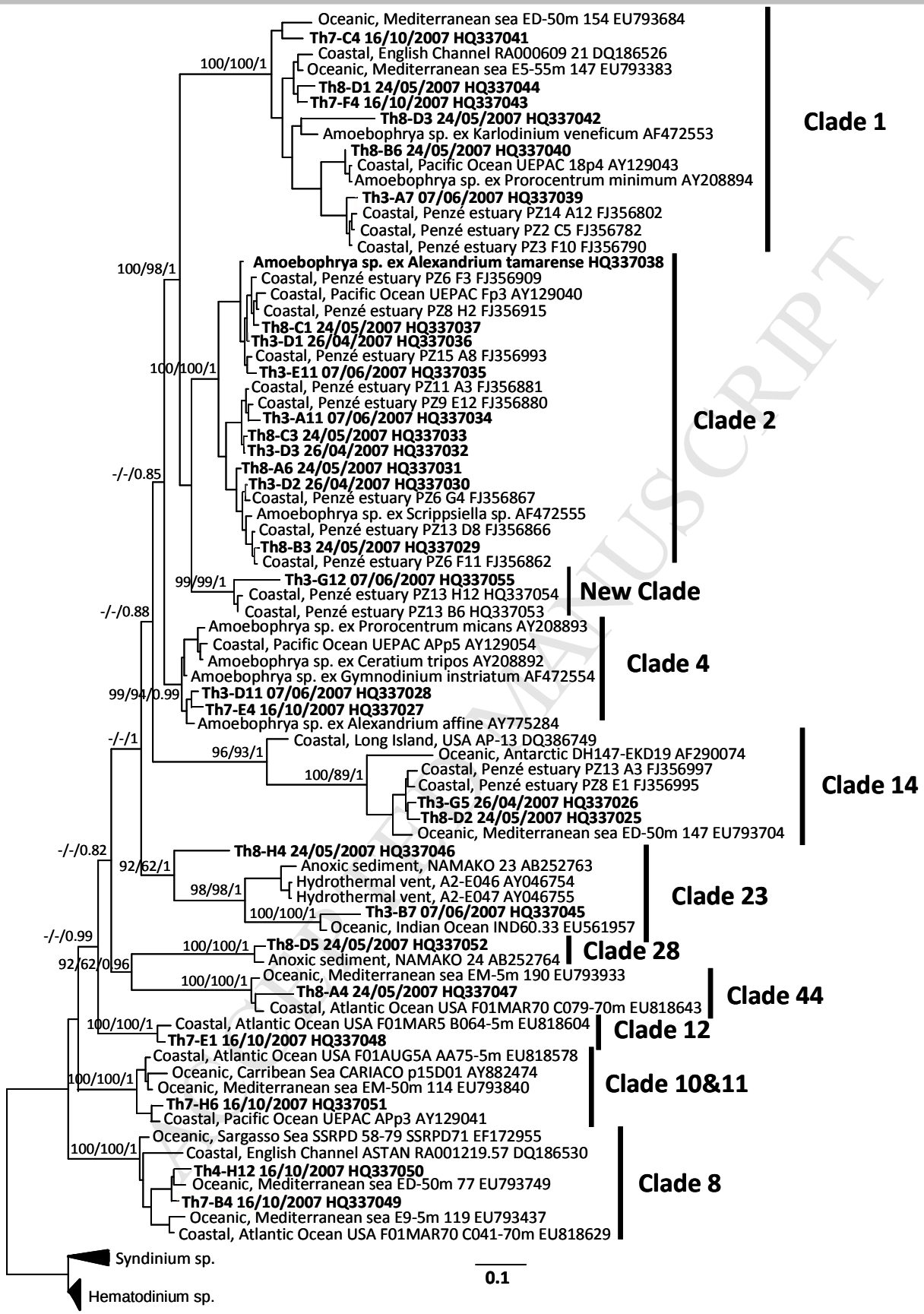
Oceanic, Mediterranean sea ED-50m 154 EU793684
Th7-C4 16/10/2007 HQ337041
Coastal, English Channel RA000609 21 DQ186526
Oceanic, Mediterranean sea E5-55m 147 EU793383
Th8-D1 24/05/2007 HQ337044
Th7-F4 16/10/2007 HQ337043
Th8-D3 24/05/2007 HQ337042
Amoebophrya sp. ex Karlodinium veneficum AF472553
Th8-B6 24/05/2007 HQ337040
Coastal, Pacific Ocean UEPAC 18p4 AY129043
Amoebophrya sp. ex Prorocentrum minimum AY208894
Th3-A7 07/06/2007 HQ337039
Coastal, Penzé estuary PZ14 A12 FJ356802
Coastal, Penzé estuary PZ2 C5 FJ356782
Coastal, Penzé estuary PZ3 F10 FJ356790
Clade 1
100/100/1
100/98/1
Amoebophrya sp. ex Alexandrium tamarense HQ337038
Coastal, Penzé estuary PZ6 F3 FJ356909
Coastal, Pacific Ocean UEPAC Fp3 AY129040
Coastal, Penzé estuary PZ8 H2 FJ356915
Th8-C1 24/05/2007 HQ337037
Th3-D1 26/04/2007 HQ337036
Coastal, Penzé estuary PZ15 A8 FJ356993
Th3-E11 07/06/2007 HQ337035
100/100/1
Coastal, Penzé estuary PZ11 A3 FJ356881
Coastal, Penzé estuary PZ9 E12 FJ356880
Th3-A11 07/06/2007 HQ337034
Th8-C3 24/05/2007 HQ337033
Th3-D3 26/04/2007 HQ337032
Th8-A6 24/05/2007 HQ337031
-/-/0.85
Th3-D2 26/04/2007 HQ337030
Coastal, Penzé estuary PZ6 G4 FJ356867
Amoebophrya sp. ex Scrippsiella sp. AF472555
Coastal, Penzé estuary PZ13 D8 FJ356866
Th8-B3 24/05/2007 HQ337029
Coastal, Penzé estuary PZ6 F11 FJ356862
Clade 2
99/99/1
Th3-G12 07/06/2007 HQ337055
Coastal, Penzé estuary PZ13 H12 HQ337054
Coastal, Penzé estuary PZ13 B6 HQ337053
New Clade
-/-/0.88
Amoebophrya sp. ex Prorocentrum micans AY208893
Coastal, Pacific Ocean UEPAC APp5 AY129054
Amoebophrya sp. ex Ceratium tripos AY208892
Amoebophrya sp. ex Gymnodinium instriatum AF472554
99/94/0.99
Th3-D11 07/06/2007 HQ337028
Th7-E4 16/10/2007 HQ337027
Amoebophrya sp. ex Alexandrium affine AY775284
Clade 4
-/-/1
Coastal, Long Island, USA AP-13 DQ386749
96/93/1
Oceanic, Antarctic DH147-EKD19 AF290074
100/89/1
Coastal, Penzé estuary PZ13 A3 FJ356997
Coastal, Penzé estuary PZ8 E1 FJ356995
Th3-G5 26/04/2007 HQ337026
Th8-D2 24/05/2007 HQ337025
Oceanic, Mediterranean sea ED-50m 147 EU793704
Clade 14
-/-/0.82
Th8-H4 24/05/2007 HQ337046
92/62/1
Anoxic sediment, NAMAKO 23 AB252763
Hydrothermal vent, A2-E046 AY046754
98/98/1
Hydrothermal vent, A2-E047 AY046755
100/100/1
Th3-B7 07/06/2007 HQ337045
Oceanic, Indian Ocean IND60.33 EU561957
Clade 23
-/-/0.99
100/100/1
Th8-D5 24/05/2007 HQ337052
92/62/0.96
Anoxic sediment, NAMAKO 24 AB252764
Clade 28
Oceanic, Mediterranean sea EM-5m 190 EU793933
100/100/1
Th8-A4 24/05/2007 HQ337047
Coastal, Atlantic Ocean USA F01MAR70 C079-70m EU818643
Clade 44
100/100/1
Coastal, Atlantic Ocean USA F01MAR5 B064-5m EU818604
Th7-E1 16/10/2007 HQ337048
Clade 12
Coastal, Atlantic Ocean USA F01AUG5A AA75-5m EU818578
Oceanic, Carribean Sea CARIACO p15D01 AY882474
100/100/1
Oceanic, Mediterranean sea EM-50m 114 EU793840
Th7-H6 16/10/2007 HQ337051
Coastal, Pacific Ocean UEPAC APp3 AY129041
Clade 10&11
Oceanic, Sargasso Sea SSRPD 58-79 SSRPD71 EF172955
100/100/1
Coastal, English Channel ASTAN RA001219.57 DQ186530
Th4-H12 16/10/2007 HQ337050
Oceanic, Mediterranean sea ED-50m 77 EU793749
Th7-B4 16/10/2007 HQ337049
Oceanic, Mediterranean sea E9-5m 119 EU793437
Coastal, Atlantic Ocean USA F01MAR70 C041-70m EU818629
Clade 8
Syndinium sp.
Hematodinium sp.
0.1

Figure 4

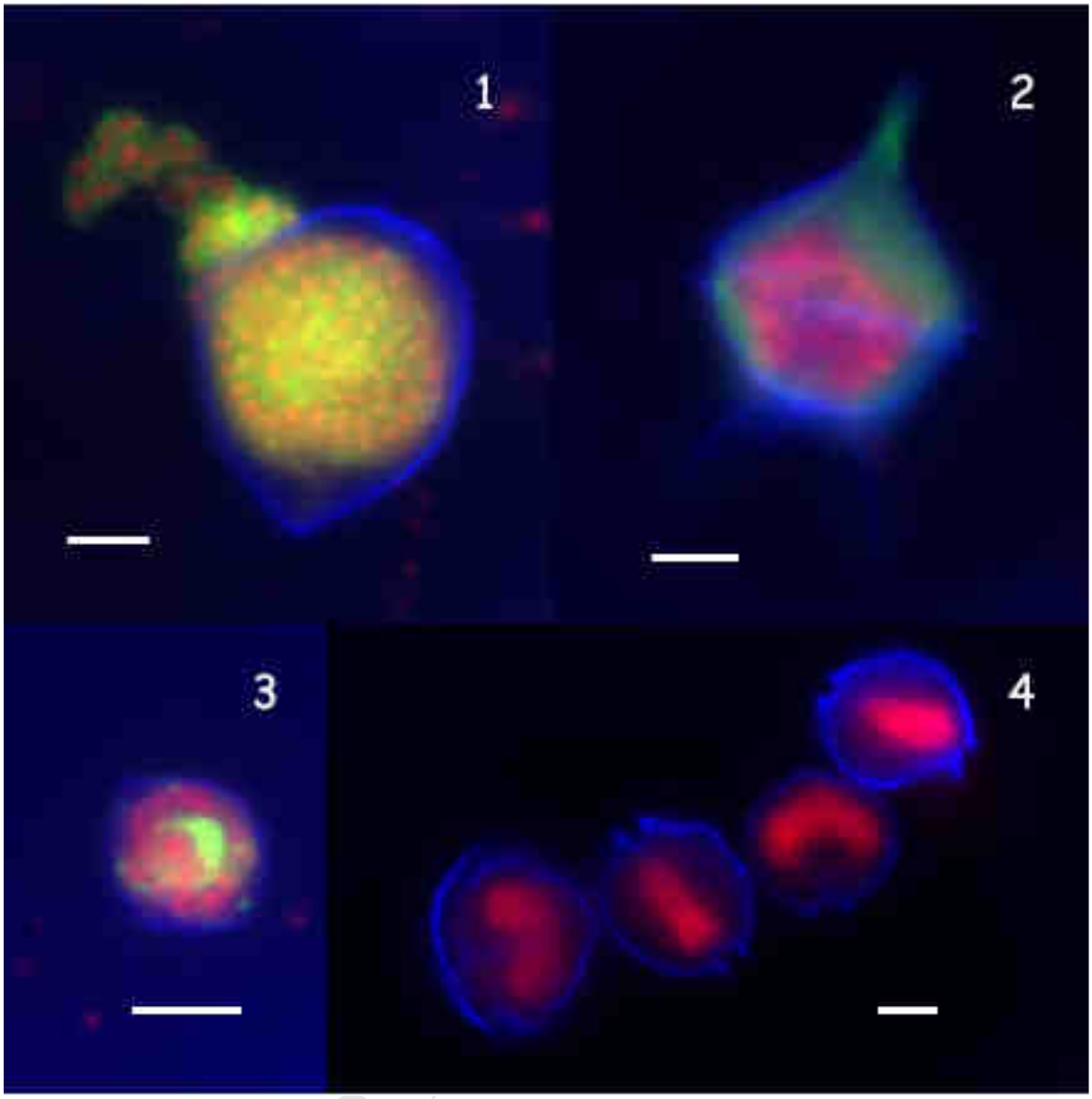

Figure 5