THE DIVERSITY OF DINOFLAGELLATES BELONGING TO THE GYMNODINIALES FROM THE CATALAN COAST (NW MEDITERRANEAN SEA)

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AGRAÏMENTS

Malgrat que aquests agraïments figuren al principi de la memòria, reflecteixen l'acabament d'una etapa; en aquest cas, la realització d'una tesi doctoral. És en aquest moment que mires enrere i te n'adones del camí recorregut al llarg dels darrers anys. El fet que la vida d'aquesta tesi no s'hagi cenyit a la seva durada habitual, sinó que ha estat molt més llarga, es tradueix en una interacció amb moltíssima gent que ha anat passant per l'ICM durant tot aquest temps i els quals, tothom en diferents graus, han tingut la seva influència sobre la tesi. Al mateix temps, aquest període ha coincidit amb grans canvis en la meva vida: m'he independitzat (parlo de mi, no del referèndum...), m'he casat, he tingut una filla, i una segona està a punt d'arribar. Per tant, aquesta tesi ha estat present, sempre mantenint-se en un discret segon pla, en tots aquests canvis. Jo no sóc home de gaires paraules i darrera de cada agraïment podria afegir-hi un munt de comentaris, anècdotes i històries. Però encara que no quedi aquí reflectit, us estic sincerament agraït a tots i guardo els millors records de tots aquells que ja no esteu a l'Institut.

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RESUM DE LA TESI

Les dinoflagel·lades són un dels grups més abundants i diversos de microalgues. Moltes espècies estan cobertes amb plaques de cel·lulosa, mentre que d'altres que no en tenen són conegudes com a atecades o nues. Les espècies atecades han estat generalment mal caracteritzades ja que es deformen quan són fixades amb mètodes tradicionals. La majoria de gèneres atecats s'inclouen dins l'ordre Gymnodinials i es distingeixen per caràcters morfològics, però la combinació recent d'observacions morfològiques amb dades filogenètiques conclou que les filogènies moleculars no suporten els criteris morfològics clàssics utilitzats per distingir els gèneres. També es coneix que l'ordre Gymnodinials no és monofilètic. Per tant, la correcta taxonomia de dinoflagel·lades requereix una combinació d'informació morfològica i molecular. Tot i que moltes dinoflagel·lades no són fàcilment cultivables i no es poden seqüenciar mitjançant tècniques estàndards, la tècnica de single-cell PCR ha permès obtenir seqüències a partir d'una sola cèl·lula, aprofitant el gran nombre de còpies dels gens ribosòmics que les dinoflagel·lades contenen.

Entre els anys 1960 i 1980 es van dur a terme identificacions extensives de les dinoflagel·lades existents al litoral català. Tanmateix, no es va caracteritzar adequadament la diversitat de dinoflagel·lades atecades degut a l'ús de mostres fixades i la manca d'eines moleculars. Aquesta memòria presenta l'estudi de la diversitat d'espècies de l'ordre Gymnodinials a la costa catalana com a representativa del NO del Mar Mediterrani, entre els anys 2010-2013. L'estudi presenta una revisió de la seva taxonomia pel que s'han combinant estudis morfològics d'exemplars vius amb la corresponent informació filogenètica. Atès que la filogènia d'alguns dels organismes estudiats no s'havia determinat amb anterioritat i l'evidència que l'ordre Gymnodinials no és monofilètic, el segon objectiu ha estat estudiar les relacions filogenètiques de les espècies. Es va seleccionar la regió D1-D2 del 28s rDNA per dur a terme els anàlisis amb SC-PCR, però també es van obtenir seqüències del 18s rDNA quan ha estat necessari.

La combinació de les dades morfològiques i moleculars ha permès la identificació inequívoca de 58 espècies atecades pertanyents a l'ordre Gymnodinials. D'aquestes, es detecten deu morfoespècies per primera vegada a la mar Mediterrània, i vuit per primera vegada al litoral català **(Capítols 1 i 2)**. A més, l'aplicació de la SC-PCR ha permès seqüenciar 43 espècies atecades, 25 de les quals per primera vegada **(Capítols 1 i 2)**. També ha permès la detecció i caracterització d'espècies no descrites prèviament, que ha resultat en la descripció de les noves espècies *Gymnodinium litoralis* **(Capítol 3)** i *Polykrikos tanit* **(Capítol 4)**. A més, es va detectar per primera vegada l'espècie tòxica *Cochlodinium polykrikoides* al litoral català. La majoria d'aquests organismes pertanyien a un nou ribotip, però d'altres quedaren inclosos en un ribotip ja conegut, demostrant la seva coexistència al Mar Mediterrani **(Capítol 5)**. Finalment, es va seqüenciar per primera vegada un espècimen del gènere *Ceratoperidinium* i es va obtenir un nou grup filogenètic, juntament amb d'altres dinoflagel·lades atecades, incloent *Ceratoperidinium margalefii*, *Gyrodinium falcatum*, que va ser transferit al gènere *Ceratoperidinium*, tres espècies de *Cochlodinium*, i dos organismes semblants a *Gymnodinium*. Això va donar lloc a l'esmena de la família Ceratoperidiniaceae i del gènere *Ceratoperidinum* **(Capítol 6)**. La correcta identificació de les espècies ha permès concloure que la costa catalana presenta una gran diversitat de dinoflagel·lades atecades, i discutir les implicacions en la distribució i biogeografia de les espècies a nivell de la Mediterrània i global.

SUMMARY OF THE THESIS

Dinoflagellates are one of the most abundant and diverse groups of microalgae. Many dinoflagellates are covered with cellulose plates, whereas others lack these plates and are hence referred to as athecate or unarmoured. Unarmoured species have been historically poorly characterized because they deform when fixed with traditional methods. Most unarmoured genera are included within the order Gymnodiniales and are differentiated by morphological characters, but the recent combination of morphological observations with phylogenetic data concludes that molecular phylogenies do not support the “classical” morphological criteria used to distinguish the genera. It is also known that the order Gymnodiniales is not monophyletic. Consequently, the taxonomy of dinoflagellates has shifted to a combination of morphology and molecular information. The widespread use of molecular techniques has enabled detailed studies on the systematics of a lot of groups. Although many dinoflagellates are not easily cultivable and cannot be sequenced by standard techniques, the single-cell PCR technique has allowed obtaining sequences from a single cell by taking advantage of the large copy number of the ribosomal genes.

Extensive identifications of dinoflagellates present in the NW Mediterranean Sea were carried out between the 1960s and 1980s. However, the diversity of unarmoured dinoflagellates was not suitably characterized because of the use of fixed samples and the lack of molecular tools. This thesis studies the diversity of dinoflagellates belonging to the Gymnodiniales order from the Catalan coast, as a representative site of the NW Mediterranean Sea. This thesis presents a revision of their taxonomy by combining morphological studies of live specimens with the respective phylogenetic information. Given that the phylogeny of most of the studied organisms had not been previously determined and the evidence that Gymnodiniales is not monophyletic, a secondary objective was to study the phylogenetic relationships of species. The LSU rDNA sequence was selected to conduct the SC-PCR, but SSU rDNA sequences were obtained when necessary.

The combination of morphological and molecular data has led to the identification of 58 unarmoured species belonging to the order Gymnodiniales. Ten morphospecies are detected for the first time in the Mediterranean Sea, and eight, for the first time along the Catalan coast **(Chapter 1 and 2)**. Additionally, the application of single-cell PCR has resulted in the sequencing of 43 unarmoured species, 25 of them for the first time **(Chapters 1 and 2)**. It has also allowed the detection and characterization of species previously undescribed, resulting in the erection of two new species: *Gymnodinium litoralis* **(Chapter 3)** and *Polykrikos tanit* **(Chapter 4)**. Additionally, the toxic species *Cochlodinium polykrikoides* was detected for the first time along the Catalan coast. Most populations formed a newly differentiated ribotype, but others were included within a previously known one, demonstrating their coexistence in the Mediterranean Sea **(Chapter 5)**. Finally, a *Ceratoperidinium* specimen was sequenced for the first time and a new phylogenetic clade was obtained, along with other unarmoured dinoflagellates, including *Ceratoperidinium margalefii*, *Gyrodinium falcatum*, which was transferred to the genus *Ceratoperidinium*, three *Cochlodinium* species, and two *Gymnodinium*-like organisms. This resulted in the emendation of the Ceratoperidiniaceae family and the genus *Ceratoperidinum* **(Chapter 6)**. The correct identification of the species has allowed to conclude that there is a high diversity of unarmoured dinoflagellates in the Catalan coast, and to discuss the implications on the distribution and biogeography of the species at a Mediterranean level or globally.

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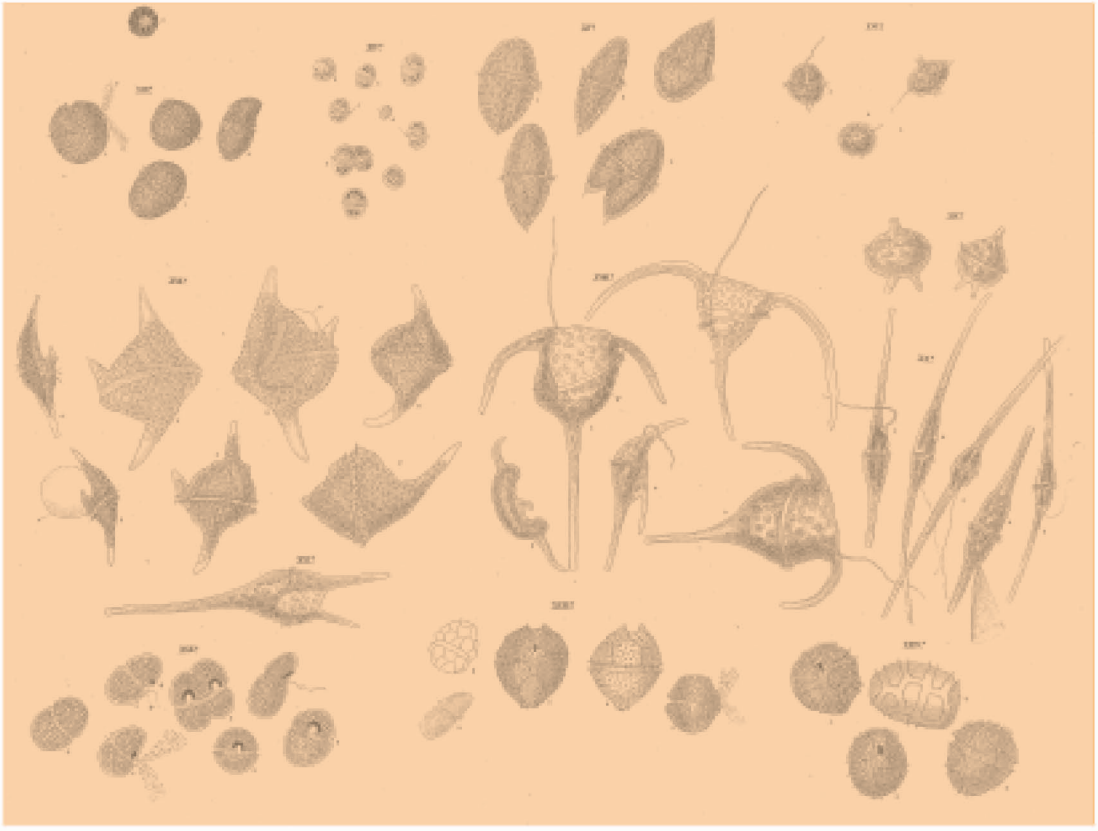


Ehrenberg (1838) Die infusionsthierchen als vollkommene organismen

General Introduction

"Nomenclature, the other foundation of botany, should provide the names as soon as the classification is made... If the names are unknown knowledge of the things also perishes... For a single genus, a single name. "
- Carolus Linneus 1751

THE DINOFLAGELLATES

Overview

Dinoflagellates are one of the most abundant and diverse groups of microplanktonic protists in the world's oceans, with more than 2,000 extant species described (Gómez, 2005; Guiry, 2012; Sournia, 1995; Taylor et al., 2008). Their morphological diversity (Fig. 1) encompasses unicellular and colonial species; phototrophic, heterotrophic, and mixotrophic species; and free-living, parasitic, and symbiont species (Taylor and Pollingher, 1987). Dinoflagellates inhabit freshwater, brackish, and marine environments, with planktonic, benthic, and psammophilic species (Taylor et al., 2008; Taylor and Pollingher, 1987). Their main accessory pigment is most commonly peridinine but in some species it is fucoxanthin or chlorophyll *b* (Zapata et al., 2012), acquired by the endosymbiosis of plastids (Figueroa et al., 2009; Tengs et al., 2000; Watanabe and Suda, 1990; Xia et al., 2013). Heterotrophic and mixotrophic species exhibit several different mechanisms of ingestion (Gaines and Elbrächter, 1987; Schnepf and Elbrächter, 1992) and have a wide range of prey.

Taxonomy

In contrast to smallest eukaryotes, which lack conspicuous morphological features (Massana and Pedrós-Alió, 2008), the broad diversity of dinoflagellate morphologies provides the basis for identifying, classifying, and differentiating the different genera and species. Many dinoflagellates are covered with cellulose plates (theca), whose number, disposition, and shape form the basis of their identification, whereas others lack these plates and are hence referred to as athecate or unarmoured. In fact, dinoflagellate orders reflect this difference (Fensome et al., 1993), with armoured members further differentiated by the morphology and configuration of their thecal plates (Fig. 2).

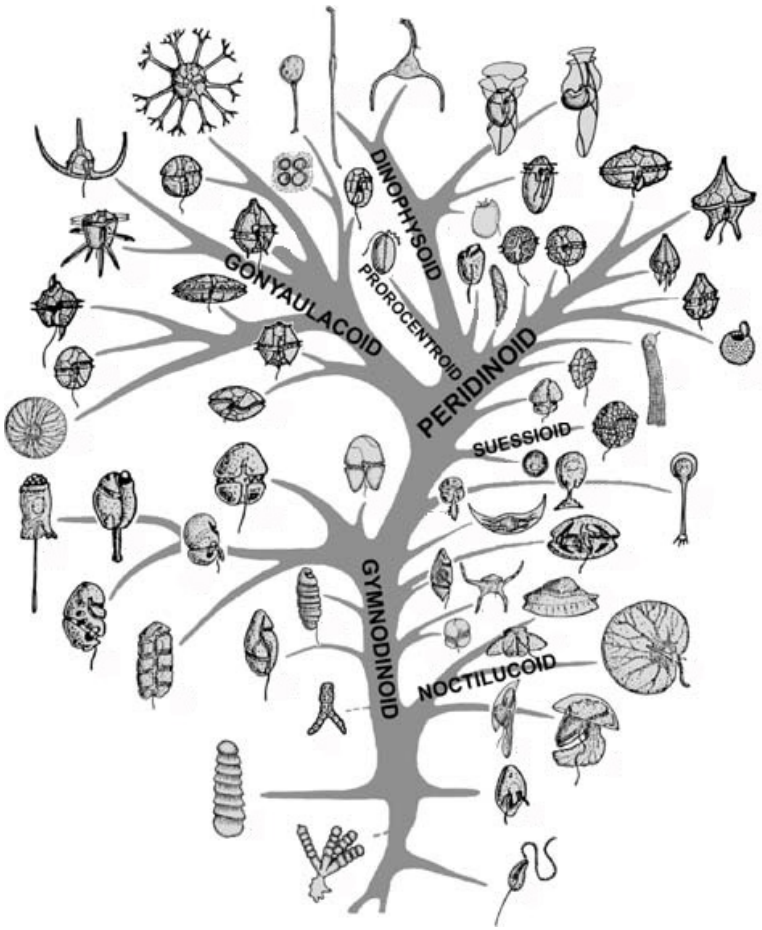


Figure 1: Extant dinoflagellate diversity. The evolutionary tree was based on the morphological characters of the examined genera. Modified from Taylor et al. (2008).

However, classical morphological taxonomy is not only time-consuming it also requires a high level of expertise and during the last decades there has been a decrease in the number of taxonomists in all fields. Thus, as an alternative approach, molecular taxonomy is gaining increasing acceptance because the same techniques can be readily applied to a wide range of taxa and the interpretation of the results is more straightforward (Caron, 2013; Lee, 2000; Medlin et al., 2007). But while morphological classifications obscure species diversity and relationships, molecular taxonomy does not allow detecting if different sequences belong to cryptic species or an understanding of their ecology and physiology. Consequently, while the taxonomy of dinoflagellates has shifted from a morphology-based to a molecular-based system, there is much information to be gained by combining these two complementary perspectives (Medlin et al., 2007).

The widespread use of phylogeny has also provided a new standpoint when establishing delimitations for the species concept. The classical morphospecies concept is based on morphology to distinguish species, but it has been proved to be insufficient in most cases to identify organisms at species level. Furthermore, it has been proved that cryptic species are very difficult to distinguish by their morphological characters (Montresor et al. 2003). Following the biological species concept, a species is defined as a population reproductively isolated from other populations. This concept could solve the problem of cryptic species, but sexuality is very difficult to study in organisms like the dinoflagellates (Taylor, 1993) because their life cycle is poorly known as well as their mating compatibility and environmental conditions required to undergo sexuality. In the phylogenetic species concept, a species is defined as the smallest group of organisms hierarchically related. However, this concept also entails controversy as there exist intraspecific variability and the limits of genetic variability to consider different populations as different species are problematic. Moreover, the different concepts are not always congruent; sometimes molecular and morphological speciation does not evolve at the same rate and morphologically identical subclades are phylogenetically distinct and reproductively isolated (Lundholm and Moestrup, 2006). Or morphologically and ecologically distant species are phylogenetically identical (Logares et al. 2007). These incongruences among the different criteria raise serious problems that must be taken into account in ecological and biogeographical distribution studies of a species.

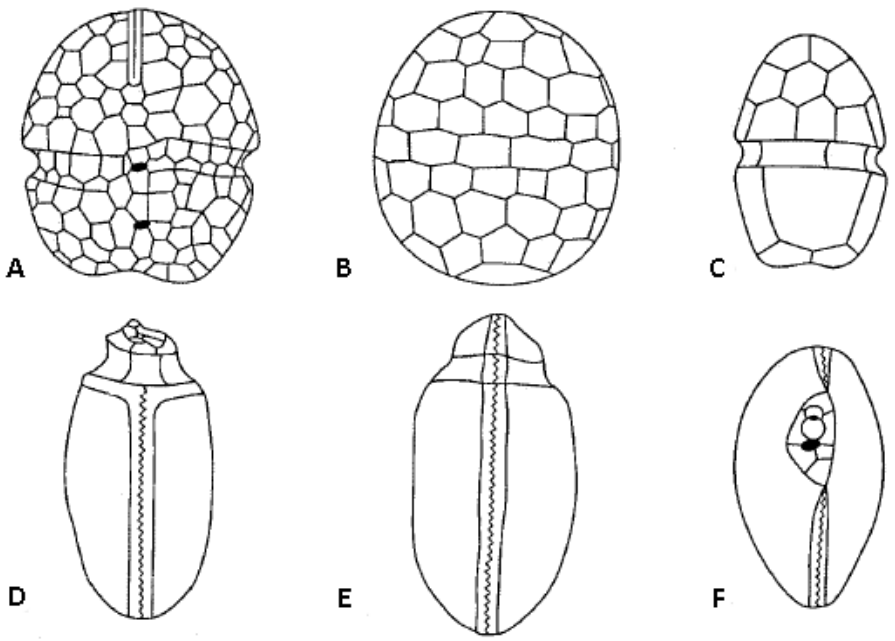


Figure 2: Tabulation types. A) Gymnodinioid in ventral view. B) Suessioid in dorsal view. C) Gonyaulacoid-Peridinioid in dorsal view. D) Nannoceratiopsioid in dorsal view. E) Dinophysioid in dorsal view. F) Prorocentroid in oblique view. From Fensome et al. (1993).

Molecular analyses

Taxonomic markers

The widespread use of the polymerase chain reaction (PCR) and molecular phylogenies during the last few decades has enabled detailed studies on the evolution and systematics of most groups of protists. The genes applied in phylogenetic studies are mitochondrial (cytochrome *b*, cytochrome c oxidase subunit I), plastidial (Rubisco), or encode common cellular proteins (actin, beta-tubulin, heat-shock protein 90). Microsatellite loci are used in species- or population-level analyses. However, most molecular taxonomy studies, whether prokaryote or eukaryote, employ ribosomal DNA (rDNA) genes as molecular markers. The rDNA genes of eukaryotes consist of three successive conserved fragments referred to by their size: 18S (small subunit, SSU), 5.8S, and 28S (large subunit, LSU). The three fragments are separated by two internal transcribed spacers (ITS1 and ITS2) (Fig. 3). The utility of these genes in molecular taxonomy is explained by their relatively long fragments, their presence in all eukaryotes, their relatively high degree of conservation, and, in some lineages, their high copy number along the genome. The different rDNA gene fragments have different rates of evolution, allowing studies at different evolutionary levels. Because SSU rDNA is highly conserved, comparisons can be made at high taxonomic levels. LSU rDNA is less conserved and is thus often used for genus- or species-level studies. In contrast to these coding fragments, the ITS are non-coding and consequently more variable; accordingly, they are commonly used for intraspecific comparisons.

Molecular techniques

The first molecular analyses on dinoflagellates were conducted using cultured photosynthetic species (Lenaers et al., 1989; Lenaers et al., 1991). The cultures of dinoflagellates provide large amounts of DNA of a single species and thus allow to apply a large number of molecular analyses and to repeat them when necessary. However, many dinoflagellates are not easily cultivable and therefore cannot be sequenced by standard techniques, such that the phylogenetic position of many genera is unknown.

The problem has been partially resolved with a technique known as single-cell PCR (SC-PCR), in which sequences from heterotrophic and not easily cultured species are obtained by taking advantage of the large copy number of the ribosomal genes. With this method, the rDNA of a single cell that has been studied morphologically can be isolated for sequencing (Bolch, 2001; Ruiz-Sebastián and O'Ryan, 2001). Indeed, since the introduction of SC-PCR a large number of sequences from uncommon and uncultivable species have been analyzed (Lynn and Pinheiro, 2009), increasing our knowledge of the phylogenetic positions of genera and species of several protist lineages. The drawback of this technique is that the molecular analyses cannot be reproduced, as the cell is lost during the process, and the morphological details are restricted to that of the studied specimen, observed under optical microscopy.
Alternatively, the diversity and relationships of dinoflagellates can be studied by environmental sequencing, in which the DNA of either the entire community or a fraction thereof is extracted. The amplification products are then cloned and sequenced, resulting in a single approach for all taxonomic groups (López-García et al., 2001; Moon-van der Staay et al., 2001). Environmental sequencing has revealed new taxonomic groups of protists that were not previously identified as such based solely on their morphological features (Guillou et al., 2008; Massana et al., 2004; Not et al., 2007). It has also made clear that because of our inability to culture or differentiate them the number of as yet unstudied and unknown taxa remains quite large. However, this technique also has its limitations, as it does not provide information about the morphology, ecology, or physiology of the sequenced organisms. Furthermore, it may lead to overestimates of diversity given that intraspecific variability cannot be determined because of the lack of

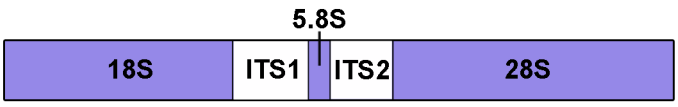


Figure 3: The fragments of ribosomal DNA (rDNA). 18S: small subunit (SSU); ITS: internal transcribed spacer; 28S: large subunit (LSU).

morphological observations, or to underestimates, because the results may be biased by differences in the extraction and amplification efficiencies of the different taxa (Caron, 2013). Consequently, a combination of molecular, morphological and physiological information is desirable when characterizing the diversity of these organisms.

Taxonomy of the dinoflagellates

Historically, dinoflagellates were classified based on morphological characters, which for the most part demonstrated the monophyly of this group (Lenaers et al., 1991; Saldarriaga et al., 2001; Saunders et al., 1997). Analyses of 18S rDNA genes subsequently showed that dinoflagellates clustered with ciliates and apicomplexans (Van de Peer and De Wachter, 1997), in a group called Alveolata Cavalier-Smith 1991. Later on, several parasitic organisms, such as the Perkinsida and the Syndiniales, were found to occupy basal positions alongside the dinoflagellates (Saldarriaga et al., 2003; Saldarriaga et al., 2004). But, overall, the interrelationships among dinoflagellate lineages remained largely unresolved because most orders were polyphyletic and some formed a core complex, e.g., Gymnodiniales – Peridiniales – Prorocentrales (Murray et al., 2005; Saldarriaga et al., 2004; Saunders et al., 1997). The dinoflagellates placed in a basal position of the phylogenetic trees resulting from molecular analyses were heterotrophic and unarmoured (Saldarriaga et al., 2004). While the absence of thecae was interpreted as a basal character (Fig. 1), the highly complex structures (piston, ocellus) of other heterotrophic unarmoured species, such as the warnowiids, led to the proposal of successive gains and losses of theca along the evolutionary history of dinoflagellates (Saldarriaga et al., 2004). However, the results of further studies, combining analyses of eight different genes, pointed to both an athecate origin of dinoflagellates, although these unarmoured members were paraphyletic, and the monophyly of most dinoflagellates orders (Orr et al., 2012). A group is considered monophyletic when it contains a common ancestor and all its descendants; a group is paraphyletic when it consists of all the descendants of the common ancestor except some groups of descendants; and a group is polyphyletic when it does not include a common ancestor.

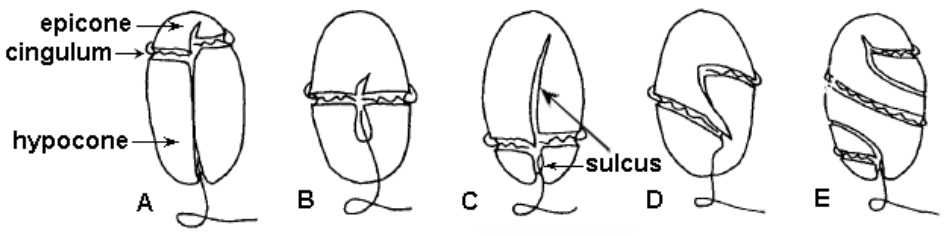


Figure 4: Position and characteristics of the cingulum used to group the genera of unarmoured dinoflagellates. A) Pre-median position (*Amphidinium*). B) Median position (*Gymnodinium*). C) Post-median position (*Katodinium*, *Torodinium*). D) Cingulum displaced (*Gyrodinium*). E) Several turns of the cingulum around the cell (*Cochlodinium*, *Warnowia*). Modified from Steidinger and Tangen (1997).

UNARMOURED DINOFLAGELLATES

Morphology-based taxonomy of unarmoured dinoflagellates

Compared to their armoured (thecated) counterparts, unarmoured species have been historically less well studied because they are fragile, easily collapse, and deform when processed with fixatives. With the exception of Noctilucales Haeckel, which species exhibit unique and characteristic features, unarmoured species have been classified into the orders Gymnodiniales Apstein, and the less diverse Ptychodiscales Fensome, Suessiales Fensome, and Brachidiniales Loeblich III ex Sournia (Fensome et al., 1993). Most genera are included within the order Gymnodiniales and are differentiated by morphological characters such as displacement of the cingulum (*Gymnodinium* Stein 1878, *Gyrodinium* Kofoid *et* Swezy 1921), differences in its position (*Amphidinium* Claparède *et* Lachmann 1859, *Torodinium* Kofoid *et* Swezy 1921), and the number of turns it makes around the cell (*Cochlodinium* Schütt 1896, *Warnowia* Lindemann 1928) (Fig. 4).

A taxonomic milestone of the group was settled early in the last century by Kofoid and Swezy (1921), who described more than 100 new species in their monograph on unarmoured dinoflagellates. Other authors, such as Lebour (1917, 1925), Schiller (1933), Hulburt (1957), Campbell (1973), and, later on, Larsen (1994, 1996), also contributed with descriptions of a large number of new unarmoured species. Unfortunately, many of the early descriptions were doubtful or incomplete, and in some cases the observations were of stressed, deformed, or fixed specimens. Thus, many species have not been observed again since their description (so-called oncers). For example, the genus *Gymnodinium* contains about 270 described species, of which 40% are oncers, what suggest that they might represent anomalous morphologies of other existing species. Some authors are especially notorious in this regard: 54% of the *Gymnodinium* species described by Schiller and 20% of those described by Kofoid & Swezy are oncers (Thessen et al., 2012). This uncertainty about the real number of existing species also occurs in other unarmoured genera, such as *Gyrodinium* and *Cochlodinium*.

In some studies differences in the shape of the acrobase or apical groove were noted (Biecheler, 1934, 1952; Takayama, 1985, 1998), although this structure, present in the apex of all unarmoured dinoflagellates, is difficult to observe using typical optical microscopy techniques. The contribution of Daugbjerg et al. (2000) was crucial in that it combined morphological and ultrastructural observations with phylogenetic data. Those authors concluded that molecular phylogenies did not support the "classical" morphological criteria used to distinguish the different dinoflagellate genera. They demonstrated that the genus *Gymnodinium* was polyphyletic and therefore instead erected the genera *Akashiwo* Hansen *et* Moestrup, *Karenia* Hansen *et* Moestrup, and *Karlodinium* Larsen. Morphologically, each genus was characterized by a distinct acrobase shape, which constituted a key marker with which to classify the different genera of unarmoured dinoflagellates (Fig. 5). The clade containing *Gymnodinium fuscum* (Ehrenberg) Stein, the type species of the genus, was referred to as *Gymnodinium sensu stricto* (*s.s.*).

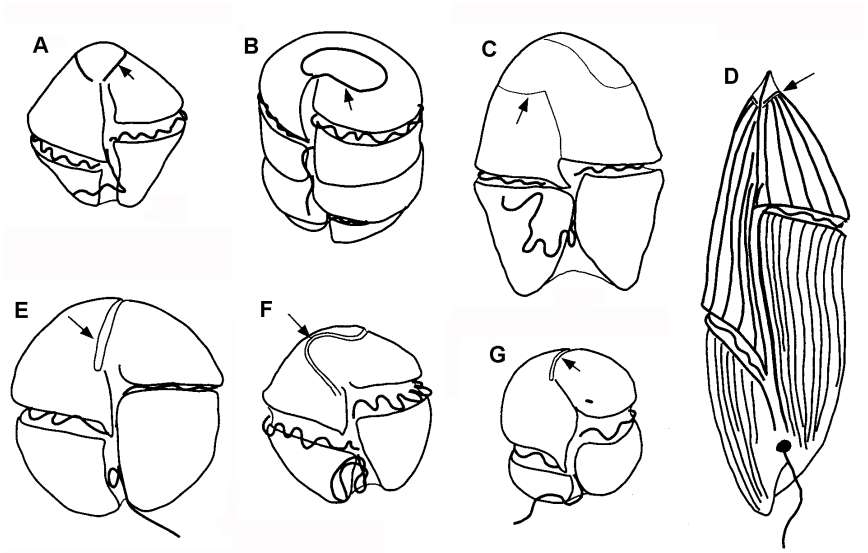


Figure 5: Apical grooves (arrows) of unarmoured dinoflagellates. A) *Gymnodinium*. B) *Polykrikos* C) *Akashiwo*. D) *Gyrodinium*. E) *Karenia*. F) *Takayama*. G) *Karlodinium*. Modified from Daugbjerg et al. (2000).

Contribution of molecular taxonomy

The historical record of molecular studies

The first sequences of unarmoured dinoflagellates were obtained at the end of the 1980s, with others gradually incorporated into the databases thereafter. During the 1990s, most of the determined sequences were those of harmful autotrophic species (Bolch et al., 1999; Costas et al., 1995; Hansen et al., 2000) because they are easily cultured and of

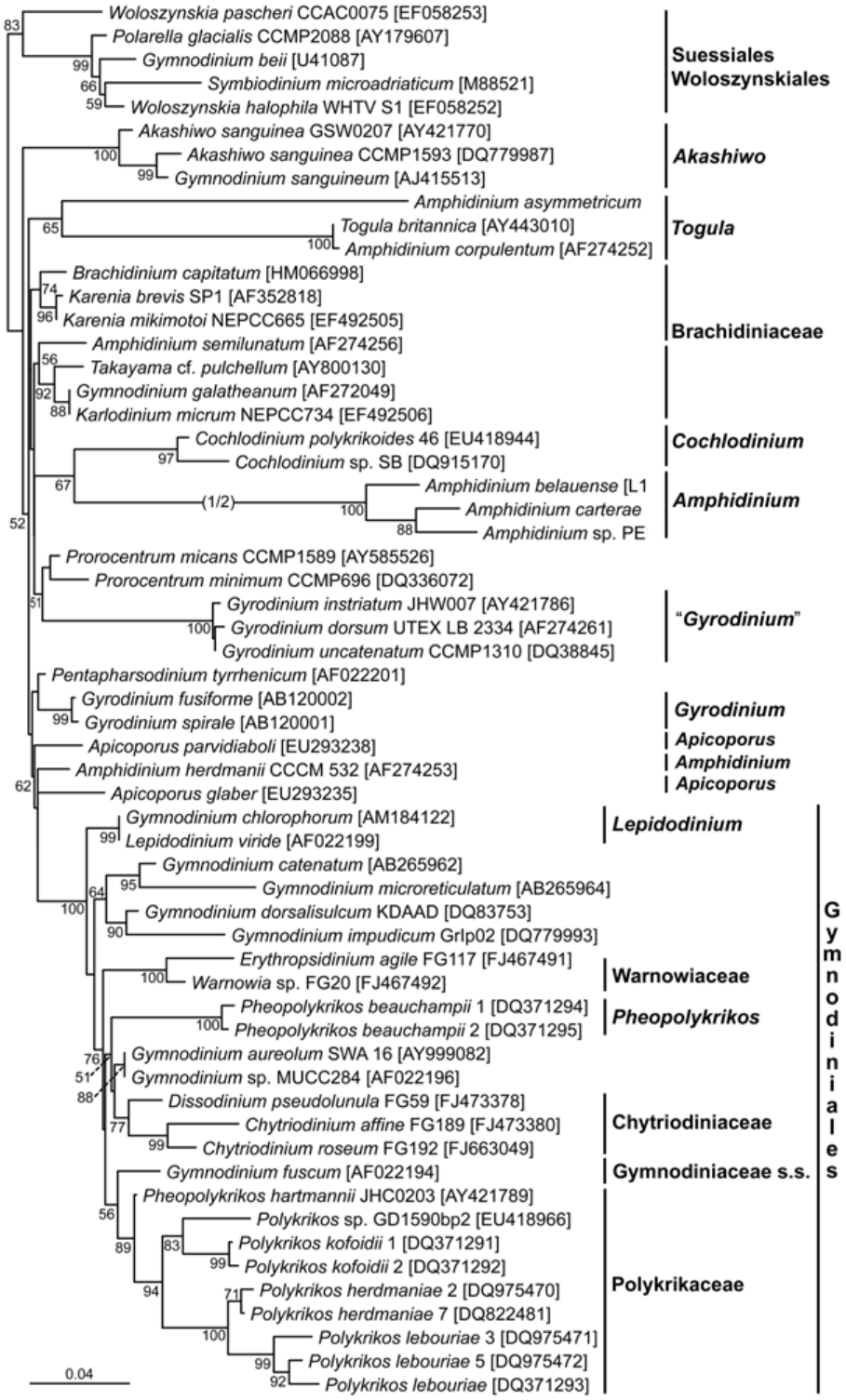


Figure 6: Maximum-likelihood phylogenetic tree of SSU rDNA sequences from unarmoured dinoflagellates. From Gómez et al. (2011).

tremendous economic impact. The study of Daugbjerg et al. (2000) was followed by descriptions of many unarmoured species, mainly those belonging to the Kareniaceae group and in most cases including phylogenetic data (Bergholtz et al., 2006; Botes et al., 2003; Chang and Ryan, 2004; de Salas et al., 2004a; de Salas et al., 2004b; de Salas et al., 2003; de Salas et al., 2005; de Salas et al., 2008; Haywood et al., 2004). Morphological and molecular studies were also performed on amphidinioid species (Flø Jørgensen et al., 2004a, 2004b) and were followed by phylogenetic studies of *Cochlodinium* species (Iwataki et al., 2007; Iwataki et al., 2008). Molecular data were also obtained for polykrikoid organisms (Hoppenrath and Leander, 2007a, 2007b; Kim et al., 2008; Matsuoka et al., 2009). In parallel, SC-PCR was successfully used to analyze unarmoured species, yielding sequences for several heterotrophic members of *Gyrodinium* (Hansen and Daugbjerg, 2004; Takano and Horiguchi, 2004). Further studies focused on the scarce and heterotrophic warnowiids (Gómez et al., 2009a; Hoppenrath et al., 2009) and the parasitic unarmoured dinoflagellates belonging to *Dissodinium* and *Chytriodinium* (Gómez et al., 2009b; Kim et al., 2008). Other species have since been described and a large number of unarmoured dinoflagellate genera have been erected. Some of these were based on distinct morphological features, e.g., *Apicoporus* (Sparmann et al., 2008), *Paragymnodinium* (Kang et al., 2010), *Barrufeta* (Sampedro et al., 2011), *Gyrodiniellum* (Kang et al., 2011), and *Bispinodinium* (Yamada et al., 2013), whereas others reflected a reclassification as a result of phylogenetic incongruencies, as was the case for *Moestrupia* (Hansen and Daugbjerg, 2011), *Testudodinium* (Horiguchi et al., 2012), and *Ankistrodinium* (Hoppenrath et al., 2012). Consequently, phylogenetic information has become mandatory in studies of the taxonomy and systematic position of dinoflagellates, especially unarmoured ones.

Current classification of unarmoured dinoflagellates

Previous studies demonstrated the necessity to obtain the sequences of the different species in determinations of their taxonomy. Indeed, as the number of sequenced species increased, so did the number of different genera and families included within the *Gymnodinium s.s.* clade. Thus, the genus *Lepidodinium* Watanabe, Suda, Inouye, Sawaguchi et Chihara, in which chlorophyll *b* is the main accessory pigment, and the newly described genera *Barrufeta* Sampedro *et* Fraga, *Paragymnodinium* Kang, Jeong, Moestrup *et* Shin, and *Gyrodiniellum* Kang, Jeong *et* Moestrup, were also included (Kang et al., 2011; Kang et al., 2010; Sampedro et al., 2011; Saunders et al., 1997). The Polykrikaceae (*Polykrikos*, *Pheopolykrikos*), Chytriodiniaceae (*Dissodinium*, *Chytriodinium*), and Warnowiaceae (*Proterythropsis*, *Warnowia*, *Nematodinium*, *Erythropsidinium*) families also clustered within this clade (Gómez et al., 2009b; Hoppenrath et al., 2009; Hoppenrath and Leander, 2007a; Kim et al., 2008), which resulted in the recognition of the order Gymnodiniales *sensu stricto* (Gómez et al., 2009a; Hoppenrath and Leander, 2007a, 2010).

Many other unarmoured genera are considered as members of the order Gymnodiniales, although its paraphyly has been demonstrated (Orr et al., 2012). These genera do not show any molecular relationship with the *Gymnodinium s.s.* clade and are regarded as Gymnodiniales *sensu lato*. The genus *Gyrodinium* is monophyletic, with some exceptions that need to be amended, but unrelated to any other unarmoured genus. Unfortunately, molecular information on *Gyrodinium* species for which their SSU or LSU rDNA sequences have been determined is restricted to less than ten.

The genera *Karlodinium*, *Karenia*, and the posteriorly erected *Takayama* de Salas, Bolch, Bores et Hallegraeff cluster independently (Fig. 6). They have in common the main accessory pigment fucoxanthin. These three genera originally comprised the family Kareniaceae (Bergholtz et al., 2006), but a recent study obtained the phylogeny of *Brachidinium*, which clustered together with *Karenia* species. The genus *Brachidinium* was formerly included within the Brachidiniaceae family and the Brachidiniales order (Henrichs et al., 2011), which have taxonomic priority over the Kareniaceae family and *Karenia* genus.

The position of the cingulum and the minute size of the epicone distinguish the genus *Amphidinium*, which is polyphyletic (Flø Jørgensen et al., 2004a). The clade containing *Amphidinium operculatum* Claparède *et* Lachmann, the type species of the genus, is considered as the *Amphidinium s.s.* clade. Although it is currently made up of several species, their absolute identification has been challenging and detailed studies on the morphology and molecular

phylogeny of some formerly *Amphidinium* species resulted in the erection of several new genera: *Togula* Jorgensen, Murray et Daugbjerg, *Apicoporus* Sparmann, Leander et Hoppenrath, *Ankistrodinium* Hoppenrath, Murray, Sparmann et Leander, *Testudodinium* Horiguchi, Tamura, Katsumata et Yamaguchi, and *Bispinodinium* Yamada et Horiguchi (Flø Jørgensen et al., 2004b; Hoppenrath et al., 2012; Horiguchi et al., 2012; Sparmann et al., 2008; Yamada et al., 2013). However, many other species not included in the *Amphidinium s.s.* clade still remain in the genus as *Amphidinium sensu lato* species.

The genus *Cochlodinium* is currently defined only by the number of turns of the cingulum around the body. Of the more than 40 member species described thus far, only a few have been studied in detail and only three have been sequenced. Of these, *C. polykrikoides* Margalef and *C. fulvescens* Iwataki, Kawami *et* Matsuoka form a cluster but it does not include *C.* cf. *geminatum* (Schütt) Schütt, which has been recently transferred to the genus *Polykrikos* (Qiu et al., 2013). This suggests that the genus is polyphyletic and thus artificial, but the lack of information on most *Cochlodinium* species prevents their further classification.

The taxonomic and phylogenetic positions of other unarmoured genera remain poorly studied. In some, such as *Pavillardia* Kofoid *et* Swezy, *Plectodinium* Biecheler, and *Ceratoperidinium* Margalef *ex* Loeblich III, the rarity of the organisms impedes their study. In others, such as *Katodinium* Fott or the warnowiids, the taxonomy remains puzzling despite several published studies (Calado, 2011; Gómez et al., 2009a; Hoppenrath et al., 2009). Even quite common genera, such as *Torodinium* Kofoid *et* Swezy, have received scarce attention by the scientific community.

Despite the advances made in the dinoflagellates and in particular in the Gymnodiniales during the last decade, there are very few sequenced species for some of these genera in molecular databases. This results in an underrepresentation of the group in comparison with armoured dinoflagellates and some other well-known genera (Fig. 7).

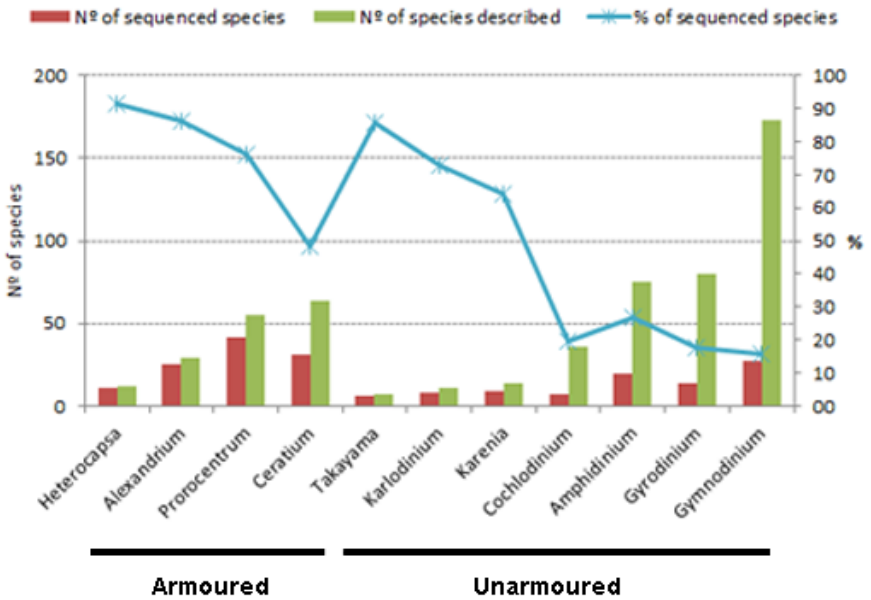


Figure 7: Number of sequences available in molecular databases for some genera of dinoflagellates. Columns (left axis) represent the number of species sequenced (red) and described (green) for each genus. The blue line (right axis) represents the percentage of sequenced species with respect to those described for each genus.

STUDY SITE: THE CATALAN COAST

The microalgal composition, and specifically, the dinoflagellate composition, of NW Mediterranean waters has been intensively studied since the early 1900s, first in the Gulf of Lions (Pavillard, 1909, 1916) and then in the Balearic Islands (Massutí, 1930). The first studies on the plankton composition of the waters of the Catalan coast

(NW Mediterranean Sea) were carried out by Margalef (Margalef, 1945a, 1945b, 1965, 1969). Other researchers contributed to this knowledge during the same period (Morales, 1956) and in the following decades (Delgado, 1987; Estrada, 1979, 1980; López and Arté, 1973; Velásquez, 1997). Those studies allowed the development of extensive checklists of dinoflagellates detected in the Mediterranean Sea, with a focus on its northwestern area. However, the use of fixed samples prevented the complete identification of most unarmoured dinoflagellates. Consequently, some species were morphologically identified only to the genus level and the true diversity of unarmoured dinoflagellates remained a black box.

The dinoflagellates directly impact on the ecosystem due to their ability to form harmful algal blooms (HABs). The harmful effects on humans and the environment are caused by the production of toxins or by the high-biomass proliferations. Since the 1990s, a monitoring programme of toxic and harmful phytoplanktonic species is conducted in the Catalan Coast, and a large number of studies have focused on their diversity, distribution, dynamics, life-cycle, and ecophysiology (Anglès et al., 2012; Garcés et al., 2004; Garcés et al., 1999; Quijano-Scheggia et al., 2005; Vila et al., 2001a; Vila et al., 2001b; Vila et al., 2005). But despite all these studies, the knowledge on unarmoured dinoflagellates present in the area was still partially unknown. Some HABs detected in the Catalan Coast were directly ascribed to unarmoured species, and recurrent fish mortalities were attributed to blooms of *Gyrodinium corsicum* (Delgado et al., 1995). A detailed study of the causative organism reported high abundances of the toxic species *Karlodinium armiger* and *K. veneficum* during those events (Garcés et al., 2006). Additionally, recurrent high-biomass blooms at La Fosca Beach have also been reported, and a detailed taxonomic study of the causative organism resulted in the erection of the new genus and species *Barrufeta bravensis* (Sampedro et al., 2011). Yet, while informative, those studies covered only a small fraction of the harmful unarmoured dinoflagellates present along the Catalan coast.

The recent taxonomic re-classifications of unarmoured dinoflagellates and the lack of knowledge on their diversity from the Catalan coast highlight the need for in-depth assessments of the Gymnodiniales. Furthermore, resolving the polyphyletic nature of the order require information on as yet poorly studied genera. Consequently, this information could be relevant not only to the study site of this thesis work, i.e., Catalan coastal waters, but also globally.

The heterogeneity and length of the Catalan coast make this site a good proxy to study the diversity of species present in the NW Mediterranean Sea. The high heterogeneity of the Catalan coast includes sandy, rocky, and open beaches, semi-enclosed bays, beaches with different degrees of fluvial influence, estuaries, and small to large harbours.

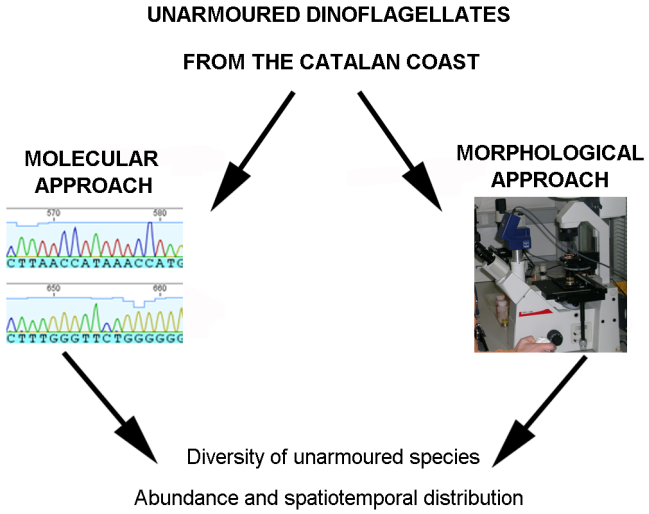
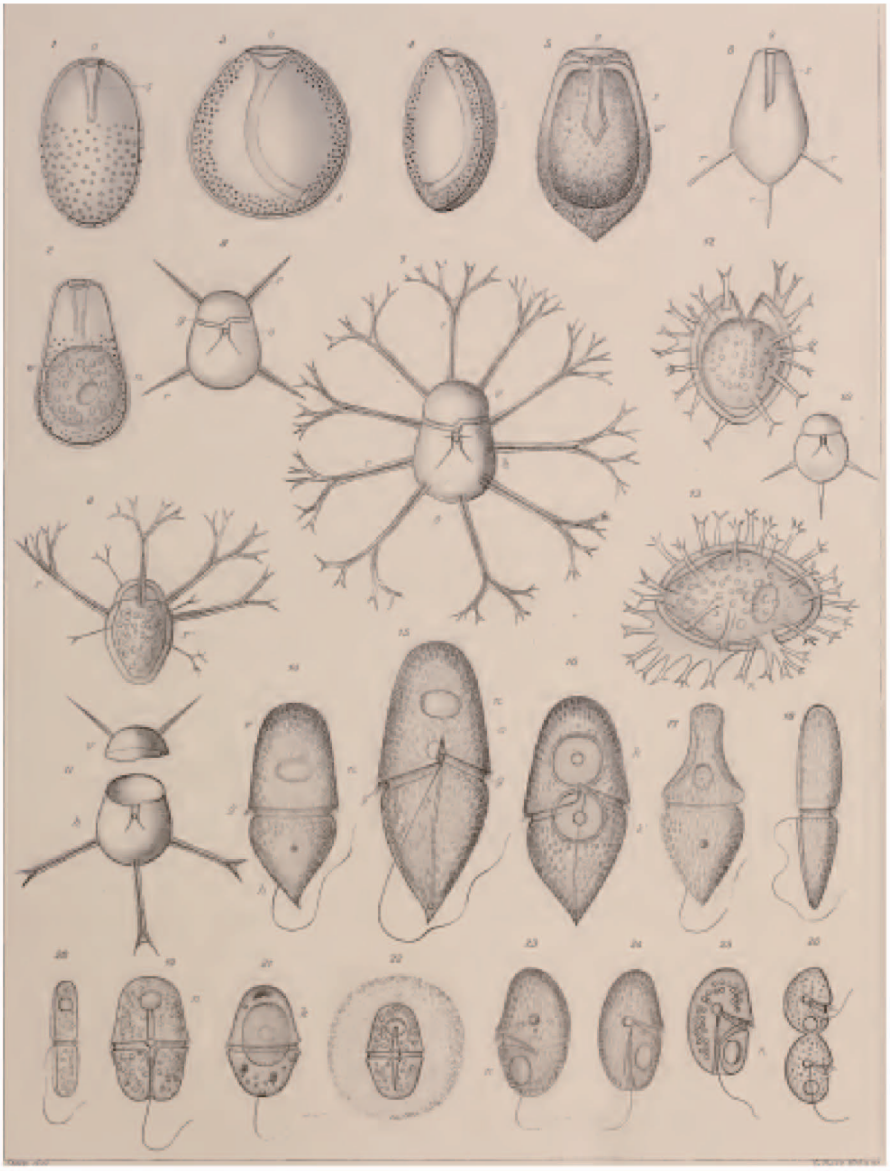


Figure 8: Approach used to study the unarmoured dinoflagellates present along the Catalan coast.

METHODOLOGICAL APPROACH

The inaccuracy of available identifications in the study area derived from fixed samples and the difficulties in culturing heterotrophic species have made it necessary to rely on the morphological and molecular information resulting from a combination of these approaches. Thus, when cultures cannot be obtained SC-PCR provides an intermediate method between the sequencing of cultured organisms and environmental sequencing (Fig. 8). The D1-D2 region of the LSU rDNA sequence was selected to carry out the SC-PCR studies of this thesis. As discussed above, ribosomal genes are the most widely used markers in molecular taxonomy. Their use allowed a comparison of the sequences collected in this study with those previously deposited in the databases. The LSU rDNA sequences of unarmoured dinoflagellates are well represented in these databases such that a good species level resolution is possible for this region (Fensome et al., 1999). Nonetheless, LSU rDNA are not effective to study phylogenetic relationships at higher levels, i.e., order or phylum. Instead, high-level taxonomic studies are mostly carried out with the SSU rDNA region, which is more well conserved and thus allows a better determination of the phylogenetic position and relationships among different genera, families, and orders (De Rijk et al., 1995), although it sometimes lacks resolution at the species level (Taylor, 2004). Accordingly, in the studies contributing to this thesis, SSU rDNA sequences were obtained when necessary to identify species relationships.



Stein (1878) Der Organismus der Infusionsthiere

Aims of the thesis

Examining this water...I found floating therein divers earthy particles, and some green streaks, spirally wound serpent-wise...and I judge that some of these little creatures were above a thousand times smaller than the smallest ones I have ever yet seen, upon the rind of cheese, in wheaten flour, mould, and the like.

- Antonie van Leeuwenhoek 1674

AIMS OF THE THESIS

The aim of this thesis was to provide a detailed study of the diversity of unarmoured dinoflagellates from the NW Mediterranean Sea.

Extensive identifications of dinoflagellates present in the NW Mediterranean Sea were carried out between the 1960s and 1980s. However, in these early efforts, unarmoured dinoflagellates were clearly misrepresented because of the use of fixed samples and the lack of phylogenetic tools. The unarmoured dinoflagellates examined in this thesis were identified by combining morphological studies of live specimens with the respective phylogenetic information. Given that the phylogeny of most of the studied organisms had not been previously determined and the evidence that Gymnodiniales is not monophyletic, a secondary objective was to study the phylogenetic relationships of unarmoured dinoflagellates. The Catalan coast is representative of the NW Mediterranean Sea and therefore served as the study site.

The specific objectives of the thesis were:

1. **To study the diversity of the unarmoured dinoflagellates belonging to the order Gymnodiniales and present along the Catalan coast, by combining morphological observations with phylogenetic data.**

2. **To define the phylogenetic position of ambiguously classified unarmoured dinoflagellates by obtaining their molecular sequences.**

3. **To detect and identify those unarmoured species responsible for the formation of harmful algal blooms (HABs) along the Catalan coast.**

4. **To fully describe new species detected.**

The studies conducted for this thesis are reported in **six chapters**, each of which addresses some of the above-defined specific objectives.

The six chapters are structured as scientific papers, some of them already published. This thesis concludes with a general discussion of its chapters and general conclusions.

CHAPTER 1: DIVERSITY OF DINOFLAGELLATES INCLUDED WITHIN THE GYMNODINIALES *SENSU STRICTO* CLADE FROM THE CATALAN COAST (NW MEDITERRANEAN SEA).
REÑÉ, A., CAMP, J., GARCÉS E. In prep.

The diversity of the dinoflagellates belonging to the Gymnodiniales *sensu stricto* clade present along the Catalan coast (NW Mediterranean) was studied during a two-year sampling of several harbours and beaches. The organisms were identified based on morphological observations and their partial LSU rDNA sequences. Eighteen morphospecies belonging to Gymnodiniales *sensu stricto* were detected, 16 of which were successfully sequenced. Nine of the sequences obtained are the first available for those species.

CHAPTER 2: DIVERSITY OF DINOFLAGELLATES BELONGING TO THE ORDER GYMNODINIALES *SENSU LATO* FROM THE CATALAN COAST (NW MEDITERRANEAN SEA).
REÑÉ, A., CAMP, J., GARCÉS E. In prep.

A combination of morphological and molecular data led to the identification of 40 species of unarmoured dinoflagellates belonging to the order Gymnodiniales *sensu lato* and present along the Catalan coast. Eight of these species are reported for the first time in the Mediterranean Sea and seven in the Catalan Coast, including three toxic *Karenia* species never previously reported. Partial LSU rDNA sequences were obtained for 27 different morphospecies, of which novel sequences represented 15 species.

CHAPTER 3: *GYMNODINIUM LITORALIS* SP. NOV. (DINOPHYCEAE), A NEWLY IDENTIFIED BLOOM -FORMING DINOFLAGELLATE FROM THE NW MEDITERRANEAN SEA.
REÑÉ, A., SATTA, C.T., GARCÉS, E., MASSANA, R., ZAPATA, M., ANGLÈS, S., CAMP, J., 2011. HARMFUL ALGAE 12, 11-25.

High-biomass blooms caused by unidentified unarmoured dinoflagellates have been recurrently detected along several beaches of the northern Catalan coast. Since unarmoured dinoflagellates can only rarely be identified from fixed samples obtained in routine samplings, the causative organism was instead isolated and cultured. Its morphology, ultrastructure, pigment profile and sequencing of the LSU rDNA led to the description of a new species, *Gymnodinium litoralis*.

CHAPTER 4: *POLYKRIKOS TANIT* SP. NOV., A NEW MIXOTROPHIC UNARMOURED PSEUDOCOLONIAL DINOFLAGELLATE FROM THE NW MEDITERRANEAN SEA.
REÑÉ A., CAMP J., GARCÉS E. (In press). PROTIST. DOI: 10.1016/j.protis.2013.12.001

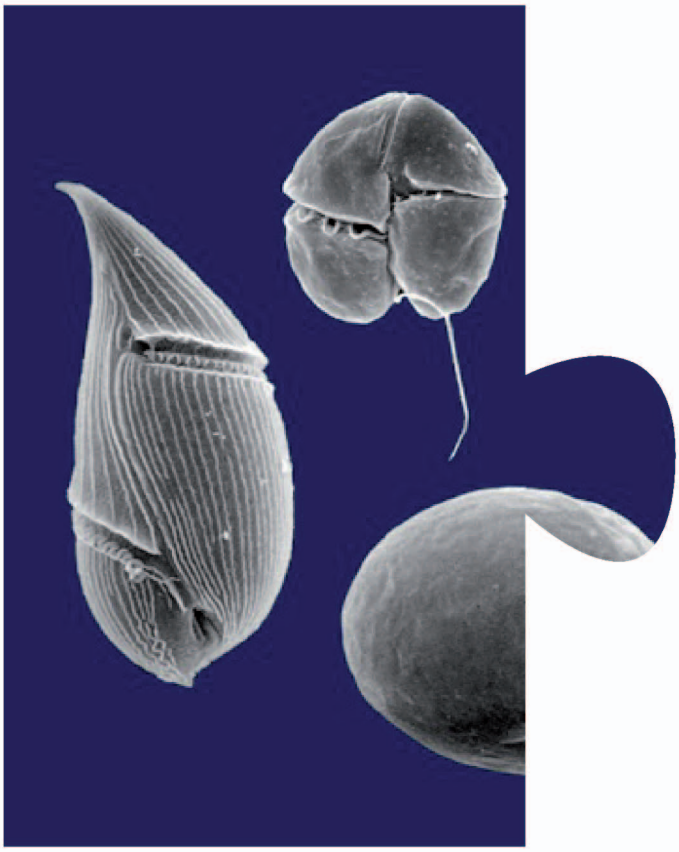
Pigmented pseudocolonies initially identified as *Polykrikos hartmannii* Zimmermann were detected at several locations along the Catalan coast (NW Mediterranean Sea). To further explore the remarkable morphological discrepancies between these organisms and *P. hartmannii*, we carried out a detailed morphological study. Their partial LSU and SSU rDNA sequences were obtained and the resulting phylogenies showed that our isolates occupy a basal position within the *Polykrikos* clade, close to *P. hartmannii*, but do not correspond to any previously described polykrikoid species. The organisms studied in this work were also similar to *P. barnegatensis* but showed significant morphological differences with its original description. Consequently, the studied organisms were described as a new species, *Polykrikos tanit* sp. nov.

CHAPTER 5: PHYLOGENETIC RELATIONSHIPS OF *COCHLODINIUM POLYKRIKOIDES* MARGALEF (GYMNODINIALES, DINOPHYCEAE) FROM THE MEDITERRANEAN SEA AND THE IMPLICATIONS OF ITS GLOBAL BIOGEOGRAPHY.
REÑÉ, A., GARCÉS, E., CAMP, J., 2013. HARMFUL ALGAE 25: 39-46.

The distribution of *Cochlodinium polykrikoides* has likely expanded worldwide during the last decade. This toxic unarmoured dinoflagellate was previously detected at low abundances in the Mediterranean Sea and resting cysts were detected along the Catalan coast. In this study, vegetative cells of *C. polykrikoides* at high abundances were detected for the first time along the Catalan coast. Partial LSU rDNA sequences obtained showed that most *C. polykrikoides* populations formed a newly differentiated ribotype, but one of them was included within the 'Philippines' ribotype, demonstrating the coexistence of the two in the Mediterranean Sea. Our findings suggest that the current biogeographic nomenclature of the ribotypes is invalid with respect to the available information on populations comprising the 'Philippines' ribotype. The phylogeny suggests the existence of cryptic species that should be evaluated for species-level status.

CHAPTER 6: A NEW CLADE, BASED ON LSU rDNA SEQUENCES, OF UNARMOURED DINOFLAGELLATES.
REÑÉ A., DE SALAS M., CAMP J., BALAGUÉ V., GARCÉS E., 2013. PROTIST 164 (5): 673-675.

LSU rDNA sequences obtained for seven species of unarmoured dinoflagellates led to the inclusion of all of them within a monophyletic clade. Despite their substantial morphotypic differentiation, these species have in common the shape of the acrobase. This study allowed the taxonomic position of *C. margalefii* to be resolved. *G. falcatum* was determined to have been erroneously assigned to the genus *Gyrodinium* and was transferred to the genus *Ceratoperidinium*. The genus *Cochlodinium* was demonstrated to be polyphyletic and thus artificial because two species belonged to the studied clade, while other species not. Finally, two *Gymnodinium*-like species were also included in the clade. They could not be morphologically or phylogenetically related to any other *Gymnodinium*-like species sequenced to date. However, it could be safely concluded that both were members of the family Ceratoperidiniaceae, amended in this study.



From Dr. Takayama's personal website

Chapter 1

"Diversity of dinoflagellates included within the Gymnodiniales *sensu stricto* clade from the Catalan coast (NW Mediterranean Sea)"

In prep.

DIVERSITY OF DINOFLAGELLATES INCLUDED WITHIN THE GYMNODINIALES *SENSU STRICTO* CLADE FROM THE CATALAN COAST (NW MEDITERRANEAN SEA)

Albert Reñé, Jordi Camp, Esther Garcés

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Abstract:

The diversity of dinoflagellates belonging to the Gymnodiniales *sensu stricto* clade was studied during a 2-year period at several coastal stations along the Catalan Coast (NW Mediterranean). Diversity was assessed based on a combination of morphology and partial LSU rDNA sequences. Of the 18 detected morphospecies belonging to the clade, sequences were obtained from 16, including 9 novel sequences. Several potentially bloom-forming species were reported, as *Gymnodinium litoralis*, *G. impudicum*, *G. aureolum*, and *Barrufeta bravensis*, although some were only detected at low abundances. *Lepidodinium viride* was detected for the first time in the Catalan Coast and *Polykrikos herdmanae* and cf. *Gyrodinium undulans* in the Mediterranean Sea. Although the latter could not be unequivocally identified because of its resemblance to *Syltodinium listii*, its phylogenetic position supports the fact that this species does not belong to the genus *Gyrodinium*. Three unidentified *Gymnodinium*-like species were also detected. *Polykrikos kofoidii* was commonly observed along with the recently described *P. tanit* and the benthic species *P. herdmanae*. Several warnowiid species were sequenced, allowing their assignment to four different clades. This result is of particular interest given that the taxonomy of the group is still unresolved and only two other LSU sequences from this group were previously available.

1. Introduction:

Dinoflagellates belonging to the order Gymnodiniales Apstein are referred to as "unarmoured" or "naked" as they lack a theca. Gymnodiniales comprises, depending on the report, 20–30 different genera and more than 450 free-living species, belonging mainly to the genera *Amphidinium*, *Cochlodinium*, *Gyrodinium*, and the highly diverse (with about 250 species described) *Gymnodinium* (Gómez 2005; Guiry and Guiry 2013). The taxonomy of this group underwent major revisions beginning with the work of Daugbjerg et al. (2000), who redefined the genera *Gymnodinium* and *Gyrodinium* and erected the genera *Akashiwo*, *Karenia*, and *Karlodinium* by combining the morphological, mainly the shape of the apical groove, and ultrastructural features of these organisms with their phylogeny. The phylogenetic clade containing *Gymnodinium fuscum* (Ehrenberg) Stein 1878, the type species of the genus, was called *Gymnodinium sensu stricto* (*s.s.*). Molecular phylogenies also showed the inclusion of other unarmoured genera within this clade: *Lepidodinium* Watanabe, Suda, Inouye, Sawaguchi et Chihara (Saunders et al. 1997); *Barrufeta* Sampedro et Fraga (Sampedro et al. 2011); *Paragymnodinium* Kang, Jeong, Moestrup et Shin (Kang et al. 2010); and *Gyrodiniellum* Kang, Jeong et Moestrup (Kang et al. 2011). Furthermore, non *Gymnodinium*-like families, such as Polykrikaceae (Hoppenrath and Leander 2007a), Chytriodiniaceae (Gómez et al. 2009b; Kim et al. 2008), and Warnowiaceae (Hoppenrath et al. 2009a) also cluster within this clade. Therefore, as this cluster contains *G. fuscum*, the first unarmoured species described, it is considered as the Order Gymnodiniales *sensu stricto* clade (Gómez et al. 2009a; Hoppenrath and Leander 2007a, 2010).

However, the assignment of a large number of unarmoured species is problematic because some of the defining morphological structures are difficult to observe and furthermore, they deform when fixed due to the absence of a theca in this group of organisms. Therefore, routine samplings using fixatives are not suitable for species identifications, which instead must rely on live specimens. Moreover, the original descriptions of some species are incomplete and doubtful, and a large number of these species, including many from the genus *Gymnodinium*, have never been observed again (Thessen et al. 2012). Some of the taxa belonging to the Gymnodiniales *s.s.* have been studied in relative detail, especially harmful algal bloom producers, whereas studies on the diversity and distribution of many other species and genera are scarce and incomplete, in addition to being hindered by a lack of molecular data. The genetic sequences of these organisms provide highly valuable information, as they allow both the discrimination of similar morphospecies and the characterization of specimens that cannot be easily assigned to a genus based on morphology alone. Because many species of unarmoured dinoflagellates are heterotrophic or mixotrophic, efforts to obtain viable and dense cultures are, at best, time consuming but often unsuccessful. The available phylogenetic information is therefore accordingly scarce and generally restricted to photosynthetic species. In these cases, single-cell PCR is a powerful tool to study organisms that are problematic to culture (Ruiz-Sebastián and O'Ryan 2001) due to heterotrophic requirements, but also on those usually found in their natural environment at low abundances.

The diversity of unarmoured dinoflagellates was intensely studied in several locations from the Mediterranean Sea during the early 20th century (Gómez 2003), but those studies were only based on morphology, commonly observing fixed specimens, and lacking phylogenetic studies. The first detailed studies on the specific composition of dinoflagellates from the Catalan Coast (NW Mediterranean Sea) were carried out during the 1940s by Margalef (1945). Even though he was able to identify a large number of dinoflagellates at the species level, this was seldom possible for the unarmoured dinoflagellates because fixed samples were used, which resulted in an underestimation of their total diversity. In the following years, a few unarmoured species found off the Catalan Coast were intensively investigated due to their harmful effects (Delgado et al. 1995; Garcés et al. 2006; Reñé et al. 2013b; Sampedro et al. 2011; Vila et al. 2001), combining in most cases morphological and phylogenetic information. However, the diversity and distribution of most unarmoured dinoflagellates has remained unknown. Thus, the aim of this work was to use morphology and partial LSU rDNA phylogeny to determine the diversity of planktonic species belonging to the Gymnodiniales *s.s.* clade inhabiting the Catalan Coast.

2. Material and methods:

2.1 Observation, isolation, single-cell PCR amplification, and sequencing:

Samples obtained among 2011-2013 from nearshore coastal stations such as beaches and harbours along the Catalan Coast (Fig. 1) were observed either live or following fixation in Lugol's fixative. Occasionally, samples from offshore and coastal sediments were collected. Sub-surface water samples were collected weekly to monthly or in some cases sporadically, depending on the season and station. Sediment samples were filtered using a 200-µm mesh and cleaned with seawater from the same locality. Further steps were conducted as follows. For fixed samples, 50 ml were settled in a settling chamber for 24 h and then aliquots thereof were examined under an inverted microscope. For live samples, a random volume was concentrated using a 10-µm mesh and observed under a Leica-Leitz DM-Il inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Organisms were filmed and photographed with a Sony NEX-5 camera (SONY, Tokyo, Japan) and their morphological features were studied when possible. Each cell was then transferred, using Pasteur pipettes, into filtered seawater drops multiple times and, after these washing steps, into a 200-µl PCR tube. Several fixed cells were also isolated for sequencing using the same method. Single-cell PCR was directly conducted with a PCR mixture containing 5 ml of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 mM of the primers D1R and D2C (Scholin et al. 1994). The PCR conditions were as follows: initial denaturation for 5 min at 95 ºC, 40 cycles of 20 s at 95 ºC, 30 s at 55 ºC, and 1 min at 72 ºC, followed by a final extension step for 7 min at 72 ºC. Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and then visualized under UV illumination. The remainder of the sample was frozen at -20 ºC and later used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using both forward and reverse primers and a 3730XL DNA sequencer.

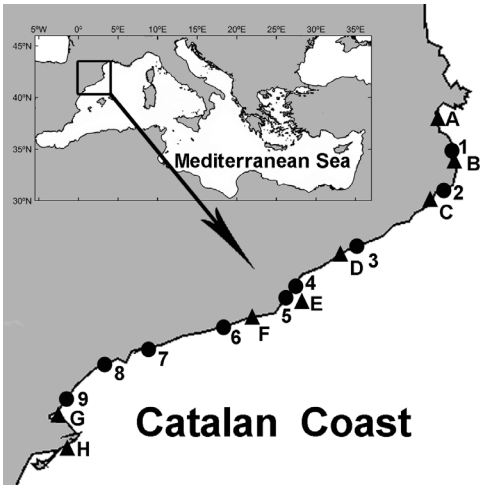

Figure 1: Sampling sites at the Catalan Coast. Dots and numbers represent harbours; triangles and letters represent beaches. 1) L'Estartit, 2) Palamós, 3) Arenys, 4) Olímpic, 5) Barcelona, 6) Vilanova, 7) Tarragona, 8) Cambrils, and 9) L'Ametlla. A) mouth of La Muga River, B) L'Estartit, C) La Fosca, D) Llavaneres, E) offshore Barcelona, F) Castelldefels, G) Fangar Bay, H) Platjola.

2.2 Phylogenetic analyses:

Sequences obtained in this study (Table 1) were aligned with those stored in GenBank using the MAFFT v.6 program (Katoh et al. 2002) under FFT-NS-i (slow; iterative refinement method) and manually checked with BioEdit v. 7.0.5 (Hall 1999), with a final alignment of the D1–D2 region, comprising ~760 positions. Phylogenetic relationships were determined using the maximum-likelihood (ML) and Bayesian inference methods. For the ML method, the GTRGAMMA evolution model was used on RAxML (Randomized Axelerated Maximum Likelihood) v.7.0.4 (Stamatakis 2006). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with RAxML software. Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al. 2012), run with a GTR model with rates set to gamma. Each analysis was conducted using four Markov chains (MCMC), with one million cycles for each chain. The consensus tree was created from post-burn-in trees and the Bayesian posterior probabilities (BPP) of each clade were examined.

3. Results:

3.1 Morphospecies detected:

During this study, 18 different morphospecies belonging to the Gymnodinium *s.s.* clade were detected from the Catalan Coast samplings (Table 2). Some of them were identified based on their morphological characteristic, while others could only be identified at the species level when their molecular phylogeny was obtained (Table 1; Fig. 4). Nonethe-

less, in some cases the morphospecies were not confidently identified despite successful LSU rDNA sequencing. In the following, morphological descriptions are provided for the organisms not unequivocally identified at the species level, for organisms whose characteristics differed from those reported in the literature, and for organisms identified only by their phylogeny. For all species, we also comment on the locations and physico-chemical parameters (temperature and salinity) measured at the detection sites, and, when quantified, the cellular abundances.

- *Barrufeta bravensis* Sampedro & Fraga (Fig. 2A)

This organism, collected during this study from La Fosca beach in June 2012 (temperature: 22.4ºC; salinity: 38.3), was recurrently present at high cell abundances (10^5–10^6 cells·L^{-1}) during the summer months (June–September) of 2002 and 2005 (Sampedro et al. 2011).

- *Gymnodinium aureolum* (Hulburt) Hansen (Fig. 2B)

This species was unequivocally detected only once, in June 2012, off the coast of Barcelona. The greenish-yellow pigmented cell was 22 µm long and 18 µm wide. Its morphology slightly differed from available descriptions of the species (Hansen et al. 2000; Hulburt 1957). It was round, but in contrast to available descriptions, the epicone was slightly shorter and narrower than the hypocone. The cingulum was located medially; it was wide and displaced by

Table 1: Sequences identification number (the sequences of *P. tanit* were already deposited in GenBank), species name (+ represents sequences obtained from cultured organisms, * represents sequences obtained from fixed organisms), date and locality of isolation of the cells from which sequences obtained in this study. Species sequences first obtained in this study are indicated in the last column (the sequences of *P. tanit* are also presented in Chapter 4).

Sequence	Species	Date	Locality	First sequence
1	*Barrufeta bravensis*	Jul-12	La Fosca Beach	-
2	*Gymnodinium aureolum*	Jun-12	Offshore Barcelona	-
3	*Gymnodinium impudicum* +	-	Tarragona Harbour	-
4	*Gymnodinium litoralis*	Jul-11	La Muga river mouth	-
5	*Gymnodinium* sp. 1	Dec-11	Olímpic Harbour	yes
6	*Gymnodinium* sp. 1	Dec-11	Olímpic Harbour	yes
7	*Gymnodinium* sp. 1	Jun-12	Arenys Harbour	yes
8	*Gymnodinium* sp. 2	Apr-13	Arenys Harbour	yes
9	*Gymnodinium* sp. 2	Apr-13	Arenys Harbour	yes
10	*Gymnodinium* sp. 3	Aug-12	Vilanova Harbour	yes
11	*Gymnodinium* sp. 3	Aug-12	Vilanova Harbour	yes
12	cf. *Gyrodinium undulans*	Feb-12	Palamós Harbour	yes
13	*Lepidodinium viride*	Oct-12	Fangar Bay	-
14	*Polykrikos kofoidii*	Dec-11	Estartit Beach	-
15	*Polykrikos kofoidii*	Oct-11	Estartit Harbour	-
16	*Polykrikos kofoidii*	Dec-11	Estartit Beach	-
KF806600	*Polykrikos tanit*	Jun-11	Arenys Harbour	yes
KF806601	*Polykrikos tanit*	May-12	Vilanova Harbour	yes
KF806602	*Polykrikos tanit*	Jun-12	Offshore Barcelona	yes
17	*Warnowia* sp. 1	Dec-11	Barcelona Harbour	yes
18	*Warnowia* sp. 1	Oct-12	Barcelona Harbour	yes
19	*Warnowia* sp. 2	Dec-11	Tarragona Harbour	yes
20	*Warnowia* sp. 2 *	Jun-12	Tarragona Harbour	yes
21	*Warnowia* sp. 2 *	Jun-12	Tarragona Harbour	yes
22	*Warnowia* sp. 3	Jun-12	Vilanova Harbour	yes
23	*Warnowia* sp. 4	Aug-12	Vilanova Harbour	yes
24	*Warnowia* sp. 4	Aug-12	Vilanova Harbour	yes
25	*Warnowia* sp. 5	Nov-12	Arenys Harbour	yes

Table 2: List of morphospecies detected during this study along the Catalan Coast. First detections in the Mediterranean Sea (Med) or along the Catalan Coast (CC) are noted. Asterisks indicate that the type location of the species is in the Mediterranean Sea. Previous detections of organisms not identified at species level are unknown and are represented by grey boxes. Potentially bloom-forming (B) species are indicated. Numbers in the Occurrence column are based on qualitative assessments and range from very rare (*) to very common (****).

Species	First detection	Bloom	Occurrence
Barrufeta bravensis	*	B	**
Gymnodinium aureolum	-	B	*
G. impudicum	*	B	***
G. litoralis	*	B	****
Gymnodinium sp. 1		-	**
Gymnodinium sp. 2		-	*
Gymnodinium sp. 3		-	*
cf. *Gyrodinium undulans*	Med	-	**
Lepidodinium viride	CC	-	*
Polykrikos herdmanae	Med	-	*
Polykrikos kofoidii	-	-	****
Polykrikos tanit	*	-	**
Erythropsidinium cf. *minor*	-	-	*
Warnowia sp.1		-	*
Warnowia sp.2		-	**
Warnowia sp.3		-	*
Warnowia sp.4		-	*
Warnowia sp.5		-	*

a distance less than once its width. The sulcus ran from the epicone to the hypocone, where it widened. An inconspicuous protuberance was observed in the apex. The nucleus was situated centrally. The cell contained several small bodies.

- *Gymnodinium impudicum* (Fraga & Bravo) Hansen & Moestrup (Fig. 2C)

This species recurrently produced high-biomass blooms (>10^6 cells·L^{-1}) in Tarragona Harbour, although during the samplings it was also detected at low cell abundances in other locations. Blooms are usually detected in Tarragona Harbour from June to September (Vila et al. 2001).

- *Gymnodinium litoralis* Reñé (Fig. 2D)

High abundances (>10^6 cells·L^{-1}) of this organism have been detected recurrently at several beaches on the northern coast of Catalonia between May and September (Reñé et al. 2011; Chapt III), although low cell abundance are found throughout the year, also in harbours.

- cf. *Gyrodinium undulans* Hulburt (Fig. 2E, F)

Three specimens were observed: in Palamós Harbour in February 2012 (temperature: 11.1ºC; salinity: 38.6), in Vilanova Harbour in June 2012 (temperature: 25ºC; salinity: 37.8), and offshore along the coast of Barcelona. The cells were 32–36 µm long and 24–32 µm wide. The epicone and hypocone were almost equal in size. The epicone was sub-spherical and the apex was round. The hypocone was trapezoidal, with slightly concave sides. The antapex was flattened. The cingulum was deep and wide, descending for a distance slightly less than its width, and overhanging slightly. The sulcus was bi-sigmoid, curving from left to right in the epicone, then to the left again in the intercingular area, where it widened, and to the right in the hypocone, forming a lobe. Cells were colourless but contained several granules. They agreed with available morphological descriptions (Hulburt 1957; Drebes and Schnepf 1998).

- *Gymnodinium* sp. 1 (Fig. 2G)

This morphospecies was detected in Olímpic Harbour in December 2011 (temperature: 16ºC; salinity: 37.5) and in Arenys Harbour in June 2012 (temperature: 20.1ºC; salinity: 37.3). Light microscopy observations did not reveal any conspicuous cellular features. The oval-shaped, yellow-brownish densely pigmented cells were 33.5–36.5 µm long and 26.5–29 µm wide. The epicone was conical and the hypocone was round. The cingulum was located medially, descending for a distance two to three times its width. The sulcus was observed near the apex and reached the antapex, where it widened. A round body was present in the hypocone. Three identical partial LSU rDNA sequences were obtained.

- *Gymnodinium* sp. 2 (Fig. 2H)

This morphospecies was detected in Arenys Harbour during April 2013 (temperature: 17.8 ºC; salinity: 36.5). It was characterized by forming two-cell chains, although three-cell chains were also observed. None single cell could be reliably assigned to this morphospecies. Cells were 13-18 µm long and 10-13 µm wide, almost equal in size. Cells were slightly dorso-ventrally compressed. The epicone of the anterior cell was conical with a rounded apex, while the hypocone was quadrangular, with a completely flattened antapex and slightly wider than the epicone. The posterior cell was ovoid, with roundish apex and antapex. The cinguli were broad, median and descending about 1-2 times its width. The sulci were narrow and deep in the epicone, where joined the horseshoe-shaped acrobase. It ran anticlockwise around the apex and its distal end was not in touch with the sulcus. The sulcus of the anterior cell broadened in the hypocone, reaching the antapex and forming a cavity which sheltered the epicone of the posterior cell. The sulcus of the posterior cell was not so broad, shallower and also reached the antapex. Chains were pigmented. Two identical partial LSU rDNA sequences were obtained.

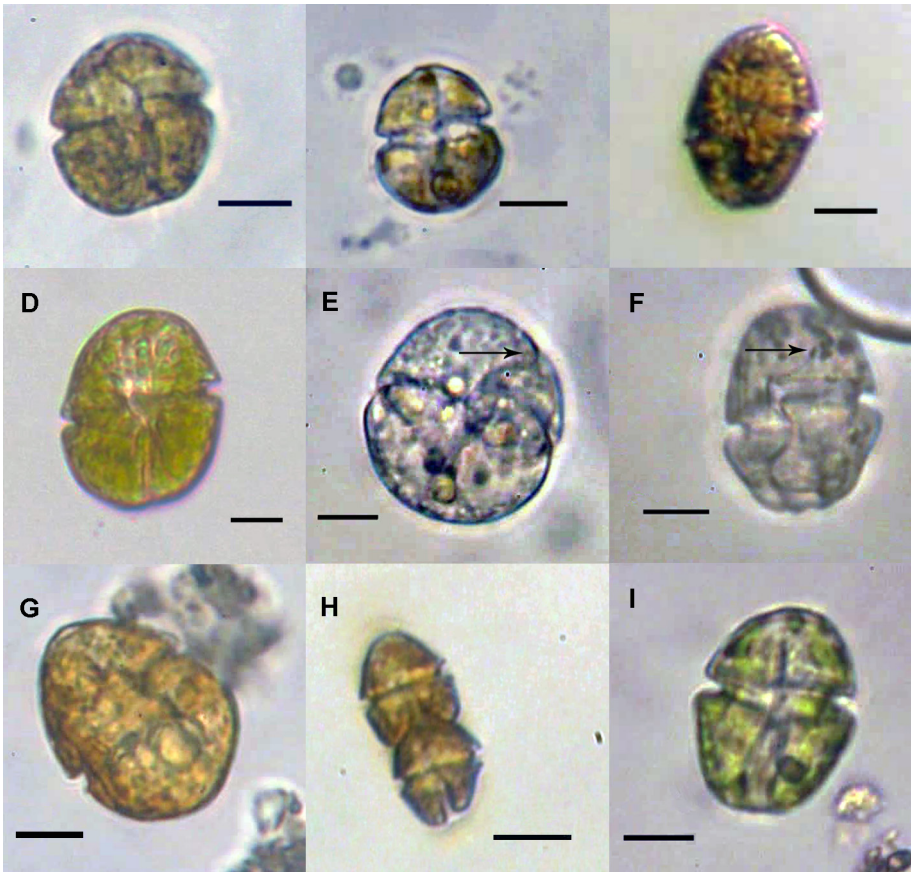

Figure 2: Light micrographs. Ventral view of A) *Barrufeta bravensis*, B) *Gymnodinium aureolum*, C) *G. impudicum*, D) *G. litoralis*, E) and F) cf. *Gyrodinium undulans*. The arrows show the characteristic outline of the sulcus, G) *Gymnodinium* sp. 1, H) *Gymnodinium* sp. 2 and I) *Lepidodinium viride*. Scale bars: 10 µm.

- *Gymnodinium* sp. 3

Specimens were collected in Vilanova Harbour in August 2012 (temperature: 27ºC; salinity: 37.8). Their morphological features could not be studied in detail because the cells collapsed during the observations; however, two partial LSU rDNA sequence were successfully obtained (99.6% similarity) and did not match that of any other sequence available in GenBank. The pigmented cells were 20–30 µm, with a *Gymnodinium*-like shape.

- *Lepidodinium viride* Watanabe, Suda, Inouye, Sawaguchi & Chihara (Fig. 2I)

One specimen was obtained from Fangar Bay in October 2012. The cell was 36 µm long and 33.5 µm wide. Its external morphology agreed with that of the original description (Watanabe and Suda 1990), except that in the latter the epicone was described as being slightly conical in shape while in our specimen it was completely round.

- *Polykrikos herdmanae* Hoppenrath et Leander (Fig. 3A)

Three specimens were observed from a sediments sample obtained in June 2013 at L'Estartit beach (temperature: 17.1ºC; salinity: 37.1). The pseudocolonies were 40-60 µm long and 21-35 µm wide and formed by 8 fused zooids. The pseudocolonies were obliquely compressed, the central zooids wider, while the distal zooids were narrower. Furthermore, the pseudocolonies were not symmetrical and the apical zooids were smaller and more pointed than the antapical ones, in contrast to available morphological descriptions (Hoppenrath and Leander 2007b). Pseudocolonies were heterotrophic and numerous thread-like extrusomes running vertically were present, as well as small granules. The nuclei could not be observed and none of the pseudocolonies showed ingestion bodies. None sequence was successfully obtained.

- *Polykrikos kofoidii* Chatton (Fig. 3B)

This species was commonly observed at abundances of up to $<10^3$ cell·L^{-1} in several locations along the Catalan Coast from spring to autumn. The cells were easily identified by the presence of longitudinal furrows in the hypocone of the posterior zooid (Matsuoka et al. 2009). Several partial LSU rDNA sequences were obtained, showing certain degree of intraspecific variability (99.2% similarity). Only 3 sequences are shown in this study.

- *Polykrikos tanit* Reñé (Fig. 3C)

This species was detected in several harbours and beaches during 2012 and 2013 from April to June, always at cell abundances $<10^3$ cell·L^{-1} (Reñé et al. 2013a; Chapt IV).

- *Erythropsidinium* cf. *minor* (Kofoid & Swezy) Silva (Fig. 3D)

One specimen was detected in Fangar Bay in October 2012. The ovoid cell was 51 µm long and 36 µm wide. It was only seen in dorsal view as it collapsed during microscopy. The epicone was small and asymmetrical. A knob was observed in the apex, probably because of the acrobase turning around it. The cingulum was pre-median, descending to the left. The junction with the sulcus was not observed. The morphology of the sulcus could only be seen in the hypocone, where it widened, forming an excavation on the right side of the cell. The antapex was flattened. A large ocellus was present on the upper left side of the epicone and the lens was hemispherical. The piston was not present in the antapex. The cell remained immobile during the observations under the light microscope. It was tentatively identified based on its original description (Kofoid and Swezy 1921). Unfortunately, it could not be sequenced.

- *Warnowia* sp. 1 (Fig. 3E)

Three specimens belonging to this morphospecies were detected in Barcelona Harbour, in December 2011 (temperature: 16.5ºC; salinity: 37.6) and in October 2012 (temperature: 21.6ºC; salinity: 38.4) and two were successfully sequenced (100% similarity). The cells were 29–33.5 µm long and 19.5–21 µm wide, ovoid, and elongated, but they could only be observed in lateral view. The epicone and hypocone were almost equal in size. The apex and antapex

were round. The cingulum was medially located, with the two ends joined ventrally, but neither their junction nor the sulcus was observed. The position of the ocellus varied in the studied specimens, as it was located near the antapex in one cell and in the hypocone near the cingulum in the two others. The lens was elongated and spherical at its end, located above the ocellus. The large, round, nucleus was centrally positioned in the epicone. The colourless cells were covered by a hyaline membrane.

- *Warnowia* sp. 2 (Fig. 3F)

Several live specimens were detected in Tarragona Harbour in December 2011 (temperature: 15.7º C; salinity: 36.7) and June 2012 (temperature: 23.8ºC; salinity: 37.6), although fixed specimens were previously collected from beaches during the summer months. The cells were 47–54.5 µm long, 20.5–27 µm wide, fusiform and elongated. The apex was blunt. The cingulum encircled the cell 2–2.5 times. The sulcus was not unequivocally observed. The antapex was asymmetrical, forming an elongated protuberance. The elongated nucleus was situated in the centre of the cell, and the elongated ocellus posteriorly. The lens was thin and long. Numerous nematocysts were present in the anterior part of the cell, radiating from the centre to the periphery. Three identical partial LSU rDNA sequences were obtained.

- *Warnowia* sp. 3 (Fig. 3G)

The only detection was in June 2012, in Vilanova Harbour (temperature: 25ºC; salinity: 37.8). The 39-µm long and 26.5-µm wide cell was only observed in lateral view and its outline was similar to that of *Warnowia* sp. 1. However, the cingulum encircled the cell twice. The nucleus was elongated, occupying 75% of the cell length. The reddish ocellus was small and elongated, situated on the ventral side of the cell. The colourless cell was covered by a hyaline membrane.

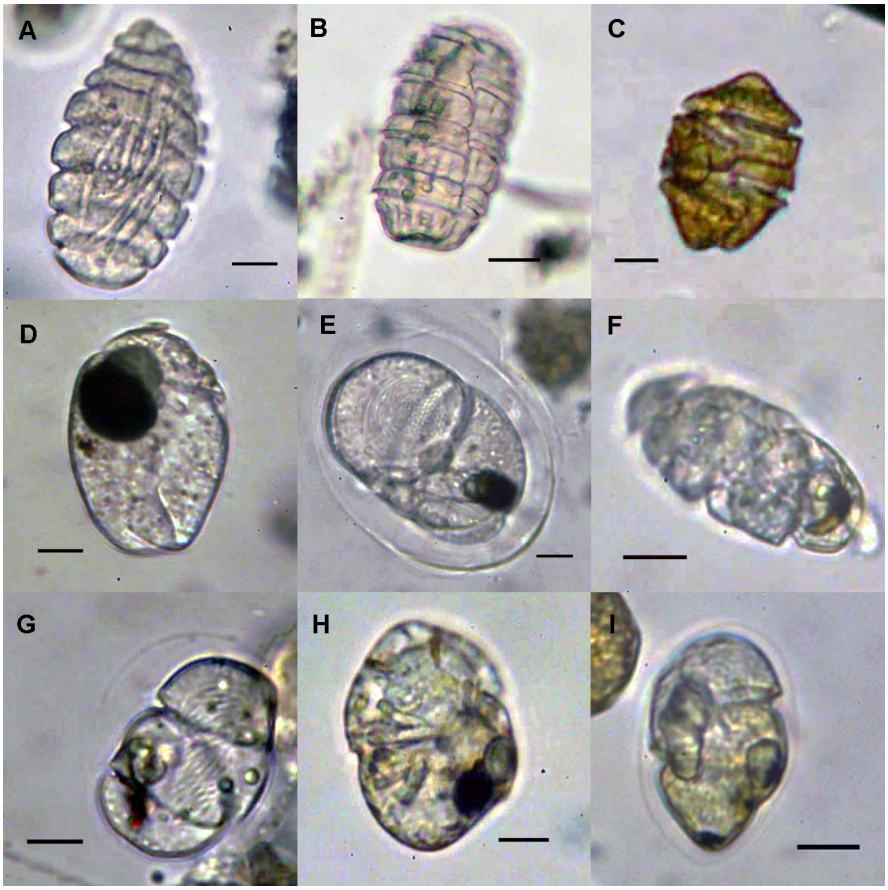

Figure 3: Light micrographs. A) Lateral view of *Polykrikos herdmanae*. Ventral views of B) *Polykrikos kofoidii* and C) *Polykrikos tanit*. D) Dorsal view of *Erythropsidinium* cf. *minor*. Lateral views of E) *Warnowia* sp. 1, F) *Warnowia* sp. 2, and G) *Warnowia* sp. 3. Ventral views of H) *Warnowia* sp. 4 and I) *Warnowia* sp. 5. Scale bars: 10 µm.

- *Warnowia* sp. 4 (Fig. 3H)

Several specimens were detected in August 2012 in Vilanova Harbour (temperature: 27ºC; salinity: 37.8). The cells were 44.5–54 µm long and 28.5–33.5 µm wide, roundish in shape, and slightly compressed dorso-ventrally. The epicone and hypocone were almost equal in size. The epicone was conical. The asymmetrical hypocone was round on its right side and less developed on its left side. The antapex was flattened. The cingulum was medially located, descending for a distance three to four times its width. The sulcus was not observed in detail but it widened in the hypocone and accounted for the asymmetry of the latter. Numerous nematocysts were present, mainly radiating from the center of the cell. The large, round nucleus had an irregular outline and was located in the epicone. The ocellus was situated in the hypocone. The lens was round, almost equal in size to the ocellus and situated to its left. Two partial LSU rDNA sequences (99.8% similarity) were obtained.

- *Warnowia* sp. 5 (Fig. 3I)

This morphospecies was detected in Arenys Harbour in November 2012 (temperature: 17.1ºC; salinity: 37.8) and again in April 2013 (temperature: 17.8 ºC; salinity: 36.5). The cells were ovoid and slightly dorso-ventrally flattened, although amorphous cells were also observed. The epicone was ovoid and the apex round. The hypocone was asymmetrical, with the right side more pointed than the left one. The cingulum was median, displaced about two times its width. The sulcus was shallow and not clearly observed. The lens and the ocellus were situated at the posterior end of the right side of the hypocone, as well as dark bodies scattered in the hypocone. An elongated refractive body was often present in the upper half of the cell. The cells showed a pale yellow-greenish colouration and were usually covered by a hyaline membrane.

3.2 Phylogenetic analyses:

The constructed phylogeny explored the diversity of the dinoflagellates included within the Gymmodiniales *sensu stricto* clade (87% Bootstrap / 1 BPP) (Fig. 4). Although weakly supported, the species were split in two clades. The first contained several *Polykrikos* species species (*P. hartmannii*, *P. tanit*, *P. schwartzii* and *P. kofoidii*) and *Cochlodinium* cf. *geminatum*. *Polykrikos lebourae* and *Pheopolykrikos beauchampii* clustered independently of the other polykrikoid species. Three different *Polykrikos* species were detected in the Catalan Coast. The sequence of the first species was consistent with the *P. kofoidii* sequences from GenBank and clearly distant from those of *P. schwartzii*. The sequence of *P. tanit* clustered with that of *P. hartmannii* from GenBank (70%/0.96) but at a substantial distance. The second group contained the remaining species, although their phylogenetic positions were unresolved. *Barrufeta bravensis*, *Gymnodinium aureolum*, *G. impudicum*, *G. litoralis* and *Lepidodinium viride* sequences obtained in this study agreed with those available in GenBank, while sequences of *Gymnodinium* sp. 1, sp. 2 and sp. 3 and cf. *Gyrodinium undulans* did not match with any other sequence previously available. A highly supported clade contained all the Warnowiaceae species (98%/1), which formed four main clades. The first branch only contained *Warnowia* sp. 4. In a sister branch, two sub-clades were obtained. The first one contained *Warnowia* sp. 1, sp. 2, and sp. 3 (100%/1), with *Warnowia* sp. 1 and sp. 3 clustering together (100%/1). Finally, *Warnowia* sp. 5 clustered with sequences from GenBank and was strongly related with the *Warnowia* sp. BS-2009a sequence (100%/1).

4. Discussion:

The genus *Gymnodinium* is phylogenetically included in a well-supported group, the Gymnodiniales *sensu stricto* clade, which also contains several other genera. We obtained sequences of several otherwise species difficult to identify as well as *Gymnodinium*-like species and members of the family Polykrikaceae and Warnowiaceae. These new LSU rDNA sequences of warnowiids are an important contribution to appreciating the diversity of these organisms. In addition, the detections of the species cf. *Gyrodinium undulans*, *Polykrikos herdmanae* and *Lepidodinium viride* were the first reports for the Mediterranean Sea and along the Catalan Coast, respectively (Table 2).

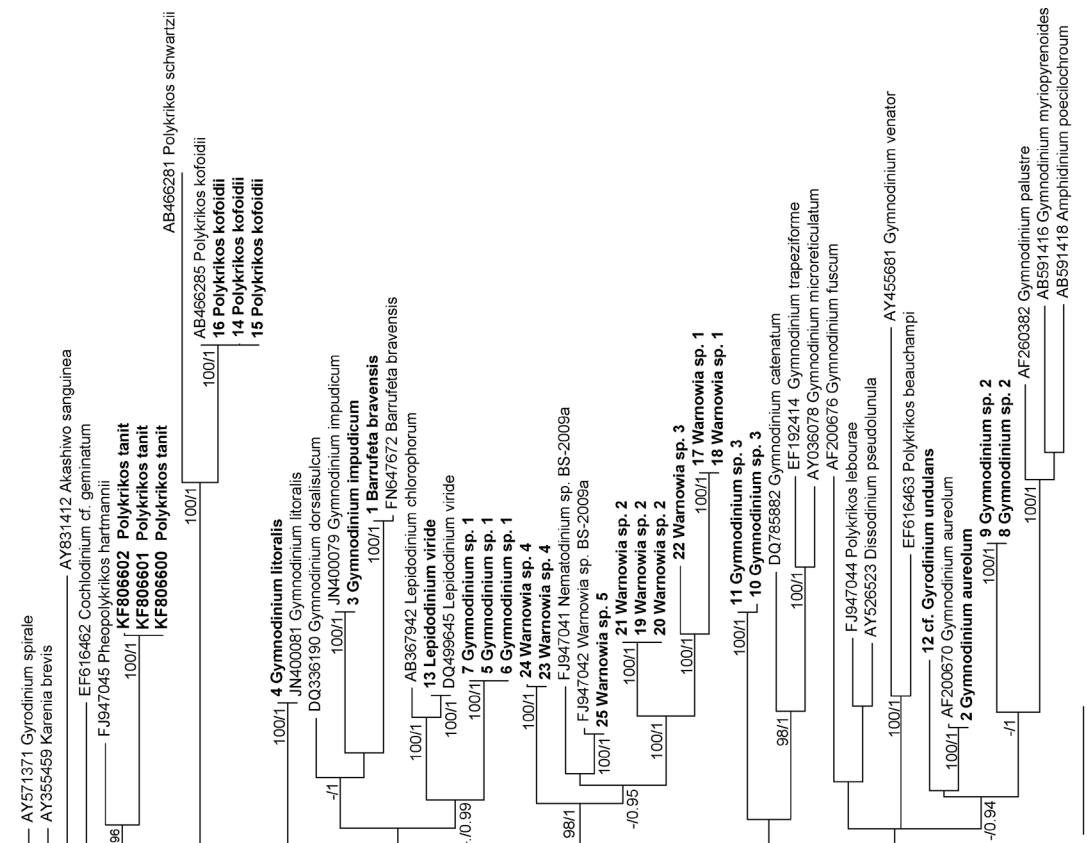

Figure 4: Maximum-likelihood phylogenetic tree of selected species based on the D1–D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap (%) values and the Bayesian posterior probability (BPP). Only bootstrap values >70 and BPP values >0.9 are shown. *Polarella glacialis* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

The most commonly detected and well-known *Gymnodinium*-like species are those that recurrently produce blooms and cause ecological and economic problems in the affected areas. They include *Gymnodinium litoralis* and *Barrufeta bravensis*, which are frequently present at beaches at high abundances (Reñé et al. 2011; Sampedro et al. 2011), and *G. impudicum*, which form blooms that extend for several kilometres and is often reported in harbours (Vila et al. 2001) and near beaches (Delgado et al. 1996). The NW Mediterranean is the type locality for *G. litoralis* (Reñé et al. 2011), *G. impudicum* (Fraga et al. 1995), and *B. bravensis* (Sampedro et al. 2011). These species usually proliferate during summer months, when water temperature rises to 25ºC and irradiance levels are high. The bloom-forming species *G. aureolum* and *L. viride* were also detected in the studied area, although only once and at low cell abundances. While *L. viride* was identified because of its green pigments, *G. aureolum* was more difficult to distinguish. However, while *G. aureolum* was previously detected in Catalan waters (Margalef 1995), *L. viride* detection was the first in our sampling area although it has been reported from other locations in the NW Mediterranean (Siano et al. 2009).

Another species, initially identified as cf. *Gyrodinium undulans*, was sporadically detected. One specimen was successfully sequenced for the first time, placing it within the Gymnodiniales *s.s.* clade. *Gyrodinium undulans* is an ectoparasite with a *Gymnodinium*-like stage in its life cycle (Drebes and Schnepf 1998). Taxonomic identification of the observed specimen according to the existing literature was not possible because of its morphological similarities with *Syltodinium listii* Drebes, another ectoparasite only known from the waters off Sylt, German Bight (Drebes 1988; Hoppenrath et al. 2009b). The lack of observations of either the organism's host or the infection process prevented us from confirming the affiliation of the studied specimen. *Gyrodinium undulans* is regarded as a cold water species because it has always been observed during the winter–spring months (water temperature from -1ºC to 6ºC). *Syltodinium listii* typically inhabits warmer waters, as it has been observed during the summer (average water temperature of 18ºC) in the waters off Sylt. Most records of *G. undulans* are from both sides of the North Atlantic whereas in those from warmer regions (Gascogne, France and Victoria, Australia) the affiliation has yet to be confirmed (Hoppenrath et al. 2009b). The organism sequenced in this study was first observed in February, presumably coinciding with the observation periods of *G. undulans*. However, the second detection was at the end of June, consistent with the observations of *S. listii* described in the literature. Regardless, our finding is the first detection of either *G. undulans* or *S. listii* in the Mediterranean Sea. Some ectoparasitic dinoflagellates, such as *Dissodinium* and *Chytriodinium*, are also included in the Gymnodiniales *s.s.* clade (Gómez et al. 2009b). All of them produce *Gymnodinium*-like cells during their life cycle. Therefore, the phylogenetic position of the sequenced representative is in agreement with those of the related ectoparasites, although our sequence did not cluster with the *Dissodinium pseudolunula* sequence available from GenBank. Since a close phylogenetic relationship between *G. undulans* and *S. listii* can be expected, we would reject *G. undulans* as a member of the *Gyrodinium* genus because it is included within the Gymnodiniales *s.s.* clade. However, since our specimen could not be precisely identified any systematic change would be premature.

Three other *Gymnodinium*-like species (*Gymnodinium* sp. 1, sp. 2 and sp. 3) were successfully sequenced. All of them were placed within the Gymnodiniales *s.s.* clade, but not coinciding with any other available sequence. *Gymnodinium* sp.1 could not be identified at the species level because the morphological observations were limited and none distinctive morphological feature was observed. The morphology of *Gymnodinium* sp. 2 was studied in detail and to the best of our knowledge, its morphology does not correspond to that of any other described species. Finally, *Gymnodinium* sp. 3 was not observed in detail, impeding its morphological description. Thus, although they could constitute new species, detailed morphological investigations are required to confirm this hypothesis. Despite the large number of described *Gymnodinium* morphospecies and the previous reports of other *Gymnodinium* species in NW Mediterranean waters (Gómez 2003; Velásquez 1997), during this study only a few were detected along the Catalan Coast. However, since most of the previous studies were based on the use of fixed samples and morphological variability is common in several species, we cannot rule out that the reported diversity of the *Gymnodinium* genus was previously overestimated or contrarily, that other species with inconspicuous features were also present in the sampled locations.

The *Polykrikos* genus is also included within the Gymnodiniales *s.s.* clade. Most polykrikoid species show a strong phylogenetic relationship, but there are discrepancies between SSU and LSU rDNA phylogenies for *P. lebourae*, which clusters independently in LSU rDNA phylogenies (Hoppenrath et al. 2009a; Hoppenrath and Leander 2007a). In the literature, both *P. kofoidii* and *P. schwartzii* have been widely reported along Catalan shores. The identification of both species is problematic because the two are similar in their external shape but distinguishable by other features, e.g., the longitudinal furrows on the hypocone of the zooids and the numbers of zooids developed in *P. kofoidii* (Matsuoka et al. 2009). In our study, *Polykrikos* specimens were commonly detected at many locations albeit always at low abundances. The presence of the furrows on the hypocone usually allowed the *P. kofoidii* specimens to be unequivocally identified, while in other specimens we were unable to observe the furrows. These cells were therefore selected for sequencing, but, according to their sequences, all of them belonged to *P. kofoidii* as they matched with sequences from publications in which a detailed morphological study was carried out (Matsuoka et al. 2009). Therefore, our inability to detect *P. schwartzii* suggests either the misidentification of *P. schwartzii* in the literature or its absence from our samplings due to its presence in lower abundances than *P. kofoidii* or its complete absence in the studied locations. Another detected *Polykrikos* species was the benthic *P. herdmanae* and to the best of our knowledge, it constitutes the first detection in the Mediterranean Sea. It was detected once from sediments obtained at L'Estartit beach. As sand samples were rarely examined, further studies should determine whether the species is common in the area and its distribution in the Catalan Coast. Finally, *P. tanit* was morphologically and phylogenetically close to *P. hartmannii* but they were demonstrated to be different species (Reñé et al. 2013a). Both were placed in a basal position within the *Polykrikos* clade and shared most of their characteristic features, pigmented, pseudocolonies of two zooids, same number of nuclei and zooids (Reñé et al. 2013a). *Polykrikos tanit* was recurrently detected in harbours and beaches but never *P. hartmannii*.

The last group of genera included within the Gymnodiniales *s.s.* clade are the members of the Warnowiaceae family. During this study, they were observed regularly but always at very low cell abundances. Six different morphospecies were distinguished. However, they were observed or manipulated under the microscope with difficulty as they were very delicate and quickly collapsed. Despite a previous report describing the presence of several species of the genus *Erythropsidinium* along the Catalan Coast (Margalef 1995), we detected only one morphospecies, identified as *Erythropsidinium* cf. *minor*, but were unable to obtain its sequence. Gómez et al. (2009a) demonstrated that, based on the SSU region, these *Erythropsidinium* species form a monophyletic clade with other warnowiid genera. Because the taxonomy of this group is extremely challenging (Hoppenrath et al. 2009a) we were unable to identify any of the other observed morphospecies at the genus level and thus refer to the different species as *Warnowia* sp. The species that we most commonly detected was *Warnowia* sp. 2. It strongly resembled *Nematodinium torpedo* Kofoid et Swezy, but we refrained from conferring this name because although the presence of this latter species along the Catalan Coast was previously reported (Margalef 1995), the size of our specimens was almost half that described in the literature. *Warnowia* sp. 1, *Warnowia* sp. 2, and *Warnowia* sp. 3 formed a strongly supported clade and all three were heterotrophic. Nematocysts were not observed for *Warnowia* sp. 1 and *Warnowia* sp. 3 but they were present in *Warnowia* sp. 2. Warnowia sp. 4 clustered independently. Its partial SSU sequence (1300 bp) differed by only one bp from that of the '*Proterythropsis*' sp. sequence from GenBank (data not shown) and the two species are morphologically similar, although the posterior cell 'extension' (Hoppenrath et al. 2009a) was not observed in our specimens. This could, however, be a variable feature and we are confident that the two species belong to the same genus, as they cluster independently of the other species in the LSU rDNA phylogeny obtained. *Warnowia* sp. 5 LSU rDNA was almost identical to *Warnowia* sp. BC-2009a sequence available from GenBank and its partial SSU sequence showed a 100% identity with that of *Warnowia* sp. BC-2009a representative (data not shown) but also clustered with the *Nematodinium* sp. sequence from GenBank. Therefore, the features used by Hoppenrath et al. (2009a) to discriminate among morphospecies belonging to the genera *Nematodinium* and *Warnowia* are not supported by the partial LSU rDNA phylogeny obtained in this study, which hinders a taxonomic clarification.

5. Conclusions:

- In this study, we reported the presence of 18 different species belonging to the Gymnodiniales *sensu stricto* clade along the Catalan Coast. *Polykrikos herdmanae* and cf. *Gyrodinium undulans* represent the first detection for the Mediterranean Sea and *Lepidodinium viride* for the Catalan Coast. Information of previous detections could not be evaluated for eight of them because they were not identified at species level.

- Partial LSU rDNA sequences were obtained for 16 of the morphospecies. Nine of these sequences were the first available for these species. They will allow comparisons with further detections of morphotypes not easily identifiable based on morphological observations.

- The detected species *B. bravensis*, *G. aureolum*, *G. impudicum*, and *G. litoralis* are reported as high-biomass bloom producers. Although proliferations of *G. aureolum* have never been detected in the Catalan Coast, we cannot exclude forthcoming blooms of this species.

- The sequences obtained for *Gymnodinium* sp. 1, sp. 2 and sp. 3 did not coincide with those of any other organism. Further morphological studies are needed to confirm if they could constitute new species. The cf. *Gyrodinium undulans* specimens could not be confidently identified because of their resemblance with *S. listii*, but the obtained sequence confirms its clustering into the Gymnodiniales *s.s.* clade.

- Three different *Polykrikos* species were detected; *P. herdmanae* for the first time in the Mediterranean Sea, *P. tanit*, which was previously described as a new species, and *P. kofoidii*, but never *P. schwartzii*, which was commonly reported in the literature from the studied area. Thus, those reports could constitute misidentifications, given its similarity with *P. kofoidii*.

- LSU rDNA sequences and morphological information were provided for five different warnowiid specimens. While they were grouped in four different clades, our data do not allow further clarification of their challenging taxonomy.

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From Dr. Takayama's personal website

Chapter 2

"The diversity of dinoflagellates belonging to the order Gymnodiniales *sensu lato* from the Catalan coast (NW Mediterranean Sea)"

In prep.

DIVERSITY OF DINOFLAGELLATES BELONGING TO THE ORDER GYMNODINIALES *SENSU LATO* (DINOPHYCEAE) FROM THE CATALAN COAST (NW MEDITERRANEAN SEA)

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Abstract

The diversity of dinoflagellates belonging to the order Gymnodiniales *sensu lato* and present along the Catalan coast (NW Mediterranean) was studied during coastal samplings during a 3-year period. The detected dinoflagellates were identified based on their morphological features together with their partial LSU rDNA sequences. This approach resulted in the detection of 40 different morphospecies, eight of which were observed for the first time in the Mediterranean Sea and seven along the Catalan coast. Among the latter were three toxic *Karenia* species. Partial LSU rDNA sequences were obtained for 27 different morphospecies, including novel sequences for 16 species, most of them belonging to the genus *Gyrodinium*. Although the presence of cryptic species related to *G. spirale* was determined, the LSU rDNA region lacked sufficient resolution to discriminate between various *Gyrodinium* morphospecies. The phylogenetic position of the genus *Torodinium* was obtained for the first time. Overall, the species detected in this study represent 30% of the Gymnodiniales species detected thus far in the entire Mediterranean Sea and 85% of the species detected in the NW Mediterranean Sea.

1. Introduction

The diversity of living marine dinoflagellates is estimated at 2,500 species (Gómez, 2005; Sournia, 1995). Traditionally, the taxonomy of dinoflagellates was based on the morphological features of the different groups and the major orders were established accordingly (Fensome et al., 1993; Taylor, 1987). However, the interrelationships among the different lineages remain unresolved and many orders have proved to be polyphyletic (Moestrup and Daugbjerg, 2007; Murray et al., 2005; Saldarriaga et al., 2004). The coupling of morphological features with molecular phylogenetic data has enabled analyses of the relationships between species (Handy et al., 2009; Murray et al., 2009; Taylor, 2004). Unfortunately, most of the organisms that have been sequenced are cultivable photosynthetic species, such that information from a large number of genera, such as those that are mixo- and heterotrophic, is scarce. However, organisms that are difficult to culture can now be studied with single-cell PCR (Ruiz-Sebastián and O'Ryan, 2001), a powerful technique that has been successfully and recurrently applied to dinoflagellates (Lynn and Pinheiro, 2009).

Most dinoflagellates that lack a theca are included in the order Gymnodiniales Apstein. These "unarmoured" or "naked" protists comprise 20–30 different genera and more than 500 free-living species, belonging mainly to the genera *Amphidinium*, *Cochlodinium*, *Gymnodinium*, and *Gyrodinium* (Gómez, 2005; Guiry and Guiry, 2013). However, the identification of unarmoured species is challenging because several of their key characters are difficult to observe and the lack of a theca often results in their deformation when fixed for microscopy studies. Complementary information about the studied organisms is obtained with phylogenetic data, which allow the discrimination of similar morphospecies and the characterization of specimens that cannot be easily identified by their morphology alone. Consequently, the taxonomy of unarmoured dinoflagellates has undergone deep revisions since Daugbjerg et al. (2000), as the combination of morphological, ultrastructural, and phylogenetic information has demonstrated the polyphyly of organisms included within the genus *Gymnodinium* in addition to redefining the genera *Gymnodinium* and *Gyrodinium* and erecting the new genera *Akashiwo*, *Karenia*, and *Karlodinium*.

Daugbjerg et al. (2000) defined the *Gymnodinium sensu stricto* clade, which contained *Gymnodinium fuscum* (Ehrenberg) Stein 1878, the first gymnodinioid species described. Since then, the phylogenetic relationships of unarmoured genera and species have been intensively studied, resulting in the further erection of more than ten new genera [e.g., de Salas et al. (2003); Flø Jørgensen et al. (2004b); Sampedro et al. (2011); Sparmann et al. (2008)]. Using single-cell PCR, several authors have obtained phylogenetic data on Gymnodiniales, which includes a large number of heterotrophic and not easily cultivable species (Hansen and Daugbjerg, 2004; Hoppenrath et al., 2009; Reñé et al., 2013a; Reñé et al., 2013b; Takano and Horiguchi, 2004). Based on this new phylogenetic information, the order Gymnodinales was recognized as either polyphyletic and thus artificial with respect to single rRNA genes (Daugbjerg et al., 2000; Gómez et al., 2011; Saldarriaga et al., 2001) or paraphyletic when multiple genes were used to reconstruct phylogenies (Orr et al., 2012). In light of the polyphyly or paraphyly of the order Gymnodiniales, the *Gymnodinium sensu stricto* clade is considered as Gymnodiniales *sensu stricto* (Gómez et al., 2009; Hoppenrath and Leander, 2007, 2010; Yamaguchi et al., 2011).

The specific composition of phytoplankton from the study area, the Catalan coast (NW Mediterranean Sea), has been intensively investigated since the 1940s (Estrada, 1979, 1980; Margalef, 1945, 1969). However, in most cases fixed samples were used, which impeded the unequivocal identification of some Gymnodiniales members, thus resulting in a probable underestimation of their total diversity. Later on, the taxonomy and distribution of unarmoured species along the Catalan coast was examined, mainly with respect to the presence of harmful algal blooms (Delgado et al., 1995; Garcés et al., 2006; Sampedro et al., 2011; Vila et al., 2001). Gómez (2003) used published data to catalogue the dinoflagellates recorded in the Mediterranean Sea. That study identified 173 morphospecies belonging to the order Gymnodiniales. The Catalan coast was included within the Balearic-Provençal sub-basin, which although it had the second highest number of reported dinoflagellate taxa, included only 55 Gymnodiniales species, suggesting that their

diversity was underestimated. The checklist compiled by Velásquez (1997) on the phytoplankton reported from the NW Mediterranean Sea included 62 Gymnodiniales species reported in the same area.

The aim of this work was to study the diversity of Gymnodiniales species present along the Catalan coast by combining information derived from studies of their morphology and partial LSU rDNA phylogeny. Underlying this aim was a recognition of the uncertainty regarding the taxonomy of the order Gymnodiniales, and the fact that its diversity along the Catalan coast had never been thoroughly studied using this combined approach. The diversity of organisms included in the Gymnodiniales *sensu stricto* clade from the Catalan coast was presented in Chapter I of this thesis. Here the focus is on organisms not included within this clade and thus referred to as the order Gymnodiniales *sensu lato*.

2. Material and methods

2.1 Microscopic observation, cell isolation, single-cell PCR amplification, and sequencing

Lugol's fixed and live samples from along the Catalan coast were examined during 2011–2013. For some species belonging to this order, information has already been published, such as *Cochlodinium polykrikoides* [Chapter V; Reñé et al. (2013c)] and the Ceratoperidiniaceae family [Chapter VI; Reñé et al. (2013b)]. Nonetheless, they have also been included in this chapter to show the total diversity of Gymnodiniales *sensu lato* in the study area. Sub-surface water samples were obtained weekly to monthly or sporadically, depending on the year and the station. Sampled locations included beaches and harbours (Fig. 1). Occasionally, samples from offshore and coastal sediments were collected. Sediment samples were filtered through a 200-µm mesh and cleaned with seawater from the same locality. Further processing consisted of the following. For fixed samples, 50 ml were settled in a settling chamber during 24 h and examined under a Leica-Leitz DM-Il inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

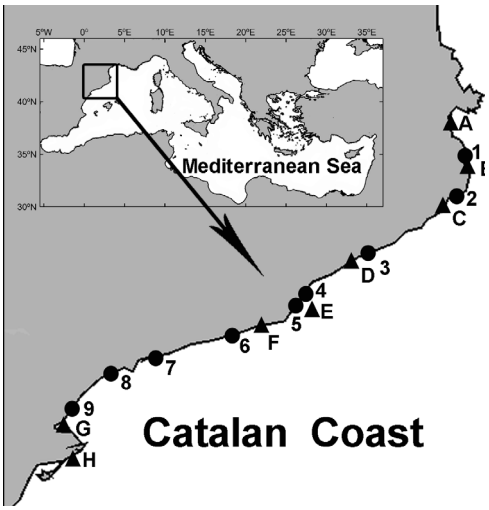

Figure 1: Sampling sites from the Catalan coast. Dots and numbers indicate harbours, and triangles and letters beaches. 1) L'Estartit; 2) Palamós; 3) Arenys; 4) Olímpic; 5) Barcelona; 6) Vilanova; 7) Tarragona; 8) Cambrils; 9) L'Ametlla; A) La Muga River mouth; B) L'Estartit; C) La Fosca; D) Llavaneres; E) offshore Barcelona; F) Castelldefels; G) Fangar Bay; H) Platjola.

Depending on cell abundances, different volumes of live samples were concentrated through a 10-µm mesh and observed under an inverted microscope. The organisms were filmed and photographed with a Sony NEX-5 camera (SONY, Tokyo, Japan) and their morphological features were studied when possible. Each cell was transferred several times into filtered seawater drops using Pasteur pipettes and finally transferred to a 200-µl PCR tube. Some fixed cells were also isolated for sequencing, using the same method. Single-cell PCR was conducted with a PCR mixture containing 5 ml of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 mM of the primers D1R and D2C (Scholin et al., 1994) for the partial LSU region and the primers EUK A (Medlin et al., 1988) and 1209R (Giovannoni et al., 1988) for the partial SSU region. The LSU PCR conditions were as follows:

initial denaturation for 5 min at 95 ºC, 40 cycles of 20 s at 95 ºC, 30 s at 55 ºC, and 1 min at 72 ºC, followed by a final extension step for 7 min at 72 ºC. The SSU PCR conditions were: initial denaturation for 5 min at 95 ºC, 30 cycles of 45 s at 95 ºC, 1 min at 55 ºC, and 3 min at 72 ºC, followed by a final extension step for 10 min at 72 ºC. Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and then visualized under UV illumination. The remainder of each sample was frozen at -20 ºC and later used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using both forward and reverse primers and a 3730XL DNA sequencer.

Table 1: List of LSU rDNA sequences obtained in this study: sequence number, species name (+sequences obtained from cultured organisms, * sequences obtained from fixed organisms), date and locality of the isolation, and whether the sequence was the first one obtained for the species.

Sequence	Species	Date	Locality	First sequence
1	*Akashiwo sanguinea* +	Aug-10	Vilanova Harbour	-
2	*Akashiwo sanguinea*	Jun-12	Ametlla Harbour	-
3	*Amphidinium carterae* +	Dec-11	Llavaneres	-
4	*Amphidinium carterae* +	Dec-11	Llavaneres	-
5	*Amphidinium crassum*	Jul-11	Tarragona Harbour	-
6	*Amphidinium crassum*	Oct-11	Tarragona Harbour	-
7	*Apicoporus* sp.	Jun-13	Castelldefels beach	yes
8	*Apicoporus* sp.	Jun-13	Castelldefels beach	yes
9	*Cochlodinium* sp.	Nov-12	Palamós Harbour	yes
10	*Gymnodinium instriatum* +	Jun-10	Arenys Harbour	-
11	*Gymnodinium instriatum*	May-12	La Muga river mouth	-
12	*Gymnodinium agaricoides*	Nov-11	Tarragona Harbour	yes
13	*Gyrodinium britannicum*	Mar-12	Barcelona Harbour	yes
14	*Gyrodinium corallinum*	May-12	Barcelona Harbour	yes
15	*Gyrodinium dominans* +	Feb-11	Barcelona offshore	-
16	*Gyrodinium dominans* +	Feb-11	Barcelona offshore	-
17	*Gyrodinium heterogrammum*	Oct-11	Tarragona Harbour	yes
18	*Gyrodinium heterogrammum*	Dec-11	Tarragona Harbour	yes
19	*Gyrodinium heterogrammum*	Dec-11	Arenys Harbour	yes
20	*Gyrodinium* cf. *ochraceum*	Oct-11	Tarragona Harbour	yes
21	*Gyrodinium* cf. *ochraceum*	Oct-11	Tarragona Harbour	yes
22	*Gyrodinium* cf. *spirale*	Dec-11	Estartit beach	yes
23	*Gyrodinium* cf. *spirale*	Dec-11	Estartit beach	yes
24	*Gyrodinium* cf. *spirale*	Dec-11	Barcelona Harbour	yes
25	*Gyrodinium* cf. *spirale*	Dec-11	Tarragona Harbour	yes
26	*Gyrodinium* cf. *spirale*	Dec-11	Tarragona Harbour	yes
27	*Gyrodinium* cf. *spirale*	Dec-11	Barcelona Harbour	yes
28	*Gyrodinium viridescens*	May-12	Castelldefels	yes
29	*Gyrodinium viridescens*	Jul-12	Estartit beach	yes
30	*Gyrodinium* sp.1	Oct-11	Tarragona Harbour	yes
31	*Gyrodinium* sp.2	Dec-11	Barcelona Harbour	yes
32	*Gyrodinium* sp.3	Dec-11	Tarragona Harbour	yes
33	*Gyrodinium* sp.4	Dec-11	Tarragona Harbour	yes
34	*Gyrodinium* sp.4	Mar-12	Arenys Harbour	yes
35	*Gyrodinium* sp.5	Oct-11	Tarragona Harbour	yes
36	*Karenia mikimotoi* *	Jun-12	Tarragona Harbour	-
37	*Karenia mikimotoi*	Oct-12	Fangar Bay	-
38	*Karenia umbella*	Oct-12	Olimpic Harbour	-
39	*Karlodinium armiger* +	Feb-11	Barcelona offshore	-
40	*Karlodinium decipiens*	May-11	L'Estartit beach	-
41	*Karlodinium veneficum* +	Jan-00	Alfacs Bay	-
42	*Katodinium glaucum*	Jan-12	Tarragona Harbour	-
43	*Takayama tasmanica*	Jul-12	Llavaneres beach	-
44	*Takayama tasmanica*	Oct-12	Fangar Bay	-
45	*Torodinium teredo*	May-11	L'Estartit beach	yes
46	*Torodinium robustum* *	Aug-12	Castelldefels beach	yes
47	*Torodinium robustum*	Oct-12	Fangar Bay	yes

Table 2: List of SSU rDNA sequences obtained in this study: sequence number, species name (+sequences obtained from cultured organisms), date and locality of the isolation, and whether the sequence was the first one obtained for the species.

Sequence	Species	Date	Locality	First sequence
48	*Apicoporus* sp.	Jun-13	Castelldefels beach	yes
49	*Apicoporus* sp.	Jun-13	Castelldefels beach	yes
50	*Apicoporus* sp.	Jun-13	Castelldefels beach	yes
51	*Ceratoperidinium falcatum*	Oct-12	Fangar Bay	yes
52	*Ceratoperidinium falcatum*	Oct-12	Fangar Bay	yes
53	*Gymnodinium instriatum* +	Aug-09	La Muga River mouth	-
54	*Gymnodinium litoralis* +	Jun-09	La Muga River mouth	yes
55	*Gyrodinium* cf. *spirale*	Mar-12	Vilanova harbour	-
56	*Gyrodinium* cf. *spirale*	Mar-12	Barcelona harbour	-
57	*Gyrodinium* cf. *spirale*	May-12	Arenys harbour	-
58	*Gyrodinium* cf. *spirale*	Jun-12	Ametlla harbour	-
59	*Gyrodinium* cf. *spirale*	Dec-12	Tarragona harbour	-
60	*Gyrodinium heterogrammum*	Feb-12	Tarragona harbour	yes
61	*Gyrodinium heterogrammum*	Feb-12	Tarragona harbour	yes
62	*Torodinium robustum*	Oct-12	Fangar Bay	yes
63	*Torodinium robustum*	Oct-12	Fangar Bay	yes

2.2 Phylogenetic analyses

The sequences obtained (Table 1 and 2) were aligned with those from GenBank using the MAFFT v.6 program (Katoh et al., 2002) under FFT-NS-i. The alignments were manually checked with BioEdit v. 7.0.5 (Hall, 1999) and the highly variable regions of the LSU alignment were removed using Gblocks v.0.91b (Castresana, 2000) under the less stringent options, obtaining a final alignment of about 630 positions for LSU sequences and 1710 positions for SSU sequences. In both cases, phylogenetic relationships were determined using maximum-likelihood (ML) and Bayesian inference methods. For the former, the GTRGAMMA evolution model was used on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006). All model parameters were estimated by RAxML. Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RAxML software. The Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al., 2012), run with a GTR model in which the rates were set to gamma. Each analysis was performed using four Markov chains (MCMC), with one million cycles for each chain. The consensus tree was created from post-burn-in trees and the Bayesian posterior probabilities (BPP) of each clade were examined.

3. Results

3.1 Morphospecies detected

During this study, 40 different species belonging to the Gymnodiniales *sensu lato* were detected along the Catalan coast. All morphospecies observed are listed in Table 3. Of these, some were identified by their characteristic morphological features, others were only unequivocally identified at the species level when their molecular phylogeny was determined (Table 1 and 2 and Figure 5 and 6), and some could not be confidently identified at all even though their LSU rDNA sequence was obtained. In the following, a detailed morphological description is provided only for organisms not unequivocally identified at the species level, for those with characteristics different than the ones reported in the literature, and for those identified only by their phylogeny. Remarks about the locations and physicochemical parameters, e.g., water temperature (temp) and salinity (sal), of the detections are provided for all species, and cellular abundances, when quantified, are reported.

- *Akashiwo sanguinea* (Hirasaka) Hansen & Moestrup (Fig. 2A).
This species was commonly detected throughout the study area, including beaches and harbours, and throughout the year, with cell abundances occasionally reaching 10^3–10^4 cells·L^{-1}. Two partial LSU rDNA sequences were obtained, showing intraspecific variability (97.5% similarity).

- *Amphidinium carterae* Hulburt (Fig. 2B)
Usually described as a benthic species, it was sporadically detected at cell abundances <10^2 cells· L^{-1} in the water column close to beaches during the summer. Two identical partial LSU rDNA sequences were obtained.

- *Amphidinium crassum* Lohmann (Fig. 2C)
Its presence in the water column was commonly observed in several harbours from May to October (temp: 21.1–25ºC; sal: 36.6–37.8) at abundances <10^2 cells·L^{-1}.

- *Amphidinium* cf. *operculatum* Claparède & Lachmann (Fig. 2D)
One fixed specimen was obtained from L'Alguer beach in July 2011 (temp: 22.5ºC; sal: 38.2). The specimen was tentatively identified based on Murray et al. (2004). The green-brown cell was 38.3 µm long and 28 µm wide, ovoid, and dorsoventrally compressed. The epicone was small and overlaid the anterior part of the hypocone, bent to the left side of the cell. Its right side formed an angle of 90° and the left side deflected to the left. The hypocone was oval and symmetrical, and the antapex was round and slightly flattened. The cingulum formed a V in ventral view, its distal end descending one-third of the cell length. The nucleus was large, occupying almost the posterior half of the hypocone. Although a round structure seemed to be present just above the nucleus, it could not be unequivocally distinguished as a pyrenoid or stigma.

- *Apicoporus* sp. (Fig. 2E)
Several specimens, 34–44 µm long and 22–29 µm wide (n=13), were observed in a sand sample from Castelldefels obtained at the beginning of June 2013 (temp: 19.7ºC; sal: 37.5). The cells were dorsoventrally flattened and their surfaces were smooth. The epicone was short, wide, and beak-shaped. The cingulum was deep, descending, and its distal end was not connected with the sulcus, which was narrow and ran through the hypocone, where it formed a semicircular indentation. The sulcus penetrated into the epicone, reaching the apex, where a protuberance was present. The shape of the hypocone varied from rectangular to ovoid and the antapex was roundish but slightly asymmetrical. No horns or protrusions in the antapical end were observed. The nucleus was large, quadrangular, and occupied almost the entire hypocone, from the ends of the cingulum to the end of the sulcus. The cells were colourless and chloroplasts were absent. Two identical partial LSU rDNA sequences and three identical partial SSU rDNA sequences were obtained.

- *Asterodinium gracile* Sournia (Fig. 2F)
Three fixed specimens were obtained in January 2009 in surface samples from off the coast of Barcelona. Cell abundances were <10^3 cells·L^{-1}.

- *Balechina coerulea* (Dogiel) Taylor (Fig. 2G)
One fixed specimen was observed in August 2011 in Montjoi beach (temp: 23.3ºC; sal: 37.9).

- *Brachidinium* sp. (Fig. 2H)
One fixed specimen was obtained in November 2010 in surface samples from off the coast of Barcelona.

- *Ceratoperidinium falcatum* (Kofoid & Swezy) Reñé et de Salas (Fig. 2I)
Several organisms were detected in October 2012 in Fangar Bay at abundances <10^3 cell·L^{-1} (Reñé et al., 2013b; Chapt VI). Two partial SSU rDNA sequences (99.7% similarity) were obtained in this study.

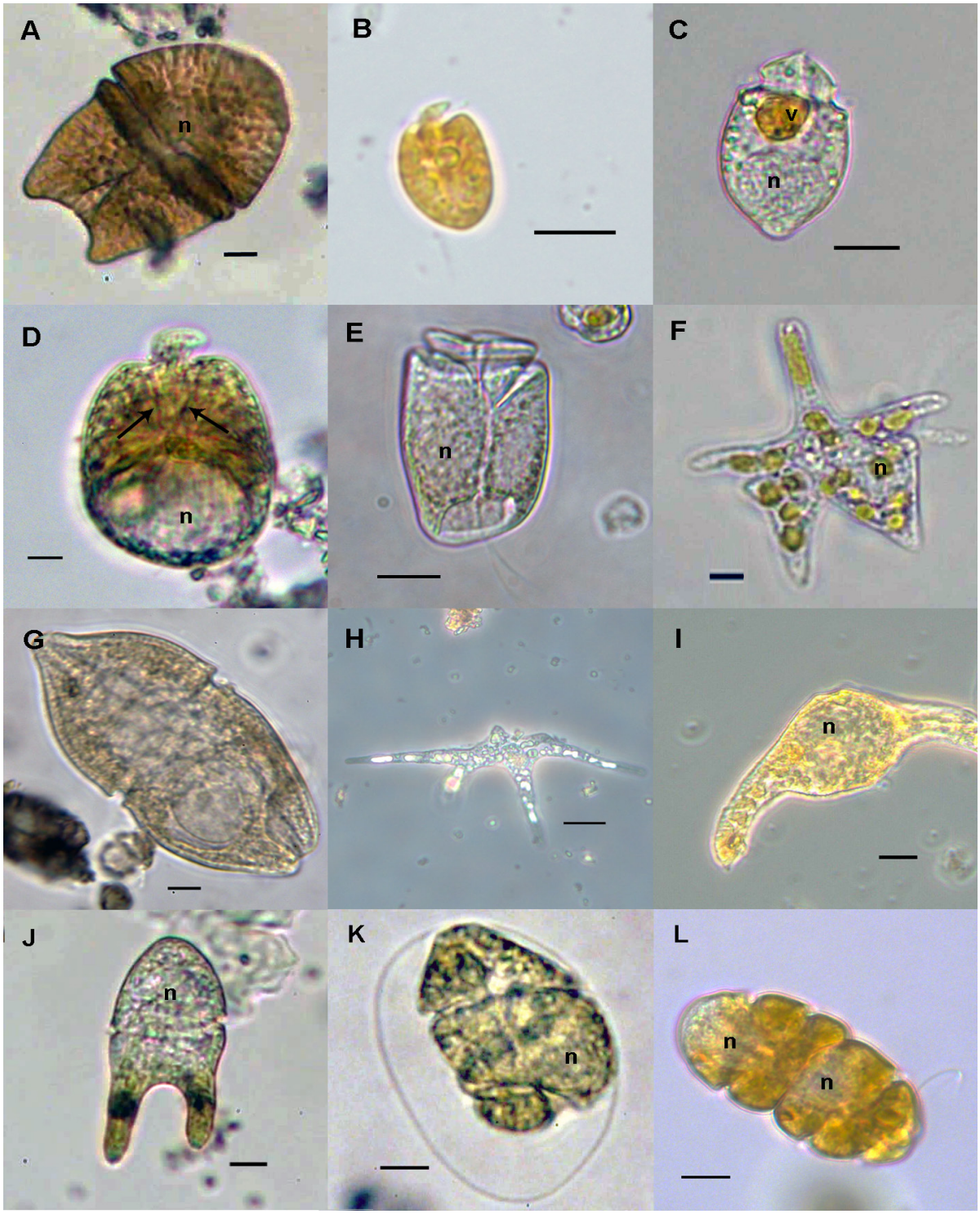

Figure 2: Light micrographs. Ventral view of A) *Akashiwo sanguinea*, B) *Amphidinium carterae*, C) *A. crassum*, D) *A.* cf. *operculatum (*arrows mark the ends of the cingulum), E) *Apicoporus* sp., F) *Asterodinium gracile*, G) *Balechina coerulea*, H) *Brachidinium* sp., I) *Ceratoperidinium falcatum*, J) *C. margalefii*, K) *Cochlodinium* cf. *convolutum*, L) *Cochlodinium polykrikoides*. Nuclei (n) and vacuoles (v) are indicated. Scale bars = 10 µm.

- *Ceratoperidinium margalefii* Margalef *ex* Loeblich III (Fig. 2J)
One specimen was collected from the mouth of the La Muga River in July 2011 (temp: 21.2ºC; sal: 30.9) (Reñé et al., 2013b; Chapt VI).

- *Cochlodinium* cf. *convolutum* Kofoid et Swezy (Fig. 2K)
Two specimens of this morphospecies were obtained, one from Barcelona Harbour in October 2012 (temp: 21.6ºC; sal: 38.4) and the other from Palamós Harbour in November 2012 (temp: 16.4ºC; sal: 38.1). Only the latter was successfully sequenced. It clustered independently of previously sequenced *Cochlodinium* species (Reñé et al., 2013b; Chapt VI).

- *Cochlodinium polykrikoides* Margalef (Fig. 2L)
This species was recurrently detected in harbours from June to September, reaching maximum cell abundances of 10^4 cells/L (Reñé et al., 2013c; Chapt V).

- *Cochlodinium* sp. (Fig. 3A)
Several specimens were detected in Palamós Harbour in November 2012 (temp: 16.4ºC; sal: 38.1), but only one cell was thoroughly observed and successfully sequenced. It was 49.5 µm long and 33.5 µm wide and ovoid in shape, with its widest transdiameter in the center of the cell. Both apex and antapex were round. The cingulum encircled the orange-pigmented cell more than two times but the sulcus was not unequivocally observed. The oval nucleus was situated in the upper half of the cell, slightly displaced to the right.

- cf. *Cochlodinium* sp.1
One specimen was obtained from Palamós Harbour in November 2012 (temp: 16.4ºC; sal: 38.1) and successfully sequenced. Its morphology could not be studied in detail because the cell collapsed during the observations, but phylogenetically it clustered within the *Ceratoperidiniaceae* family together with *C.* cf. *convolutum* (Fig. 5). We cannot rule out that the cell was *C.* cf. *convolutum* (Reñé et al., 2013b; Chapt VI).

- '*Gymnodinium*' sp. (Fig. 3B)
This specimen was obtained from Palamós Harbour in November 2012 (temp: 16.4ºC; sal: 38.1). It was referred to as '*Gymnodinium*' sp. 2 in Reñé et al. (2013b) and clustered within the *Ceratoperidiniaceae* family (Chapt VI).

- *Gymnodinium agaricoides* Campbell (Fig. 3C)
One specimen was collected from Tarragona Harbour in November 2011 (temp: 20.5ºC; sal: 35.9). The elliptical cell was 24 µm long and 16 µm wide. The epicone was round and its length was less than one-third of the total cell length. The hypocone was elliptical and as wide as the epicone. The cingulum was wide and weakly impressed in ventral view. In dorsal view, its anterior margin was well defined. The cingulum was displaced less than once its width. The sulcus was straight and narrow, running from the antapex to the apex. The nucleus was centrally located, on the ventral side of the hypocone. The cell was colourless but contained numerous granules. It agreed with its original description (Campbell, 1973).

- *Gymnodinium instriatum* (Freudenthal & Lee) Coats (Fig. 3D)
This organism was widespread along the Catalan coast, producing high biomass blooms (>10^6 cells·L^{-1}) in several harbours and northern beaches, mainly during the warmer months (June–September). Two identical partial LSU rDNA sequences were obtained.

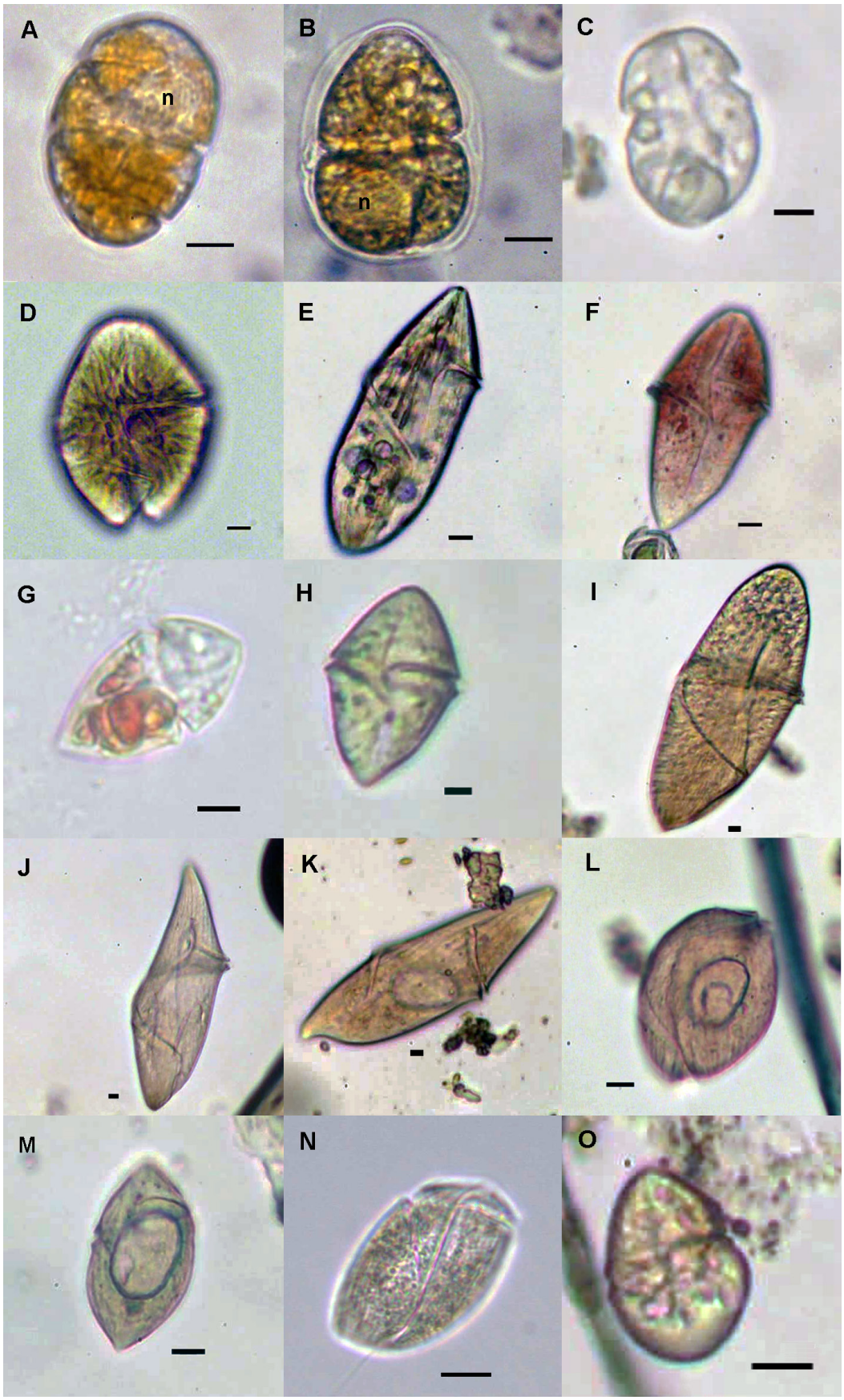

Figure 3: Light micrographs. Ventral view of A) *Cochlodinium* sp., B) '*Gymnodinium*' sp. C) *Gymnodinium agaricoides*, D) *Gymnodinium instriatum*, E) *Gyrodinium* cf. *britannicum*, F) *G. corallinum*, G) *G. dominans*, H) *G. heterogrammum*, I) *G.* cf. *ochraceum*, J), K) L) and M) *G.* cf. *spirale*, N) *G. viridescens*, O) *Gyrodinium* sp. 1. Nuclei (n) are indicated. Scale bars = 10 µm.

- *Gyrodinium* cf. *britannicum* Kofoid & Swezy (Fig. 3E)

A few specimens were detected in Barcelona Harbour in March 2012 (temp: 14.3ºC; sal: 38.4) and in Tarragona Harbour in June 2012 (temp: 23.8ºC; sal: 37.6). The greenish cells were 120–134 µm long and 45–52 µm wide. They were fusiform in shape, widest in the middle and tapering towards both apices, which were blunt. The highly vacuolated hypocone was larger than the epicone and the antapex less pointed. The descending cingulum began anteriorly and was slightly displaced. The sulcus ran from the apex to the antapex, widening in the cingular region. The nucleus was round and central. The cells had a striated surface, with bright elongated granules following the striae in the epicone. The specimens were tentatively identified based on available morphological descriptions (Kofoid and Swezy, 1921; Elbrächter, 1979)

- *Gyrodinium corallinum* Kofoid & Swezy (Fig. 3F)

Three specimens were detected in Barcelona Harbour in May 2012 (temp: 18.6ºC; sal: 38.4). The cells were 82–98 µm long and 44–53 µm wide, fusiform, and striated, but the apex was round. The epicone was shorter than the hypocone and the antapex was pointed. The pre-median cingulum descended more than four times its width and was overhanging. The sulcus ran from near the apex to the antapex. It was relatively wide in the epicone but narrowed through the cell. In the antapex it was almost not visible. The round nucleus was located centrally, in the junction between cingulum and sulcus. It was covered by a double membrane. The cell was pale red, with numerous coral-red droplets scattered mainly in the epicone. The posterior side of the hypocone was less coloured. They mostly agreed with its original description (Kofoid and Swezy 1921).

- *Gyrodinium dominans* Hulburt (Fig. 3G)

This species was obtained in February 2011 along the coast of Barcelona. It was also collected from other harbours along the Catalan coast, including recurrently in Tarragona Harbour throughout the year, although at low abundances.

- *Gyrodinium heterogrammum* Larsen (Fig. 3H)

This morphospecies was collected from many harbours but most commonly from Tarragona Harbour (during summer and autumn). The cells were 31-45 µm long and 21.5–31.5 µm wide and spindle-shaped, broader in their central part of the body than in their rounded apices. The epicone was equal in size or slightly shorter than the hypocone. The cingulum was descending, displaced by about twice its width and overhanging. The sulcus was narrow in the epicone, almost reaching the apex, and deflected in the intercingular region following the overhang. It widened in the hypocone, slightly displaced to the left, which caused an asymmetry in the hypocone, with the antapex pointed on its right side. Striations were observed in both the epicone and hypocone, although less abundant in the former. The nucleus was almost central, slightly displaced to the left. It was spherical in ventral view but elongated diagonally in lateral view. The cells were yellow-greenish, without chloroplasts. Some had a large ingestion body occupying almost the entire hypocone. Their morphology agreed with that of its original description (Larsen 1996). Three partial LSU rDNA sequences (99.9% similarity) and two partial SSU rDNA sequences (99.9% similarity) were obtained.

- *Gyrodinium* cf. *ochraceum* Kofoid & Swezy (Fig. 3I)

This morphospecies was isolated recurrently throughout the year from most harbours and beaches within the study area but always at cell abundances $<10^2$ cell·L^{-1}. The large, elongated, brownish cells were 97–133 µm long and 36–55.5 µm wide, with numerous thin striations. Granulation was usually observed in the epicone. The apex was round. The hypocone was round posteriorly but the antapex was pointed. The cingulum descended, with a severe displacement about one-third the cell length. The distal end formed a 45º angle with the sulcus, which deeply penetrated the epicone and descended straight to reach the hypocone, where it formed an excavation. The sulcus twisted towards the left. The large nucleus was spherical to subspherical and situated in the epicone. A long extension in the antapex, probably the feeding apparatus, was observed several times. The tentative identification was based on Kofoid and Swezy (1921) and Elbrächter (1979). Two partial LSU rDNA sequences were obtained (99.0% similarity).

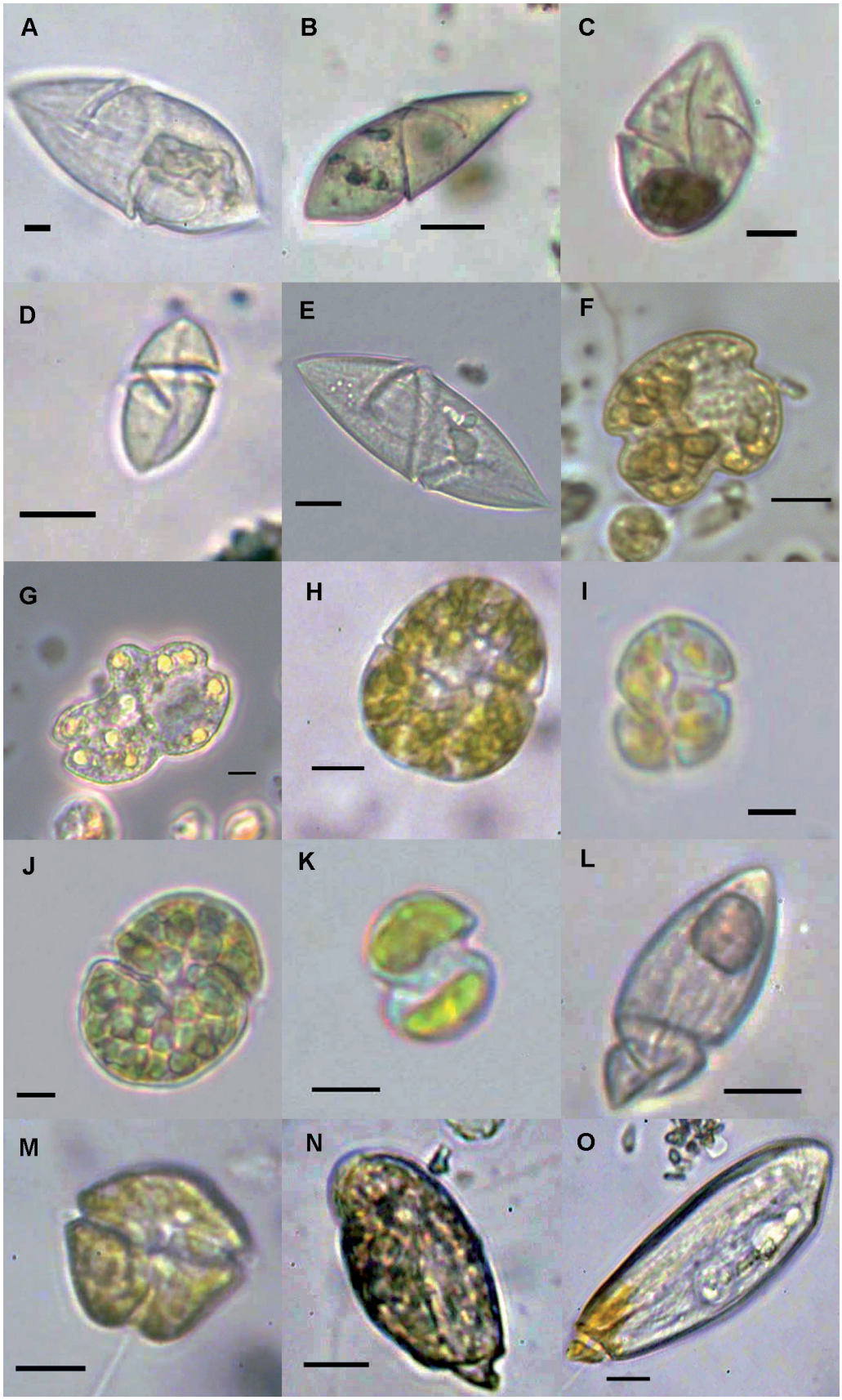

Figure 4: Light micrographs. Ventral views of A) *Gyrodinium* sp. 2, B) *Gyrodinium* sp. 3. C) *Gyrodinium* sp. 4, D) *Gyrodinium* sp. 5, E) *Gyrodinium* sp. 6, F) a fixed specimen of *Karenia mikimotoi*, G) a fixed specimen of *K.* cf. *papilionacea*, H) *K. umbella*, I) *Karlodinium armiger*, J) *K. decipiens* and K) *K. veneficum*, L) *Katodinium glaucum* and M) *Takayama tasmanica*, N) left lateral view of *Torodinium robustum*, O) right lateral view of *Torodinium teredo*. Scale bars = 10 µm.

- *Gyrodinium* cf. *spirale* (Bergh) Kofoid & Swezy (Fig. 3J, K, L, M)

This species was obtained from most harbours and beaches throughout the year but in abundances always $<10^3$ cell·L^{-1}. It showed a high morphological plasticity. The cells whose features agreed with those of *G. spirale* were 110–130 µm long and 20–30 µm wide. In other specimens the morphologies differed. In most cases, the cingular displacement and surface striations were maintained, but in others the cells were completely round or had a short and round hypocone or were spindle-shaped. Nonetheless, the phylogenetic data confirmed that all of them belonged to the same species. Up to 16 partial LSU rDNA sequences were obtained, but only 6 are shown in this study (99.1% similarity), as well as 5 partial SSU rDNA sequences. Four were identical and one slightly differed (99.9% similarity).

- *Gyrodinium viridescens* Kofoid & Swezy (Fig. 3N)

Several specimens were collected at Castelldefels in May 2012 (temp: 25.4ºC; sal: 38.4) and from along L'Estartit beach in July 2012 (temp: 22.2ºC; sal: 38.3) and June 2013 (temp: 17.1; sal: 37.1). The elongated and dorsoventrally flattened cells were colourless or slightly greenish, 35–50 µm in length, 24–29 µm in width, and featuring a striated surface. The epicone was small and blunt and the apex was slightly pointed. The cingulum turned left and its distal end joined the sulcus approximately in the middle of the cell, forming a 30–45º angle, but they were not connected. The sulcus ran the whole length of the cell, turning left as it reached the apex and running straight and turning right as it reached the antapex, where it widened. The hypocone was asymmetrical. Its left side was tilted toward the antapex while the right side ran straight until reaching it. The antapex was also asymmetrical, with the left side being smaller and rounder than the larger and usually pointed right side. The elongated nucleus was situated just below the cingulum. Large ingestion bodies located posteriorly and scattered refractive were seen in some specimens. Their morphology agreed with the original description (Kofoid and Swezy 1921). Two partial LSU rDNA sequences were obtained (99.3% similarity).

- *Gyrodinium* sp. 1 (Fig. 3O)

This species was detected only in samples from Tarragona Harbour obtained in October 2011 (temp: 21.1ºC; sal: 36.6). The greenish, vacuolated cells were 36.5–41.5 µm long and 27–30 µm wide, with a conical epicone and rounded apex. The hypocone was hemispherical and both wider and slightly longer than the epicone. The cingulum was wide and located medially, descending about a quarter of the cell length. The intercingular area was protuberant. The sulcus was narrow, running from the apex to the antapex. The round nucleus was situated in the epicone.

- *Gyrodinium* sp. 2 (Fig. 4A)

One specimen belonging to this morphospecies was obtained from Barcelona Harbour in December 2011 (temp: 16.5ºC; sal: 37.6). The colourless, fusiform cell was 112 µm long and 48 µm wide. The apex was pointed but the antapex was more rounded, with a pointed protuberance. The surfaces of the epicone and hypocone were striated, with 7–8 striae in the epicone on ventral view. The cingulum was deep, descending, highly displaced, and slightly overhanging. The distal end of the cingulum joined the sulcus perpendicularly. The sulcus, which ran in a straight line from the epicone to the antapex, was not very apparent. A ridge extending from the apex to the proximal end of the cingulum was clearly visible. The oval nucleus was situated on the right side of the hypocone.

- *Gyrodinium* sp. 3 (Fig. 4B)

One specimen was isolated from Tarragona Harbour in December 2011 (temp: 15.3ºC; sal: 37.2). The fusiform cell was 39.5 µm long, 13 µm wide, and had slender striations. The apex was pointed. The cingulum was descending, running from the epicone to almost the antapex, with the distal end joining the sulcus to form a 45º angle. The sulcus was hardly apparent and it widened near the antapex, slightly penetrating the dorsal side of the cell. It was slightly displaced to the left of the hypocone, such that it caused an asymmetry in the latter. The left side was round and the right side less developed and pointed. The nucleus was spherical, dorsal, and situated in the centre of the cell. The yellow-greenish cell lacked chloroplasts.

Table 3: List of morphospecies detected during this study of the Catalan coast. Whether they were sequenced and first detections in the Mediterranean Sea (Med) or along the Catalan coast (CC) are noted. Previous detections of organisms not identified at species level are unknown and are represented by grey boxes. Bloom-forming (B) and toxic (T) species are indicated. Numbers in the "Occurrence" column indicate the frequency of detections, ranging from very rare (*) to very common (****).

Species	Sequenced	First detection	Harmful	Occurrence
Akashiwo sanguinea	yes	-	B	****
Amphidinium carterae	yes	-	T	***
A. crassum	yes	-	-	***
A. cf. *operculatum*	-	-	T	*
Apicoporus sp.	yes	Med	-	*
Asterodinium gracile	-	-	-	*
Balechina coerulea	-	CC	-	*
Brachidinium sp.	-	-	-	*
Ceratoperidinium falcatum [a]	yes	-	-	*
C. margalefii [a]	yes	-	-	*
Cochlodinium cf. *convolutum* [a]	yes	-	-	**
C. polykrikoides [b]	yes	CC	T	**
Cochlodinium sp.	yes		-	*
cf. *Cochlodinium* sp. 1 [a]	yes		-	*
'*Gymnodinium*' sp. [a]	yes		-	*
Gymnodinium instriatum	yes	-	B	****
Gymnodinium agaricoides	yes	CC	-	*
G. cf. *britannicum*	yes	Med	-	*
G. corallinum	yes	Med	-	*
G. dominans	yes	-	-	***
G. heterogrammum	yes	Med	-	***
G. cf. *ochraceum* [c]	yes	CC	-	***
G. cf. *spirale*	yes	-	-	****
G. viridescens	yes	Med	-	**
Gyrodinium sp. 1	yes		-	*
Gyrodinium sp. 2	yes		-	*
Gyrodinium sp. 3	yes		-	*
Gyrodinium sp. 4	yes		-	*
Gyrodinium sp.5	yes		-	***
Gyrodinium sp.6	-		-	**
Karenia mikimotoi	yes	CC	T	**
K. umbella	yes	Med	T	*
K. cf. *papilionacea*	-	CC	T	*
Karlodinium armiger	yes	-	T/B	***
K. decipiens	yes	Med	-	**
K. veneficum	yes	-	T/B	***
Katodinium glaucum	yes	CC	-	**
Takayama tasmanica	yes	Med	-	**
Torodinium robustum	yes	-	-	**
Torodinium teredo	yes	-	-	**

[a] Their presence was previously reported by Reñé et al. (2013b).
[b] Its presence was previously reported by Reñé et al. (2013c).
[c] The closely related *G. contortum* was previously reported along the CC by Margalef (1995) and we cannot exclude that our specimens belong to that species.

- *Gyrodinium* sp. 4 (Fig. 4C)

Two specimens were collected from Tarragona Harbour in December 2011 (temp: 15.3ºC; sal: 37.2) and from Arenys Harbour in March 2012 (temp: 14.5ºC; sal: 36.8). The ovoid and spherical cells were 42.5-52.5 µm long and 29-31.5 µm wide. The epicone was conical and the apex flattened. The hypocone was slightly elongated and the antapex was round. The surface was thinly striated. The cingulum was median, descending, and highly displaced, with its distal end curved toward the antapex. The sulcus ran from the apex to the antapex. It was straight in the epicone, then turned slightly to the left to join the distal end of the sulcus, finally widening in the hypocone. The large nucleus was sub-spherical, located in the centre of the cell. The cells were colourless, but a large ingested body was visible in the hypocone of one of them. Two identical partial LSU rDNA sequences were obtained.

- *Gyrodinium* sp. 5 (Fig. 4D)

This small morphospecies was commonly obtained throughout the year from a few harbours and beaches. The colourless cells were 20–25 µm long, 10–15 µm wide, ovoid, and slightly dorsoventrally compressed. Slender striations were present in the epicone and hypocone. The epicone was about three times smaller than the ovoid, tapering hypocone and conical in shape. A knob was observed in the apex. The antapex was round. The cingulum was deeply impressed in its pre-median portion, descending, and its distal end joined the sulcus approximately in the center or the cell. A narrow sulcus ran from the apex to the antapex, where it widened. The nucleus was ellipsoidal, occupying the intercingular area. Ingestion bodies were commonly seen in the posterior part of the cell.

- *Gyrodinium* sp. 6 (Fig. 4E)

This morphospecies was obtained from the mouth of the La Muga River in May 2011 (temp: 20.2ºC; sal: 34.7) and from Tarragona Harbour in December 2012 (temp: 14.8ºC; sal: 38) but all attempts at sequencing were unsuccessful. The colourless cell was 60–70 µm long and 20–25 µm wide, fusiform, wider in its central part and marked with slender striations in the equally sized epicone and hypocone. Both apices were pointed, but the antapex was more elongated. The cingulum was displaced, beginning and ending at a distance from the apex and antapex that was about a quarter of the cell length. It had a slight overhang and the distal end joined the sulcus at an angle of 45º. The sulcus was narrow, running from near the apex to the antapex, with some curvature in the intercingular zone. The ellipsoid nucleus was situated on the left side of the cell, just below the proximal end of the cingulum.

- *Karenia mikimotoi* (Miyake & Kominami ex Oda) Hansen & Moestrup (Fig. 4F)

This species was isolated sporadically from beaches and harbours along the Catalan coast throughout the year at abundances < 10^2 cells·L^{-1}. Two identical partial LSU rDNA sequences were obtained.

- *Karenia* cf. *papilionacea* Haywood & Steidinger (Fig. 4G)

Two fixed specimens were obtained from La Fosca beach in June and August 2010. (temp: 21.0 and 24.0ºC; sal: 37.8 and 38.3, respectively). Live specimens were collected from Cambrils Harbour in January 2012 (temp: 11.9ºC; sal: 37.1) and offshore of Barcelona in June 2012, but sequencing was unsuccessful. Their tentative identification was based on Haywood et al. (2004). The fixed cells were 20–30 µm long, 30–40 µm wide, and dorsoventrally compressed. The epicone was flattened, with an apical process in the apex. The hypocone was bilobed. The cingulum was slightly displaced. The sulcus extended into the epicone and reached the antapex. The nucleus was round, situated on the left side of the hypocone. Many roundish chloroplasts were seen in the cell periphery.

- *Karenia umbella* de Salas, Bolch & Hallegraeff (Fig. 4H)

One cell was collected from Olímpic Harbour in October 2012 (temp: 21ºC; sal: 38.4). It was 40.5 µm long and 33 µm wide, almost round, with a hemispherical epicone. The antapex was slightly flattened. The cingulum was displaced about twice its width. The sulcus broadened into the hypocone. The nucleus was round, located centrally. Chloroplasts were situated at the cell periphery.

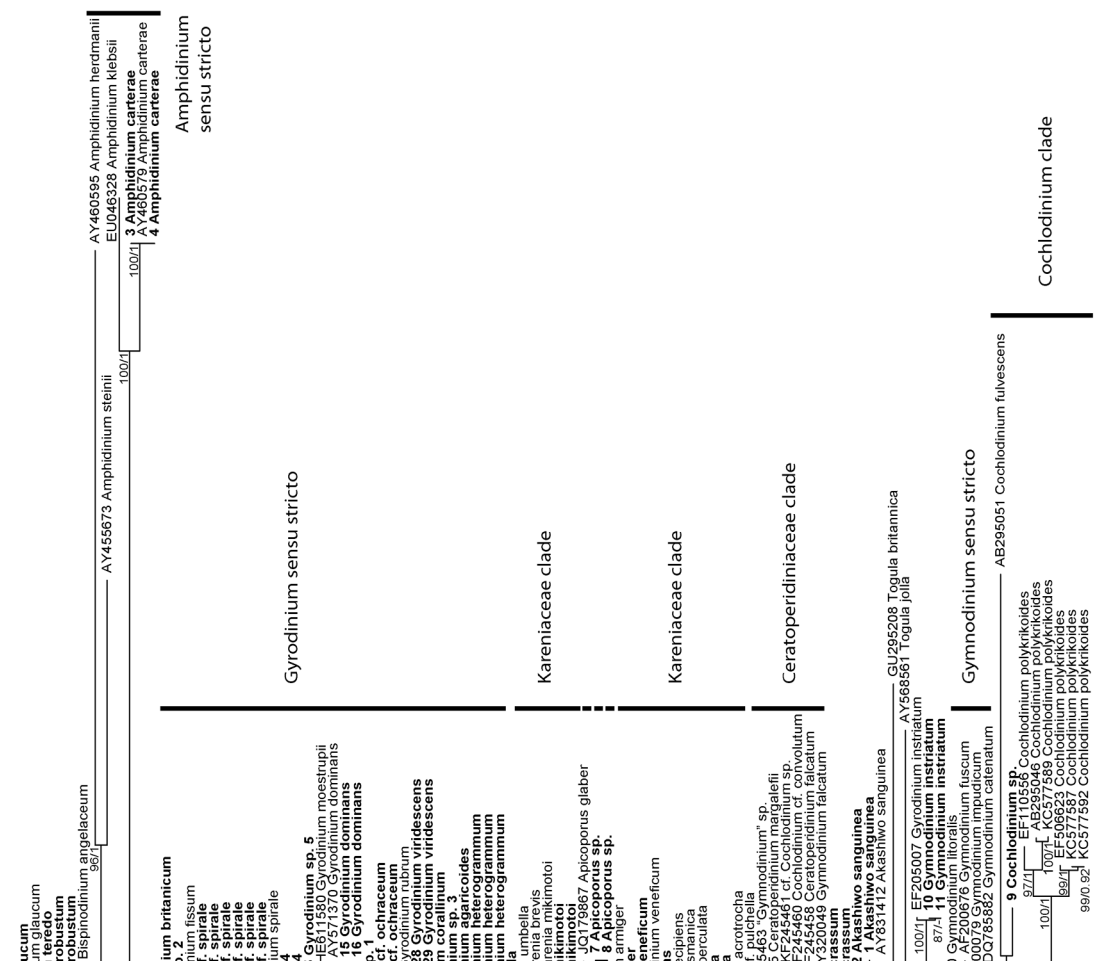

Figure 5: Maximum-likelihood phylogenetic tree of selected species based on the D1–D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap (%) and the Bayesian posterior probability (BPP) values. Only BPP values >0.7 and bootstrap values >50 are shown. *Perkinsus marinus* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

- *Karlodinium armiger* Bergholtz, Daugbjerg & Moestrup (Fig. 4I)
This mixotrophic organism was reported from Alfacs Bay together with *K. veneficum* and mainly during the winter months (Garcés et al., 2006). The studied specimens were obtained along the coast of Barcelona in February 2011.

- *Karlodinium decipiens* de Salas & Laza-Martínez (Fig. 4J)
This species occurred in low abundances along L'Estartit beach in May 2011 (temp: 20.2ºC; sal: 37.3). The oval and slightly dorsoventrally compressed cells were 28 µm long, 22 µm wide, and densely pigmented. The epicone and hypocone were round and almost equal in length. The cingulum was wide and largely displaced. The sulcus widened into the hypocone. The nucleus was large, central, and oval. They agreed with its original description (de Salas et al. 2008).

- *Karlodinium veneficum* (Ballantine) Larsen (Fig. 4K)
This species was detected at low densities at several beaches off the northern Catalan coast, although high densities during the winter in Alfacs Bay were reported (Garcés et al., 2006).

- *Katodinium glaucum* (Lebour) Loeblich III (Fig. 4L)
This species was detected in several harbours and recurrently in Tarragona Harbour throughout the year at cell abundances $<10^2$ cell·L^{-1}.

- *Takayama tasmanica* de Salas, Bolch & Hallegraeff (Fig. 4M)
One specimen belonging to this species was obtained in July 2012 from Llavaneres beach (temp: 23.4ºC; sal: 38.1) and from Fangar Bay in October 2012 at abundances of 10^3 cell·L^{-1}. The cells were pentagonal in shape, slightly dorsoventrally compressed, 20.5–27 µm long, and 20–28 µm wide. The epicone was hemispherical and the hypocone was truncated. The cingulum was wide and displaced by about twice its width. The sulcus ran from the intercingular region to the antapex, widening in the hypocone. A short intrusion into the epicone was observed, forming a 45º angle with the rest of the sulcus. The apical groove ran from below and to the right of the sulcal intrusion. The nucleus was situated in the epicone but its shape could not be unequivocally determined. As many key features, e.g., the presence of a ventral pore, the acrobase outline, the shape of the nucleus, and the presence a pyrenoid, could not be observed, these cells were identified at the species level by their partial LSU rDNA sequences.

- *Torodinium robustum* Kofoid & Swezy (Fig. 4N)
Cells belonging to this species were detected along beaches of the Catalan coast from spring to autumn, but always at low abundances. The green-brownish cells were 41.4–51.2 µm long and 17.6–21.6 µm wide and agreed with its original description (Kofoid and Swezy 1921). Two identical partial LSU rDNA sequences and two identical partial SSU rDNA sequences were obtained.

- *Torodinium teredo* (Pouchet) Kofoid & Swezy (Fig. 4O)
Cells of this species were collected from beaches along the Catalan coast from spring to autumn, but always at low abundances. The elongated nucleus was situated in the central part of the cell and a large pusule ran straight through the right side of the epicone. The elongated cells were colourless, except at the yellow-greenish ends of the epicone and hypocone. Although they matched the descriptions of *T. teredo* (Kofoid and Swezy, 1921; Gómez, 2009), our specimens were shorter (59–76.5 µm in length and 20–24 µm in width) than their commonly reported dimensions (100–130 µm long).

3.2 Phylogenetic analyses: The unarmoured dinoflagellates are not a monophyletic group. Consequently, the Gymnodiniales order is not supported phylogenetically by the LSU rDNA region, as clearly demonstrated by the phylogenetic tree (Fig. 5). Besides the well characterized Gymnodiniales *sensu stricto* clade, whose members were not

included in this study (see Chapter I), several groups or genera form well-supported clades. All *Gyrodinium* species (excluding the *Gymnodinium instriatum* / *G.uncatenum* / *Gyrodinium dorsum* clade) cluster together (65% bootstrap / 0.73 BPP). Species were split in two main branches. The first contained *G.* cf. *britannicum*, *Gyrodinium* sp. 2, and the group of specimens identified as *G.* cf. *spirale*, which clustered with the *G. fissum* sequence from GenBank. The second contained the remaining sequences of *Gyrodinium* species. *Gyrodinium* sp. 4 and *G. spirale* clustered together (79% / 0.97). A monophyletic clade was obtained for *Gyrodinium* sp. 5, *G. moestrupii*, and *G. dominans* (100% / 1). The remaining sequences did not show any supported relationship, except *Gyrodinium* sp. 3, *G. heterogrammum*, and *Gymnodinium agaricoides*, which had identical LSU rDNA sequences. Members of the Kareniaceae family (*Karenia*, *Karlodinium*, and *Takayama*) clustered together, although good support was obtained only for branches representing some of the genera. All sequences of species belonging to this family were almost identical to those previously available in GenBank. The *Apicoporus* genus also clustered within this clade, although with no support. The sequence of *Apicoporus* sp. clustered with *A. glaber* (98% / 1). The so-called *Amphidinium sensu stricto* clade was also obtained (95% / 1), including the sequences of *A. carterae* determined in this study. However, the sequence of *A. crassum* clustered independently. Another clade with high support comprised *Ceratoperidinium* and related species (Reñé et al., 2013b), as well as some *Cochlodinium* species (94% / 1). *C. polykrikoides*, *C. fulvescens*, and *Cochlodinium*

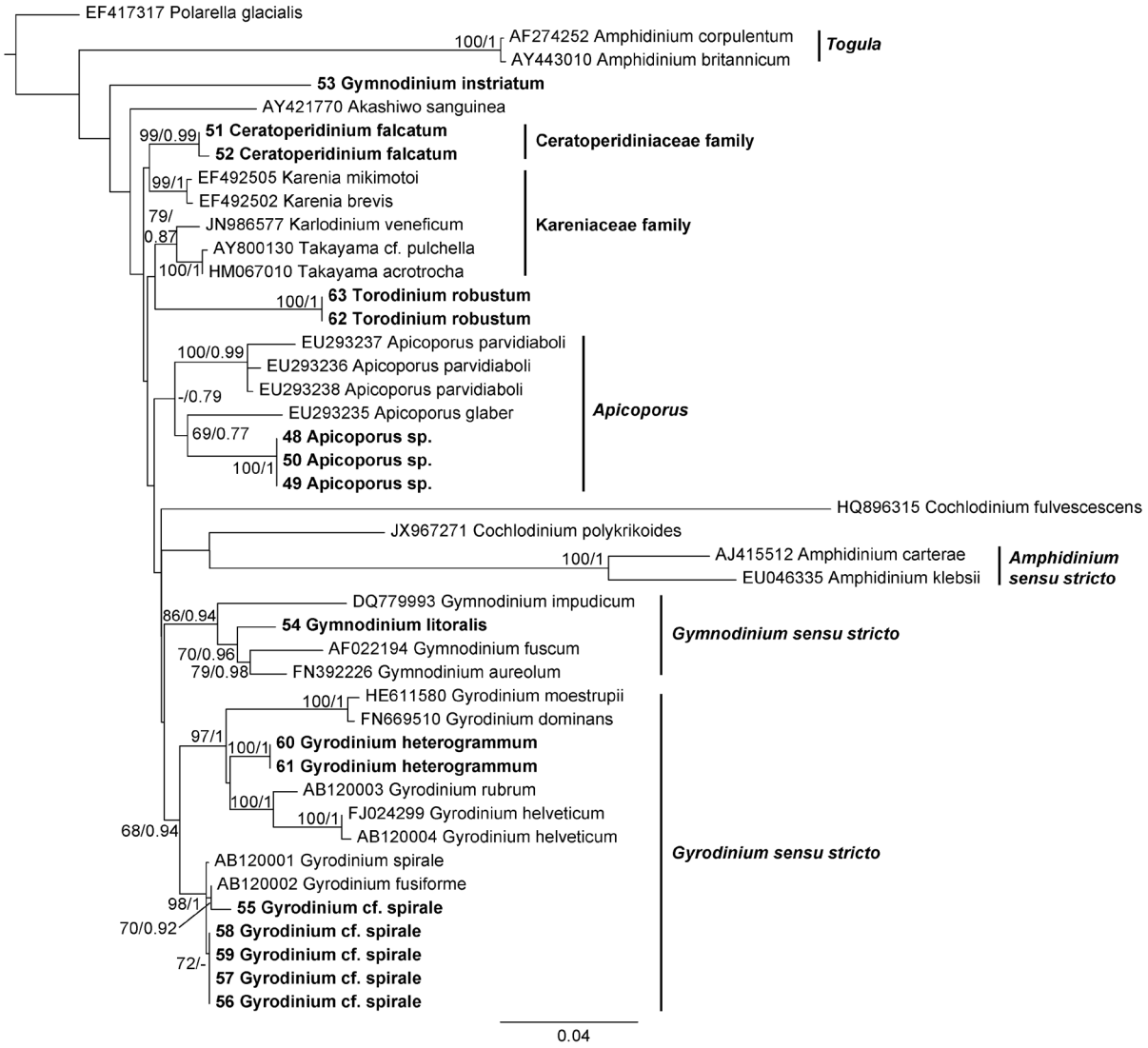

Figure 6: Maximum-likelihood phylogenetic tree of selected species based on the partial SSU rRNA. Numbers on the nodes are the bootstrap (%) and the Bayesian posterior probability (BPP) values. Only BPP values >0.7 and bootstrap values >60 are shown. *Polarella glacialis* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

sp. clustered together (98% / 1) and independently of previously obtained *Cochlodinium* sequences. Finally, the sequences of *Torodinium* specimens and *K. glaucum* clustered together, although with low support (59% / 0.76). The clade containing both *Torodinium* species was highly supported (100% / 1). The sequences obtained for *A. sanguinea* and *G. instriatum* were not phylogenetically related to any other unarmoured dinoflagellates sequenced to date.

Some partial SSU rDNA sequences were also obtained in order to contrast them with the corresponding LSU rDNA sequences (Fig. 6). Several organisms identified as *G. spirale* were sequenced. Those sequences clustered with the available sequences of *G. spirale* and *G. fusiforme* (98% / 1), which are almost identical. The SSU rDNA sequences of *Apicoporus* sp. confirmed that they clustered with *A. glaber* (69% / 0.77) and independently of *A. parvidiaboli*. Finally, some specimens of *Torodinium robustum* were also sequenced. They clustered independently of any other unarmoured organism. Unfortunately, *T. teredo* specimens could not be sequenced, impeding characterization of the phylogenetic relationship with *T. robustum*. Similarly, the SSU rDNA sequence of *Katodinium glaucum* is not available and the relationship determined with the genus *Torodinium* based on their LSU rDNA sequences could therefore not be confirmed.

4. Discussion

The combination of morphological observations with the phylogenetic information of the studied specimens allowed their unequivocal identification at least at the genus level. However, the limited morphological observations and the similarity of several unarmoured species prevented species-level determinations for some organisms. Furthermore, the scarcity of genetic information available in the databases for some organisms limited the interpretation of phylogenetic data obtained from their partial LSU rDNA sequences.

4.1 Gyrodinium sensu stricto group

Fourteen different *Gyrodinium* species were distinguished during this study, 13 of which were successfully sequenced. At the time of this study, GenBank contained only five LSU rDNA sequences corresponding to identified *Gyrodinium* species belonging to the *sensu stricto* clade. In addition, our morphological observations were limited, because many morphospecies were only detected once, impeding their species-level identification. This problem was further compounded by the lack of sequences in the databases.

The most commonly detected morphospecies was initially identified as *G. spirale*. Although those specimens were highly variable morphologically, they were confirmed to be the same species. Surprisingly, their partial LSU rDNA sequences were identical to that of *G. fissum* from GenBank and only 86% similar to that of *G. spirale*. No morphological description or image was provided in the report of the sequenced representative of *G. fissum* (Kim and Kim, 2007), but our specimens did not match the morphological descriptions available (Kofoid and Swezy, 1921; Lebour 1925). In that study, the sequence was obtained by applying single-cell PCR to one specimen, and the observation of an anomalous morphology of *G.* cf. *spirale* cannot be ruled out. Another possibility is that the discrepancy is related to the fact that, as previously discussed, the LSU rDNA region does not have enough resolution to discriminate between the two species. Regarding the *G. spirale* specimen used to obtain the GenBank sequence (Hansen and Daugbjerg, 2004), we found no remarkable morphological differences suggesting that our specimens did not belong to the same species, based on the differences in the LSU sequences. The partial SSU sequence of our specimens showed a 99.9% similarity with the available sequence of *G. fusiforme* and *G. spirale*. The sequenced species could be differentiated only by their size (Takano and Horiguchi, 2004), but our measurements overlap with the dimensions of both species. Given the molecular data obtained and the difficulties in distinguishing those close species, we propose that we are dealing with cryptic species, as previously discussed and suggested by Elbrächter (1979). Therefore, the wide distribution of *G. spirale* must be reconsidered and it highlights the need to re-investigate that group of species.

We also obtained a sub-clade (100% / 1) containing three different morphotypes (*G. heterogrammum*, *Gyrodinium* sp. 3, and *Gymnodinium agaricoides*) with almost identical partial LSU rDNA sequences. However, here we reject the

possibility that they belong to the same species as they were morphologically very different. Therefore, in this case the LSU fragment proved to be useless in discriminating these species. As previously noted, a morphospecies identified as *Gymnodinium agaricoides* was successfully sequenced, placing it within the *Gyrodinium sensu stricto* group. It lacked some of the key characters of the genus (surface striation, acrobase shape) and a detailed morphological study is still needed, but its phylogenetic position suggests that it was incorrectly assigned to the genus *Gymnodinium* and actually belongs to the genus *Gyrodinium*. The features of *G. heterogrammum* specimens agreed well with the original description (Larsen, 1996). To the best of our knowledge, this species was only detected once before, in the waters of Australia (Larsen, 1996). Thus, the sequences obtained in this study are the first available for the species.

The *G. corallinum* specimens observed in this study slightly differed from the original description (Kofoid and Swezy, 1921) thereof in that the apex was less pointed and the cingulum was overhanging. Those features were similar to *Gyrodinium rubrum* (Kofoid & Swezy) Takano & Horiguchi, but our specimens shared with *G. corallinum* its typical red pigments and the more pointed apices than those of *G. rubrum* (Kofoid and Swezy, 1921). Also, the sides of the hypocone were not concave and the nucleus was round in ventral view. *Gyrodinium rubrum* was previously sequenced and the sequence obtained in this study had a 94.2% similarity with that one, which rules out the possibility that our specimens belong to that species (Takano and Horiguchi, 2004). A high plasticity was observed for *G. rubrum* (Takano and Horiguchi, 2004), while *G. corallinum* was described from a few specimens. To the best of our knowledge, the latter has only been detected in its type location (Kofoid and Swezy, 1921), in the Russian Arctic (Okolodkov 1998), and in Skagerrak waters (Gollasch et al., 2009).

The specimens we identified as *G.* cf. *britannicum* agreed with available morphological descriptions (Kofoid and Swezy, 1921; Elbrächter, 1979) but the typical carmine-coloured granules following the striae were lacking; however, colourless cells were described in an early study of that species (Lebour, 1925). The specimens identified as *G.* cf. *ochraceum* differed from the original description in the absence of a pointed apex, the presence of an antapical loop of the sulcus, and with respect to colour (Kofoid and Swezy, 1921). Although some variability in those characters was reported (Elbrächter, 1979), this was not the case for colouration. The morphology of our specimens was compared with that of the closely related *G. contortum* (Schütt) Kofoid & Swezy. However, whereas the shape and the coloration of the cells agreed, other characters, such as the distance between the apex and the anterior end of the cingulum, the shape and position of the nucleus and the shape of the sulcus were clearly different. Therefore, we stand by the identifications of our specimens as *G.* cf. *ochraceum*. This species was previously reported in central areas of the Mediterranean Sea but never along the Catalan coast (Innamorati et al., 1989; Schiller, 1933). Since *G. contortum* had been reported in many areas, including the Catalan coast (Gómez, 2003), we cannot exclude the possibility that our specimens and those previously identified as *G. contortum* from the literature were actually the same species. Finally, the detection of *G. viridescens* is the first in the Mediterranean Sea and the sequence of this species was successfully obtained, likewise for the first time.

4.2 Karenia / Karlodinium / Takayama group

Three *Karenia* species were identified during this study but they were always detected at low abundances. The most frequently detected species was *K. mikimotoi*. Its presence has been reported in several Mediterranean sites, such as the Tyrrhenian Sea (Zingone et al., 2006) and the Aegean Sea (Ignatiades and Gotsis-Skretas, 2010), but never along the Catalan coast. Some of the other observed specimens were morphologically similar to *K. papilionacea*, but all sequencing attempts failed. This species was previously detected in the NW Mediterranean (Puigserver et al., 2010; Zingone et al., 2006) but never along the Catalan coast. Finally, we obtained a sequence with 99.7% similarity to that of the GenBank sequence of *K. umbella*. However, our morphological observations did not allow an unequivocal identification. To the best of our knowledge, prior to our study *K. umbella* had only been detected in Australian, Tasmanian, and New Zealand waters (de Salas et al., 2004; Guiry and Guiry, 2013). All detected species are toxic. Therefore, despite the low cell abundances, the detection of these species is of great importance as they are responsible for serious health problems in humans and negative effects on aquaculture industry.

The taxonomy of the genus *Karenia* has been a recent issue of discussion. The genera *Asterodinium*, *Brachidinium*, and *Microceratium* were formerly included within the order Brachidiniales. Morphological variations are common for the respective species and they were transferred into the Gymnodiniales because of the similarities between their morphology and those of *Karenia* species (Gómez, 2006, 2011; Gómez et al., 2005). Henrichs et al. (2011) studied the phylogeny of *Brachidinium capitatum* and placed that species within the *Karenia* genus, confirming that it belongs to the Gymnodiniales order. The phylogenies of *Asterodinium* and *Microceratium* have not been obtained yet, but we assume that they are phylogenetically closely related to *B. capitatum*. Although not sequenced, the observed *Asterodinium gracile* specimens were unequivocally identified. That genus has already been detected in the NW Mediterranean (Gómez, 2003) and along Catalan shores (Estrada, 1979) although its rarity explains the scarcity of the detections. A fixed specimen of the genus *Brachidinium* was also observed but its sequence could not be obtained.

Three different *Karlodinium* species from the Catalan coast were detected and sequenced. They include *Karlodinium decipiens*, which previously had only been detected in the temperate to subpolar Southern Ocean (from coastal Tasmania southward to the north polar front) and in the temperate western European Atlantic waters of Spain (de Salas et al., 2008). Consequently, ours is the first report of *K. decipiens* in the Mediterranean Sea. Two other species belonging to the *Karlodinium* genus and detected in the Mediterranean, i.e., *K. armiger* and *K. veneficum*, have been well studied because both are considered toxic. Importantly, they produced high biomass blooms in several locations of the Catalan coast (Bergholtz et al., 2006; Garcés et al., 2006), killing off fishes and damaging the regional aquaculture industry (Fernández-Tejedor et al., 2003). However, other species previously reported in the Mediterranean Sea, such as *K. ballantinum* (Siano et al., 2009), were not detected during our study, suggesting that additional *Karlodinium* species might be present along Catalan shores.

Several specimens attributed to the genus *Takayama* were detected during the study, but several of the key features needed to identify them at the species level could not be observed in detail. However, sequences with a 99.5% and 99.3% similarity to *T. tasmanica* and *T. tuberculata*, respectively, were obtained. The two species are easily distinguishable by their size, the presence of irregularities on the cell surface, and the shape of the sulcal intrusion (de Salas et al., 2008). Accordingly, the studied specimens were identified as *T. tasmanica*. This is the first report of this species in the Mediterranean Sea, as to the best of our knowledge its presence was known only in New Zealand (Guiry and Guiry, 2013), Tasmania (de Salas et al., 2003), and North America (Haywood et al., 2007). *T. pulchella* was previously reported along the Catalan coast at abundances of $<10^4$ cells·L^{-1} during 1998–1999 in a location close to Fangar Bay (Delgado et al., 1999; Vila et al., 2001). But as *T. pulchella* was not detected during the course of this study, we cannot exclude the option that those proliferations were actually caused by *T. tasmanica*.

4.3 Amphidinium sensu stricto group

Few *Amphidinium* species were observed during this study, as they are common in benthic and sand-dwelling but not in planktonic communities. We mainly observed them during summer, in areas where macroalgae occur. Therefore, the diversity of *Amphidinium* species along the Catalan coast is no doubt higher than determined in this study, which did not focus on the benthos. *A. carterae* is seldom detected in planktonic samples. It is known to harbour cryptic species (Murray et al., 2012) and our phylogenetic analyses placed it in clade 2 (Murray et al., 2004), which contains strains from the Mediterranean but also from Mexico, Brazil, Australia, and New Zealand. Some of the strains of this clade are toxic (Houdai et al., 2001). A specimen morphologically similar to *A. operculatum* was detected in fixed samples. However, key features, such as the lack of a pyrenoid, the shape of the plastids, and the presence of a stigma just above the nucleus, could not be observed and its LSU sequence was not obtained. This species is difficult to distinguish from other, similar species like *A. massartii* and *A. trulla* (Murray et al., 2004). It was previously reported along the Catalan coast (Palau et al., 1991) but no morphological description was provided. Since it is a toxic species, confirmation of its presence along the Catalan coast would be of great interest.

A. crassum occurs commonly and has a wide distribution. Its distinctive features facilitate its identification but its phylogeny had not been studied. Based on the partial LSU rDNA sequence obtained as part of this work, *A. crassum* cannot be placed within the *Amphidinium* "sensu stricto" clade. This is also the case for species such as *A. semilunatum*, *A. latum*, and *A. poecilochroum*, which, as pointed out by Flø Jørgensen et al. (2004a), could represent new genera.

4.5 Other Gymnodiniales genera

Apicoporus sp. specimens were obtained from sediment samples. Two different *Apicoporus* species have been described. *A. parvidiaboli* Sparmann, Leander & Hoppenrath was observed only in British Columbia, Canada (Sparmann et al., 2008), and *A. glaber* (Hoppenrath & Okolodkov) Sparmann, Leander & Hoppenrath only from far northern and polar areas (Hoppenrath and Okolodkov 2000) and Kuwait's intertidal sand flats (Saburova et al., 2009). Therefore, we are the first to report the presence of any member of this genus in the Mediterranean Sea. In the studied specimens, the non-parallel sides of the hypocone and the asymmetry of the antapex were in concordance with the respective features of *A. parvidiaboli*. However, posterior horns in the hypocone were absent and some specimens resembled *A. glaber.* The LSU rDNA sequences obtained in this study differed from the available for *A. glaber* (sequences for *A. parvidiaboli* are not available). The SSU rDNA sequences clustered independently of *A. parvidiaboli*, forming a cluster with *A. glaber*. Thus, the specimens observed probably represent a new species.

Several organisms within the *Ceratoperidiniaceae* clade were observed. As discussed by Reñé et al. (2013b), the species were morphologically distant, including *C. margalefii*, *C. falcatum*, *Cochlodinium* cf. *convolutum*, cf. *Cochlodinium* sp. 1, and a *Gymnodinium*-like species. In contrast to the more commonly detected *C. falcatum*, the other organisms were rarely detected and always at very low abundances.

The toxic species *Cochlodinium polykrikoides* was isolated several times from harbour samples, with maximum abundances of 10^4 cells·L^{-1} (Reñé et al., 2013c). These are the first reports of this species along the Catalan coast. Moreover, two different ribotypes were detected, one comprising only specimens from the Catalan coast and referred to as group II, following Reñé et al. (2013c), and the other included within what was formerly called the Philippine ribotype, now renamed as group IV (Reñé et al., 2013c). Another, unidentified *Cochlodinium* species was detected and sequenced; it clustered with *C. polykrikoides* and *C. fulvescens*.

Akashiwo sanguinea is a cosmopolitan species easily recognizable by its morphological features, but its LSU rDNA sequences show intraspecific variability. It is not phylogenetically related to any other known species. *Gymnodinium instriatum* has been detected recurrently along the Catalan coast. *G. instriatum*, *G. uncatenum*, and *Gyrodinium dorsum* have almost identical SSU and LSU sequences (Saldarriaga et al., 2004) and form a clade distinct from *Gyrodinium* and *Gymnodinium sensu stricto* species. A new genus containing the three species should be erected, as already pointed out in the literature (Kim and Kim, 2007). The sequences obtained for *G. instriatum* and those from GenBank showed a certain degree of intraspecific variability. However, morphological discrimination between *G. instriatum* and *G. uncatenum* is problematic and hinders a clarification of their taxonomy, distribution, and ecology.

Katodinium glaucum is a widespread cosmopolitan species commonly found in estuarine waters. It forms high biomass blooms and has been detected sporadically along the Catalan coast. This species is included within the *Katodinium* genus, an artificial genus previously named *Massartia* Conrad, comprising marine, brackish, and freshwater species characterized by a maximum hypocone length that is one-third the total length of the cell (Conrad, 1926). Based on this criterion, several unrelated species have been assigned to this genus but some were later assigned to other genera. For example, Calado (2011) transferred some of them to *Opisthoaulax* Calado and Murray et al. (2007) transferred *K. dorsalisulcum* to the genus *Gymnodinium*. Nonetheless, *Katodinium* remains an artificial genus (Calado, 2011). As previously suggested by Kim and Kim (2007), some taxonomically related *Katodinium* species should be sequenced in order to study their relationship and phylogeny. The sequence obtained during this study agrees with the one available from GenBank.

Torodinium specimens were detected several times during this study. This genus is commonly detected worldwide, including along the Catalan Coast (Margalef, 1969), but its sequence had not been previously obtained. The LSU rDNA sequences of *Torodinium* cluster with those of *Katodinium glaucum*, although with low support. According to the SSU rDNA sequences obtained, the genus *Torodinium* clusters independently of any other unarmoured organism, although this conclusion is qualified by the current unavailability of *Katodinium glaucum* sequences. Both genera are characterized by the post-median position of the cingulum and a common ancestor seems probable. We observed two different *Torodinium* morphotypes; their occurrence was also reflected in the LSU rDNA sequences obtained. The specimens identified as *T. teredo* agreed with the morphological descriptions available in the literature but they were much shorter. *T. teredo* had been reported once along the Catalan coast, although neither an image nor morphological characteristics were provided (Palau et al., 1991). The authors also reported the presence of *T. robustum* and of a third morphotype (*Torodinium* sp.).

Finally, a specimen of *Balechina coerulea* was observed for the first time in the Catalan coast. The members of this genus are characterized by having a rigid amphiesma, in contrast to *Gymnodinium* members. *Balechina coerulea* has been reported in other Mediterranean locations (Gómez, 2003) and also in the Atlantic and Pacific Ocean (Licea et al. 2004; Maciel-Baltazar and Hernández-Becerril, 2013; Su-Myat et al. 2012). However, this genus is poorly known and the validity of the transfer of this species, previously included within the genus *Gymnodinium*, to the genus *Balechina* is doubious (Gómez, 2007). Furthermore, there is not molecular information available for any of its species and thus, their phylogenetic position remains unknown.

4.6 Species distribution

A large number of species detected during this study were previously reported from other areas of the NW Mediterranean Sea. Those observations confirm the heterogeneity of dinoflagellate species along the Catalan coast and that this study site is representative in determinations of the diversity of unarmoured dinoflagellates from the area as a whole. Furthermore, in this study, eight species were detected for the first time in the Mediterranean Sea. They are probably not exclusive to the Catalan coast, but they have yet to be detected in other locations of the Mediterranean, evidence of a lack of detailed studies on unarmoured dinoflagellates. A few of the species were previously detected in Australian, Tasmanian, and New Zealand waters, i.e., *Karenia*, *Takayama*, and *Karlodinium* species, and some Ceratoperidiniaceae members. The latitudes of those locations in the southern hemisphere are close to those of the Catalan coast and NW Mediterranean Sea (35–45º), suggesting that these species, like most dinoflagellates, occur within similar climatic zones in the two hemispheres, a phenomenon that has been termed "modified latitudinal cosmopolism" (Taylor et al., 2008).

5. Conclusions

- In this study, we identified 40 different species belonging to the Gymnodiniales *sensu lato* order and present along the Catalan coast. Eight of the species were reported for the first time in the Mediterranean Sea and seven of them in Catalan waters, confirming that the total diversity of unarmoured species present in the studied area was only partially known.

- If we include the 18 detected species belonging to Gymnodiniales *sensu stricto* (presented in Chapter I), then 58 different Gymnodiniales species were detected as part of this thesis research. Comparing our results with those from Velásquez (1997) and Gómez (2003), the species described in this study account for 30% of the Gymnodiniales species ever detected in the Mediterranean Sea and 85% of the species detected in the NW Mediterranean Sea.

- Some of the detected species probably represent new, yet to be described species, e.g., *Apicoporus* sp. and *Cochlodinium* sp.

- Partial LSU rDNA sequences were obtained for the first time for 16 species, most of them belonging to the *Gyrodinium* genus. They will allow comparisons with further detections of morphotypes not easily identifiable based solely on morphological characteristics.

- Some of the sequences obtained for *Gyrodinium* species point to the existence of cryptic species, including the type species of the genus. Consequently, further studies are needed to clarify this issue.

- Partial LSU rDNA sequences cannot be used to discriminate among some of the different *Gyrodinium* morphospecies. In those cases, the use of other markers may better show whether this limitation is restricted to the LSU regions or also includes SSU rDNA and other regions.

- Many unarmoured dinoflagellate species are toxic or bloom-forming. We reported three toxic species never previously detected along the Catalan coast. Detailed studies on the distribution of toxic and noxious unarmoured species in the Mediterranean Sea will improve their detection and control and thereby avoid possible economic losses to the aquaculture industry.

- Given that we mainly sampled coastal locations, thus largely excluding benthic communities and those of brackish and offshore waters, the number of species present in the Catalan coast is almost certainly much larger than currently estimated.

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L'Estartit beach (Catalan coast)

Chapter 3

"*Gymnodinium litoralis* sp. nov. (Dinophyceae), a newly identified bloom-forming dinoflagellate from the NW Mediterranean Sea"

Harmful Algae (2011)

GYMNODINIUM LITORALIS SP. NOV. (DINOPHYCEAE), A NEWLY IDENTIFIED BLOOM-FORMING DINOFLAGELLATE FROM THE NW MEDITERRANEAN SEA

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Abstract

Recurrent high-biomass blooms of a gymnodinioid species have been periodically recorded at different sites in the NW Mediterranean Sea (Catalan and Sardinian coast), causing intense discolorations of the water. In this study, several strains of the causative organism were isolated and subsequently studied with respect to the morphology of the vegetative cells and different life cycle stages, pigments profile, and molecular phylogeny. Based on phylogenetic analyses, the strains were placed within the *Gymnodinium sensu stricto* clade. The species possessed a horseshoe-shaped apical groove running anticlockwise around the apex and the major accessory pigment was identified as peridinin. These characteristics place the organism within the *Gymnodinium* genus, as defined today, although some other characteristics, such as vesicular chambers in the nuclear envelope and a nuclear fibrous connective were not observed. Morphologically, the isolates highly resemble *Gyrodinium vorax* (Biecheler) but major differences with the latter suggest that they comprise a new species, *Gymnodinium litoralis* sp. nov. The resting cyst of this species is described herein from field samples of the Catalan and Sardinian coast; pellicle cysts were observed in field samples and also in cultures. This species recurrently produces high biomass blooms (>10^6 cell L^{-1}) in summer along several beaches and coastal lagoons in the NW Mediterranean Sea (L'Estartit, La Muga River mouth, and Corru S'Ittiri). Knowledge about its geographic distribution is limited, since the precise identification of *G. litoralis* from the field or fixed samples can be difficult. Therefore we expect that molecular studies will reveal a much wider distribution of the species.

1. Introduction:

Harmful algal blooms (HABs) are caused by organisms that either produce toxins, with adverse effects on the ecosystem and humans (through the ingestion of toxin-contaminated food), or generate high biomass proliferations that alter the habitat (water discoloration, anoxia, foam, and mucilage scum production) and thus impede commercial and recreational activities in the affected area (Zingone and Enevoldsen, 2000). In the Mediterranean Sea, small-scale localized proliferations of diverse microalgae are frequently observed in the nearshore coastal waters. They may recur annually or emerge in a seemingly random manner and some of these proliferations result in the formation of HABs. Moreover, along the coast, HABs occur with greater frequency and with higher cell abundances than in offshore Mediterranean waters (Garcés and Camp, 2011). For example, discolorations of Greek coastal waters by high abundances of *Noctiluca scintillans*, *Gymnodinium* sp., and *Alexandrium insuetum* have been reported (Nikolaidis et al., 2005) ; there have been anomalous discolorations caused by high-biomass proliferations of *Alexandrium balechii* and *Prorocentrum triestinum* in the Campania region of Italy (Zingone et al., 2006); blooms of *Alexandrium taylori* frequently produce intensive water discolorations along the coasts of Sicily, Sardinia, Catalonia, and the Balearic islands (Basterretxea et al., 2005; Garcés et al., 1999, 2002; Giacobbe et al., 2007; Satta et al., 2010b).

High abundances (>10^6 cells L^{-1}) of gymnodinioid organisms have been recorded in the NW Mediterranean area. These organisms lack a theca and are therefore referred to as “unarmored” or “naked” dinoflagellates. Previously, the taxonomy of the gymnodinioids was based on several morphological characteristics, but those used to distinguish the different genera were clearly problematic. Improved observations resulting in the identification of additional morphological aspects, such as the shape of the apical groove, led to the proposal of new characteristics with which to distinguish dinoflagellate genera (Biecheler, 1934; Takayama, 1985). Thus, a combination of morphological and ultrastructural features along with the phylogeny of the organisms resulted in a redefinition of the genera *Gymnodinium* and *Gyrodinium*, and the establishment of the genera *Akashiwo*, *Karenia*, and *Karlodinium* (Daugbjerg et al., 2000). Since then, numerous studies have focused on revising the identification of certain species (Bergholtz et al., 2006; Hansen et al., 2007; Jorgensen et al., 2004; Kremp et al., 2005; Takano and Horiguchi, 2004), thus introducing new genera, such as *Takayama* (De Salas et al., 2003), *Togula* (Jorgensen et al., 2004), *Prosoaulax* (Calado and Moestrup, 2005), *Apicoporus* (Sparmann et al., 2008), *Paragymnodinium* (Kang et al., 2010), and *Barrufeta* (Sampedro et al., 2011). Routine coastal water samplings are typically performed with Lugol fixation. However, this procedure causes the deformation and degradation of athecate and delicate dinoflagellates, making their identification difficult. Partly due to this limitation and the fact that most species are not toxin producers, gymnodinioids have remained poorly characterized, which has hampered studies of the dynamics, ecophysiology, and potential toxicities of the different species. Several studies conducted in the Mediterranean Sea aimed at resolving the taxonomy of these organisms by combining morphological observations with physiology, phylogeny and life cycle studies. This has allowed, for example, the identification of two *Karlodinium* species causing recurrent fish mass mortality in Alfacs Bay (Ebro River delta) (Garcés et al., 2006), the identification of several unarmored species from the Gulf of Naples (Italy) (Siano et al., 2009) and the identification of *Barrufeta bravensis* and *Lepidodinium chlorophorum* as the causative organisms of blooms detected at La Fosca beach (Sampedro et al., 2011) and Balearic Islands (Illoul et al., 2008), respectively.

The recurrent observations of high-biomass blooms, attributed to a gymnodinioid dinoflagellate, at several beaches in the NW Mediterranean Sea motivated the present study. Its specific aims were, first, to identify the causative organism; second, to describe different life cycle stages; and third, to determine its temporal abundance in a given site. These efforts led to the identification of a new species, *Gymnodinium litoralis*, based on light and scanning-electron microscopy examinations of its external morphology, phylogenetic analysis of its LSU rDNA, and analyses of its ultrastructural and pigment composition.

2. Material and methods:

2.1 Sample collection, cell isolation, and culture: Strains were isolated from single cells of live samples and from resting cysts. Vegetative cells were collected during June 2009 from La Muga, and L'Estartit beaches. Resting cysts were isolated from surface sediment samples collected in March 2009 in Fangar Bay (Ebre delta, Spain) and in May 2010 in Corru S'Ittiri lagoon (Gulf of Oristano, Sardinia, Italy) (Figure 1). The surface layer of the collected sediment cores were processed following the density gradient method proposed by Bolch (1997), and later modified by Amorim *et al.* (2001) and Bravo *et al.* (2006). Isolated cysts were photographed and transferred into IWAKI tissue-culture multi-wall plates filled with L1 medium adjusted to 31 psu. Plates containing resting cysts were checked for germination every 2–4 days. All established cultures were maintained at 20ºC under a 12:12 light:dark cycle. Illumination was provided by fluorescence tubes (Gro-lux, Sylvania, Germany), generating a photon irradiance of 100 µmol photons $m^{-2} s^{-1}$, in enriched L1 seawater media without silicate (Guillard and Hargraves, 1993) at a salinity of 34 (La Muga and L'Estartit strains) or 31 (Fangar Bay and Corru S'Ittiri strains).

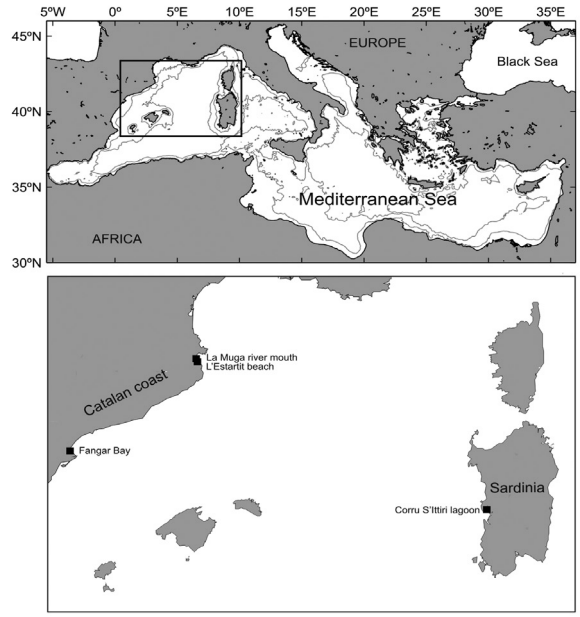

Figure 1: Locations of isolated strains.

2.2 Morphological characterization

2.2.1 Light microscopy: Live vegetative cells were observed and measured using a Leica–Leitz DMIRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and bright-field, phase-contrast, or epifluorescence (50W mercury lamp). Cell size of the strains growing in exponential phase was measured using the ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik, Systeme GmbH). The cells were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2 µg mL^{-1} to determine the position of the nuclei under epifluorescence microscopy with the aid of a UV filter set. A blue excitation filter was used to reveal the chloroplast's autofluorescence in the same cell. Resting cysts were observed and measured with Leica DMIRB (Fangar Bay strain) and Axiovert 10 (Corru S'Ittiri strains).

2.2.2 Scanning electron microscopy (SEM): Samples were prepared following the protocol of Jung et al. (2010), using L1 medium to obtain the desired concentration of osmium tetraoxide. Cells were filtered onto 3.0 µm Whatman polycarbonate filters. Cacodylate buffer was adjusted to a pH 7.4. The filters were examined with a Hitachi S-3500N (Nissei Sangyo Co. Ltd., Tokyo, Japan) SEM operating at 5 kV.

2.2.3 Transmission electron microscopy (TEM): Ten mL of exponentially growing culture were concentrated (500 rpm, 1 min) and immediately immersed in the freezing medium 1-hexadecene. The sample was directly mounted into the cavity of a standard platelet filled with the same medium. The platelet was inserted into a holder of high-pressure liquid-nitrogen freezer (EM Pact, Leica) under a pressure of about 2000 bars, and stored in liquid nitrogen before freeze substitution (Leica AFS2) in acetone containing molecular sieves at -90ºC for 72 h. The samples were then serially re-warmed to -60ºC for 30 min, -30ºC for 1 h, and finally room temperature and washed in acetone before being embedded in Spurr epoxy resin (Soyer-Gobillard et al., 2002). These embedded samples were sectioned on an Ultracut E (Reichert-Jung) microtome using a diamond knife (Diatome), collected on a 200-mesh grid, and placed on a Formvar film for staining in 2% uranyl acetate. The sections were examined in a JEOL JEM-1010 electron microscope (JEOL-USA Inc., Peabody, MA, USA) operated at 80kV. Micrographs were taken using a Gatan, BioScan model 792 (Gatan, Inc. Corporate Headquarters, Pleasanton, CA, USA) digital camera.

2.3 Pigment analyses: Fifteen mL of exponentially growing cultures were concentrated on GF/F filters (25 mm diameter) under reduced pressure and frozen immediately at -20ºC. Frozen filters were extracted under low light in Teflon-lined screw capped tubes containing 5 mL of 90% acetone, using a stainless steel spatula for filter grinding. The tubes were chilled in a beaker of ice and sonicated for 5 min in an ultrasonic bath. Extracts were then filtered through 25 mm diameter syringe filters (MFS HP020, 25 mm, 0.20 µm pore size, hydrophilic PTFE) to remove cell and filter debris. A 0.5 mL aliquot of the 90% acetone extract was mixed with 0.2 mL of water and 200 µL were injected immediately. This procedure avoids peak distortion of early-eluting peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to injection (Latasa et al., 2001). Pigments were separated using a Waters (Waters Corporation, Milford, MA) Alliance HPLC System, consisting of a 2695 separations module, a Waters 996 diode-array detector, and a Waters 474 scanning fluorescence detector, and following the method of Zapata et al. (2000), with a reformulated mobile phase A. The column was a C8 monomeric Waters Symmetry (150 × 4.6 mm, 3.5

Table 1. List of the strains, sampling locations, Genbank accession numbers, and strain identifiers of species used in LSU rDNA phylogeny. [1] Sequences obtained in this study. * Cultures obtained from a resting cyst.

Species	Origin	Accession nº	Strain
Gymnodinium litoralis[1]	Muga River mouth, Catalonia (Spain)	JN400080	ICMB224
Gymnodinium litoralis[1]	Muga River mouth, Catalonia (Spain)	JN400081	ICMB225
Gymnodinium litoralis[1]	L'Estartit beach, Catalonia (Spain)	JN400082	ICMB226
Gymnodinium litoralis[1*]	Fangar Bay, Catalonia (Spain)	JN400083	ICMB232
Gymnodinium litoralis[1*]	Corru S'Ittiri lagoon, Sardinia (Italy)	JN400084	UNISS1
Gymnodinium litoralis[1*]	Corru S'Ittiri lagoon, Sardinia (Italy)	JN400085	UNISS2
Akashiwo sanguinea	Great South Bay (USA)	AY831412	CCMP1321
Alexandrium minutum	Gulf of Naples (Italy)	EU707487	SZN030
Barrufeta bravensis	La Fosca, Catalonia (Spain)	FN647674	VGO859
Barrufeta bravensis	La Fosca, Catalonia (Spain)	FN647676	VGO866
Gymnodinium aureolum	Namibian coast (Angola)	AY999082	SWA16
Gymnodinium catenatum	Jindong (South Korea)	DQ779989	GCCW991
Gymnodinium dorsalisulcum	Darwin (Australia)	DQ837533	KDAAD
Gymnodinium fuscum	Melbourne (Australia)	AF200676	CCMP1677
Gymnodinium cf. *impudicum*	Swan River estuary (Australia)	EF616465	GISR01
Gymnodinium impudicum	Gulf of Naples (Italy)	AF200674	JL30
Gymnodinium impudicum	Ría de Vigo (Spain)	DQ785884	CCMP1678
Gymnodinium impudicum	Yosu (South Korea)	DQ779992	Gi1cp
Gymnodinium impudicum	Hase (South Korea)	DQ779993	GrIp02
Gymnodinium impudicum[1]	Valencia harbor (Spain)	JN400079	Gy2VA
Gymnodinium microreticulatum	Uruguay	AY916539	GMUR02
Gymnodinium nolleri	Kiel blight (Baltic Sea)	AY036079	GNKB03
Gymnodinium palustre	-	AF260382	AJC14-732
Gymnodinium trapeziforme	Gulf of Oman (Iran)	EF192414	GYPC02
Gyrodinium instriatum	South Korea	EF613354	JHW0007
Gyrodinium rubrum	Ballen harbor, Samsø (Denmark)	AY571369	-
Gyrodinium spirale	Ballen harbor, Samsø (Denmark)	AY571371	-
Karenia brevis	Florida (USA)	EU165310	CCMP2281
Karenia mikimotoi	Sutton harbor, Plymouth (England)	EU165311	CCMP429
Karlodinium armiger	Alfacs Bay, Catalonia (Spain)	DQ114467	K0668
Karlodinium veneficum	Gulf of Naples (Italy)	FJ024701	MC710-A1
Lepidodinium viride	South Africa	DQ499645	-
Lepidodinium chlorophorum	East China Sea	AB367942	NIES1867
Polykrikos kofoidii	-	EF192411	PKHK00
Polykrikos schwarzii	-	EF102409	PSSH00
Takayama achrotrocha	Singapore	DQ656116	GT15
Takayama cf. *pulchellum*	East China Sea	AY764178	TPXM

µm particle-size, 100 Å pore-size). Eluent A was methanol: acetonitrile: 0.025 M aqueous pyridine (50:25:25 v/v/v). Eluent B was methanol: acetonitrile: acetone (20:60:20 v/v/v). The elution gradient (time: %B) was: t0: 0%, t22: 40%, t28: 95%, t37: 95%, t40: 0%. The flow rate was 1.0 mL min^{-1} and the column temperature 25ºC. Solvents were HPLC grade (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with standards obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). The peaks were checked for purity and the spectral information was then compared with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see Jeffrey and Wright (1997)).

2.4 DNA extraction, PCR amplification, and sequencing: Genomic DNA was extracted from approximately 10 mL of a clonal culture collected at the exponential growth phase. Cells were harvested by centrifugation at 3000 rpm for 15 min. The pellet was transferred to a 2 ml Eppendorf tube and centrifuged again at 10,000 rpm for 5 min. Total genomic DNA was extracted from the resulting pellet using a DNeasy Plant MiniKit (Qiagen), following the instructions of the manufacturer. The extracted DNA was immediately frozen at -80ºC. PCR primers D1R and D2C (Scholin et al., 1994) were used to amplify the D1-D2 regions of the LSU rRNA gene. PCR was carried out in 50 µL reactions containing 1 µL of genomic DNA, 0.25 µM of each primer, 600 µM of dNTPs (Qiagen mix), PCR Buffer 1x (Qiagen) containing 1.5 mM of $MgCl_2$, 3 µl of $MgCl_2$ (25mM) and 1.25 U of Taq DNA polymerase. Thermocycling included one initial cycle of 95ºC for 5 min followed by 40 cycles of 95ºC for 20 sec, 55ºC the PCR products were electrophoresed in 1% agarose gels and the remaining product frozen at -20ºC until sequenced. Purification and sequencing were carried out by an external service (Macrogen Inc., Korea) using the D1R primer and a 3730XL DNA sequencer.

2.5 Phylogenetic analyses: Sequences obtained in this study were aligned with those obtained from GenBank (Table 1) using the MAFFT v.6 program (Katoh et al., 2002) under FFT-NS-i (slow; iterative refinement method). Alignments were manually checked with BioEdit v. 7.0.5 (Hall, 1999). Phylogenetic relationships were done with the maximum-likelihood (ML) method and the GTRGAMMA evolution model on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with MrBayes (Huelsenbeck and Ronquist, 2001). All analyses were done through the freely available University of Oslo Bioportal, http://www.bioportal.uio.no (Kumar et al., 2009).

2.6 Temporal distribution: During 2008, 2009 and 2010, surface seawater samples were collected on a monthly base during the whole year and biweekly from May to September, at a fixed station in L'Estartit beach (water depth 1 m). During 2010 (February to September), samples were obtained monthly from Corru S'Ittiri lagoon. Sampling always included *in situ* measurements of temperature and salinity. Samples (150 mL) of phytoplankton were preserved with Lugol's iodine solution A 50 mL aliquot was settled in a counting chamber for 1 day. For phytoplankton enumeration, the appropriate area of the chamber was scanned at 200-400x magnification using a Leica-Leitz DMII (L'Estartit) and an Axiovert 25 (Corru S'Ittiri) inverted microscope. Although the distinction of gymnodinioid species is difficult in fixed samples, we are able to identify the species in field samples by observing its conspicuous size, shape and color characteristics.

3. Results:

3.1 Gymnodinium litoralis A. Reñé sp. nov.

Diagnosis: Cellulae nudae, ovoideae et dorsoventraliter paululum compressae, 19-42 µm longae, 14-37 µm latae. Cellulae solitariae, vulgo binae. Cingulum bene definitum, descendens, in dextro latere cellulae. Sulcus in hypocono dilatat, intra antapicem penetrans. Nucleus in epicono sphaericus est, interdum leviter in sinistro latere cellulae

dislocatus, ad medium cellulae consistens. In prioribus cellulis catenarum nucleus centralis est. Chloroplasti sunt aureo-fusci, porrectis, locati peripherice. Cystidii quiescientia rotundi et ovales sunt, leves cum pallido granorum continente et unum aut duo corpora luteae cumulationis.

Cells naked, ovoid, and slightly dorsoventrally compressed. Length: 19–42 µm; width: 14–37 µm. Cells single, often in pairs. Cingulum well-defined, descending, displaced 1/3–1/4 of the cell length, overhanging about 1/3–1/4 of the cell width. Sulcus deep, extending slightly and narrowly through the epicone. The acrobase is horseshoe-shaped, running anticlockwise on the right side of the cell. Sulcus broadens on the hypocone, penetrating until the antapex. Nucleus spherical, in the epicone, sometimes slightly displaced to the left side of the cell, running towards the center of the cell. For cells in pairs, nucleus is located central in anterior cells. Chloroplasts golden-brown, elongated, located peripherally. Resting cysts are round to oval, smooth with pale granular content and one or two orange accumulation bodies.

Holotype: Figure 11A from culture ICMB226, isolated from the beach at the mouth of the La Muga River (Catalonia) and deposited in a culture collection (CCMP3294). LSU rDNA sequence deposited in Genbank under accession number:JN400082.
Etymology: Latin *litoralis*, coastal, referring to the habitat of the organism.
Type locality: La Muga River mouth (Catalonia) Spain (Fig. 1)
Isotype: Fig. 2A
Distribution: Catalan coast, Gulf of Oristano, Gulf of Naples, and Western Australia
Habitat and ecology: Along the Catalan coast, the organism is detected in coastal plankton in spring, summer, and fall, producing high-biomass blooms (>10^6 cells L^{-1}) from May to September, when the water temperature reaches >22ºC. It is observed in salinities from 33 to 38.5. In the Corru S'Ittiri coastal lagoon, the maximum abundances are observed in June (>6 x 10^6 cells L^{-1}).

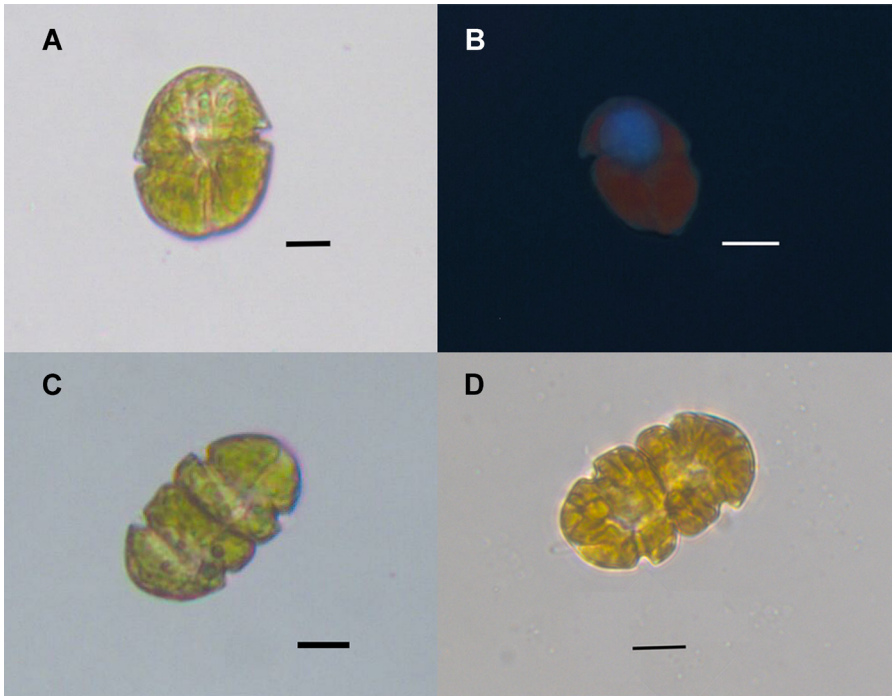

Figure 2. Light micrographs of vegetative cells of *Gymnodinium litoralis*. Ventral view of a single vegetative cell under bright-field (A) and epifluorescence (B) microscopy. Note the presence of the DAPI-stained nucleus (blue) and autofluorescent chloroplasts (red) in (B). Cell pairs of *G. litoralis* (C) and *G. impudicum* (D) under bright-field microscopy. Scale bar = 10 µm.

Table 2: Mean (± standard deviation) length, width and ratio length:width of cells measured for each strain, and mean values of all measured cells.

Strain	ICMB224	ICMB225	ICMB226	ICMB227	ICMB228	Mean
Length	30.9 ± 4.9	29.7 ± 2.8	28.5 ± 5.0	30.8 ± 4.5	29.7 ± 3.3	29.4 ± 4.7
Width	25.3 ± 3.3	26.0 ± 3.1	23.9 ± 3.8	24.6 ± 3.7	24.1 ± 3.0	24.5 ± 3.6
Ratio	1.23 ± 0.11	1.15 ± 0.07	1.19 ± 0.1	1.25 ± 0.08	1.24 ± 0.11	1.20 ± 0.13
n	26	25	25	26	25	127

3.2 Description: Gymnodinium litoralis is usually found as single cells, but pairs of cells are found as well. The mean cell sizes of strains isolated from the type locality are presented in Table 2. The mean cell size of all strains is 29.4 µm ± 4.7 S.D long and 24.5 µm ± 3.6 S.D wide, with a length:width ratio of 1.2 ± 0.1 S.D. The epicone is almost equal in size to the hypocone, with a round to oval shape (Figs. 2A, 3A, 3B). The hypocone is convex and the antapex slightly flattened and slightly bilobate on dorsal view, with the right lobe being prominent (Figs. 2A, 3C). The cells are compressed dorsoventrally, mainly in the epicone (Fig. 3B). The cingulum is well-defined, with a descending displacement of about twice its width. It overlaps about 1/3 to 1/4 of the cell width. The sulcus is narrow, deeply excavated, and shallowly penetrates the epicone directly to the apex forming a ventral ridge on its right side (Fig. 3A). The apical groove is horseshoe-shaped, running anticlockwise, and displaced to the right side of the cell. The loop is elongated, with the distal end more closely approximating the anterior border of the cingulum than the proximal end of the loop (Fig. 4A). It contains three vesicles, none of which appears to possess knobs (Fig. 4B). The sulcus widens through the hypocone, reaching the antapex (Fig. 3A, D). The cells in pairs have a different shape. Both cells are shorter and wider, usually with smaller dimensions than solitary cells. The anterior cell has a concave hypocone whose surface completely contacts the flattened epicone of the second cell (Figs. 2C, 3D). In some cells, SEM showed an amphiesmal polygonal pattern covering the cell surface (Fig. 4C, D). A row of pentagonal polygons surrounds only the cingulum, while the rest of the cell is covered by irregularly shaped, mostly hexagonal polygons (Fig. 4C). Several pores are observed on the polygon sutures (Fig. 4D). The nucleus is spherical, situated in the central part of the cell, and oriented towards the epicone (Fig. 2B). The nucleus occupies most of the epicone and is sometimes displaced to the left side of the cell. Chloroplasts are numerous, with a golden color. Elongated chloroplasts are located peripherally all around the cell but multilobated forms are observed on the hypotheca (Fig. 2B). The cells swim fast, in a straight direction, turning on their own axis and changing direction quickly and randomly.

Resting cysts from the field (Fig. 5A, B) are round to oval (22–31 µm long and 22–30 µm wide, n=6), with a thick wall, and somewhat irregular in appearance. The resting cyst content is pale to greenish, with numerous starch grains and one or two orange accumulation bodies. The shape of some of the resting cysts is similar to that of the vegetative cells, but with a visibly thickened wall and a dark-orange accumulation body (Fig. 5C). Cells covered by a hyaline membrane—thus, following the nomenclature of Bravo et al. (2010), considered to be pellicle cysts—were observed in field sediments and in the cultures. These cysts resemble vegetative cells, as the cingulum and sulcus are maintained, but they are non-motile and become round (Fig. 5D). They are produced by single cells or cells in pairs (Fig. 5D-E) and maintain the capacity to produce planktonic cells (Fig. 5F). Pellicle cysts containing dividing cells have also been observed, evidencing the capacity for cell division at this stage (Fig. 5G). The DNA content of the different types of cysts and cells is as yet unknown.

3.3 Ultrastructure: The nucleus (diameter 7.5–9.5 µm) is situated in the epicone. It is round on frontal view (Fig. 6A) but dorsoventrally flattened on lateral view and displaced to the dorsal side of the cell (Fig. 6B). Condensed chromosomes and a nucleolus are present in the nucleus (Fig. 6A, C). The nuclear membrane is regular and smooth, without the presence of nuclear chambers (Fig. 6C). The amphiesma is composed of flattened amphiesmal vesicles containing plate-like material (Fig. 6D), although the presence of external plates was not observed under light microscopy by using calcofluor white M2R (Fritz and Triemer, 1985) dye on live and fixed samples. The chloroplasts are numerous, elongated, and radially distributed, except in some larger chloroplasts, observed in the central part of the hypocone

(Fig. 7A). Chloroplasts contain several lamellae that consist of 4–10 thylakoids (Fig 6E). The cells possess a pusular system displaced across the sulcus (Fig. 6A, 7A) and consisting of a large collecting chamber surrounded by pusular vesicles that open into it (Fig. 7C); two independent collecting chambers were observed in several cells (Fig. 7A, B). The numerous elongated trichocysts contain dense material (Fig. 7D) and are rhomboid in cross-section (Fig. 7E).

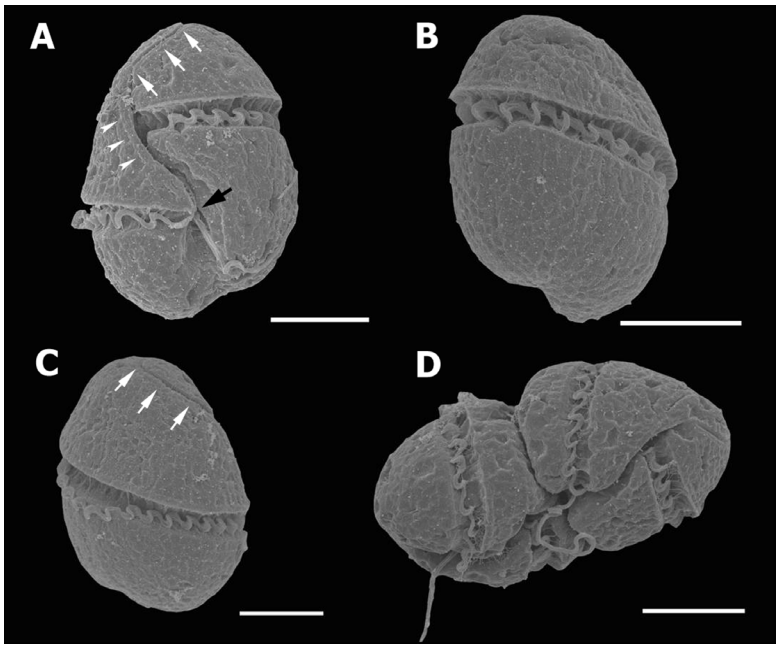

Figure 3. Scanning electronic micrographs of strain *Gymnodinium litoralis* ICMB226. (A) Ventral view of the cells showing the apical groove (white arrows), ventral ridge (arrowheads), and longitudinal flagellar insertion (black arrow). (B) Dorsal and (C) lateral views, showing the loop of the apical groove (white arrows). (D) A two-cell chain. Scale bar = 10 µm.

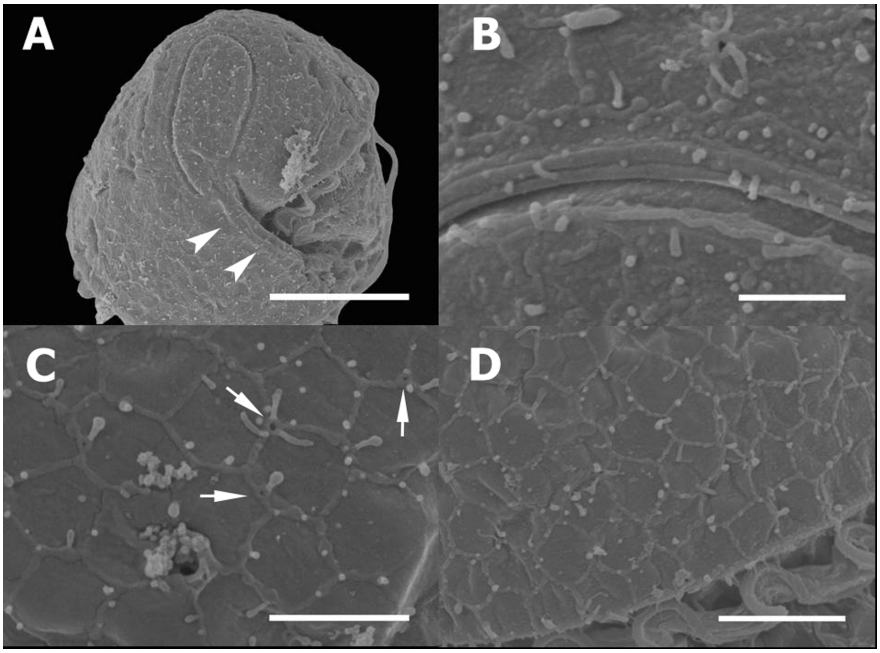

Figure 4. Scanning electronic micrographs of strain *Gymnodinium litoralis* ICMB226. (A) Apical view of the cell shows the elongated, anticlockwise loop of the apical groove and the ventral ridge (arrowheads). (B) Detail of the apical groove. Note the presence of three vesicles and the lack of knobs in any of them. (C) Detail of the cell surface, showing the typical gymnodinioid amphiesmal pattern and the presence of several pores (white arrows). (D) General view of the amphiesma. Note the polygonal, irregular pattern of the structures and the pentagonal line just above the cingulum. Scale bars: (A) 10 µm, (B) 1 µm, (C) 2 µm, (D) 3 µm.

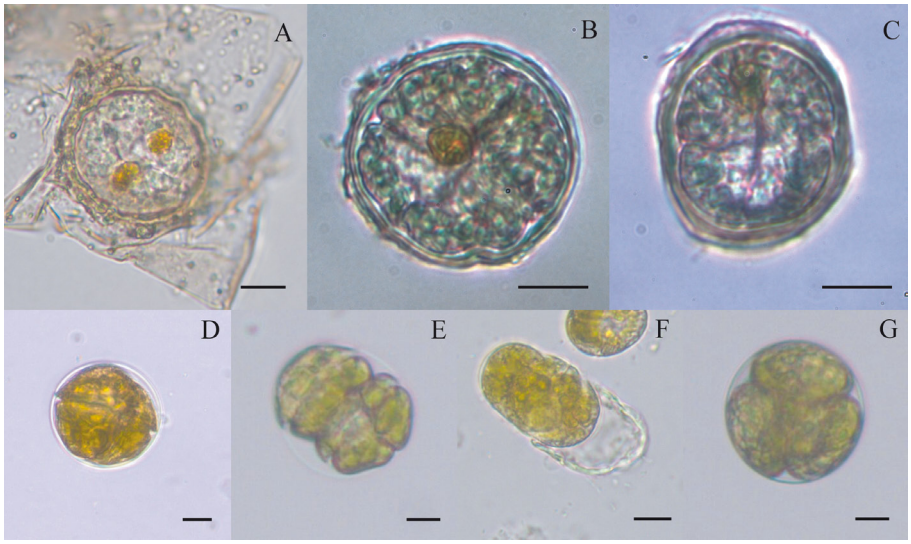

Figure 5. Light micrographs of resting cysts obtained from Fangar bay (A) and from Corru S'Ittiri lagoon (B). Wild cysts resembling a vegetative cell (C) and pellicle cyst produced by a single cell (D) or by a cell chain (E). Cell emerging from a pellicle cyst (F). Dividing cells within the hyaline membrane (G). Scale bar =10 µm.

3.4 Pigment profile: The HPLC chromatogram (Fig. 8) shows the standard peridinin (Per)-containing chloroplast, with chl c_2 and peridnin as the major accessory pigments. Diadinoxanthin (Diadino) and dinoxanthin (Dino) were also identified as relevant pigments, with a similar quantitative contribution to the carotenoid pool (Dino/Diadino=0.70). Pigment to chl *a* molar ratios varied: Per/chl *a* = 0.47, Diadino/chl *a* = 0.40, Dino/chl *a* = 0.28, chl c_2/chl *a* = 0.27. The Per/chl c_2 ratio (1.74) is in the lower value among Gymnodiniales.

3.5 Phylogeny: Six partial LSU rDNA sequences (D1/D2 regions, 635 base pairs) were obtained from the cultures. These sequences were aligned with a selection of GenBank sequences from different genera belonging to the Gymnodiniales order and using *Alexandrium minutum* as outgroup. The LSU rDNA phylogeny resulted in two main groups (Fig. 9). The first comprised *Gyrodinium* species (*G. spirale*, *G. rubrum,* both freshwater species, and *G. instriatum*), the *Kareniaceae* genera (*Takayama*, *Karlodinium*, *Karenia*), and *Akashiwo sanguinea*. All species of the same genera grouped with high bootstrap values (> 89), except *G. instriatum*, which was unrelated with the other *Gyrodinium* species. The second group, supported by a bootstrap value of 85, comprised the *Gymnodinium sensu stricto* clade. It included a basal branch, with the two *Polykrikos* species, and a large clade, supported by a moderated bootstrap value (66), containing all *Gymnodinium* species (including *G. fuscum*, the type species of the genera) and the genera *Lepidodinium* and *Barrufeta*. These genera formed independent branches, and among these there was the clade formed by *Gymnodinium litoralis* sequences. The six *G. litoralis* sequences, together with *G. impudicum* JL30 and *G.* cf. *impudicum* GisR01, were almost identical at the LSU rDNA level. Molecular phylogeny was also analyzed using ITS rDNA sequences with a subset of species (data not shown). The results did not significantly differ from those obtained with LSU rDNA phylogeny.

3.5 Temporal distribution and detection localities: Along the Catalan coast, high abundances of the organism later identified as *G. litoralis* was noted in 2008 at l'Estartit beach and at La Muga river mouth. In 2010, high abundances of the same organism were detected at Corru S'Ittiri Lagoon (Sardinia). L'Estartit beach was intensively monitored during 2008, 2009 and 2010 (Fig. 10). In winter, the organism was not detected in the water column; rather, it first appeared in spring, when water temperature reached about 14ºC. In summer, at water temperature ≥22ºC, *G. litoralis* abundances increased to more than 10^5 cells L^{-1}, reaching >10^6 cells L^{-1}. In September, as the water temperature decreased, cell abundances declined dramatically but it can still be detected until the end of the year. The species was found at low densities in Corru S'Ittiri lagoon as early as February and March (water-temperature range: 11–14°C).

In June, with temperatures typically around 24°C, *G. litoralis* reached its highest densities (>10^6 cells L^{-1}). In July, August, and September, as the salinity increased to 40, the species disappeared from the water column (data not shown). Resting cysts of the species were obtained in 2009 from El Fangar Bay, a location with estuarine characteristics but no blooms are reported.

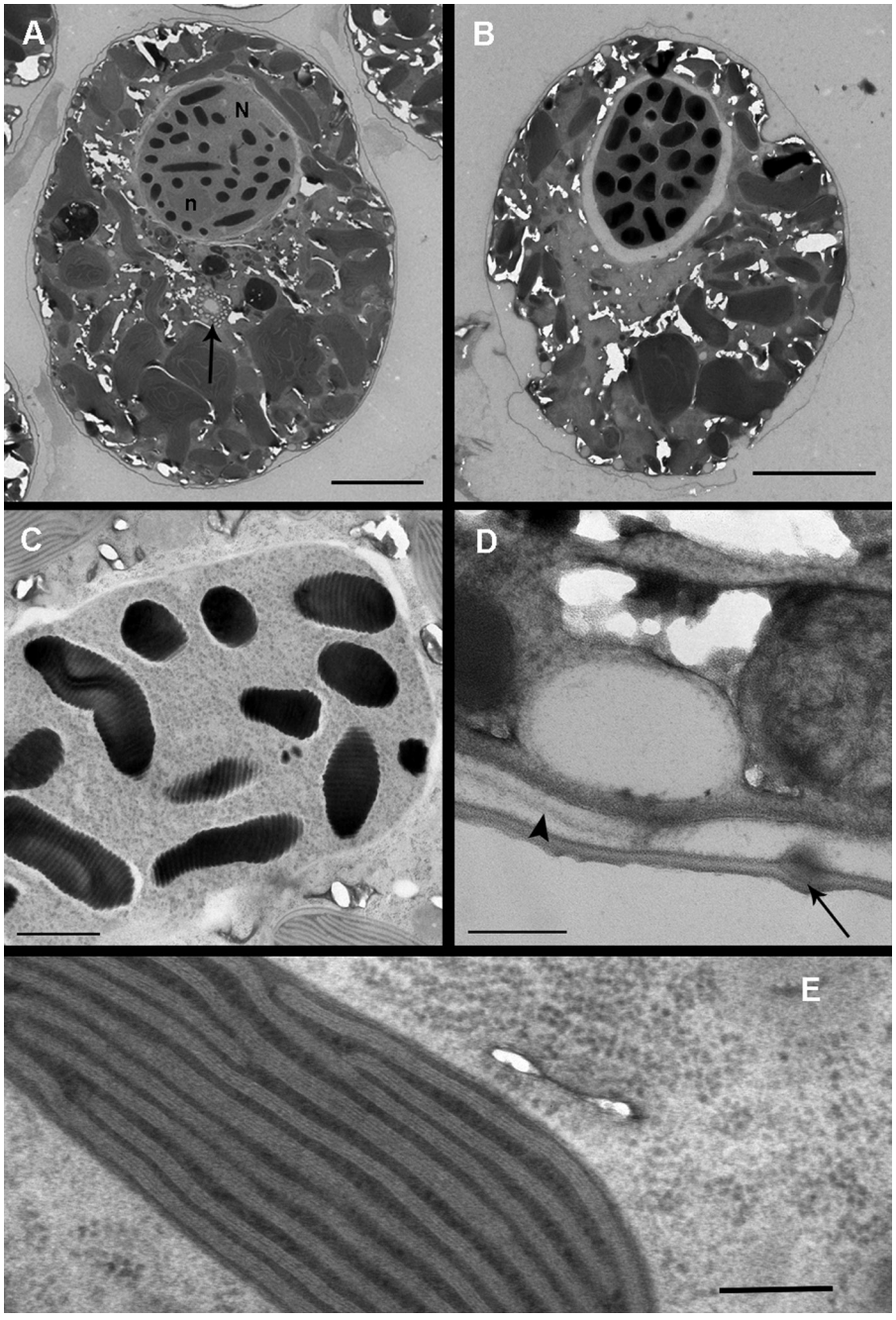

Figure 6. Ultrastructure of strain *Gymnodinium litoralis* ICMB226. Longitudinal section of a cell in (A) frontal and (B) lateral views. Chromosomes (dark bodies) within the nucleus (N) and the nucleolus (n) are seen. A pusule is indicated by the arrow. (C) Detail of the chromosomes. Note the smooth structure of the nuclear membrane. (D) Amphiesmal section, showing the junction of two amphiesmal vesicles (arrow) and a plate-like material (arrowhead). (E) A chloroplast containing 10 lamellae. Scale bars: (A, B) 5 µm, (C) 1 µm, (D, E) 200 nm.

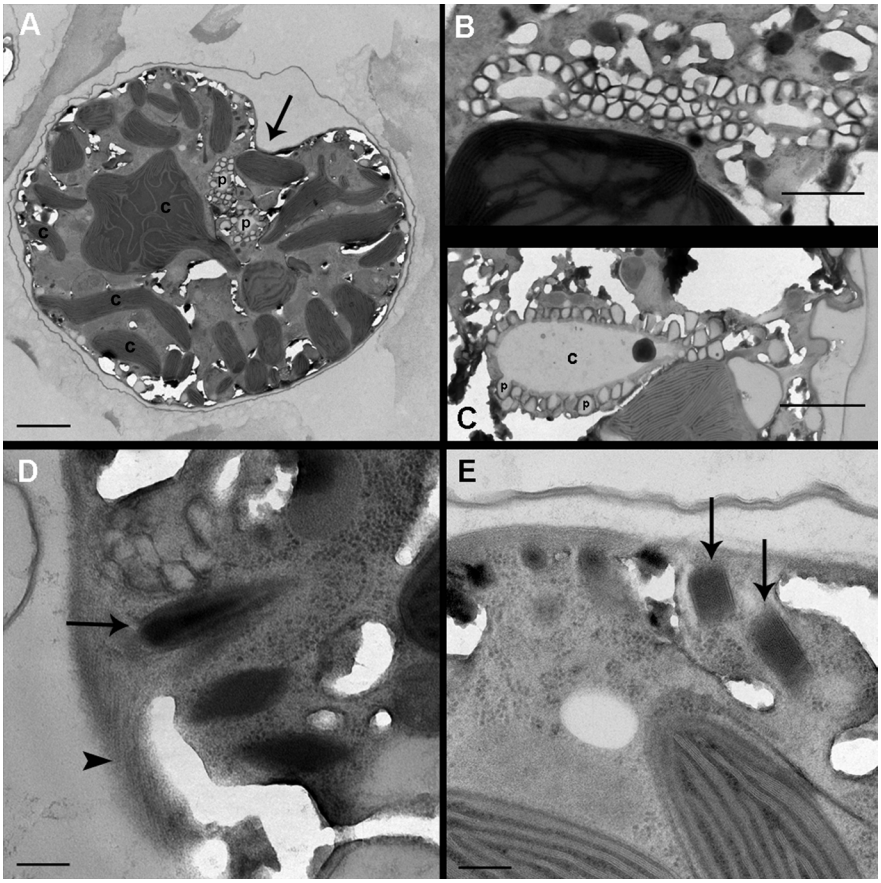

Figure 7. Ultrastructure of strain *Gymnodinium litoralis* ICMB226. (A) Transverse section of the hypocone. Numerous chloroplasts (c), and the pusule (p), with two separated collecting chambers, are situated beneath the sulcus (arrow). (B) Detail of the pusule in transverse section shows two collecting chambers. (C) Longitudinal section of the pusule, with a large collecting chamber (c) and numerous pusule chambers around it (p). (D) Longitudinal section of trichocysts (arrow) near the cell membrane, where several microtubules (arrowhead) are present. (E) Cross-section of the trichocysts (arrows), showing a rhomboid shape. Scale bars: (A) 2 µm, (B, C) 1 µm, (D, E) 200 nm.

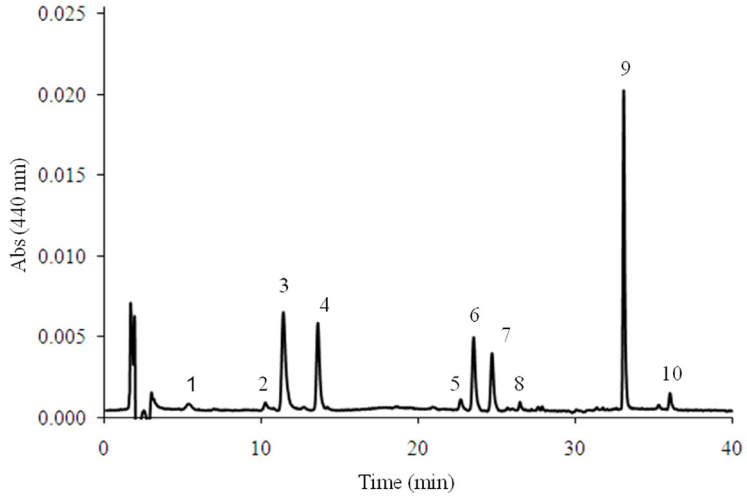

Figure 8. HPLC chromatogram of strain *Gymnodinium litoralis* ICMB226. Peak identification: (1) peridininol, (2) divinyl protochlorophyllide (MgDVP), (3) chl c2, (4) peridinin, (5) diadinochrome, (6) diadinoxanthin, (7) dinoxanthin, (8) diatoxanthin, (9) chl a, (10) β, β-carotene. Detection by absorbance (Abs) at 440 nm.

4. Discussion:

The genus *Gymnodinium*, erected by Stein in 1878, is one of the largest dinoflagellate genera, with more than 150 described species. The type species is *G. fuscum* (Ehrenberg) Stein, a freshwater organism, but the genus comprises both freshwater and marine species. A large number of species were described during the early 20th century, based on the observation of a single organism (Kofoid and Swezy, 1921) and resulting in vague descriptions of the specimens. The shape plasticity of the athecate organisms has led to misidentification of similar specimens as different species (e.g., *Gymnodinium gracile* Bergh). Most descriptions were based on morphological characters observed under light microscopy. Currently, these characters are known to be insufficient and the development of more sophisticated microscopy techniques, such as SEM or TEM, has allowed description of the external morphology and ultrastructure of gymnodinioids and thus new taxonomic detail.

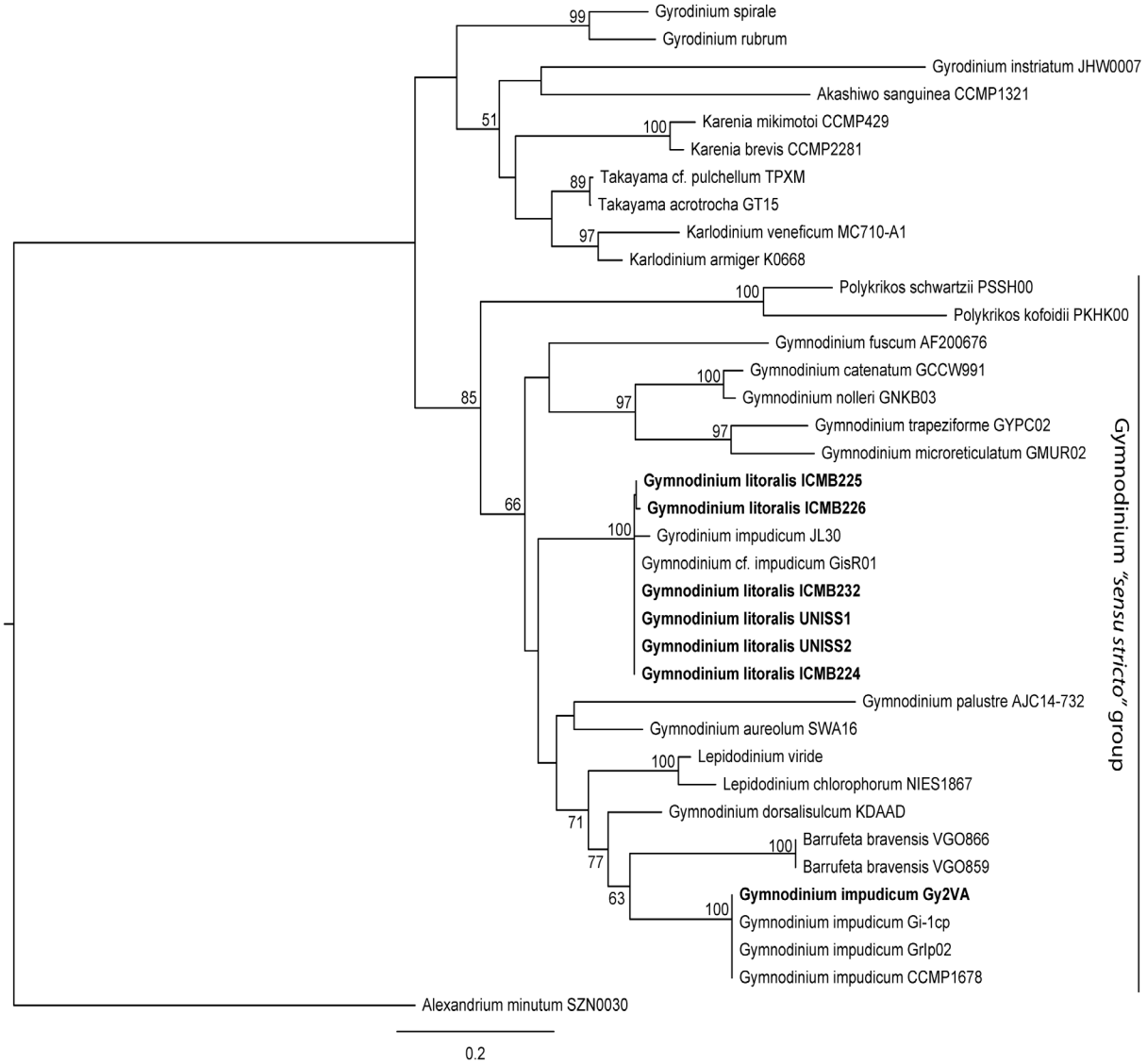

Figure 9. Maximum-likelihood phylogenetic tree of selected species based on the D1-D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap values obtained after 1000 replicates. Only bootstrap values >50 are shown. *Alexandrium minutum* was used as outgroup. Organisms sequenced in this study are highlighted in bold.

Daugbjerg et al. (2000) redefined the genus *Gymnodinium* as unarmored dinoflagellates possessing a horseshoe-shaped apical groove running in an anticlockwise direction, cingulum displacement, vesicular chambers in the nuclear envelope, and the presence of a nuclear or dorsal fibrous connective. Although these characters were proposed to be essential to species identification, some of them have not been observed in most species belonging to the genus; rather, as further studies progress, they have been confirmed in other genera. The horseshoe-shaped apical groove is a character observed for all the studied *Gymnodinium* species, despite the slight differences in shape observed in *G. uncatenum* and the *G. catenatum/nolleri/microreticulatum* group (Bolch et al., 1999; Ellegaard and Moestrup, 1999). A horseshoe-shaped apical groove is, however, also seen in *Polykrikos* species, such as *P. hartmanii* (= *Pheopolykrikos hartmanii*), *P. kofoidii*, and *P. schwartzii* (Nagai et al., 2002; Takayama, 1985), the two species belonging to the *Lepidodinium* genus, *L. viride* and *L. chlorophorum* (Elbrächter and Schnepf, 1996; Watanabe and Suda, 1990), and some warnowiids, such as *Nematodinium* and *Proterythropsis* (Hoppenrath et al., 2009). The presence of nuclear chambers has been observed in some *Gymnodinium* species, such as *G. fuscum* (Dodge and Crawford, 1969), *G. aureolum* (Hansen, 2001), *G. nolleri* (Ellegaard and Moestrup, 1999), and *G. corollarium* (Sundström et al., 2009), as well as in other genera, including *Lepidodinium* (Hansen et al., 2007), *Barrufeta* (Sampedro et al., 2011), and *Polykrikos* (Hoppenrath and Leander, 2007). In this study, we confirmed two key characters of the genus in *G. litoralis*, the shape of the apical groove and cingulum displacement, while others, including nuclear envelope chambers or a nuclear fibrous connective, are apparently absent.

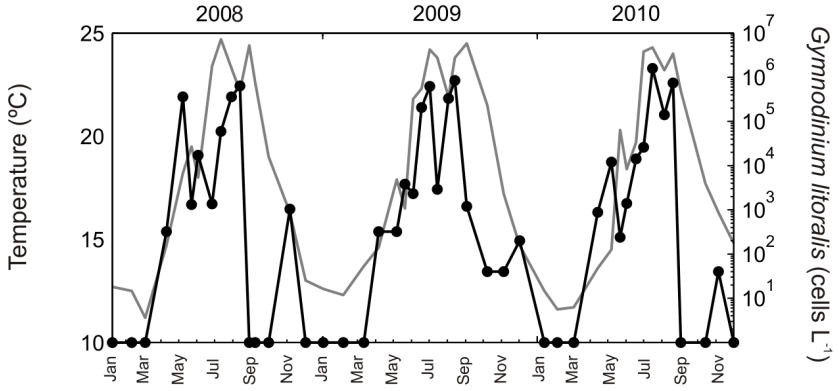

Figure 10. Temporal distribution of *Gymnodinium litoralis* at l'Estartit beach during 2008–2010. Temperature (ºC) is shown on the left axis (gray line) and cell abundance (cells L-1) on the right axis (dark line).

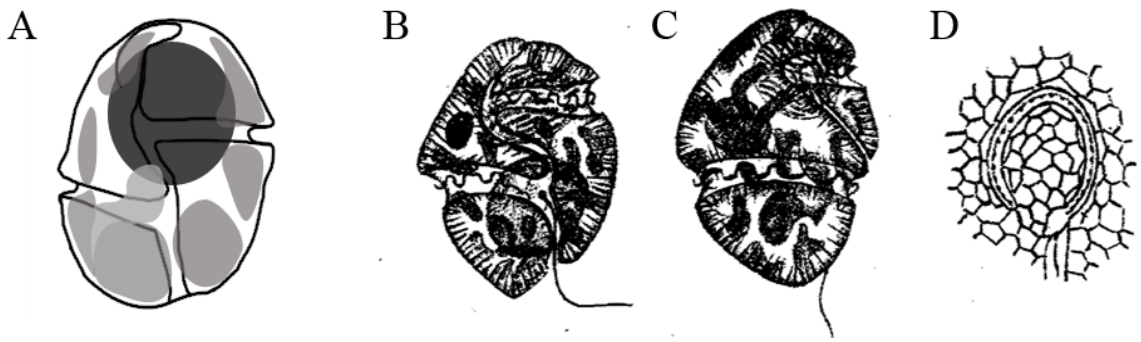

Figure 11. Schematic representation of *Gymnodinium litoralis* (A) and the images of *G. vorax* drawn by Biecheler (1952) showing ventral (B) and lateral (C) views and the structure and shape of the acrobase (D) (not drawn to scale).

Table 3. Comparative morphological characters of species similar to *Gymnodinium litoralis* and other species belonging to the Gymnodinium sensu stricto group. n.a.: not available Table 3. Comparative morphological characters of species similar to *Gymnodinium litoralis* and other species belonging to the *Gymnodinium sensu stricto* group. n.a.: not available

	Gymnodinium litoralis	*Gyrodinium vorax*	*Gyrodinium impendens*	*Gymnodinium impudicum*	*Gymnodinium aureolum*	*Gymnodinium microreticulatum*	*Barrufeta bravensis*	*Lepidodinium chlorophorum*	*Lepidodinium viride*
Cell shape	Ovoid. Slightly dorsoventrally compressed	Oval	Episome cone-shaped. Hyposome obliquely rounded	Pointed	Globular. Slightly dorsoventrally flattened	Ovoid to biconical. Slightly laterally compressed	Oval. Dorsoventrally flattened	Subglobular to ovoid. Dorsoventrally compressed	Subglobular. Dorsoventrally compressed
Length (µm)	19-46	30-35	20-35	14-37	27-34	20-34	22-35	18-33	22-52
Width (µm)	14-37	22-25	13-19	16-32	17-32	15-22	16-25	12-18	19-38
Cingulum overhang	Yes	Yes	Pronounced overhang	No	Yes	No	No	No	No
Chains	Cell pairs	No	No	4-16 cells	No	No. Cell pairs rare	No	No	No
Acrobase	Elongated horseshoe-shaped, running anticlockwise	Rounded, deep, narrow, running anticlockwise	From the proximal end of the girdle to the apex, following a sigmoid curve	Deep, turning anticlockwise. Indentation in side view	Horseshoe-shaped, counterclockwise coming close to the origin	Anticlockwise hoseshoe-shaped, encircling the apex	Diagonally smurf cap-shaped. Counterclockwise	Narrow and shallow apical loop that encircles the apex	Narrow and shallow. Turning left around the apex, encircling it almost completely
Nucleus	Centered, running into the epicone	On the dorsal side of the epicone	In the intercingular region, extending into the episome	Central. Displaced depending on the chain position	Central. Wider than long	Large, spherical. In epicone	Centered in the epicone	Centrally located	Subspherical or ovoid. Location ranges from the center to the apex
Chloroplasts	Elongated. Golden-brown. Peripherally located without pyrenoids	Variables. Yellow-green, mainly in the hypocone	Several small, each with a distinct pyrenoid	Numerous, small and elongated	Numerous yellow-browh. Elliptical. Radiating.	Greenish-brown. Peripherally located. Multilobed	Numerous, yellow-brown. Radiating from pyrenoids	Green. Lens-shaped to lobed	Green. Irregular. Peripherally located.
Resting cysts	Smooth. Round to oval. Pale to green with orange accumulation bodies	n.a.	n. a.	Smooth. Yellow-greenish to brownish. Spherical	Smooth. Dark brown. Spherical. Mucus layer	Reticulated. Pale purple-brown.	Smooth. Circular to oval. One or more orange spots. Mucoid	n. a.	n. a.
Source	This study	Biecheler (1952)	Larsen (1996)	Fraga et al. (1995)	Hulburt (1957), Tang (2008)	Bolch et al. (1999)	Sampedro et al. (2011)	Elbrachter & Schnepf (1996)	Watanabe et al. (1990)

4.1 Morphological comparison with other species

A comparison between species with morphological characters similar to those of G. *litoralis* is provided in Table 3. *G. litoralis* shows a high morphological similarity to *Gyrodinium vorax* Biecheler. According to the morphological description of *G. vorax* (Biecheler, 1952), most of its characters are shared with *G. litoralis*, but there are also significant differences between them. In *G. vorax*, the hypocone is described as pointed, the nucleus is small and completely situated within the epicone, and the cingulum overhang depicted is highly pronounced. The *G. litoralis* hypocone is rounded, the nucleus is larger and often observed in a central location (Fig. 11A-C), and the cingulum overhang is less pronounced. The acrobase of *G. vorax* describes a round loop and Biecheler reports the presence of knobs in the central line of vesicles. In *G. litoralis*, the apical groove is an elongated loop (Fig. 11D) possessing three elongated vesicles without knobs. Cells in pairs are not reported in *Gyrodinium vorax*, while they are frequently observed both in cultures and in field samples of *G. litoralis*. *G. vorax* is described as mixotrophic, possessing chloroplasts but also ingestion bodies. Its chloroplasts are pale green-yellow, with an irregular disposition, and the author considers it as a species with plastids in regression. By contrast, cells of *G. litoralis* are densely colored with numerous chloroplasts. This is observed both in cultured strains and in organisms from the field. Light microscopy observations of several wild cells failed to demonstrate ingestion bodies.

Given these many differences, we conclude that our organism is not *G. vorax*. However, a reexamination of *G. vorax* in its type location would be of great interest, as a detailed study of this species could result in the consideration of *G. litoralis* and *G. vorax* as synonymous. Some morphological features of *G. litoralis* are also shared with *Gyrodinium impendens* Larsen, although in the former the cingulum overhang is less marked, pyrenoids are not observed, and the cells are cone shaped. There is also a resemblance with *G. impudicum* (= *Gyrodinium impudicum* (Fraga & Bravo)) Hansen & Moestrup. This species forms chains of 4–8 cells, but cells in pairs that are very similar to those found in *G. litoralis* are also observed (Fig. 2 C, D). Single cells of *G. impudicum* have a pointed epitheca and hypotheca (Fraga et al., 1995) that differ from the rounded epitheca and flattened hypotheca of *G. litoralis*. Mucus production of *G. impudicum* was never observed in *G. litoralis*. There are also some resemblances between *G. litoralis* and *G. microreticulatum* (Bolch & Hallegraeff), including their size, the presence of cell pairs, and several morphological characters, such as the shape and position of the nucleus. However, *G. microreticulatum* has a concave hypotheca, its cyst is reticulated, and it is described as non-bloom forming.

Resting cyst production is not rare in marine and brackish *Gymnodinium* species. *G. catenatum*, *G. nolleri*, *G. microreticulatum*, and *G. trapeziforme* are known for the production of characteristic reticulated cysts (Anderson et al., 1988; Attaran-Fariman et al., 2007; Bolch et al., 1999; Ellegaard and Moestrup, 1999). Furthermore, different types of resting cysts have been observed in some species, including *G. impudicum*, *G. corollarium*, and *G. aureolum* (Kobayashi et al., 2001; Sundström et al., 2009; Tang et al., 2008). *G. litoralis* resting cysts are found in the sediments of Fangar Bay and Corru S'Ittiri lagoon but have not yet been studied in the type location (Estartit and Muga beaches). The round to oval shape of its cysts is shared with *G. impudicum* and *G. aureolum*, while *G. corollarium* cysts are definitely oval. *G. corollarium* produces wild cysts that resemble the vegetative cell, with a cingular groove and sulcal depression, as observed in *G. litoralis* (Fig. 5C). Another common character of the cysts of *G. impudicum* and *G. litoralis* is the presence of obvious accumulation bodies; this is in contrast to *G. aureolum* and *G. corollarium*, both of which have yellow-greenish residual bodies. The *G. litoralis* resting cyst significantly resembles the Gymnodiniales cyst described from another area of Sardinia (Satta et al. (2010a); Plate V Fig. a and b). Unfortunately, the authors did not provide detailed information on the morphology of the vegetative cells and it is therefore not possible to compare *G. litoralis* with that specimen. Efforts to cross cultures of *G. litoralis* (n=10) of different localities and strains failed in the observation of resting cyst formation. By contrast, pellicle cysts are frequently observed in *G. litoralis* cultures and occasionally in sediments. The formation of pellicle or 'hyaline cysts' has also been observed for *Cochlodinium polykrikoides* (Kim et al., 2002), *Polykrikos lebourae* (Hoppenrath and Leander, 2007), *Nematodinium* sp., and *Warnowia* sp. (Hoppenrath et al., 2009). Pellicle cysts maintain the capacity to divide at this stage, a capacity also observed for other gymnodiniales species, such as *Nematodinium* sp. (Hoppenrath et al., 2009).

4.2 Pigment profiles

Members of the Gymnodinales are the most diverse group among the Dinophyta in terms of pigment composition, ranging from the green chl *a*- and *b*-containing *Lepidodinium chlorophorum* to the haptophyte-containing chloroplasts of *Karenia mikimotoi* and *Karlodinium veneficum*. However, most *Gymnodinium* species are typically peridinin-containing dinoflagellates. The closely related *G. impudicum* (Fraga et al., 1995), *G. aureolum* (Hansen et al., 2000), *G. nolleri*, and *G . microreticulatum* (Bolch et al., 1999), and *Barrufeta bravensis* (Sampedro et al., 2011) have a similar pigment profile than *G. litoralis*.

4.3 Phylogenetic relationships

Based on LSU rDNA phylogeny, *G. litoralis* is included within *Gymnodinium sensu stricto*. It groups with other *Gymnodinium* species (including the type species) and the genera *Polykrikos*, *Lepidodinium*, and *Barrufeta*, as also determined in other studies (Hansen et al., 2007; Kim et al., 2008; Sampedro et al., 2011). Genera not included in our phylogenetic tree, such as *Paragymnodinium*, *Nematodinium*, and *Warnowia*, also belong to the *Gymnodinium sensu stricto* group (Hoppenrath et al., 2009; Kang et al., 2010). The presence of several genera within this group evidences the need to redefine the nomenclature. Furthermore, several *Gymnodinium* species, such as *G. nolleri*, *G. catenatum*, *G. trapeziforme*, and *G. microreticulatum*, are monophyletic, while *G. aureolum*, *G. impudicum*, and *G. fuscum* are included in different branches, pointing out the need to establish new genera for several species currently considered as *Gymnodinium*.

Our sequences have a 99% similarity with GenBank sequences AF200674 and EF616465. The first sequence (JL30 strain) was identified as *G. impudicum*, cultured from an isolated cyst from the Gulf of Naples (M. Montresor pers. com.), and the second as *G.* cf. *impudicum,* isolated from the Swan River Estuary (Western Australia). However, they are distant from other *G. impudicum* sequences obtained from GenBank and the LSU sequence of *G. impudicum* strain GY2VA, the type culture of the species sequenced as part of this study. Therefore, the organisms with AF200674 and EF616465 sequences were certainly misidentified and we can affirm that they belong instead to *G. litoralis*. The sequence AF200674 has been frequently used to construct phylogenetic trees in a large number of publications, causing that references to the relationship of different species with *G. impudicum* are erroneous. Misidentification of sequence AF200674 was previously suggested by Murray et al. (2007) and by Sundström et al. (2009).

4.4 *Ecology and distribution:*

The development of *G. litoralis* blooms is linked to specific conditions of temperature and salinity. The locations from the Catalan coast and the Corru S'Ittiri lagoon are areas characterized by freshwater influence, which provides nutrients to the ecosystems. Cell density of the species increases in early summer when water temperature reaches high values. In summer months hydrodynamism is low and the weather is under high pressures. The water column stability under high water temperatures can favour dinoflagellate growth. In the Catalan coast, the end of the blooms appears to be due to the decrease of temperature, but in the Corru S'Ittiri lagoon seems to be related to the strong increase of salinity. However, in spite of these concrete environmental factors that could trigger the *G. litoralis* blooms, low abundances of the species are observed under a wider range of physicochemical conditions along the Catalan coast demonstrating an adaptation to a range of environmental conditions.

5. Conclusions:

A new bloom-forming dinoflagellate is described. The horseshoe-shaped apical groove running anticlockwise around the apex, in addition to the displacement of the cingulum and peridinin as the major accessory pigment, place *G. litoralis* within the *Gymnodinium sensu stricto* genus, as currently defined. Phylogenetic relationships also place the organism within the *Gymnodinium sensu stricto* clade. However, as pointed out by several authors, e.g. Hansen

(2001), Yamaguchi et al. (2011), differences between some species and the polyphyletic nature of the genus suggest its division into new and different genera. The similarities of the morphological characters from different species together with difficulties in studying them using light microscopy highlight the need for a combination of taxonomic studies to distinguish similar organisms. Molecular phylogeny is a powerful tool when morphological characters do not easily differentiate between organisms. The robust results obtained in this study clearly show that *G. litoralis* and *G. impudicum* are distinct species. The detection of *G. litoralis* in different sites of the NW Mediterranean and in W Australia suggests a wide distribution of this organism. The fact that *G. litoralis* coastal blooms are frequent, intense and noticeable implies that the species is relevant in coastal waters.

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Barcelona (Catalan coast)

Chapter 4

"*Polykrikos tanit* sp. nov., a new mixotrophic unarmoured pseudocolonial dinoflagellate from the NW Mediterranean Sea"

Protist (Accepted)

POLYKRIKOS TANIT SP. NOV., A NEW MIXOTROPHIC UNARMOURED PSEUDOCOLONIAL DINOFLAGELLATE FROM THE NW MEDITERRANEAN SEA

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Abstract:

Pigmented pseudocolonies initially identified as *Polykrikos hartmannii* Zimmermann were detected at several locations of the Catalan coast (NW Mediterranean Sea) in April–June of 2012 and April–May of 2013. To further explore the several remarkable morphological discrepancies between these organisms and *P. hartmannii*, we carried out a detailed morphological study and used single-cell PCR to obtain partial LSU and SSU rDNA sequences. The resulting phylogenies showed that our isolates occupy a basal position within the *Polykrikos* clade, close to *P. hartmannii*, but do not correspond to any described polykrikoid species. *P. barnegatensis* Martin is controversially considered to be synonymous with *P. hartmannii*. The organisms studied in this work were similar to *P. barnegatensis* but showed significant morphological differences with its original description such as the torsion of the pseudocolony, more pronounced overhanging of the cingula, stepped fusion border of the zooids, and number and shape of nuclei. Consequently, we propose that the isolates constitute a new species, which we named *Polykrikos tanit* sp. nov. The observed characters, pigmented, same number of zooids and nuclei, sulci not fused, and its phylogeny suggest that the species is an early evolutionary *Polykrikos* species.

1. Introduction:

The polykrikoid organisms are included within the Gymnodiniales sensu stricto clade (Hoppenrath and Leander 2007a, b; Kim et al. 2008) and are grouped within two genera: *Polykrikos*, erected by Bütschli (1873), and *Pheopolykrikos*, erected by Chatton (1933) and later emended by Matsuoka and Fukuyo (1986). Both genera comprise unarmoured multinucleated pseudocolonial organisms and autotrophic as well as heterotrophic species are known.

Polykrikos species were primarily characterized on the basis of their even number of zooids, with half the number of nuclei. Every zooid has its own cingulum and a pair of flagella. The sulci of both zooids are fused. In addition to the type species of the genus, *P. schwartzii* Bütschli, members include *P. kofoidii* Chatton, *P. lebourae* Herdman emend. Hoppenrath et Leander, *P. herdmanae* Hoppenrath et Leander, and *P. hartmannii* Zimmermann. The validity of *P. grassei* Lecal is highly dubious and *P. auricularia* Bergh is considered to be synonymous with *P. schwartzii*. *P. barnegatensis* was erected by Martin (1929) following observations of a single specimen from Barnegat Bay (USA). This two-zooid pseudocolonial photosynthetic organism contains only a single, large nucleus and lacks nematocysts. It was synonymized with *P. hartmannii* by Chatton (1952), although he described two nuclei. However, this nomenclature was not adopted by Hulburt (1957) because of the difference in the number of nuclei. Nevertheless, the synonymy of *P. barnegatensis* with *P. hartmannii* is accepted (Gómez 2012; Guiry and Guiry 2013), albeit with uncertainty (Hoppenrath and Leander 2007a).

The genus *Pheopolykrikos* comprises *Ph. beauchampii* Chatton as the type species, characterized by having four zooids and four nuclei. Although the validity of the genus has been discussed by several authors (Dodge 1982; Loeblich III 1980; Sournia 1986), *Pheopolykrikos* species are defined as having the same number of nuclei as zooids and forming phototrophic pseudocolonies that are able to dissociate (Matsuoka and Fukuyo 1986). *P. hartmannii* was transferred into *Pheopolykrikos* because it agreed with all previously cited characteristics (Matsuoka and Fukuyo 1986). However, phylogenetic analyses of LSU rDNA (Hoppenrath and Leander 2007a; Kim et al. 2008) and SSU rDNA (Hoppenrath and Leander 2007a, b) sequences subsequently showed that *P. hartmannii* clusters with *Polykrikos* species independently of *Ph. beauchampii*, thereby confirming the validity of the *Pheopolykrikos* genus. Moreover, those results together with ultrastructural studies led to the re-classification of *Pheopolykrikos hartmannii* as *Polykrikos hartmannii* (Hoppenrath et al. 2010), although this nomenclature has not been adopted by all authors (Tang et al. 2013)

Recently, in samplings carried out at several locations along the Catalan coast (NW Mediterranean Sea), we detected pigmented pseudocolonies comprising two zooids. Detailed morphological observations and partial LSU and SSU rDNA sequencing were used to determine whether these organisms were *P. hartmannii* or *P. barnegatensis* or constituted a new species. In this study, we show that our isolates indeed belong to a new species, which we have named *Polykrikos tanit* sp. nov.

2. Material and methods:

2.1 Detection locations, isolation, and morphological observations:

Observations. The target specimens were detected in sub-surface live samples collected from Arenys (41º51’29’’ N; 2º33’20.5’’ E) and Vilanova (41º12’55’’ N; 1º 43’ 50’’ E) Harbours as well as L’Estartit beach (42º 2’ 47’’ N; 3º 11’ 53’’ E) and offshore of Barcelona (41º 22’ 35’’ N; 2º 12’ 41’’ E) (Catalan Coast, NW Mediterranean Sea) at the end of May–June 2012 and from Arenys Harbour in April–May 2013. Random volumes of live samples were concentrated using a 10-µm mesh. The organisms in these filtered samples were observed in a settling chamber under a Leica-Leitz DM-Il inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Sony NEX-5 digital camera (Sony, Tokyo, Japan) and under a phase-contrast Leica DM IRB inverted microscope connected to a ProgRes C10 (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) digital camera.

Culturing. Several attempts were made to culture the organisms. Specimens were isolated and placed in culture wells filled with either L1 medium at a salinity of 37 or filtered seawater from the same sample, or non-filtered seawater to provide potential prey. However, all of these attempts were unsuccessful. A set of prey ranging from 1 to 20 µm was also added to the isolated specimens, with same unsuccessful results.

Epifluorescence microscopy. To determine the number of nuclei in each pseudocolony, live specimens were placed in a slide, stained with 1:100 Sybr Green (Molecular Probes, Eugene, OR, USA) in 0.01 M PBS, pH 7.4, for 20 min, and observed in an epifluorescence Leica-Leitz DM-Il inverted microscope through a blue filter. Chloroplast autofluorescence was observed directly on unstained live specimens through the same blue filter.

Scanning electron microscopy. Concentrated natural samples (5–10 ml) were fixed for 15 min at room temperature with an adequate volume of 4% osmium tetroxide to reach a final concentration of 2%. The sample was gravity-filtered through a Nucleopore (Pleasanton, CA, USA) polycarbonate filter (13 mm diameter, pore size 8 µm). The filtered cells were then washed in distilled water, dehydrated for 10 min each in a 25, 50, 75, 95, and 100% ethanol series, and critical-point dried. The filters were mounted on stubs, sputter-coated with gold, and examined with a JEOL JSM-6500F scanning electron microscope (JEOL-USA Inc., Peabody, MA, USA).

2.2 Single-cell PCR amplification, sequencing, and phylogenetic analyses:

Each target pseudocolony was transferred several times into filtered seawater drops using Pasteur pipettes, then transferred to 200-µl PCR tubes, adding the minimum volume of seawater, subjected to several rounds of freezing/thawing, and finally stored at -80 ºC until processed. Single-cell PCR was conducted with a PCR mixture containing 5 ml of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 mM of the primers D1R and D2C (Scholin et al. 1994) for the partial LSU region and the primers EUK A (Medlin et al. 1988) and 1209R (Giovannoni et al. 1988) for the partial SSU region. The PCR conditions for LSU were as follows: initial denaturation for 5 min at 95 ºC, 40 cycles of 20 s at 95 ºC, 30 s at 55 ºC, and 1 min at 72 ºC, followed by a final extension step for 7 min at 72 ºC. The PCR conditions for SSU were: initial denaturation for 5 min at 95 ºC, 30 cycles of 45 s at 95 ºC, 1 min at 55 ºC, and 3 min at 72 ºC, followed by a final extension step for 10 min at 72 ºC. Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and then visualized under UV illumination. The remainder of the sample was frozen at -20 ºC and later used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using both forward and reverse primers and a 3730XL DNA sequencer.

The obtained sequences were aligned with those from GenBank using the MAFFT v.6 program (Katoh et al. 2002) under G-INS-i and manually checked with BioEdit v. 7.0.5 (Hall 1999), obtaining a final alignment of about 830 positions for LSU sequences and 1760 positions for SSU sequences. In both cases, phylogenetic relationships were determined using maximum-likelihood (ML) and Bayesian inference methods. For the former, the GTRGAMMA evolution model was used on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis 2006). All model parameters were estimated by RAxML. Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RAxML software. The Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al. 2012), run with a GTR model in which the rates were set to gamma. Each analysis was performed using four Markov chains (MCMC), with one million cycles for each chain. The consensus tree was created from post-burn-in trees and the Bayesian posterior probabilities (BPP) of each clade were examined.

3. Results:

***Polykrikos tanit* sp. nov.**

3.1 Morphology

Unarmoured pseudocolonies consisted of two zooids. They were 46–76 µm long and 26–50 µm wide with a length:width ratio of 1.4–2 (n=20). Pseudocolonies were ovate and nearly circular in cross-section. The sides of the pseudocolonies were convex, with a constriction at the junction of the two zooids (Fig. 1A, B; 2A). The border of this junction was stepped, with the left side being higher than the right one (Fig. 1A, B; 2A, B). The epicone of the anterior zooid was round (Fig. 1A) to conical (Fig. 1F; 2A, B). The apex was blunted and protuberant (Fig. 2A, B). The hypocone of the posterior zooid was round, slightly bilobated, and the antapex was flattened. Pseudocolonies showed torsion of the cell body to the left (Fig. 2A). The cingula were displaced about two to three times their width, with overhanging ends (Fig. 1A, B; 2A, B). A large peduncle was always present in each zooid, emerging from the upper intercingular area (the area where both ends of the cingulum meet) (Fig. 2A, B, C). Peduncles were present in all specimens observed by SEM (n=30), but we never observed them under LM. The sulci ran obliquely from right to left (Fig. 2B) and had a sigmoid outline resulting from the overhanging cingula (Fig. 1A, B; 2A). The sulcus of the posterior zooid reached the antapex, where it widened. The sulcal anterior end of the anterior zooid penetrated the epicone, in contact with the acrobase, which formed a closed anti-clockwise loop around the apex that re-joined the sulcus, although at a lower position than its proximal end (Fig. 2A, B, D). The sulci of both zooids were not fused and ran independently. Pseudocolonies had two nuclei, one for each zooid, located in the hypocone of the anterior zooid and the epicone of the posterior zooid (Fig. 1C, E). The nuclei were ovate to horizontally lenticular and nearly touched. Some pseudocolonies contained only a single nucleus, located centrally (Fig. 1D). Pseudocolonies had a yellow-greenish colouration, apparently with numerous small ovate chloroplasts (Fig. 1F, G). Neither nematocysts nor taeniocysts were observed. Large ingestion bodies were commonly seen in the posterior zooids (Fig. 1H), in some cases displacing the nuclei. Cyst formation was never observed.

The pseudocolonies differed in width among specimens. Some were wider, with a lower length:width ratio (≤ 1.5), while others were narrower, with a higher length:width ratio (> 1.6). In the wider pseudocolonies (Fig. 1H; 2A) the cingula were more deeply overhanging and torsion was greater than in the narrower pseudocolonies (Fig. 1A; 2B). Both morphologies were observed in natural samples, but "healthy" specimens and those possessing visible ingestion bodies tended to be wider, whereas isolated specimens maintained for a few weeks and exhibiting a loss of pigmentation tended to be narrower. Single zooids were only observed once, when an isolated pseudocolony dissociated. These were 29–32 µm long and 26–27 µm wide (Fig. 1I), almost round but with a blunted apex. The cingulum was median, displaced three times its width, with overhanging ends. The sulcus was sigmoid, widening in the antapex.

Table 1: GenBank accession numbers, locations, and dates of isolation of *P. tanit* and the region targeted for SC-PCR for each DNA sequence obtained in this study.

Accession number	Species	Location	Isolation Date	Target region
KF806598	*Polykrikos tanit*	Arenys Harbour	May-2012	SSU rDNA
KF806599	*Polykrikos tanit*	Arenys Harbour	April- 2013	SSU rDNA
KF806600	*Polykrikos tanit*	Vilanova Harbour	May-2012	LSU rDNA
KF806601	*Polykrikos tanit*	Arenys Harbour	May-2012	LSU rDNA
KF806602	*Polykrikos tanit*	Offshore Barcelona	June-2012	LSU rDNA

3.2 Phylogeny:

The sequences obtained for the partial LSU rDNA region were ~690 bp (KF806602 was ~585 bp) and ~1340 bp for the partial SSU rDNA region (Table 1). All the sequences of each region were identical, except KF806602, which differed in 1 position with the other LSU rDNA sequences obtained. The ML phylogenetic trees were constructed with representatives of the Gymnodiniales sensu stricto clade and other unarmoured species not included in it. *Polarella glacialis* was used as outgroup for both SSU rDNA and LSU rDNA phylogenetic trees.

The tree obtained for SSU rDNA sequences showed that all polykrikoid species were included within the Gymnodiniales *sensu stricto* clade (100% bootstrap / 1 BPP) (Fig. 3). All *Polykrikos* species clustered together (94%/1) and only *Ph. beauchampii* clustered independently. *P. tanit* occupied a basal position in the clade, as did *P. hartmannii*, although its position was not totally resolved. The remaining species were included in a subclade (87%/0.99) containing on the one hand *P. kofoidii* and *P. schwartzii* (100%/1) and on the other *P. lebourae* and *P. herdmanae* (100%/1).

The phylogenetic position of *P. tanit* based on its LSU rDNA sequences (Fig. 4) agreed with that obtained for the SSU region. All polykrikoid species were included within the Gymnodiniales *sensu stricto* clade (95%/1). In this case though, the *Polykrikos* clade was not obtained. *Pheopolykrikos beauchampii* (sequence named in GenBank as *Polykrikos beauchampi*) clustered independently, as well as *Polykrikos lebourae*. The clade containing the remaining species was not consistently supported. Species were grouped in two sub-clades, one containing *P. kofoidii* and *P. schwartzii* (100%/1) and the other containing *P. hartmannii* (sequence named in GenBank as *Pheopolykrikos hartmannii*) and the sequences obtained during this study.

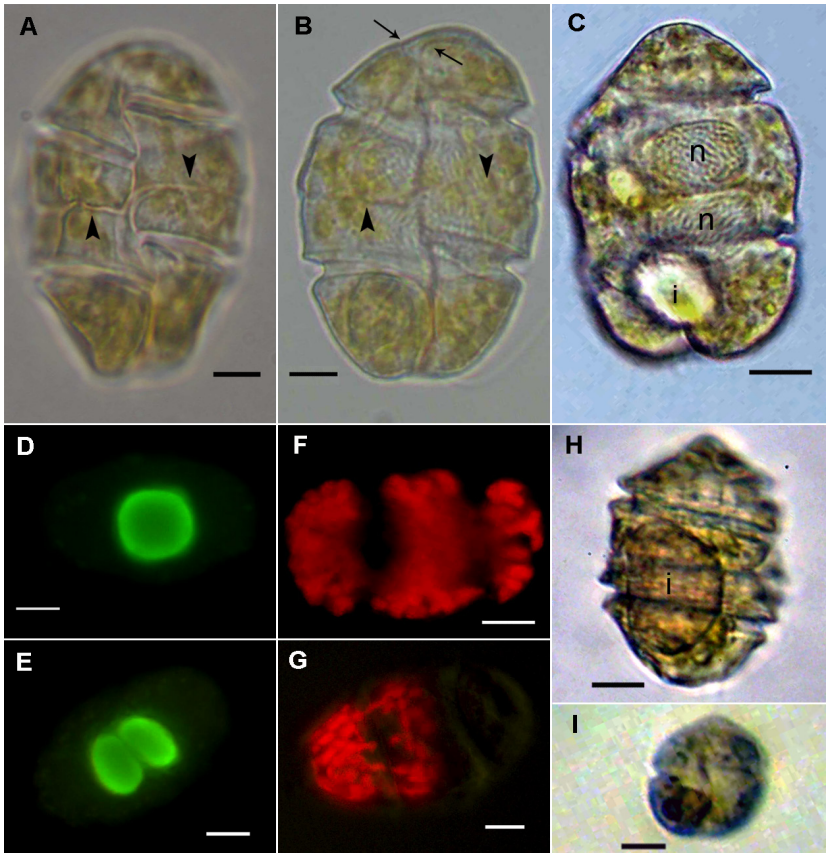

Figure 1: Light microscopy images of *P. tanit* sp. nov. A) and B) Ventral view of pseudocolonies. Note the sigmoid outline of the sulci and the stepped junction of the two zooids (arrowheads). Arrows indicate the acrobase. C) Left lateral view of a pseudocolony showing two nuclei (n) and an ingestion body (i). Epifluorescence images of *P. tanit* sp. nov. D) Pseudocolony with one nucleus stained with Sybr Green. E) Pseudocolony with two nuclei stained with Sybr Green. F) and G) Images showing the shape and distribution of the autofluorescent plastids. Light microscopy images of *P. tanit* sp. nov. H) Right lateral view of a pseudocolony showing a large ingestion body (i). I) Ventral view of a single zooid. Scale bars = 10 µm.

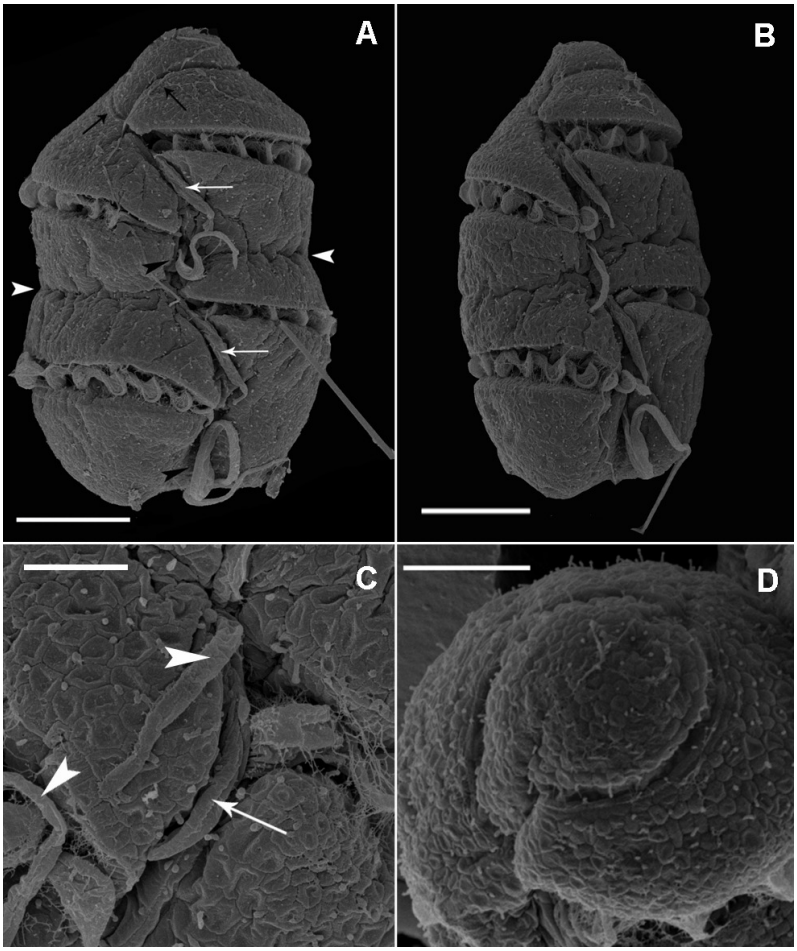

Figure 2: Scanning electron microscopy images of *P. tanit* sp. nov. A) and B) Ventral view of pseudocolonies, showing the acrobase (black arrows), the stepped junction of the two zooids (white arrowheads), and the presence of peduncles in both zooids (white arrows). Black arrowheads point to the longitudinal flagella. C) Detail of the intercingular area of the posterior zooid of a pseudocolony. The peduncle (arrow) is inserted in the proximal end of the cingulum. Arrowheads point to both longitudinal flagella. D) Apical view of the apex, showing the detail of the acrobase. Scale bars = A) and B) 10 µm; C) 5 µm; D) 2.5 µm.

3.3 Occurrence:

Specimens primarily identified as P. *hartmannii* were detected occasionally at abundances <100 cells•L^{-1} during the spring and summer months in samplings carried out along the Catalan coast as part of the Monitoring of Harmful Phytoplankton Programme. However, the fact that they deformed when fixed prevented the confirmation of their identity. In this study, further observations of live specimens allowed the unequivocal detection of *P. tanit* during 2012 and 2013. In 2012, the species was detected at Arenys and Vilanova harbours as well as at L'Estartit beach and 1.5 km offshore of Barcelona during May–June. In 2013, we only sampled Arenys Harbour but detected *P. tanit* from the beginning of April well into May. Abundances at all locations never reached 10^3 cells•L^{-1}. The water temperature from all localities ranged from 14 to 22 °C, with a salinity of 31.2–37.8.

4. Discussion:

4.1 Morphological comparison:

A detailed comparison of similar pigmented polykrikoid species is provided in Table 2. Studies of a large number of pseudocolonies of *P. tanit* distinguished two morphologies, probably related to "fed" vs. "starved" states, but only pseudocolonies consisting of two zooids were observed, albeit they were able to dissociate. Consequently, our newly isolated specimens resembled *P. hartmannii* or *P. barnegatensis* in the number of zooids.

P. tanit differs from *P. hartmannii* in several characters. The pseudocolonies of these species are different in shape. The left torsion of *P. tanit* pseudocolonies creates an oblique outline of its sulci (Fig. 5A). This curvature is a common feature of most *Polykrikos* species but according to morphological descriptions of *P. hartmannii* (Hoppenrath et al. 2010; Hulburt 1957; Matsuoka and Fukuyo 1986; Zimmermann 1930) the outline of the sulci is commonly straight and there is no torsion (Fig. 5D), although some images from the literature show an outline slightly sigmoid (Fig. 1C in Kim et al. (2008), Fig. 2A in Hoppenrath et al. (2010), Fig. 2B in Tang et al. (2013)). *Ph. beauchampii* (Chatton 1933), which is characterized by four zooids and four nuclei, also show a straight outline of the sulci, with no torsion. Furthermore, for *P. tanit*, overhanging of the ends of the cingula by a variable degree was common, especially in pseudocolonies exhibiting greater torsion, and resulted in the sigmoid outline of the sulci. None of the *P. hartmannii* depictions show overhanging cingula. The presence of food vacuoles has been observed in *P. hartmannii* (Omura et al. 2012), as well as the presence of nematocyst-taeniocyst complexes (Hoppenrath et al. 2010). However, the presence of peduncles has never been observed, while its presence in the intercingular area of zooids was confirmed in scanning electron microscopy (SEM) observations of all *P. tanit* pseudocolonies. Ingestion bodies were also frequently seen in these specimens. Furthermore, our inability to obtain cultures from newly isolated pigmented specimens suggested that it might be an obligated mixotroph organism, as also assumed for the pigmented *P. lebourae* (Hoppenrath and Leander 2007b). Peduncles are feeding appendages (Gaines and Elbrächter 1987), and they have been described in pigmented species such as *Amphidinium cryophilum* (Wedemayer et al. 1982), *Akashiwo sanguinea* or *Gyrodinium instriatum* (Gaines and Elbrächter 1987), in heterotrophic species such as *Gyrodinium lebourae* (Lee 1977) or *Gyrodiniellum shiwhaense* (Kang et al. 2011), and in parasitic species such as *Amyloodinium* spp. (Landsberg et al. 1994). The constant presence of the peduncles supports the mixotrophy of this newly established species. A finger-like structure was observed in the flagellar area of each *P. kofoidii* zooid, but only in gametes; it was therefore assumed to be a "copulation globule", involved in supporting the contact and fusion of gametes (Tillmann and Hoppenrath 2013).

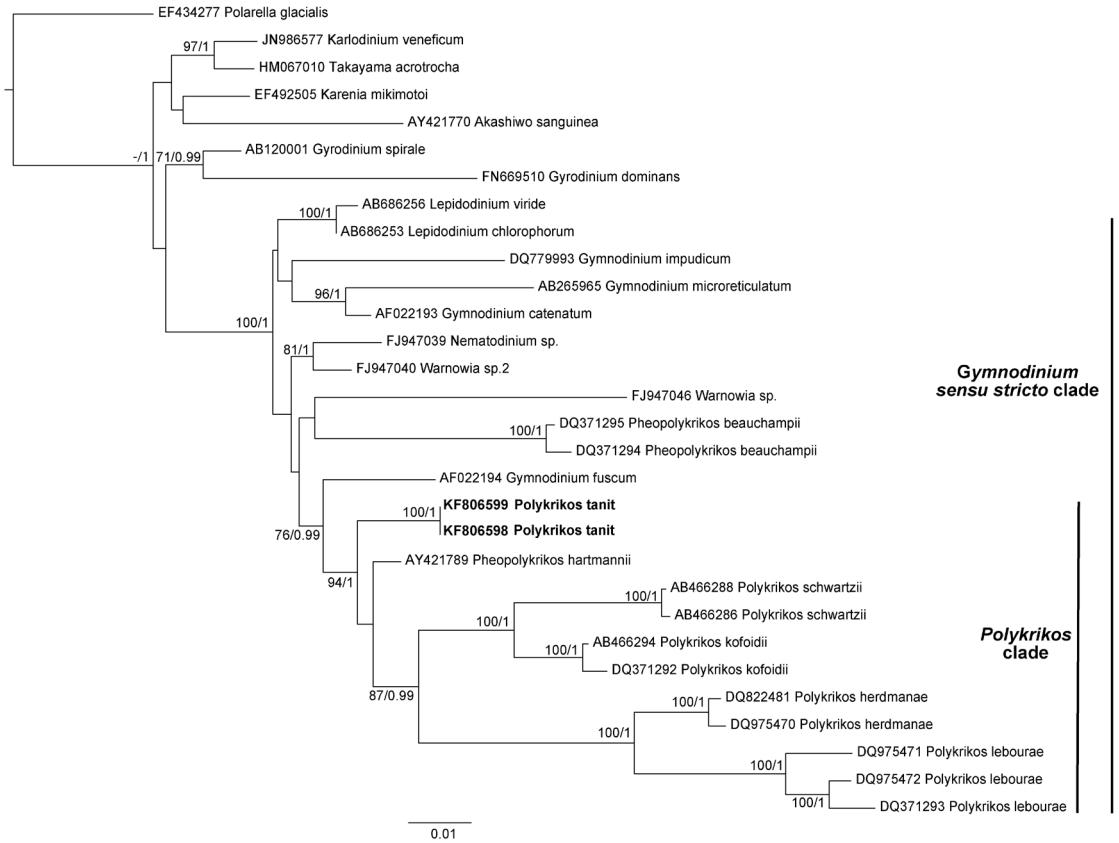

Figure 3: Maximum-likelihood phylogenetic tree of selected species based on the partial SSU rRNA. Numbers on the nodes are the bootstrap values (%) followed by the Bayesian Posterior Probabilities (BPP). Only bootstrap values >70 and BPP >0.9 are shown. *Polarella glacialis* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

P. barnegatensis was described after the observation of only one specimen but it significantly differs from our specimens (Fig. 5C). There was no torsion in the pseudocolony described by Martin (1929), although the sulci had an oblique outline. A slightly overhanging cingulum was depicted albeit only for the posterior zooid. All specimens of *P. tanit* showed a stepped fusion border of the zooids, while in *P. barnegatensis* the fusion border was not stepped. Other polykrikoid species showed a slight degree of stepped borders, as *Ph. beauchampii* (Chatton 1933) or *P. kofoidii* and *P. schwartzii* (Matsuoka et al. 2009). *P. barnegatensis* was described as having one large beaded nucleus and two zooids. Pseudocolonies of *P. tanit* possess two nuclei, but specimens with only one nucleus were occasionally seen. The life cycles of polykrikoid species are largely unknown, but the complex life cycle of *P. kofoidii*, including stages with only one nucleus, was recently described (Tillmann and Hoppenrath 2013). Thus, in *P. tanit* pseudocolonies the different number of nuclei could represent different life cycle stages. Nevertheless, the two nuclei were nearly in contact with one another and overlapped with the fusion border of the two zooids. In specimens with only one nucleus, it was never vertically elongated but spherical and centrally located. Correct nuclear discrimination is problematic using common light microscopy methods; consequently, the original description of *P. barnegatensis* may be incorrect. Nonetheless, the depicted shape of the nucleus clearly differs from the nuclei of *P. tanit*. Finally, *P. barnegatensis* is thought to be autotrophic and ingestion bodies are absent. Thus, considering the differences between *P. tanit* and *P. barnegatensis*, i.e., the stepped junction of the zooids, the number and shape of the nuclei, the torsion of the cell body, and the more pronounced overhanging of *P. tanit* cingula, they cannot be considered as the same species.

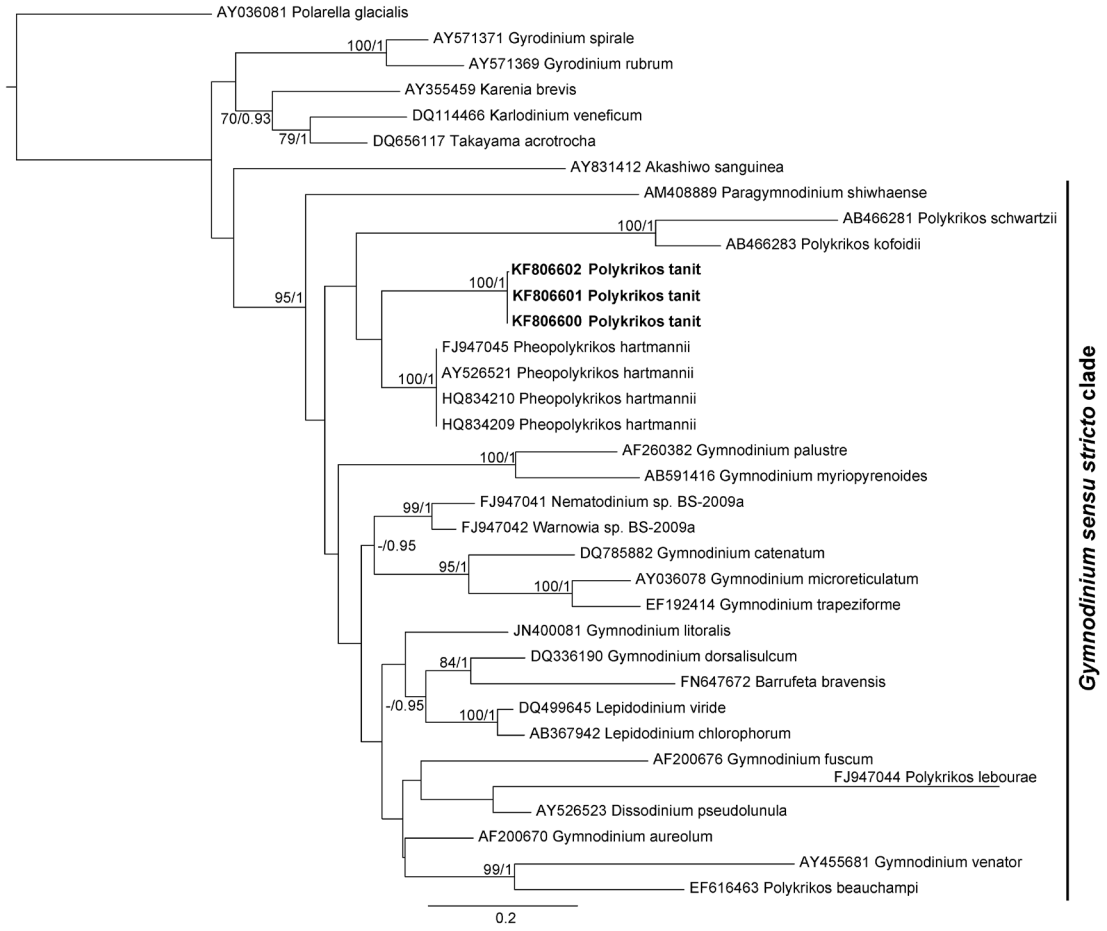

Figure 4: Maximum-likelihood phylogenetic tree of selected species based on the D1–D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap values (%) followed by the Bayesian Posterior Probabilities (BPP). Only bootstrap values >70 and BPP >0.9 are shown. *Polarella glacialis* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

P. barnegatensis was also reported by Chatton (1952) (Fig. 5B), who did not provide a description of this species, but only a depiction. Although the species was originally described as having one nucleus and two zooids, his drawing showed an organism with two nuclei and two zooids, based on his assumption that the original description was incorrect. Erroneously *P. barnegatensis* was considered to be synonymous with *P. hartmannii* (Chatton 1952). The depicted pseudocolony showed torsion of the cell body, slightly overhanging cingula and a stepped fusion border of zooids. The sulci of the anterior and posterior zooids of the specimen depicted by Chatton (1952) were not connected one another, as demonstrated for *P. hartmannii* (Tang et al. 2013). Based on light microscopy observations, that was our initial impression of *P. tanit* (Fig. 1A) and SEM images confirmed that the sulci were not fused, in contrast to other *Polykrikos* species as *P. kofoidii*, *P. schwartzii* and *P. lebourae*. The presence and shape of the acrobase was also shown in the depiction provided by Chatton (1952) but it was not shown in that published by Martin (1929). In *P. barnegatensis sensu* Chatton the acrobase forms a horizontally elongated closed loop around the apex, as observed for *P. kofoidii* and *P. schwartzii* (Nagai et al. 2002), *P. hartmannii* (Takayama 1985), and *P. tanit*. However, the acrobase of *P. lebourae* (Hoppenrath and Leander 2007b) and *Ph. beauchampii* (Omura et al. 2012) is droplet-shaped. According to Chatton (1952), *P. barnegatensis* contains two large ingestion bodies and its nuclei are displaced to one side of the cell. The position of the food vacuoles and the possibility to displace the nucleus was in agreement with our observations of *P. tanit*. He also showed an invagination in the intercingular area, possibly misinterpreting the structure of the peduncles. Thus, although Chatton (1952) considered his organism as *P. barnegatensis*, its morphology better suggests *P. tanit*.

The studied specimens showed too many discrepancies with *P. barnegatensis* to consider *P. barnegatensis* and *P. tanit* as the same species. However, the similarities of both species and their differing characters with *P. hartmannii* suggest that *P. barnegatensis* represent a different species and it should not be considered as a synonym of *P. hartmannii*.

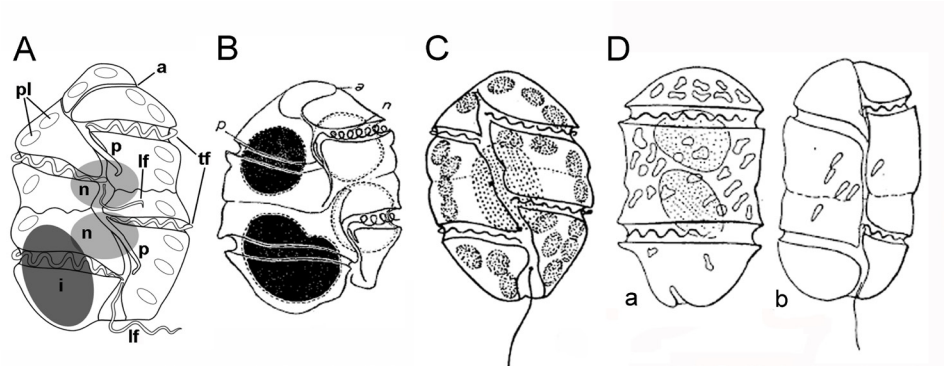

Figure 5: Schematic drawings of A) *Polykrikos tanit* sp. nov. Note the acrobase (a), nucleus (n), chloroplasts (pl), peduncle (p), ingestion body (i), longitudinal flagellum (lf) and transverse flagellum (tf). B) *P. barnegatensis* [from Chatton (1952)]. Note the acrobase (a), nucleus (n) and ingestion body (p). C) *P. barnegatensis* [from Martin (1929)]. D) Dorsal (a) and ventral (b) views of P. *hartmannii* [from Zimmermann (1930)]. Drawings are not to scale.

4.2 Distribution:

Chatton (1952) did not provide any information about where the specimen of *P. barnegatensis* was obtained, but we can assume that it was near Thau Lagoon (France, NW Mediterranean Sea), located about 200 km northern of Arenys Harbour. The type locality of *P. hartmannii* is Naples Bay (Mediterranean Sea) and that of *Ph. beauchampii* is Thau Lagoon. To the best of our knowledge, the original descriptions of both species refer to the unique detections from the Mediterranean Sea reported in the literature (Gómez 2003), although resting cysts similar to those of *P. hartmannii* have been also reported in southeastern Italy (Moscatello et al. 2004). Nevertheless, our observations of *P. tanit* agree with the fact that small, pigmented polykrikoid species are well represented in the NW Mediterranean Sea. The relatively wide ranges of salinity and temperature recorded during *P. tanit* sample collection are the characteristics of

the spring and summer along the Catalan coast and are in contrast with the restricted period of detection. Consequently, and given the different characteristics of the locations where *P. tanit* has been reported, this species may also be present along the Catalan coast during the summer months, but at very low abundances.

4.3 Evolutionary characters:

The phylogenies obtained in this study are in agreement with those of previous studies that focused on the phylogenetic relationship of polykrikoid organisms (Hoppenrath and Leander 2007a, b; Matsuoka et al. 2009). In this study, both the LSU and the SSU rDNA phylogenies unequivocally support our specimens as a different species of those previously sequenced. It is phylogenetically distant from *Ph. beauchampii*, the unique current representative of the genus *Pheopolykrikos*, and therefore belongs to the genus *Polykrikos*, as previously demonstrated for *P. hartmannii*. However, a common character of *Polykrikos* species is the presence of taeniocyst-nematocyst complexes. Although these have yet to be observed for *P. tanit* their presence cannot be ruled out because their detection in pigmented species is challenging (Hoppenrath et al. 2010).

P. tanit is phylogenetically and morphologically close to *P. hartmannii* and both species occupy basal positions, conforming to an early sister clade within the *Polykrikos* clade (Hoppenrath et al. 2010). While the LSU rDNA phylogeny does not completely resolve this clade, the SSU rDNA sequences place *P. tanit* in a basal position. Like *P. hartmannii*, the sulci of the two *P. tanit* zooids are not fused and the dinoflagellate contains chloroplasts, which were lost in subsequent species along with the fusion process of the sulci of the zooids. Previous studies suggest that photosynthesis was regained in *P. lebourae* (Hoppenrath and Leander 2007b). Furthermore, *P. tanit* has the same number of nuclei and zooids, although specimens with only one nucleus were occasionally observed, in agreement with the hypothesis of zooid doubling during evolution (Hoppenrath and Leander 2007a). The lack of nematocyst-taeniocyst complexes, which as noted above could not be confirmed for *P. tanit*, can also be considered as an early evolutionary character. The mixotrophy of *P. tanit* supports the assumption suggested by Hoppenrath and Leander (2007a) that during evolution the development of heterotrophy was accompanied by a loss of photosynthetic capability in polykrikoid organisms.

Provided that studied specimens differ from previously described *Polykrikos* species both morphologically and phylogenetically, we describe the studied specimens as a new species.

Polykrikos tanit sp. nov. Reñé

(= *Polykrikos barnegatensis sensu* Chatton 1952, Fig. 243b)

Description: Unarmoured pseudocolonies (46–76 µm long; 26–50 µm wide) consisting of two zooids and usually two (sometimes one) nuclei located centrally in the pseudocolonies, which are ovate, almost circular in cross-section, and exhibit torsion to the left. The fusion border of the two zooids is visible and stepped. Closed loop-shaped acrobase. Sulci not fused, with sigmoid outline. Descending and overhanging cingula, displaced two–three times their width. Each zooid has its own longitudinal and transverse flagellum and a peduncle in the intercingular area. Mixotrophic.

Etymology: named after Tanit, a Punic goddess worshiped in the Western Mediterranean until the 2nd century A.D., in reference to both the early evolutionary position of the species within the genus and its type locality.

Holotype: Figure 5A. A SEM-stub was deposited in the Electronic Microscopy Laboratory of the Institut of Ciències del Mar (ICM-CSIC) from Barcelona, under the code 20130423-AR.

Isotype: Figure 2A.

Type habitat: Marine planktonic.

Type locality: Arenys Harbour, Catalonia, NW Mediterranean Sea (41º51’29’’ N; 2º33’20.5’’ E).

Distribution: NW Mediterranean Sea.

Gene sequences: Sequences have been deposited in GenBank under the accession numbers KF806598-KF806599 for SSU rDNA and KF806600 TO KF806602 for LSU rDNA.

Table 2: Morphological traits of *Polykrikos tanit* sp. nov. and related species, as provided by available studies.

	P. tanit sp. nov.[a]	*P. barnegatensis*[b]	*P. barnegatensis*[c]	*P. hartmannii*[d, e, f]	*Ph. beauchampii*[g, h]	*P. lebourae*[i]
Pseudocolony length (µm)	46-76	46	-	60-100	100-120	37.5-90
Pseudocolony width (µm)	26-50	31.5	-	42-59	60-75	20-50
Pseudocolony shape	ovate	ovate	ovate	barrel-shaped	barrel-shaped	ovate
Pseudocolony compression	no	no	-	dorsoventral, sometimes longitudinal[j]	dorsoventral	obliquely flattened
Number of zooids per pseudocolony	2	2	2	2	4	8
Number of nuclei per pseudocolony	usually 2	1	2	2	4	2
Dissociation into single zooids	yes	-	-	yes	yes	no
Displacement of cingula	2-3 times its width	2 times its width	2-3 times its width	1-2 times its width	1-2 times its width	1-2 times its width
Overhanging cingula	yes	no	yes	no, sometimes yes[f, k]	no	no
Apical groove	loop-shaped	-	loop-shaped	loop-shaped	loop-shaped[l]	loop-shaped
Fusion border of zooids	stepped	straight	stepped	straight	slightly stepped	fused
Fusion of sulci	no	yes (?)	no	no	no	yes
Plastids	yes	yes	yes	yes	yes	yes
Mixotrophic	yes	no	yes	probably yes[k]	no	yes
Peduncle	yes	-	-	no[j, l]	no	no
Taeniocyst-nematocyst complexes	-	-	-	yes	yes, but not confirmed	sometimes
Cysts	-	-	-	yes	-	hyaline vegetative

[a] This study; [b] Martin (1929); [c] Chatton (1952); [d] Hulburt (1957); [e] Matsuoka and Fukuyo (1986); [f] Hoppenrath et al. (2010); [g] Chatton (1933); [h] Hoppenrath and Leander (2007a); [i] Hoppenrath and Leander (2007b); [j] Tang et al. (2013); [k] Omura et al. (2012); [l] Takayama (Personal website). – not observed or not detailed.

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The mouth of the river Fluvià (Catalan coast)

Chapter 5

"Phylogenetic relationships of *Cochlodinium polykrikoides* Margalef (Gymnodiniales, Dinophyceae) from the Mediterranean Sea and the implications of its global biogeography"

Harmful Algae (2013)

PHYLOGENETIC RELATIONSHIPS OF *COCHLODINIUM POLYKRIKOIDES* MARGALEF (GYMNODINIALES, DINOPHYCEAE) FROM THE MEDITERRANEAN SEA AND THE IMPLICATIONS OF ITS GLOBAL BIOGEOGRAPHY

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Abstract:

Although the diversity of dinoflagellates has been intensively studied in several locations in the Mediterranean Sea since the 1950s, it is only during the last two decades that the morphotype of the toxic unarmoured dinoflagellate *Cochlodinium polykrikoides* Margalef has been detected, coinciding with its apparent worldwide expansion in marine coastal waters. In this study, vegetative cells of *C. polykrikoides* morphotype from the Catalan coast (NW Mediterranean Sea) were detected and isolated, and the DNA from collected cells was sequenced. While in the Mediterranean Sea, detections are scarce and *C. polykrikoides* is consistently present at low concentrations, we reported exceptional blooms of this species, in which the maximum abundance reached $2 \cdot 10^4$ cells$\cdot L^{-1}$. Partial LSU rDNA region sequences showed that most *C. polykrikoides* populations from the Catalan coast formed a new differentiated ribotype, but others were included within the 'Philippines' ribotype, demonstrating their coexistence in the Mediterranean Sea. Thus, the current biogeographic nomenclature of the ribotypes is likely to be invalid with respect to the available information from populations comprising the 'Philippines' ribotype. The phylogeny suggests the existence of cryptic species that should be evaluated for species-level status. Accordingly, the ribotype determination must be carefully evaluated for all detections and bloom events, since accurate characterization of the morphology, ecophysiology and distribution of the ribotypes are not well resolved.

1. Introduction:

In phytoplankton, there are several examples in which high genotypic and phenotypic variability has been evidenced within what is considered to be the same species. Consequently, biogeographic information for phytoplankton is thus far limited since it requires accurately defined species. Phylogenetic studies on diverse microalgae have revealed that several traditional morphospecies are genetically distinct at a geographic scale, e.g., *Emiliania huxleyi* (Hagino et al., 2011), while cryptic species coexist in some locations, as shown for the *Pseudo-nitzschia delicatissima* complex (Quijano-Scheggia et al., 2009). Proper identification of the target species, including those that are toxic or noxious, is therefore often hindered. However, their detection is crucial due to the worldwide threat posed by harmful algae to human health, aquaculture, wild life, and ecosystem functioning. Furthermore, the identification of some species is extremely difficult when based only on fixed samples, which highlights the importance of using molecular methods to identify and quantify toxic and noxious organisms.

Cochlodinium polykrikoides Margalef is a bloom- and chain-forming unarmoured dinoflagellate responsible for high mortalities of wild and farmed fish (Kim, 1998). The first blooms of *C. polykrikoides* were reported prior to 1990 in Southeast Asia (Kim, 1998; Yuki and Yoshimatsu, 1989) and along the east coast of North America (Ho and Zubkoff, 1979; Tomas and Smayda, 2008). Since then, blooms of this species have expanded to the East China Sea, the Philippines, Malaysia, the west coast of North America, Costa Rica, and, in the last decade, in south-western Asia and Europe (Kudela and Gobler (2012) and references therein). In the Mediterranean Sea, *C. polykrikoides* was first detected in the late 1990s, initially in eastern Sardinia, the Gulf of Naples (Italy) (Sannio et al., 1997; Siano et al., 2002; Zingone et al., 2006), and, later, in the Adriatic Sea (Saracino and Rubino, 2006). Resting cysts of this species in sediments from the Mediterranean have been reported in the Adriatic Sea (Saracino and Rubino, 2006), the Ionian Sea (Rubino et al., 2010), and recently by Satta et al. (accepted) in Alfacs Bay (Catalan Coast, NW Mediterranean Sea).

The detection of *C. polykrikoides* resting cysts in Catalan waters and the observation of fixed chains of unarmoured dinoflagellates in samplings from the region's monitoring program carried out in the area, raised our suspicions that *C. polykrikoides* was established along the Catalan coast. Confirmation of these suspicions, as described herein, was based on the detection of vegetative cells of *C. polykrikoides*, the first such report involving the western Mediterranean. Misidentification of this species was avoided by using single-cell PCR to sequence the partial large subunit (LSU) rDNA region from the isolated vegetative cells. This approach resulted in the first elucidation of the phylogenetic relationship between the organism isolated from the Mediterranean Sea and *C. polykrikoides* populations from other geographic regions. It also allowed an analysis of the biogeographic implications for this species.

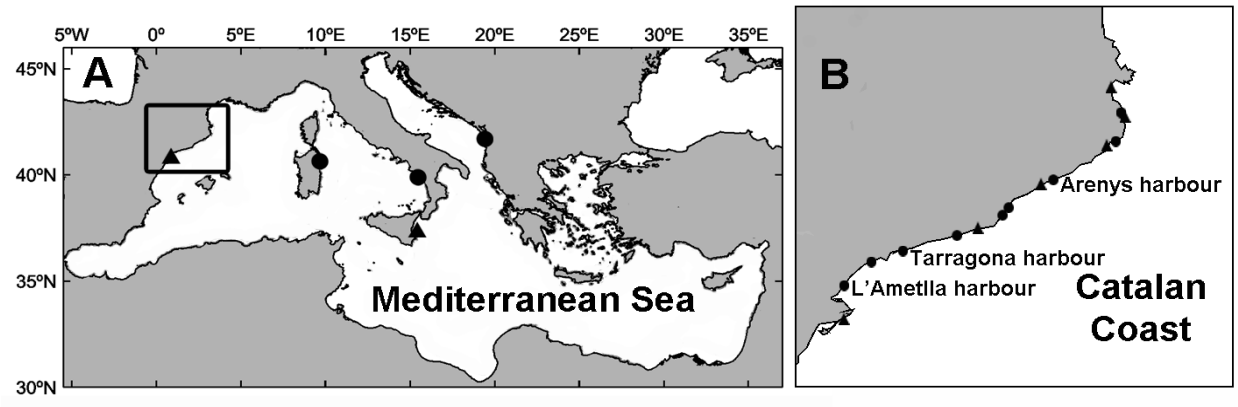

Figure 1: Sampling locations. A) Sites from the Mediterranean Sea where *Cochlodinium polykrikoides* has been previously reported. Dots indicate sites of vegetative cell detections and triangles those of resting cysts. B) Locations sampled during this study. Dots indicate harbours, and triangles beaches. Only the locations where *C. polykrikoides* was detected are labelled.

2. Material and methods:

2.1 Sampling and isolation: During 2011 and 2012, fresh samples were obtained from coastal stations (9 harbours and 6 beaches along the Catalan coast, NW Mediterranean) (Fig. 1), with monthly to weekly samplings throughout the year. From each sample, one sub-sample was fixed with Lugol's iodine and 50 ml were allowed to settle for 24 h in a settling chamber. *C. polykrikoides* cell abundances were determined based on observations at a 200x magnification of an appropriate area, made using a Leica-Leitz DM-Il inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). The remaining live sample was concentrated through a 10-µm mesh, allowed to settle in a settling chamber, and then observed under a Leica-Leitz DM-Il inverted microscope. Since all efforts to obtain cultures from the isolated specimens were unsuccessful, microscopy observations were performed directly on living cells from the concentrated samples. Target organisms were filmed and photographed with an Alpha NEX5 camera (Sony) adapted to the microscope and subsequently isolated with a micropipette, washed in several drops of filtered seawater, and placed in a 200-µl PCR tube for further analysis as described below.

2.2 Extraction, amplification, and sequencing: For some samples, DNA was extracted from the cells following the method of Kai et al. (2006). Briefly, 5 µl of lysis buffer (0.005% SDS with 400 ng proteinase K μl^{-1}) was added to each 200-µl tube after which the tubes were frozen at -80ºC for at least 10 min. The samples were then incubated first at 60ºC for 30 min, then at 95ºC for 10 min to inactivate the proteinase K, and finally stored at -80ºC until processed. For other samples, the cells were transferred directly to the PCR tube, adding the minimum volume of seawater, followed by several rounds of freezing/thawing. The PCR mixture contained 5 µl of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 µM of the primers D1R and D2C (Scholin et al., 1994). The PCR conditions were as follows: an initial denaturation for 5 min at 95ºC, 40 cycles of 20 s at 95ºC, 30 s at 55ºC, and 1 min at 72ºC, followed by a final extension step for 7 min at 72ºC. Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and visualized under UV illumination. The remainder was frozen at -20ºC until used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using the D1R primer and a 3730XL DNA sequencer.

2.3 Phylogenetic analyses: Sequences obtained in this study were deposited in Genbank and aligned with those obtained from GenBank (Table 1) using the MAFFT v.6 program (Katoh et al., 2002) under FFT-NS-i (slow; iterative refinement method) and manually checked with BioEdit v. 7.0.5 (Hall, 1999), obtaining a final alignment of the D1–D2 region of about 760 positions. Phylogenetic relationships were determined using the maximum-likelihood (ML) and Bayesian inference methods. For the ML method, the GTRGAMMA evolution model was used on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RaxML software. The Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al., 2012), run with a GTR model with rates set to gamma. Each analysis was performed using four Markov chains (MCMC), with one million cycles for each chain. The consensus tree was created from post-burn-in trees and the Bayesian posterior probabilities (BPP) of each clade were examined.

3. Results:

3.1 Detection: C. polykrikoides was detected in three harbours (Fig. 1) during 2011 and 2012 but never in samples from beaches. The cell abundances were low ($<10^4$ cells$\cdot L^{-1}$) and the detections were restricted to the summer months (from June to September), with water temperatures of 23.4–24.8 ºC and salinities of 31.4–38.2 (Table 2). However, in Arenys Harbour, *C. polykrikoides* was detected in 2011 over a period of 2 months, reaching a maximum abundance of $2 \cdot 10^4$ cells$\cdot L^{-1}$ in July 2011, but it was not detected again in that location in 2012.

The cells occurred individually or in two-cell chains. Individual cells were ovoid, with a rounded apex and a truncate or bilobulate antapex (Fig. 2A, B). Individual specimens ranged in size, with a length of 27.8-48.5 µm and a width of 21–38.1 µm; the mean values were 34.9 µm and 24.5 µm, respectively (n=17). Cells in chains were smaller and shorter, with lengths of 18.4–30.8 µm and widths of 18.9–28 µm; their mean values were 25 µm and 23.8 µm respectively (n=22). The cingulum encircled the cell 1.5–2 times (Fig. 2A). The yellow-brownish coloured cells contained many rod-shaped chloroplasts running vertically (Fig. 2C). A small pigmented body was seen dorsally in the epicone (Fig. 2B, D) and a large red body was commonly observed in the hypocone (Fig. 2D). The spherical nucleus was situated in the epicone (Figs. 2B, D). In some of the samples, individual cells were covered by a hyaline membrane (pellicle cysts) (Fig. 2D). A different morphotype, co-occurring with the common one, was observed among cells in chains detected in L'Ametlla Harbour. It was smaller (20-22 µm long; 19-21.5 um wide) and less intensely coloured (Fig. 2E, F). However, for individual cells it was difficult to discriminate between the two morphotypes.

Table 1: *Cochlodinium polykrikoides* sequences used to construct the phylogenetic tree. Sequences obtained during this study are indicated in bold.

Accession number	Isolation location	Ribotype
AB295042	Mishima Island, Japan	East Asian
AB295044	Inokushi Bay, Japan	(Group I)
AB295045	Katagami Bay, Japan	
AB288383	Inokushi Bay, Japan	
AB288384	Tachibana Bay, Japan	
AB288385	Kamigoto Island, Japan	
AB288386	Usuka Bay, Japan	
AY725423	Korea	
AB295043	Isahaya Bay, Japan	
EF506614	Namhae, Korea	
EF506616	Tongyong, Korea	
EF506618	Busan, Korea	
EF506620	Busan, Korea	
EF506622	Namhae, Korea	
EF506623	Hong Kong	
AF067861		
DQ779984	Tongyong, Korea	
DQ779985	Narodo, Korea	
DQ779986	Hakdong, Korea	
AY347309	Sarangdo, Korea	
KC577587	Arenys Harbour, Spain	Mediterranean
KC577588	Arenys Harbour, Spain	(Group II)
KC577590	L'Ametlla Harbour, Spain	
KC577591	Tarragona Harbour, Spain	
KC577592	Tarragona Harbour, Spain	
KC577593	Tarragona Harbour, Spain	
AB295048	Sabah, Malaysia	American/
AB295049	Sabah, Malaysia	Malaysian
EF110556	Long Island, NY, USA	(Group III)
EF506625	Cotuit Bay, MA, USA	
EF506627	Bahía de La Paz, México	
AB295050	Phosphorescence Bay, Puerto Rico	
AB609750	Qeshm Island, Iran	
GQ500117	Arabian gulf, United Arab Emirates	
AB609749	Bandar Marina, Oman	
AB295046	Manila Bay, Philippines	Philippines
AB295047	Omura Bay, Japan	(Group IV)
KC577589	L'Ametlla Harbour, Spain	

3.2 Phylogeny: The tree constructed from the sequences obtained during this study together with those from Genbank and using *Cochlodinium fulvescens* sequences as outgroup resulted in the formation of two clades and four highly supported sub-clades or ribotypes (Fig. 3, 4). Clade 1 (100% bootstrap/1 BPP) was formed by the East Asian ribotype (86%/-), made up of 20 sequences with 99.9% identical sites, and a new sub-clade (98%/1) comprising six sequences from this study (99.6% identical sites). As this sub-clade only contains sequences from the Mediterranean Sea it is referred to herein as the 'Mediterranean' ribotype. However, the topology of the trees obtained for the two inference methods slightly differed. While in the ML tree (Fig. 3) the two ribotypes were clearly independent, this was not the case in the Bayesian inference tree (Fig. 4), although the Mediterranean ribotype was also obtained, with a high BPP. Clade 2 (83%/0.96) consisted of both the American/Malaysian ribotype (100%/1), consisting of nine sequences with 99.9% identical sites, and the Philippines ribotype (99%/1), with two sequences from Genbank and one from this study (98.8% identical sites). The distance between sequences included within this ribotype was greater than those within other ribotypes.

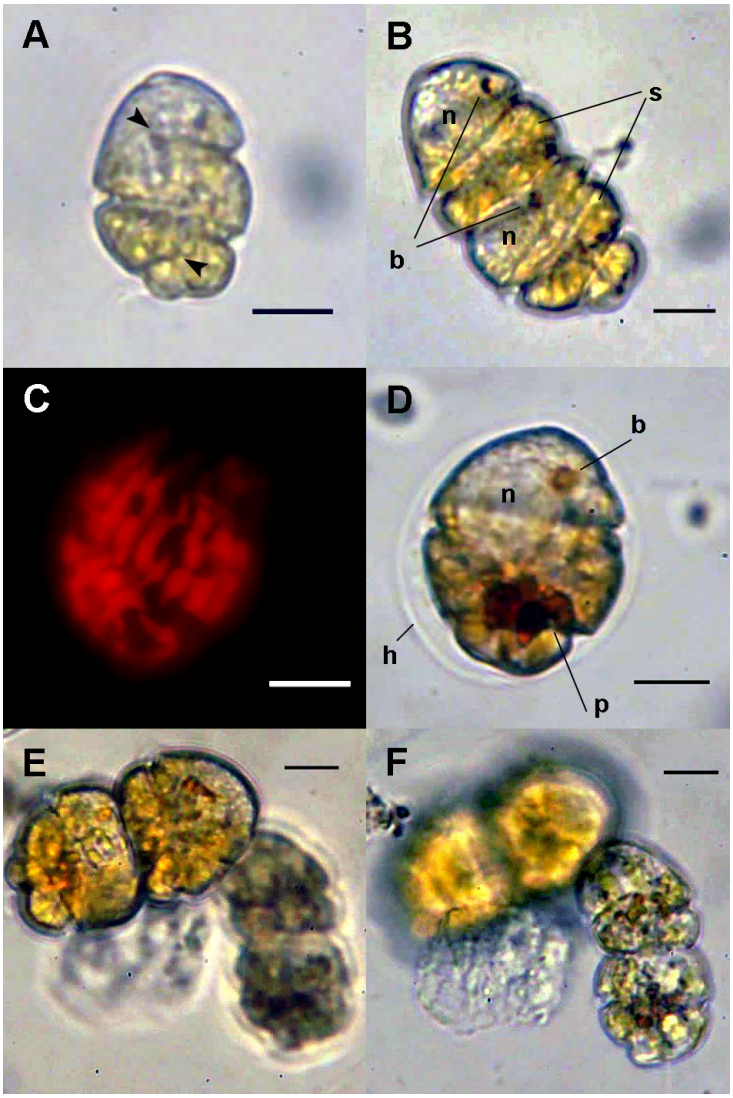

Figure 2: Light micrographs of *Cochlodinium polykrikoides* cells. A) Ventral view of a single vegetative cell. The cingulum encircles the cell twice (arrowheads). B) Dorsal view of a 2-cell chain. Nuclei (n) and pigmented bodies (b) are located in the epicone. The sulcus (s) runs just below the cingulum. C) Fluorescence micrograph showing rod-shaped chloroplasts extending vertically. D) Cell covered by a hyaline membrane (h). The nucleus (n) and pigmented body (b) are clearly visible. A large pigmented body (p) is present in the hypocone. E) and F) show different morphotypes of *C. polykrikoides* chains observed in L'Ametlla Harbour. Their phylogeny indicates that morphotype E) belongs to the Mediterranean ribotype and morphotype F) to the Philippines ribotype. Scale bar= 10 µm.

Table 2: Detections of *Cochlodinium polykrikoides* between 2011 and 2012. The asterisk indicates samplings in which the cells were isolated for sequencing.

Location	Date	Temperature (ºC)	Salinity	Cells·L^{-1}
Arenys Harbour	16/06/2011 *	23.4	36.2	<100
	04/07/2011	24.6	36.9	440
	17/07/2011	24.2	32.1	20408
	08/08/2011	24	31.4	7266
	16/08/2011	24.8	36.3	880
Tarragona Harbour	08/07/2011	24	37.1	600
L'Ametlla Harbour	25/06/2012 *	23.6	35.5	480
Tarragona Harbour	17/09/2012 *	24.8	38.2	<100

4. Discussion:

4.1 C. polykrikoides in the Mediterranean Sea:

The composition of phytoplankton in several locations of the Mediterranean Sea has been exhaustively studied since the late 1950s. Prior to the first detection of *Cochlodinium polykrikoides* in Sardinian waters (Sannio et al., 1997), other species belonging to this genus, but not *C. polykrikoides*, were reported in the Mediterranean Sea (Gómez, 2003). During the last decade, the *C. polykrikoides* morphotype was reported in Mediterranean waters but these detections were relatively rare and the cell abundances were consistently low. Nonetheless, these observations suggest that over the last two decades this morphospecies has expanded its distribution within the Mediterranean Sea.

Ours is the first report of two distinct *C. polykrikoides* ribotypes (Mediterranean and Philippine ribotypes) in the Mediterranean Sea and of a bloom of this species, formed by the Mediterranean ribotype. Although the toxicology of this ribotype has not yet been studied, detection of the toxic dinoflagellate *C. polykrikoides* along the Catalan coast implies the need for efforts to control and avoid its harmful effects on wild and aquacultured fauna in the area.

4.2 C. polykrikoides ribotypes:

The sequences obtained from the Catalan coast form a new (Mediterranean) ribotype that is clearly distinct from ribotypes previously defined based on sequences from all over the world. One sequence of *C. polykrikoides* in our samples matched the 'Philippines' ribotype, which to date includes only two other sequences: one from the Philippines (South China Sea) and one from southern Japan (East China Sea). There are two other established ribotypes, American/Malaysian, comprising sequences from America, the Arabian Sea, and Malaysia (South China Sea), and East Asian, made up of sequences from Japan and Korea. A morphotype referred to as *Cochlodinium* sp. type 'Kasasa' (Matsuoka et al., 2010), occurring in the East China Sea, was reported but it has not been completely characterized morphologically nor are sequences available, preventing further analysis of its phylogenetic position.

Our current understanding of geographic distribution of all ribotypes is circumscriptive. According to the prevailing conceptual model, in which ribotypes are related to geographic areas, there is no evidence to support the clade 'Philippine'. Iwataki et al. (2008) discussed that the population from Japan was apparently unrelated to the Philippines one. The detection of this latter ribotype in the Mediterranean Sea raises reasonable uncertainties about whether it actually represents a biogeographic population, in which case the name 'Philippines' would be inappropriate. Furthermore, populations belonging to the East Asian and Philippine ribotypes, along with the possibly new 'Kasasa' ribotype, have been detected within the East China Sea. Recently, a seasonal fluctuation of the 'Philipine' and 'East Asian' ribotypes has been observed during blooms in South Korea (Han, 2012). Nagai et al. (2009), in a more detailed study on the structure of populations from Japan and South Korea using microsatellites, concluded that there are two distinct populations in the area, both of which are probably expanding, driven by current-mediated transport from South Korea. Consequently, studies at regional and fine scales are of great importance because they

further our understanding of the mechanisms of global diversification and expansion. However, to date, no other study has determined the structure and origin of *C. polykrikoides* populations from other locations. Populations belonging to the American/Malaysian and the Philippine ribotypes have been confirmed in the South China Sea. In the Mediterranean, two co-occurring ribotypes have been detected in the same location. We therefore suggest that geographically based names are no longer indicative of the range occupied by members of a given ribotype as further incongruences will increasingly become apparent as more sequences from other locations become available and new ribotypes are distinguished among populations not yet sequenced. A similar case was described by Lilly (2007) for the *Alexandrium catenella / tamarense / fundyense* complex. Although the three morphotypes were considered as different species, this was not supported phylogenetically, biologically, or morphologically. The three morphotypes scattered into different ribotypes that were initially considered as geographic clades. Some of the ribotypes were found to be distributed worldwide, while others had a restricted distribution. Accordingly, the use of a numerical scheme instead of the geographic one was suggested. Here, we propose that since the taxonomy of *C. polykrikoides* ribotypes is not completely resolved, a group-numbering scheme (Groups I, II, III, IV) (Fig. 3, 4) would be preferable when referring to the different ribotypes.

4.3 Morphotypes and life cycle stages:

Different morphotypes attributed to *C. polykrikoides* are described in the literature. *C. catenatum* Okamura was described from Japan in 1916. These sub-spherical to ovoidal cells had a length of 24–27 µm. There were additional detections of this morphotype along the western coasts of Mexico (Cortes-Altamirano et al., 2004), Costa Rica, and Panama (Guzman et al., 1990; Hargraves and Víquez, 1981). However, a morphological description and a comparison

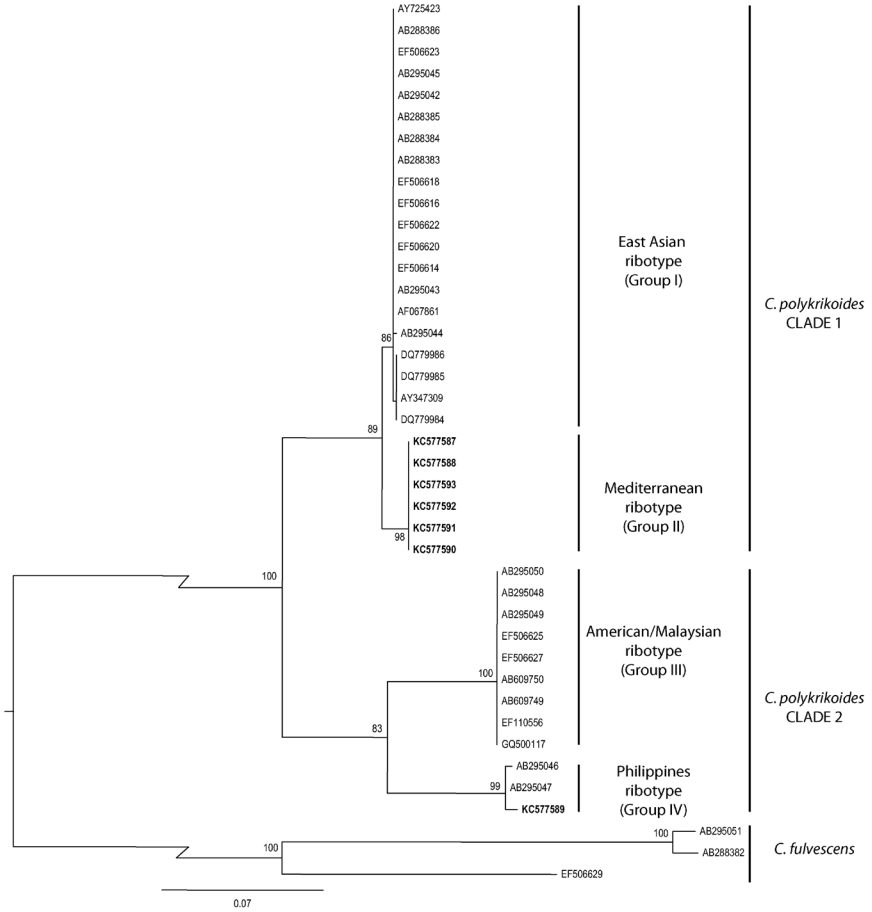

Figure 3: Maximum-Likelihood phylogenetic tree of selected species based on the D1–D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap (%) values. Only bootstrap values >80 are shown. *Cochlodinium fulvescens* sequences were used as outgroup. Organisms sequenced in this study are shown in bold.

with the *C. polykrikoides* morphotype were provided only for the Costa Rica occurrence. *C. polykrikoides* Margalef was described from Puerto Rico in 1961, differing from *C. catenatum* mainly in its ovoidal shape and larger size (ca. 50 µm). However, Margalef himself emphasized that the measurements were approximate, as the observations were based on live specimens and without a micrometer in the microscope (Margalef, 1961). A third species, *C. heterolobatum* Silva, was described from New Jersey (USA) but it does not differ markedly from *C. polykrikoides*. The populations of *C. polykrikoides* from East Asia have a length of 30–40 µm (Matsuoka et al., 2008), similar to those from America (Gárate-Lizárraga et al., 2004), the Arabian Gulf (Richlen et al., 2010), and Malaysia (Anton et al., 2008) and to the measured cells from this study. Although the different species exhibit slight morphological differences, they are considered as conspecific (Matsuoka et al., 2008).

Regarding the life cycle stages of *C. polykrikoides*, resting cysts of this species obtained from many locations have been reported in the literature, although in most cases the identity of the cysts or of their germinated cells was not fully confirmed. Tang and Gobler (2012) found that strains of *C. polykrikoides* from North America ('American/Malaysia' ribotype) produce resting cysts. However, the morphology of these cysts differs from that of the most commonly reported morphocyst of *C. polykrikoides*, detected in locations where the presence of the East Asian ribotype has been confirmed (Kim et al., 2007; Matsuoka, 1985; Matsuoka and Fukuyo, 2000, 2003). The same 'East Asian' morphocyst has been reported from the western coast of India (D'Silva et al., 2012), where the only ribotype detected thus far is the 'American/Malaysian' one. The only reports of resting cysts in the Mediterranean Sea (Rubino et al., 2010; Satta et al., accepted) describe a morphology similar but not identical to that of the 'East Asian' clade but with clear

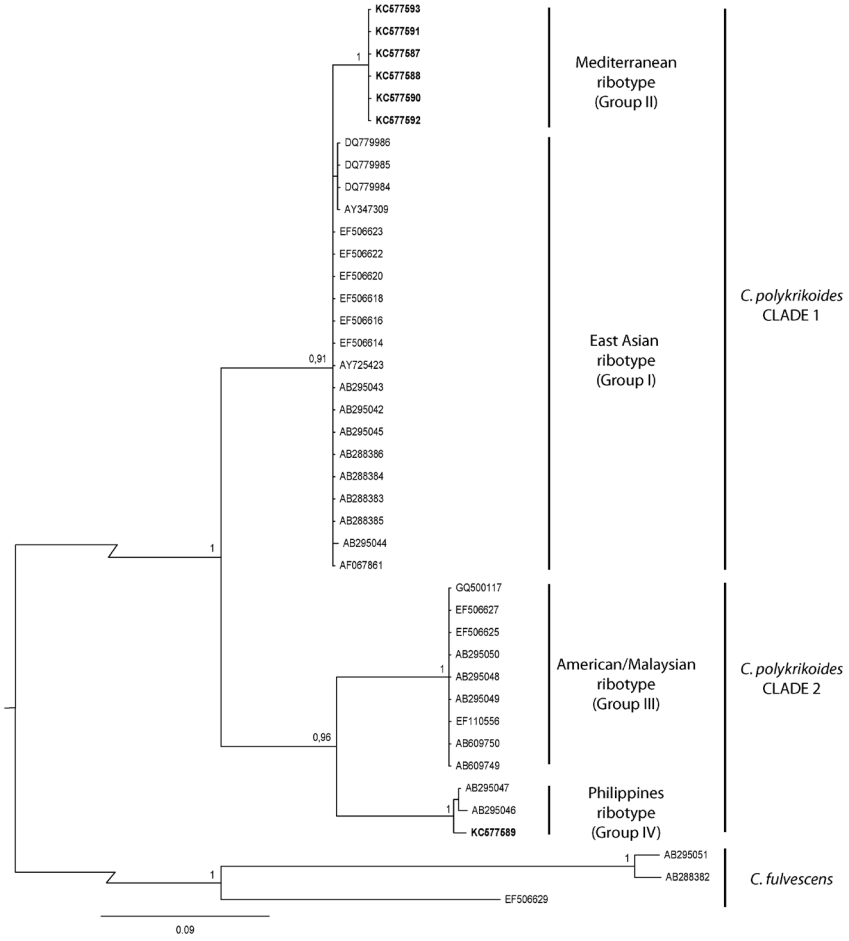

Figure 4: Bayesian inference phylogenetic tree of selected species based on the D1–D2 domain of LSU rRNA. Numbers on the nodes are the Bayesian posterior probability. Only values >0.9 are shown. *Cochlodinium fulvescens* sequences were used as out-group. Organisms sequenced in this study are shown in bold.

differences from the morphology described by Tang and Gobler (2012). The germinated cell described in Rubino et al. (2010) could not be unequivocally identified whereas a germinated cell illustration was not included in the study of Satta et al. (accepted). Kim et al. (2007) examined the life cycle of *C. polykrikoides* isolated from Korean waters, reporting the presence of thecate life stages and a possible resting cyst originating from those stages. The same morphocyst was observed again in Korean waters (Pospelova and Kim, 2010). Finally, an ornamented morphocyst observed in Guatemala, W Central America (Rosales-Loessener et al., 1996), and similar to those described as *Cochlodinium* sp. 2 by (Matsuoka and Fukuyo, 2000) was suggested to be produced by *C. polykrikoides*, although it differed from other morphocysts discussed in the literature and therefore might have been produced by another species. Observed differences among the morphotypes of vegetative cells can be attributed to morphological plasticity or to methodological problems related to observations of athecate organisms. Nonetheless, the detection of different morphocysts and the existence of different ribotypes point out the gaps in our knowledge regarding the relationship between morphotypes and their life cycle, such that a complex of species cannot be ruled out.

4.4 Ecophysiology:

The Mediterranean ribotype described herein was observed in several locations from the Catalan coast and during the 2 years of the study, confirming that the species is well established in the area and capable of occasionally producing blooms. The abiotic range of the Mediterranean ribotype is completely unknown, but our samples contained only single cells or 2-cell chains. Tomas and Smayda (2008) reported the presence of single cells and short chains under suboptimal growth conditions, which could actually have been the case with the populations from the Catalan coast.

The relationship between the environmental parameters determined in bloom events and in culture experiments from the literature suggests that *C. polykrikoides* is well adapted to temperatures >20ºC and to salinities of 30–33. However, this species has a wide tolerance for both temperature and salinity (Kudela and Gobler, 2012), with the main differences between ribotypes being the ability to grow at lower temperatures and salinities in the east coast of USA (Tomas and Smayda, 2008). With rare exceptions (Gobler et al., 2008; Richlen et al., 2010), studies on the ecophysiology of *C. polykrikoides* blooms have not included phylogenetic analyses, and morphological characterizations are often lacking. These deficits prevented confident identification of the ribotype/s under examination. Instead, the relationships among the different morphotypes/ribotypes and environmental variables remain poorly understood due to the wide tolerance range of *C. polykrikoides*, potentially reflecting mixed ribotypes or different species.

While the morphology, ecophysiology, and distribution of the clades determined thus far are not well resolved, the phylogeny suggests the presence of cryptic species that should be evaluated for species-level status. Meanwhile, ribotype assignment must be evaluated for all detections and bloom events in order to characterize each ribotype, considering the important economic threat posed by the toxicity of *C. polykrikoides*.

Acknowledgements

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Fangar Bay, Ebre river delta (Catalan coast)

Chapter 6

"A new clade, based on LSU rDNA sequences, of unarmoured dinoflagellates"

Protist (2013)

A NEW CLADE, BASED ON PARTIAL LSU rDNA SEQUENCES, OF UNARMOURED DINOFLAGELLATES

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Abstract:

The order Gymnodiniales comprises unarmoured dinoflagellates. However, the lack of sequences hindered determining phylogenetic positions and systematic relationships of several gymnodinioid taxa. In this study, a monophyletic clade was defined for the species *Ceratoperidinium margalefii* Loeblich III, *Gyrodinium falcatum* Kofoid & Swezy, three *Cochlodinium* species, and two *Gymnodinium*-like dinoflagellates. Despite their substantial morphotypic differentiation, *Cochlodinium* cf. *helix*, *G. falcatum* and '*Gymnodinium*' sp. 1 share a common shape of the acrobase. The phylogenetic data led to the following conclusions: (1) *C. margalefii* is closely related to several unarmoured dinoflagellates. Its sulcus shape has been observed for the first time. (2) *G. falcatum* was erroneously assigned to the genus *Gyrodinium* and is transferred to *Ceratoperidinium* (*C. falcatum* (Kofoid & Swezy) Reñé & de Salas comb. nov.). (3) The genus *Cochlodinium* is polyphyletic and thus artificial; our data support its separation into three different genera. (4) The two *Gymnodinium*-like species could not be morphologically or phylogenetically related to any other gymnodinioid species sequenced to date. While not all studied species have been definitively transferred to the correct genus, our study is a step forward in the classification of inconspicuous unarmoured dinoflagellates. The family Ceratoperidiniaeceae and the genus *Ceratoperidinium* are emended.

1. Introduction

Although approximately $5 \cdot 10^5$ species of protists are currently known and described (Adl et al. 2007), extensive molecular analyses indicate an estimated diversity several orders of magnitude higher (Adl et al. 2007; López-García et al. 2001; Savin et al. 2004), including numerous cryptic species (Amato et al. 2007; Katz et al. 2005; Montresor et al. 2003; Quijano-Scheggia et al. 2009). Occasionally, morphological diversity is not reflected at the molecular level. For example, Kareniaceae species that are morphologically distinctive have been shown to have little-differentiated LSU genes, and conversely, morphologically similar species are well-differentiated in their LSU genes (de Salas et al. 2008). Recently diversified organisms may be morphologically distinct but the differences may not be discernible at the molecular level. In phylogenetic studies of dinoflagellates, the common use of highly conserved molecular markers, such as the ribosomal RNA genes, may partially explain this paradox (Edvardsen et al. 2003; Logares et al. 2007).

Dinoflagellate diversity has been estimated at approximately 2,000 species. Historically, these organisms were classified based on morphological features, including the presence and arrangement of their thecal plates (Saldarriaga et al. 2004). With the development of molecular approaches and sequence analysis, studies of the evolution and taxonomic position of many organisms, including in some cases their re-classification, have been possible (Daugbjerg et al. 2000; Hackett et al. 2004; Saldarriaga et al. 2004; Saldarriaga et al. 2001). However, when examining the systematic relationships of dinoflagellates an obvious complication is the fact that the phylogenetic positions of many species and genera are unknown because the respective sequences have yet to be obtained.

The order Gymnodiniales comprises organisms lacking a theca (Fensome et al. 1993). This criterion has been used as the basis of classification of a large variety of dinoflagellates with few other shared characters, and resulted in a situation where the Gymnodiniales can be shown to be polyphyletic based on their rRNA genes (Daugbjerg et al. 2000; Moestrup and Daugbjerg 2007), or paraphyletic when examining a larger number of genes (Orr et al. 2012). Moreover, a critical assessment of the morphological and ultrastructural features of unarmoured dinoflagellates and a re-evaluation of their phylogeny resulted in the redefinition of several existing genera (Daugbjerg et al. 2000). With the help of molecular tools and improved morphological observations, several new dinoflagellate genera have been introduced, i.e. *Akashiwo*, *Karenia* and *Karlodinium* (Daugbjerg et al. 2000), *Takayama* (de Salas et al. 2003), *Togula* (Flø Jørgensen et al. 2004b), *Apicoporus* (Sparmann et al. 2008), *Testudodinium* (Horiguchi et al. 2012) and *Ankistrodinium* (Hoppenrath et al. 2012).

The genus *Ceratoperidinium* Margalef was erected in 1969 with *Ceratoperidinium yeye* Margalef, bearing retractile apical and antapical appendices, as the type species. However, the original description of the genus *Ceratoperidinium* Margalef (1969) was invalid, as it was not accompanied by a Latin diagnosis (International Code of Nomenclature for algae, fungi and plants, 2011 Melbourne, Article 39, Section 1). The species *C. yeye* was automatically invalid under Article 35, Section 1, since any species described in a genus that itself is not validly described is automatically invalidated. Loeblich III (1980) corrected this deficiency by validly describing the genus *Ceratoperidinium* Margalef ex Loeblich III. He renamed the species as *C. margalefii* Loeblich III, making *C. yeye* a synonym of *C. margalefii*. Nonetheless, the taxonomic position of the genus *Ceratoperidinium* has long been uncertain (Fensome et al. 1993; Sournia 1986) and its two species, *C. margalefii* and *C. mediterraneum* Abboud-Abi Saab, have been considered as morphological variants of a single one (Gómez et al. 2004). The original description of *C. margalefii* was based on a single specimen isolated from Spanish Mediterranean coastal waters. Although the species was also detected offshore in the tropical and western Equatorial Pacific Ocean (Gómez et al. 2004) and in Acapulco Bay (Mexico) (Meave-del Castillo et al. 2012), further detections have been extremely rare and only in Mediterranean coastal waters (France and Mozetic 2009, and references therein).

The species under the current name of *Gyrodinium falcatum* is commonly detected in temperate and warm waters of both hemispheres (Konovalova 2003). Its cells are fusiform but they also develop long extensions that vary in size during the organism's life cycle. Such variability has given rise to several names in both *Gyrodinium* and *Gymnodinium*. In addition, a stage in the life history of *G. falcatum* was formerly described as *Pseliodinium vaubanii* Sournia (Konovalova 2003). However, according to the available partial LSU rDNA sequence this species is not placed within known clades that include *Gymnodinium* and *Gyrodinium* species (de Salas et al. 2003; Kim and Kim 2007).

The genus *Cochlodinium* thus far consists of about 40 species that are characterized by cellular torsion and a cingulum that makes 1.5–4.0 turns around the cell. Most currently known species are heterotrophic and only four are phototrophic (Kudela and Gobler 2012). Toxic species among the latter include *C. polykrikoides* Margalef and *C. fulvescens* Iwataki, Kawami et Matsuoka, both of which have been extensively studied (Iwataki et al. 2007; Iwataki et al. 2008; Kudela and Gobler 2012; Reñé et al. 2013). However, for the majority of *Cochlodinium* species their identification has been challenging because of the scarcity of heterotrophic species, the absence of molecular information, and the few studies of their taxonomy, distribution and ecology.

Finally, while the gross morphology of many gymnodinioid organisms has resulted in their inclusion within *Gymnodinium*, in some cases, such as *Gymnodinium instriatum* (Freudenthal & Lee) Coats, they were determined to be phylogenetically unrelated to other unarmoured species (Saldarriaga et al. 2004). Conversely, several species in other genera have *Gymnodinium*-like stages during their life cycles, as shown for *Polykrikos kofoidii* Chatton (Tillmann and Hoppenrath 2013). While new genera of gymnodinioid organisms have recently been erected, i.e. *Gyrodiniellum* (Kang et al. 2011), *Barrufeta* (Sampedro et al. 2011) and *Paragymnodinium* (Kang et al. 2010), all of them are within the phylogenetic clade *Gymnodinium sensu stricto* (as defined by Daugbjerg et al. 2000).

Given the unreliability of gross external morphology in determining the true phylogenetic and taxonomic affinities of unarmoured dinoflagellates, we carried out a detailed investigation of the morphology and LSU rDNA phylogeny of *C. margalefii*, *G. falcatum*, three '*Cochlodinium*' morphospecies and two '*Gymnodinium*' morphospecies. Our findings clarify the phylogenetic positions and relationships between the dinoflagellate species studied and other genera within the order Gymnodiniales.

2. Material & Methods

2.1 Sampling and isolation: Live organisms were isolated from Port Lincoln (South Australia), Pirates Bay and Nubeena (Tasmania), as well as the Catalan coast (NW Mediterranean Sea) (Table 1). Surface seawater from the Catalan coast was concentrated through a 10-µm mesh. A settling chamber was used to observe the live organisms under a Leica-Leitz DM-Il inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Target organisms were filmed and photographed with a Sony NEX5 digital camera (Sony, Tokyo, Japan) adapted to the microscope. They were subsequently isolated with a micropipette, washed in several drops of filtered seawater and a single cell was placed in a 200-µl PCR tube adding the minimum volume of seawater, followed by several rounds of freezing/thawing and finally stored at -80ºC until processed for further single-cell PCR as described below. Clonal cultures were established from the Australian dinoflagellates by single-cell isolation using a micropipette and maintained in GSe culture medium, as detailed in de Salas et al. (2003). The cultures were for a while deposited in the University of Tasmania's microalgae culture collection (codes GFPL01 for *Ceratoperidinium falcatum*, CPNU01 for *Cochlodinium* cf. *helix* and GspTRA01 for "*Gymnodinium*" sp. 1), but have since been de-accessioned as they were lost. Scanning electron microscopy (SEM) images were obtained from the cultures following the method described in de Salas et al. (2003). Briefly, culture material was fixed for 1 hour with 4% osmium tetroxide that was dissolved in sterile culture medium, rinsed with sterile-filtered seawater and deionised water, and dehydrated using a methanol/acetone series (10, 30, 50, 70, 80, 90 and 100% MeOH, followed by 2x rinses in dry acetone). The cells were critical-point

Table 1: Locations, isolation dates and GenBank accession numbers of studied organisms. The method used to obtain the partial LSU rDNA sequence is provided in the last column; SC-PCR = single-cell PCR. * indicates that the sequence was previously available in GenBank.

Organism	Isolation date	Location	Coordinates	GenBank accession number	Method
Ceratoperidinium margalefii	July 2011	La Muga river mouth (Mediterranean Sea)	42º 14'3.83'' N 3º 7'39.96''E	KF245455 (clone 19) KF245456 (clone 24)	SC-PCR and cloning
Ceratoperidinium falcatum	November 2002	Port Lincoln (South Australia)	34º 43'02.97''S 135º 51'43.54''E	AY320049 *	PCR from culture
Ceratoperidinium falcatum	October 2012	Fangar Bay (Mediterranean Sea)	40º 46'29.31''N 0º 45'16.67''E	KF245457 KF245458	SC-PCR
Cochlodinium cf. *helix*	September 2002	Nubeena (Tasmania)	43º 05'50.62''S 147º 44'22.10''E	KF245459	PCR from culture
Cochlodinium cf. *convolutum*	November 2012	Palamós Harbour (Mediterranean Sea)	41º 50'47.02''N 3º 8'8.64''E	KF245460	SC-PCR
Cochlodinium sp.1	November 2012	Palamós Harbour (Mediterranean Sea)	41º 50'47.02''N 3º 8'8.64''E	KF245461	SC-PCR
'*Gymnodinium*' sp.1	September 2002	Pirates Bay (Tasmania)	43º 01'19.03''S 147º 55'44.30''E	KF245462	PCR from culture
'*Gymnodinium*' sp.2	November 2012	Palamós Harbour (Mediterranean Sea)	41º 50'47.02''N 3º 8'8.64''E	KF245463	SC-PCR

dried and sputter-coated with gold/palladium, then observed and photographed using a JEOL 35C scanning electron microscope.

2.2 Extraction and PCR: Total genomic DNA of Australian dinoflagellates was extracted using gentle lysis and two phenol:chloroform extractions as detailed in Bolch et al. (1998). Extracted DNA was used as a template to amplify a fragment of the LSU ribosomal gene approximately 1400 bp long, using the primers D1R (Scholin et al. 1994a) and 28:1483R (Daugbjerg et al. 2000). PCR conditions were as described in de Salas et al. (2003). Primers D1R, D2C and D3Ca (Scholin et al. 1994b) were used to determine the nucleotide sequence of approximately 850 bp of the amplified fragment. Single-cell PCR was directly conducted on dinoflagellates from the Catalan coast. The PCR mixture contained 5 µl of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 µM of the primers D1R and D2C (Scholin et al. 1994a). The PCR conditions were as follows: an initial denaturation for 5 min at 95ºC, 40 cycles of 20 s at 95ºC, 30 s at 55ºC, and 1 min at 72ºC, followed by a final extension step for 7 min at 72ºC. Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and visualized under UV illumination. The remainder was frozen at -20ºC until used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using the D1R primer and a 3730XL DNA sequencer.

2.3 Cloning: Initial attempts to obtain the *C. margalefii* sequence failed because two different sequences were amplified during the PCR. Therefore, the PCR product was cloned in order to distinguish the sequence of our target from that of the other organism. The PCR product was first purified using the QIAquick PCR purification kit and then cloned using the StrataClone PCR cloning kit (Agilent Technologies, Inc., USA) according to the manufacturer's recommendations. Putative positive colonies were selected, grown in a multi-well plate containing LB medium, kanamycin and 7% glycerol and stored at -80°C. The presence of the LSU rDNA insert was verified by PCR amplification of each colony, using the same primers and PCR procedure as described above. PCR products from positive clones were sent to Genoscreen for purification and sequencing with the D1R primer. The resulting 650-bp sequences were submitted to a NCBI BLAST analysis (Altschul et al. 1997) for an approximate assessment of their phylogenetic affiliations based on comparisons with sequences in the GenBank database.

2.4 Phylogenetic analyses: Sequences obtained in this study were aligned with those obtained from GenBank using the MAFFT v.6 program (Katoh et al. 2002) under FFT-NS-i (slow; iterative refinement method), resulting in an alignment of about 1100 positions. Alignments were manually checked with BioEdit v. 7.0.5 (Hall 1999) and the highly variable regions removed using Gblocks v.0.91b (Castresana 2000), with a final alignment of 840 positions. Phylogenetic relationships were determined using the maximum-likelihood (ML) method and the GTRGAMMA evolution model of RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis 2006). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap (BS) ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RAxML software. The Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al. 2012), run with a GTR model in which the rates were set to gamma. Each analysis was performed using four Markov chains (MCMC), with three million cycles for each chain. The consensus tree was created from postburn-in trees and the posterior probabilities (BPP) of each clade were examined.

3. Results:

3.1 Morphology

- Ceratoperidinium margalefii: One live specimen of *C. margalefii* was obtained from the mouth of the La Muga River (Table 1). The cell was 42.9 µm long (excluding its antapical appendices) and 31.4 µm wide, with a characteristic morphology: The epicone was semi-oval in outline, with a rounded apex, and larger than the hypocone (Fig. 1A, B). No tubular apical process was observed. The hypocone was characterized by the presence of two retractile appendices

(Fig. 1A, B). During observations of the specimen, the shape of the appendices changed, from large and thin to short and thick. The cell was highly dorsoventrally compressed, with a longitudinal excavation in its right dorsal side (Fig. 1C). The cingulum was descending, more than twice its width (Fig. 1A), clearly impressed in the dorsal side of the cell. Its junction with the sulcus was displaced to the right side of the cell (Fig. 1A, 2A). The narrow, weakly depressed sulcus ran sigmoidally through the epicone, reaching the cingulum and continuing through the right side of the hypocone until the right antapical appendix (Fig. 1D, 2A). The apical groove was not unequivocally observed. The elongated, reniform nucleus was positioned centrally on the left side of the cell (Fig. 1B, 2A). The observed cell was colourless whereas the antapical appendices had a yellow-brownish colour, with a dark band located anteriorly in each appendix (Fig. 1A, 2A). The organism swam along a straight line, turning around its own axis. A comparative plate of drawings including previous observations of *C. margalefii* is provided in Fig. 2.

- *Ceratoperidinium falcatum* (Kofoid et Swezy) Reñé et de Salas comb. nov.
As will be discussed below, the morphological features and phylogenetic position of this species do not support its inclusion within the genus *Gyrodinium*. Since the genus *Ceratoperidinium* Margalef ex Loeblich III already exists for a species that is unambiguously located within this clade, *C. margalefii*, we suggest that *Gyrodinium falcatum* properly belongs in the genus *Ceratoperidinium*.

A monoclonal culture of *Ceratoperidinium falcatum* was established with organisms from Port Lincoln (South Australia) and several cells of this species were detected in Fangar Bay (NW Mediterranean Sea) (Table 1). Despite the high plasticity of the cells, overall their morphology agreed with the available descriptions in the literature. Some cells were elongated and fusiform while others were ovoid to conical (Fig. 3A, B and C). The cingulum was displaced by about two to four times its own width (Fig. 3A, B; 4D). The sulcus was broad, running from the epicone to the hypocone but not reaching the apices (not shown). The nucleus was central (Fig. 3C). The cells had an orange pigmentation near their apices (Fig. 3A, 4D), but a pale colouration in their centres. A hyaline membrane covering the cell was sometimes observed (Fig. 3B). The acrobase made a circular loop around the apex (Fig. 4E).

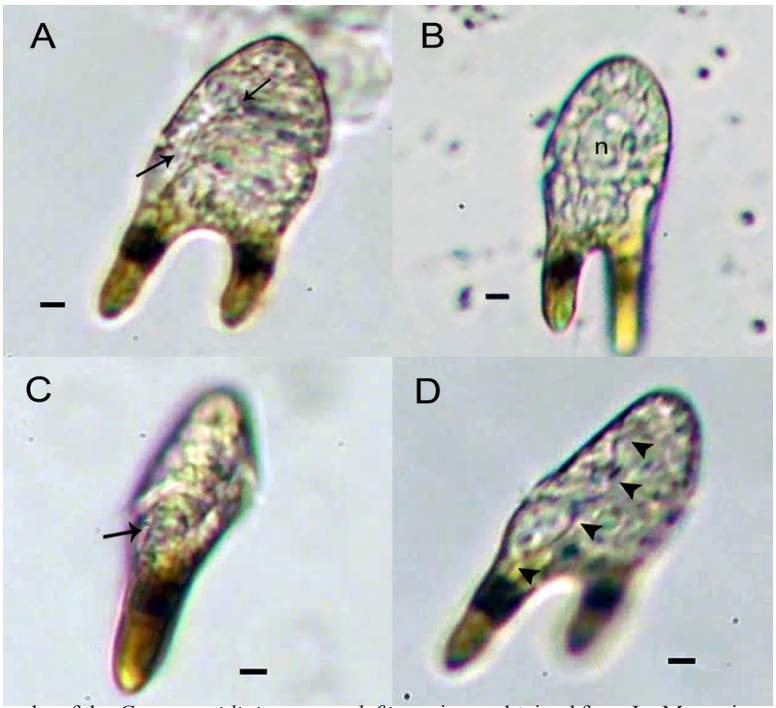

Figure 1: Light micrographs of the *Ceratoperidinium margalefii* specimen obtained from La Muga river mouth. A) Ventral view of the cell. The arrows indicate displacement of the cingulum. The sulcus runs through the epicone to the hypocone. B) The reniform nucleus (n) as seen in a dorsal view of the cell. C) Lateral view of the right side of the cell; an excavation is indicated by the arrow. D) Ventral side of the cell in a right lateral view; the arrowheads point to the sigmoid sulcus. Scale bar: 5 µm.

- *Cochlodinium* spp.: Three different morphotypes were detected and successfully sequenced (Table 1). One specimen of *Cochlodinium* cf. *convolutum* Kofoid et Swezy was detected in Palamós Harbour. The cell was bullet-shaped, 47 µm long and 32.5 µm wide. The apex was tapering and flattened (Fig. 3D). The cingulum made 1.5 turns around the cell, joining the sulcus near the antapex on the dorsal side of the cell (Fig. 3E). The sulcus penetrated the epicone in straight line and ran through the cell, making 0.5 turns and ending centrally, resulting in a bilobated hypocone. The nucleus was large, elongated, situated dorsally and filling nearly the entire cell length. The cell had a pale-yellow coloration and was covered by a hyaline membrane much larger than the cell (Fig. 3D, E). *Cochlodinium* cf. *helix* (Pouchet) Lemmermann was isolated and cultured from coastal waters off Nubeena, SE Tasmania. The cells were 50–60 µm long and 30–50 µm wide. The epicone was conical, with a rounded apex (Fig. 4A). The cingulum made 1.5 turns around the cell, joining the sulcus near the antapex on the dorsal side of the cell (Fig. 4B), although cells with a cingulum making just one turn were also observed. The sulcus was narrow in the epicone. It reached the apex, running from left to right, and then joined the proximal end of the cingulum, where it turned left to run deeply through the cell, making 0.5 turns (Fig. 4A) before ending centrally, thus creating a slightly bilobated hypocone (Fig. 4B). The acrobase formed a circular loop around the apex, with both ends in contact with the sulcus (Fig. 4C). The nucleus was centrally-located, dense and highly refractive. All cells were pigmented. At any given time only a proportion of the cells in the culture would be actively swimming, the rest would form a hyaline membrane and rest. One specimen of *Cochlodinium* sp. 1 was also obtained from Palamós Harbour. Unfortunately, it quickly collapsed such that its morphology could not be studied in detail.

- '*Gymnodinium*' spp.: *Gymnodinium* sp. 1 was isolated and cultured from the coastal waters off Pirates Bay (Tasmania) (Table 1). The cells were 22–31 µm long and 19–24 µm wide, widest medially. Epicone and hypocone were almost equal in length. The epicone was conical and the apex flattened. The hypocone was hemispherical (Fig. 4F, G). The wide and deep cingulum was displaced by a distance approximately equal to its width (Fig. 4F). The sulcus slightly penetrated into the epicone and a narrow and weakly impressed sulcal extension joined it with the acrobase; in the junction with the cingulum, the sulcus made a sigmoid curve to the left and widened in the hypocone but not reaching the antapex. The acrobase made a circular loop around the apex (Fig. 4F, G), with both ends ventrally joining the sulcus extension. The cells were pigmented. One specimen of *Gymnodinium* sp. 2 was obtained from Palamós Harbour (Table 1). This cell was ovoid, 38 µm long and 26.5 µm wide, widest posteriorly. The epicone was conical, with a flattened apex (Fig. 3F), longer than the hypocone. The hypocone was slightly bilobated and the antapex flattened. The cingulum was median, displaced by a distance approximately equal to its width. The sulcus reached the antapex, where it widened. The nucleus was situated on the right side of the hypocone (Fig. 3F). The brownish pigmented cell was covered by a hyaline membrane.

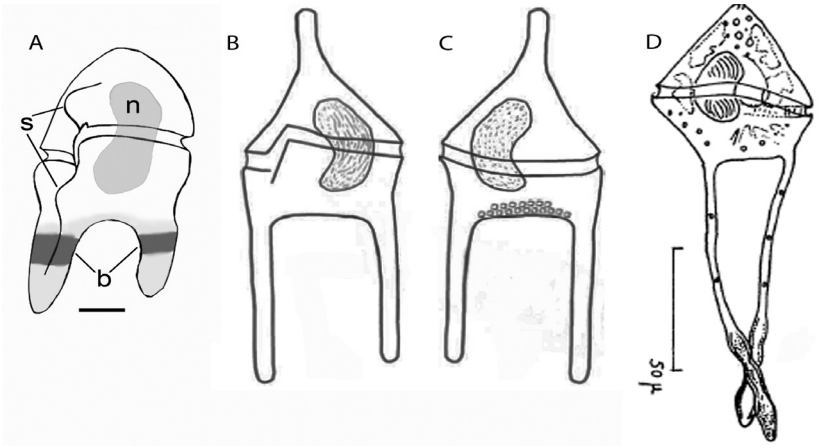

Figure 2: Schematic drawings of *Ceratoperidinium margalefii* according to different authors. A) Ventral view. The reniform nucleus (n), the pigmented bodies (b), the sigmoid sulcus (s) and the pigmented areas of the appendices (grey shades) are depicted (this study). Scale bar = 10 µm. B) Ventral and C) dorsal views according to Gómez et al. (2004) (scale bar not provided). D) Dorsal view from the original description of Margalef (1969). Scale bar = 50 µm.

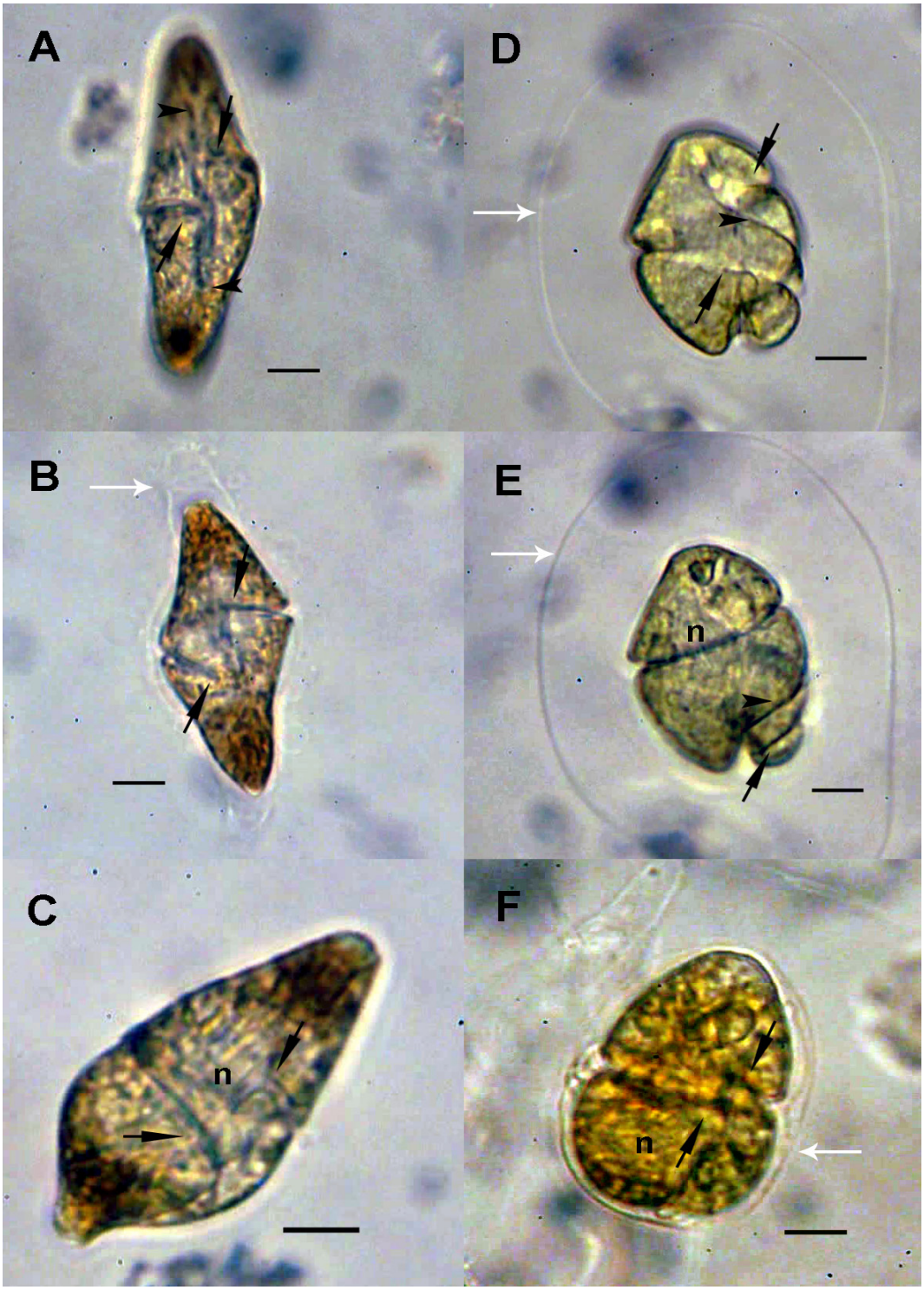

Figure 3: Light micrographs of the studied species. A), B) and C) Ventral view of *Ceratoperidinium falcatum* cells obtained from Fangar Bay. D) Ventral and E) dorsal views of *Cochlodinium* cf. *convolutum* specimen obtained from Palamós Harbour. F) Ventral view of '*Gymnodinium*' sp. 2 specimen obtained from Palamós Harbour. The nuclei are indicated (n). Black arrows indicate the cingulum, arrowheads the sulcus and white arrows the hyaline membrane that covers the cells. Scale bars = 10 µm.

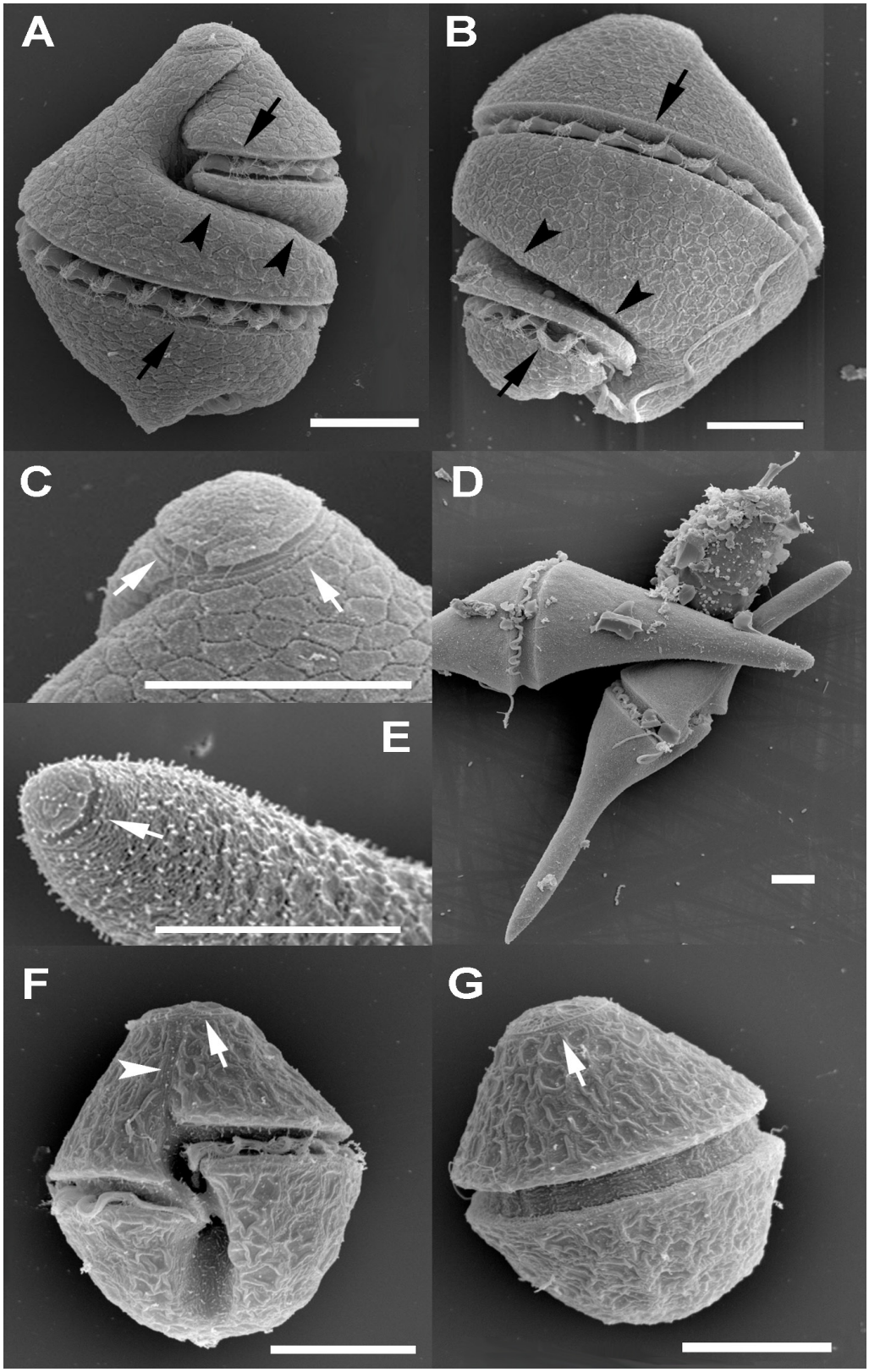

Figure 4: Scanning electron micrographs. A) Ventral and B) dorsal views of cultured *Cochlodinium* cf. *helix* from Nubeena. C) Detail of the acrobase of *Cochlodinium* cf. *helix* in lateral view. D) Two cells of cultured *Ceratoperidinium falcatum* in lateral (left) and ventral (right) views obtained from Port Lincoln. E) Detail of the acrobase of *C. falcatum* in dorsal view. F) Ventral and G) dorsal views of cultured '*Gymnodinium*' sp. 1 from Pirates Bay. Black arrows indicate the cingulum, black arrowheads the sulcus, white arrows the acrobase and white arrowheads the sulcal extension. Scale bars= 10 µm.

3.2 Phylogeny

Nine partial LSU rDNA sequences assigned to *C. margalefii* were obtained by cloning. Of these, seven were identical and they differed from the other two, which were also identical, only at one position. Three morphotypes assigned to the genus *Cochlodinium* on the basis of their morphology were successfully sequenced, as were two '*Gymnodinium*' species. Two sequences of organisms initially identified as *C. falcatum* were also originally determined. Sequences obtained from single-cell PCR were ~650 bp, except that of *Cochlodinium* sp. 1 which was ~500 bp, while those obtained from cultures were ~850 bp.

The ML phylogenetic tree was made up of representative species of most of the dinoflagellate orders. The alveolates *Toxoplasma gondii* and *Perkinsus marinus* were used as outgroups (Fig. 5). Among the organisms belonging to the polyphyletic Gymnodiniales order, several well-supported clades were obtained (*Gymnodinium sensu stricto* (*s.s.*), *Gyrodinium s.s.*, *Amphidinium s.s.*). The remaining genera and species of unarmoured dinoflagellates clustered independently under distinct supported clades, as did the organisms sequenced in this study. All of them, including the *C. falcatum* sequence from GenBank, clustered within a highly supported clade (93% BS / 1 BPP) that was not

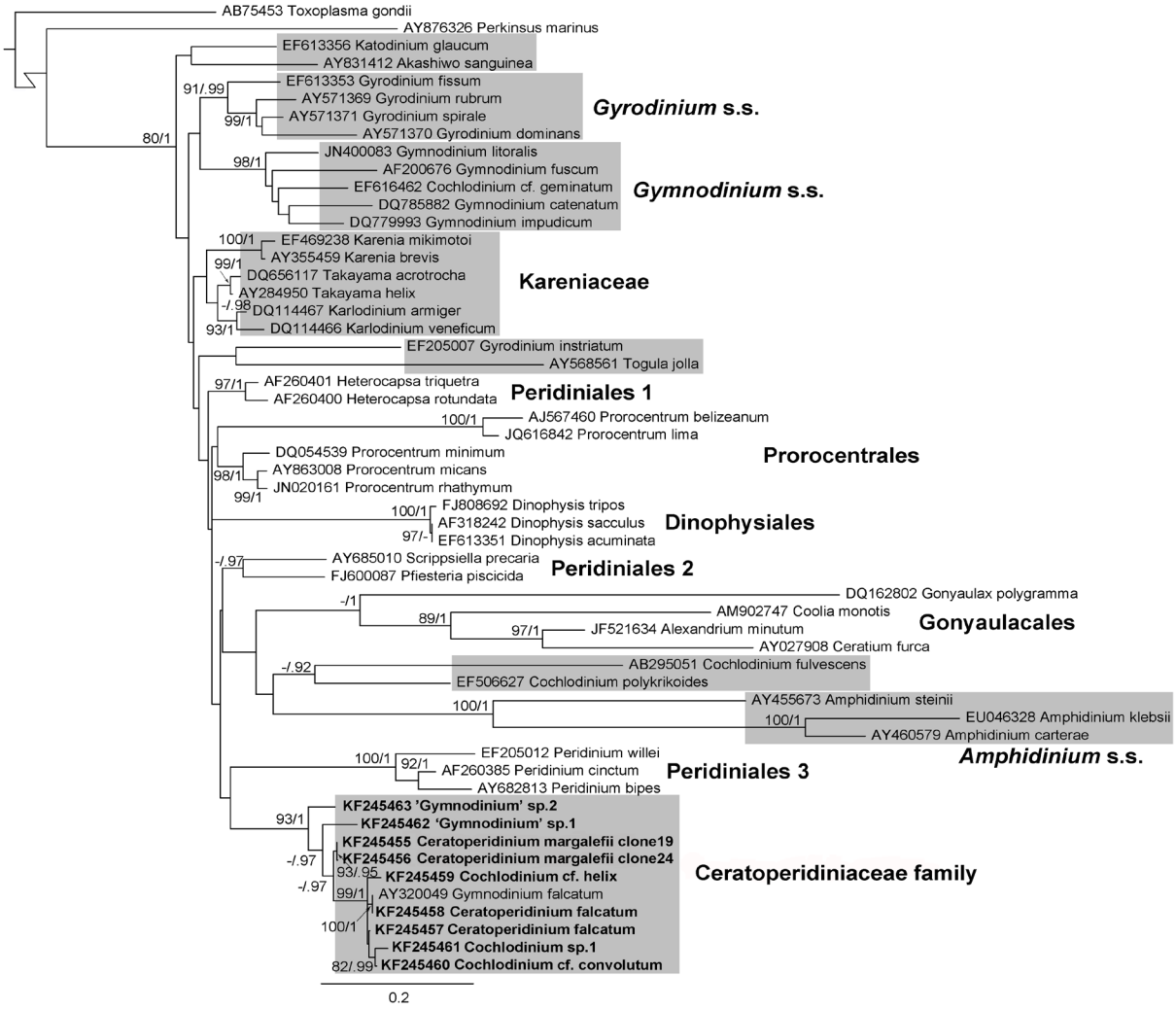

Figure 5: Maximum-likelihood phylogenetic tree of selected species based on 840 positions of the D1–D3 domain of LSU rDNA. Numbers on the nodes are the bootstrap values obtained after 1000 replicates and the Bayesian posterior probabilities (BPP). Only bootstrap values >80 and BPP >0.9 are shown. *Toxoplasma gondii* and *Perkinsus marinus* were used as outgroups. The code before each species corresponds to the GenBank accession numbers. Organisms sequenced in this study are highlighted in bold; shaded areas indicate the unarmoured species.

related to other organisms belonging to the Gymnodiniales order nor to any other clade of armoured dinoflagellates. Both "*Gymnodinium*" species occupied basal positions. A subclade was obtained, although not supported, containing *C. margalefii* sequences, and a cluster (99% / 1) comprising sequences of all *Cochlodinium* species identified in this study (*Cochlodinium* cf. *convolutum*, cf. *helix* and sp. 1) and those of *C. falcatum*. One of the *C. falcatum* sequences determined in this study (KF245458) obtained from Mediterranean specimens was identical to that available from GenBank (obtained from the culture of Australian specimens), but the second sequence (KF245457) also obtained from Mediterranean specimens differed from the others, with a 98.9% similarity.

4. Discussion:

The partial LSU rDNA sequences obtained in this study are evolutionarily very close and form a highly supported new clade, despite substantial differences in the morphologies of the respective unarmoured dinoflagellates. Historically, dinoflagellates have been distinguished and classified based on morphological features, with the shape of the acrobase recently proposed as a key feature to distinguish genera comprising unarmoured organisms (Daugbjerg et al. 2000; de Salas et al. 2003; Takayama 1985). However, molecular phylogeny has led to extensive revisions of dinoflagellate taxonomy as it has revealed, on the one hand, the classification of numerous species within the wrong genera and, on the other, relationships between organisms that a priori are morphologically unrelated. While some genera of unarmoured dinoflagellates form well-supported clades, i.e. that of *Gymnodinium s.s*., which contains several genera (Daugbjerg et al. 2000), and that of *Amphidinium s.s.* (Flø Jørgensen et al. 2004a), there are also organisms that do not cluster with any other group of unarmoured dinoflagellates, for example, *Akashiwo sanguinea* (Hirasaka) Hansen & Moestrup (Kim and Kim 2007) and *G. instriatum* (Saldarriaga et al. 2004). Additionally, monophyletic clades of unarmoured dinoflagellates contain morphologically distant genera; thus, the *Gymnodinium s. s.* clade consists not only of *Gymnodinium*-like species but also of polykrikoids (pseudocolonial organisms) and warnowiids (ocelloid-bearing organisms) (Hoppenrath et al. 2009). It appears that in several clades, such as that of *Ceratoperidinium*, studied here, the rapidly evolving morphologies of their member species result in gross morphological variations that betray the underlying conservative phylogenetic affinities.

The shape of the acrobase was successfully observed for *C. falcatum*, *Cochlodinium* cf. *helix* and '*Gymnodinium*' sp. 1. It formed a circular loop around the apex with its two ends in contact, as was previously observed for *C. falcatum* and *Cochlodinium convolutum* (Takayama 1998, Personal website). The phylogeny obtained in this study showed the close relationship between *C. margalefii, C. falcatum*, some *Cochlodinium* species and *Gymnodinium*-like dinoflagellates. While prior to our study, the sequence of *C. falcatum* available from GenBank clustered independently (de Salas et al. 2003), we were able to demonstrate that this species is strongly related to other unarmoured species. This was also the case for *C. falcatum*, *C. convolutum* and two *Gymnodinium*-like species, based on SSU rDNA sequences (Iwataki et al. 2005; Matsuoka 2006). Therefore, a number of species whose sequences have yet to be obtained might be included within clades of unarmoured species that currently are not well represented. Nonetheless, for the studied organisms there are several considerations, discussed in the following.

Ceratoperidinium margalefii was described as a thecate free-living photosynthetic species, with a pentagonal shape, dorsoventrally compressed and characteristic flexible extensions in the apex and antapex (Loeblich III 1980). Other authors also described this species as pigmented (Margalef 1969; Nincevic et al. 2006) but the cell observed in this study was colourless, During our observations of the *C. margalefii* specimen the sizes of the antapical appendices varied and the apical appendix was completely absent. Reports in the literature also note a broad range of apical lengths (France and Mozetic 2009; Gómez and Abboud-Abi Saab 2003; Gómez et al. 2004; Nincevic et al. 2006). *Ceratoperidinium falcatum* exhibits retractile appendices, which have been observed in other genera as well, including *Brachidinium* Taylor (Gómez 2006, 2011). However, *C. margalefii* and *C. falcatum* are not phylogenetically related to *B. capitatum*

Taylor, as the latter clusters with the *Karenia* genus (Henrichs et al. 2011). Therefore, species that share a particular morphological trait are not necessarily phylogenetically related. We were able to observe the sulcus outline of the studied cell. Margalef (1969) observed a single cell from its dorsal side, which impeded visualization of the cingulum junction and the sulcus (Fig. 2D). Gómez et al. (2004) depicted the cell from observations of fixed specimens and was therefore unable to characterize the outline of the sulcus (Fig. 2B, C). Among the several illustrations of this organism, there are also notorious differences related to the apical and antapical appendices.

The distinctively different morphologies that occur during the different life cycle stages of *C. falcatum* have led to their erroneous description as different species (Gómez 2007; Konovalova 2003). Accordingly, Gómez (2007) discussed the need to re-assess the systematic position of *C. falcatum*. The phylogenetic characterization of *C. falcatum* does not support its placement within the *Gyrodinium* genus, as it is not included in the clade containing other species of the genus. Furthermore, *C. falcatum* contains chloroplasts while *Gyrodinium* species are defined as heterotrophic. Additionally, the shape of its acrobase differs from that defined for the *Gyrodinium* genus (Daugbjerg et al. 2000). The two different sequences representing the *C. falcatum* morphotypes reflect at least a large degree of intraspecific variability, if not the presence of cryptic species.

Our results and the other *Cochlodinium* sequences available in GenBank (*C.* cf. *geminatum*, *C. polykrikoides* and *C. fulvescens*) provide evidence that the genus *Cochlodinium* is polyphyletic and should be divided into at least three different genera. However, the realization of this modification is hindered by the lack of phylogenetic and detailed morphological information for *C. strangulatum* (Schütt) Schütt, the type species of the genus. Consequently, any genus transfer should be avoided until the phylogenetic position of the type species is obtained. The acrobase of *C. polykrikoides* (Iwataki et al. 2010) clearly differs from that of *Cochlodinium* cf. *helix*, an observation that supports the assignment of these two organisms to different genera. *Cochlodinium* cf. *convolutum* was only tentatively identified because some characters differed from its original description. They agreed with their length, cingulum turns, notched antapex, nucleus shape and the presence of a hyaline membrane around the cells. However, *C. convolutum* was defined as being wider posteriorly, with a round apex and greenish, while our specimen was yellowish, with a flattened apex and wider in the central area. Regarding the existing reports of *C. convolutum* from the literature, specimens observed by Gárate-Lizárraga et al. (2011) were similar to our specimen but the antapex were less notched and the epicone more pointed for some of their specimens. Matsuoka et al. (2008) observed pigmented specimens with an elongated epicone, clearly differing to the original description of *C. convolutum* and having a better agreement with *C. pirum* (Schütt) Lemmermann. Finally, our specimen agreed with the specimen identified as *C. convolutum* by Meave-del Castillo et al. (2012). *Cochlodinium* cf. *helix* was morphologically very similar to *C.* cf. *convolutum* but the epicone was conical and the apex rounded, with a less notched antapex. The sulcus turned to the left in the epicone. In this case, although the original description of *C. helix* is highly dubious, our specimens showed a great similarity with those depicted by Schütt (1895). Available information for *C. helix* is scarce, but organisms identified as *C.* cf. *helix* were reported to produce harmful algal blooms in Australia (Hallegraeff 1992). The high similarity observed for LSU rDNA sequences of *Cochlodinium* cf. *convolutum* and sp. 1, and the lack of morphological traits of *Cochlodinium* sp. 1 arise the possibility that it probably represents intraspecific variability for the same species, as observed for *Ceratoperidinium falcatum*. However, although in the clade composed of *C. falcatum* and *Cochlodinium* spp. the relationship among subclades is not resolved, the morphological differences observed for *Cochlodinium* cf. *convolutum* and *Cochlodinium* cf. *helix* and the similarity of both sequences (88%) support that they are different species.

Two *Gymnodinium*-like dinoflagellates are also included within the Ceratoperidiniaceae clade. Monoclonal cultures were obtained for '*Gymnodinium*' sp. 1 and under the culture conditions neither different life cycle stages nor different morphologies were detected. Therefore, although we cannot reject the possibility that we observed only one stage of the '*Gymnodinium*' sp. 1 life cycle, i.e. the asexual vegetative stage, this species differs from those in other

genera with respect to acrobase shape and phylogeny. However, as '*Gymnodinium*' sp. 1 and '*Gymnodinium*' sp. 2 are morphologically similar, additional information is needed to determine their relationship. Although it is safely concluded that both species belong to the family Ceratoperidiniaceae, only unambiguously identified species should be assigned to a given genus, in order to avoid classification errors.

The results obtained in this study lead to the following conclusions:
1. A new monophyletic clade of unarmoured dinoflagellates was determined that includes the apparently morphologically unrelated species *C. margalefii*, *C. falcatum* comb. nov., *Cochlodinium* spp. and *Gymnodinium*-like species. In all of the examined species, the acrobase formed a closed circular loop around the apex and all species presumably possessed pigments. The family Ceratoperidiniaceae Loeblich III, 1980 is emended to reflect this.

2. Historically, the phylogenetic and taxonomic position of *Ceratoperidinium margalefii* has been doubtful. However, we showed that this species is closely related to other unarmoured dinoflagellates, confirming it as a member of the Gymnodiniales sensu lato order. We were also able to provide the first description of the morphology of the sulcus of this organism.

3. *Gyrodinium falcatum* was erroneously assigned to the genus *Gyrodinium* and, based on our findings, it has now been transferred to *Ceratoperidinium falcatum* (Kofoid & Swezy) Reñé et de Salas comb. nov. The sequences obtained suggest a large degree of intraspecific variability, if not the presence of 'cryptic' species.

4. The genus *Cochlodinium* is polyphyletic, and thus artificial, and should be separated into at least three different genera. However, the phylogenetic position of the type species of the genus must be clarified prior to any taxonomic change.

5. The two *Gymnodinium*-like species are not phylogenetically related to any other gymnodinioid species sequenced to date. While in one of them the acrobase forms a circular loop, the two species have some differing features and the relationship between them is not clear. They probably belong to a new genus to be erected but it must be preceded by the unequivocal description of their characteristic traits.

Taxonomic summary

- Family Ceratoperidiniaceae Loeblich III, 1980, emend. Reñé et de Salas
Unarmoured dinoflagellates possessing chloroplasts. Acrobase making a closed circular loop around the apex.

- Genus *Ceratoperidinium* Loeblich III, 1980, emend. Reñé et de Salas
Unarmoured dinoflagellates possessing chloroplasts. Acrobase making a circular loop around the apex. Retractile appendices (both apical and antapical) present at least during some life-cycle stages. Cingulum descending, displaced 2-3 times its own width, not overhanging.

- *Ceratoperidinium falcatum* (Kofoid et Swezy) Reñé et de Salas comb. nov.
Basionym: *Gyrodinium falcatum* Kofoid & Swezy (1921) The free-living unarmored Dinoflagellata, p. 299. Memoirs of the University of California, v. 5. University of California Press, Berkeley, California, U.S.A. 28 June 1921. 562 pp.

Synonyms: *Gymnodinium fusus* Schütt (1895) per parte, incl. only Fig. 81, Pl. 25.
Pseliodinium vaubanii Sournia (1972)

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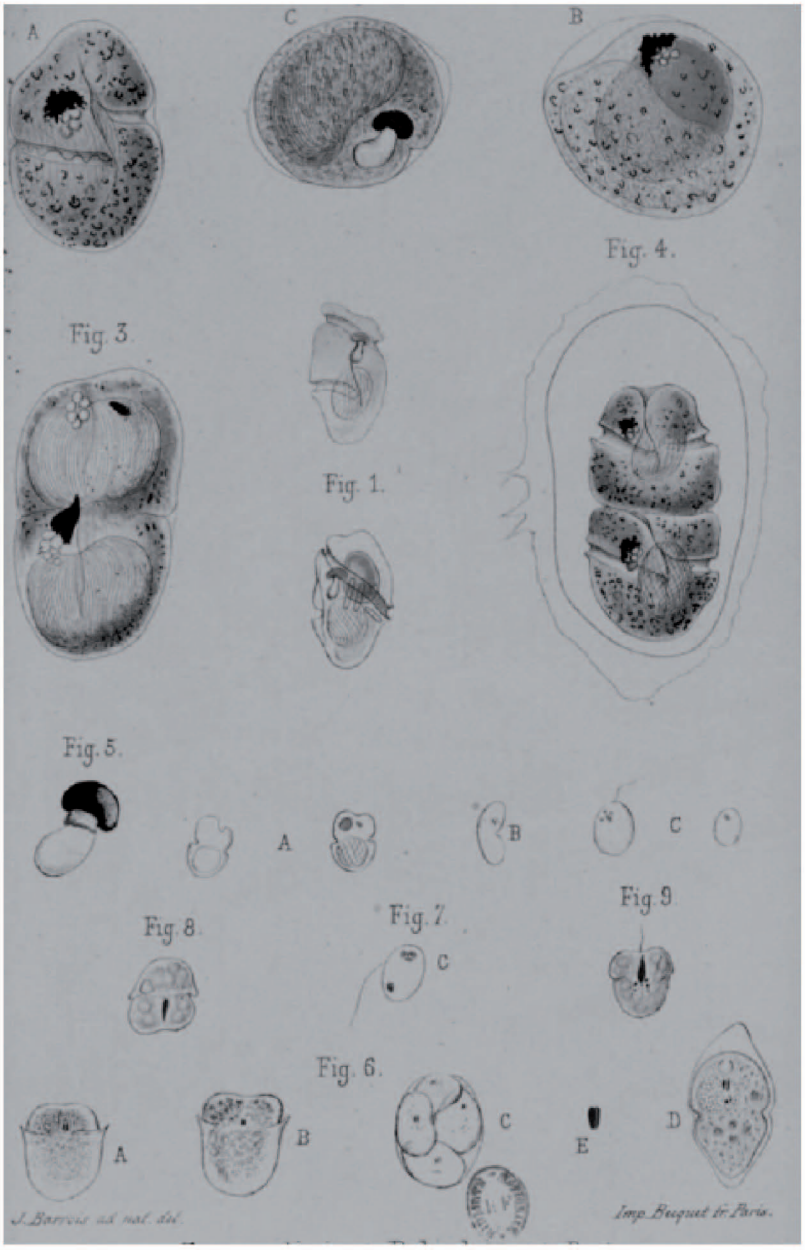

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General Discussion

"Biology is a science of three dimensions. The first is the study of each species across all levels of biological organization, from molecule to ecosystem. The second dimension is the diversity of all species in the biosphere. The third dimension is the history of each species in turn, comprising both its genetic evolution and the environmental change that drove the evolution. Biology, by growing in all three dimensions, is progressing toward unification and will continue to do so."

- Edward O. Wilson 2005

GENERAL DISCUSSION

The species-level identification of unarmoured dinoflagellates is challenging because of their plasticity, the artifacts that limit the study of fixed specimens, and the difficulties in culturing most species. Moreover, uncertainties arise from the often incomplete and doubtful original descriptions, which were based solely on morphological observations of a few specimens. Nevertheless, in this thesis many unarmoured genera were detected and successfully identified by combining morphological observations with phylogenetic data. Most of the existing genera of unarmoured dinoflagellates; 18 morphospecies belonging to the Gymnodiniales *sensu stricto* clade and 40 belonging to the order Gymnodiniales *sensu lato* (**Chapters 1 and 2**) were observed and classified. However, because of the large number of species described in the literature and the incompleteness and uncertainty of some of those descriptions, the species-level identification of some specimens belonging to the genera *Gymnodinium* (**Chapter 1**) and *Gyrodinium* (**Chapter 2**) was not possible. Furthermore, the delimitations of some genera are confusing, such that in some cases the genus-level classification of an organism was also hindered, as shown for Warnowiaceae members (**Chapter 1**).

Although about 200 *Gymnodinium* species have been described worldwide (Gómez, 2005; Thessen et al., 2012), 38% have not been observed again since their description (Thessen et al., 2012). It is therefore likely that some descriptions based on the morphology of a single cell were in fact observations of damaged or teratological specimens (Thessen et al., 2012). The *Gyrodinium* genus contains about 90 species (Gómez, 2005; Guiry and Guiry, 2013) and, although it has not been quantified in detail, the controversy resembles that associated with *Gymnodinium*. A high phenotypic plasticity was observed for some morphospecies, while the features often used to distinguish very similar species were in many cases irrelevant. Consequently, some reportedly distinct morphospecies may actually refer to a single one, as previously noted in the literature (Elbrächter, 1979; Gómez and Souissi, 2007). The Warnowiaceae group is composed of 35 described species divided into five accepted genera (Gómez, 2005). However, while two of them have unique characteristics, the boundaries between the remaining genera are doubtful. Furthermore, most species are rare and the specimens easily collapse when they are manipulated, impeding thorough characterization of the organisms.

Consequently, morphological characters are often insufficient to discriminate among the different species, thus preventing their classification. Nonetheless, several molecular studies suggest that the current expected diversity of dinoflagellates is underestimated (Stern et al., 2010). Molecular techniques allow us to study and confirm the taxonomy of unarmoured dinoflagellates and to clarify several issues impossible to resolve based on morphology alone.

Contribution of molecular taxonomy to identify and classify unarmoured dinoflagellates

Combination of morphological and molecular data

Single-cell PCR has been successfully applied to decipher the molecular taxonomy of dinoflagellates. This technique, in contrast to the use of DNA extracted from cultures, has allowed studies of the morphological characters of rare, heterotrophic, and uncultivable species as well as determinations of their systematic position and phylogenetic relationships. In this thesis, it resulted in the sequencing of 43 different species, 25 of which are the first sequences available for the target species. Among the most remarkable contributions are those enhancing our knowledge of Warnowiaceae members and the genus *Gyrodinium* **(Chapters 1 and 2)**, given the low number of sequences previously available.

Molecular information on Warnowiaceae members is scarce, despite recent efforts to redress this situation (Gómez et al., 2009; Hoppenrath et al., 2009). The sequences and morphological features of some morphospecies collected and studied during this thesis provide a step forward to the reclassification of these organisms. While the characters historically used to discriminate among the different genera are frequently inconsistent, the addition of the molecular

phylogenies of these organisms might represent a turning point in the reclassification of all the organisms included within this family. However, the morphological characteristics of the organisms must be studied in depth and the inclusion of other molecular markers could provide new insights for their classification.

The *Gyrodinium* genus is quite common and can be found worldwide, but the LSU rDNA sequences of only five identified species and very few SSU rDNA sequences were previously available. The LSU rDNA sequences of 13 *Gyrodinium* morphospecies are provided in this thesis, allowing further comparisons with sequences obtained from other studies, although some of them could not be identified at species level.

Detection of new species

In other cases, the combination of morphological characters and phylogenetic information allowed the detection and characterization of species previously undescribed, resulting in the erection of two new species: *Gymnodinium litoralis* **(Chapter 3)** and *Polykrikos tanit* **(Chapter 4)**, and a possible new species of the genus *Apicoporus* remains to be described **(Chapter 2)**. *Gymnodinium litoralis* is a common coastal species. It has been detected along the Catalan coast throughout the year and is a producer of high-biomass proliferations. *P. tanit* is recognizable by its characteristic morphology and has been detected at moderate abundances, although only in certain locations, and for restricted periods of time. Therefore, while neither species is rare the presence of both has often been overlooked. Other detected organisms could represent new species, as *Gymnodinium* sp. 2 **(Chapter 1)** or the *Gymnodinium*-like species included in the Ceratoperidiniaceae family **(Chapter 6)**. However, the currently available information is too scarce to affirm it.

Going deeper into the species delimitations

The results obtained in this thesis highlighted some difficulties to clearly delimit the different species studied. The biological species concept could not be used because of the limitations to induce sexuality in these organisms under laboratory conditions. The classical morphological concept was proved to be useless in cases when morphological features were very difficult to observe or morphological differences among species were nearly inexistent (cryptic species). The use of the phylogenetic species concept helped in solving some of these problems. However, intraspecific variability and the lack of resolution of the region used to construct the phylogenies observed for some species also highlighted the limits when applying this species concept.

The sequences obtained for some organisms revealed the existence of cryptic and pseudo-cryptic species, i.e., *Gyrodinium spirale* complex **(Chapter 2)**, and the presence of different ribotypes of *Cochlodinium polykrikoides* in Catalan waters **(Chapter 5)**. The cosmopolitan species *Gyrodinium spirale* is the type species of the genus and its morphology, ultrastructure, and phylogeny have been well studied (Hansen and Daugbjerg, 2004; Takano and Horiguchi, 2004). However, all the sequences obtained during this thesis from specimens firstly identified as *G. spirale* differed from the only LSU rDNA sequence previously available, suggesting the existence of cryptic species. The SSU sequences of *G.* cf. *spirale* obtained in this thesis agreed with those of *G. spirale* and *G. fusiforme* available in GenBank, impeding a clarification about this issue. Accordingly, some of the detections of *G. spirale* reported in the literature might instead refer to different species, with all of the ecological implications that conclusion entails. Cryptic species are common in dinoflagellates (Lilly, 2007; Montresor et al., 2003) but they also occur in other microplanktonic groups, such as diatoms (Kooistra et al., 2008; Quijano-Scheggia et al., 2009).

Cochlodinium polykrikoides is a toxic species that occurs worldwide and is responsible for fish mortality (Kim, 1998; Kudela and Gobler, 2012; Richlen et al., 2010). Although detected in high abundances during this thesis, the presence of *C. polykrikoides* had never been previously confirmed along the Catalan coast, mostly because of problems in distinguishing this species in fixed samples and in identifying *Cochlodinium* members at the species level. Surprisingly, our molecular analyses confirmed the coexistence of two different ribotypes in Catalan waters: one considered exclusive to Philippine waters and the other characterized for the first time. These results not only suggest

that the current biogeographic interpretation of ribotypes is invalid but also imply the possible existence of pseudo-cryptic species, potentially differing in their behaviour, toxicity, and autoecology. Given the economic impacts of this species on the aquaculture industry, these findings merit careful consideration.

In some cases, different sequences were obtained for a single species and showed enough level of divergence to be considered as different species, i.e. *Akashiwo sanguinea* **(Chapter 2)**, *Ceratoperidinium falcatum* **(Chapter 2 and 6)**. However, the morphological observations did not provide any evidence that the specimens belonged to different species. In contrast, other morphospecies showed almost identical partial LSU rDNA sequences, i.e. *Takayama tasmanica* and *T. tuberculata*, or some *Gyrodinium* species **(Chapter 2)**. Consequently, a fixed limit of divergence cannot be established to delimit the different species of dinoflagellates.

Determination of phylogenetic relationships

The molecular information obtained for newly sequenced organisms pointed out incongruences in morphology-based taxonomy, as shown for *Gymnodinium agaricoides* **(Chapter 2)**, and resulted in new combinations for species previously classified within the wrong genus, i.e., *Ceratoperidinium falcatum* **(Chapter 6)**. Moreover, ours were the first sequences obtained for some genera, which allowed their phylogenetic position and relationships to be determined. Specimens belonging to the genus *Torodinium* were sequenced and found to cluster independently of other unarmoured dinoflagellates sequenced to date except to the morphologically similar species *Katodinium glaucum*, although with low support **(Chapter 2)**. A specimen belonging to the genus *Ceratoperidinium*, whose taxonomic position was doubtful, was also sequenced for the first time, which confirmed its close relationship with other unarmoured dinoflagellates **(Chapter 6)** but also with morphologically distant species (*Cochlodinium* spp., *Gymnodinium*-like, *C. falcatum*), resulting in the emendation of the family Ceratoperidiniaceae and the genus *Ceratoperidinium* **(Chapter 6)**. In addition, the polyphyly of the genus *Cochlodinium* was demonstrated as was the existence of *Gymnodinium*-like organisms phylogenetically unrelated to this genus. Those results reinforce the frequent inability of morphology alone to determine either the taxonomic position or the relationships of an organism. Instead, a combined morphological and phylogenetic approach is most likely to yield an accurate characterization.

Adequacy of the molecular method used

Although attempts were commonly made to culture the specimens, cultures could only be established for a few autotrophic species. Ideally, cultures reveal the morphological variability of the species and enable detailed studies, for example, scanning or transmission electron microscopy, analyses of pigment composition, and epifluorescence staining. Moreover, the availability of large amounts of DNA ensures the reproducibility of the molecular analyses and the sequencing of different DNA regions.

In this thesis, the use of environmental sequencing to study the diversity of unarmoured dinoflagellates was dismissed. Environmental sequencing is a useful approach to estimate the total diversity of a community. However, interpretation of the results is based on the existing molecular information available for each group. While this method can lead to new insights into the diversity of the target group, it does not provide crucial morphological, functional, and physiological information (Caron, 2013). In this thesis, environmental sequencing was not applied because the aim was a morphological and molecular characterization of the diversity of unarmoured dinoflagellates. However, the results obtained in this thesis provide basic information to supplement current databases, which in turn will facilitate the interpretation of environmental sequencing data.

Single-cell PCR (SC-PCR) was applied successfully for the wide range of species studied in this thesis; however, it has several limitations. Most importantly, perhaps, it only allows one attempt to obtain the sequence of the isolated

specimen. If this attempt fails, it is impossible to repeat the process. Consequently, some morphospecies were detected but their sequences could not be obtained. Furthermore, the morphological description and the sequence are obtained from the same cell. Therefore, observations are limited to one specimen and the morphological variability of the species, which is common in dinoflagellates, remains unrecognized.

In our hands, the success rate of SC-PCR varied from nearly 100% of the samples processed to very a low percentage of them. The same protocol was applied to all the species but probably, not all of the cells were equally disrupted during the freezing-thawing process. Furthermore, the amount of cellular DNA varied greatly among different species, which clearly affected the final results in single-cell DNA amplifications. In addition, universal primers were used to amplify the selected regions. Although the whole range of unarmoured species was successfully sequenced, for some of them the performance of those primers might not be optimal, affecting the overall success of the process. Therefore, the failure to obtain the sequences of some specimens was probably due to methodological limitations.

Sequencing of the LSU rDNA fragments allowed the species-level characterization, discrimination, and classification of most of the studied organisms. However, this fragment lacks the resolution needed to characterize some species of the *Gyrodinium* genus **(Chapter 2)**, as their D1-D2 regions are almost identical. Thus, the entire LSU rDNA should be sequenced, if not a different gene. This conclusion would not have been possible in the absence of a morphological characterization of the specimens. A similar lack of resolution was previously reported for other groups of dinoflagellates, i.e., some species of *Dinophysis* (Edvardsen et al., 2003). The SSU sequence of some unarmoured species was also obtained in this thesis, and in general, the phylogenies agreed with those obtained for the LSU rDNA region.

The diversity of unarmoured dinoflagellates along the Catalan coast

The composition and diversity of dinoflagellates from the Catalan coast has been relatively well studied compared to other areas of the Mediterranean Sea (Delgado, 1987; Estrada, 1979, 1980; López and Arté, 1973; Margalef, 1945a, 1945b, 1965, 1969; Morales, 1956). Some studies included checklists of dinoflagellates either from the whole Mediterranean Sea (Gómez, 2003) or its NW part (Velásquez, 1997), obtained from studies based on morphological observations because molecular techniques were inexistent or still being developed.

The 58 different unarmoured species of dinoflagellates detected as part of this thesis cannot be compared directly with already reported species because of the recent erection of several new genera and species. Furthermore, some species were shown to be synonymous and some genera have been reorganized. Even so, the number of species reported in this thesis accounts for 85% of the diversity of unarmoured species reported in the NW Mediterranean Sea (Velásquez, 1997) and 30-40% of those ever reported in the Mediterranean Sea (Gómez, 2003). Ten species in the Mediterranean Sea and eight along the Catalan coast were detected for the first time **(Chapters 1 and 2)**. This is quite surprising considering that in this thesis the characterized species were from a relatively well-studied coastal location. It can thus be assumed that the number of new genera and species detected does not reflect the diversity of rare species from remote or offshore areas but quite common, as yet uncharacterized organisms from a nearby environment. Additionally, some of the detected genera and species were those mostly inhabiting benthic and psammophilic habitats, i.e., *Amphidinium (*Flø Jørgensen et al., 2004), *Apicoporus* (Sparmann et al., 2008). The number of benthic species previously reported from the Catalan coast is relatively low, but in a few of the sediment samples acquired during this thesis species never previously observed in the area were detected, such as *Gyrodinium viridescens*, *Apicoporus* sp., and *Polykrikos lebourae*, highlighting the lack of knowledge related to their diversity and taxonomy. Thus, if rather than focusing on planktonic samples from coastal environments all the habitats of the Catalan coast and offshore waters had been studied, the total diversity obtained would have probably surpassed that

previously stated for the whole NW Mediterranean Sea.

The diverse characteristics of the locations studied (open and semi-closed, sandy and rocky beaches, large and small harbours, estuarine and freshwater-influenced areas) facilitate comparisons with other areas of the Mediterranean Sea and explain our detection of a high percentage of species previously reported in the NW Mediterranean. For instance, high-biomass blooms of *G. litoralis* developed along the Catalan and Sardinian coasts **(Chapter 3)**. However, some of the species detected during this thesis had previously only been reported in Australian waters **(Chapter 2)** and in several cases a close phylogenetic relationship between them was established **(Chapter 6)**. Those results support the "modified latitudinal cosmopolism" phenomenon (Taylor et al., 2008), which suggest that most dinoflagellates occur within similar climatic zones in the two hemispheres.

Harmful algal blooms (HABs) and routine monitoring programmes

Harmful algal blooms (HABs) are proliferations of algae that cause harmful effects on humans or the environment. Dinoflagellates comprise 70% of the harmful species and are divided in two groups, toxic species and high-biomass producers. There are about 80 known toxic species of dinoflagellates, affecting humans and wild fauna (Moestrup et al., 2009 onwards). About 200 dinoflagellates species produce high-biomass blooms (Smayda and Reynolds, 2003; Sournia, 1995), which negatively impact the environment by producing discolorations, foams, and mucilage and by causing anoxia or clogging the gills of fish, thus killing off marine fauna. In addition to the environmental effects, coastal economic activities such as tourism or aquaculture are hampered. Accordingly, substantial efforts have been made worldwide to detect, identify, and understand the behaviour of HAB-forming species.

Seven of the species detected as part of this thesis were toxic, with three of them detected for the first time along the Catalan coast (*Cochlodinium polykrikoides*, *Karenia mikimotoi*, and *K.* cf. *papilionacea*) and one in the Mediterranean Sea (*Karenia umbella*); eight bloom-forming species were identified as well **(Chapters 1, 2, 3 and 5)**. Thus, despite the seemingly exhaustive studies of harmful species, our results point out the lack of knowledge regarding their identification, distribution, and intraspecific variability, as demonstrated for the new ribotype of *C. polykrikoides* detected along the Catalan coast **(Chapter 5)**. Finally, routine monitoring samplings should be adapted to properly detect and identify toxic species, bearing in mind that fixed samples, which are commonly used, do not allow their discrimination. Rather, several molecular techniques, such as qPCR (Andree et al., 2011; Galluzzi et al., 2004) and microarrays (Galluzzi et al., 2011; McCoy et al., 2013), have proved to be effective to detect and quantify some species difficult to identify under microscopy.

Recommendations and future work

The following recommendations run from general issues to specific for unarmoured dinoflagellates or methodological issues.

- The endemism or ubiquity of a species can only be demonstrated when representative samplings from all environments are available (Mann and Vanormelinger, 2013). Consequently, a detailed discussion about the distribution of the species detected in this thesis has been avoided because of the scarce information available not only for some Mediterranean areas (both coastal and offshore), but also for some areas of the world's oceans.

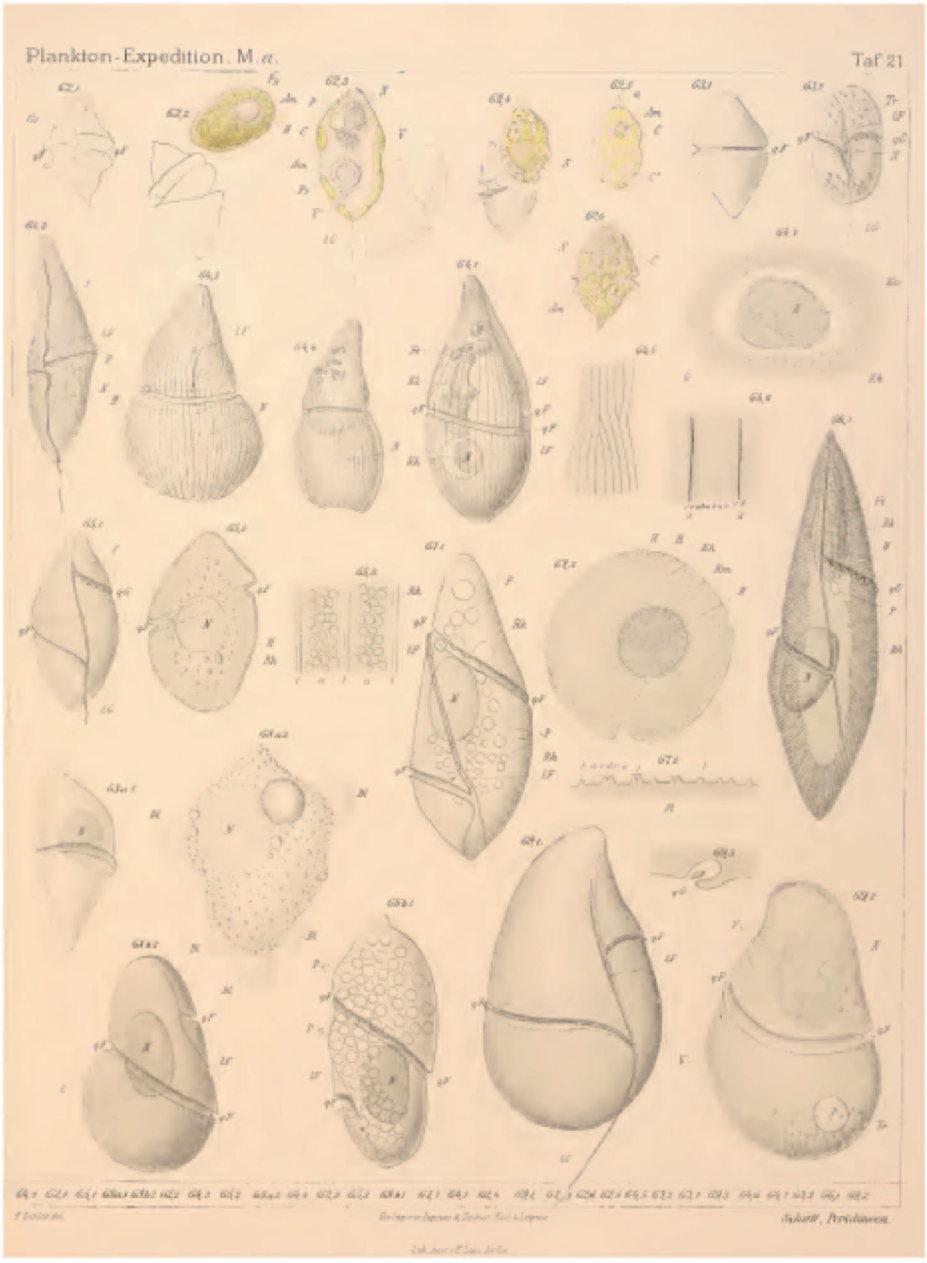
- For a better understanding of unarmoured dinoflagellates, accurate investigations on both planktonic and benthic species are needed, given that diversity estimates of the Gymnodiniales will be partial if only planktonic members are included.

- The global distribution of some unarmoured species must be considered with caution, e.g., *Cochlodinium polykrikoides*, as some cosmopolitan species have been shown to represent different cryptic or pseudo-cryptic species,

as also demonstrated for some other species of dinoflagellates (Lilly et al. 2007) and diatoms (Kooistra et al. 2008).

- Early phylogenetic studies indicated that the order Gymnodiniales was polyphyletic (Daugbjerg et al., 2000; Fensome et al., 1999; Saunders et al., 1997), but phylogenies were constructed using only one or a few ribosomal genes. Further studies, combining eight different genes, demonstrated the paraphyly of the group, a monophyletic origin of the theca, and the monophyly of most groups of thecated dinoflagellates (Orr et al., 2012). Accordingly, phylogenetic assumptions at high taxonomic levels must be made cautiously if they are based only on a few ribosomal genes.

- The application of further molecular techniques, such as DNA barcoding, on dinoflagellates is being explored elsewhere (Lin et al., 2009; Stern et al., 2010; Stern et al., 2012), in attempts to overcome the limitations of currently used techniques. Single-cell genomic techniques have been applied to bacteria and archaea to recover draft genomes. Genome-based phylogenetics are able to resolve the relationships among organisms that are not apparent by single gene analysis (Blainey, 2013; Rinke et al., 2013).



Schütt (1895) Die peridineen der Plankton Expedition

Main conclusions

"Taxonomy is described sometimes as a science and sometimes as an art, but really it's a battleground."
- Bill Bryson 2004

MAIN CONCLUSIONS

1. Eighteen morphospecies belonging to the Gymnodiniales *sensu stricto* clade and 40 belonging to the Gymnodiniales *sensu lato* order were detected along the Catalan coast. Together, they represent 85% of the total diversity reported in the NW Mediterranean Sea. Ten morphospecies were detected for the first time in the Mediterranean Sea, and eight, including several toxic species, for the first time along the Catalan coast. Among all detected species, eight were toxin-producers and another eight were high-biomass bloom producers.

2. Single-cell PCR was successfully applied in most organisms, resulting in the sequencing of 43 unarmoured species. For 25 of them, these are the first available sequences.

3. Five different warnowiids species were successfully sequenced and morphologically characterized, whereas only two LSU rDNA sequences were previously available (*Nematodinium* sp. and *Warnowia* sp.). The newly obtained sequences clustered in four clades, one of them including sequences previously available in GenBank. Consequently, the existing criteria for the discrimination of the two genera are not suppported by the obtained phylogeny.

4. The existence of cryptic species was demonstrated for the *Gyrodinium spirale* complex, which includes the type species of the genus. However, the D1-D2 LSU rDNA region lacked sufficient resolution to discriminate among other *Gyrodinium* morphospecies.

5. A new bloom-forming species, *Gymnodinium litoralis*, and a pigmented pseudocolonial species, *Polykrikos tanit*, were described. A new *Apicoporus* species was detected and it remains to be described. Other *Gymnodinium* organisms and the *Gymnodinium*-like species belonging to the Ceratoperidiniaceae family probably represent new species but it remains to be confirmed in further studies.

6. The toxic species *Cochlodinium polykrikoides* was detected for the first time along the Catalan coast. Most populations formed a newly differentiated ribotype, but others were included within the 'Philippines' ribotype, demonstrating their coexistence in the Mediterranean Sea. Thus, the former biogeographic nomenclature of the ribotypes appears to be invalid.

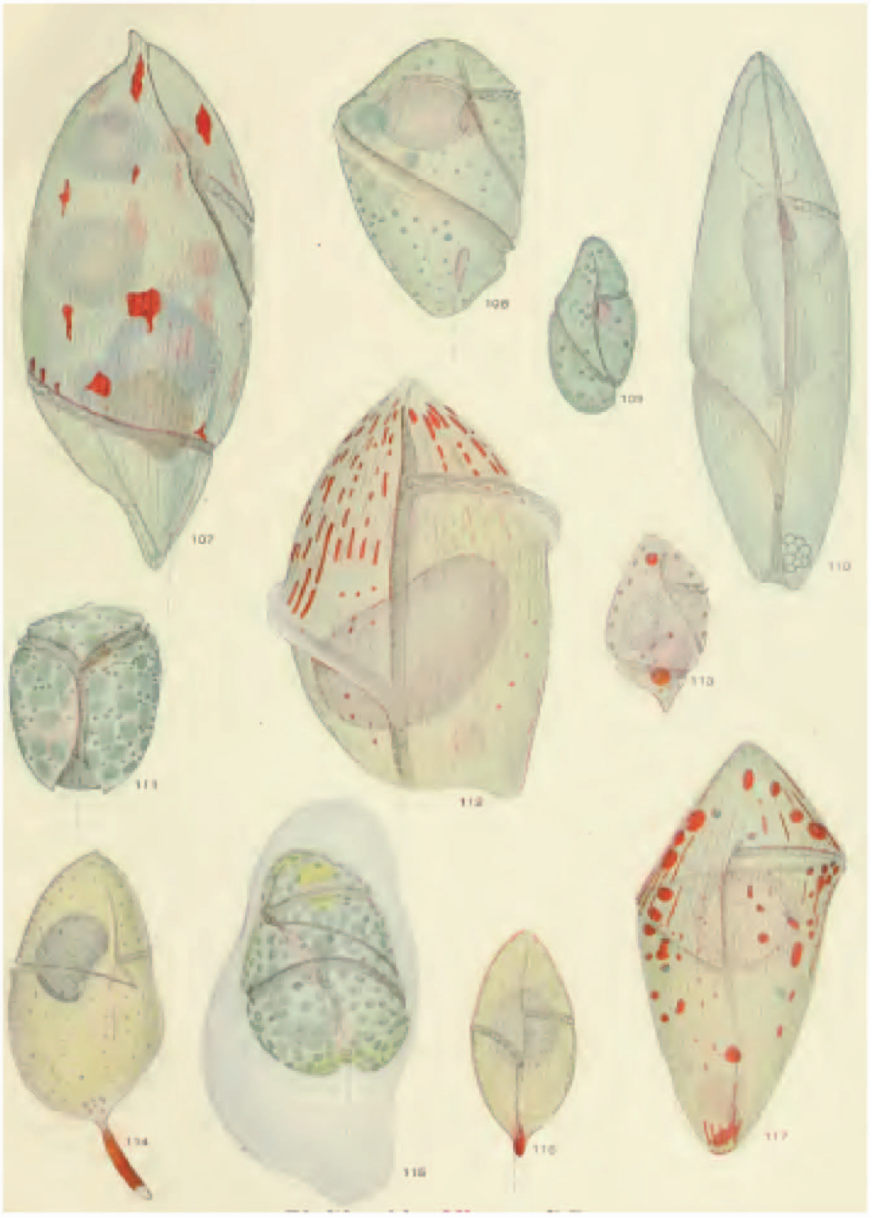
7. An incorrect generic assignation was demonstrated for two species (*Gyrodinium falcatum*, *Gymnodinium agaricoides*), resulting in the reclassification of *Ceratoperidinium falcatum* (Kofoid & Swezy) Reñé et de Salas comb. nov. Erroneous assignments were also demonstrated for *Cochlodinium* species belonging to the Ceratoperidiniaceae family, but they could not be reclassified.

8. A *Ceratoperidinium* specimen was sequenced for the first time and a new phylogenetic clade was obtained for several newly sequenced unarmoured dinoflagellates, including *Ceratoperidinium margalefii*, *C. falcatum*, three *Cochlodinium* species, and two *Gymnodinium*-like organisms. This resulted in the emendation of the Ceratoperidiniaceae family and the genus *Ceratoperidinum*.

9. LSU rDNA sequences obtained for *Torodinium* specimens are the first sequences available for the genus and allow studies of its phylogenetic relationship with other unarmoured dinoflagellates. The sequences clustered independently, albeit with a poorly-supported relationship with *Katodinium glaucum*.

10. Despite the high number of species detected, some habitats were not studied during this thesis. Consequently, the total diversity of unarmoured dinoflagellates will certainly be even greater than that recognized thus far. Furthermore, the distribution of some species as described in the literature must be regarded with caution because in most marine areas the presence of these organisms is generally overlooked.

11. The classifications and relationships of unarmoured dinoflagellates are still unknown or are dubious in some cases. The application of new molecular knowledge acquired will greatly aid in clarifying the phylogenetic relationships of unarmoured dinoflagellates.



Kofoid & Swezy (1921) The free-living unarmoured dinoflagellata

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of General Introduction and Discussion

"How inappropriate to call this planet Earth when it is quite clearly Ocean."
- Arthur C. Clarke

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