

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

Running title: A new clade 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 Ceratoperidiniaceae and the genus *Ceratoperidinium* are emended.

Keywords: acrobase; *Ceratoperidinium*; Gymnodiniales, LSU rDNA; phylogeny; unarmoured

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 (Edwardsen 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.

Results

- 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).

- *Cochlodinium* spp.: Three different morphotypes were detected and successfully sequenced (Table 1). One specimen of *Cochlodinium* cf. *convolutum* Kofoed 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.

- 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 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.

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 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. Retractable 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)

Material & Methods

- 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- μ m mesh. A settling chamber was used to observe the live organisms under a Leica-Leitz DM-II 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- μ l PCR tube adding the minimum volume of seawater, followed by several rounds of freezing/thawing and finally stored at -80°C 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 dried and sputter-coated with gold/palladium, then observed and photographed using a JEOL 35C scanning electron microscope.

- 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 95°C, 40 cycles of 20 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension step for 7 min at 72°C. 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 -20°C 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.

- 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.

- **Phylogenetic analyses:** Sequences obtained in this study were aligned with those obtained from GenBank using the MAFFT v.6 program (Kato 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 Accelerated 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.

Acknowledgments

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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. * indicates that the sequence was previously available in GenBank.

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

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 μ m.

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 μ m. 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 μ m.

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 μ m.

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 μ m.

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.

Figure 1

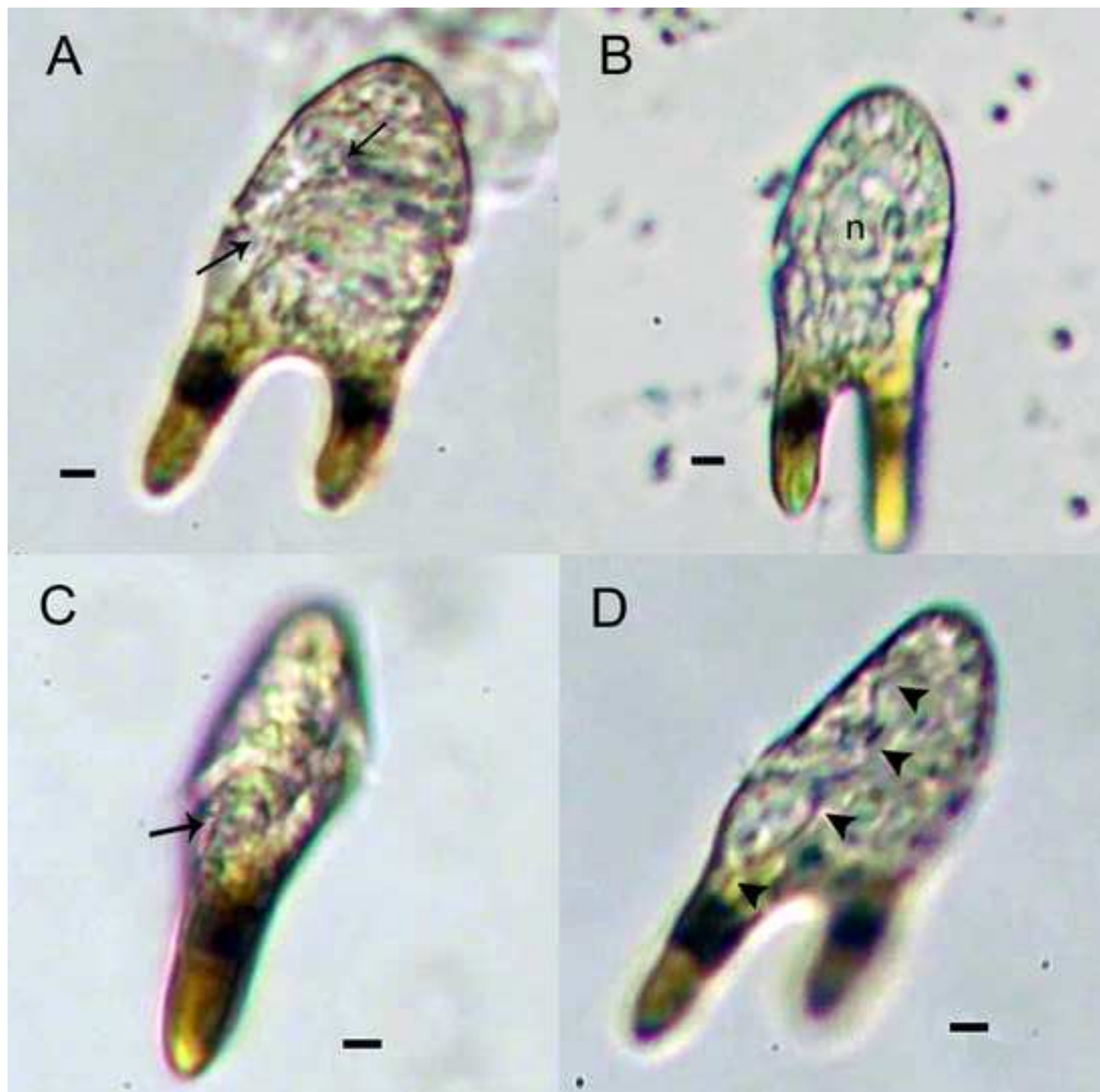


Figure 2

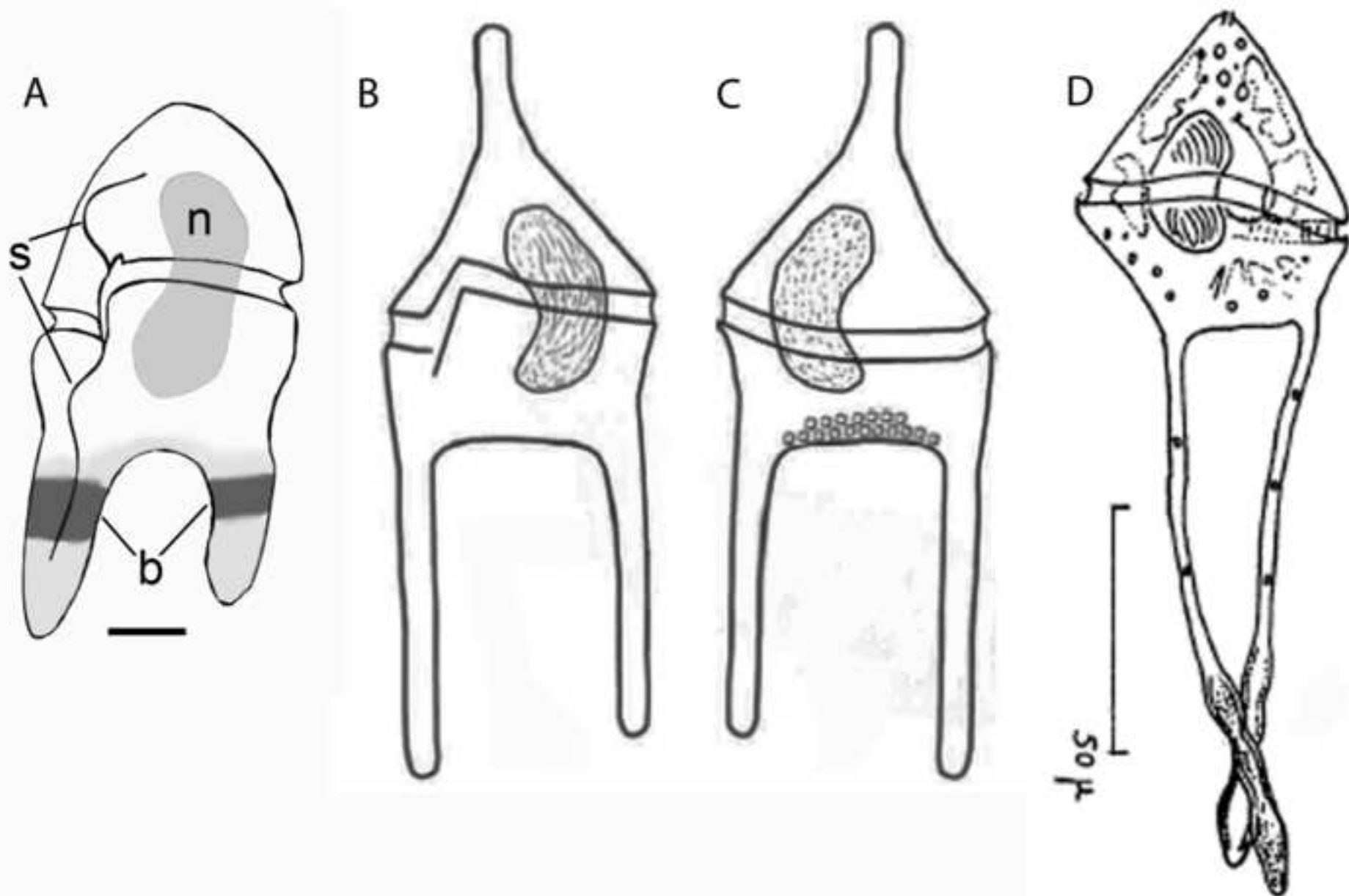


Figure 3

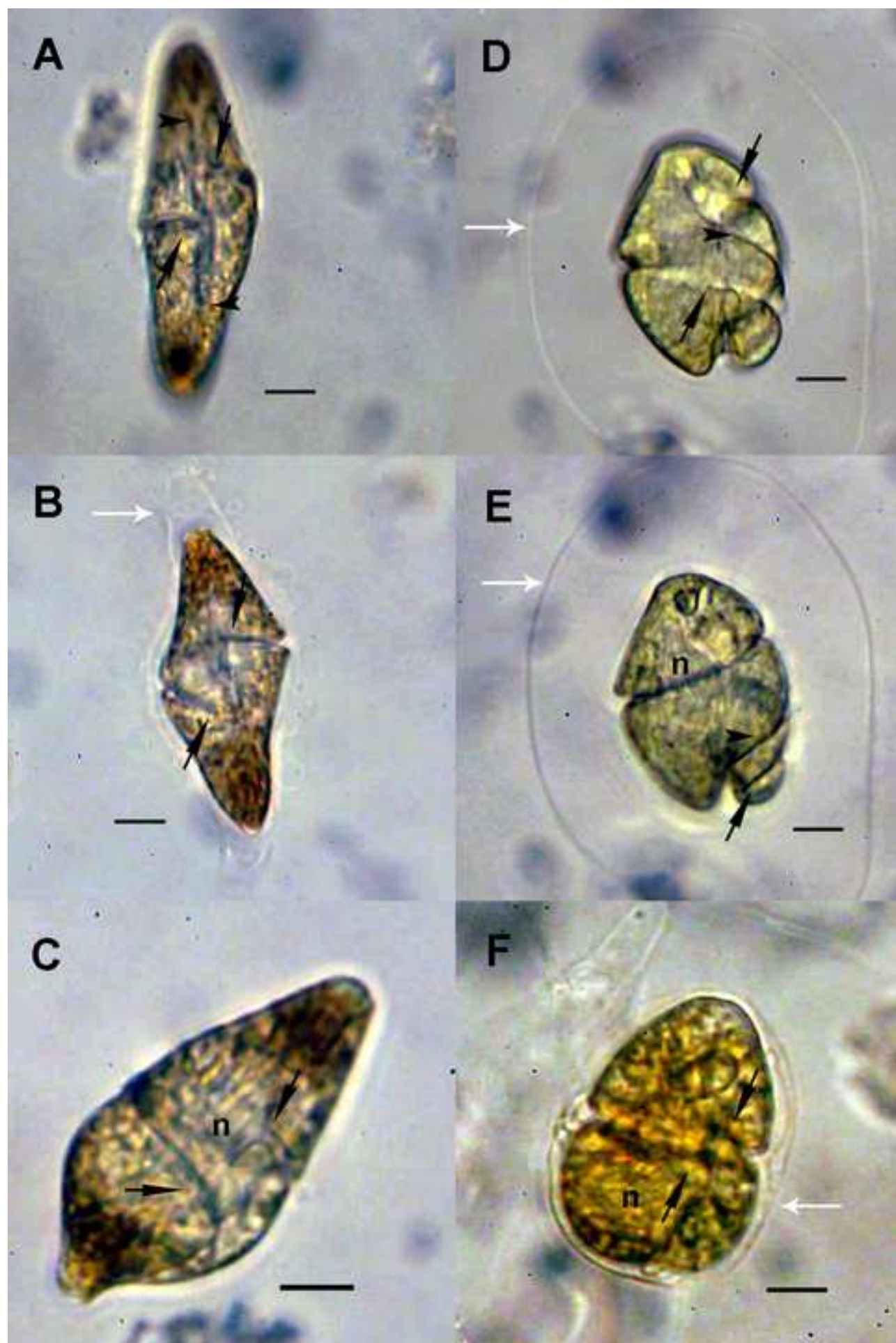


Figure 4

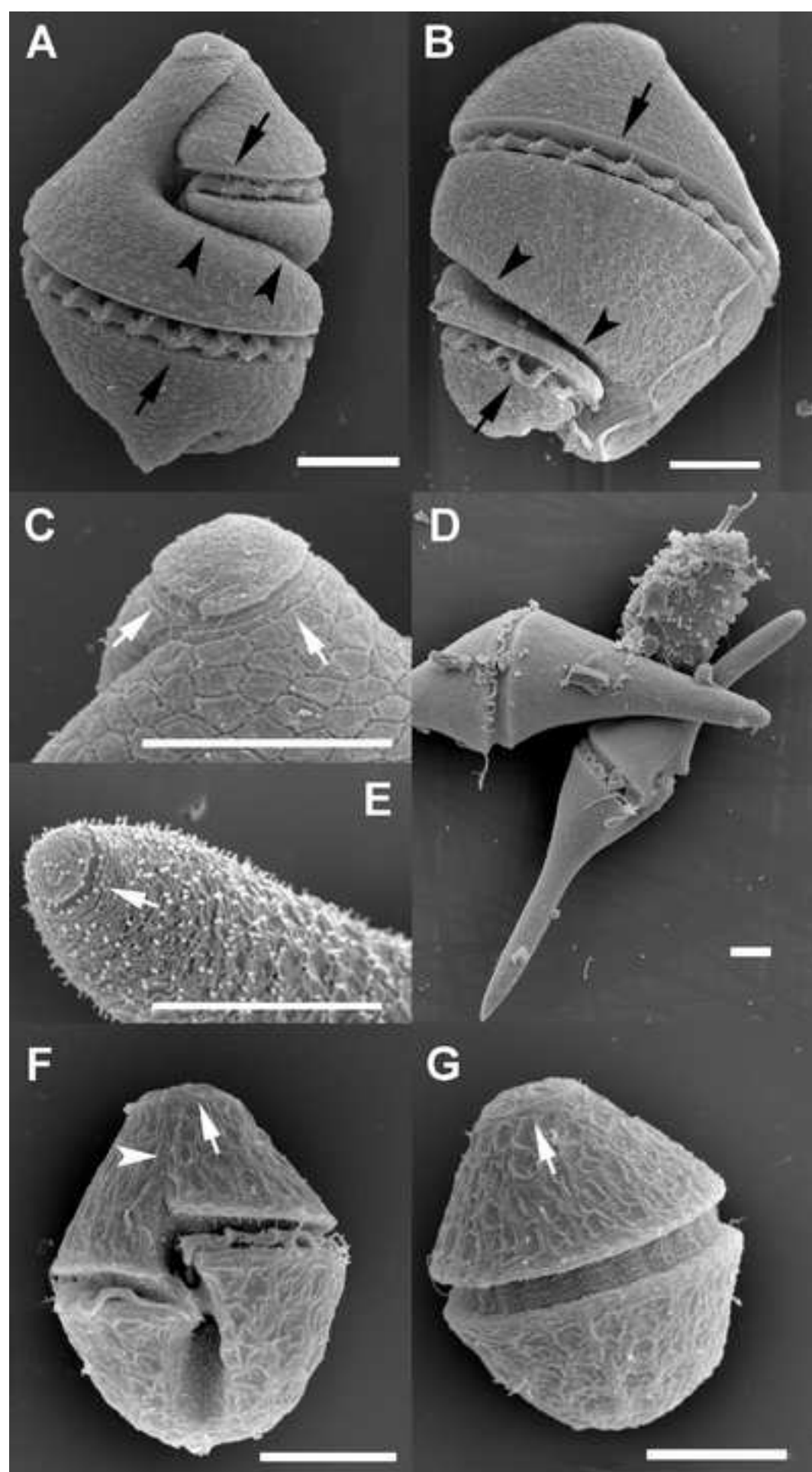


Figure 5

