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Molecular Phylogeny of Noctiluroid Dinoflagellates (Noctilucales, Dinophyceae)

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The order Noctilucales or class Noctiluciphyceae encompasses three families of aberrant dinoflagellates (Noctilucaceae, Leptodiscaceae and Kofoidiniaceae) that, at least in some life stages, lack typical dinoflagellate characters such as the ribbon-like transversal flagellum or condensed chromosomes. *Noctiluca scintillans*, the first dinoflagellate to be described, has been intensively investigated. However, its phylogenetic position based on the small subunit ribosomal DNA (SSU rDNA) sequence is unstable and controversial. *Noctiluca* has been placed either as an early diverging lineage that diverged after *Oxyrrhis* and before the dinokaryotes -core dinoflagellates- or as a recent lineage branching from unarmoured dinoflagellates in the order Gymnodiniales. So far, the lack of other noctiluroid sequences has hampered the elucidation of their phylogenetic relationships to other dinoflagellates. Furthermore, even the monophyly of the noctiluroids remained uncertain. We have determined SSU rRNA gene sequences for Kofoidiniaceae, those of the type *Spatulodinium* (=Gymnodinium) *pseudonoclituca* and another *Spatulodinium* species, as well as of two species of *Kofoidinium*, and the first gene sequence of Leptodiscaceae, that of *Abedinium* (=Leptophyllus) *dasypus*. These taxa were collected from their type localities, the English Channel and the NW Mediterranean Sea, respectively. Phylogenetic analyses place the Noctilucales as a monophyletic group at a basal position close to parasites of the Marine Alveolate Group I (MAGI) and the Syndiniales (MAGII), before the core of dinokaryotic dinoflagellates, although with moderate support.

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Introduction

Noctiluca scintillans (Macartney) Kofoid is the first known dinoflagellate (Baker 1753; Slabber 1771). Initially classified as a jelly fish, Haeckel (1873) proposed that *Noctiluca* Suriray ex Lamarck should be included in the Cystoflagellata within

the dinoflagellates. Based on detailed observations of the trophonts, Kofoid (1920) homologized

Abbreviations: bp, base pairs; BV, bootstrap value; DAPI, 4,6-diamidino-2-phenylindole; MAGI, Marine Alveolate Group I; PCR, Polymerase chain reaction; SSU, small subunit; rDNA, ribosomal deoxyribonucleic acid

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the structures recognised in *Noctiluca* with the sulcus, cingulum, longitudinal and transverse flagella of the dinoflagellates, and since then, *Noctiluca* was classified within dinoflagellates as the type of the order Noctilucales. In current taxonomic schemes, dinoflagellates (Dinoflagellata) are divided into two subdivisions: Dinokaryota and Syndinea (Fensome et al. 1993). Syndinea comprises one single order, Syndiniales, of exclusively non-photosynthetic parasitic species that, among other features, lack most characters defining the typical nucleus of the Dinokaryota. The syndinean nucleus contains a low number (usually four to ten) of non-fibrillar and V-shaped chromosomes (Cachon 1964; Hollande 1974). The chromatin of Dinokaryota has fibrillar chromosomes (~20-300) condensed throughout the cell cycle (Pfiester 1984; Rizzo 2003). Likewise, the diploid *Noctiluca* trophonts do not retain characters shared by typical dinoflagellates, such as a transverse flagellum and condensed chromosomes. In contrast, the haploid zoospores maintain primitive dinoflagellate-like characteristics including two grooves, slightly differentiated flagella with different lengths and paraxial rod, and condensed chromosomes (Afzelius 1963; Fukuda and Endoh 2006; Höhfeld and Melkonian 1995; Melkonian and Höhfeld 1988; Soyer 1969, 1970, 1972; Zingmark 1970). Zingmark (1970) considered *Noctiluca* not to be a true dinoflagellate because its vegetative nucleus is of conventional eukaryotic type and not dinokaryotic. However, Fensome et al. (1993), based on the dinokaryotic nature of the gamete nucleus and the intergradational nature with other Noctilucales (such as *Kofoidinium* Pavillard), placed the noctiluroids in the class Noctiluciphyceae within the subdivision Dinokaryota (Fensome et al. 1993).

In the 1990s, the phylogenetic position of *Noctiluca* was re-evaluated using molecular information. The first phylogenetic analyses based on the large subunit (LSU) and small subunit (SSU) rDNA sequences suggested that *Noctiluca* represented a basal lineage within the dinoflagellates (Lenaers et al. 1991; Saunders et al. 1997). Recent studies based on other markers (light-emitting enzyme luciferase gene, editing density of mitochondrial *cox1* mRNA and Spliced-Leader RNA trans-splicing groups, β -tubulin and heat shock protein 90 -Hsp90-) also suggested that *Noctiluca* might belong to an early diverging dinoflagellate lineage that diverged after that leading to *Oxyrrhis* Dujardin and before the core dinoflagellates, although the corresponding phylogenies were most often poorly supported (Fukuda and Endoh

2008; Liu and Hastings 2007; Zhang and Lin 2008). Similarly, in the SSU rDNA phylogeny, the most extended marker for protist phylogenetic and diversity studies, the placement of *Noctiluca* was unstable, its position likely influenced by the number of nucleotides used in the alignment (Saldarriaga et al. 2004). An analysis using diverse alveolate SSU rDNA sequences as outgroup suggested that *Noctiluca* occupies a basal position among dinoflagellates, while another analysis rooted using sequences only from *Perkinsus* Levine and Syndiniales placed *Noctiluca* within one of the clades of the order Gymnodiniales (Saldarriaga et al. 2004). The SSU rDNA phylogeny turned out to be too weak to place *Noctiluca* with confidence. Since the enrichment of taxonomic sampling is well known to improve the resolution of phylogenetic analyses, the sequencing of SSU rDNA from other noctiluroids might help to resolve the phylogenetic position of this group within the dinoflagellates.

In addition to *Noctiluca*, which is easily accessible from cultures and widespread in eutrophic coastal waters, other noctiluroids have a predominantly tropical to warm-temperate oceanic distribution (Cachon and Cachon 1967, 1969; Gómez and Furuya 2005, 2007). Most of the Noctilucales have been described from the NW Mediterranean and knowledge on their morphology and life cycle is almost completely restricted to the studies by Cachon and Cachon (1967, 1969). More than 100 nucleotide sequences are publicly available for *N. scintillans*, but sequences from other noctiluroid protists are lacking. *Spatulodinium pseudonoctiluca* (Pouchet) Cachon et Cachon, the type of the family Kofoidiaceae, was first named *Gymnodinium pseudonoctiluca* due to the intermediate morphological characters between *Gymnodinium* Stein and *Noctiluca*. Pouchet (1885) already noted that members of this taxon undergo a complex ontogenetic process from gymnodinioid or amphidinioid immature stages to tentacle-bearing noctiluroid trophonts and he showed that *G. pseudonoctiluca* is a single species with two different morphotypes. The immature morphotype has two differentiated flagella, including the ribbon-like transverse flagellum, and the cingulum and the sulcus, whereas the mature morphotype, which also keeps these dinoflagellate morphological features, has radiating contractile fibrils from the perinuclear region and a tentacle that resembles the one of *Noctiluca*. Other authors described the immature stages of *Spatulodinium* J. Cachon et M. Cachon as separate species (i.e., *Gymnodinium conicum*

Kofoid et Swezy, *G. fulgens* Kofoid et Swezy, and *G. lebouriae* Pavillard). Pouchet's observations were confirmed by Cachon and Cachon (1967) and more recent studies (Gómez and Souissi 2007; Konovalova and Selina 2002). Nevertheless, *G. lebouriae* and *S. pseudonociluca* often appear as a separate species in the literature (Hansen and Larsen 1992; Hoppenrath 2004). The family Leptodiscaceae is the least known among the Noctilucales due to their delicacy and warm-water oceanic distribution. Their cell bodies are strongly antero-posteriorly flattened with a bilateral symmetry or with equatorial wing-like expansions, being able to contract suddenly (Cachon and Cachon 1967; Cachon and Cachon-Enjumet 1964, 1966).

The present study provides the first SSU rRNA gene sequences for representatives of the families Kofoidiaceae and Leptodiscaceae. This allows us to test the monophyly of the noctiluroid dinoflagellates and to study the placement of *Noctiluca* in the SSU rDNA phylogeny, namely, to test whether the noctiluroid protists are an early diverging lineage or a recent lineage derived from typical dinokaryotes phylogenetically related to Gymnodiniales.

Results

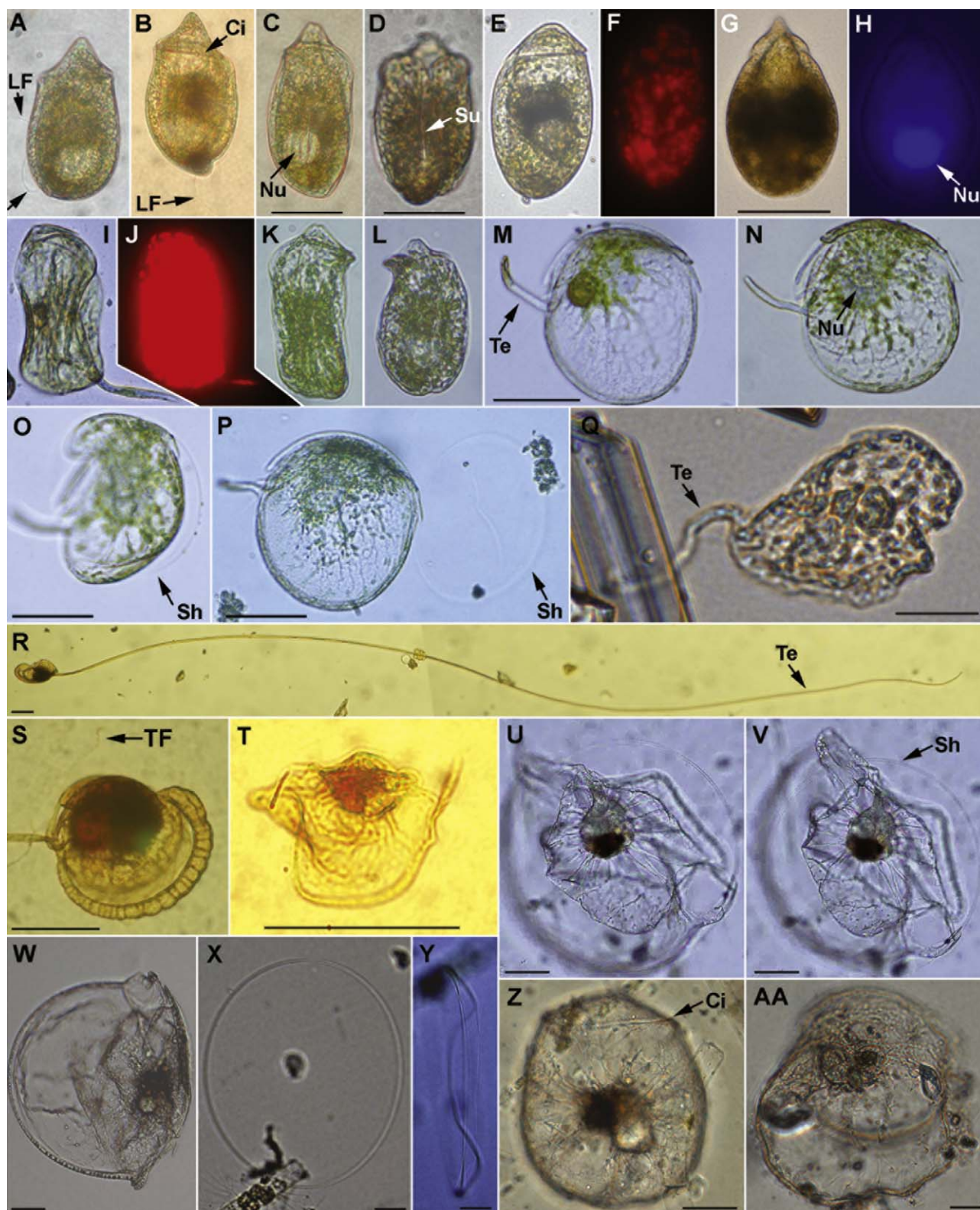
Observations of *Spatulodinium*

Despite one year of intensive sampling in the coast of Marseille, NW Mediterranean Sea, from October 2007 to September 2008, the records of *Spatulodinium* were very scarce. We observed only eight specimens on May 10-11th, which corresponded to *Gymnodinium lebouriae*, stage 'D' in the life cycle of *S. pseudonociluca* according to Cachon and Cachon (1967) (Fig. 1A-D). The sampling continued from October 2008 to July

2009 in the port of Banyuls sur Mer, NW Mediterranean Sea. The first specimens of *G. lebouriae* appeared on April 29th and the records were numerous throughout May (Fig. 1E-H). One specimen collected on June 11th showed the shape of *G. lebouriae* and the tentacle of the mature stage (Fig. 1I-J). No specimens of the tentacle-bearing mature stage were observed in the coasts of Marseille or Banyuls sur Mer, and only one specimen was observed in summer from Lugol-fixed samples collected from the shallow coastal Berre Lagoon, near Marseille (43°30'N, 5°10'E). In contrast, there were numerous mature specimens of *Spatulodinium* with different morphologies in Lugol-fixed samples collected in open waters during the BOUM cruise. Some specimens showed an extremely long tentacle and a cell body that clearly differed from the type species (Fig. 1R-S). We succeeded in determining the sequence of one specimen of *Spatulodinium* (120 µm in diameter) that was highly distorted, but recognizable by its tentacle (Fig. 1Q).

We also observed and isolated for single-cell PCR four specimens of the tentacle-bearing *Spatulodinium* and six specimens of *G. lebouriae* (Fig. 1K-P) from samples collected in early summer at Wimereux, coastal English Channel. At this location, the stage known as *Gymnodinium lebouriae* co-occurred with the tentacle-bearing specimens in nearly all the samples. The immature specimens, *G. lebouriae*, from the Mediterranean and Atlantic waters showed a subovate cell shape (100-120 µm long, 50-60 µm wide) with an anterior cingulum that resembled a large pigmented *Amphidinium*-like cell. The hyposome showed several longitudinal grooves and the sulcus. The longitudinal flagellum was clearly visible and a ribbon-like flagellum was inserted in the cingulum (Fig. 1A-B). The specimens showed numerous dispersed small globular chloroplasts. All the specimens showed chlorophyll *a* under blue light

Figure 1. Light micrographs of *Spatulodinium* and *Kofoidinium*. **A-L.** Immature specimens of *S. pseudonociluca* (*Gymnodinium lebouriae*) from Marseille, collected on 10-11 May 2008 (**A-D**), from Banyuls sur Mer, collected on 24 April and 23 May 2009 (**E-J**) and from Wimereux, NE English Channel, collected on 6-8 July 2008 (**K-L**). *Gymnodinium lebouriae* with a tentacle from Banyuls sur Mer (**I-J**). Arrows in Figures **A-B** indicate the longitudinal flagellum. Figures **F** and **J** illustrate the red fluorescence of the chlorophyll *a* illuminated with UV light of the specimens on the left adjacent micrographs. **H.** Note the nucleus stained with DAPI. **M-P.** Mature specimens from Wimereux collected on 6-8 July 2008. Arrows in Figures **O-P** indicate the shell or "coque". **Q.** Ethanol-fixed *Spatulodinium* sp. from the open Gulf of Lions. **R-S.** Lugol-fixed unidentified *Spatulodinium* with a long tentacle. **T.** Lugol-fixed specimen of *Kofoidinium* sp. **U-Y.** *Kofoidinium pavillardii*. **U-V.** Two frames of the movement onto the shell. **X-Y.** Detail of the shell of the same specimen. **Z.** Immature stage of *Kofoidinum*. **AA.** Ethanol fixed *Kofoidinum* cf. *pavillardii* from the open Gulf of Lions. Ci=cingulum; LF=longitudinal flagellum; Nu=nucleus; Te=tentacle; TF=transversal flagellum; Sh=shell; Su=sulcus. Scale bar=50 µm.



epifluorescence microscopy (Fig. 1F, J). The nucleus appeared as a pale area in the posterior hyposome (Fig. 1A, C), as confirmed by DAPI staining (Fig. 1H). The mature specimens of *S. pseudonociluca* showed a rotund flattened hyposome (100-120 μm in diameter) with a moveable tentacle (Fig. 1M-P). From the episome emerged a highly transparent extracellular hemispherical dome, known as shell or 'coque' according to Cachon and Cachon (1967), which easily detached during the manipulation. The diameter of the shell was slightly similar to that of the cell (Fig. 1O-P).

Observations of *Kofoidinium*

Several live specimens of *Kofoidinium pavillardii* J. Cachon et M. Cachon were observed from the port of Banyuls sur Mer. The cell (350 μm in diameter) rotated on the shell attached by two hook-like anchorages (Fig. 1U-V, see video 1 as supplementary material <http://www.youtube.com/watch?v=E-LabKdIlbE>). During the manipulation, the specimen releases the shell, which has a slightly ellipsoidal shape (300-360 μm diameter) (Fig. 1W-Y). These mature specimens coincided with numerous immature stages, the morphology of which resembled the stage *Gymnodinium lebouriae* of *Spatulodinium*, but they were larger (200 μm long), non-pigmented, lacking the longitudinal grooves and the structure of fibrils was more marked (Fig. 1Z). We successfully obtained the SSU rDNA sequence from one small specimen (40 μm in diameter) ascribed to genus *Kofoidinium* (this specimen was not photographed due to a camera failure). It did not correspond to any of the three larger described species and it may likely go unnoticed due to its small size. The specimen had the pointed extension that arises from the episome towards the ventral side of the cell. This pointed extension was flexible and tended to be in a different plane from the flattened hyposome. This species was also observed in Lugol-fixed samples from Mediterranean open waters (Fig. 1T) and illustrated as *Kofoidinium* sp. in the figures 7-9 by Gómez and Furuya (2007) from samples of the Pacific Ocean. From the ethanol-fixed samples of the Mediterranean Sea open waters, we successfully obtained the SSU rDNA sequence from a large specimen of *Kofoidinium* (400 μm in diameter) that was highly distorted. In the replicate Lugol-fixed sample, numerous specimens of *K. pavillardii* appeared. Based on this feature, general morphology and size and despite the cell

was highly distorted (Fig. 1AA), we ascribed this sequence to *K. cf. pavillardii*.

Observations of *Abedinium dasypus*

The specimens of the family Leptodiscaceae were hyaline and extremely fragile, disintegrating very easily. In the coast of Marseille, several specimens of *Scaphodinium mirabile* Margalef and *Abedinium dasypus* (J. Cachon et Cachon-Enjumet) Loeblich Jr. et A.R. Loeblich III were observed along the autumn of 2008, but they were dead and highly deteriorated. Only three specimens of the leaf-shaped *A. dasypus* (200 μm in length, 80 μm in width) were observed alive. In their anterior part, they showed a tentacle with an orange-pigmented distal extreme (Fig. 2A-F). The tentacle oscillated from one side to the other as a tactile organ while the cell moved forward (Fig. 2A-D). At the other extremity, a long flagellum served to regulate the forward movement. About ten transparent filaments were visible in a radial orientation to the periphery of the nucleus (Fig. 2A, E). These filaments are considered to be ejectile bodies based on the observations by Cachon and Cachon-Enjumet (1964). The specimens were able to contract suddenly when meeting an obstacle or if the surrounding water was disturbed (Fig. 2F). They were then able to roll up immediately and the tentacle folded from the base against its superior face. Relaxation was reached soon after. We were unable to determine the SSU rDNA sequences of these specimens from the coast of Marseille, but we successfully obtained those from two single specimens of *A. dasypus* collected in October 2008 from the port of Banyuls sur Mer (Table 1, Fig. 2G).

Molecular Phylogeny

We obtained SSU rDNA sequences of representatives of the families Kofoidiniaceae and Leptodiscaceae, which were determined after PCR amplification from cells identified, photographed and collected under the microscope (Table 1). We carried out phylogenetic analyses including the new sequences and a variety of dinoflagellate sequences, using perkinsozoan sequences as outgroups. Since the Noctilucales possess quite divergent SSU rDNA sequences, we did not include the sequences of several extremely divergent alveolates, in particular *Oxyrrhis* and ellobiopsid species, to decrease the probability of phylogenetic artefacts, especially 'long branch attraction'. In our SSU rDNA phylogeny, the new

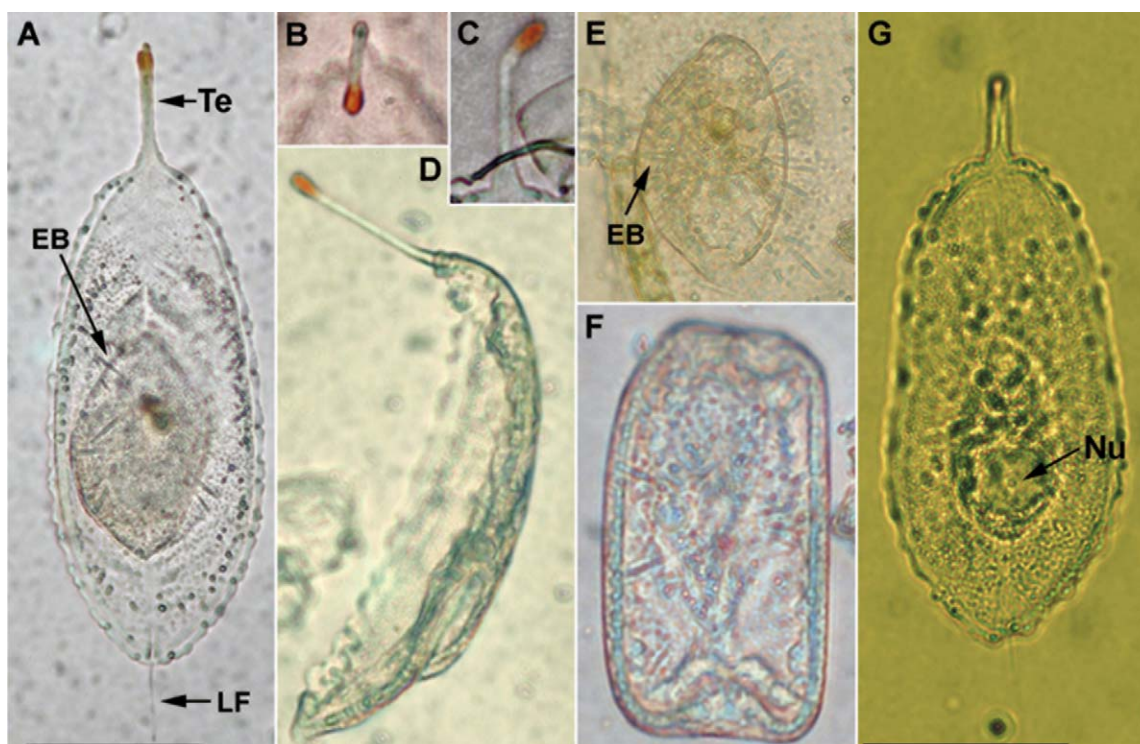


Figure 2. *Abedinium dasypus* from Marseille, collected on 17 December 2007. **A-D.** Different positions of the distal part of the tentacle. **E.** Detail of the ejectile bodies. **F.** Contracted specimen. **G.** *Abedinium dasypus* from Banyuls sur Mer, collected on 31 October 2008. EB=ejectile body; LF=longitudinal flagellum; Nu=nucleus; Te=tentacle. Scale bar=50 μm .

Table 1. List of new SSU rDNA sequences of Noctilucales.

Taxa	GenBank No.	Sequence length (bp)	GC content (%)	Geographical origin (date)	Figure
<i>Spatulodinium pseudonoclituca</i> f. <i>Gymnodinium lebouriae</i> FG126	GU355684	1497	45	Marseille (10 May 2008)	Fig. 1B
<i>Spatulodinium pseudonoclituca</i> f. <i>Gymnodinium lebouriae</i> FG127	GU355685	1576	45	Marseille (11 May 2008)	Fig. 1C
<i>Spatulodinium pseudonoclituca</i> FG200	GU355683	1206	45	Wimereux (7 Jul 2008)	Fig. 1M
<i>Spatulodinium</i> sp. FG541	GU355682	1194	46	open Gulf of Lions (14 Jul 2008)	Fig. 1Q
<i>Kofoidinium</i> sp. FG256	GU355681	1600	47	Banyuls (13 Oct 2008)	–
<i>Kofoidinium</i> cf. <i>pavillardii</i> FG540	GU355680	1208	47	Open Gulf of Lions (14 Jul 2008)	Fig. 1AA
<i>Abedinium dasypus</i> FG257	GU355679	1212	44	Banyuls (31 Oct 2008)	Fig. 2G
<i>Abedinium dasypus</i> FG239	GU355678	1222	44	Banyuls (7 Oct 2008)	–

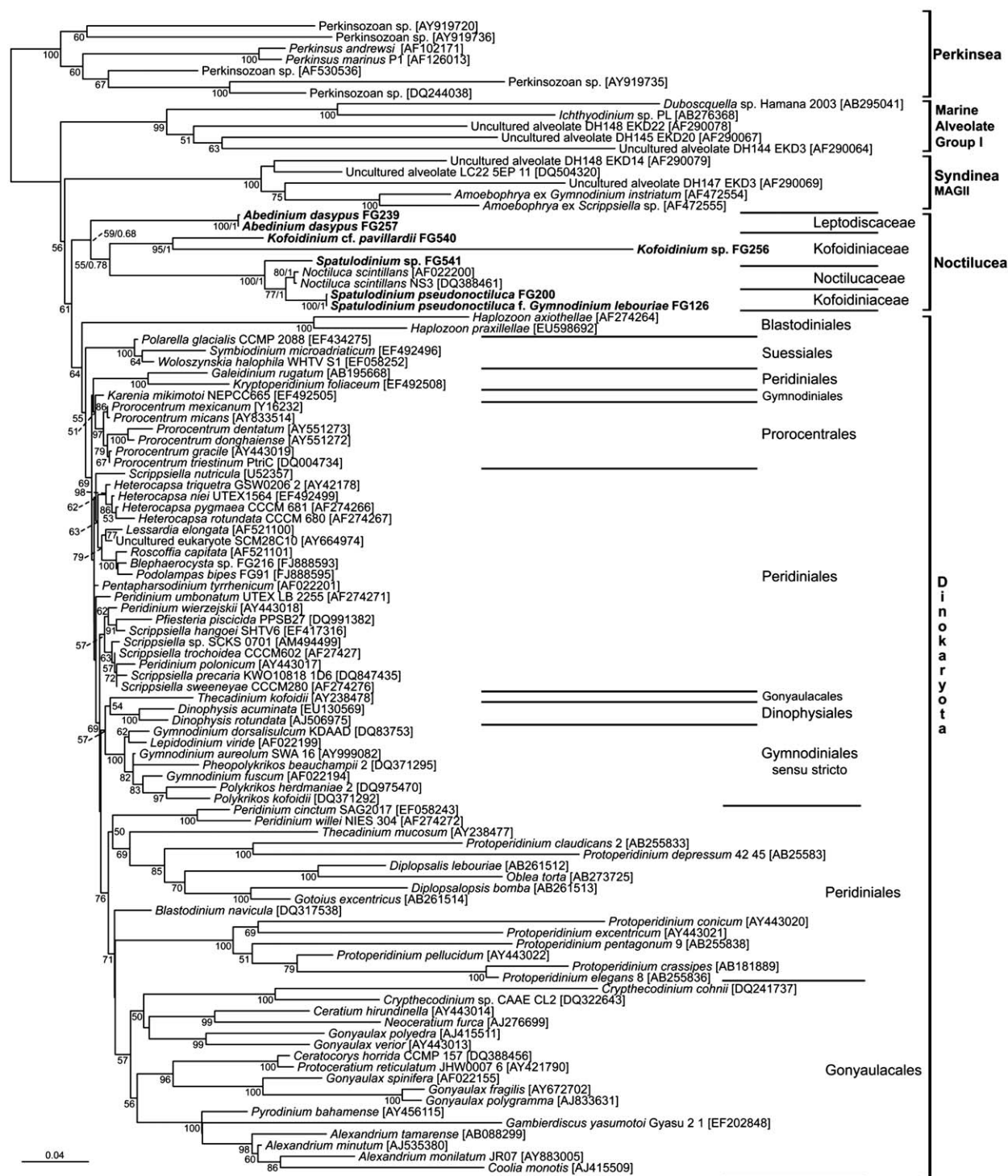


Figure 3. Maximum likelihood phylogenetic tree of alveolate SSU rDNA sequences, based on 1,194 aligned positions. Names in bold represent sequences obtained in this study. Numbers at the nodes are bootstrap proportions (values under 50% are omitted). For the nodes concerning the position of the Noctilucales, Bayesian posterior probability values are also indicated. Accession numbers are provided in brackets. The scale bar represents the number of substitutions for a unit branch length. The epithet of *Polykrikos herdmaniae* misspelled as ‘herdmanae’ is corrected according to ICBN: Art. 60.11; Recom. 60C.1., Art. 32.7.

sequences, together with those of *Noctiluca scintillans*, branched with a moderate support (bootstrap values -BV- between 61 and 64%) between the Syndiniales (Marine Alveolate Group II) and the core dinoflagellates (Fig. 3). The monophyly of the Noctilucales was poorly supported (BV of 59%). They appeared subdivided into three well-supported clades, one for the sequences of leptodiscaceans (*Abedinium dasyopus*), the second for the *Kofoidinium* sequences and the third for the rest of Noctilucales (*Noctiluca* and *Spatulodinium*).

Our study confirmed using molecular data that *Gymnodinium lebouriae* is a life stage of *S. pseudonociluca*. In fact, the sequences of *G. lebouriae* from the coastal Mediterranean Sea (Fig. 1A-D) and the tentacle-bearing *S. pseudonociluca* from the coastal Atlantic Ocean (Fig. 1M-P) were identical. Although the genus *Spatulodinium* is considered as monotypic, our molecular data evidenced that there is at least a second species in open waters within the genus (*Spatulodinium* sp. FG541, Fig. 1Q). The genera *Spatulodinium* and *Kofoidinium* share the lateral flattening and the shell, whereas the sac-like *Noctiluca* and *Spatulodinium* share the occurrence of a tentacle. In our SSU rDNA phylogeny, *Noctiluca* branched among the species of the genera *Spatulodinium* (Fig. 3). Therefore, our results do not support the split of the Noctilucales into the families Noctiluaceae and Kofoidinaceae, especially in what refers to the conservation of *Noctiluca* as the only representative of its own family.

Discussion

Based on SSU rDNA phylogenetic analyses rooted using sequences from *Perkinsus* and Syndiniales as outgroup, Saldarriaga et al. (2004) found that *Noctiluca* branched close to the *Amphidinium* Claparède et Lachmann species within one of the clades of the order Gymnodiniales, suggesting a common gymnodinioid ancestor for the entire group. Saldarriaga et al. (2004, p. 97) reported that “the basal position of *Noctiluca* within the dinokaryotic dinoflagellates should be reexamined: the two main arguments for proposing such a basal position have been shown to be either very weak (SSU-based phylogenetic analyses), or probably wrong (the ostensible presence of histones in the nuclei of feeding stages)”. They also reported that the immature stages of noctiluroids (i.e., *Spatulodi-*

nium) are practically indistinguishable from a number of Gymnodiniales, especially *Amphidinium*. According to Saldarriaga et al. (2004), *Noctiluca* shares with members of the *Gymnodinium* sensu stricto group two rare morphological features: 1) peculiar chambers (ampullae) in which the nuclear pores are situated, observed in the trophont of *Noctiluca* (Afzelius 1963; Soyer 1969) and in other noctiluroids, such as *Petalodinium* (Gómez and Furuya 2005), but also in several representatives of the *Gymnodinium* sensu stricto group (Daugbjerg et al. 2000; Hansen and Moestrup 2005; Hoppenrath and Leander 2007); and 2) the absence of a transverse striated flagellar root in *Gymnodinium* and in the zoospores of *Noctiluca*, otherwise a feature typical of most dinoflagellates (Hansen et al. 2000).

An additional feature that may suggest a relationship between noctiluroids and the Gymnodiniales is the presence of ejectile bodies. In fact, dinoflagellate representatives of the *Gymnodinium* sensu stricto group, like *Polykrikos* Bütschli and *Nematodinium* Kofoid et Swezy, possess ejectile bodies, such as nematocysts, used for prey capture (Greuet 1987; Hoppenrath and Leander 2007), and Cachon and Cachon-Enjumet (1964) observed that stressed specimens of *Abedinium dasyopus* are able to project 200 µm-long trichocysts whose function may be convergent with the ejectile bodies of *Polykrikos*. However, ejectile bodies or extrusomes used for food capture or escape from predation are known in other alveolates groups such as the ciliates (Wessenberg and Antipa 1970). Moreover, despite all those putative common characters relating Noctilucales and Gymnodiniales, our SSU rDNA phylogeny does not reveal a close phylogenetic relation between both groups (Fig. 3). This was also the case in phylogenetic trees constructed using the relatively short-branching *Abedinium* sequences as the only representatives of the Noctilucales, which still retrieved a basal emergence for these species, although poorly supported (data not shown).

In our SSU rDNA phylogeny, the Noctilucales emerge as a clade of free-living species in a basal position close to a variety of lineages of parasitic alveolates (perkinsoids, Marine Alveolate Group I and Syndiniales-MAGII) lacking chloroplasts and as sister to a large clade containing the non-photosynthetic parasite *Haplozoon* Dogiel and the rest of dinokaryotes. In contrast, some typical dinokaryotes with a parasitic lifestyle possess plastids, e.g. *Blastodinium* Chatton and *Dissodinium* Klebs (Chatton 1920; Gómez et al. 2009a).

Though the Noctilucales, including *Spatulodinium*, have been traditionally considered as exclusively heterotrophic (Larsen and Sournia 1991), we show in this study the occurrence of chloroplasts in *S. pseudonociluca* using epifluorescence microscopy (Fig. 1F, J). Chloroplasts are present in other species of *Spatulodinium* as illustrated in Gómez and Furuya (2007). *Pomatodinium* J. Cachon et Cachon-Enjument may contain zooxanthellae according to Cachon and Cachon-Enjument (1966). In the tropical to sub-tropical areas of Southeast Asia a green form of *Noctiluca* harbours thousands of free-swimming cells of the chlorophyte *Pedinomonas noctilucae* (Subrahmanyam) Sweeney inside the cell (Sweeney 1976). Hence, the Noctilucales appear to behave like other dinoflagellates in their ability to incorporate, replace or lose chloroplasts, a common phenomenon in the dinoflagellate core (Saldarriaga et al. 2001; Shalchian-Tabrizi et al. 2006) that contrasts with the rare occurrence of chloroplasts in other alveolate groups (Moore et al. 2008). Studies on the characteristics of the *Spatulodinium* plastids (pigmentation, ultrastructure and molecular sequences) will be necessary to determine whether their chloroplasts are keptoplastids (Koike et al. 2005) or if they derive from ancient endosymbiosis as in other dinoflagellate families.

Most published phylogenies have placed *Noctiluca* as an early diverging lineage within dinoflagellates, branching after *Oxyrrhis* and before the core dinoflagellates (Cavalier-Smith and Chao 2004; Fukuda and Endoh 2008; Gómez et al. 2009b; Lenaers et al. 1991; Liu and Hastings 2007; Moore et al. 2008; Saldarriaga et al. 2001; Saunders et al. 1997; Shalchian-Tabrizi et al. 2006; Zhang and Lin 2008). In the present SSU rDNA phylogeny, with more representatives, the Noctilucales appear to maintain their basal position (Fig. 3). Several morphological and ultrastructural features apparently agree with the placement of *Noctiluca* and the other noctiluroids as early diverging dinoflagellates, between the dinoflagellate core and the clades of Marine Alveolate Group I (*Duboscquella* Chatton and *Ichthyodinium* Hollande et Cachon) and Group II-Syndiniales (*Amoebophrya* Koeppen). The process of cell division in Marine Alveolate Groups I and II is very fast and distinctive (Cachon 1964; Harada et al. 2007). *Duboscquella* shows no evidence of condensed interphase chromosomes at any stage of its life cycle (Harada et al. 2007) and, during sporogenesis, a long, rosary-like chain of transparent sporocytes forms (Cachon 1964; Harada et al. 2007). Likewise, during sporogenesis,

Kofooidinium sporocytes distribute helicoidally in a way that recalls that of Syndiniales such as *Amoebophrya* (Cachon and Cachon 1967). In *Spatulodinium*, Gómez and Souissi (2007) illustrated the formation of peculiar sporocyte clusters that are unknown in typical dinoflagellates. Cachon and Cachon (1967, p. 28) also observed that the division of the leptodiscacean *Leptodiscus* Hertwig was similar to that of Syndiniales as illustrated in Cachon (1964). Cachon and Cachon (1969, p. 15) illustrated a large (700 μm long) multiflagellate and multinucleate spororont of the leptodiscacean *Scaphodinium mirabile*. This resembles the multiflagellate stage that is released at the end of the intracellular phase of *Amoebophrya*. The vermiform stage differentiates and breaks apart releasing the new infective dinospores (Cachon and Cachon 1987, p. 586). All these observations converge to suggest that the sporogenesis in Noctilucales involves a helicoidal disposition of the sporocytes or vermiform stages, which resembles more the one of Syndiniales than that of typical dinokaryotic dinoflagellates.

A peculiar characteristic of several noctiluroid genera, such as *Noctiluca*, *Spatulodinium* and *Abedinium*, is the presence of a highly moveable ventral tentacle, which is missing in typical dinoflagellates and other alveolates. The tentacle of *Noctiluca* moves slowly and shows a contraction-relaxation cycle helping in food capture and used only to convey the prey to the cytostome located at its base (Soyer 1970). It does not play any role in keeping the cell in suspension, as it appears to be the case also in *Spatulodinium*, which has a tentacle involved in the concentration of food particles (Cachon and Cachon 1967). Our study illustrates for the first time an extremely long tentacle in *Spatulodinium* specimens, suggesting that in oceanic conditions it may have other functions (Fig. 1R). The ventral tentacle of *Noctiluca* is striated, whereas there is not striation in *Spatulodinium*. The tentacle of *Leptophyllus* differs from those of *Noctiluca* and *Spatulodinium*. The cell moves the tentacle in all directions while it advances forward, like a tactile structure. The extreme of the tentacle has an intense orange pigmentation that suggests a putative photoreceptor function (Fig. 2A-D). It could be speculated that the extremity of the tentacle provides information to trigger the ejectile bodies for prey capture, but evidence for this potential role is missing at present. In contrast with all these noctiluroids, no tentacle has been reported in the three known species of *Kofooidinium*. However, in this study we have illustrated a species, likely

unnoticed until now due to its delicacy and small size, showing a short pointed appendix that may be homologous to an atrophied tentacle (Fig. 1T). Another undescribed kofoidiniacean has two tentacles (Gómez and Furuya 2007).

In addition to the morphological features mentioned above regarding sporogenesis and distinctive tentacles in several genera, all Noctilucales have in common cytoplasmic strands that extend radially from the nucleus to the membrane. They are involved in the locomotion, short-time shape changes and the complex ontogenetic process of these organisms (Figs 1 and 2; see <http://www.youtube.com/watch?v=WdHJ6laills>). While these general traits can be useful for the diagnosis of the group, the cell shape has little taxonomic value. For instance, in our SSU rDNA phylogeny, the sac-like *Noctiluca* species appeared among the laterally flattened *Spatulodinium* species. Furthermore, the cell morphology changes along the life cycle and very different shapes can be unambiguously attributed to the same species using molecular data. Such was the case of *Gymnodinium lebouriae*, unambiguously shown to be a life stage of *S. pseudonociluca*.

From an evolutionary point of view, although our study suggests a basal position for the Noctilucales after the emergence of several parasitic alveolate lineages, this position is only weakly supported. Similarly, the monophyly of the otherwise three well-supported noctiluroid clades is weakly supported itself. Future analyses of additional phylogenetic markers and an extended taxon sampling should help to clarify their actual phylogenetic position.

Methods

Collection of organisms: Mediterranean specimens were collected by slowly filtering surface seawater taken from the end of the pier (depth 3 m) of the Station Marine d'Endoume, Marseille (43°16'48"N, 5°20'57"E) from October 2007 to September 2008. A strainer with netting of 20, 40 or 60- μ m mesh-size was used to collect the organisms and the filtered volume varied between 10 and 100 liters, according to the concentration of particles. The concentrated sample was examined in Utermöhl chambers at 100 \times magnification with a Nikon inverted microscope (Nikon Eclipse TE200) and was photographed at 200 \times or 400 \times magnification with a digital camera (Nikon Coolpix E995). Sampling continued from October 2008 to May 2009 in the surface waters of the port (depth of 2 m) of Banyuls sur Mer, France (42°28'50"N, 3°08'09"E). The samples were prepared with the same procedure described above. The specimens were observed with an Olympus inverted microscope (Olympus IX51) and photographed with an Olympus DP71 digital camera. Some specimens were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) that specifically binds to double-stranded DNA,

and when excited with ultraviolet (UV) light the DAPI-DNA complex fluoresces as bright blue. In addition, open-water samples were collected from the Gulf of Lions, NW Mediterranean Sea (39°5'57"N, 5°21'E) on July 14th 2008 within the context of the BOUM (Biogeochemistry from the Oligotrophic to the Ultra-oligotrophic Mediterranean) cruise. Ten liters were collected from the surface with a bucket and filtered by using a strainer of 20- μ m netting aperture. The retained material was fixed with ethanol. Several replicate samples of 0.5 l were fixed with acid Lugol's solution. At the laboratory, the ethanol sample was examined following the procedure described above. In contrast to the live specimens, the ethanol-fixed samples showed a precipitate of elongate crystals and the specimens appeared highly distorted and non-pigmented. When compared with the Lugol-fixed sample, all the unarmoured dinoflagellates disappear in the ethanol samples, except for a few of the larger forms of Noctilucales.

The type locality of *Spatulodinium pseudonociluca* is the English Channel, NE Atlantic Ocean (Pouchet 1885). A previous study based on Lugol-fixed plankton collected from an 8-year time-series in the NE English Channel, revealed a higher abundance of *Spatulodinium* in early summer (Gómez and Souissi 2007). The Atlantic specimens were collected with the method described above from the surface of a large tidal pool (~30 m diameter, 1 m maximal depth) at the beach of Wimereux, France (50°45'60"N, 1°36'21"E) from 5-8 July 2008. The concentrate was scanned in settling chambers at 100 \times magnification with an inverted fluorescence microscope (Olympus IX71) and was photographed at 200 \times or 400 \times magnification with a digital camera (Olympus DP70-BSW). In all cases, each specimen was micropipetted individually with a fine capillary into a clean chamber and washed several times in serial drops of 0.2- μ m filtered and sterilized seawater (live specimens from coastal waters) or ethanol (ethanol pre-fixed specimens from open waters). Finally, the specimen was deposited into a 0.2 ml Eppendorf tube filled with several drops of 100% ethanol. The sample was kept at room temperature and in darkness until the molecular analysis could be performed.

PCR amplification of small subunit rRNA genes (SSU rDNAs) and sequencing: The specimens fixed in ethanol were centrifuged gently for 5 min at 3,000 rpm. Ethanol was then evaporated in a vacuum desiccator and single cells were resuspended directly in 25 μ l of Ex TaKaRa (TaKaRa, distributed by Lonza Cia., Levallois-Perret, France) PCR reactions were done in a volume of 30 to 50 μ l reaction mix containing 10-20 pmol of the eukaryotic-specific SSU rDNA primers EK-42F (5'-CTCAARGAYTAAGCCATGCA-3') and EK-1520R (5'-CYGCAGGTTACCTAC-3'). The PCR reactions were performed under the following conditions: 2 min denaturation at 94 °C; 10 cycles of 'touch-down' PCR (denaturation at 94 °C for 15 s; a 30 s annealing step at decreasing temperature from 65 down to 55 °C employing a 1 °C decrease with each cycle-, extension at 72 °C for 2 min); 20 additional cycles at 55 °C annealing temperature; and a final elongation step of 7 min at 72 °C. A nested PCR reaction was then carried out using 2-5 μ l of the first PCR reaction in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the eukaryotic-specific primers EK-82F (5'-GAAACTGCGAATGGCTC-3') and EK-1498R (5'-CACCTACG-GAAACCTTGTTA-3') and similar PCR conditions as described above. A third, semi-nested, PCR was carried out using the dinoflagellate specific primer DIN464F (5'-TAACAATACAGGG-CATCCAT-3'). Negative controls without template DNA were used at all amplification steps. Amplicons of the expected size (~1,200 bp) were then sequenced bidirectionally using primers DIN464F and EK-1498R (Cogenics, Meylan, France).

Phylogenetic analyses: The new sequences were aligned to a large multiple sequence alignment containing 1,100 publicly available complete or nearly complete (>1,300 bp) dinoflagellate SSU rDNA sequences using the profile alignment option of MUSCLE 3.7 (Edgar 2004). The resulting alignment was manually inspected using the program ED of the MUST package (Philippe 1993). Ambiguously aligned regions and gaps were excluded in phylogenetic analyses. Preliminary phylogenetic trees with all sequences were constructed using the Neighbour Joining (NJ) method (Saitou and Nei 1987) implemented in the MUST package (Philippe 1993). These trees allowed identifying the closest relatives of our sequences, which were selected, together with a sample of other dinoflagellate species, to carry out more computationally-intensive Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. ML analyses were done with the program TREEFINDER (Jobb et al. 2004) applying a GTR + Γ + I model of nucleotide substitution, taking into account a proportion of invariable sites, and a Γ -shaped distribution of substitution rates with four rate categories. Bootstrap values were calculated using 1,000 pseudoreplicates with the same substitution model. The BI analyses were carried out with the program PHYLOBAYES applying a GTR + CAT Bayesian mixture model (Lartillot and Philippe 2004), with two independent runs and 1,000,000 generations per run. After checking convergence (maximum difference between all bipartitions <0.01) and eliminating the first 1,500 trees (burn-in), a consensus tree was constructed sampling every 100 trees. Sequences were deposited in GenBank with the following accession numbers: GU355678- GU355685 (see also Table 1).

The phylogenetic position of the noctiluroid dinoflagellates was analyzed by means of a global alignment of 103 taxa representing sequences of alveolates: perkinsoids (*Perkinsus*), Marine Alveolate Group I (*Duboscquella*, *Ichthyodinium*), syndineans / Alveolate Group II (*Amoebophrya*), environmental sequences and representative species of the dinokaryotes, with the perkinsoid sequences used as outgroup.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.protis.2009.12.005](https://doi.org/10.1016/j.protis.2009.12.005).

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