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Pelagodinium gen. nov. and *P. béii* comb. nov., a Dinoflagellate Symbiont of Planktonic Foraminifera

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The taxonomic status of the free-living stage of the dinoflagellate *Gymnodinium béii*, symbiont of the foraminifer *Orbulina universa*, was reassessed on the basis of detailed morpho-genetic analyses. Electron microscopy observations revealed previously undescribed morphological features of the cell that are important for species recognition. The presence of a single elongated apical vesicle (EAV) ornamented with a row of small knobs, absent in species of the genus *Gymnodinium*, calls into question the current taxonomic position of the symbiont. The presence of a type E extraplastidial eyespot, the arrangement of the amphiesmal vesicles in series and the absence of trichocysts confirm the affiliation with other symbiotic dinoflagellates and certain genetically related non-symbiotic genera, all belonging to the order Suessiales. The arrangement of the series of vesicles of the analyzed strain is unique within the Suessiales, and the ultrastructure of the pyrenoid is different from other symbiotic dinoflagellates. A large subunit (LSU) rDNA phylogenetic analysis confirmed that the analyzed pelagic symbiont clusters in an independent, well-supported clade within the Suessiales with other sequences of symbiotic dinoflagellates extracted from planktonic foraminifera. Hence a novel genus, *Pelagodinium* gen. nov., is erected for this pelagic, symbiotic dinoflagellate, and *Gymnodinium béii* is reclassified as *Pelagodinium béii*.

Key words: dinoflagellates; foraminifera; *Orbulina universa*; *Pelagodinium*; Suessiales; symbionts.

Introduction

Dinoflagellates show an extraordinary variety of modes of life, biological traits, and morphological adaptations that make them unique among protists. They can be autotrophic, mixotrophic, kleptoplastidic, strictly heterotrophic, as well as parasitic or symbiotic. As swimming cells they can make vertical migrations, they can be bioluminescent, and they can produce toxins noxious to humans and to other components of the marine food web. They can have cellulose plates forming a rigid, inflexible cell wall, or they may have a single layer of flattened, empty vesicles surrounding the plasmalemma, meaning cells are more fragile (Hackett et al. 2004).

Mutualistic associations involving photosynthetic dinoflagellates are common in both benthic and pelagic ecosystems and are essential for establishing and maintaining the structure of marine communities (Caron 2000). Symbiotic dinoflagellates are presently attributed to seven different genera: *Amphidinium* Claperède et

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Lachmann, *Aureodinium* Dodge, *Gloeodinium* Ehrenberg, *Gymnodinium* (Stein) Hansen et Moestrup, *Prorocentrum* Ehrenberg, *Scrippsiella* Balech ex Loeblich III, and *Symbiodinium* Freudenthal (Banaszak et al. 1993). These are hosted by a wide range of phylogenetically distant organisms, including protists (e.g. foraminifers, radiolarians, ciliates), sponges, flatworms, cnidarians (corals and jellyfish), and molluscs (tridacnid bivalves) (Caron 2000; Farmer et al. 2001; Gast and Caron 2001; Leggat et al. 2002; Lopes and Silveira 1994; Schonberg and Loh 2005; Stoecker et al. 2009; Trench 1993). Host organisms likely benefit from this association by acquiring photosynthetically fixed carbon from the symbionts, whereas the microalgae find in the hosts a microenvironment with higher nutrient concentrations than surrounding waters and a refuge to escape from predation, parasitism, and/or viral infection (Caron 2000). Dinoflagellate symbionts are characterized by complex life cycles with alternation of free-living and non-motile stages that can differ considerably in terms of morphology and physiology. Within the host, the symbionts are typically coccoid without flagella, and the cingulum and sulcus are no longer apparent (Trench and Blank 1987). During the free-living stage, cells regain their original morphology (Freudenthal 1962; Spero 1987). Symbiotic species of the genus *Amphidinium* are an exception to this, since they retain *in hospite* the morphology of the free-living stage, including the flagellar apparatus (Trench 1993).

Symbiosis between the dinoflagellate genus *Symbiodinium* and corals is fundamental for the survival and ecological success of coral reef ecosystems. Studies on this benthic, coastal symbiotic relationship significantly increased when the coral-bleaching phenomenon was brought to global attention and associated to increases in sea surface temperature, enhanced light intensity, and ocean acidification (Hoegh-Guldberg et al. 2007). Species of the genus *Symbiodinium*, commonly known as zooxanthellae, have been intensively studied with regards to their life cycle (Freudenthal 1962), morphology (Loeblich III and Sherley 1979; Trench and Blank 1987), and genetic diversity (Apprill and Gates 2007; Hunter et al. 2007; LaJeunesse 2001; LaJeunesse et al. 2005; Manning and Gates 2008; van Oppen 2007). Phylogenetic analyses based on nuclear ribosomal internal transcribed spacer (ITS) and large subunit (LSU) DNA sequences have classified *Symbiodinium* strains into six (A-F) (LaJeunesse 2001) and subsequently eight (A-H) (Coffroth and Santos 2005) subgroups. Strains belonging to different clades can be differentially beneficial for coral growth (Stat et al. 2008) and show different sensitivity to thermal stress (Tchernov et al. 2004).

Symbiotic interactions in pelagic environments have received less attention despite the fact that they are widespread in the photic layer of the world ocean, where they play a fundamental role in the ecology of the planktonic ecosystem (Stoecker et al. 2009). In particular, symbiotic relationships between pelagic foraminifera and dinoflagellates are poorly known. *Orbulina universa* D'Orbigny is a cosmopolitan planktonic spinose foraminifer (Globigerininae) with a photosymbiotic mode of life that may explain its ecological prominence in oligotrophic subtropical and tropical photic zone waters (Arnold and Parker 1999; Spero 1987). Combined genetic and biometric data of specimens from the Atlantic, Indian, and Pacific Oceans demonstrated the presence of three cryptic species within the morphospecies *O. universa* (de Vargas et al. 1999; Morard et al. 2009), whereas the morphological description of its symbiont recognized a single species, *Gymnodinium béii* Spero (Spero 1987). On the basis of morphological and ultrastructural observations, the symbiont of *O. universa* was shown to be more similar to dinoflagellates of the order Gymnodiniales than to those of the Suessiales (to which *Symbiodinium* belongs) (Spero 1987). However, SSU-, LSU- and ITS rDNA-based phylogenies of symbiotic dinoflagellates from several planktonic foraminiferal species (Gast and Caron 1996; Shaked and de Vargas 2006) suggest that they are part of the Suessiales. LSU and ITS rDNA data from specimens collected in various oceanic regions revealed a significant biodiversity of foraminiferal pelagic symbionts, but no clear correlation between the symbiont genetic types and the host genetic and morphological species was observed (Shaked and de Vargas 2006).

Here we examined the morphology, ultrastructure, and phylogenetic position of a cultured strain of the free-living stage of the athecate dinoflagellate endosymbiont of the foraminifer *O. universa.* Morphological and ultrastructural features match those of the endosymbiotic dinoflagellate *G. béii* (Spero 1987) and the LSU rDNA-based molecular phylogeny places this strain in one of the previously described clades of dinoflagellate endosymbionts of planktonic foraminifera (Shaked and de Vargas 2006). However, the redefinition of *Gymnodinium* (Daugbjerg et al. 2000) does not support the classification of this endosymbiotic dinoflagellate in this genus. Both the phylogenetic

analysis and the comparison of morphological features of our strain with those of other closely related species support the erection of the new genus *Pelagodinium* gen. nov. and the recombination of *G. béii* as *P. béii* comb. nov.

Results

Microscopy Observations

Cells are small: 8.8-11.4 μm in length (average 10.0±0.8 μm, *n*=30) and 6.0-7.5 μm in width (average 6.6±0.4 μm, *n*=30). The epicone and the hypocone are of approximately the same size. Observed under LM, cells have a round to elliptical episome (Fig. 1A–D). Depending on the cell position, the hypocone appears rounded (Fig. 1B), at times flattened at the antapex (Fig. 1C, D), and at other times asymmetrical, with the right portion more pronounced than the left (Fig. 1A). The nucleus is round and large, and occupies the centre of the cells (Fig. 1A–D). One or two golden-yellow chloroplasts are present around the cell periphery, sometimes appearing as a single plastid bordering the cell periphery (Fig. 1A–D). One or two round pyrenoids are often visible in LM, as is an eyespot appearing as a small and shiny brown-orange spot in the antapical portion of the sulcal region (Fig. 1A, B).

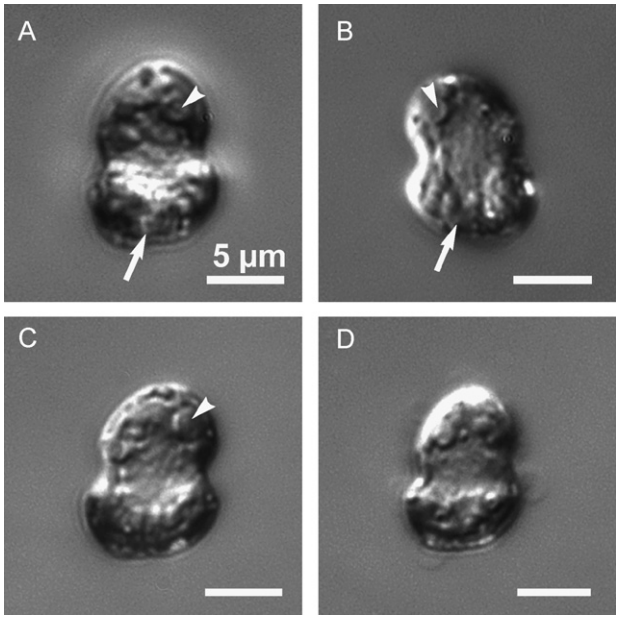

Figure 1. Light micrographs of *Pelagodinium béii* comb. nov. **A, B.** Ventral view of the cell showing the large nucleus in the central portion of the cell; the eyespot appears as a small orange-brown dot in the sulcal region (arrow), arrowheads indicate the large pyrenoids. **C, D**. Dorsal view of the cell, arrowhead indicates the large pyrenoid.

Cells swim fast in a straight line, rotating around the transapical axis. They suddenly stop, change direction at different angles from the original path, often back-tracking. When cells change direction they typically rapidly accelerate their swimming speed, followed by a gradual slowing down. A few minutes after slide preparation, cells stop swimming, the amphiesma detaches, and cells lose their original shape.

In SEM, the epicone appears elliptical (Fig. 2A) to rounded (Fig. 2B), whereas the hypocone appears clearly asymmetrical when cells are observed in either ventral (Fig. 2A) or dorsal (Fig. 2B) view. The cingulum is rather wide and shallow. It is located in the median portion of the cell and it is descending displaced by approximately once its own width (Figs 2A and 3A). The sulcus is deep, narrow at the anterior end, enlarging towards the posterior end (Fig. 2A, G). The hypoconal flange is clearly visible in the upper, left hypocone; in some cells it is short and rounded (Fig. 2A), while in others it is longer and more pointed, reaching the terminal point of the right epicingulum (Fig. 2G). Flagella emerge from flagellar pores in the sulcal region, and no peduncle is visible (Fig. 2A, E, G). The thin amphiesmal vesicles are clearly visible on the cell surface. Although a certain variability in the number and shape of vesicles was observed, some regular features can be recognized (Figs 2A–G, 3A–D). A straight single elongated apical vesicle (EAV) is present at the cell apex (Figs 2C, D, 3C); this structure resembles a zip, it is ornamented with a single row of small globular knobs. The EAV lies in between 3 vesicles (epiconal series 1) (Figs 2D, 3C). A small, squared to rectangular vesicle (X vesicle) is present at the ventral tip of the EAV, slightly displaced towards the right side of the cell (Figs 2D, arrow, and 3C). Two longitudinal series of vesicles follow the apical series in the epicone (epiconal series 2 and 4) constituted of 7 and 8 vesicles, respectively (Figs 2C, 3A–C). Two or three intercalary vesicles are interposed between the two series (epiconal series 3), two to the right side of the epicone, one on the dorsal side, slightly displaced towards the left side of the cell (Figs 2A–C, 3A–C). This character varies between different cells. The ventral part of the epicone is occupied by two large five-sided vesicles of the second series, extending from the upper margin of the ventral epicingulum to the shorter, ventral side of the EAV

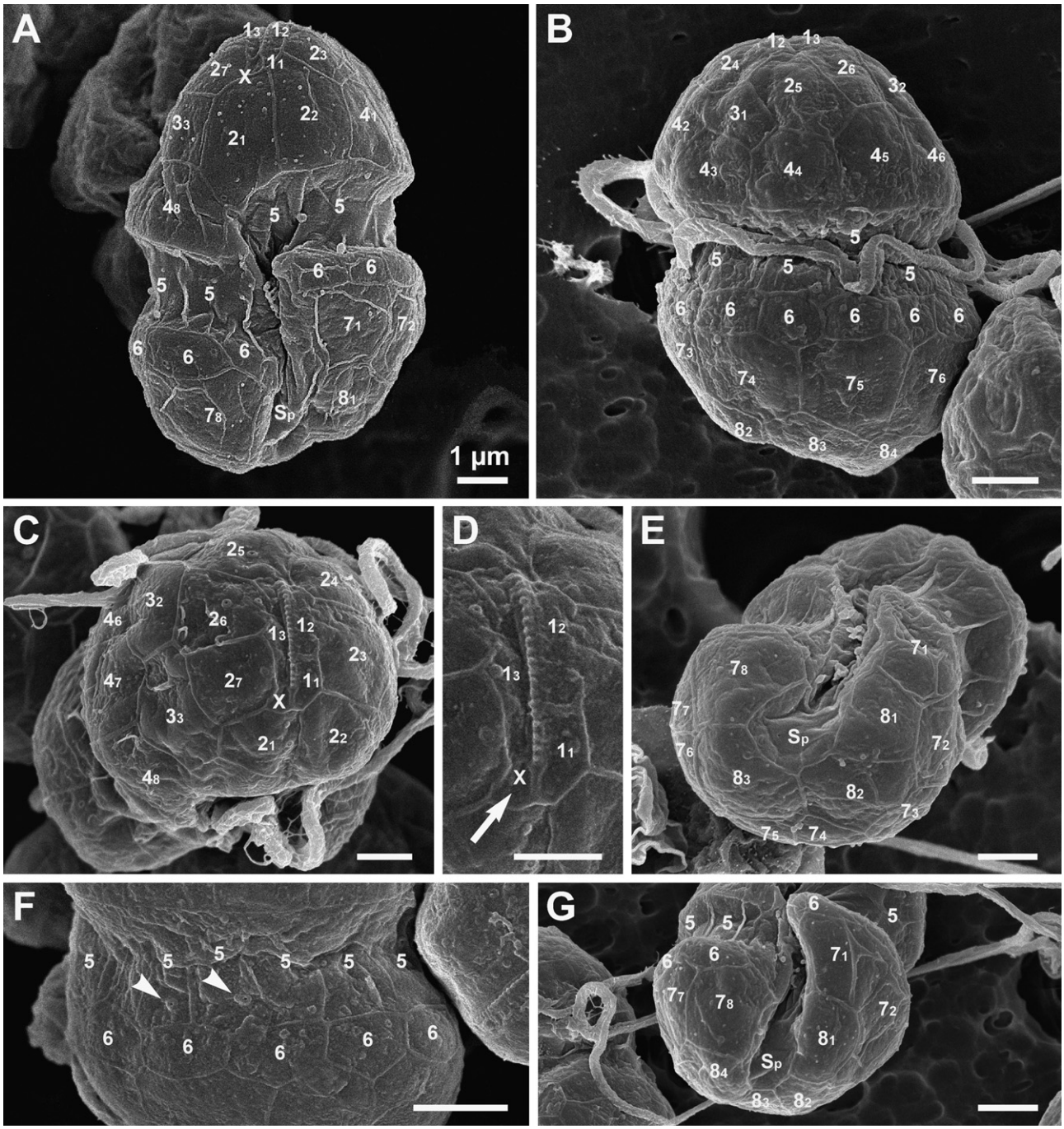

Figure 2. Scanning electron micrographs of *Pelagodinium béii* comb. nov. Different numbers mark the latitudinal series of vesicles; subscript numbers identify the vesicles within a series. **A.** Ventral view of a cell (flagella lost during fixation). **B.** Dorsal view. **C.** Apical view, note the elongated apical vesicle (EAV). **D.** Detail of the EAV of cell of Figure 1C, the arrow indicates the X vesicle. **E.** Antapical view. **F.** Detail of the dorsal part of the cingulum constituted by one series of quadrangular vesicles; a series of four- or five-sided post-cingular vesicles is visible in the hypocone. Pores on cell surface are arrowed. **G**. Detail of the sulcal region and of the hypoconal flange.

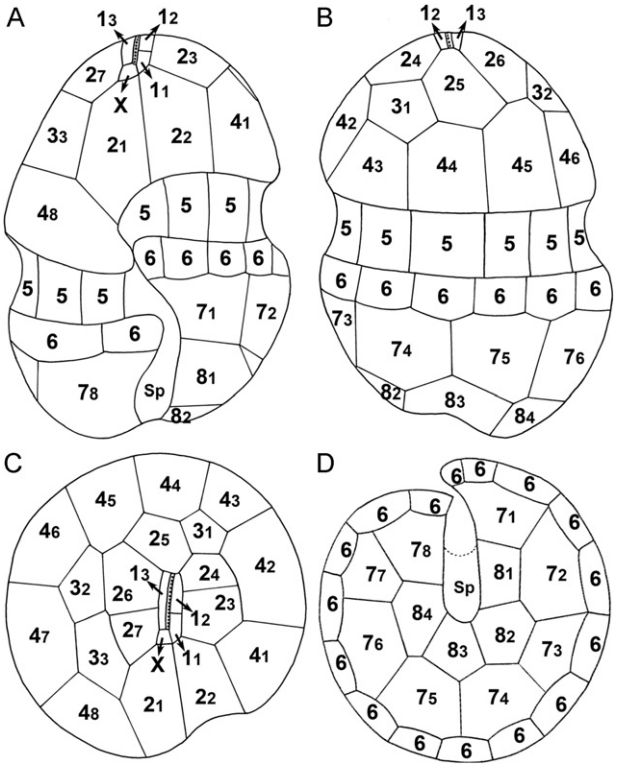

Figure 3. Line drawing of the amphiesmal vesicles of *Pelagodinium béii* comb. nov. **A.** Ventral view. **B.** Dorsal view. **C.** Apical view. **D.** Antapical view. Some variability in the number of vesicles was at time observed (see text); we illustrate the most common pattern.

(Figs 2A, C, 3A, C). In some cells, the left vesicle is not in contact with the EAV. The cingulum is constituted by a single series of mostly rectangular vesicles (series 5), whose number is variable (Figs 2A, F, 3A, B). In the sulcal area, a relatively big sulcal posterior vesicle is clearly visible, whereas the other vesicles are completely hidden in the sulcal furrow (Fig. 2A, E, G). In the hypocone, a series of 16 to 20 small, four- or five-sided post-cingular vesicles border the cingulum (hypoconal series 6) (Figs 2F, 3A, B, D). This series of small vesicles is followed by a series of 8 vesicles (hypoconal series 7) (Figs 2E, 3A, B, D) and by 4 antapical vesicles (hypoconal series 8) (Figs 2G, 3D). Some changes in the pattern of the amphiesmal vesicles has been observed in the hypocone. In some cells, only 3 antapical vesicles were detected (Fig. 2E) and one intercalary vesicle has been detected between the series 7 and 8. The cell surface is mostly smooth, with scattered globular knobs and some pores (Fig. 2F arrowed).

Thin sections of cells observed with TEM reveal the typical ultrastructure of a dinoflagellate, with a large nucleus (n) with condensed chromosomes (Fig. 4A) within which a small nucleolus (nl) is present (Fig. 4B). Chloroplasts (ch) are peripheral, with 1 (Fig. 4A) or 2 (Fig. 4B) stalked pyrenoids (p). Pyrenoids are penetrated by thylakoid lamellae (th), which can appear open (Fig. 4C) or closed (Fig. 4D) depending on the angle of the section. The chloroplast stroma is present within the thylakoid lamellae (Fig. 4D). Pyrenoids are enclosed in a starch sheath (s) (Fig. 4C, D). The number of membranes surrounding chloroplasts and pyrenoids is not clearly detectable from our thin sections. The eyespot (e) is a multi-vesiculate body containing packed crystalline blocks. It is located at the cell periphery, outside the chloroplasts, near the flagellar roots (fl) (Fig. 4E, F). The Golgi apparatus (gl) is located near the eyespot and comprises many dictyosomes (Fig. 4F). Lipid accumulation bodies (ab) and mitochondria (mt), sometimes rather large, are scattered in the cytoplasm (Fig. 4A, C). Trichocysts are absent. The fixation protocol employed most probably induced ecdysis because amphiesmal vesicles were not observed surrounding the cell. The cell appears to be surrounded by two membrane layers, which at times are in tight contact (Fig. 4H), at times detached (Fig. 4G). In accordance with Höhfeld and Melkonian (1992), we interpret the inner layer as being the cytoplasmic membrane (cm) and the outer layer as being the pellicular layer (pl) and pellicle membrane (pm).

Phylogenetic Analysis

The LSU rDNA sequence of the studied strain clusters within a well-supported clade including other '*G. béii*' sequences, here called the *Pelagodinium* clade. Two distinct sub-clades, P1 and P2, are recognized at the 99% identity threshold, and the studied sequence belongs to sub-clade P1 (Fig. 5). The *Pelagodinium* clade is part of a larger phylogenetic group including 8 other dinoflagellate genera (*Baldinia* Hansen et Daugbjerg, *Biecheleria* Moestrup, Lindberg et Daugbjerg, *Biecheleriopsis* Moestrup, Lindberg et Daugbjerg, *Borghiella* Moestrup, Hansen et Daugbjerg, *Polarella* Montresor, Procaccini et Stoecker, *Protodinium* Lohmann, *Woloszynskia* Thompson, and *Symbiodinium*) which are all part of the order Suessiales. The Suessiales are clearly distinct from other dinoflagellate orders, the Gymnodiniales, Peridiniales, Prorocentrales, Dinophysiales, and Gonyaulacales

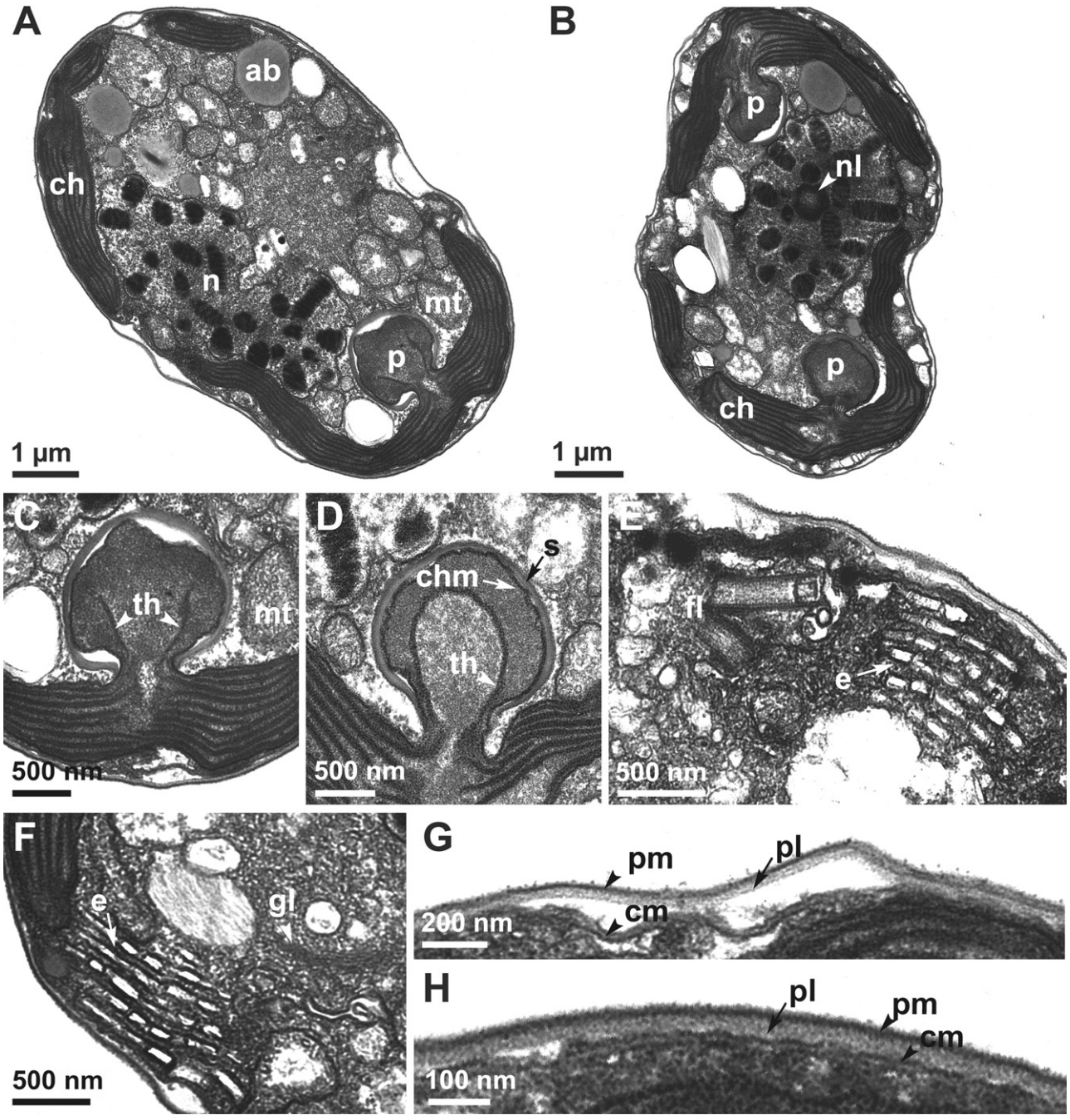

Figure 4. Transmission electron micrographs of *Pelagodinium béii* comb. nov. **A.** General view of the ultrastructure of the cell showing the nucleus (n) with condensed chromosomes, the parietal chloroplasts (ch) with a single stalked pyrenoid (p), lipid accumulation body (ab) and mitochondria (mt) scattered in the cell cytoplasm. **B.** Cell showing the nucleolus (nl) within the nucleus and two stalked pyrenoids (p). **C.** Detail of the stalked pyrenoid of Figure 3A showing the thylakoid lamella (th) penetrating the pyrenoid. **D.** Stalked pyrenoid with closed thylakoid lamella within which the chloroplast stroma is visible. The pyrenoid is surrounded by the chloroplast membrane (chm) and by a starch sheath (s). **E.** Detail of the type E eyespot (e), constituted by packed crystalline blocks, located in the cell periphery, near the flagellar roots (fl). **F.** Golgi apparatus (gl) near the eyespot (e). **G, H.** Detail of the cell external membranes of the cell showing the absence of amphiesmal vesicles and the presence of two layers sometimes in contact (H), at times detached (G). The inner layer is interpreted as the cytoplasmic membrane (cm), the outer layers as the pellicular layer (pl) and pellicle membrane (pm).

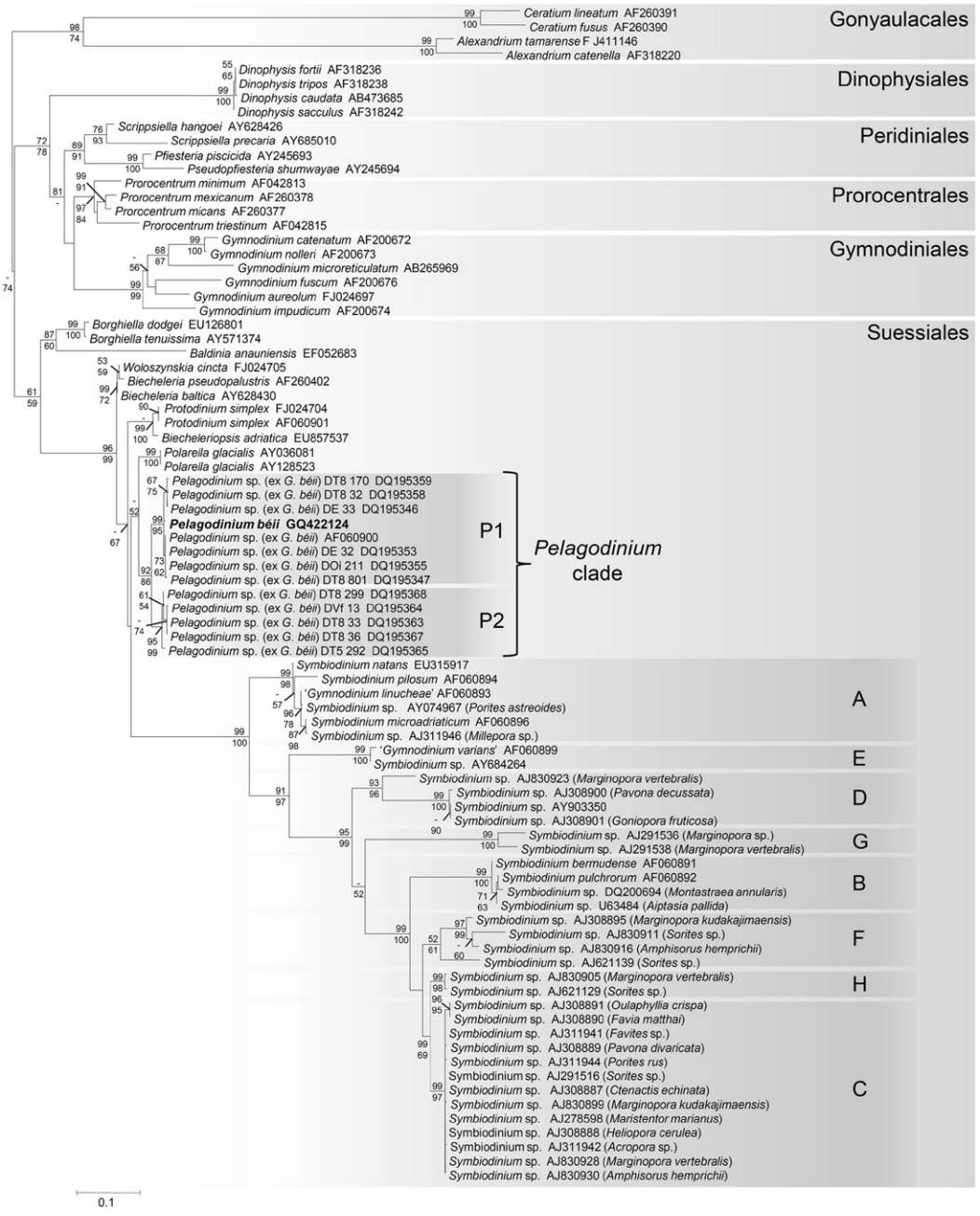

Figure 5. LSU rDNA phylogenetic tree inferred by Maximum Likelihood (ML) analysis. 582 unambiguously aligned positions were considered from an alignment of 83 sequences, including *Pelagodinium béii* gen. nov., comb nov. (bold). The tree was rooted with Gonyaulacales (*Alexandrium* spp. and *Ceratium* spp.) as outgroup. Bootstrap values $>50\%$ are shown at nodes from top (NJ, 1000 replicates) to bottom (ML, 500 replicates); '-' indicates that lower or no bootstrap value were obtained for the corresponding node. Subclades P1 and P2 are labeled according to Shaked and de Vargas (2006).The eight sub-clades (A-H) of *Symbiodinium* are labeled according to LaJeunesse (2001) and Coffroth and Santos (2005). Names in brackets for *Symbiodinium* spp. indicate the invertebrate host or the foraminifer species from which the sequence was obtained.

(Fig. 5). *Baldinia* and *Borghiella* form a cluster at the base of the order Suessiales in both Neighbor Joining (NJ) and Maximum Likelihood (ML) topologies. The other genera within the Suessiales show slightly different branching patterns in the two topologies. The clade including *Biecheleria* and *Woloszynskia* and the clade with *Biecheleriopsis* and *Protodinium* are sisters in NJ, but not in ML. *Polarella* and *Pelagodinium* are sister clades in both NJ and ML, however, very low bootstrap supports were obtained in both topologies. In both NJ and ML trees, the eight *Symbiodinium* spp. clades (A-G) are well separated from the *Pelagodinium* clade and the other genera within the Suessiales. The sequences attributed to *Gymnodinium linucheae* and *G. varians* cluster in the *Symbiodinium* clades A and E, and thus these species, as already pointed out by LaJeunesse (2001), should be referred to as *Symbiodinium linucheae* and *Symbiodinium varians*, respectively.

Discussion

In this study, the morphostructural features and the phylogenetic position of a symbiotic dinoflagellate isolated from the foraminifer *Orbulina universa* collected offshore Puerto Rico (Atlantic Ocean) were analyzed. The external characteristics (shape, dimensions, hypoconal flange) and ultrastructural features of the cell (stalked pyrenoids penetrated by thylakoid lamellae, absence of trichocysts), as well as its swimming behaviour, indicated that the dinoflagellate matches the morphological description of *Gymnodinium béii* (Spero 1987). The new morphological and phylogenetic information gathered on this species provoke a reassessment of the taxonomy of the dinoflagellate symbiont of planktonic foraminifera. A new genus is erected, *Pelagodinium* gen. nov. Siano, Montresor, Probert et de Vargas and the species *Gymnodinium béii* Spero is reclassified as *Pelagodinium béii* (Spero) Siano, Montresor, Probert et de Vargas (see taxonomic appendix).

SEM images revealed the presence on the cell surface of a single and straight elongated apical vesicle (EAV) ornamented with a row of small globular knobs (Fig. 3C, D). This character is sufficient to state that '*G. béii*' does not belong to the genus *Gymnodinium* (order Gymnodiniales), which is characterized by possessing a horseshoe-shaped acrobase running in an anticlockwise direction on the cell apex (Daugbjerg et al. 2000). Phylogenetic analyses inferred from LSU rDNA sequences support this conclusion, showing that *Pelagodinium béii* is phylogenetically distant from the Gymnodiniales, being rather a member of the order Suessiales. According to the morphological criteria proposed by Fensome et al. (1993), the order Suessiales encompasses dinoflagellates with 7 to 10 latitudinal series of amphiesmal vesicles, although an emendation of the order has been suggested to accommodate species with more than 10 latitudinal series (Kremp et al. 2005). Being characterized by 8 latitudinal series of amphiesmal vesicles, the genus *Pelagodinium* fulfils the diagnostic character of the order Suessiales.

The Order Suessiales

The order Suessiales is presently divided into three families: Borghiellaceae, Suessiaceae and Symbiodiniaceae (Fensome et al. 1993; Moestrup et al. 2009a). The family Borghiellaceae was erected recently and the diagnosis of the family Suessiaceae emended accordingly (Moestrup et al. 2009a). The two families are distinguished on the basis of the ultrastructure of the eyespot and the arrangement of the apical furrow. In the Borghelliaceae, the eyespot is ordinary with the carotenoid globules located within the chloroplast (type B of Moestrup and Daugbjerg 2007) and the apical furrow is constituted by a pair of elongated vesicles (PEV). In the Suessiaceae, the eyespot comprises a series of cisternae with brick-like content (type E of Moestrup and Daugbjerg 2007) and the apical furrow is a single elongated apical vesicle (EAV) (Moestrup et al. 2009a) (Table 1). Moreover, the Suessiaceae have clearly identifiable latitudinal series of amphiesmal vesicles, ranging in number between 7 and 15, while in the Borghiellaceae the number of latitudinal series is not specified in the description of the family (Moestrup et al. 2009a). The Borghelliaceae includes the genera *Baldinia* and *Borghiella*, although *Baldinia* does not have the PEV, whereas the Suessiaceae comprises *Biecheleria*, *Biecheleriopsis, Symbiodinium* and *Polarella* (Moestrup et al. 2009a), although *Polarella* does not have an EAV.

The genus *Symbiodinium* was attributed to the family Symbiodiniaceae by Fensome et al. (1993) based mainly on its occurrence as coccoid cells in symbiosis with benthic organisms. However, Moestrup et al. (2009a) transferred the genus to the family Suessiaceae due to the fact that both the type species *S. microadriaticum* Freudenthal and the recently described *S. natans* Hansen et

Table 1. Selected morphological features of genera included in the order Suessiales. The type species of the genus *Woloszynszkia* (*W. reticulata*) is included for comparison.

Features	*Pelagodinium*[1]	*Symbiodinium*[2]	*Baldinia*[3]	*Biecheleria*[4]	*Biecheleriopsis*[5]	*Borghiella*[6]	*Polarella*[7]	*Protodinium*[8]	*Woloszynskia*[9]
External morphology									
Apical furrow	EAV	EAV	absent	EAV	EAV	PEV	absent	EAV	carina running over the apex, across the whole epicone
Number of apical vesicles surrounding the furrow	3+X	2+X	-	ca. 14	2 or 4+X	6	-	2 or 4+X	?
Number of longitudinal series	8	7	not defined (>100 vesicles)	not defined (many vesicles)	8-10	16	9	8	9-10
Number of cingular series	1	2	1	3-4	2	2	2	2	1?
Postcingular series of small vesicles	present	absent	absent	Absent (a post cingular rim is present)	present	absent	absent	absent	absent
Hypoconal flange	present	absent	absent	absent	present	absent	absent	absent	absent
Ultrastructure									
Eyespot type	E	E	B	E	E	B	E	?	present, V-shaped, type unknown
Chloroplasts	1 or more peripheral	1 or more peripheral	1 central. radiating from the pyrenoid	many, forming a peripheral network	many, peripheral	many, peripheral forming a loose network	1 or many, central	2-4, peripheral	many
Pyrenoids	1-2, stalked, penetrated by thylakoid lamellae	1-2, stalked, not penetrated by thylakoid lamellae	1 central	many, stalked penetrated by thylakoid lamellae	many, stalked penetrated by thylakoid, swollen ends	absent	many, central, stalked, penetrated by thylakoid lamellae	?	?
Nuclear connector or rhizoplast	absent	absent	absent	absent	present	absent	absent	?	?
Peduncle	absent	present	present	present	absent	absent	absent	?	?
Trichocysts	absent	absent	absent	absent	absent	absent	absent	?	?
Pusule	absent	present (1)	present (1)	present (1)	Present (2)	Present (1-2)	?	?	?

Abbreviations: EAV: Elongated apical vesicle; PEV: pair of elongated amphiesmal vesicles. Eyespot types according to Moestrup and Daugbjerg (2007).

Literature references: [1]Spero (1987), present work; [2]Features of the free living stage are considered: Freudenthal (1962), Loeblich III and Sherley (1979), Trench and Blank (1987), Hansen and Daugbjerg (2009); [3]Hansen et al. (2006); [4]Kremp et al. (2005), Moestrup et al. (2009a); [5]Moestrup et al. (2009b); [6]Moestrup et al. (2008); [7]Montresor et al. (1999); [8]Siano et al. (2009); [9]The type species *W. reticulata*, Thompson (1951).

Daugbjerg have a type E eyespot and an EAV (Hansen and Daugbjerg, 2009; Loeblich III and Sherley 1979). A consequence of this new classification is that no genera are presently ascribed to the Symbiodiniaceae, making this family redundant. The genus *Protodinium* could also belong to the Suessiaceae since it has an EAV (Siano et al. 2009), but no information on the eyespot ultrastructure is presently available.

The affiliation of the genus *Woloszynskia* to the Suessiales is uncertain. The morphology of the type species, *W. reticulata* Thompson, is clearly different from that of other Suessiales species (Table 1). In contrast to all Suessiales species described to date, *W. reticulata* has thin amphiesmal plates on the episome and notably thick ones on the hypocone, and a large crest or 'carina' extending across the apical end along the whole episome (Thompson 1951). Unfortunately, no ultrastructural or molecular data are available for the type material. Many species previously identified as *Woloszynskia* have been reclassified in the genera *Tovellia* Moestrup, Lindberg et Daugbjerg, *Jadwigia* Moestrup, Lindberg et Daugbjerg (Lindberg et al. 2005; Moestrup et al. 2006) and *Biecheleria* (Moestrup et al. 2009a). *W. cincta* Siano, Montresor et Zingone, recently described from the Mediterranean Sea (Siano et al. 2009), should most probably also be transferred to the genus *Biecheleria* based on the presence of an EAV and its phylogenetic position (Fig. 5). Ultrastructural information, especially on the type of eyespot, is however needed to confirm this recombination.

Distinctive Features of the Genus *Pelagodinium*

Pelagodinium béii fulfils the recently designated morphological criteria of the family Suessiaceae, having a type E eyespot, an EAV, and 8 latitudinal series of amphiesmal vesicles. The molecular phylogenetic relationship of *P. béii* with *Biecheleria*, *Biecheleriopsis*, *Symbiodinium* and *Polarella* corroborates this affiliation.

Pelagodinium béii shares some morphological characters with the other genera assigned to the Suessiales, while other features make this genus unique within the order (Table 1). The EAV of *P. béii* resembles those described for *Biecheleria baltica* (Elbrächter et Kremp) Moestrup, Lindberg et Daugbjerg (Moestrup et al. 2009a) (=*Woloszynskia halophila* Elbrächter et Kremp (Kremp et al. 2005), *B. pseudopalustris* (Schiller) Moestrup, Lindberg et Daugbjerg (Moestrup et al. 2009a) *Biecheleriopsis adriatica* Moestrup, Lindberg et Daugbjerg (Moestrup et al. 2009b), *Protodinium simplex* Lohmann (Siano et al. 2009), *S. natans* Hansen et Daugbjerg (Hansen and Daugbjerg, 2009) and *W. cincta* (Siano et al. 2009). The EAV of *P. béii* is surrounded, however, by 3 elongated vesicles and a small X vesicle (Fig. 2D), distinguishing it from that of *Biecheleria* (ca. 14 vesicles), *Biecheleriopsis* (2 or 4+X) and *Protodinium* (2 or 4+X). The presence of 3+X vesicles around the EAV in *Pelagodinium* is a unique feature within the Suessiales (Table 1). *Pelagodinium béii* is characterized by a single series of vesicles within the cingulum and the arrangement of a series of small four- or five-sided vesicles below the cingulum is a peculiar feature within the Suessiales. Both *Biecheleria pseudopalustris* (Moestrup et al. 2009a) and *Biecheleriopsis adriatica* (Moestrup et al. 2009b) have a post-cingular rim of very small vesicles, but these are much smaller than those of *Pelagodinium* and are located on the posterior rim of the cingulum and not in the hypocone.

Like *Symbiodinium*, *Pelagodinium* is an endosymbiotic dinoflagellate. *Pelagodinium béii* and at least some *Symbiodinium* species (*S. microadriaticum* and *S. natans*) can, however, live as motile stages and these somewhat resemble each other when observed in light microscopy. There are nevertheless clear differences between *P. béii* and the free-living stages of *Symbiodinium* species (Table 1). As observed by Spero (1987), *P. béii* has a hypoconal flange, a cingulum displaced by once its width, no peduncle, and pyrenoids penetrated by thylakoid lamellae, whereas in *S. microadriaticum* no hypoconal flange is present, the cingulum is displaced by less than once its width, a peduncle is present, and the pyrenoids are not penetrated by thylakoid lamellae (Freudenthal 1962; Loeblich III and Sherley 1979 as *Zooxanthella microadriatica*; Trench and Blank 1987). Our new SEM and TEM images of *P. béii* show important new characters useful for distinguishing the symbiotic genera. The number of latitudinal series of vesicles differs between *P. béii* and *S. microadriaticum,* the former having 8, the latter 7. This difference is due to the number of series of vesicles in the epicone: 4 in *P. béii* and 3 in *S. microadriaticum*. Moreover, *S. microadriaticum* is described as having two series of vesicles within the cingulum, whereas *P. béii* has only one series within the cingulum as well as the series of small four- or five-sided vesicles in the hypocone, immediately below the cingulum. These differences between the genera are confirmed by the recent description of the free-living stage of a

new *Symbiodinium* species, *S. natans*, which is characterized by having 7 series of latitudinal series of vesicles and two cingular series, and by the absence of the hypoconal flange (Hansen and Daugbjerg 2009). *P. béii* is characterized by the presence of an eyespot with a peculiar vesiculate ultrastructure classifiable as type E of Moestrup and Daugbjerg (2007); this organelle was not shown at the time of the first description of this dinoflagellate (Spero 1987). The eyespot is not clearly described in the original description of *S. microadriaticum* (Freudenthal 1962), but in the thin sections provided by Loeblich III and Sherley (1979) it is clearly visible. Overall, the morphological differences between *Pelagodinium* and *Symbiodinium*, together with their genetic differences, fully support the fact that the studied strain is not a *Symbiodinium*, confirming the first intuition of Spero (1987).

Phylogenetic Position of *Pelagodinium béii*

The LSU rDNA sequence of the studied strain groups in clade P1, sister of clade P2, sensu Shaked and de Vargas (2006). Both clades include dinoflagellates recorded only as endosymbionts in various planktonic foraminifera sampled worldwide, and named '*Gymnodinium béii*' (Shaked and de Vargas 2006). These two clades form a well-supported, independent group, distinct from sequences of the genus *Gymnodinium* (Gymnodiniales). This result clearly indicates that '*G. béii*' was wrongly classified in the genus *Gymnodinium*, corroborating the conclusion obtained from the comparison of morphological features. Given the significant genetic diversity observed both between and within clades P1 and P2 (Shaked and de Vargas 2006), only LSU rDNA sequences identical to our type sequence should be attributed to *P. béii*. All other sequences of '*G. béii*' belonging to either clades P1 or P2 should be attributed to *Pelagodinium* sp. awaiting further morphological, genetic, and ecological studies to verify their actual taxonomic status.

Symbiotic Relationships

Shaked and de Vargas (2006) demonstrated that a high flexibility characterizes the photosymbiotic relationship between foraminifers and dinoflagellates in open oceanic plankton. The four *Pelagodinium* subgroups detected (containing in total 21 unique phylotypes) were found without any specificity in association with the four different foraminifera morphospecies (*Globigerinoides ruber*, *G. sacculifer*, *G. conglobatus*, and *O. universa*), each of which also harbours cryptic diversity (de Vargas et al. 1999; Morard et al. 2009). Unfortunately, we do not have information on the host genotype from which our strain of *P. béii* was isolated, but combined symbiont culture isolation and recovery of DNA from the crushed host cell should be possible in the future, allowing testing of whether different *Pelagodinium* phylotypes show any host specificity.

Symbiotic dinoflagellates of pelagic organisms have previously been studied from either the morphological (Banaszak et al. 1993; Lee 1980; Spero 1987; Trench 1993; Trench and Thinh 1995), or the molecular (Gast and Caron 1996; Shaked and de Vargas 2006) point of view. The matching morpho-genetic information gathered here for the first time on a pelagic dinoflagellate endosymbiont revealed clear differentiation from dinoflagellate symbionts of benthic organisms. Thus, within the order Suessiales, two different and ancient lineages are involved in photosymbiotic associations, the *Symbiodinium* spp. in coastal benthic ecosystems, and the *Pelagodinium* spp. in open oceanic waters. Previous phylogenetic analysis of the Suessiales suggested either independent endosymbiotic transitions from a free-living dinoflagellate lineage (*Polarella*) into coastal benthic (*Symbiodinium*) and pelagic (*Pelagodinium*, named as *G. béii*) photosymbioses, or a single symbiotic event (*Symbiodinium*, *Pelagodinium*) involving a free-living lineage (*Polarella*), followed by a loss of symbiotic behaviour in *Protodinium* and *Woloszynskia* (Shaked and de Vargas 2006). However, LSU rDNA data are currently not robust enough to confirm either of these hypotheses and further morpho-genetic data are needed to resolve the evolutionary paths that led to the emergence of major photosymbiotic lineages within the Suessiales.

Finally, our study illustrates the utility of establishing clonal cultures to conduct morpho-molecular characterization of symbiotic microalgae. This would be useful, for example, to resolve cases where dinoflagellate symbionts hosted by phylogenetically distant organisms (hydrozoans and radiolarians) have been shown to be genetically similar (Gast and Caron 1996), but their morphological affinities are still to be demonstrated. It is also relevant in light of the fact that a number of described dinoflagellate symbionts warrant morphological reexamination in order to clarify their systematic positions. For example, the morphology of *Symbiodinium linucheae* (Trench and Thinh) LaJeunesse, the symbiont of the

jellyfish *Linuche unguiculata* (Trench and Thinh 1995), should be reexamined since this species was assigned to *Symbiodinium* only on the basis of molecular data (LaJeunesse 2001), and *Gymnodinium vertebralis* Lee, the symbiont of the foraminifer *Marginopora vertebralis* (Lee 1980; Trench 1993), is probably not a member of the genus *Gymnodinium*. A fundamental question will be to determine whether open oceanic photosymbiosis involving *Pelagodinium* spp. has developed in host organisms other than the foraminifers, like its coastal benthic counterpart, where *Symbiodinium* has become associated with many protistan and metazoan lineages. Symbiotic associations are likely to be a rich source of yet unsuspected biodiversity, and cultured strains of symbiotic dinoflagellates are good candidates for analysis of the molecular and physiological mechanisms underlying photosymbiotic associations.

Taxonomic Appendix

***Pelagodinium* Siano, Montresor, Probert, et de Vargas gen. nov.**

Diagnosis: cellulae photosynteticae ad Dinophita pertinentes. Cellulae in libera vita octo seriebus vesicularum amphiesmatis contectae. Quattor in epicono, tres in hypocono, una in cingulo. Longa recta vesicula linea recta tuberum globosorum constituta in cellulae apice. Series parvarum vesicularum quadriangularum vel pentagonarum sub cingulo proxime est et hypoconum circumdat. Chloroplasti colore flavente cum pyrenoidis adherentibus. Pyrenoidi thylachoidorum lamellis invasi. Stigma extra plastidium ad typum E pertinens. Trichocisti absunt.

Photosynthetic dinoflagellate. Free-living cells covered by eight series of amphiesmal vesicles: four in the epicone, three in the hypocone, and one in the cingulum. A straight single elongated apical vesicle constituted of a single row of globular knobs is present on the cell apex. A series of small quadrangular or pentagonal vesicles is present immediately below the cingulum and encircles the hypocone. Chloroplasts golden-yellow in colour, with stalked pyrenoids. Pyrenoids penetrated by thylakoid lamellae. Extraplastidial eyespot present belonging to type E. Trichocysts absent.

Type species: *Pelagodinium béii* (Spero) Siano, Montresor, Probert et de Vargas comb. nov.

Etymology: the genus name derives from the life strategy of this dinoflagellate: dinoflagellate (=*dinos*) symbiont of pelagic (=*pelagos*) protists.

***Pelagodinium béii* (Spero) Siano, Montresor, Probert et de Vargas comb. nov. (Fig. 1 A-D; Fig. 2 A-G, Fig. 3 A-C)**

Basionym: *Gymnodinium béii* Spero in Spero (1987): 316, fig. 7 (holotype), Fig. 3a-d (isotypes, designated herein)

Diagnosis: cells are small: 10.0 ± 0.8 μm in length, 6.6 ± 0.4 μm in width, with a round to elliptical epicone and a slightly asymmetrical hypocone of almost the same dimensions. A flange is present on the left side of the epicone, projecting over the sulcus, it can be short and rounded to more pointed and elongated. Cingulum wide and shallow, descending and displaced one cingulum width. Sulcus deep and narrow, enlarging only at cell antapex. Flagella emerging from the sulcal region, no peduncle is evident. When cells are observed in SEM, amphiesmal vesicles are visible on the cell surface, arranged in 8 longitudinal series. A single elongated apical vesicle (EAV) ornamented with a row of globular knobs is present on the cell surface, surrounded by a series of 3 quadrangular vesicles and a small squared vesicle (X vesicle). Another 3 series of vesicles are present in the epicone constituted respectively of 7, 2-3 (intercalary), and 8 vesicles. Cingulum with one series of vesicles. Hypocone with a series of 16-20 small vesicles, anterior to another series of 8 vesicles and 3-4 antapicals. One or two peripheral golden-yellow chloroplasts, with one or two stalked pyrenoids. Pyrenoids are penetrated by thylakoid lamellae. An extraplastidial eyespot of type E is present near the flagellar roots. Trichocysts absent.

Taxonomic Note: *Gymnodinium béii* was suggested to resemble the free-living dinoflagellate *Aureodinium pigmentosum* Dodge (Anderson and Bé 1976; Hemleben and Spindler 1983; Spindler and Hemleben 1980). This latter species was described based on LM and TEM observations as being 10 μm in length and 7 μm in width, with an irregular hypoconal outline, peripheral chloroplasts with two stalked pyrenoids penetrated by thylakoid lamellae, without trichocysts, and with a theca composed of thin polygonal plates (Dodge 1967). Believing that the 'theca' described for *A. pigmentosum* was not typical of a thecate dinoflagellate, Loeblich III (1969) transferred the species to *Gymnodinium*, that, at that time, encompassed all species with an entirely membranous amphiesma, and he recombined the species as *G. pigmentosum* (Dodge) Loeblich III. In light of the redescription of the genus *Gymnodinium* (Daugbjerg et al. 2000) this assignment appears questionable, and the actual

taxonomic position of *G. pigmentosum* is unclear. *Gymnodinium pigmentosum* was not obtained from a symbiotic organism, but was isolated in a free-living stage directly from a seawater sample (Dodge 1967). We cannot rule out the hypothesis that *G. pigmentosum* might represent the free-living stage of an endosymbiontic dinoflagellate, but this species might also be a free-living dinoflagellate of the genera *Biecheleria*, *Biecheleriopsis*, *Protodinium* or *Woloszynskia*. We therefore did not consider the possibility of using the name *Aureodinium* for the endosymbiontic dinoflagellates of the foraminifer *Orbulina universa*.

Methods

Culture origin and maintenance: The *Orbulina universa* specimen from which the algal culture originated was isolated from a sample collected off the coast of Puerto Rico, Caribbean Sea (Atlantic Ocean; 14°49′N 67°03′W) in November 2005. The foraminiferal specimen was identified under a binocular microscope at $\times 100$ magnification, cleaned by successive transfers into sterile seawater in Petri dishes, before being crushed with a fine needle under the binocular microscope. The dinoflagellate culture was obtained by micropipette isolation of a single cell released from the crushed specimen. The resulting monoclonal culture was maintained in filter-sterilized seawater with K/2(-Tris, -Si) medium supplements (Keller et al. 1987) at 21 °C with an irradiance of 70–80 μmol photons m^{-2} s^{-1} in a 12:12 light:dark regime. The culture was deposited in the Roscoff culture collection (Roscoff Culture Collection, http://www.sb-roscoff.fr/Phyto/rcc) as RCC1491.

Microscopy preparations and observations: Live cells were observed and measured with a Nikon Eclipse TS100 inverted light microscope (Nikon, New York, USA). Light micrographs were taken with a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss AxioCam digital camera system (Carl Zeiss, Oberkochen, Germany).

For scanning electron microscopy (SEM), cells were fixed in 1% (v:v) OsO_4 for 5–10 min at room temperature. Samples were gently filtered onto 3 μm pore-size Nucleopore polycarbonate filters (Pleasanton, CA, USA), washed with distilled water, dehydrated in an ethanol series (25%, 50%, 75%, 95%, 100%), and critical point dried. The filters were mounted on stubs, sputter coated with gold, and examined with a JEOL JSM-6500F SEM (JEOL-USA Inc., Peabody, MA, USA). The stub of the analyzed sample is deposited in the museum of the Stazione Zoologica Anton Dohrn in Naples and it is available on request.

For observations of thin sections with transmission electron microscopy (TEM), cells were concentrated by gentle centrifugation (800 r.p.m. for 7 min), fixed with cold 1% (v:v) gluteraldehyde for 1 h on ice, rinsed with filtered seawater (FSW), and post-fixed with 1% (v:v) osmium tetroxide for 30 min on ice. After two rinses with FSW, the sample was dehydrated in an ethanol series (25%, 50%, 75%, 95%, 100%), transferred to propylene oxide, and embedded in Epon resin (v:v, 1:1). After polymerization at 70 °C for 24 h, thin sections were cut using a Reichert Ultracut ultramicrotome (Depew, NY, USA), stained with uranyl acetate and lead citrate, and examined with a LEO 912AB EF-TEM (LEO, Carl Zeiss, Oberkochen, Germany).

DNA extraction and phylogenetic analysis: DNA was extracted from an exponentially growing culture of the *Pelagodinium béii* strain using the method described in de Vargas et al. (2002). The D1-D2 part of the nuclear large subunit ribosomal DNA (LSU rDNA) was PCR amplified and sequenced using the methods described in Shaked and de Vargas (2006). The partial LSU rDNA sequence of the analyzed strain is deposited in Genbank (http://www.ncbi.nlm.nih.gov) with the accession number GQ422124.

The sequence generated from the studied strain was aligned with other LSU rDNA sequences downloaded from GenBank and attributed to '*G. béii*', with sequences of species of the order Suessiales and with sequences undoubtedly attributable to other main dinoflagellate orders, the Gonyaulacales, Peridiniales, Prorocentrales, Dinophysiales, Gymnodiniales. An alignment of 83 sequences was generated using MAFFT (Katoh et al. 2002;) and manually edited in Bioedit v.7.0.9.0 (Hall 1999). The 582 positions used in phylogenetic analyses were determined using the Gblocks method (Castresana 2000) for selecting conserved blocks (minimum block length=5; allowed gap positions=with half).

Phylogenetic analyses were conducted with Neighbor Joining (NJ) and Maximum Likelihood (ML) methods. The NJ phylogenetic analysis was inferred using pair wise p-distance in MEGA (v. 4.1, Tamura et al. 2007) and bootstrap values were calculated from 1000 replicates. The ML analysis was carried out using PhyML v. 3.0 aLRT (Guindon and Gascuel 2003), performed on the web portal Phylogeny.fr (Dereeper et al. 2008). The General Time Reversible (GTR) model of nucleotide substitution and the number of substitution rate categories, the shape parameter (α) of the Gamma (Γ) distribution and the proportion of invariable sites (I) were estimated from the dataset using default options in Phylogeny.fr. Bootstrap supports for the tree were obtained after 500 replicates. The tree was visualized and edited in MEGA (v. 4.1, Tamura et al. 2007).

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