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Molecular Phylogeny of the Sand-dwelling Dinoflagellates *Amphidiniopsis hirsuta* and *A. swedmarkii* (Peridiniales, Dinophyceae)

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Summary. *Amphidiniopsis* is one of the most specious genera of sand-dwelling dinoflagellates. However, its systematic position and the affinities to other genera remain unresolved. We obtained the first SSU rDNA sequences of two species of *Amphidiniopsis*, *A. hirsuta* and *A. swedmarkii* collected from the French coasts of the English Channel. In the corresponding SSU rDNA phylogeny, the sand-dwelling *Amphidiniopsis* spp., *Thecadinium dragescoi*, *Herdmania litoralis* and the planktonic *Archaeperidinium* (= *Protoperidinium*) *minutum* formed a strongly supported clade (100% bootstrap support), with a *Scrippsiella*-like species occupying a basal position. This clade branched close to families of Peridiniales such as Calciodinellaceae (*Scripssiella*), Podolampadaceae (*Podolampas*, *Roscoffia*), Heterocapsaceae (*Heterocapsa*) and other peridinioids. The members of the clade of *Amphidiniopsis* are placed in the family Amphidiniopsidaceae, within the Peridiniales.

Key words: *Archaeperidinium*, benthic peridinioids, Dinoflagellata, Gymnodiniales-Peridiniales-Prorocentrales lineage, *Herdmania litoralis*, *Protoperidinium minutum*, SSU rDNA phylogeny, *Thecadinium*.

Abbreviations: bp – base pairs; BV – bootstrap value; PCR – polymerase chain reaction; LSU – large subunit; SSU – small subunit; rDNA – ribosomal deoxyribonucleic acid.

INTRODUCTION

Sand-dwelling dinoflagellates possess unusual morphologies and atypical tabulation patterns that are subjected to different interpretations. Hence, assigning them to existing families, or even orders, and determining their evolutionary relationships is usually very difficult on the basis of morphological characters. Woloszyńska (1928) was the first to report the presence of thecal plates in sand-dwelling dinoflagellates with the description of the new genus *Amphidiniopsis* from the Polish coasts. She illustrated the type species *A. kofoidii* by partly incomplete drawings and gave the plate formula: 3′ three apical plates, 7″ seven precingular plates, 5‴ five postcingular plates and 2″″ two antapical plates. Balech (1956) created the new family Thecadiniaceae within the Peridiniales for the type of *Thecadinium* Kofoid et Swezy, *T. kofoidii* (Herdman) Larsen, and described four new species: *T. dragescoi* Balech, *T. inclinatum* Balech, *T. hirsutum*

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Balech and *T. swedmarkii* Balech. He mentioned that *T. hirsutum* and *T. swedmarkii* may belong to the genus *Amphidiniopsis*. Later, Dodge (1982) transferred these two species into *Amphidiniopsis.* Based on scanning electron microscopy, Dodge and Lewis (1986) reported for *A. kofoidii* the plate formula 4′ 3a 7″ 3c 3s? 5‴ 2⁗. The type species was not re-examined in further studies. Currently, there are 14 species of *Amphidiniopsis*. They are heterotrophic marine species, except *A. sibbaldii* Nicholls, which occurs in freshwater lakes (Nicholls 1998). *Amphidiniopsis* cells are bilaterally, almost circular or dorso-ventrally compressed, with a relatively small epitheca and a larger hypotheca. The cingulum is nearly horizontal or ascending, with the left ventral part running posteriorly into the sulcus. The sulcus has a characteristic curved left side and reaches the antapex (Dodge 1982, Hoppenrath 2000, Yoshimatsu *et al*. 2000, Toriumi *et al*. 2002, Murray and Patterson 2002, Hoppenrath *et al*. 2009). Determining the precise number of cingular and sulcal, and even precingular plates for several species is not without difficulties (Hoppenrath 2000, Murray and Patterson 2002).

The species of *Amphidiniopsis* have been classified with the gonyaulacoid dinoflagellates of the family Gonyaulacaceae (Schiller 1937). Balech (1956) placed his species of *Amphidiniopsis* (described as *Thecadinium hirsutum* and *T. swedmarkii*) in the family Thecadiniaceae within the Peridiniales. Dodge (1982) and Sournia (1986) placed *Amphidiniopsis* in the order Peridiniales, family Oxytoxaceae. Later, Dodge (1984) proposed the family Amphidiniopsidaceae for *Amphidiniopsis* and *Adenoides* Balech. These different classifications prompted us to study the systematic position of *Amphidiniopsis* using molecular data.

MATERIALS AND METHODS

Sampling and isolation of material

The specimens of *Amphidiniopsis* were isolated from sand samples collected in 2010 in a tidal pool on Wimereux beach (50°45′60″N; 1°36′21″E). The upper centimetre of sand was collected with a spoon and deposited into a bottle. There, the sand was rinsed with seawater and stirred vigorously, and the suspension settled in a composite Utermöhl chamber. The settled material was examined with a Nikon inverted microscope (Nikon Eclipse TE2000-S) and photographed with a Nikon Digital Sight DS-2M camera.

Each specimen was micropipetted individually with a fine capillary into a clean chamber and washed several times in serial drops of 0.2-µm filtered and sterilized seawater. Finally, each specimen was deposited into a 0.2 mL Eppendorf tube filled with several drops of absolute 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 and sequencing

The specimens fixed in ethanol were centrifuged gently for 5 min. at 3000 rpm. Ethanol was then evaporated in a vacuum desiccator and single cells were resuspended directly in 25 µL of Ex TaKaRa buffer (TaKaRa, distributed by Lonza Cia., Levallois-Perret, France). PCRs were done in a volume of 30 to 50 µL reaction mix containing 10–20 pmol of the eukaryotic-specific SSU rDNA primers EK-42F (5’-CTCAARGAYTAAGCCATGCA-3’) and EK-1520R (5’-CYGCAGGTTCACCTAC-3’) (López-García *et al*. 2001). PCRs 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 was then carried out using 2–5 µL of the first PCR products in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the eukaryotic-specific primers EK-82F (5’-GAAACTGCGAATGGCTC-3’) and EK-1498R (5’-CACCTACGGAAACCTTGTTA-3’) (López-García *et al*. 2001) and similar PCR conditions as described above. A third, semi-nested PCR was carried out using the dinoflagellate specific primer DIN464F (5’-TAACAATACAGGGCATCCAT-3’) (Gómez *et al*. 2009) and keeping the reverse primer EK-1498R. Negative controls without template DNA were used at all amplification steps. Amplicons of the expected size (~ 1200 bp) were then sequenced bidirectionally using primers DIN464F and EK-1498R using an automated 96-capillary sequencer ABI PRISM 3730xl (BC Genomics, Takeley, UK). New sequences were deposited in GenBank under accession numbers JN587281-JN587284.

Phylogenetic analyses

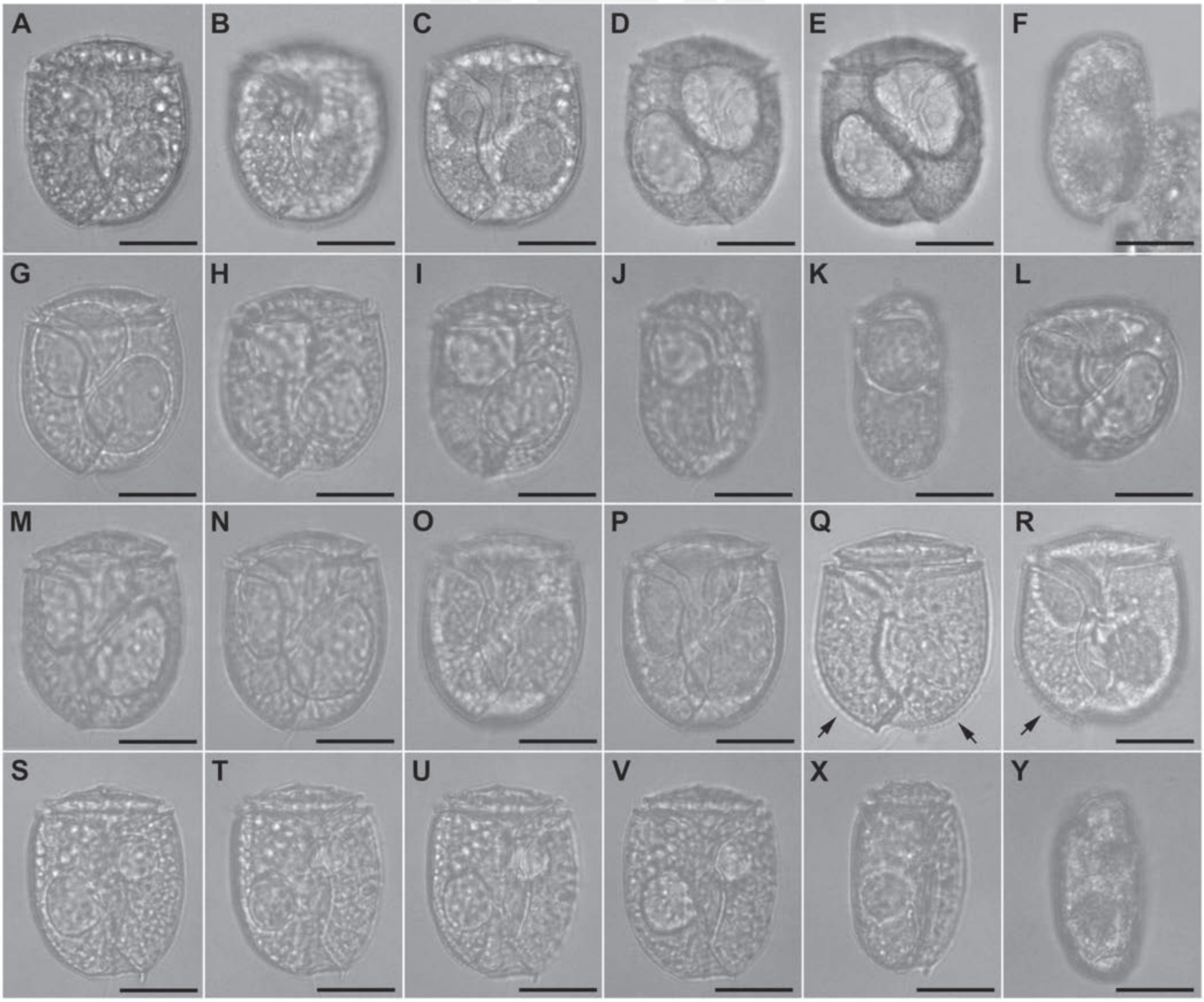
The new SSU rDNA sequences were aligned to a large multiple sequence alignment containing 1300 publicly available complete or nearly complete (> 1300 bp) dinoflagellate sequences using the profile alignment option of MUSCLE 3.7 (Edgar 2004). The resulting alignment was manually inspected using the program 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 identification of the closest relatives of our sequences together with a sample of other dinoflagellate species, which were selected to carry out more computationally-intensive Maximum Likelihood analyses. These 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 1000 pseudoreplicates with the same substitution model.

The phylogenetic position of *Amphidiniopsis* was analyzed by means of a data set of 119 dinoflagellate taxa (1214 sites), including sequences of representatives of the Gymnodiniales, Prorocentrales, Dinophysales, Gonyaulacales, Suessiales and Peridiniales, with an especial focus on peridinioids and the benthic thecate dinoflagellates.

RESULTS

Observations of *Amphidiniopsis*

The SSU rDNA sequences of *Amphidiniopsis* were obtained from four live specimens of two species, *A. hirsuta* and *A. swedmarkii*, collected at Wimereux beach, NE English Channel. The specimens of *A. hirsuta* were dorso-ventrally flattened, 38–42 μm long and 35–37 μm wide (Figs 1A–P). The cell shape was rectangular, squarish at the anterior end and rounded at the posterior hypotheca. The very small epitheca was a flattened cap-like structure of less than one tenth of the cell length and narrower than the hypotheca. The cingulum was deeply impressed and the sulcus was broad and characteristically curved. The round to oval nucleus was situated in the lower left lateral part of the hypotheca and two pusules lied in the lower right and upper left lateral



Figs 1A–Y. Photomicrographs of live specimens of *Amphidiniopsis* from the beach of Wimereux, France used for the single-cell PCR analysis, bright field optics. **A–F** – *A. hirsuta* isolate FG1768 (10 May 2010), accession number #JN587281; **G–L** – *A. hirsuta* isolate FG1793 (4 Oct 2010) #JN587283; **M–P** – *A. hirsuta* isolate FG1794 (4 Oct 2010), #JN587284; **Q–R** – another specimen (1 Oct 2010). The arrows point the row of small spines; **S–Y** – *A. swedmarkii* isolate FG1787 (28 Sep 2010), #JN587282. Scale bars: 20 μm.

part of the hypotheca. Epifluorescence microscopy observations suggested that the specimens were devoid of chloroplasts. The antapical row of spines was visible in some specimens under light microscopy (Figs 1Q–R). The hypotheca of the second species studied, *A. swedmarkii* (Figs 1S–Y), was slightly narrower than in *A. hirsuta*, whereas the epitheca was more flattened than in *A. hirsuta*. The most distinctive character of *A. swedmarkii* was the conspicuous antapical spine (Fig. 1S).

Molecular phylogeny

We examined the phylogenetic position of *Amphidiniopsis* spp. using maximum likelihood tree reconstruction with a data set including a variety of dinoflagellate SSU rDNA sequences and rooted using perkinsozoan and syndinean sequences as outgroup. The sequences from the three *A. hirsuta* specimens were nearly identical. *Amphidiniopsis hirsuta* and *A. swedmarkii* branched together with a strong support (bootstrap value, BV, 100%) but the relatively long distance between them confirmed that they are distinct species. *Amphidiniopsis* spp. emerged within a clade with a strong support (BV 100%) also containing *Thecadinium dragescoi*, *Archaeperidinium* (= *Protoperidinium*) *minutum* and the type of the genus *Herdmania* (= *Dodgeia* Özdikmen, non *Herdmania* Lahille), *H. litoralis* Dodge. A sequence named 'thecate dinoflagellate UDTSW0701' and the environmental clone TAGIRI-6 from a marine sediment sample formed a robust group with the clade of *Amphidiniopsis* and related genera (BV 100%). This group branched in an unresolved position among the short-branched sequences of the poorly resolved Gymnodiniales-Peridiniales-Prorocentrales lineage, close to families of Peridiniales such as Calciodinellaceae (*Scripssiella* Balech), Podolampadaceae (*Podolampas* Stein, *Roscoffia* Balech), Heterocapsaceae (*Heterocapsa* Stein) and other peridinioids (Fig. 2).

DISCUSSION

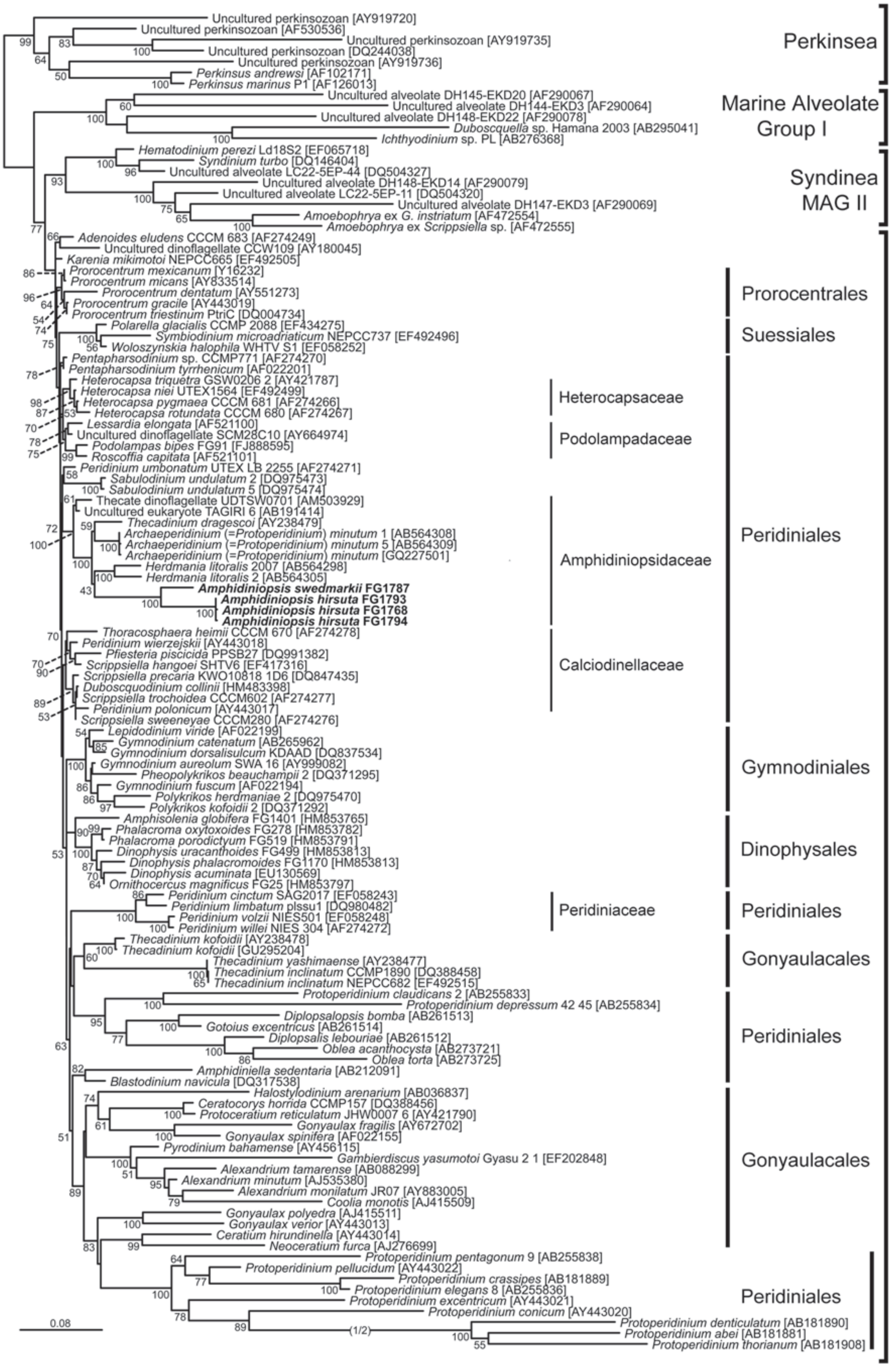
The four sequences of *Amphidiniopsis* spp. formed a strongly supported clade with the sand-dwelling species *Herdmania littoralis*, *Thecadinium dragescoi* and the planktonic *Archaeperidinium* (= *Protoperidinium*) *minutum*. In most recent classifications *Amphidiniopsis*, *Thecadinium* and *Roscoffia* have been placed in the family Thecadiniaceae (Steidinger and Tangen 1997). However, *Roscoffia* is a member of the peridinioid family Podolampadaceae (Gómez *et al.* 2010) and the type of *Thecadinium* and other congeneric species tended to branch in a basal position of the gonyaulacoid lineage (Fig. 2). An exception is *Thecadinium dragescoi*, which has conspicuous morphological differences with respect to the other *Thecadinium* species and a close affinity to *Amphidiniopsis*, as has been pointed out repeatedly (Sournia 1986, Hoppenrath 2000, Murray and Patterson 2002, Yamaguchi *et al*. 2011). In fact, *T. dragescoi* is heterotrophic and has a totally different plate arrangement from the photosynthetic type of *Thecadinium*. In agreement with these morphological differences, the molecular phylogeny results support the transfer of *T. dragescoi* into a separate genus. Nevertheless, we hesitate to erect a new genus based on this single species or to transfer it into *Amphidiniopsis*, *Herdmania* or *Archaeperidinium*. In our phylogeny, *T. dragescoi* appears to be closer to *Archaeperidinium minutum*, but the low bootstrap values and the strong differences in the general appearance and habitats preclude us to propose both species under the same genus.

Tabulation determination in sand-dwelling dinoflagellates is subjected to different interpretations, as illustrated by the example of *T. dragescoi* and *H. litoralis* (Yamaguchi *et al*. 2011). In contrast, there are not discrepancies in the tabulation of the planktonic *Archaeperidinium minutum* (Ribeiro *et al*. 2010, Yamaguchi *et al*. 2011). The robust placement of *A. minutum* in the same clade as *T. dragescoi* and *Herdmania* in the SSU rDNA tree can be considered as a support for the recent interpretations of the tabulation of these species, which share a common tabulation for the main plate series. However, this tabulation is not a distinctive character for this clade, being common for several peridinioid families (4′ 1–3a 7″ 5‴ 2⁗).

Ribeiro *et al*. (2010) analysed the phylogenetic position of *Archaeperidinium minutum* in LSU and SSU

▸▸

Fig. 2. Maximum likelihood phylogenetic tree of dinoflagellate SSU rDNA sequences, based on 1214 aligned positions. Names in bold represent sequences obtained in this study. Numbers at the nodes are bootstrap proportions (values under 50% are omitted). Accession numbers are provided between brackets. A long branch shortened to one half of its original length is indicated by (1/2). The scale bar represents the number of substitutions for a unit branch length.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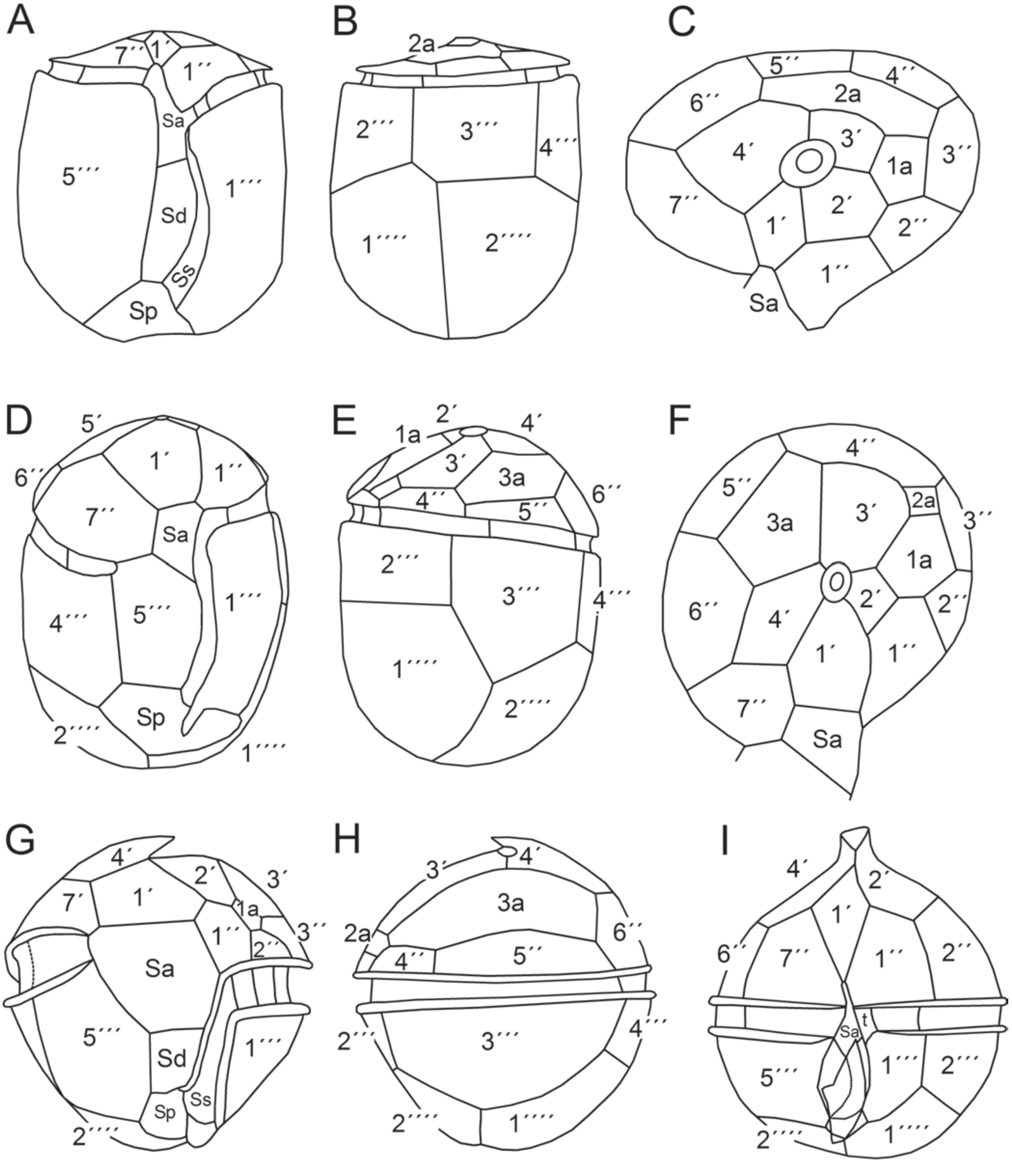
Uncultured perkinsozoan [AF530536]
Uncultured perkinsozoan [AY919735]
Uncultured perkinsozoan [DQ244038]
Uncultured perkinsozoan [AY919736]
Perkinsus andrewsi [AF102171]
Perkinsus marinus P1 [AF126013]
Uncultured alveolate DH145-EKD20 [AF290067]
Uncultured alveolate DH144-EKD3 [AF290064]
Uncultured alveolate DH148-EKD22 [AF290078]
Duboscquella sp. Hamana 2003 [AB295041]
Ichthyodinium sp. PL [AB276368]
Hematodinium perezi Ld18S2 [EF065718]
Syndinium turbo [DQ146404]
Uncultured alveolate LC22-5EP-44 [DQ504327]
Uncultured alveolate DH148-EKD14 [AF290079]
Uncultured alveolate LC22-5EP-11 [DQ504320]
Uncultured alveolate DH147-EKD3 [AF290069]
Amoebophrya ex G. instriatum [AF472554]
Amoebophrya ex Scrippsiella sp. [AF472555]
Adenoides eludens CCCM 683 [AF274249]
Uncultured dinoflagellate CCW109 [AY180045]
Karenia mikimotoi NEPCC665 [EF492505]
Prorocentrum mexicanum [Y16232]
Prorocentrum micans [AY833514]
Prorocentrum dentatum [AY551273]
Prorocentrum gracile [AY443019]
Prorocentrum triestinum PtriC [DQ004734]
Polarella glacialis CCMP 2088 [EF434275]
Symbiodinium microadriaticum NEPCC737 [EF492496]
Woloszynskia halophila WHTV S1 [EF058252]
Pentapharsodinium sp. CCMP771 [AF274270]
Pentapharsodinium tyrrhenicum [AF022201]
Heterocapsa triquetra GSW0206 2 [AY421787]
Heterocapsa niei UTEX1564 [EF492499]
Heterocapsa pygmaea CCCM 681 [AF274266]
Heterocapsa rotundata CCCM 680 [AF274267]
Lessardia elongata [AF521100]
Uncultured dinoflagellate SCM28C10 [AY664974]
Podolampas bipes FG91 [FJ888595]
Roscoffia capitata [AF521101]
Peridinium umbonatum UTEX LB 2255 [AF274271]
Sabulodinium undulatum 2 [DQ975473]
Sabulodinium undulatum 5 [DQ975474]
Thecate dinoflagellate UDTSW0701 [AM503929]
Uncultured eukaryote TAGIRI 6 [AB191414]
Thecadinium dragescoi [AY238479]
Archaeperidinium (=Protoperidinium) minutum 1 [AB564308]
Archaeperidinium (=Protoperidinium) minutum 5 [AB564309]
Archaeperidinium (=Protoperidinium) minutum [GQ227501]
Herdmania litoralis 2007 [AB564298]
Herdmania litoralis 2 [AB564305]
Amphidiniopsis swedmarkii FG1787
Amphidiniopsis hirsuta FG1793
Amphidiniopsis hirsuta FG1768
Amphidiniopsis hirsuta FG1794
Thoracosphaera heimii CCCM 670 [AF274278]
Peridinium wierzejskii [AY443018]
Pfiesteria piscicida PPSB27 [DQ991382]
Scrippsiella hangoei SHTV6 [EF417316]
Scrippsiella precaria KWO10818 1D6 [DQ847435]
Duboscquodinium collinii [HM483398]
Scrippsiella trochoidea CCCM602 [AF274277]
Peridinium polonicum [AY443017]
Scrippsiella sweeneyae CCCM280 [AF274276]
Lepidodinium viride [AF022199]
Gymnodinium catenatum [AB265962]
Gymnodinium dorsalisulcum KDAAD [DQ837534]
Gymnodinium aureolum SWA 16 [AY999082]
Pheopolykrikos beauchampii 2 [DQ371295]
Gymnodinium fuscum [AF022194]
Polykrikos herdmaniae 2 [DQ975470]
Polykrikos kofoidii 2 [DQ371292]
Amphisolenia globifera FG1401 [HM853765]
Phalacroma oxytoxoides FG278 [HM853782]
Phalacroma porodictyum FG519 [HM853791]
Dinophysis uracanthoides FG499 [HM853813]
Dinophysis phalacromoides FG1170 [HM853813]
Dinophysis acuminata [EU130569]
Ornithocercus magnificus FG25 [HM853797]
Peridinium cinctum SAG2017 [EF058243]
Peridinium limbatum plssu1 [DQ980482]
Peridinium volzii NIES501 [EF058248]
Peridinium willei NIES 304 [AF274272]
Thecadinium kofoidii [AY238478]
Thecadinium kofoidii [GU295204]
Thecadinium yashimaense [AY238477]
Thecadinium inclinatum CCMP1890 [DQ388458]
Thecadinium inclinatum NEPCC682 [EF492515]
Protoperidinium claudicans 2 [AB255833]
Protoperidinium depressum 42 45 [AB255834]
Diplopsalopsis bomba [AB261513]
Gotoius excentricus [AB261514]
Diplopsalis lebouriae [AB261512]
Oblea acanthocysta [AB273721]
Oblea torta [AB273725]
Amphidiniella sedentaria [AB212091]
Blastodinium navicula [DQ317538]
Halostylodinium arenarium [AB036837]
Ceratocorys horrida CCMP157 [DQ388456]
Protoceratium reticulatum JHW0007 6 [AY421790]
Gonyaulax fragilis [AY672702]
Gonyaulax spinifera [AF022155]
Pyrodinium bahamense [AY456115]
Gambierdiscus yasumotoi Gyasu 2 1 [EF202848]
Alexandrium tamarense [AB088299]
Alexandrium minutum [AJ535380]
Alexandrium monilatum JR07 [AY883005]
Coolia monotis [AJ415509]
Gonyaulax polyedra [AJ415511]
Gonyaulax verior [AY443013]
Ceratium hirundinella [AY443014]
Neoceratium furca [AJ276699]
Protoperidinium pentagonum 9 [AB255838]
Protoperidinium pellucidum [AY443022]
Protoperidinium crassipes [AB181889]
Protoperidinium elegans 8 [AB255836]
Protoperidinium excentricum [AY443021]
Protoperidinium conicum [AY443020]
Protoperidinium denticulatum [AB181890]
Protoperidinium abei [AB181881]
Protoperidinium thorianum [AB181908]
(1/2)
0.08
Perkinsea
Marine Alveolate Group I
Syndinea MAG II
Prorocentrales
Suessiales
Heterocapsaceae
Podolampadaceae
Peridiniales
Amphidiniopsidaceae
Calciodinellaceae
Gymnodiniales
Dinophysales
Peridiniaceae
Peridiniales
Gonyaulacales
Peridiniales
Gonyaulacales
Peridiniales
Dinokaryota



Figs 3A–I. Line drawings of the morphology and tabulation of members of Amphidiniopsidaceae. **A–C** –ventral, dorsal and apical views of *Amphidiniopsis hirsuta* (redrawn from Hoppenrath *et al.* 2009); **D–F** – *Thecadinium dragescoi* (redrawn from Hoppenrath 2000); **G–H** – *Herdmania litoralis* and **I** – *Archaeperidinium minutum* (redrawn from Yamaguchi *et al.* 2011).

rDNA trees with all the available sequences of Protoperidiniaceae but a limited representation of other dinoflagellate groups. *A. minutum* was found to be sister group of the Diplopsaloideae, supported by 100% Bayesian posterior probability but low maximum parsimony (56%) and maximum likelihood (< 50%) bootstrap support. However, in the LSU rDNA phylogeny, *A. minutum* emerged as an isolated branch in a poorly resolved region of the tree. Yamaguchi *et al*. (2011) reported that *P. minutum* formed a well-supported clade with the sand-dwelling *H. litoralis* and *T. dragescoi*. They proposed *P. minutum* under a separate genus and reinstated the genus *Archaeperidinium* for the type species, while other species of *Archaeperidinium* were distantly related and branched with Protoperidiniaceae. Our molecular data support these results, rejecting the placement of the clade of *Amphidiniopsis* in the family Protoperidiniaceae (Fig. 2).

Schiller (1937) placed *Amphidiniopsis* in the order Peridiniales, family Gonyaulacaceae, Balech (1956) placed the family Thecadiniaceae (*Thecadinium*, *Adenoides* and *Roscoffia*) within the Peridiniales. Dodge (1982) thought that the genera *Amphidiniopsis*, *Adenoides* and *Roscoffia* belong in the order Peridiniales, family Oxytoxaceae. This proposal was followed by Sournia (1986), who placed *Amphidiniopsis* and *Roscoffia* in the Oxytoxaceae. Dodge (1984) proposed, lacking Latin diagnosis, the family Amphidiniopsidaceae for *Adenoides* and *Amphidiniopsis*. However, the molecular phylogeny reveals that *Adenoides*, which strongly differs in the morphology and tabulation (Hoppenrath *et al.* 2003), is not related to the clade of *Amphidiniopsis* (Fig. 2).

In our analyses, the clade of *Amphidiniopsis* spp., *Thecadinium dragescoi*, *Herdmania litorialis* and the planktonic *Archaeperidinium minutum* and an undescribed species formed a strongly supported clade that could eventually be considered as a separate family within the Peridiniales. Nevertheless, we prefer to keep the existing family Amphidiniopsidaceae before proposing a new family, as it is difficult to define the unifying and distinctive characters of this family composed of genera with different morphologies (Fig. 3). The tabulation (4′ 1–3a 7″ 5‴ 2⁗) is common to most of peridinioid dinoflagellates (Protoperidiniaceae, Calciodinellaceae, and the type and many members of Peridiniaceae). The morphology, ecology and habitat are variable in the species of Amphidiniopsidaceae, as in other peridinioid families (Calciodinellaceae, Podolampadaceae). Further studies are necessary to determine the morphological and structural characters that support the phylogenetic classification of these peridinioid families.

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