



MORPHOLOGY AND MOLECULAR PHYLOGENY OF A NEW MARINE, SAND-DWELLING DINOFLAGELLATE GENUS, *PACHENA* (DINOPHYCEAE), WITH DESCRIPTIONS OF THREE NEW SPECIES¹

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Marine benthic dinoflagellates are interesting not only because some epiphytic genera can cause harmful algal blooms but also for understanding dinoflagellate evolution and diversification. Our understanding of their biodiversity is far from complete, and many thecate genera have unusual tabulation patterns that are difficult to relate to the diverse known phytoplankton taxa. A new sand-dwelling genus, *Pachena* gen. nov., is described based on morphological and DNA sequence data. Three species were discovered in distant locations and are circumscribed, namely, *P. leibnizii* sp. nov. from Canada, *P. abriiliae* sp. nov. from Spain, and *P. meriddae* sp. nov. from Italy. All species are tiny (about 9–23 µm long) and heterotrophic. Species are characterized by their tabulation (APC 4' 3a 6'' 5c 5s 5''' 2'''), an apical hook covering the apical pore, an ascending cingulum, and a sulcus with central list. The first anterior intercalary plate is uniquely “sandwiched” between two plates. The species share these features and differ in the relative sizes and arrangements of their plates, especially on the epitheca. The ornamentation of thecal plates is species-specific. The new molecular phylogenies

based on SSU and LSU rDNA sequences contribute to understanding the evolution of the planktonic relatives of *Pachena*, the Thoracosphaeraceae.

Key index words: benthic; distribution; morphology; Peridinales; protists; taxonomy; Thoracosphaeraceae

Abbreviations: AICc, corrected Akaike information criterion; APC, apical pore complex; (B)PP, (Bayesian) posterior probability; BS, bootstrap; DIC, differential interference contrast; HMDS, hexamethylsilazane; ML, maximum likelihood; Po, apical pore plate; 1', first apical plate; 2', second apical plate; 3', third apical plate; 4', fourth apical plate; 1a, first intercalary plate; 2a, second intercalary plate; 3a, third intercalary plate; 1'', first precingular plate; 2'', second precingular plate; 3'', third precingular plate; 4'', fourth precingular plate; 5'', fifth precingular plate; 6'', sixth precingular plate; c1, first cingular plate; c2, second cingular plate; c3, third cingular plate; c4, fourth cingular plate; c5, fifth cingular plate; Sa, anterior sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sp, posterior sulcal plate; Sm, median sulcal plate; 1''', first postcingular plate; 2''', second postcingular plate; 3''', third postcingular plate; 4''', fourth postcingular plate; 5''', fifth postcingular plate; 1''''', first antapical plate; 2''''', second antapical plate

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The first studies on sand-dwelling dinoflagellates were conducted in the early twentieth century (Kofoid and Swezy 1921, Herdman 1922, 1924a,b, Balech 1956), even though they were not studied comprehensively until the 2000s (Hoppenrath 2000a, Murray 2003, Tamura 2005, Mohammad-Noor et al. 2007, Al-Yamani and Saburova 2010). The studies showed that the species composition is distinct from planktonic communities and the species diversity was largely unexplored (Hoppenrath et al. 2014). Epiphytic species have received more attention from the scientific community, mainly because many of them are toxin producers and are toxic to humans (Berdalet et al. 2017). Still there is undiscovered biodiversity among benthic, especially sand-dwelling, dinoflagellates with new taxon descriptions nearly every year, including new genera: *Vulcanodinium* (Nézan and Chomérat 2011), *Moestrupia* (Hansen and Daugbjerg 2011), *Ankistrodinium* (Hoppenrath et al. 2012), *Testudodinium* (Horiguchi et al. 2012), *Bispinodinium* (Yamada et al. 2013), *Ailadinium* (Saburova and Chomérat 2014), *Madanidinium* (Chomérat and Bilien 2014), *Aduncodinium* (Kang et al. 2015), *Fukuyoa* (Gómez et al. 2015), *Pellucidodinium* (Onuma et al. 2015), *Laciniporus* (Saburova and Chomérat 2019), and *Psammodinium* (Reñé and Hoppenrath 2019).

Benthic, sand-dwelling species seem to have morphological adaptations reflecting their life in the interstitial habitat, such as smooth (i.e., without striking extensions like wings, spines, or horns) and flattened cell shapes (Hoppenrath et al. 2014). Several taxa cover their apical pore with thecal extensions (e.g., *Rhinodinium* has a large apical hook; Murray et al. 2006); some *Amphidiniopsis* species and *Herdmania* have a small hook (Hoppenrath 2000b, Murray and Patterson 2002, Toriumi et al. 2002, Yamaguchi et al. 2011, Reñé et al. 2020); *Apicoporus* has finger-like projections (Sparmann et al. 2008); *Laciniporus* has a small flap-shaped projection (Saburova and Chomérat 2019); and *Sinophysis* has parallel upright projections (Hoppenrath 2000c, Chomérat 2016). Many thecate, benthic dinoflagellate taxa have unusual tabulation patterns that are difficult to relate to the known tabulations in planktonic taxa (Hoppenrath et al. 2014). For example, *Madanidinium* has no apical pore (Chomérat and Bilien 2014); *Plagiodinium* has no precingular or no apical plate series (Faust and Balech 1993, Wakeman et al. 2018), depending on interpretation; *Thecadinium* sensu stricto and *Pseudothecadinium* have only incomplete precingular plate series and other special plate arrangements (Efimova et al. 2019, Selina et al. 2019); *Pseudadenoides* is the only known genus with a complete posterior intercalary plate series (Hoppenrath et al. 2003, 2017).

A species diversity survey of marine sandy sediments in British Columbia, Canada, revealed species richness including new taxa (Hoppenrath and Leander 2007, 2008, Sparmann et al. 2008, Hoppenrath et al. 2014, 2017; M. Hoppenrath unpub. data).

Studies on benthic dinoflagellates from the Mediterranean Sea have mainly focused on epiphytic toxic species (Vila et al. 2001, Aligizaki and Nikolaidis 2006, Aligizaki et al. 2009, Penna et al. 2012), whereas sand-dwelling dinoflagellates have been poorly studied and information is scarce (Reñé et al. 2020). Here, a new genus is described that was first discovered on the western shoreline of Vancouver Island, Canada and further species were recorded in Spanish and Italian Mediterranean Sea samples.

METHODS

Sampling, cell extractions, and microscopy. Sand samples from Canada were collected with a spoon during low tide at Pachena Beach (48°47'34.6" N, 125°07'19.0" W), Vancouver Island, British Columbia, in May and June 2005, April and June 2006, and May and June 2007. The sand samples were transported directly to the laboratory, and dinoflagellates were separated from the sand by extraction through a fine filter (mesh size 45 µm) using melting seawater ice (Uhlig 1964). Cells of the new taxon were observed directly with a Leica DMIL inverted microscope (Wetzlar, Germany) and isolated by micropipetting for the preparations described below. For differential interference contrast (DIC) light microscopy, pipetted cells were viewed with a Zeiss Axioplan 2 imaging microscope (Carl-Zeiss, Oberkochen, Germany) connected to a Leica DC500 color digital camera.

Mediterranean sediment samples from the Catalan Coast were obtained at Castelldefels Beach (41°15'37.0" N; 1°55'48.8" E) during spring and summer months from 2015 to 2017. Sediment samples from Sardinian beaches were obtained at Platamona Beach (40°49'27.1" N; 8°31'36.4" E) and La Speranza Beach (40°29'43.1" N; 8°22'12.1" E) during summer months in 2015 and 2018. Surface samples were taken by snorkeling at a depth of approximately 1.5–2 m with plastic bottles. The distance to the shore depended on the underwater slope of each beach. The sediments were kept at room temperature, in the dark, and immediately taken to the laboratory. Once there, cells were extracted from the sediment using the melting seawater-ice method (Uhlig 1964). Subsamples were fixed with Lugol's iodine or formaldehyde (2%) and preserved in the dark at 4°C. Live and fixed samples from the Catalan coast were observed under a phase-contrast Leica DM-IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) connected to a ProgRes C10 (Jenoptik Laser, Optik Systeme GmbH, Jena, Germany) digital camera. Cell measurements were conducted using the ProgRes CapturePro software (Jenoptik Laser; Optik Systeme GmbH). Live samples from the Sardinian coast were observed under a Zeiss 100 inverted microscope (Carl Zeiss, Oberkochen, Germany), equipped with DIC. Digital photos were taken using a Zeiss Axiocam (Carl Zeiss). Cell measurements were obtained from LM and SEM images using the ImageJ software (1.47v; W. Rasband, USA).

For scanning electron microscopy observations (1) the Canadian mixed-extraction samples were fixed overnight with acidic Lugol's solution. Cells were transferred onto a 5 µm polycarbonate membrane filter (Corning Separations Div., Acton, MA, USA), washed with distilled water, dehydrated with a graded series of ethanol, rinsed twice in hexamethyldisilazane (HMDS), and oven-dried at 65°C. Filters were mounted on stubs, sputter-coated with gold, and viewed under a Hitachi S4700 scanning electron microscope; (2) the fixed Mediterranean subsamples were filtered into a 3.0–

5.0 µm polycarbonate filter, and washed in seawater and distilled water for 15 min. A subsequent dehydration was carried out in a 25, 50, 75, 90, 96, and 100% ethanol series for ca. 10 min. The final step of 100% ethanol was repeated twice. The filters were critical-point dried or rinsed twice in HMDS and dried for 5 min at 60°C. The dried filters were then mounted on stubs, sputter coated with gold-palladium. Catalan samples were examined with a HITACHI S-3500N scanning electron microscope (Hitachi High Technologies Corp., Tokyo, Japan) at the Servei de Microscopia Electrònica (ICM-CSIC) in Spain and a Tescan VEGA3 microscope (Elektronen-Optik-Service GmbH, Dortmund, Germany) in Germany. Sardinian samples were examined with a Tescan VEGA3 microscope (Elektronen-Optik-Service GmbH) in Germany.

Polymerase chain reactions and phylogenetic analyses. Canadian specimens isolated from a raw sample were washed with filtered (eukaryote-free) seawater and 100 cells deposited in 35 µL distilled water in a 1.5 mL Eppendorf tube (Dia-Med Lab Supplies Inc., Mississauga, ON, Canada) heated at 65°C and afterwards stored in a freezer. DNA amplification was carried out using 10 µL of this cell preparation and puRe-Taq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA). The protocol using universal eukaryotic primers (PF1-R4) was described in Hoppenrath and Leander (2007, 2010). PCR products corresponding to the expected size were gel isolated and cloned into the pCR2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). One clone from *Pachena leibnizii* sp. nov. was sequenced with ABI big-dye reaction mix (Applied Biosystems, Foster City, CA, USA) using the vector primers and internal primers oriented in both directions (See Table 1 for GenBank accession numbers).

Around 40 specimens from the Catalan Coast were isolated from a raw sample from May 2017, washed with filtered and autoclaved seawater, and transferred to a 1.5 mL Eppendorf tube containing 200 µL of seawater. Genomic DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer instructions. Two microliters of this extract was subjected to a first 25 µL PCR using EK-82F – 28S-1611R primers with an amplification mixture containing 2 µL of 10X buffer (TaKaRa Bio), 1.5 mM MgCl₂, 1 U of TaKaRa Taq DNA polymerase (TaKaRa Bio), 0.2 mM of each dNTP, and 0.4 mM of each primer. PCR amplification conditions were as follows: initial denaturation for 3 min at 95°C, followed by 6 cycles of 15 s at 95°C, 30 s at 58–53°C, decreasing 1°C each cycle, and 2 min at 72°C, and 34 additional cycles at annealing temperature of 52°C, followed by a final extension step for 5 min at 72°C. The resulting product was used as template for semi-nested PCRs to amplify the SSU and LSU rDNA regions independently, using primers EK-82F and EK-1520R, and 28S-1F – 28S-1611R, respectively. Each amplification reaction contained 1 µL of template, 2.5 µL of 10X buffer (Invitrogen, Thermo Fisher Scientific Corp.) containing 15 mM MgCl₂, 1.25 U of Platinum Taq DNA polymerase (Invitrogen, Thermo Fisher

Scientific Corp.), 0.2 mM of each dNTP, and 0.4 mM of each primer. PCR conditions were as follows: initial denaturation for 2 min at 94°C, 35 cycles of 15 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension step for 5 min at 72°C. A second semi-nested, using 1 µL of the previous PCR product as template was performed for each region using primers DIN-464F and EK-1520R, and 28S-1F and 28S-803R, respectively. Four µL of PCR products were electrophoresed in an agarose gel and visualized under UV illumination. Purification and Sanger sequencing was carried out by external services (Genewiz, Takeley, UK) using both forward and reverse primers. See Table 1 for GenBank accession numbers.

Several specimens from Platamona Beach, Italy were isolated from samples collected in June 2015 and 2018 and one specimen from La Speranza Beach, Italy, was isolated from a sample collected in June 2018. Isolated cells were washed in several drops of filtered seawater and transferred to a 0.2 mL PCR tubes containing 5 µL of lysis buffer (400 ng · µL⁻¹ Proteinase K and 0.005% SDS). Tubes were subjected to freezing at -20°C for 10 min, heating at 60°C for 30 min, and then at 95°C for 10 min to facilitate cell lysis. Resulting lysates were directly used as a template for amplification of SSU and LSU rDNA fragments.

The lysate from two specimens of Platamona (sample June 2015) was amplified for the LSU rDNA using D1R and D2C primers. The 50 µL PCR mixture contained 5 µL of 10X buffer, 1.5 µL of MgCl₂, 0.25 µL of Hot start Taq DNA polymerase and 1 µL of dNTP 0.2 mM each (Qiagen), and 0.4 mM of each primer. PCR amplification conditions were as follows: initial denaturation for 5 min at 95°C, 40 cycles of 20 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension step for 10 min at 72°C.

The lysate of one specimen from Platamona and one from La Speranza (samples June 2018) were used as template for amplification of SSU rDNA fragment using EukA and EukB primers. Each 50 µL PCR amplification reaction contained 5 µL of 10X buffer, 1.5 µL of MgCl₂, and 0.25 µL of Hot start Taq DNA polymerase (Biotech rabbit), 1 µL of dNTP 0.2 mM each (Qiagen), and 0.4 mM of each primer. PCR conditions were as follows: initial denaturation for 5 min at 94°C, 34 cycles of 1 m at 94°C, 1:30 m at 55°C, and 2 min at 72°C, followed by a final extension step for 7 min at 72°C. The resulting product was used as template for nested PCRs using primers Dino18SF1 and 18S-comR1. Each PCR amplification reaction contained 5 µL of 10X buffer, 1.5 µL of MgCl₂, and 0.25 µL of Hot start Taq DNA polymerase (Biotech rabbit), 1 µL of dNTP 0.2 mM each (Qiagen), 0.4 mM of each primer, and 1 µL of template. PCR conditions were as follows: initial denaturation for 5 min at 94°C, 29 cycles of 45 s at 94°C, 1 m at 55°C, and 3 min at 72°C, followed by a final extension step for 10 min at 72°C. All information regarding the primers used during the amplification protocols can be found in Table S1 in the Supporting Information.

TABLE 1. List of rDNA sequences obtained in this study, including their location and date of isolation, length in base pairs (bp), and GenBank accession number.

Species	Origin	Year of isolation	rDNA region	Length (bp)	Acc. no.	Isolate
<i>Pachena leibnizii</i>	Pachena Beach (Canada)	2007	SSU	1804	MN707939	Vancouver Island
<i>Pachena abriiliae</i>	Castelldefels (Catalonia)	2017	SSU	1140	MN707940	Castelldefels
<i>Pachena abriiliae</i>	Castelldefels (Catalonia)	2017	LSU	689	MN703810	Castelldefels
<i>Pachena abriiliae</i>	La Speranza (Sardinia)	2018	SSU	1571	MN707941	Speranza6
<i>Pachena merididae</i>	Platamona (Sardinia)	2018	SSU	1552	MN707942	Platamona19
<i>Pachena</i> sp.	Platamona (Sardinia)	2015	LSU	656	MN703811	Platamona28

Four μL of all PCR products were electrophoresed in an agarose gel and visualized under UV illumination. Purification and Sanger sequencing was carried out by external services (Macrogen Europe, Amsterdam, Netherlands in 2015 and Genoscreen, Lille, France in 2018) using both forward and reverse primers. See Table 1 for GenBank accession numbers.

The SSU and LSU rDNA sequences were aligned using MUSCLE (Edgar 2004) and viewed in Mesquite v3.11 (Maddison and Maddison 2015). Highly variable regions were eliminated using Gblocks (Castresana 2000, Talavera and Castresana 2007). The final alignments of the SSU and LSU rDNA dataset consisted of 48 with 1768 and 49 taxa with 1167 sites, respectively. The best-fit model for each dataset was chosen by ModelFinder in IQ-TREE under AICc (Trifinopoulos et al. 2016, Kalyaanamoorthy et al. 2017). Maximum-likelihood (ML) analyses were run with IQ-TREE using TIM2 + F + R4 and GTR+F+R5, as the model of evolution for the SSU and LSU rDNA, respectively. Bootstrap analyses were run for each dataset with 1000 replicates to evaluate statistical reliability. MrBayes v3.2.5 was used to perform Bayesian analyses (Ronquist and Huelsenbeck 2003) with the GTR + I + G and four Monte-Carlo–Markov chains starting from a random tree. For the SSU dataset a total of 2,500,000 and the first 6,250 trees were discarded; for the LSU rDNA dataset 1,000,000 generations were calculated and the first 2,500 trees in each run were discarded. Trees were sampled every 100 generations in both analyses. Posterior probabilities (PP) correspond to the frequency at which a given node was found in the post-burn-in trees.

RESULTS

Pachena gen. nov. Hoppenrath, Satta & Reñé

Description: Thecate, heterotrophic dinoflagellate without stigma. Thecal tabulation: APC 4' 3a 6'' 5c 5s 5''' 2'''. Dorsoventrally flattened cells; with apical hook covering the apical pore, pointing to the left dorsal cell side; ascending cingulum; sulcus

reaching the antapex. First anterior intercalary plate “sandwiched” between two plates.

Etymology: The genus is named after the sampling area of its first discovery, “Pachena Beach” near Bamfield, Vancouver Island, British Columbia, Canada.

Registration: <http://phycobank.org/102113>

Type: *Pachena leibnizii* sp. nov. Hoppenrath

Pachena leibnizii sp. nov. Hoppenrath (Figs. 1, A–E, 2 and 3)

Description: Cells slightly dorsoventrally flattened, roughly oval to pentagonal in shape, 13–23 μm long, and 12–20 μm wide. Cells without chloroplasts. Epitheca and hypotheca are nearly of equal size, cingulum is ascending about one cingulum height, and sulcus is reaching the antapex. An apical hook covers the apical pore and points to the left dorsal cell side. It possesses a characteristic small, first anterior intercalary plate with only two plate borders that is sandwiched between the second anterior intercalary plate and the second precingular plate. Thecal plates are smooth or faintly ornamented except for the cingular and some sulcal plates that are always smooth.

Holotype: The SEM stub containing the type (specimen shown in Fig. 2A) is deposited at the dinoflagellate type collection in the Centre of Excellence for Dinophyte Taxonomy (CEDiT, Wilhelmshaven, Germany), which is part of the Herbarium Senckenbergianum Frankfurt/M. (FR) with the designation CEDiT2019H105.

Molecular characterization: nuclear ribosomal SSU (MN707939)

Etymology: The species is named in honor of the German Federal Ministry of Education and

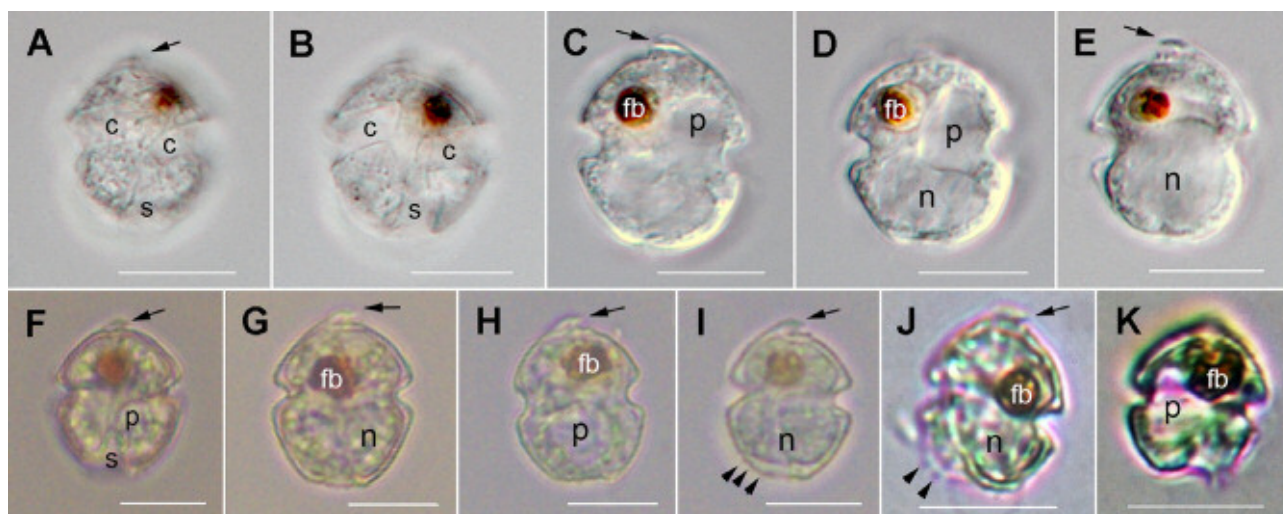


FIG. 1. Light micrographs of the investigated *Pachena* gen. nov. species. (A–E) *Pachena leibnizii* sp. nov. from Canada. (F–I) *Pachena abriilae* sp. nov. from Catalonia, Spain. J, K) *Pachena meriddae* sp. nov. from Sardinia, Italy. The ascending cingulum (c) and the sulcus (s) reaching the posterior cell end can be recognized (A, B, F). All cells contain a colored food body (fb). The nucleus (n) is located in the hyposome and sometimes the pusule (p) is visible. Note the apical hook (arrow) and posterior spines (arrowheads) in the Mediterranean species. Scale bars = 10 μm . [Color figure can be viewed at wileyonlinelibrary.com]

Research: as funding body for institutes of the Leibniz Association the ministry enabled the renovation and relocation of the scientific collections and research infrastructure into the new Senckenberg buildings in Frankfurt/Main. This new research infrastructure allows safeguard Senckenberg's invaluable scientific collections, promotes Senckenberg's international visibility and scientific excellence, and opens new avenues for international collaboration and transfer of knowledge into the society.

Registration: <http://phycobank.org/102114>

Type locality: Pachena Beach, Vancouver Island, British Columbia, NE Pacific (48°47'34.6" N, 125°07'19.0" W)

Habitat: marine, benthic, sandy sediment

Cells slightly dorsoventrally flattened, roughly oval to pentagonal in shape and small: 13.1–22.5 µm long and 11.5–20.0 µm wide (n = 20; Fig. 1, A–E). Cells are not pigmented but often contain a colored (green, orange to red) food body of varying size in the episome (Fig. 1, C and E). The relatively large nucleus is located in the hyposome (Fig. 1, D and E). A pusule can be visible in the cingular area and partly in the episome (Fig. 1, C and D). Epitheca and hypotheca are nearly of equal size (Figs. 1, C–E and 2, A, C), the cingulum is ascending about one cingulum height, and the sulcus is reaching the antapex and slightly extending into the epitheca (Figs. 1, A and B; 2, A and B and 3, A–D). The thecal tabulation is APC 4' 3a 6'' 5c 5s 5''' 2'''' (Figs. 2 and 3). The thecal plates can be ornamented with faint structures (Figs. 2, D–F and 3, B, D and F) or are smooth (Figs. 2, A and C and 3, A and E) with conspicuous pores of two size classes, partly in irregular patterns (groups; Fig. 3, E–H) and marginal rows of pores at the cingulum borders of the pre- and postcingular plates (Fig. 2); except for the cingular and some sulcal plates that are always smooth (Figs. 2 and 3). All plates have scattered thecal pores that are surrounded by an obvious rim (like a ring; Figs. 2 and 3). An apical hook covers the apical pore complex and points to the left dorsal cell side (Figs. 2, A, C and D and 3, G and H). The apical pore is not directly visible but seems to be surrounded by a rim (Fig. 3, G and H). It is unclear whether the apical pore is rounded (Fig. 3G) or slit like (Fig. 3H). The four apical plates are of very different size, with plates 2' and 3' being small and the hook being part of plate 4' (Figs. 2, C and D and 3, G and H). The three anterior intercalary plates are in contact to each other (Fig. 2, C and D). The characteristic small, first anterior intercalary plate (1a) has only two plate borders and is sandwiched between the second anterior intercalary plate (2a) and the second precingular plate (2''; Figs. 2D and 3G). From the six precingular plates, plate 2'' is large and 3'' is noticeably narrow and relatively small located centrally on the dorsal side (Fig. 2, A–D). The hypothecal plates have an

unusual asymmetric arrangement. The first (1''') and fifth (5''') postcingular plates are on the ventral side (Fig. 2, A and B) and the small, second (2''') and third (3''') postcingular plates are rectangular (Fig. 2, C–F). The fourth postcingular plate (4''') is relatively large covering most of the right dorsal cell side (Fig. 2, C and F). The two antapical plates are of different size, with the first (1''') being very large (Fig. 2, C–F). The sulcus widens toward the posterior cell end and five sulcal plates were recognized. The anterior sulcal plate (Sa) is narrow, elongated, and extends into the epitheca, and has a short left posterior list (Figs. 2, A and B; 3, A–D). The narrow and elongated right sulcal plate (Sd) strikingly widens posteriorly through a wide, smooth, wing-like left list (Figs. 2, A and B; 3, B–F) that covers the sulcal center. The left (Ss) and middle (Sm) sulcal plates are mostly hidden and cannot be described in detail (Fig. 3, D–F). The posterior sulcal plate (Sp) is wide, anteriorly elongated on its left side and has no special structures (Figs. 2, A and B and 3, E and F).

Pachena abriiiae sp. nov. Reñé, Satta & Hoppenrath (Figs. 1, F–I; 4 and 5)

Description: Cells slightly dorsoventrally flattened, roughly oval to pentagonal in shape, 16–21 µm long and 13–16 µm wide. Cells without chloroplasts. Epitheca and hypotheca are nearly of equal size, cingulum is ascending about one cingulum height, and sulcus is reaching the antapex. An apical hook covers the apical pore and points to the left dorsal cell side. It possesses a characteristic small, first anterior intercalary plate with only two plate borders that is sandwiched between the second anterior intercalary plate and the third precingular plate. Thecal plates are ornamented with small spines except for the cingular and some sulcal plates that are smooth.

Holotype: The SEM stub containing the type (specimen shown on Fig. 4B) is deposited at the dinoflagellate type collection in the Centre of Excellence for Dinophyte Taxonomy (CEDiT, Wilhelmshaven, Germany), which is part of the Herbarium Senckenbergianum Frankfurt/M. (FR) with the designation CEDiT2019H106.

Molecular characterization: nuclear ribosomal SSU (MN707940) and LSU (MN703810)

Etymology: The species is named after the daughter of the second author of this study, Abril Reñé.

Registration: <http://phycobank.org/102115>

Type locality: Castelldefels beach, Catalonia, NW Mediterranean Sea (41°15'37" N; 1°55'48.8" E)

Habitat: marine, benthic, sandy sediment

Cells slightly dorsoventrally flattened, roughly oval to pentagonal in shape, and small: 16.4–21.3 µm long and 12.9–16.4 µm wide (n = 37; Fig. 1, F–I). Cells are not pigmented but often contain a colored (orange to red) food body of varying size in the episome (Fig. 1, F–I). The nucleus is located in the hyposome (Fig. 1, G and I). A pusule can be visible

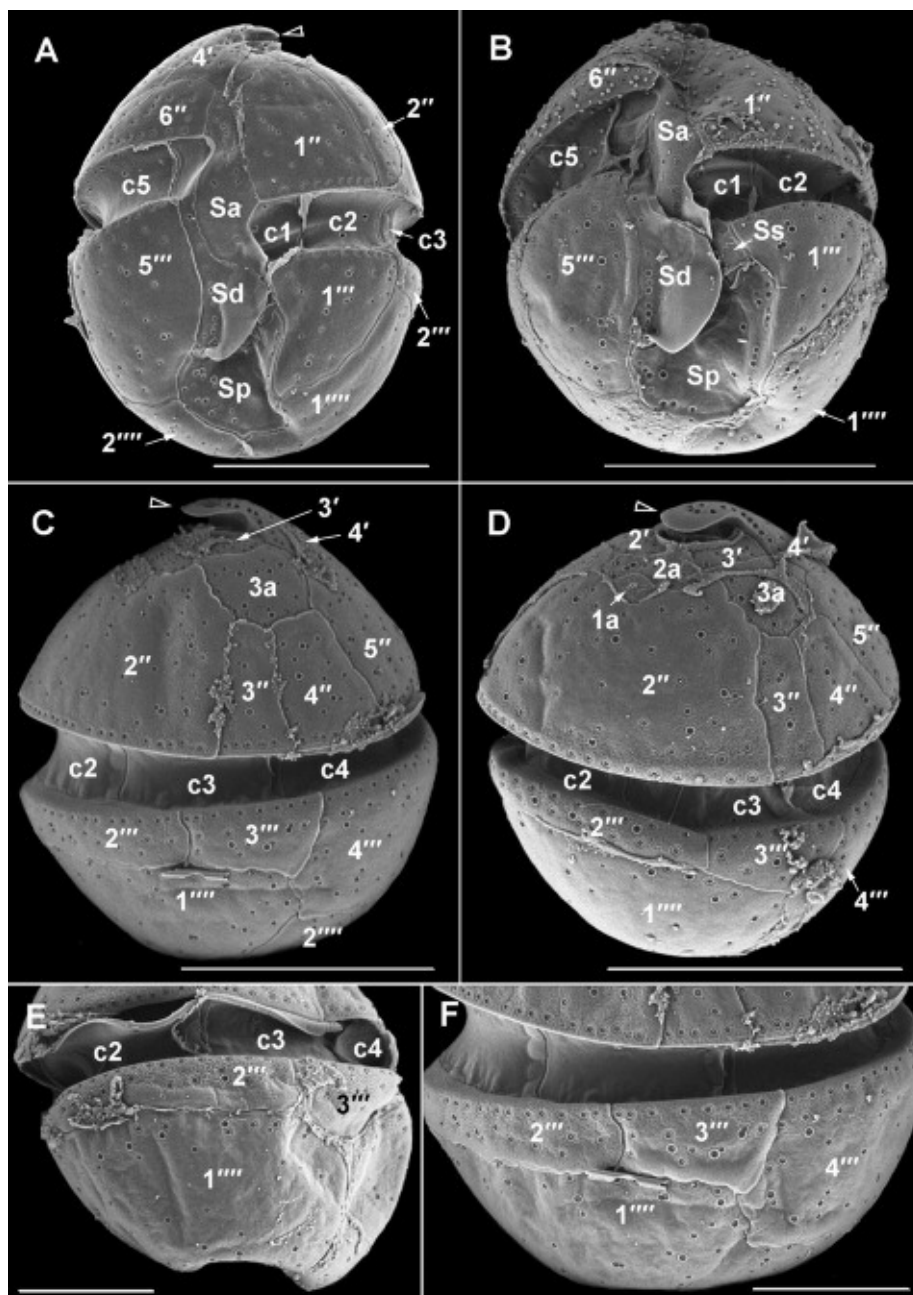


FIG. 2. Scanning electron micrographs showing the tabulation of the type species *Pachena leibnizii* sp. nov. from British Columbia, Canada. (A, B) Ventral views. (C, D) Dorsal views. (E, F) Dorsal hypotheca and cingulum. The apical hook is marked by an arrowhead. 1'-4', apical plates; 1a-3a, anterior intercalary plates; 1''-6'', precingular plates; 1'''-5''', postcingular plates; 1''''-2'''', antapical plates; c1-c5, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sp, posterior sulcal plate; Scale bars = 10 μ m (A-D), 5 μ m (E, F).

in the hyposome (Fig. 1, F and H). Epithea and hypotheca are nearly of equal size (Figs. 1, G-I and 4, A-F), the cingulum is ascending about one cingulum height, and the sulcus is reaching the antapex (Figs. 1F and 4, A and B). The thecal tabulation is APC 4' 3a 6'' 5c 5s 5''' 2'''' (Figs. 4 and 5). The thecal plates are ornamented with small spines except for the cingular and some sulcal plates that are smooth (Figs. 4, 5). In some cells cingular plates

can have a faint bar-like ornamentation (Fig. 5, F and H). All plates have scattered thecal pores that are surrounded by a narrow rim (like a ring; Figs. 4, A-D and 5, A and H). An apical hook covers the apical pore complex and points to the left dorsal cell side (Fig. 4, A-F). The apical pore is not directly visible but seems to be surrounded by a raised rim (Fig. 5, B, D and E). The apical pore plate possesses a row of thecal pores at its margin

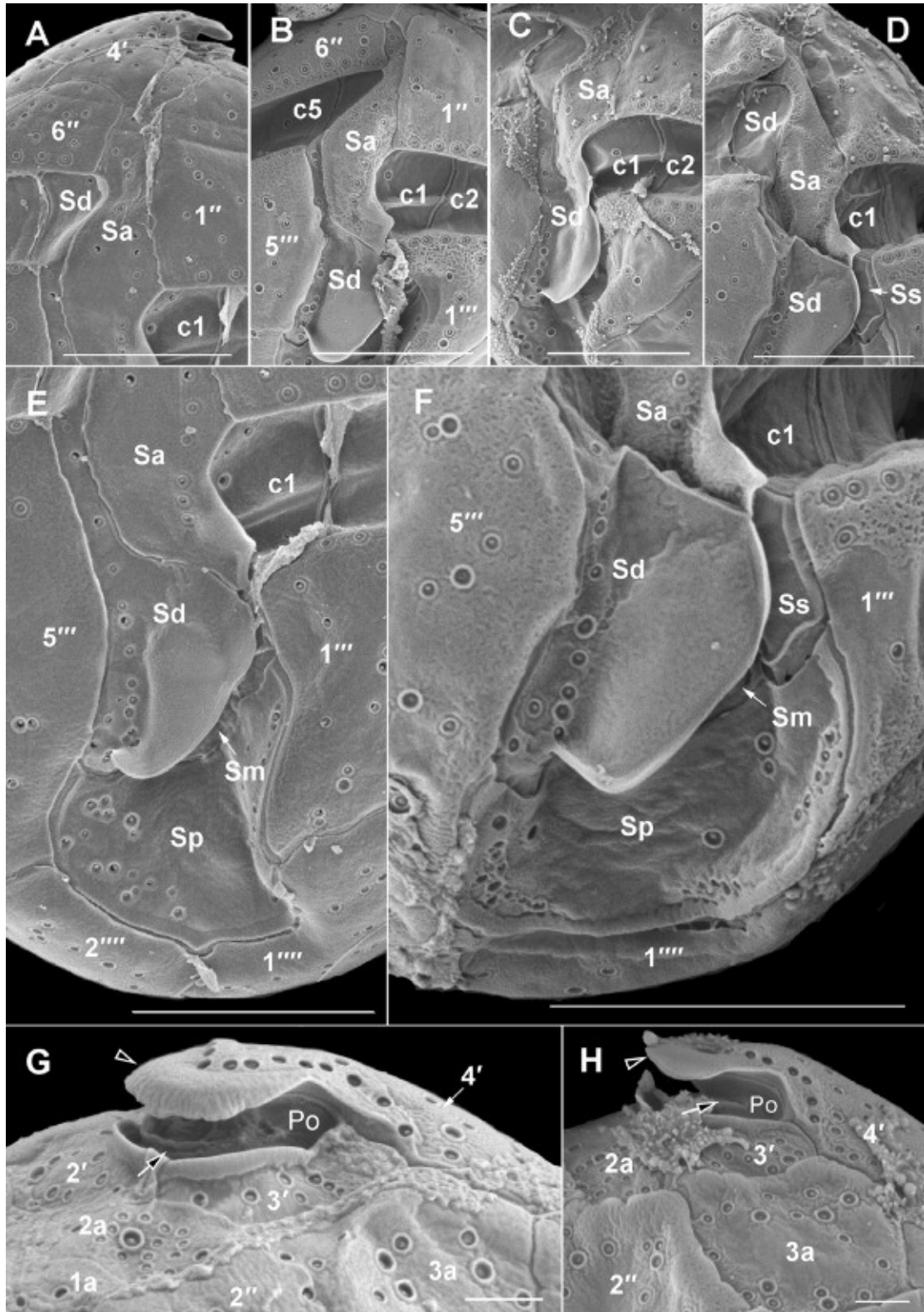


FIG. 3. Scanning electron micrographs showing the sulcal plates and the apex with the apical pore complex below the apical hook (arrowhead) of the type species *Pachena leibnizii* sp. nov. from British Columbia, Canada. Note the thecal ornamentation and the apical pore plate (Po) with the apical pore (arrow). 1'-4', apical plates; 1''-6'', precingular plates; 1'''-5''', postcingular plates; 1''''-2''', antapical plates; c1-c5, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sm, median sulcal plate; Sp, posterior sulcal plate; 1a-3a, anterior intercalary plates; Scale bars = 5 μ m in A-F, 1 μ m in G, H.

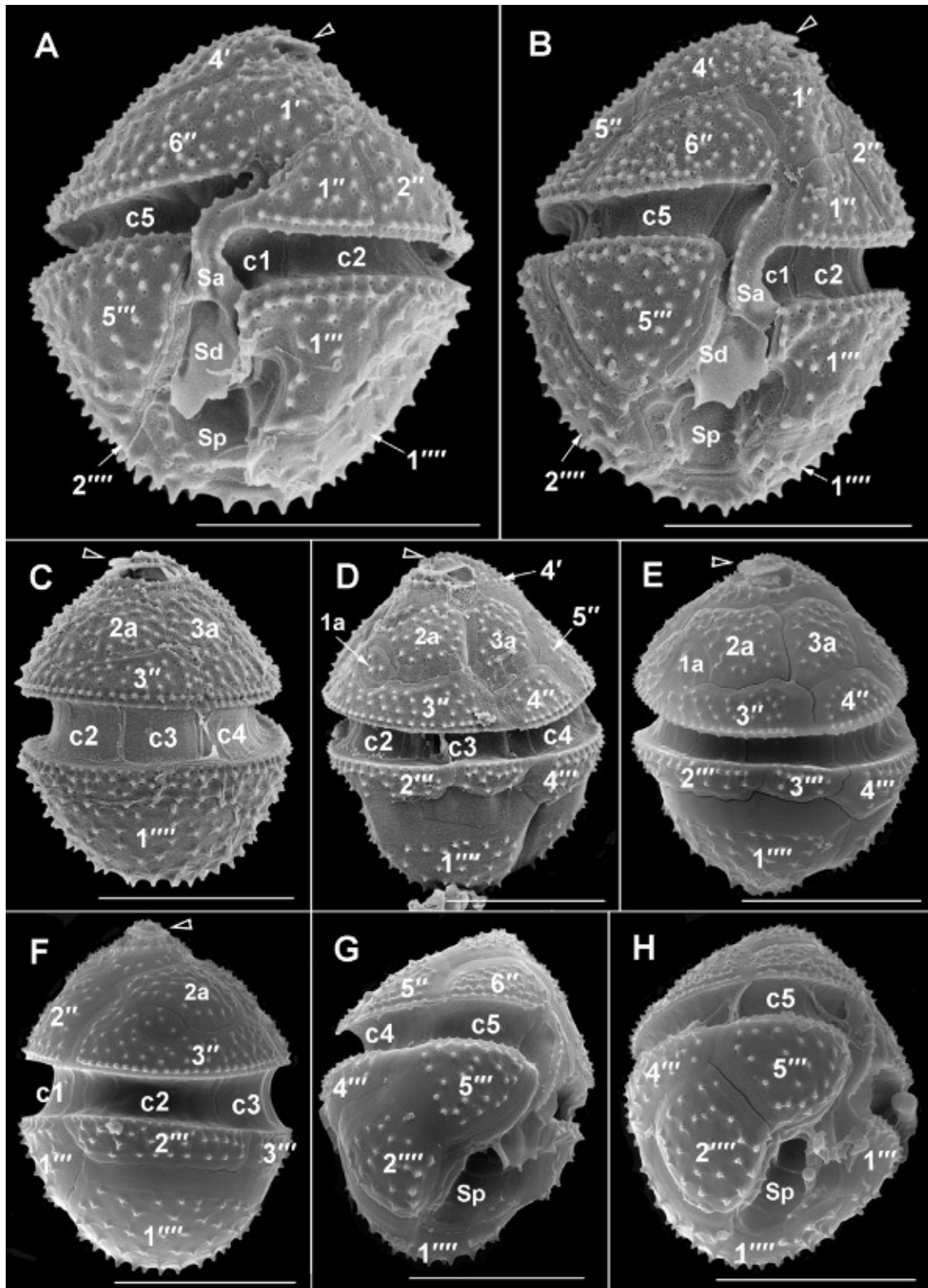


FIG. 4. Scanning electron micrographs showing the tabulation of the new species *Pachena abriiliae* sp. nov. from Catalonia, Spain. (A, B) Ventral views. (C–E) Dorsal views. (F) Left lateral view. (G, H) Right lateral view, mainly of the hypotheca. The apical hook is marked by an arrowhead. 1'–4', apical plates; 1a–3a, anterior intercalary plates; 1''–6'', precingular plates, 1'''–5''', postcingular plates; 1''''–2'''', antapical plates; c1–c5, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Sp, posterior sulcal plate; Scale bars = 10 μ m.

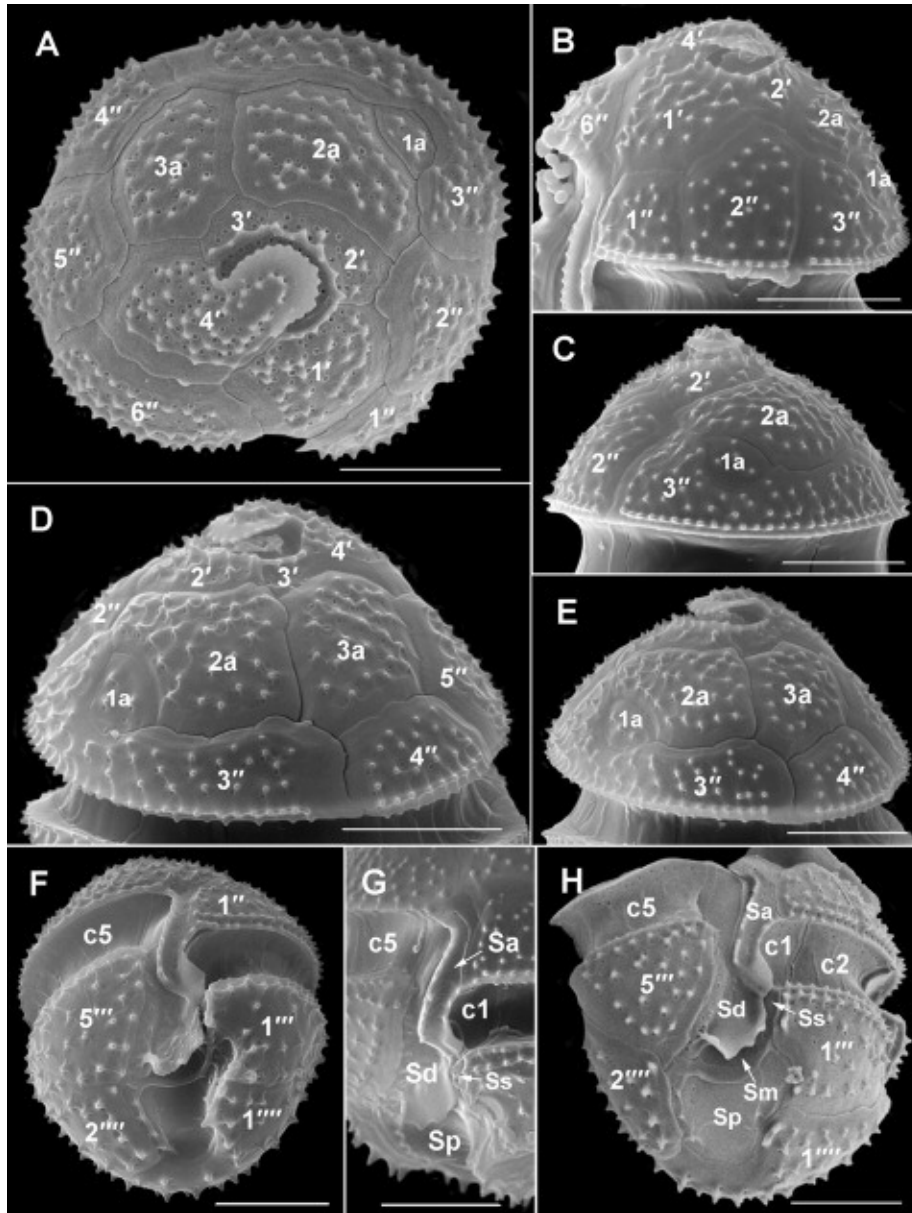


FIG. 5. Scanning electron micrographs showing the tabulation of the new species *Pachena abriiliae* sp. nov. from Catalonia, Spain. (A) Apical view of the epitheca. (B) Ventral to left lateral view of the epitheca. (C) Left lateral view of the epitheca. (D, E) Dorsal view of the epitheca. (F) Antapical to ventral view. (G) Detail of the sulcus. (H) Hypotheca with cingulum in ventral view showing all sulcal plates. 1'-4', apical plates; 1a-3a, anterior intercalary plates; 1''-6'', precingular plates; 1'''-5''', postcingular plates; 1''''-2'''', antapical plates; c1-c5, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sm, median sulcal plate; Sp, posterior sulcal plate; Scale bars = 5 μ m.

(Fig. 5A). The four apical plates are of very different size, with plate 3' being the smallest and the hook being part of plate 4' (Figs. 4, A and B and 5, A-D). The three anterior intercalary plates are in contact to each other and cover a large part of the dorsal epitheca (Figs. 4, C-E; 5, A, D and E). The characteristic small, first anterior intercalary plate (1a) has only two plate borders and is sandwiched between the second anterior intercalary plate (2a) and the third precingular plate (3''); Fig. 5, A, C-E). From the six precingular plates,

plate 3'' is noticeably elongated (Figs. 4, A-F and 5, A-E). The hypothecal plates have an unusual asymmetric arrangement. The first (1''') and fifth (5''') postcingular plates are on the ventral side (Figs. 4, A and B and 5, F and H) and the small, second (2'') and third (3'') postcingular plates are rectangular (Fig. 4, C-F). The two antapical plates are of different size, with the first (1''') being very large and covering most of the dorsal hypotheca (Figs. 4, C-H and 5, F and H). The sulcus widens toward the posterior cell end and five sulcal plates were

recognized (Figs. 4, A, B, G and H and 5, G and H). The anterior sulcal plate (Sa) is narrow and elongated, with a spiny right margin and a short left posterior list (Figs. 4, A and B and 5, G and H). The narrow and elongated right sulcal plate (Sd) strikingly widens posteriorly through a wide, serrated, wing-like left list (Figs. 4, A and B, 5, G and H) that covers the sulcal center. The left (Ss) and middle (Sm) sulcal plates are mostly hidden and cannot be described in detail (Fig. 5, G and H). The posterior sulcal plate (Sp) is visible mainly between the two antapical plates and has no special structures (Figs. 4, A, B, G and H and 5, F–H).

Pachena meriddae sp. nov. Satta, Reñé & Hoppenrath (Figs. 1, J and K, 6 and 7)

Description: Cells slightly dorsoventrally flattened, pentagonal, with triangular epitheca and trapezoid hypotheca, 9–13 μm long and 6–10 μm wide. Cells without chloroplasts. Epitheca and hypotheca are nearly of equal size, cingulum is ascending about one cingulum height, and sulcus is reaching the antapex. An apical hook covers the apical pore and points to the left dorsal cell side. Epithelial plates are ornamented with ridges and some spines, hypothecal plates with spines, cingular and sulcal plates are smooth.

Holotype: The SEM stub containing the type (specimen shown on Fig. 6B) is deposited at the dinoflagellate type collection in the Centre of Excellence for Dinophyte Taxonomy (CEDiT, Wilhelmshaven, Germany), which is part of the Herbarium Senckenbergianum Frankfurt/M. (FR) with the designation CEDiT2019H107.

Molecular characterization: nuclear ribosomal SSU (MN707942)

Etymology: The species is named to honor Mr. Marcello Meridda, for his tireless support in taking field samples.

Registration: <http://phycobank.org/102116>

Type locality: Platamona Beach, Sardinia, Mediterranean Sea (40°49'27.1" N; 8°31'36.4" E)

Habitat: marine, benthic, sandy sediment

Cells slightly dorsoventrally flattened, with triangular epitheca and trapezoid hypotheca, pentagonal in shape and small: 9.4–13.2 μm long and 6.3–10.2 μm wide ($n = 20$; Figs. 1, J and K and 6, A–F). Cells are not pigmented but often contain a colored (orange to red) food body of varying size in the eposome (Fig. 1, J and K). The nucleus is located in the hyposome (Fig. 1J). A pusule can be visible centrally (Fig. 1K). Epitheca and hypotheca are nearly of equal size (Figs. 1, J and K and 6, A–F), the cingulum is ascending about one cingulum height (Fig. 6, A and B). The sulcus characteristically extends the end of the transverse furrow anteriorly and is reaching the antapex (Fig. 7, A–C). The thecal tabulation is APC 4' 3a 6'' 5c 3 + s 5''' 2'''' (Figs. 6 and 7). The epithelial plates are ornamented with ridges and some spines, the hypothecal plates with spines, and cingular and sulcal plates are

smooth (Figs. 6 and 7). All plates have scattered thecal pores that are surrounded by a narrow rim (like a ring; Figs. 6, G and H and 7). An apical hook covers the apical pore complex and points to the left dorsal cell side (Fig. 6). The apical pore is not directly visible. The four apical plates are of very different size, with a narrow plate 1', small plates 2' and 3', and the hook being part of plate 4' (Fig. 7). The three anterior intercalary plates are in contact to each other (Fig. 7, B–D) but their shapes could not reliably be determined. Further observations are necessary to clarify whether plate 1a is triangular (Fig. 7C) or pentagonal (Fig. 7B) and not sandwiched between two plates. Plate 2a is in contact with plates 3', 1a, 2'', 3'', 3''', and 3a (Fig. 7, B–D) but the outline needs verification. It is uncertain whether plate 3a is in contact with plate 3' (Fig. 7D) or not (Fig. 7B). To understand whether the anterior intercalary plates are very variable in this species much more cell observations are needed. From the six precingular plates, plate 2'' is relatively large and 3''' is noticeably smaller and narrower and located centrally on the dorsal side (Figs. 6, E, F and H and 7C). The hypothecal plates have an unusual asymmetric arrangement. The postcingular plates 1''' and 5''' are on the ventral side (Fig. 6, A–C) and the small plates 2'' and 3''' are rectangular (Fig. 6, D and E). The two antapical plates are of different size, with plate 1'''' being very large and covering most of the dorsal hypotheca (Fig. 6, D–F). The sulcus widens toward the posterior cell end and only three sulcal plates were recognized. The anterior sulcal plate (Sa) is narrow and elongated, with a smooth ridge on the right margin and a short left posterior list (Fig. 6, A–C). The narrow and elongated right sulcal plate (Sd) optically extends the end of the transverse furrow anteriorly and it widens posteriorly through a wide, smooth left list that is posteriorly elongated and tapered (Fig. 6, B and C). A left (Ss) and middle (Sm) sulcal plate were not recognized. The posterior sulcal plate (Sp) is visible mainly between the two antapical plates (Fig. 6, A and B).

Phylogenetic relationships. SSU rDNA sequences were obtained for the three species described. The sequences corresponding to *Pachena leibnizii* show a 97.5% and a 98.1% pairwise similarity with those from *P. abriiliae* (Castelldefels) and *P. meriddae*, respectively, and the sequences of these two latter species show a 96.2% pairwise similarity between them. Both sequences corresponding to *P. abriiliae* were 99.8% similar. A phylogenetic tree inferred from SSU rDNA sequences (Fig. 8) showed that all of the *Pachena* sequences form a clade (94% BS / 1 BPP), where *P. leibnizii* and *P. meriddae* (100%/1) are more closely related to each other than to *P. abriiliae* (100%/1). The *Pachena* clade clusters with Thoracosphaeraceae (Peridinales) representatives, even though the clade shows low statistical support (63%/–). The Thoracosphaeraceae cluster includes

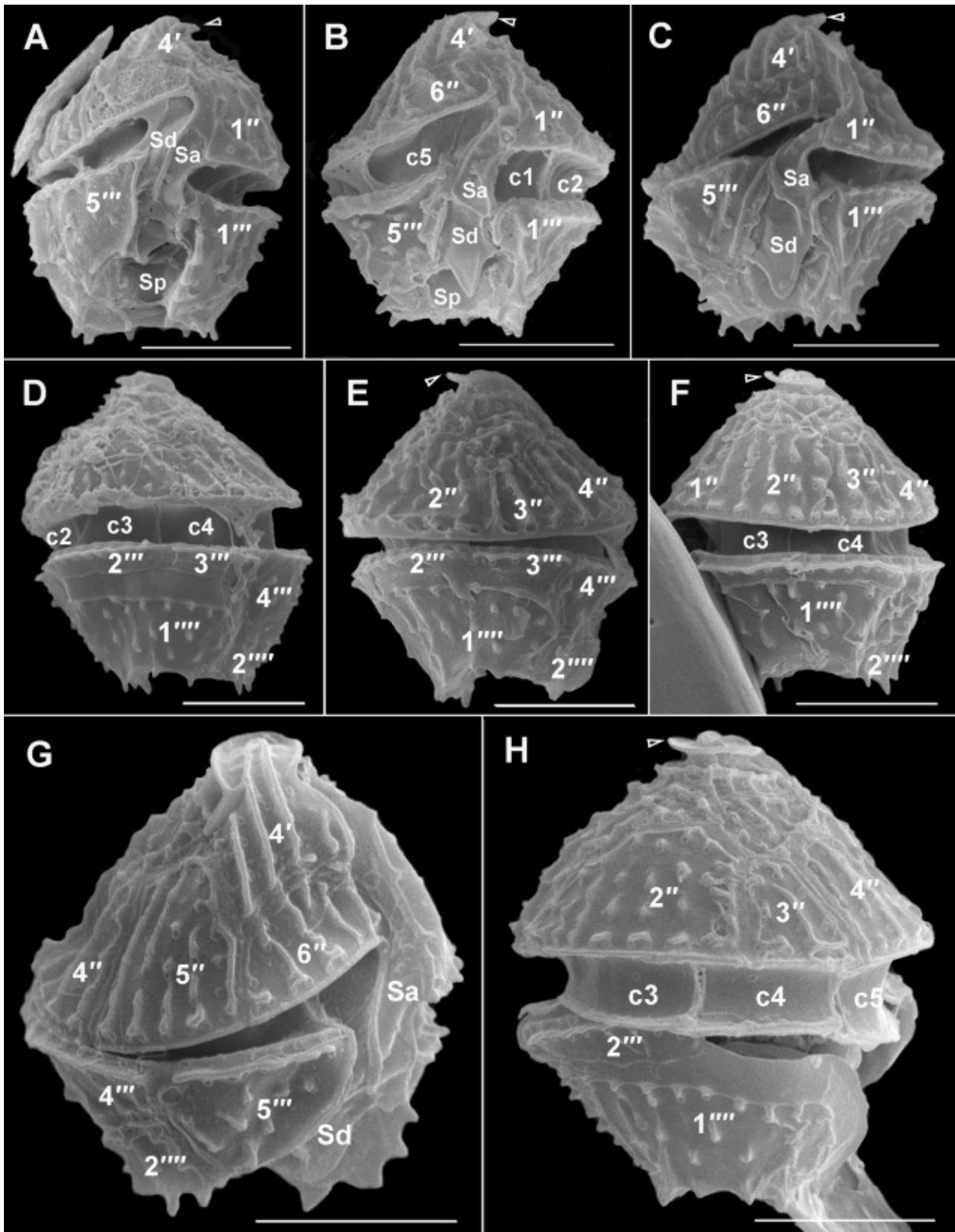


FIG. 6. Scanning electron micrographs showing the tabulation of the new species *Pachena meriddae* sp. nov. from Sardinia, Italy. (A–C) Ventral views. (D, E) Dorsal views. (F) Left lateral view. (G) Right lateral view. (H) Dorsal view. The apical hook is marked by an arrowhead. 1'-4', apical plates; 1a-3a, anterior intercalary plates; 1''-6'', precingular plates; 1'''-5''', postcingular plates; 1''''-2'''', antapical plates; c1-c5, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Sp, posterior sulcal plate; Scale bars = 5 μ m.

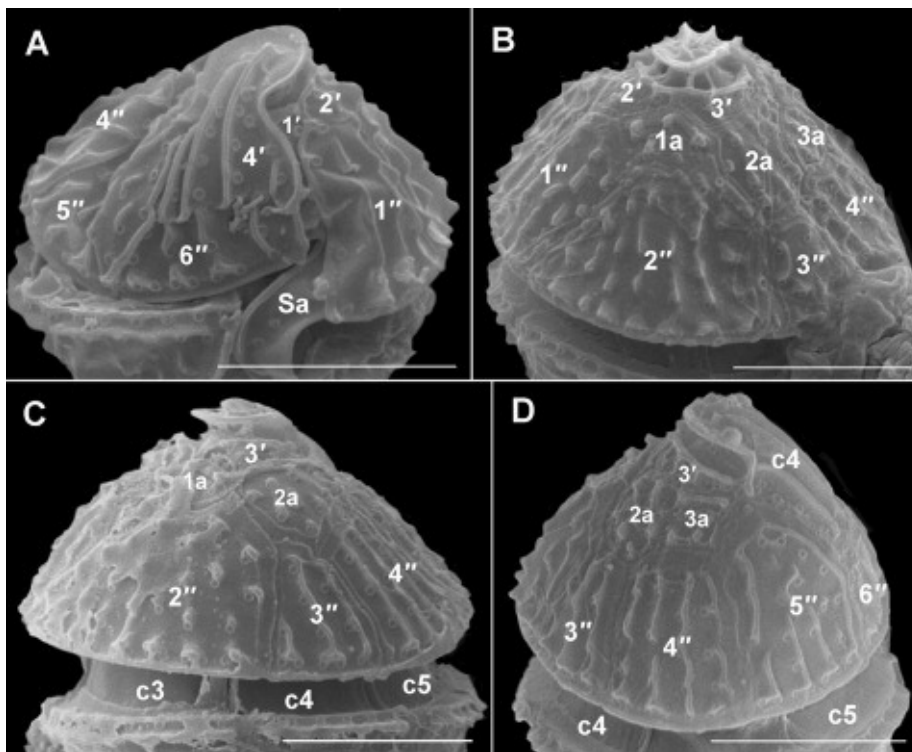


FIG. 7. Scanning electron micrographs showing the epithecal tabulation of the new species *Pachena meriddae* sp. nov. from Sardinia, Italy. (A) Ventral view. (B) Left lateral view. (C) Dorsal view. (D) Dorsal to right lateral view. 1'-4', apical plates; 1a-3a, anterior intercalary plates; 1''-6'', precingular plates; c1-c5, cingular plates; Sa, anterior sulcal plate; Scale bars = 5 μ m.

the *Scripsiella* sensu lato clade (84%/1) (containing *Scripsiella* spp. and *Duboscquodinium collinii* sequences), the sequence of *Apocalathium aciculiferum*, *Tintinnophagus acutus*, and a clade, showing low support, including pfiesteriaceans, namely, *Aduncodinium glandulum*, *Stoeckeria* spp., *Paulsenella vonstoschii*, *Pfiesteria* spp., *Luciella masanensis*, and *Cryptoperidiniopsis* spp. sequences. Other peridinioid taxa, like *Ensiculifera/Pentapharsodinium*, *Heterocapsa* spp., *Podolampacae*, or *Kryptoperidiniaceae* clustered unrelated to *Pachena* sequences.

A phylogenetic tree inferred from LSU rDNA sequences (Fig. 9) shows a clade consisting of *Pachena abriiliae* and *Pachena* sp. (100%/1). Their phylogenetic position is consistent with the SSU rDNA phylogeny in that they cluster with Thoracosphaeraceae representatives, forming a clade with moderate support (78%/1). The *Pachena* species occupy a basal position within this clade. The subsequent basal position is occupied with the sand-dwelling species *Laciniporus arabicus*, followed by a clade (83%/0.99) including *Naiadinium polonicum*, *Calciodinellum operosum*, *Scripsiella* spp., and *Duboscquodinium collinii* sequences, and a second clade (79%/1) including *Thoracosphaera heimii*, *Fusiperidinium wisconsinense*, *Chimonodinium limnickii*, *Apocalathium* spp. sequences, and pfiesteriacean representatives (*Aduncodinium glandulum*, *Pfiesteria piscicida*, and *Stoeckeria algicida*). *Pachena* sequences

cluster distantly related to other peridinioid taxa, like Protoperidiniaceae, as well as to members of Amphidomataceae, Gymnodiniales, Gonyaulacales, or Dinophysales.

DISCUSSION

Morphology. *Pachena* is characterized by its tabulation (APC 4' 3a 6'' 5c 5s 5''' 2'''''), the apical hook (part of the fourth apical plate) covering the apical pore and pointing to the left dorsal cell side, and the ascending cingulum (Fig. 10). The sulcus has an internal list in its center that is part of the anterior and right sulcal plate. The first anterior intercalary plate is uniquely sandwiched between two plates. The three species share these features and differ in the relative sizes and arrangements of their plates, especially on the epitheca (Fig. 10). The first apical plate is small and narrow in *P. leibnizii* and *P. meriddae*. Conversely, it is wide in *P. abriiliae*. Consequently, the first precingular plate is wider in *P. leibnizii* and *P. meriddae*, than in *P. abriiliae*. The second anterior intercalary plate is smaller in *P. leibnizii* than in *P. abriiliae* and occupies a lateral position, rather than dorsal. *Pachena leibnizii* shows a large second precingular plate, like *P. meriddae*, while this plate is narrower in *P. abriiliae*. The arrangement and shape of the remaining precingular plates is unique for each species. In particular, *P. abriiliae*

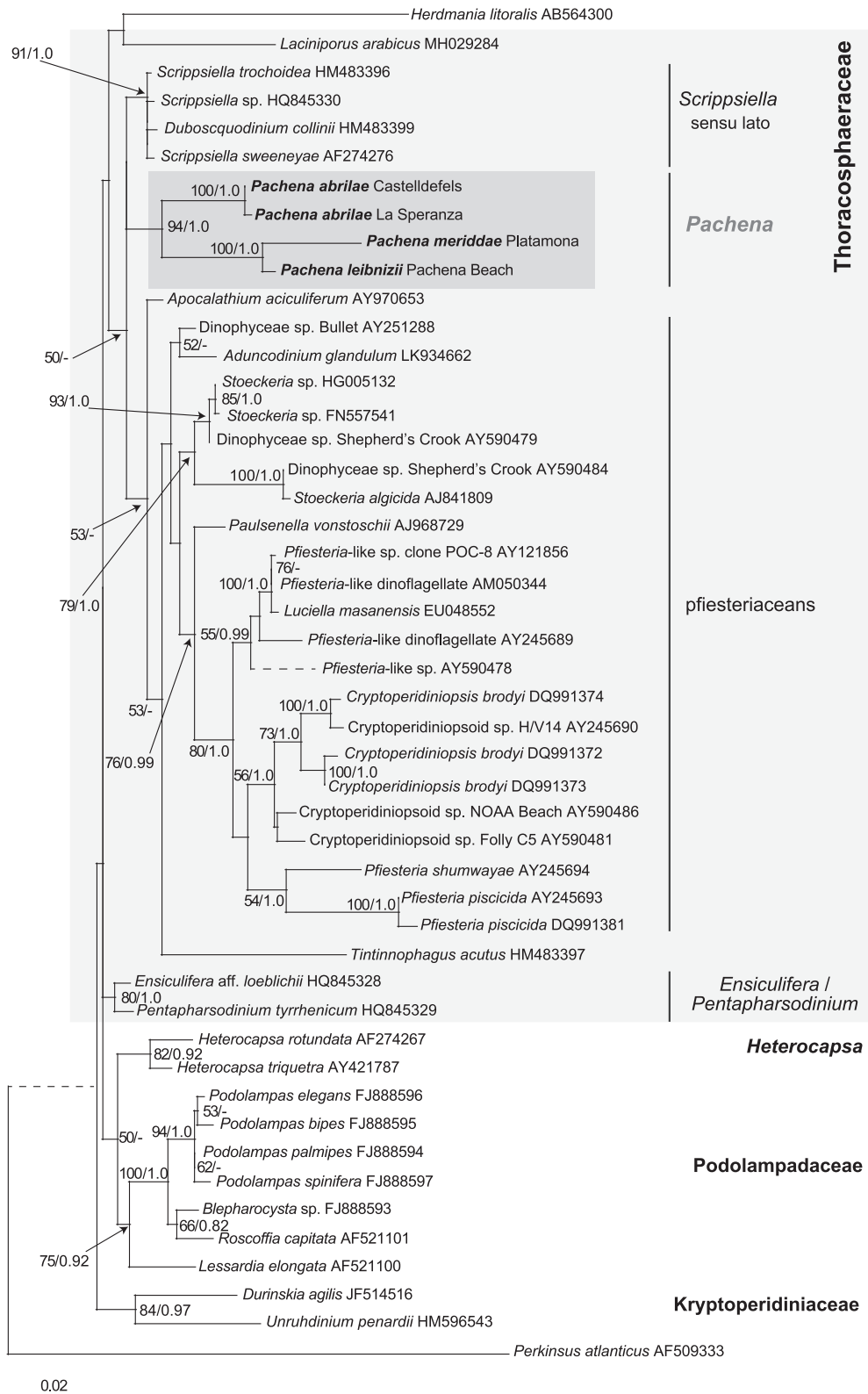


FIG. 8. Maximum likelihood phylogenetic tree inferred from SSU rDNA sequences, including representative Thoracosphaeraceae sequences and a selection of other dinoflagellate taxa. The sequence of the Perkinsea *Perkinsus atlanticus* was used as outgroup. Sequences from this study are in bold and highlighted by the gray box. The other clades are marked with vertical lines on the right. The branches leading to the fast-evolving taxa are indicated by dashed and shortened by one quarter. The scale bar represents inferred evolutionary distance in changes/site. Bootstrap (BS) values and Bayesian posterior probabilities (BPP) are provided at each node (% BS/BPP), only showing those > 50% and > 0.7, respectively.

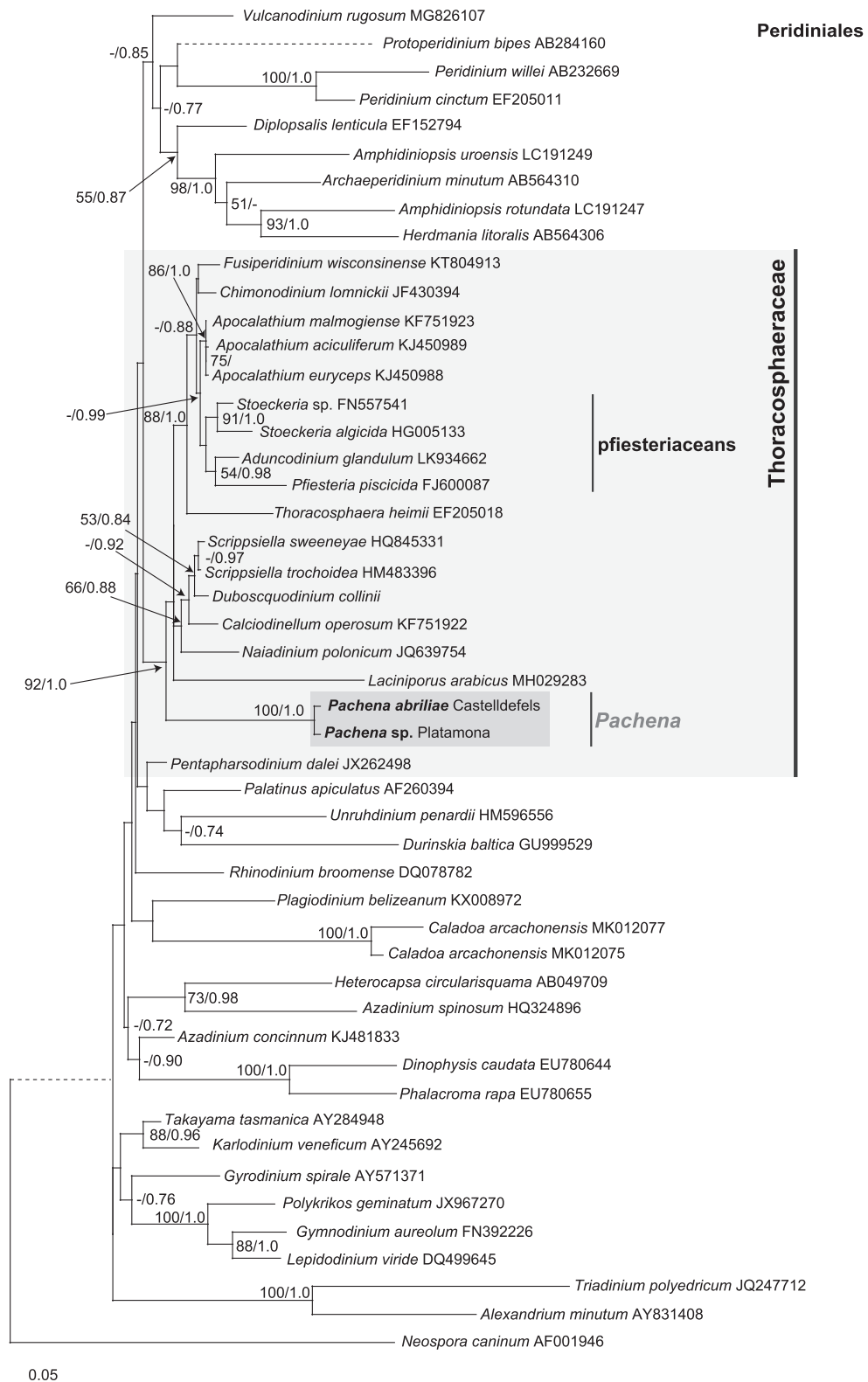


FIG. 9. Maximum likelihood phylogenetic tree inferred from LSU rDNA sequences, including a representation of Thoracosphaeraceae sequences as well as sequences belonging to main dinoflagellate orders and families. The sequence of the Apicomplexa *Neospora caninum* was used as outgroup. Sequences from this study are in bold and highlighted by the gray box. The other clades are marked with vertical lines on the right. The branches leading to the fast-evolving taxa are indicated by dashed and shortened by half. The scale bar represents inferred evolutionary distance in changes/site. Bootstrap (BS) values and Bayesian posterior probabilities (BPP) are provided at each node (% BS/BPP), only showing those >50% and >0.7, respectively.

shows a distinctive elongated third precingular plate. The ornamentation of the thecal plates of the species is different. While *P. leibnizii* is faintly ornamented, *P. abriliae* shows small spines and *P. merididae* ridges and some spines. In addition, there are slight variations among the species in the size ranges and cell shapes. *Pachena leibnizii* and *P. abriliae* are oval to pentagonal, with cell length between 13 and 22 μm , but *P. merididae* is pentagonal, with triangular epitheca, and cells are smaller (<15 μm long).

Despite the relative asymmetric tabulation, especially of the hypotheca, and the absence of a canal plate in the apical pore complex, the genus is molecular phylogenetically related to peridinoid genera (Figs. 8 and 9; discussion below). The presence of anterior intercalary plates and five postcingular plates is typical for genera of the Peridinales (Fensome et al. 1993). Most peridinoid taxa have seven precingular plates, but some have only six (e.g., *Aduncodinium*, *Luciella*, *Pseudopfiesteria*, *Tyrannodinium*; all pfiesteriaceans), which is usually characteristic for gonyaulacoids (Fensome et al. 1993, Hoppenrath 2017).

Covering the apical pore with part of a thecal plate (hook-, finger-, and flap-like projection) is a special feature of some benthic sand-dwelling dinoflagellate taxa that evolved multiple times independently (Hoppenrath et al. 2014). In *Rhynodinium*, it is a prominent morphological character. The hook is part of the second apical plate and is pointing to the dorsal cell side (Murray et al. 2006). In contrast, *Pachena* has a hook that points to the left dorsal (to lateral) side and is part of the fourth apical plate, similar to the hook in *Aduncodinium* (Kang et al. 2015), *Laciniaporus* (Saburova and Chomérat 2019), and *Herdmania* (e.g., Reñé et al. 2020) that points to the left lateral side. The apical pore complex of *Aduncodinium* and *Laciniaporus* comprises a canal plate that is typical for peridinoid taxa (Kang et al. 2015, Saburova and Chomérat 2019), a trait not detected for *Pachena* species. *Aduncodinium* further differs in having only two anterior intercalary plates and a relative symmetric tabulation (Kang et al. 2015). *Laciniaporus* has also only two anterior intercalary plates, like *Aduncodinium*, seven precingular plates, and is photosynthetic (Saburova and Chomérat 2019). *Herdmania* differs from *Pachena* by its more symmetrical tabulation, especially of the hypotheca, seven precingular plates, and the sulcal construction (Hoppenrath 2000d, Yamaguchi et al. 2011, Reñé et al. 2020). Eight *Amphidiniopsis* species have an apical hook, seven belonging to morphogroup 3 and one to morphogroup 1 (Reñé et al. 2020). A hook originating from either the third or the fourth apical plate on the right lateral side and pointing to the left over the apical pore has been described for *A. bulla*, *A. cristata*, *A. uroensis*, *A. elongata*, *A. hoppenrathae*, *A. korewalensis*, and *A. pectinaria* of morphogroup 3

(Hoppenrath 2000b, Murray and Patterson 2002, Toriumi et al. 2002, Selina and Morozova 2017, Reñé et al. 2020). *Amphidiniopsis galericulata* has a small hook as part of the first apical plate pointing dorsally (Hoppenrath 2000b). The apical hook morphology of *Pachena* species is most similar to that of *Laciniaporus arabicus* (Saburova and Chomérat 2019).

The sulcal construction of *Pachena* resembles that of *Amphidiniopsis*. Striking is the central list that is mainly part of the right sulcal plate (Hoppenrath 2000b, Murray and Patterson 2002, Toriumi et al. 2002, Selina and Hoppenrath 2013, Selina and Morozova 2017, Reñé et al. 2020). A second smaller part of this list is formed by the anterior sulcal plate. Species in morphogroup 3 of the *Amphidiniopsis* genus complex have a similar Sa- and Sd-plate arrangement to *Pachena* with the Sd-plate connecting to the end of the cingulum and the Sa-plate touching the last precingular, the first apical and the first precingular plate (Hoppenrath 2000b, Murray and Patterson 2002, Selina and Hoppenrath 2013, Selina and Morozova 2017, Reñé et al. 2020). Also *Herdmania* has this internal sulcal list, but the anterior sulcal plate is in contact with the last cingular plate and the fifth postcingular plate different from *Pachena* (Yamaguchi et al. 2011, Reñé et al. 2020). A complex sulcal list has been described as distinctive feature of *Laciniaporus* (Saburova and Chomérat 2019). It is equivalent to these internal sulcal lists. The arrangement of the involved sulcal plates (Sa and Sd) is like for *Pachena* but the plate shapes are very different, wide, and short in *Laciniaporus* and narrow and elongated in *Pachena*. A similar sulcal plate arrangement has been described for photosynthetic *Scrippsiella* species (e.g., Montresor and Zingone 1988, Janofske 2000, Zinssmeister et al. 2012, Kretschmann et al. 2015, Luo et al. 2016, Lee et al. 2019). In contrast to *Pachena*, the Sd-plate has a less pronounced list, the Sa-plate has no list and the Sp-plate is in contact with the first cingular plate in *Scrippsiella* species. The tide pool species *Scrippsiella hexapraecingula* possesses a peduncle and is likely mixotrophic (Horiguchi and Chihara 1983, Hoppenrath et al. 2014). *Apocalathium* is a putative photosynthetic sister clade to the pfiesteriaceans with sulcal plates like *Scrippsiella* and without microtubules associated with a peduncle (Larsen et al. 1995, Craveiro et al. 2017). Heterotrophic pfiesteriaceans (like *Cryptoperidiniopsis*, *Luciella*, *Pfiesteria*, *Pseudopfiesteria*, *Tyrannodinium*) also have a comparable sulcal construction, but with a plate specific for them, the peduncle cover plate (Litaker et al. 2005, Marshall et al. 2006, Steidinger et al. 2006, Mason et al. 2007, Calado and Craveiro 2009) that could be interpreted as Sd-plate. Its morphology is especially similar to the Sd-plate in *Pachena*. Nothing is known about the feeding behavior of *Pachena* yet. The species had colored food bodies in the episome like pfiesteriaceans. Comparing the Sd-plate

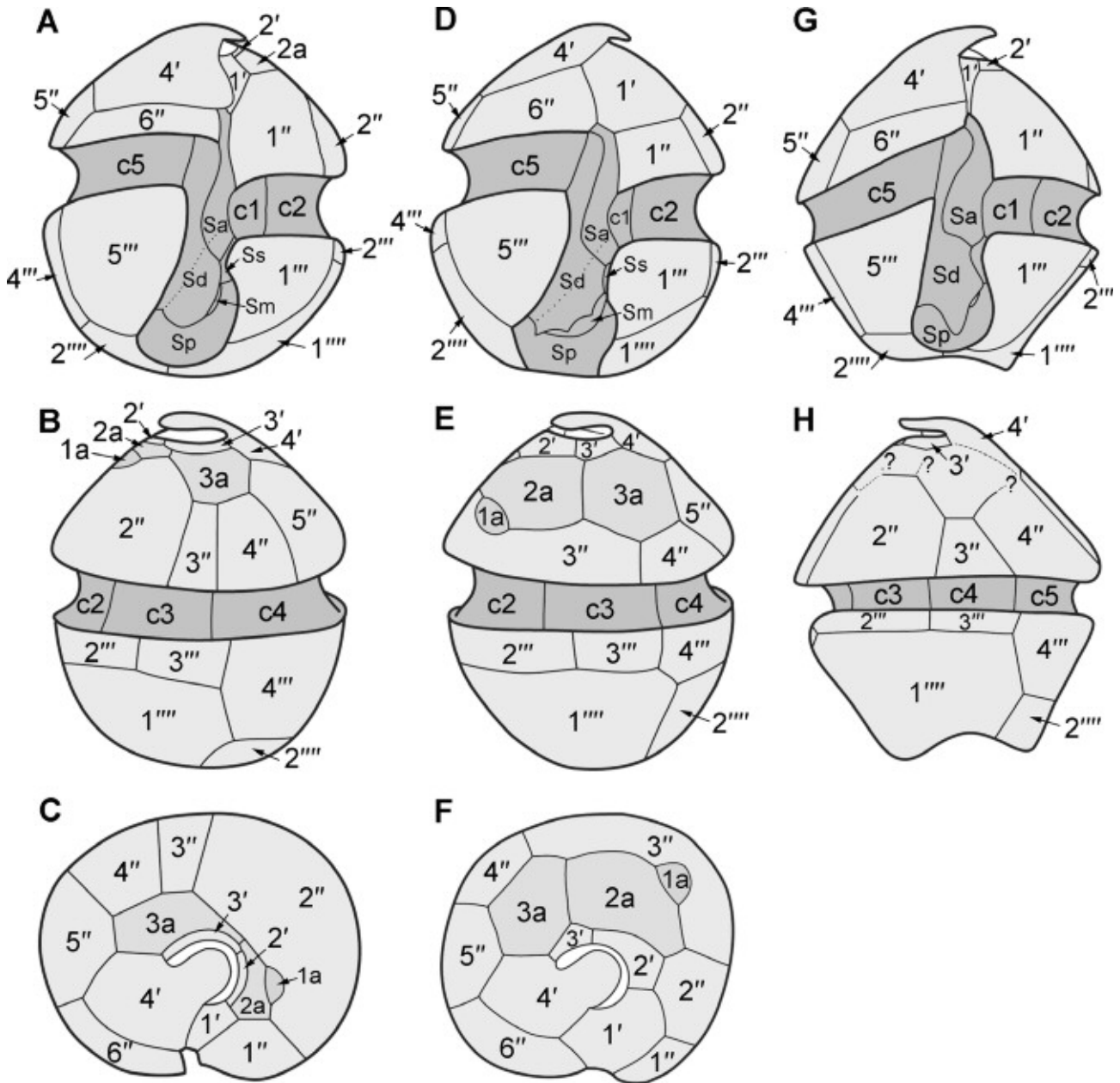


FIG. 10. Comparative tabulation drawings of the three *Pachena* gen. nov. species. (A–C) *Pachena leibnizii* sp. nov. (D–F) *Pachena abriiliae* sp. nov. (G, H) *Pachena meriddae* sp. nov. (A, D, G) Ventral cell side. (B, E, H) Dorsal cell side. (C, F) Epitheca. 1'–4', apical plates; 1a–3a, anterior intercalary plates; 1''–6'', precingular plates; 1'''–5''', postcingular plates; 1''''–2'''', antapical plates; c1–c5, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sm, median sulcal plate; Sp, posterior sulcal plate.

morphology to the peduncle cover plate, it can be hypothesized that *Pachena* is a benthic peduncle feeder, like the related *Aduncodinium* (Kang et al. 2015). A similar food body in the epi- or hyposome is characteristic for *Cabra*, *Rhinodinium*, and *Roscofia*, benthic heterotrophic genera related to the family Podolampadaceae (Hoppenrath et al. 2014). Their feeding mode has not yet been documented. Planktonic Podolampadaceae are pallium feeders (Schütt 1895, Carbonell-Moore 2004).

The ascending cingulum is a character known from *Amphidiniopsis*, *Herdmania*, and some *Protoperidinium* sensu stricto species (subgenus and section *Protoperidinium*), taxa from the order Peridinales (Balech 1974, Faust 2002, Yamaguchi et al. 2011, Reñé et al. 2020).

Three anterior intercalary plates can occur in the Peridinales. The major *Protoperidinium* species (i.e., *Protoperidinium* sensu stricto, including the sections *Protoperidinium*, *Avellana*, *Conica*, *Excentrica*, and

Divergentia) are known to have two or three anterior intercalary plates (Yamaguchi and Horiguchi 2005, Yamaguchi et al. 2006). Among these sections *Avelana* and *Excentrica* have two anterior intercalary plates (Yamaguchi et al. 2006). These sections are not monophyletic in the *Protoperdinium* s.s. clade (Yamaguchi et al. 2006). It is assumed that the reduction of the number of anterior intercalary plates from three to two occurred in different lineages (Yamaguchi et al. 2006). *Archaeperidinium* has only two anterior intercalary plates, but the closely related *Amphidiniopsis* species (except morphogroup 2) and *Herdmania* have three anterior intercalary plates (Yamaguchi et al. 2011, 2016, Reñé et al. 2020). *Scrippsiella* species have three anterior intercalary plates and similar sulcal plates, see above (e.g., Janofske 2000, Zinssmeister et al. 2012, Kretschmann et al. 2015). All *Heterocapsa* species, except for the type, have three anterior intercalary plates and are photosynthetic and characterized by specific body scales (Iwataki 2008, Tillmann et al. 2017). All these genera differ in the special arrangement of the three anterior intercalary plates and the shape of the 1a plate, plus possessing seven precingular plates. Nearly all species of *Azadinium* (family Amphidomataceae) possess three anterior intercalary plates (e.g., Tillmann et al. 2018, and references therein). This genus has four apical and six precingular plates like *Pachena* but *Azadinium* is distinct from it by the APC, the sulcal construction, a descending cingulum with six plates, and six postcingular plates (e.g., Tillmann et al. 2014, 2018).

Phylogenetic considerations. SSU rDNA sequences clearly separated the species in accordance with the morpho-species concept. *Pachena leibnizii* and *P. merididae* formed a sister clade to *P. abriiliae*, in agreement with their morphological similarities, mostly regarding the epithelial plate pattern.

Sequences from *Pachena* occupied a basal position within the Thoracosphaeraceae, even though this clade showed low statistical support. However, and as observed for the phylogenetically closely related sand-dwelling species *Laciniporus arabicus* (Saburova and Chomérat 2019), its phylogenetic position based on SSU rDNA sequences was unstable and varied depending on the taxon sampling used for phylogenetic inference. This situation is not unusual for benthic sand-dwelling species, which commonly represent diverging lineages clustering unrelated to other known (mostly planktonic) representatives, e.g., *Madanidinium adanidinium loirii* (Chomérat and Bilien 2014), *Apicoporus* spp. (Sparmann et al. 2008), or *Plagiodinium belizeanum* (Wakeman et al. 2018).

LSU rDNA sequences could only be obtained for *Pachena abriiliae* and an unidentified *Pachena* specimen. However, their phylogenetic placement at the base of the Thoracosphaeraceae was more robust and in agreement with the position obtained in the phylogenetic analysis of SSU rDNA sequences. The

molecular phylogenetic data suggest that planktonic Thoracosphaeraceae evolved from benthic taxa like *Pachena* and *Laciniporus*.

The Thoracosphaeraceae includes heterotrophic, autotrophic, and parasitic representatives, from marine and freshwater environments, and some showing noncalcareous or calcareous cysts (Elbrächter et al. 2008, Gottschling and Soehner 2013, Saburova and Chomérat 2019). *Pachena* could be the most basal taxon of the Thoracosphaeraceae with a heterotrophic nutrition and possible peduncle feeding, even though we could not verify the peduncle presence or prey source. Furthermore, the inability to establish cultures impeded the observation of cyst-production for *Pachena* representatives, unknown so far for heterotrophic sand-dwelling species. The sand-dwelling *Laciniporus arabicus* also occupies a basal position within the clade, and in this case, it is autotrophic and produces non-calcareous cysts.

Diversity and biogeography. In this study, cells of a *Pachena* species were observed in a benthic sample from the German Bight, but they could not be identified to species (M. Hoppenrath, unpub. data). Also, in the Mediterranean Sea, cells were recorded that likely belong to one or two further undescribed species (M. Hoppenrath, A. Reñé, C.T. Satta, unpub. data). Additional observations and data are needed for their delimitations. Selina (2016) documented a sand-dwelling species from Peter the Great Bay, Sea of Japan, and identified it as cf. *Herdmania*. Judged from the cell shape, the apical hook, cingulum path, and food body location (Selina 2016, p. 465, fig. 4, k and l) it could be a *Pachena* species. Thus, the genus shows a wide distribution, and several species probably coexist at the same location. However, the overall distribution of the different species remains to be determined.

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AUTHOR CONTRIBUTIONS

M.H. conceptualization, sampling, microscopy, molecular work, original draft preparation, writing, and editing; A.R. sampling, microscopy, molecular work, phylogenetic analyses, and writing; C.T.S. sampling, microscopy, and molecular work; A.Y. phylogenetic

- analyses and data discussion; B.S.L. funding acquisition (infrastructure and salary support), review, and editing. All authors read and approved the final manuscript.
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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Primers used during the amplification protocols.