MOLECULAR DIVERSITY AND PHYLOGENY OF THE CALCAREOUS DINOPHYTES (THORACOSPHAERACEAE, PERIDINIALES)

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zur Begutachtung vorgelegt von Sylvia Söhner

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“IF THERE IS LIFE ON MARS, IT MAY BE DISAPPOINTINGLY ORDINARY COMPARED TO SOME BIZARRE EARTHLINGS.”

Geoff McFadden 1999, NATURE

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SATURATORY DECLARATION

ERKLÄRUNG

Diese Dissertation wurde im Sinne von §12 der Promotionsordnung von Herrn PD Dr. Marc Gottschling betreut. Ich, Sylvia Söhner, erkläre hiermit, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

EIDESSTATTLICHE ERKLÄRUNG

Ich, Sylvia Söhner, erkläre hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Beihilfe angefertigt ist.

München, 28.02.2013 …….…….....

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PUBLICATIONS & MANUSCRIPTS

PAPER 1 (PUBLICATION)

Zinssmeister, C., **Soehner, S.**, Kirsch, M., Facher, E., Meier, K.J.S., Keupp, H. & Gottschling, M. (2012) Same but different: Two novel bicarinate species of extant calcareous dinophytes (Thoracosphaeraceae, Peridiniales) from the Mediterranean Sea. J. Phycol. 48 (5): 1107-1118.

Own contributions: Fieldwork (40%); molecular lab work (100%); molecular phylogenetic analysis (incl. images 80%); total manuscript preparation (20%).

PAPER 2 (MANUSCRIPT)

Gu, H., Kirsch, M., Zinssmeister, C., **Soehner, S.**, Meier, K.J.S., Liu, T. & Gottschling, M. Waking the dead: Morphological and molecular characterization of extant *Posoniella tricarinelloides* (Thoracosphaeraceae, Dinophyceae). Protist (in press).

Own contributions: Fieldwork (20%); molecular lab work (50%); molecular phylogenetic analysis (incl. images 10%); total manuscript preparation (10%).

PAPER 3 (PUBLICATION)

Tillmann, U., **Soehner, S.**, Nézan, E. & Krock, B. (2012) First record of the genus *Azadinium* (Dinophyceae) from the Shetland Islands, including the description of *Azadinium polongum* sp. nov. Harmful Algae 20: 142-155.

Own contributions: Molecular lab work (50%); molecular phylogenetic analysis (incl. images 100%); total manuscript preparation (20%).

PAPER 4 (PUBLICATION)

Zinssmeister, C., **Soehner, S.**, Facher, E., Kirsch, M., Meier, K.J.S. & Gottschling, M. (2011) Catch me if you can: The taxonomic identity of *Scrippsiella trochoidea* (F.Stein) A.R.Loebl. (Thoracosphaeraceae, Dinophyceae). Syst. Biodivers. 9: 145-157.

Own contributions: Fieldwork (40%); molecular lab work (100%); molecular phylogenetic analysis (incl. images 60%); total manuscript preparation (20%).

PAPER 5 (PUBLICATION)

Soehner, S., Zinssmeister, C., Kirsch, M. & Gottschling, M. (2012) Who am I – and if so, how many? Species diversity of calcareous dinophytes (Thoracosphaeraceae, Peridiniales) in the Mediterranean Sea. Org. Divers. Evol. 12 (4): 339-348.

Own contributions: Fieldwork (40%); molecular lab work (100%); network analysis (incl. images 100%); total manuscript preparation (70%).

PAPER 6 (PUBLICATION)

Gottschling, M., **Soehner, S.**, Zinssmeister, C., John, U., Plötner, J., Schweikert, M., Aligizaki, K. & Elbrächter, M. (2012) Delimitation of the Thoracosphaeraceae (Dinophyceae), including the calcareous dinoflagellates, based on large amounts of ribosomal RNA sequence data. Protist 163: 15-24.

Own contributions: Molecular lab work (50%); molecular phylogenetic analysis (incl. images 30%); total manuscript preparation (20%).

GENERAL INTRODUCTION

THE STUDY GROUP – THE DINOPHYCEAE

The Dinophyceae ('Dinoflagellata' under the zoological code of nomenclature: ICZN) are an ecologically important and evolutionarily fascinating group of protists. The majority of Dinophyceae, comprising approximately 2000 described extant species, are free-living, marine organisms, occuring from polar through temperate to tropical waters (Taylor et al. 2008; Gómez 2012). These unicellular algae are ancient organisms with fossil record dated back into the Silurian, over 400 million years ago (Steidinger & Tangen 1996). In the second half of the 18th century, the first extant dinophytes have been described (Moestrup & Daugbjerg 2007). From the end of the 1950s onwards, the Dinophyceae increasingly attracted the researchers' interest (Figure 1), which emphasises the ecological and economical importance of this group.

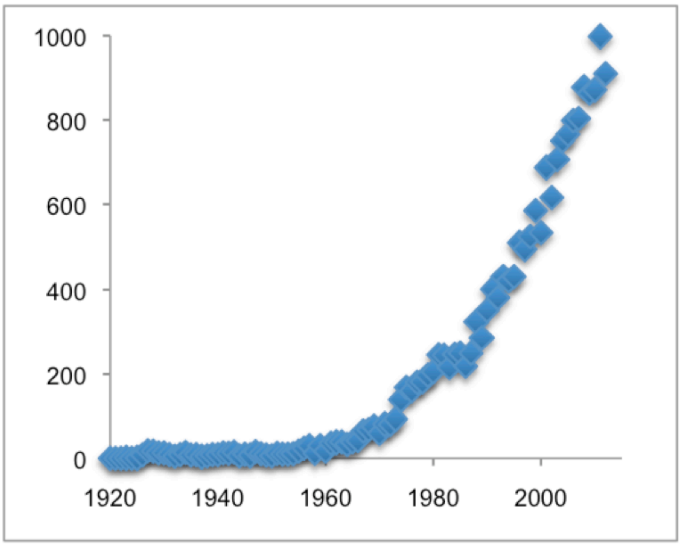

Figure 1: Number of publications per year (Web of Knowledge data base search on "dinofl*" and "dinophy*").

Together with the Apicomplexa and the Ciliata, the Dinophyceae belong to the Alveolata, which are characterised by specialised vesicles located directly beneath the cell surface and called alveoli (Cavalier-Smith 1993; Fast et al. 2002; Leander & Keeling 2004). Morphological apomorphies of the dinophytes are the unique dinokaryon with permanently condensed, liquid-crystalline chromosomes and the lack of typical eukaryotic histones or nucleosomes (Spector 1984; Moreno Díaz de la Espina et al. 2005; Lin 2011). The biflagellate cells possess a coiled transversal flagellum positioned in an encircling groove coined "cingulum" (allowing locomotion) and a longitudinal flagellum (giving propulsive force) (Taylor 1980; Hackett et al. 2004). The monophyly of the Dinophyceae is highly supported based on several molecular loci (Maroteaux et al. 1985; Saldarriaga et al. 2003; Shalchian-Tabrizi et al. 2006a; Zhang et al. 2007; Minge et al. 2009; Hoppenrath & Leander 2010).

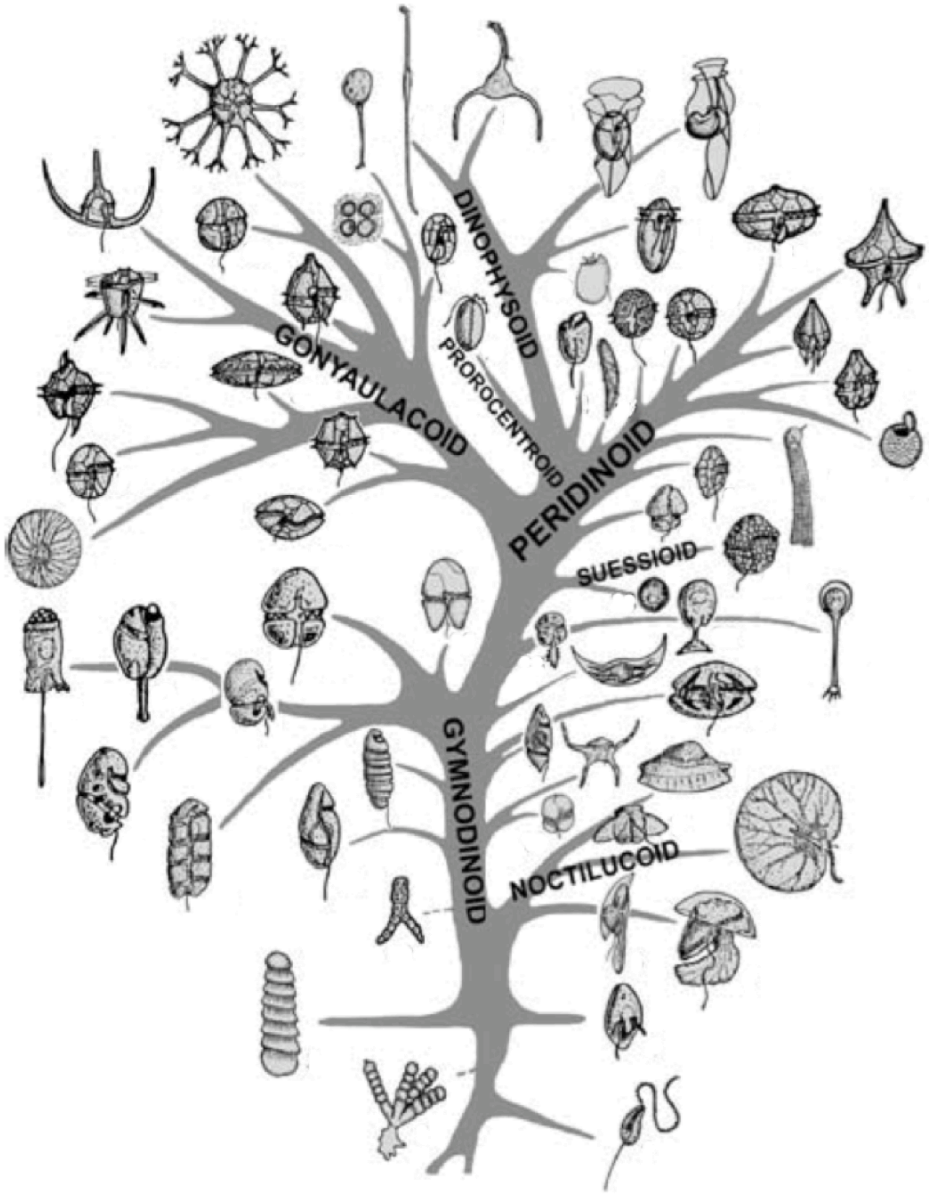

Figure 2: The Dinophyceae are highly diverse in morphology. This simplified phylogeny indicates the classification based on the arrangement of thecal plates: dinophysioid, gonyaulacoid, gymnodinoid, noctilucoid, peridinioid, prorocentroid, suessioid and is explained in detail in the text. Modified after Taylor et al. 2008.

DINOPHYCEAE – MORPHOLOGICAL AND MOLECULAR CLASSIFICATION

Many species of the Dinophyceae develop two morphologically different stages during their life history, namely motile ("vegetative") cells and immotile coccoid cells (usually termed as "cysts": Pfiester & Anderson 1987; Elbrächter et al. 2008). Motile cells (Figure 3) are morphologically divided by the cingulum into epi- and hypotheca, occasionally exhibiting apical or antapical protrusions such as horns. The cortex is composed of the surrounding cell membrane with the amphiesmal vesicles (alveoli) beneath, which might contain cellulose plates (thecate or armoured taxa, the vegetative cell is then termed "theca"). Species without such thecal plates are termed naked, athecate or unarmoured. Some athecate species form

a thin, continuous layer within the amphiesmal vesicles referred to as “pellicle”. The arrangement, number and shape of the thecal plates in armoured taxa and the amphiesmal vesicles in naked species provide important diagnostic characters for Dinophyceae taxonomy and species determination (Taylor 1980; Dodge 1985; Fensome et al. 1993).

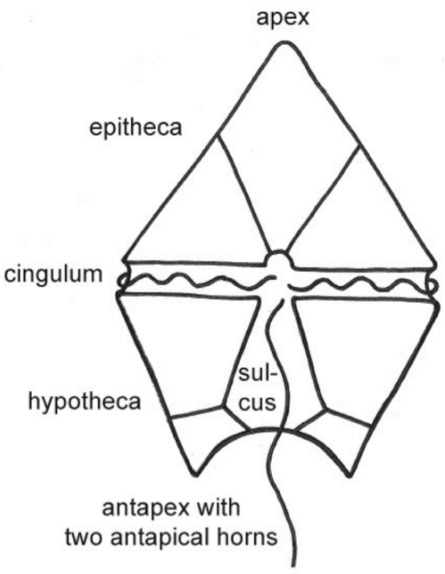

Figure 3: Schematic sketch of a theca. Modified after Fensome et al. 1993.

The Dinophyceae display a high morphological diversity (Figure 2). Depending on the specific arrangement of amphiesmal vesicles with or without cellulose plates, seven groups are readily distinguishable: dinophysioid, gonyaulacoid, gymnodinoid, noctilucoid, peridinioid, prorocentroid and suessioid (Fensome et al. 1993; Taylor 1987, et al. 2008). Taxa of the gymnodinoid type exhibit numerous, randomly arranged amphiesmal vesicles and lacking or exhibiting delicate or well developed cellulose plates. Suessiales possess only delicate thecal plates, arranged in six to eleven latitudinal plate series. Taxa of the peridinioid and gonyaulacoid tabulation type are dominating the known extant dinophytes. The well developed thecal plates are arrayed in a varying but consistent plate pattern, that is characteristic for species (groups) following the Kofoid labelling system (Kofoid 1909). Members of the Dinophysiales exhibit a vertical suture, which forms a bilateral theca, whereas the plates are arranged in four latitudinal series. The aberrant thecas of prorocentroids are reduced to two large plates and the anterior insertion of the flagella; however, cingulum and sulcus are absent. The Noctilucales are likewise unusual in being pelliculate, achieving a large size and extraordinary shapes such as leaf, butterfly or medusa.

Not all morphological circumscriptions show correspondence to molecular analyses. The ambiguous basal lineages are the non-dinokaryotic, heterotrophic and parasitic groups such as the Noctilucales, Oxyrrhinaceae, Perkinsea and Syndiniales (Taylor et al. 2008; Orr et al. 2012). Within the “core” Dinophyceae, the Suessiales, Dinophysiales and Gonyaulacales are each monophyletic based on morphological and molecular data (Daugbjerg et al. 2000; Saldarriaga et al. 2004). The Peridiniales and Prorocentrales, respectively, are monophyletic based on morphology; however, these taxa constitute monophyletic groups only when concatenated sequences are applied (Zhang et al. 2007; Tillmann et al. 2012). The athecate Gymnodiniales, though, are always paraphyletic (Daugbjerg et al. 2000; Saldarriaga et al. 2004; Moestrup & Daugbjerg 2007; Zhang et al. 2007; Tillmann et al. 2012).

The non-motile coccoid cells have diverse biological functions (Fensome et al. 1993). Merely 13 to 16% of the extant Dinophyceae are known to form resistant resting stages emerging from sexual fusion (Head 1996). The majority of fossil “cysts” investigated primarily in micropalaeontology is assumed to consist of these dormancy stages (Dale 1983). Under suboptimal conditions, a temporary cyst is formed apart from sexual reproduction. The third form is a vegetative cyst, which is not a coccoid resting stage, but metabolically and reproductively active. Rarely among dinophytes, digestion cysts are developed after the organism has consumed food (Fensome et al. 1993). The morphology of coccoid stages is highly diverse in few groups such as *Scrippsiella* Balech ex A.R.Loebl. *sensu lato*, however, the morphological characteristics are not applicable at high taxonomic level within the Dinophyceae.

Historically, two different taxonomical concepts for classification have been established for the Dinophyceae: The “neontological” system based on thecate morphology of extant Dinophyceae and the “paleontological” system using characters from “cyst” morphology. The latter system was initially developed by palaeontologists to describe fossil species. Wall and Dale (1968) successfully used cultivation experiments of Recent sediments and detected cyst-theca-relationships as motile and immotile cells being part of a species` life processes. This pioneering work has been the initial starting point to investigate cyst-theca-relationships in detail.

DINOPHYCEAE – NUTRITION AND LIFESTYLE

Nutrition modes and lifestyles are remarkably diverse within the Dinophyceae. Being roughly half primary producers and half predators makes the dinophytes an important part of the global ecosystem (Taylor et al. 2008; Gómez 2012). Beside autotrophic and heterotrophic nutrition, mixotrophy is not specifically quantifiable, but widely distributed (Stoecker 1999; Glasgow et al. 2001; Burkholder et al. 2008). Furthermore, about 7% of all species are parasites, e.g., on fish (Glasgow et al. 2001; Levy et al. 2007), copepods (Gómez et al. 2009; Skovgaard et al. 2012), ciliates (Coats et al. 2010) and other Dinophyceae (Gunderson et al. 2002). The symbiotic lifestyle of mainly *Symbiodinium* species Freud. is of primarily importance for the subsistence of many Cnidarian species such as corals, gorgonians and jellyfish, but also of bivalve molluscs (McNally et al. 1994; LaJeunesse 2001; Baker 2003; Davy et al. 2012). Other species are endosymbionts of Foraminifera and Radiolaria (Gast & Caron 1996; Decelle et al. 2012).

Approximately 100 dinophyte species are assumed to produce toxic secondary metabolites (Orr et al. 2012). The impact of such algae on the marine environment becomes particularly evident when they accumulate to mass occurrence (harmful algae bloom, HAB) (Hallegraeff 1993, 2010; Anderson 2007). In the United States, the economical consequences of HABs account for at least 82 million US$ loss per year (Hoagland & Scatasta 2006). In 1989, a single blooming event of *Gymnodinium* spec. F.Stein has caused 40 million US$ loss for Chinese shrimp aquaculture (Wang & Li 1998). As a result of toxin accumulation along the food chain, the top predators such as seabirds, marine mammals and humans are highly affected by paralytic, diarrhetic, neurotoxic and azaspiracid shellfish poisoning (PSP, DSP, NSP, AZP) as well as ciguatera fish poisoning (CFP). Azaspiracids are the most recently detected group of dinophyte toxins, identified soon after the first poisoning incident in the Netherlands in 1995 (Satake et al. 1998). Still, it took considerable twelve years until the small dinophyte *Azadinium spinosum* Elbr. & Tillmann has been identified as the source of the toxin (Tillmann et al. 2009). Subsequently, additional species of *Azadinium* Elbr. & Tillmann have been described that produce further, but non-toxic azaspirid derivates (Tillmann et al. 2010, 2011; Nézan et al. 2012).

IN FOCUS – THE THORACOSPHAERACEAE

Approximately 35 extant species of dinophytes have the ability to produce calcified coccoid cells (Vink 2004; Zonneveld et al. 2005). The potential to form calcareous structures is considered to be an apomorphic trait within the Dinophyceae, unifying and characterising the Thoracosphaeraceae (Wall & Dale 1968; Janofske 1992; Kohring et al. 2005; Elbrächter et al. 2008). Preliminary molecular analyses corroborate the monophyly of the group, if some (presumably secondarily) non-calcareous relatives such as *Cryptoperididiopsis* Steid., Landsberg, P.L.Mason, Vogelbein, Tester & Litaker, *Pentapharsodinium* Indelicato & A.R.Loebl. and *Pfiesteria* Steid. & J.M.Burkh. are included (D`Onofrio et al. 1999; Gottschling et al. 2005a; Kremp et al. 2005; Gu et al. 2008). Despite these preliminary results, the monophyly of the Thoracosphaeraceae and their delimitation still need to be tested rigorously based on large molecular datasets.

The Thoracosphaeraceae segregate into three major lineages, namely the E/Pe-clade (including *Ensiculifera* Balech, 1967 and *Pentapharsodinium*), the T/Pf-clade (including calcareous *Thoracosphaera* Kamptner and non-calcareous *Pfiesteria*) and *Scrippsiella sensu lato*, with the latter two clades closely related (Gottschling et al. 2005a). Furthermore, *Scrippsiella s.l.* segregates into a number of lineages such as *Pernambugia tuberosa* (Kamptner) Janofske & Karwath, the CAL-clade (including *Calciodinellum operosum*

Deflandre, 1947), the LAC-clade (*Scrippsiella lachrymosa* J.A.Lewis), the PRE-clade (*Scrippsiella precaria* Montresor & Zingone and relatives) and the *Scrippsiella trochoidea* (F.Stein) Balech ex A.R.Loebl. species complex (STR-SC; Gottschling et al. 2005b). Three major assemblages of the STR-SC can be identified based on molecular data, namely STR1, STR2 and STR3.

Morphological characters are not sufficient to distinguish between different species of the STR-SC, as many strains share the same thecal tabulation pattern and exhibit similar spiny coccoid stages. Contrarily, the coccoid cells occasionally exhibit a high variability within monoclonal strains (Montresor et al. 2003; Gottschling et al. 2005b; Gu et al. 2008). Morphologically indistinguishable, but genetically heterogenic specimens are referred to as "cryptic species", whose recognition increased exponentially during the last 40 years (Bickford et al. 2006). Besides *Scrippsiella*, other dinophytes such as *Alexandrium* Halim, *Dinophysis* Ehrenb. and *Symbiodinium* likewise show cases of cryptic speciation (Genovesi et al. 2011; Gómez et al. 2011; LaJeunesse et al. 2012). The existence of cryptic species challenges the precise determination of species, as species are usually described based on their morphology. Thus, a debate on the taxonomic identity of *Scrippsiella trochoidea* has been raised (Montresor et al. 2003; Gottschling et al. 2005b; Gu et al. 2008).

Friedrich von Stein described *S. trochoidea* in 1883, illustrating the motile stage with only three drawings. However, strains from the type locality Kiel Fjord (Baltic Sea) have never been investigated in detail, and neither morphological nor molecular data from the 'true' *S. trochoidea* have been available so far. Since Greuter et al. (1994), the ICN provides a tool to designate "…a specimen or illustration selected to serve as an interpretative type when the holotype, lectotype, or previously designated neotype, or all original material associated with a validly published name, is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name to a taxon" (Article 9.8 of the *International Code of Nomenclature for algae, fungi and plants* (*Melbourne Code*), McNeill et al. 2012). After clarification of the taxonomic identity of *S. trochoidea*, a rough estimation of the species number within the STR-SC based on molecular data will be possible.

THORACOSPHAERACEAE – FOSSIL AND EXTANT DIVERSITY

The coccoid cells of the calcareous dinophytes have a high potential to fossilise (Keupp 1981, 1987, 1991; Kohring 1993; Hildebrand-Habel & Streng 2003; Streng et al. 2004). Approximately 260 species are described from the fossil record (Fensome & Williams 2004; Streng et al. 2004). The altering abundance of morphologically rapidly evolving taxa

make the calcareous dinophytes valuable proxies for paleo-oceanographic and paleo-environmental reconstructions (Versteegh 1997; Keupp 2001; Hildebrand-Habel & Streng 2003; Meier et al. 2004; Masure & Vrielynck 2009). The first calcareous dinophytes have been reported from the late Triassic (Janofske 1992), but the correct affiliation of such fossils has been questioned (Elbrächter et al. 2008; Gottschling et al. 2008). The initial diversification of extant Thoracosphaeraceae might has taken place during the late Jurassic, which is in agreement with a more reliable fossil record since then (Gottschling et al. 2008).

The optical crystallography describes the orientation of the crystallographic c-axis of calcite crystals constituting the shell of the coccoid cells and is considered a key character to identify calcareous dinophytes at the subfamily level (Keupp 1981, 1991; Streng et al. 2004; Elbrächter et al. 2008). Among extant species, three types are readily distinguished (Kohring et al. 2005). Irregularly ("oblique") arranged crystals are present in the E/Pe-calde, e.g. in *Calcicarpinum bivalvum*. Regular crystals with a radial c-axis orientation can be observed in *Leonella* Janofske & Karwath from the T/Pf-clade. Regularly crystals with tangential c-axis orientation are predominant by far in extant species and are today found in *Scrippsiella s.l.* as well as in *Thoracosphaera* from the T/Pf-clade. Nevertheless, the crystallography as a key character remains questionable owing to the fact that the crystal orientation and the taxonomic grouping of the subfamilies based on molecular data are not clearly correlated.

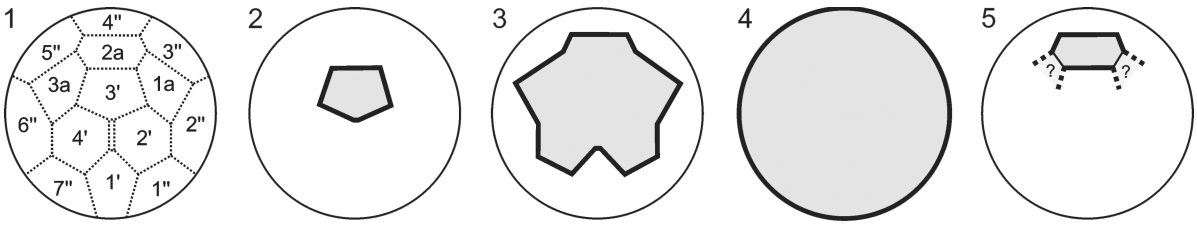

Figure 4: Major types of archeopyle among extant Thoracosphaeraceae. Schematic overview, modified with authors permission from Streng et al. 2004. 1: Apical view of the plate pattern. 2: Simple apical archeopyle (E/Pe- & T/Pf-clade). 3: Mesoepicystal archeopyle (*Scrippsiella s.l.*). 4: Epitractal archeopyle (*Pernambugia*). 5: Intercalary archeopyle (*Calciperidinium asymmetricum*).

Another important character trait of dinophytes is the morphology and composition of the archeopyle, the thecate cell excystment aperture of the coccoid cell (Keupp & Versteegh 1989; Streng et al. 2004). Today, the simple, apical archeopyle type is known from the only distantly related E/Pe- and T/Pf-clades, whereas an operculum corresponding to a single apical plate equivalent is displaced (Figure 4.2). The more complex mesoepicystal and epitractal archeopyles are composed of several epithecal plate equivalents and are considered an apomorphy of extant *Scrippsiella s.l.* (Figure 4.3 and 4.4) (Streng et al. 2004; Gottschling et al. 2005a). An intercalary archeopyle (Figure 4.5) has been described for the fossil based taxon *Calciperidinium asymmetricum* G.Versteegh, which is also known from Recent sediments.

As indicated above, two naming systems for both extant and fossil species have been developed independently, applying characteristics of the two developmental stages, theca and cyst, respectively. Furthermore, both the *International Code of Zoological Nomenclature* (ICZN) and the *International Code of Nomenclature* (ICN) have been applied historically to the Dinophyceae. Consequently, this has resulted in taxonomic conflicts. For example, *Calciodinellum operosum* has been described paleontologically under the ICZN seeing the fossil as a calcified theca (Deflandre 1947). Wall and Dale (1968) have revealed the cyst-theca-relationship by the cultivation of coccoid cells from Recent sediments and corrected Deflandres interpretations. Finally, D`Onofrio et al. (1999) conducted a molecular analysis and placed *C. operosum* in the dinophyte tree. More fossil based taxa are known from late Pleistocene as well as from Recent sediments and are colloquially termed “living fossils”.

A number of those living fossils have been brought into cultivation and have been investigated in detail, including *Calcicarpinum bivalvum* G.Versteegh [= *Pentapharsodinium tyrrhenicum* (Balech) Montresor, Zingione & D.Marino], *Calcigonellum infula* Deflandre, 1947, *Leonella granifera* (Fütterer) Janofske & Karwath and *Pernambugia tuberosa*. However, other living fossils such as *Calciperidinium asymmetricum*, *Caracomia arctica* (Gilbert & Clark) Streng, Hildebrand-Habel & H.Willems, *Follisdinellum splendidum* G.Versteegh, *Melodomuncula berlinensis* G.Versteegh and *Posoniella tricarinelloides* (G.Versteegh) Streng, Basanová, D.Reháková & H.Willems have likewise been found in Recent sediments, but have not been brought into cultivation so far. For a better understanding of the evolution, taxonomy and phylogeny of the Thoracosphaeraceae, those species are of particular importance. Laborious investigations are necessary to make strains available, including the collection of field samples, the cultivation of preferably monoclonal strains and a thorough molecular and morphological survey. At present, the knowledge on the diversity of extant Thoracosphaeraceae is still scarce (Elbrächter et al. 2008).

MOLECULAR PHYLOGENY – CHALLENGE AND CHANCE

The phylogenetic relationships within the Dinophyceae are not sufficiently resolved at present. More than three decades ago, the first rRNA sequence has been obtained from the dinophyte *Crypthecodinium cohnii* (Seligo) Javorn. and the species has been set into a phylogenetic framework (Hinnebusch et al. 1981). Since the early 1990ies, analytical techniques and methods have improved constantly (Moestrup & Daugbjerg 2007). Accordingly, a higher number of taxa, nuclear loci (e.g., rRNA, actin, α- and β-tubulin, hsp90) and mitochondrial loci (e.g., cob, cox1) have been applied to investigate the phylogenetic relationships within the Dinophyceae (Maroteaux et al. 1985; Cavalier-Smith 1993; Daugbjerg et al. 2000; Saldarriaga et al. 2003; Shalchian-Tabrizi et al. 2006a; Zhang et al.

2007; Minge et al. 2009; Hoppenrath & Leander 2010). However, single-locus approaches do not uncover the evolutionary history reliably, and some trees provide contradictory results, while basal nodes are hardly statistically supported (Moestrup & Daugbjerg 2007). The difficulties result from manifold problems such as a limited taxon sample and limited availability of molecular data (for less than 25% of the Dinophyceae sequences are available, often merely a single locus). An additional challenge is the rather high evolutionary rate heterogeneity (Saldarriaga et al. 2004; Hoppenrath & Leander 2010).

The use of chloroplast loci is less advantageous with respect to molecular phylogenies of dinophytes at a high taxonomic level because of multiple endosymbiosis events (Morden & Sherwood 2002; Shalchian-Tabrizi et al. 2006b; Keeling 2010) and horizontal gene transfer (Morden & Sherwood 2002; Bhattacharya & Nosenko 2008; Minge et al. 2010). Without doubt, particular groups within the dinophytes are analysable by the application of homologous plastid genes (Yoon et al. 2005). As a result of ongoing gene transfer from the endosymbiont to the hosts nucleus only few genes remain in the chloroplasts, whereat zero to two protein coding genes are organised in minicircles (Bachvaroff et al. 2004; Howe et al. 2008; Barbrook et al. 2010). Together with the phylogenetic sister group Apicomplexa, the Dinophyceae possess the smallest mitochondrial genome known, consisting of three protein-coding genes (cytochrome b, cytochrome oxidase subunit I and III) and being organised and expressed in an extraordinary way (Jackson et al. 2007; Nash et al. 2008; Waller & Jackson 2009). Even though the gene content is most reduced, the genes are duplicated, fragmented and re-arranged. By extensive RNA editing and trans-splicing of at least cox3, functional transcripts are generated (Jackson et al. 2007), rendering problematic to use for any phylogenetic analysis.

The concatenation of different nuclear and mitochondrial loci and the inclusion of more and more taxa have improved the results in phylogenetic analysis (hsp90, SSU, LSU, Shalchian-Tabrizi et al. 2006a; SSU, cob, cox1, Zhang et al. 2007; hsp90, SSU, LSU, Hoppenrath & Leander 2010; SSU, LSU, Tillmann et al. 2012; SSU, ITS, LSU, cob, cox1, actin, β-tubulin, hsp90, Orr et al. 2012). Hence, the creation of large data sets is highly desirable for a better understanding of the dinophycean phylogeny.

To identify morphologically relatively character poor unicellular organisms such as the Dinophyceae, a reliable link between scientific name, protologue, and distribution is highly desirable. Unquestionably, appropriate genetic loci are required for a molecular analysis of phylogenetics and evolution. Due to the large economical and evolutionary importance of the marine biota, plenty of effort has been given to the documentation of the marine biodiversity in general (Beaugrand et al. 2010; Tittensor et al. 2010; Williams et al. 2010; http://www.coml.org). The sheer mass of morphological diversity and life forms necessitates

a quick and reliable way of identification. The ideal barcode marker is applicable to any taxon and enables identification to species level (Stern et al. 2012). For Dinophyceae, the mitochondrial cob and cox1 have been discussed as universal barcode markers (Lin et al. 2009; Stern et al. 2010). However, amplification efficiency and resolution at species level have not been satisfactory. Additionally, the nuclear ITS region has been proposed as a barcode marker (Gottschling et al. 2005b; Litaker et al. 2007; Genovesi et al. 2011; Stern et al. 2012). Despite the good resolution of this marker to discriminate between species, the presence of paralagous copies has to be considered with caution (Qui et al. 2011; Stern et al. 2012). The secondary structures of helices I and II of the internal transcribed spacer 1 are suggested as a structural identification marker (Gottschling et al. 2005a).

To reveal the relationships within cryptic species, ribotyping may be an appropriate approach. This fingerprint method uses sequence information encoded in the nuclear ribosomal RNA operon to create a minimum-spanning tree allowing reticulations. A bifurcate gene tree is not always sufficient to illustrate all the phylogenetic information present in a molecular data set (Posada & Crandall 2001), since evidence for recombination and homoplasy is forced into non-reticulated tree topologies. By allowing loops and the graphical display of extinct or unsampled variants, networks can represent phylogenetic information more extensively.

AIMS OF THE THESIS

Five internationally published paper and one submitted manuscript included in this cumulative thesis (see publications & manuscripts, p. 6 and Appendix, p. 47) encompass three overarching aspects: The knowledge of diversity of the Dinophyceae in general and the Thoracosphaeraceae in particular is scarce at present; the taxonomy of important taxa needs to be clarified and the deep nodes within the dinophyte molecular phylogeny are insufficiently resolved. In particular, my specific objectives are:

1. Inventory the **diversity of the Thoracosphaeraceae collected from coastal waters**: Extensive **fieldwork** is mandatory to obtain material for detailed molecular and morphological analysis. Coastal environments have been continuously undersampled with respect to the diversity of the Thoracosphaeraceae, although access to such localities is principally facile. The material obtained is used for investigations shedding light on the evolution of the group. The focus of my thesis is the comprehensive **molecular investigation** of cultivated strains in order to:
   1.1. generally determine species (**barcoding**) and to place them in the dinophyte tree (paper 1, 2, 3, 4, 5, 6);
   1.2. particularly place fossil-based species in the dinophyte tree that have been brought into cultivation (**living fossils**) (paper 2);
   1.3. clarify the taxonomy of **cryptic species** (especially in the STR-SC), including the designation of epitypes where necessary, and to estimate the minimal number of morphologically indistinguishable species in particular clades (paper 4 and 5);
   1.4. describe **new species** (paper 1 and 3).
2. **Improvement** of the resolutions of dinophyte **molecular phylogenies** by the concatenation of ribosomal RNA sequences (own and those available in the NCBI database) and other loci as well as by the application of optimisation techniques such as the *in silico* exclusion of poorly arranged positions in an alignment. Emphasis is placed in testing the **monophyly of the Thoracosphaeraceae** and the intra-familial delimitation (paper 2 and 6).

DISCUSSION

THE BASIS FOR BIOLOGICAL STUDIES – FIELDWORK

In recent years, the marine biodiversity has been studied extensively (Beaugrand et al. 2010; Tittensor et al. 2010; Williams et al. 2010; http://www.coml.org). Nonetheless, exact data on species numbers and reliable classifications are still deficient for many taxa. This is especially true for unicellular organisms such as the Thoracosphaeraceae. Currently encountering 260 described fossil species and approximately 35 extant (morpho-)species (Vink 2004; Zonneveld et al. 2005), the diversity of the extant Thoracosphaeraceae is not sufficiently recorded at present because of several reasons (Elbrächter et al. 2008). Frequently, samples have been taken in pelagic environments, while the species at coastal sites have been relatively rarely investigated (Montresor et al. 1998; Godhe et al. 2001; Gottschling & Kirsch 2009; **paper 5**). The compact cells of calcareous dinophytes accumulate in great numbers in the sediments of shallow coastal waters (Kohring 1997; Zonneveld et al. 1999) and are accessible without extensive logistics. With respect to species composition, the Mediterranean Sea is rather well investigated, particularly off Italy (Montresor et al. 1994, 1998; Meier et al. 2002; Meier & Willems 2003; Tommasa et al. 2004; Penna et al. 2010), whereas the coastal waters of Greece have been largely disregarded.

During the course of my study, sediment samples from coastal waters have been taken in the Eastern Mediterranean Sea at 64 localities (Italy and Greece, including Crete, **paper 5, Figure 1**). Eighteen distinct morphospecies of Thoracosphaeraceae have been identified (**paper 2** and **5**), representing two thirds of the previously known diversity in the Mediterranean Sea (Montresor et al. 1998; Meier et al. 2002; Gómez 2003; Satta et al. 2010). Furthermore, several fossil-based species (living fossils) have been reported from Recent sediments of the Mediterranean Sea (listed in Elbrächter et al. 2008), including *Calciperidinium asymmetricum* and *Follisdinellum splendidum* (**paper 5, Figure 2b-c**). Following the pioneering work of Wall and Dale (1968) numerous strains of Thoracosphaeraceae have been established, however, not all morphologically identified species have been brought into cultivation yet. The species diversity of Thoracosphaeraceae appears much higher in the Mediterranean in comparison to colder environments such as the North Sea, where less than ten morphospecies occur (Persson et al. 2000; Godhe et al. 2001; Gottschling & Kirsch 2009). Concluding, ecologically differentiated regions discern in species diversity, whereas the warmer Mediterranean Sea seems to provide biotic or abiotic environmental factors, which may accelerate diversification.

INCREASING KNOWLEDGE OF DIVERSITY – NEW SPECIES AND LIVING FOSSILS

In my thesis, I contributed to the description of new species. Based on molecular sequence data using a multilocus alignment (**paper 1, Figure 4**) *Scrippsiella bicarinata* and *S. kirschiae* from Italian and Greek coastal waters are closely related and maximal supported taxa. They are clearly assigned to *Scrippsiella s.l.*, one of the three distinct clades of the Thoracosphaeraceae (Montresor et al. 2003; Gottschling et al. 2005b; Gu et al. 2011; **paper 2, Figure 4** and **paper 6, Figure 2**).

Morphologically, the presence of six cingular plates in *Scrippsiella bicarinata* and *S. kirschiae* is indicative for their correct phylogenetic placement in *Scrippsiella s.l.* However, particularly the morphology of the coccoid cells has discriminative potential among calcareous dinophytes. Both of the new species are unique among *Scrippsiella s.l.* because of the presence of two prominent ridges on the surface of the coccoid cells corresponding to the cingular contour of the thecate cells (**paper 1, Figure 1A-I** and **Figure 2D-F**). From an evolutionary perspective, *S. bicarinata* and *S. kirschiae* derive from species with spiny coccoid stages present in *S. trochoidea* (**paper 1, Figure 4**). At a first glance, the morphology of *Scrippsiella bicarinata* and *S. kirschiae* is similar to *Bicarinellum* Deflandre, 1949 known from the Mesozoic and Paleogene, but this taxon is considered to be extinct since 50 Ma (Willems 1988). *Bitorus tubiformis* Keupp from the late Cretaceous resembles likewise to *S. kirschiae* with respect to its coccoid cell morphology. However, both taxa have never been reported from Recent sediments, and seeing their distinct morphology, it is unlikely that *Bicarinellum* and *Bitorus* simply have been overlooked.

The morphology of the excystment aperture (i.e., the operculum) is of key importance for classification of calcareous dinophytes (Keupp & Versteegh 1989; Streng et al. 2004) and helps to distinguish fossils from the extant bicarinate species: All fossil bicarinate species exhibit (as known so far) an apical archeopyle corresponding to a single thecal plate equivalent (Introduction, Figure 4.2), while both new extant species have a combination (mesoepicystal) operculum, an apomorphy of the extant *Scrippsiella s.l.* clade (Introduction, Figure 4.3). In summary, the extant bicarinate taxa from the Mediterranean Sea cannot be assigned to any fossil-based taxa, but they are well distinguishable morphologically and molecularly as new species.

Unlike the previous example, coccoid cells displaying the morphology of *Posoniella tricarinelloides* in fact have been reported from Recent sediments (Montresor et al. 1994; Meier et al. 2009; Rubino et al. 2010) as well as from the fossil record of the late Pliocene and Miocene (Versteegh 1993; Bison et al. 2007; Streng et al. 2009). In the course of my study, it has been possible to cultivate living *P. tricarinelloides* not only from the

Mediterranean Sea, but also from the South China Sea (**paper 2**). The thecal morphology of this species has been previously undescribed and unusually consists of a hemispherical epitheca and a conical hyoptheca (**paper 2, Figure 1F**). However, the most striking observation of the cultivated strains is the development of two distinct morphotypes of coccoid cells, one type corresponding to the typical appearance of *P. tricarinelloides* (**paper 2, Figure 3B-C**) and another, smaller type with a thinner calcareous shell (**paper 2, Figure 3F**). This is the first record of a calcareous dinophyte species exhibiting more than one coccoid cell morphology, which are further differentiated by optical crystallography. The first type shows an irregular orientation of calcite crystals, known at present from members of the E/Pe-clade such as *Calcicarpinum bivalvum* (Montresor et al. 1997). The ultrastructure of the second type astonishingly reveals a regular arrangement of crystals with radial orientations of the crystallographic c-axes, which is known today from the fossil taxon *Caracomia* Streng, Hildebrand-Habel & Willems and the extant *Leonella* only (the latter belonging to the T/Pf-clade). Both morphologies of coccoid cells exhibit an apical archeopyle corresponding to a single thecal plate equivalent (**paper 2, Figure 3C** and **3F**, and also see Introduction, Figure 4.2), today occurring in the only distantly related E/Pe- and T/Pf-clades of the Thoracosphaeraceae (Streng et al. 2004). A reliable classification of *P. tricarinelloides* based on morphology alone thus has not been possible.

In the case of *P. tricarinelloides*, four loci have been applied to determine its phylogenetic position within the calcareous dinophytes (**paper 2, Figure 4**). The Thoracosphaeraceae segregate into the basal E/Pe-clade and the more derived *Scrippsiella s.l.* and T/Pf-clade. All strains of *P. tricarinelloides* assemble distinctly and with maximal support within the T/Pf-clade. This result is rather surprising as the phylogenetic position was expected within the E/Pe-clade based on the irregular orientated calcite crystals detected in fossil samples (Meier et al. 2009). However, the molecular data concur with the archeopyle morphology as well as with the ultrastructure and crystallography of the second type of coccoid cells. In conclusion, the cultivated strains from the Mediterranean Sea and the South China Sea can be reliably determined as the fossil-based taxon *P. tricarinelloides*, the only species described so far exhibiting two distinct morphotypes of coccoid cells.

Although not focus of my doctoral research, the increasing threat for marine organisms as well as for human health deserves especial attention and necessitates basic research on harmful algae (Hallegraeff 1993; Anderson et al. 2012) such as *Alexandrium* (Anderson et al. 2012), *Azadinium* (Tillmann et al. 2012; **paper 3**), *Karenia* G.Hansen & Ø.Moestrup (Brand et al. 2012) or *Pfiesteria* (Burkholder et al. 2012). *Azadinium* comprises five species at present (Tillmann et al. 2009, 2010, 2011; Nézan et al. 2012; **paper 3**), and together with *Amphidoma languida* Tillmann, R.M.Salas, Elbr. it constitutes the highly supported

Amphidomataceae in molecular trees (Tillmann et al. 2012). The new species *A. polongum* from waters off the Shetland Islands is described with respect to both morphological and molecular data and clusters within *Azadinium* based on a three loci phylogenetic analysis (**paper 3, Figure 10**). Additionally, the genetic ITS distances of p=0.052 to 0.247 (**paper 3, table 1**) support the species delimitations. An intergenomic distance up to 0.02 substitutions per site argues for intraspecific variation, whereas distances exceeding 0.04 are indicative for dinophyte species divergence (Litaker et al. 2007). *Azadinium polongum* is not confirmed to be a toxic species. Only *A. spinosum* produces toxic derivates of azaspirid within the Amphidomataceae. However, the assembly of azaspirids seems to be common within *Azadinium* (Krock et al. 2012), and the presence of yet unknown toxic derivates cannot be excluded and requires further research. The phylogenetic analysis of *A. spinosum* and its non-toxic relatives as a case study might help to understand the evolutionary origination of toxin production within the dinophytes in general.

CLARIFYING TAXONOMY – BARCODING AND CRYPTIC SPECIES

An appropriate way for an unambiguous identification of character-poor unicellular organisms such as (calcareous) dinophytes is necessary. Moreover, a reliable and quick designation of new samples is required. DNA barcoding has become a comparatively reasonable and fast, but also vigorously discussed technique for species identification (Herbert et al. 2003; Tautz et al. 2003; http://www.barcodinglife.com). Recently, the diagnoses of new species (LaJeunesse et al. 2012) and studies on biodiversity (Krishnamurthy & Francis 2012) have become additional applications of barcoding. However, such an integrative genetics-based approach has to be considered with caution (DeSalle et al. 2005; DeWalt 2011): Only the comparison of new sequences to an existing large database assures accurate classifications and avoids misinterpretations such as the over- or underestimation of species diversity. A proper barcoding locus and approach has been debated for various groups of organisms (Mitchell 2008; Schoch et al. 2012; Hollingsworth 2011; Pawlowski et al. 2012). For Dinophyceae, mitochondrial loci have been proposed previously (Lin et al. 2009; Stern et al. 2010), but the two main prerequisites of a suitable marker, as being universally applicable on the one hand, and providing resolution to species level on the other hand, have not been satisfactory (Stern et al. 2012). As for other eukaryotic microorganisms such as fungi (Schoch et al. 2012), the ITS region has been verified as a suitable dinophyte barcode marker (Gottschling et al. 2005b; Litaker et al. 2007; Genovesi et al. 2011; Qui et al. 2011; Stern et al. 2012). Both above mentioned requirements are provided; additionally, immense numbers of ITS sequences of dinophytes have been

accumulated in GenBank, tendering for taxonomic comparison. Consequently, the ITS region served as the barcode marker during my study.

The taxonomy of species such as *Scrippsiella trochoidea* has been challenging through the past decade. By virtue of molecular analysis, the existence of several subclades among numerous morphologically similar *S. trochoidea* strains has been documented (D`Onofrio et al. 1999; Gottschling et al. 2005b; Gu et al. 2008; **paper 5**). Hence, the taxon *S. trochoidea* appears not as a single species but rather constitutes a species complex (STR-SC) consisting of genetically distinct, but morphologically indistinguishable cryptic species (Montresor et al. 2003). Cryptic speciation has been also reported from other dinophytes such as *Alexandrium*, *Dinophysis* and *Symbiodinium* (Genovesi et al. 2011; Gómez et al. 2011; LaJeunesse et al. 2012). For the *Alexandrium tamarense* (M.Lebour) Balech species-complex five geographically distinct ribotypes have been detected (Scholin et al. 1994; Genovesi et al. 2011). In contrary, the ribotypes of the three morphologically indistinguishable subclades of the STR-SC co-occur worldwide (Gottschling et al. 2005b; **paper 5, Figure 3**). The lack of any apparent distribution pattern excludes the biogeography as a delimitating factor for the *S. trochoidea*-like species. In *Symbiodinium* LaJeunesse et al. (2012) provided ecological and physiological discriminative criteria (e.g., host specificity and temperature tolerances). Ecophysiological, morphological and genetic information are prerequisite for a robust species description (Sites & Marshall 2004) and need to be worked out rigorously for the members of STR-SC.

The existence of the distinct clades STR1, 2 and 3 within the STR-SC has brought up the question on the taxonomic identity of the “true” *S. trochoidea*. The only available type material of *S. trochoidea* consists of three rather simple sketches (Stein 1883; **paper 4, Figure 1** to **3**), which do not allow an unambiguous identification. Thus, the detailed examination of material from the type locality Kiel Fjord (Baltic Sea, Germany) is the most sensible way of clarifying the taxonomic identity of *S. trochoidea*. During the course of my study, several morphologically *S. trochoidea*-like strains originating from the type locality have been cultivated by Monika Kirsch (Bremen, Germany). The strain GeoB*185 has been chosen for epitypification due to deriving from a single cell and accordingly being monoclonal. Moreover, the morphology of *S. trochoidea* from the type locality does not oppose the protologue. Based on ITS sequence information GeoB*185 clusters within the genetically distinct STR2 (**paper 4, Figure 18**).

After the taxonomic identity of *S. trochoidea* is clarified, it remains unclear whether the STR2 clade and related groups are equivalent to a single or rather several species. Thus, a rough species number for the STR-SC has to be estimated. Network analyses of ribotypes

allow the illustration of reticulations and intermediate mutational steps for investigations on population genetics and intraspecific variability (Posada & Crandall 2001). The occurrences of cryptic species have been investigated by network analyses for fungi, animals and plants (e.g., Baloch & Grube 2009; Daniels & Ruhberg 2010; Peng et al. 2010). Conducting ITS ribotype analyses for the three distinct clades within the STR-SC and additionally for the LAC and PRE clades (**paper 5, Figure 3**), a continuum of gradually varying ribotypes within the clades is not perceived. On the contrary, classes of similarity are detected, which account for reproductive isolation and therefore several distinct species (i.e., biological species) within these clades. A minimal number of species is estimated referring to the STR3 clade, which includes two morphologically and ecologically distinct morphospecies, namely *S. trochoidea* and "*Calciodinellum*" *levantinum* S.Meier, Janofske & H.Willems (Meier & Willems 2003; Gottschling et al. 2005b; Meier et al. 2007). Doubtlessly, these taxa are reproductively isolated from another. Assuming that *C. levantinum* represents a single species with one class of similar ribotypes (i.e., biological species), seven additional classes of similarities of *Scrippsiella*-like species can be detected within the STR3 clade. The same approach enables the assumption of four and two species in STR1 and STR2, respectively. For *S. lachrymosa*, a minimal species number of four is evaluated. As these closely related species occur sympatrically, another driving force than geographical isolation has to account for species diversification among these cryptic species. Such criteria might be ecological or physiological specifications, whereas the knowledge on *S. trochoidea* and *S. trochoidea*-like species is still fragmentary. In conclusion, the six investigated morphospecies segregate into the considerably high number of 21 species based on molecular data.

INCREASING DATA AVAILABILITY – IMPROVING APPROACHES FOR BETTER CONCLUSIONS

The Dinophyceae exhibit several problematic specifics molecular biologists are faced with in phylogenetic reconstructions, resulting from, for example, various types of nutrition and lifestyle. Because of being unicellular predators, parasites or symbionts, genomes of both dinophytes and prey or host, respectively, have to be taken into consideration. Furthermore, genetically extremely reduced organelles and multiple endosymbiosis events complicate the phylogenetic analysis of dinophytes at a high taxonomic level. Moreover, the reconstruction of the relationships within the Dinophyceae is a complex challenge for three main reasons that are discussed below.

First, the taxon sample available at present is very limited (approximately a quarter of all Dinophyceae at the generic level only have sequence depositions from at least one locus in GenBank). Generally, an increased number of taxa included in alignments improve the resolution of phylogenetic trees (Dunn et al. 2008; Heath et al. 2008; Sanderson 2008).

Constant and reliable research on diversity and taxonomy combined with new molecular techniques such as next generation sequencing will counteract unbalanced sequence data availability.

Second, the application of a single genetic locus is not powerful enough to infer higher taxonomic relationships of the Dinophyceae (Keeling et al. 2005; Gottschling et al. 2005b). A gene tree based on merely one marker does not necessarily reflect the phylogeny of a taxon, but also the single gene history. Critical comparison regarding the congruence of gene trees resulting from various loci is prerequisite. Considering the demonstrated lateral gene transfer within the dinophytes (Morden & Sherwood 2002; Bhattacharya & Nosenko 2008; Howe et al. 2008; Minge et al. 2010), the combination of several loci has been shown to improve the resolution of dinophycean phylogenetic trees (Saldarriaga et al. 2004; Zhang et al. 2007; Hoppenrath & Leander 2010; Tillmann et al. 2012; **paper 2** and **6**). The impressive application of eight different nuclear and mitochondrial loci has suggested a single origin of thecate Dinophyceae diverging from an athecate ancestor (Orr et al. 2012). However, the technical limitations of this study are evident: The two applicable loci of the three in total available mitochondrial loci have been applied as well as the maximum of available nuclear sequences, partly to a rather limited taxon sample. The lack of any chloroplast marker owed to multiple endosymbiosis events within the Dinophyceae (Howe et al. 2008; Keeling 2010) and hence their inappropriateness for broad phylogenetic analyses. In addition, for a small number of dinophyte species the extensive editing of the mitochondrial messenger RNA is known (Zhang et al. 2008; Waller & Jackson 2009). Consequently, mRNA sequence information should be excluded from phylogenetic analyses, or only be used in comparison with the corresponding genomic mitochondrial sequence information.

Third, the rate heterogeneity of different molecular loci and taxa causes branch length variations. While ITS is known to evolve quickly resulting in highly divergent sequences over a broad taxonomic range, the SSU provides lower resolution in dinophyte phylogenetic trees (Taylor 2004). Considering the high morphological diversity together with the poor resolution of the deep nodes within the Dinophyceae, a rapid radiation in younger geological time might be inferred (Moestrup & Daugbjerg 2007; Hoppenrath & Leander 2010). An extreme case is the shared ribotype of the morphologically and ecologically differentiated species *S. hangoei* (J.Schiller) J.Larsen from brackish water environments and *Peridinium aciculiferum* Lemmerm. with fresh water preferences (Gottschling et al. 2005a; Logares et al. 2007a, 2007b) On the contrary, several taxa have extremely long branches, seemingly being isolated for a long time or having extraordinary high substitution rates such as *Amphidinium* Claperède & J.Lachm., *Bysmatrum* M.A.Faust & Steid. (**paper 6**), *Cochlodinium* F.Schütt, *Coolia* Meunier and *Gambierdiscus* Adachi & Fukuyo (**paper 2, Figure S1**). A further aspect of presumably high mutational rates is the incidence of the above discussed cryptic species,

whereat the molecular species diversification seems to be rather rapid (**paper 5**). Concluding, the reconstruction of ancient relationships within the Dinophyceae has been and remains challenging and demands the reflection on rate heterogeneity and data coverage.

Being aware of the above discussed problems, the concatenation of the rich pool of rRNA sequences and the inclusion of as much taxa as possible has been shown to be promising during the course of my study, to analyse the phylogenetic relationships within the Dinophyceae and address specific taxonomic issues (**paper 2** and **6**). Both Maximum Likelihood and Bayesian approaches were applied resulting in largely congruent tree topologies. Neither the use of various automatic alignment programs, nor the exclusion of poorly aligned positions improved the results significantly. Thus, topology and stability of resulting trees rely on the amount of input data only (Saldarriaga et al. 2004; Tillmann et al. 2012; Orr et al. 2012; Gottschling & McLean 2013). The monophyly of the Dinophyceae is highly supported (**paper 6, Figure 2** and **S1**), as proven by other studies (e.g., Maroteaux et al. 1985; Shalchian-Tabrizi et al. 2006a; Minge et al. 2009; Hoppenrath & Leander 2010) although early studies had a poor resolution on account of limited taxon sample and low amount of genetic data (see above). However, the phylogenetic relationships within the Dinophyceae based on molecular data do not always agree with the morphological groups. The Suessiales, the Gonyaulacales and the Dinophysiales each form monophyletic groups based on both morphological and molecular data (Daugbjerg et al. 2000; Saldarriaga et al. 2004; Zhang et al. 2007; **paper 2, Figure S1** and **paper 6, Figure S1**). Contrary, the Peridiniales and the Prorocentrales have been problematic in being para- and polyphyletic (Zhang et al. 2007; Kremp et al. 2005; **paper 6, Figure 2**), or, if monophyletic, only in phylogenies using concatenated sequences (Zhang et al. 2007; Tillmann et al. 2012; **paper 2, Figure S1**). The monophyly of the Gymnodiniales has never been shown (Saldarriaga et al. 2004; Kremp et al. 2005; **paper 2, Figure S1** and **paper 6, Figure S1**). Hence, morphological or ecophysiological characters have to be worked out to discriminate among the paraphyletic Gymnodiniales.

Fensome et al. (1993) provide a comprehensive classification of both extant and fossil Dinophyceae for the first time. Following Tangen et al. (1982), the Thoracosphaerales comprising vegetative calcareous coccid stages, have been separated from the Calciodinelloidae, having calcareous resting cells. Molecular studies have demonstrated the close relationship of the Thoracosphaerales and the Calciodinelloidae. Including non-calcareous taxa, the monophyly of the calcareous dinophytes has been shown (D`Onofrio et al. 1999; Kremp et al. 2005; Gottschling et al. 2005a). The ability to produce calcareous structures has been considered to be a morphological apomorphy within the Dinophyceae (Wall & Dale 1968; Janofske 1992; Kohring et al. 2005). Moreover, considering the vast

diversity of more than 10.000 alveolate species (Fast et al. 2002), the calcareous structures exhibited by the Thoracosphaerales and the Calciodinelloidae only, are most likely homologous. However, these suggestions have never been tested rigorously (Elbrächter et al. 2008) as either the molecular data sets (Gottschling et al. 2005b) or the taxon sample have been limited (Tillmann et al. 2009; Zhang et al. 2007). During the course of my study, the monophyly of the calcareous Dinophyceae (i.e., Thoracosphaeraceae) including some non-calcareous taxa such as *Pfiesteria piscicida* Steid. & J.M.Burkh. and *Ensiculifera* aff. *loeblichii* El.R.Cox & H.J.Arn. is clearly demonstrated (**paper 2, Figure 4** and **paper 6, Figure 2**, Tillmann et al. 2012). Accordingly, the assumptions of Fensome et al. (1993) and Tangen et al. (1982) to separate Thoracosphaerales and Calciodinelloidae can be clearly rejected.

Although the Thoracosphaeraceae are monophyletic, the assumption of a single origin of the ability to produce calcareous structures within the Dinophyceae is challenged again. The molecular phylogenies of the Thoracosphaeraceae become astonishingly complex by the recent affiliation of parasitic or endosymbiotic species such as *Duboscquodinium collinii* Grassé, *Tintinnophagus acutus* Coats (Coats et al. 2010), *Blastodinium contortum* Chatton, *B. crassum* Chatton (Skovgaard et al. 2012) and *Zooxanthella nutricula* K.Brandt (Gottschling & McLean 2013) (**paper 2, Figure 4**). Opposed to several losses of the ability to calcify, an independent repeated acquirement to produce calcareous structures within the Thoracosphaeraceae has to be considered possible.

In conclusion, the applications of large rRNA data sets confirm the monophyly of both, the Dinophyceae and the Thoracosphaeraceae. Moreover, despite the possibly paraphyletic Gymnodiniales, the dinophytes diverge into each monophyletic Suessiales, Gonyaulacales, Dinophysiales, Peridiniales and Prorocentrales. However, the requirement of further research and the necessity to combine morphology and molecular methods for meaningful interpretations regarding the evolution of the Dinophyceae in general and the Thoracosphaeraceae in particular, is evident.

LIST OF ABBREVIATIONS

AZP	azaspiracid shellfish poisoning
CFP	ciguatera fish poisoning
cob	cytochrome b
cox1	cytochrome oxidase subunit I
cox3	cytochrome oxidase subunit III
DSP	diarrhetic shellfish poisoning
EMBO	European Molecular Biology Organization
HAB	harmful algae bloom
hsp90	heat shock protein 90
ICN	International Code of Nomenclature for algae, fungi and plants
ICZN	International Code of Zoological Nomenclature
ITS	5.8S rRNA plus flanking internal transcribed spacers 1 and 2
JSPS	Japanese Society for the Promotion of Science
LSU	28S rRNA, large subunit
NCBI	National Center for Biotechnology Information
NSP	neurotoxic shellfish poisoning
PSP	paralytic shellfish poisoning
s.l.	sensu lato
SSU	18S rRNA, small subunit

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Figure 1: Number of publications per year (Web of Knowledge data base search on “dinofl*” and “dinophy*”).....9

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APPENDIX

PAPER 1 (PUBLICATION)

SAME BUT DIFFERENT: TWO NOVEL BICARINATE SPECIES OF EXTANT CALCAREOUS DINOPHYTES (THORACOSPHAERACEAE, PERIDINIALES) FROM THE MEDITERRANEAN SEA

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SAME BUT DIFFERENT: TWO NOVEL BICARINATE SPECIES OF EXTANT CALCAREOUS DINOPHYTES (THORACOSPHAERACEAE, PERIDINIALES) FROM THE MEDITERRANEAN SEA[1]

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The diversity of extant calcareous dinophytes (Thoracosphaeraceae, Dinophyceae) is currently not sufficiently recorded. The majority of their coccoid stages are cryptotabulate or entirely atabulate, whereas relatively few forms exhibit at least some degree of tabulation more than the archeopyle. A survey of coastal surface sediment samples from the Mediterranean Sea resulted in the isolation and cultivation of several strains of calcareous dinophytes showing a prominent tabulation. We investigated the morphologies of the thecate and the coccoid cells and conducted phylogenetic analyses using Maximum Likelihood and Bayesian approaches. The coccoid cells showed a distinct reflection of the cingulum (and were thus cingulotabulate), whereas thecal morphology corresponded to the widely distributed and species-rich *Scrippsiella*. As inferred from molecular sequence data (including 81 new GenBank entries), the strains belonged to the *Scrippsiella sensu lato* clade of the Thoracosphaeraceae and represented two distinct species. Morphological details likewise indicated two distinct species with previously unknown coccoid cells that we describe here as new, namely *S. bicarinata* spec. nov. and *S. kirschiae* spec. nov. Cingulotabulation results from the fusion of processes representing the pre- and postcingular plate series in *S. bicarinata*, whereas the ridges represent sutures between the cingulum and the pre- and postcingular series in *S. kirschiae*, respectively. Bicarinate cingulotabulation appears homoplasious among calcareous dinophytes, which is further supported by a comparison to similar, but only distantly related fossil forms.

Key index words: **coccoid cell; cytochrome b; distribution; molecular systematics; morphology; phylogeny; ribosomal RNA; thecate cell**

Knowledge about the diversity of extant dinophytes producing calcified cells during their life history (Thoracosphaeraceae, Dinophyceae) is limited at present. More than 250 species of great morphological

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variety have been described based on fossil material (Streng et al. 2004), vastly exceeding the diversity known from the today recognized species in the global oceans. Among myriad species of the Alveolata, the potential to produce calcareous structures is restricted to (i.e., has been considered apomorphic for) the Thoracosphaeraceae, arguing for the monophyly of this group (Wall and Dale 1968, Janofske 1992, Elbrächter et al. 2008). Molecular data, however, indicate that the Thoracosphaeraceae also include (presumably secondarily) noncalcareous relatives, such as species of *Pentapharsodinium* Indel. & A.R.Loebl. and *Pfiesteria* Steid. & J.M.Burkh. (D′Onofrio et al. 1999, Gottschling et al. 2005a, 2012, Zhang et al. 2007, Tillmann et al. in press) and even parasites, namely *Duboscquodinium* Grassé, 1952 and *Tintinnophagus* Coats, 2010 (Coats et al. 2010). The Thoracosphaeraceae may segregate into three lineages, including the E/Pe-clade (i.e., *Ensiculifera* Balech, 1967 and *Pentapharsodinium*; marine and possibly also fresh water environments), the T/Pf-clade (including *Thoracosphaera* Kamptner and *Pfiesteria*; marine, brackish and fresh water environments), and *Scrippsiella* Balech ex A.R.Loebl. *sensu lato* (*s.l.*; predominantly marine and also brackish environments), with the latter two clades showing a close relationship (Gottschling et al. 2005a, 2012, Tillmann et al. in press).

Life histories of calcareous dinophytes include (at least) two principally different developmental stages, namely a motile cell (usually thecate, with a distinct tabulation pattern of cellulose plates) and an immotile coccoid cell. The coccoid stage is frequently referred to as ''cyst'' and may retain various degrees of expressed tabulation (formerly described as paratabulation), frequently restricted to the archeopyle. Key characters used to circumscribe calcareous dinophyte species based on the motile cells are number and shape of epi- and hypothecal, cingular, and sulcal plates, whereas diagnostic characters of the coccoid stages comprise the shape, tabulation (if present), archeopyle/operculum morphology, and ultrastructure of the calcareous shell (including the optical crystallography; Elbrächter et al. 2008). The thorough investigation of the link between the two developmental stages goes back to the pioneering work of Wall and Dale (1966, 1968), who have performed cultivation experiments with coccoid cells collected from modern sediments. Later, a series of studies have been published, clarifying the cyst-theca-relationships of such fossil-based taxa as *Calcicarpinum bivalvum* G.Versteegh (= ''*Pentapharsodinium*'' *tyrrhenicum* [Balech] Montresor, Zingone & D.Marino: Montresor et al. 1993), *Calciodinellum operosum* Deflandre, 1947 (Montresor et al. 1997), and *Pernambugia tuberosa* (Kamptner) Janofske & Karwath (Janofske & Karwath in Karwath 2000).

Attempts have been made to classify calcareous dinophytes into various subgroups based on several character traits. In the coccoid stage, the number of shell layers as well as the ultrastructure of the constituent calcitic crystals appear consistent within species and informative for the inference of phylogenetic relationships. It is, however, particularly the orientation of the calcitic crystals forming the shell with their crystallographic main axis (c-axis), which has been considered important for classification. Three types are readily distinguished, namely ''irregularly oblique'', ''regularly radial'', and ''regularly tangential'' (each in relation to the cell surface: Keupp 1981, 1987, 1991, Kohring 1993a, Young et al. 1997, Meier et al. 2009). However, none of such types appears to be congruent to monophyletic groups of molecular trees (Gottschling et al. 2005a, 2012), and their importance for the classification of the entirety of the Thoracosphaeraceae remains at least questionable.

During germination, the archeopyle of the coccoid cell is the aperture, from which a new thecate cell emerges. This process takes place after removal of the operculum comprising a variable number of thecal apical plate equivalents (Evitt 1967). Archeopyle and operculum morphology has great importance to indicate relationships within calcareous dinophytes (Keupp and Versteegh 1989, Streng et al. 2004). The different types of archeopyles that are currently distinguished (Streng et al. 2004) correlate with molecular phylogenies of calcareous dinophytes (Gottschling et al. 2005a). A simple apical archeopyle is considered the ancestral condition and is today found in the two, only distantly related clades E/Pe and T/Pf. The more complex (mesoepicystal and epitractal) compound opercula include a greater number of plate equivalents and are found today in *Scrippsiella s.l.*

Only few calcareous dinophytes are entirely atabulate, whereas, the majority of forms exhibits at least some degree of tabulation in the coccoid stage (for terminology, we refer to Sarjeant 1982, Streng et al. 2009). Many forms belong to the cryptotabulate type (Streng et al. 2004), in which tabulation is restricted to the archeopyle. Relatively few species belong to the holotabulate type (e.g., *Calciodinellum operosum*), the intratabulate type (e.g., *Alasphaera* Keupp, *Wallidinellum* Keupp), and the cingulotabulate type. If the latter type is present, then the cingulum is reflected either as one (monocarinate; e.g., *Carinasphaera* Kohring, *Carinellum* Keupp) or two ridges (bicarinate; e.g., some species of *Bicarinellum* Deflandre, 1949, *Bitorus* Keupp). Two ridges are frequently developed by the fusion of processes representing pre- and postcingular plate equivalents, respectively, and they are thus not cingulotabulate in a strict sense. Occasionally, intermediates between cingulotabulate and intratabulate forms are found [e.g., in *Bicarinellum jurassicum* (Deflandre) Keupp: Keupp 1984]. Species exhibiting coccoid cells with tabulation are likely polyphyletic, and the degree of tabulation may vary between individuals of the same strain in cultivation (e.g., *Calciodinellum*

Deflandre, 1947: Gottschling et al. 2005b). Tabulation in the coccoid cells as character trait is, thus, highly homoplasious and appears to be of limited importance for the inference of phylogenetic relationships at high taxonomic level (i.e., rather at the species level if at all).

In this study, we report on two novel species of calcareous dinophytes that we have collected at various sites in the Mediterranean Sea and that we have brought into cultivation. Among extant species, they are unique, exhibiting calcareous coccoid stages with two distinct ridges reflecting the cingulum. We herein provide morphological descriptions of both stages, thecate and coccoid and investigate their phylogenetic position using molecular data of three loci (mitochondrially encoded cytochrome b: *cob*, MT-CYB; nuclear Internal Transcribed Spacer: ITS and large subunit of the ribosomal RNA: LSU). We have compiled all protologues of those Thoracosphaeraceae showing the cingulo- or intratabulate type of tabulation to delimitate the novel from the known species reliably. Our aim is an improved knowledge about extant (calcareous) dinophyte diversity, with relevance also for the fossil species.

MATERIALS AND METHODS

Morphology. Nine strains of calcareous dinophytes (GeoB 408, GeoB 411, GeoB*414, GeoB 416, GeoB 432, GeoB 453, GeoB 454, GeoB 456, and GeoB 458; see Table S2 in the Supporting Information) were established by isolation of few coccoid cells from sediment samples collected at the Italian and Greek coast as previously described in detail (Soehner et al. in rev.). Cultivation took place in a climate chamber Percival I-36VL (CLF PlantClimatics; Emersacker, Germany) at 23°C, 80 μmol photons $m^{-2} \cdot s^{-1}$ and a 12:12 h light:dark photoperiod using K-Medium without silicate (Keller et al. 1987) and 35 psu artificial seawater (hw marinemix professional: Wiegandt; Krefeld, Germany) at pH 8.2. The strains are currently held in the culture collections at the Institute of Historical Geology/Palaeontology (University of Bremen, Germany) and the Institute of Systematic Botany and Mycology (University of Munich) and are available upon request.

Cells were directly observed in an Olympus CKX41 inverted microscope, equipped with the camera DX 20H-FW (Kappa optronics; Gleichen, Germany) supplied with Calypso software. For the identification of thecal plate patterns, cells were stained with calcofluor white M2R (Sigma-Aldrich; Munich, Germany; Fritz and Triemer 1985) and observed in a Leica fluorescence microscope, equipped with the camera PS/DX40-285FW (Kappa optronics). The Kofoidean system (Taylor 1980, Fensome et al. 1993) was used for the designation of the thecal plate formula. The preparation of the type material followed the protocol as described in Zinssmeister et al. (2011). Double-staining was performed using astra blue (Fluka; Buchs, Switzerland) and eosin (Merck; Darmstadt, Germany). Ethanol-based Technovit 7100 (Heraeus; Wehrheim, Germany) was used for embedding. The types are deposited at the Centre of Excellence for Dinophyte Taxonomy (CEDiT; Wilhelmshaven, Germany), copies are available in the herbaria of Berlin and Munich.

For thin sections, cultivated coccoid cells were fixed with 2.5–3% glutaraldehyde (Plano; Wetzlar, Germany) in media, desalinated in artificial seawater with reduced salinity and dehydrated in a graded acetone p.a. (Roth; Karlsruhe, Germany) series (30, 50, 70, 90, 100, 100, and 100%). The samples were embedded in a synthetic resin (Spurr 1969) using the Embedding Medi Kit (Science Services; Munich, Germany) and following standard protocols (Meier et al. 2002). A 1:1 mixture of acetone and resin was used in a first embedding step for better infiltration of the resin into the cells. After 1 h, the mixture was replaced by pure Spurr's resin and hardened at 70°C for 48 h. The Zeiss microtome, equipped with a steel knife, was used to cut 3 μm ultra-thin sections that were examined using the Axiophot light microscope (Zeiss; Oberkochen, Germany). The method for identifying the crystallographic orientation of the calcite crystals based on standard methods (Bloss 1999) in thin sections was described in detail previously (Janofske 1996, 2000, Montresor et al. 1997, Karwath 2000). Briefly, the orientation of the c-axis is perpendicular to the cell surface, if the quadrants I and III of a conoscopic image show yellow interference colors and quadrants II and IV show blue interference colors. Conversely, the orientation of the c-axis is tangential to the cell surface, if the quadrants II and IV show yellow interference colors and quadrants I and III show blue interference colors.

For scanning electron microscopy (SEM) preparation, coccoid cells were desalinated in bi-distillate water and air-dried on a glass slide that was fixed on a SEM stub. Thecate cells were fixed using 2.5–3% glutaraldehyde in media, and further steps were performed following standard protocols as previously described (Gottschling et al. 2012). Samples were sputter-coated with platinum and documented using an electron microscope LEO 438 VP (Zeiss). For each species, the number of cells measured (thecate or calcareous coccoid cells) ranged between 5 and 101. Lengths of thecate cells were measured from the top of the apex to the antapex. Widths were measured as the largest distance in transversal view (i.e., points between the upper cingular plate boundaries of the pre-cingular plates). Ridges and processes present in the coccoid cells were likewise included.

Molecular analyses. Genomic DNA was extracted from fresh material using the Nucleo Spin Plant II Kit (Machery-Nagel, Düren, Germany). Both ITSs including the 5.8S rRNA region, the first two domains of the LSU and *cob* were amplified using the primer listed in Table S1 (see Supporting Information) following standard protocols (Gottschling and Plötner 2004, Zhang et al. 2005). Forty-four dinophyte strains were investigated (Table S2). The data matrix comprising a systematically representative set of *Scrippsiella s.l.* was assembled from sequences downloaded from GenBank. It included 81 new ITS, LSU, and *cob* sequences from strains out of our own culture collection (Table S2). The sequences were separately aligned in three partitions using ''MAFFT'' v6.624b (Katoh et al. 2005, Katoh and Toh 2008; freely available at http://mafft.cbrc.jp/alignment/software/index.html) and were concatenated afterwards. The alignment is available *via* nexus file upon request.

Phylogenetic analyses were carried out using Maximum-Likelihood (ML) and Bayesian approaches, as described in detail previously (Gottschling et al. 2012). The Bayesian analysis was performed using ''MrBayes'' v3.1.2 (Ronquist and Huelsenbeck 2003; freely available at http://mrbayes.sourceforge.net/download.php) under the GTR+Γ substitution model and the random-addition-sequence method with 10 replicates. We ran two independent analyses of four chains (one cold and three heated) with 20,000,000 cycles, sampled every 1,000th cycle, with an appropriate burn-in (10%, after checking convergence). For the ML calculation, ''RaxML'' v7.2.6 (Stamatakis 2006; freely available at http://www.kramer.in.tum.de/exelixis/software.html) was applied using the GTR + CAT substitution model to search for the best-scoring ML tree and a rapid bootstrap analysis of 1,000 non-parametric replicates. Statistical support values (LBS: ML bootstrap

support, BPP: Bayesian posterior probabilities) were drawn on the resulting, best-scoring ML tree.

RESULTS

Morphology. We herein describe two new dinophyte species and currently assign them to *Scrippsiella* (Thoracosphaeraceae, Peridiniales):

1 *Scrippsiella bicarinata* Zinssmeister, S.Soehner, S.Meier & Gottschling, spec. nov. Type: Mediterranean Sea, off Italy. Lazio, Latina, Formia, 41°15′N, 13°36′E, 17 Apr 2009 [extant]: *M. Gottschling, S. Soehner & C. Zinssmeister ITA00044* [*GeoB 416*] (holotype: CEDiT-2011H18; isotypes: B-40 0040762, M-0178306). Figures 1A–I, 3A.

Latin description: Cellulae oviformae epithecam conicam et hypothecam rotundam habent, 17 usque ad 35 μm longae, 13 usque ad 31 μm latae. Cingulum medium excavatum in media cellula est. Cellulae primam tabulam apicalem angustam habent. Tabularum formula haec: Po, x, 4′, 3a, 7″, 6c, 5s, 5‴, 2⁗. Rotundae cellulae coccoidae, quae corpus rubrum continent, bicarinatae sunt propterea quod tabulae procingulares et tabulae postcingulares tubercula formant. Diametrus est 27 usque ad 37 μm. Paries calcaratus compositus est ex una lamina cum crystallis, quarum axes crystallarum sunt ad perpendiculum. Paries calcaratus intriseus obtectus est strato, quod ex matzeria organica constat. Operculum compositum est ex tabula apicalibus et intercalaribus.

Etymology: The epithet refers to the development of two distinct ridges in the coccoid cells that result from the fusion of pre- and postcingular plate equivalents, respectively.

Distribution: *S. bicarinata* was found in the Mediterranean Sea at coastal sites of Italy and Greece (strains GeoB 411, GeoB*414, GeoB 416, GeoB 453, GeoB 454, GeoB 456, and GeoB 458; see Table S2 for details).

Motile thecate cells (Fig. 1A–D) were predominant in strain GeoB 416, whereas coccoid cells developed after a few weeks and increased slowly in number. The thecate cells were photosynthetically active, variously golden-brown in color and differed greatly in size, ranging from 17 to 35 μm in length (median: 21 μm, SD: 5 μm, $n = 101$) and from 13 to 31 μm in width (median: 19 μm, SD: 4 μm, $n = 101$). The surface was smooth and exhibited some irregularly distributed trichocyst pores. The shape of the thecate cells was spherical through ovoid, with a rounded through conical apex, and consistently showed the plate formula Po, x, 4′, 3a, 7″, 6c, 5s, 5‴, 2⁗. The outlines of the plates were variable, and in some cells additional plates could be observed (Fig. 1D). The 1′ plate was hexagonal and strongly widening in apical direction, whereas the shape was narrow near cingulum and sulcus (Fig. 1, A and D). The excavate cingulum was located in the equatorial plane, took 15–20% of the cell height and was 1–1.5 μm deep. Two flagella originated from the sulcal region (Fig. 1C), which was composed of five plates.

Coccoid cells (Fig. 1E–I) showed a red accumulation body, were spherical and ranged from 27 to 37 μm in length (median: 33 μm, SD: 4 μm, $n = 7$) and from 27 to 33 μm in width (median: 32 μm, SD: 2 μm, $n = 8$). Below the single calcareous layer, an inner organic membrane was present (Fig. 1I). The shell exhibited irregularly thickened processes that corresponded to seven pre- and five postcingular as well as two antapical plate equivalents (Fig. 1E–H). In some cells, the processes were weakly developed, or pre- and postcingular plate equivalents were more or less fused to two distinct ridges (Fig. 1H). Moreover, one through three apical processes at the top of the operculum were present and did not correspond to plate equivalents. The orientation of the crystals and their crystallographic main axis (c-axis) was ''regularly tangential'' (Fig. 3A). The operculum was mesoepicystal compound and consisted of the fused apical plate 2′–4′ and intercalary plate equivalents (Fig. 1E–F).

2 *Scrippsiella kirschiae* Zinssmeister, S.Soehner, S.Meier & Gottschling, spec. nov. Type: Mediterranean Sea, off Italy. Campania, Salerno, Salerno, 40°40′N, 14°46′E, 17 Apr 2009 [extant]: *M. Gottschling, S. Soehner & C. Zinssmeister ITA00040* [*GeoB 408*] (holotype: CEDiT-2011H19; isotypes: B- 40 0040763, M-0178307). Figures 2A–F, 3B–D.

Latin description: Cellulae epithecam conicam et hypothecam rotundam habent, 23 usque ad 40 μm longae, 17 usque ad 37 μm latae. Cingulum medium excavatum in media cellula est. Cellulae primam tabulam apicalem angustam habent. Tabularum formula haec: Po, x, 4′, 3a, 7″, 6c, 5s, 5‴, 2⁗. Rotundae cellulae coccoidae, quae corpus rubrum continent, bicarinatae sunt imaginem cinguli exprimentes. Cellulae 28 usque ad 40 μm longae, 26 usque ad 35 μm latae sunt. Paries calcaratus compositus est ex una lamina cum crystallis, quarum axes crystallarum sunt ad perpendiculum. Paries calcaratus intrinsecus obtectus est strato, quod ex materia organica constat. Operculum compositum est ex tabulis apicalibus et intercalaribus.

Etymology: The species is named in honor of Monika Kirsch, who curates Germany's largest calcareous dinophyte collection at the University of Bremen for many years and who has brought numerous calcareous dinophytes into cultivation, including this one.

Distribution: *Scrippsiella kirschiae* was found in the Mediterranean Sea at coastal sites of Italy and Greece (strains GeoB 408, GeoB 432; see Table S2 for details) and may also be present in Japanese water (*pers. comm.* K. Matsuoka, Nagasaki).

Motile thecate cells of the strain GeoB 408 (Fig. 2A–C) were photosynthetically active and variously golden-brown in color. They showed at

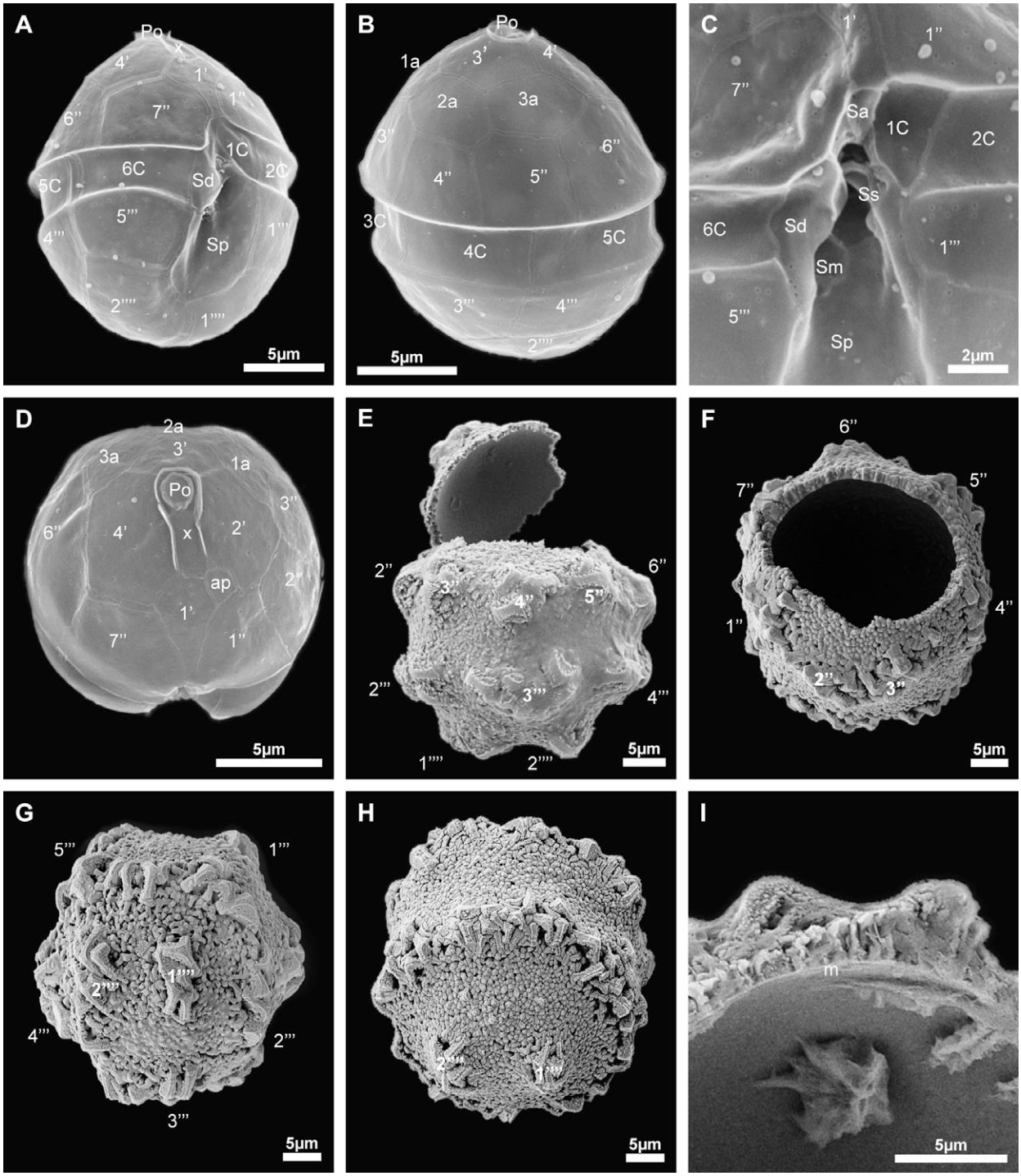

FIG. 1. *Scrippsiella bicarinata* spec. nov. showing an intratabulate tabulation in the coccoid stage as reflection of pre- and postcingular plates (SEM images; A–G: strain GeoB 416, H–I: strain GeoB 411). A, ventro-lateral view of the thecate cell, with epitheca, cingulum, and hypotheca. B, dorsal view of thecate cell, with epitheca, cingulum, and hypotheca. C, ventral detail of thecate cell with the sulcal region exhibiting five plates. D, apical view of the epitheca, with an additional plate between 1′ and 1″. E, empty coccoid cell with mesoepicystal compound operculum; note the tabulation reflected as processes corresponding to seven pre- and five postcingular plate equivalents. F, apical view of empty coccoid cell with mesoepicystal archeopyle and tabulation of seven precingular plate equivalents. G, antapical view of coccoid cell with intratabulate tabulation of five postcingular and two antapical plate equivalents. H, antapical view of coccoid cell with intratabulate tabulation comprising two antapical plate equivalents and pre- and postcingular plate equivalents (the latter fused to two distinct ridges). I, shell ultrastructure of the coccoid cell, with a single calcareous layer and an inner organic membrane. Abbreviations: ap, additional plate; m, inner organic membrane; n', apical plates; n'', precingular plates; n''', postcingular plates; na, anterior intercalary plates; nC, cingular plates; Po, apical pore plate; Sa, anterior sulcal plate; Sd, right sulcal plate; Sm, median sulcal plate; Sp, posterior sulcal plate; Ss, left sulcal plate.

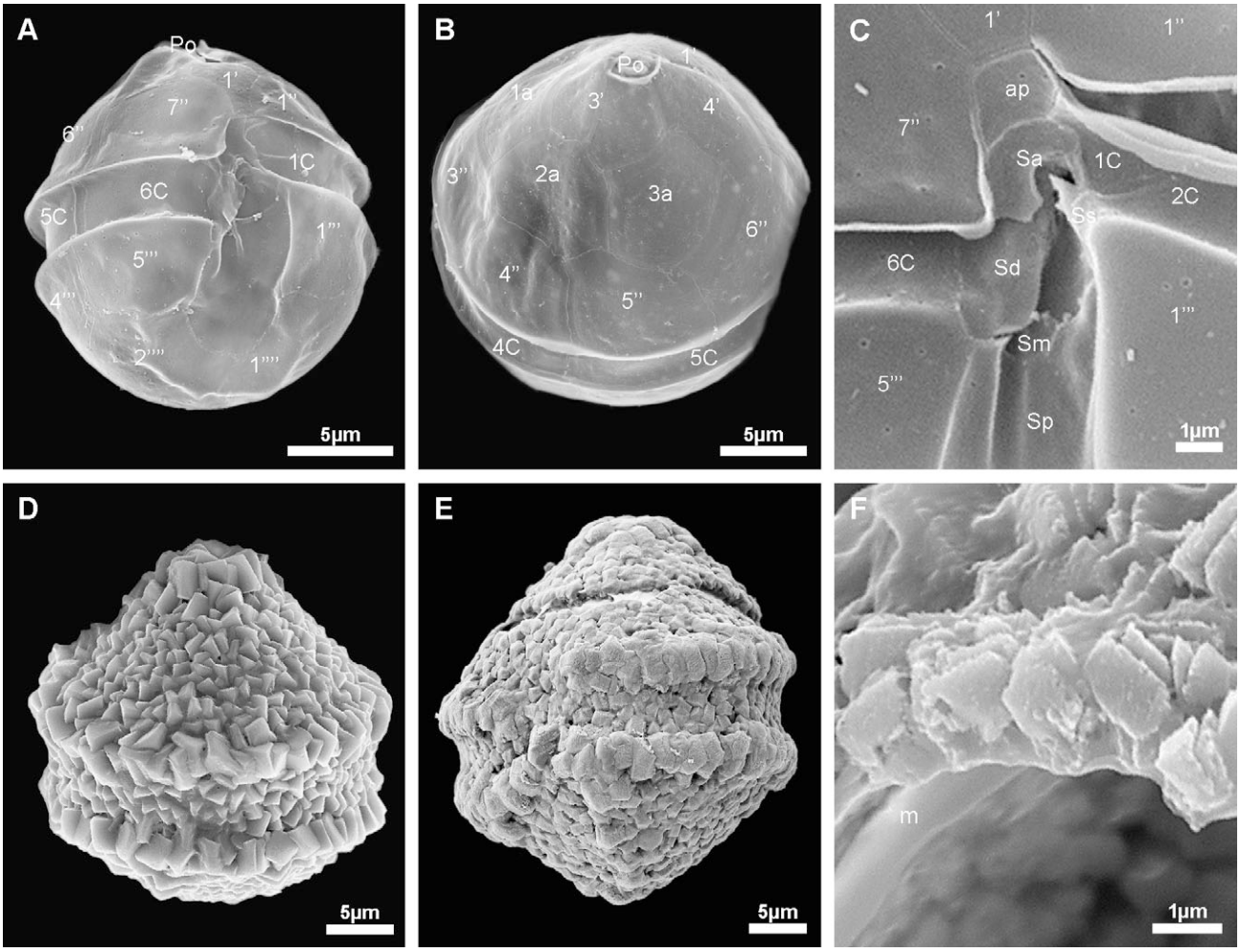

FIG. 2. *Scrippsiella kirschiae* spec. nov. showing a cingulotabulate tabulation in the coccoid stage (SEM images; strain GeoB 408). A, ventral view of thecate cell, with epitheca, cingulum, and hypotheca. B, latero-apical view of thecate cell. C, ventral detail of thecate cell with the sulcal region exhibiting five plates; note the decomposition of plate 1′ into two pieces. D, dorsal view of coccoid cell. E, ventro-lateral view of coccoid cell, with cingulotabulate tabulation reflecting cingular and sulcar sutures as well as the mesoepicystal compound operculum. F, shell ultrastructure of the coccoid cell, with a single calcareous layer and an inner organic membrane. Abbreviations: ap, additional plate; m, inner organic membrane; n', apical plates; n'', precingular plates; n''', postcingular plates; nC, cingular plates; s1′, satellite plate of 1′; Sa, apical sulcal plate; Sd, right sulcal plate; Sm, median sulcal plate; Sp, posterior sulcal plate; Ss, left sulcal plate.

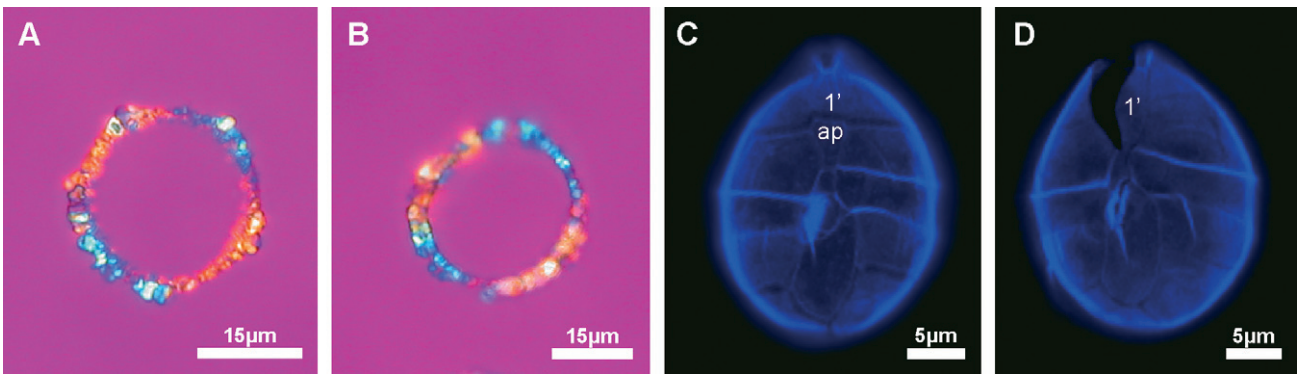

FIG. 3. Cells in polarized and fluorescent light microscopy exhibiting more traits. A–B, optical crystallography of the two new species showing the ''regularly tangential'' ultrastructure type (light microscopy with employed gypsum plate under polarized light, 400× magnification). A, *Scrippsiella bicarinata*. B, *Scrippsiella kirschiae*. C–D, ventral thecate cells of *Scrippsiella kirschiae* (fluorescent light microscopy with calcofluor white). C, indication of 1′ and additional plate close to 1′. D, indication of single 1′ plate.

least two distinct size classes, with most of the cells ranging from 23 to 32 μm in length (median: 28 μm, SD: 3 μm, $n = 19$) and from 17 to 25 μm in width (median: 22 μm, SD: 3 μm, $n = 19$), and the larger cells ranging from 30 to 40 μm in length (median: 36, SD: 3, $n = 5$) and 29 to 37 μm in width (median: 31, SD: 3, $n = 5$). The surface was smooth and exhibited some irregularly distributed trichocyst pores. The shape of the thecate cells was spherical through ovoid, with a rounded through conical apex, and consistently showed the plate formula Po, x, 4′, 3a, 7″, 6c, 5s, 5‴, 2⁗. The 1′ plate was narrowly parallel-sided and rarely widened toward the apex (Fig. 2A). In approximately half of all thecate cells examined, the apical plate 1′ was divided into two pieces (Figs 2C, 3C), and additional precingular plates were observed on the dorsal side in few cases. The excavate cingulum was located in the equatorial plane, took 12–15% of the cell length and was 1–1.5 μm deep. Two flagella originated from the sulcal region (Fig. 2C), which was composed of five plates.

The majority of cells in strain GeoB 408 were coccoid (Fig. 2D–F) and were developed very quickly after establishing a new subculture from solitary thecate cells. They showed a red accumulation body and were ovoid, ranging from 28 to 40 μm in length (median: 34 μm, SD: 5 μm, $n = 6$) and from 26 to 35 μm in width (median: 33 μm, SD: 3 μm, $n = 6$). Below the single calcareous layer, an inner organic membrane was present (Fig. 2F). The epitract was conical and the hypotract rounded, whereas the equatorial region showed a distinct, bicarinate reflection of the cingulum. The imprint of the cingulum was broad, exceeding to one-fourth of the cell length. All ridges observed reflected the sutures between epitheca/cingulum and cingulum/hypotheca, respectively, and any fusion of pre- or postcingular plate equivalents was not observed. The ridges were occasionally interrupted at the ventral side and the imprint of the sulcus, whose outline then was also reflected by a ridge (Fig. 2E). The calcareous crystals were massive and predominantly rhombohedral (Fig. 2D). The orientation of the crystals and their crystallographic main axis (c-axis) was ''regularly tangential'' (Fig. 3B). The operculum was mesoepicystal compound and consisted of the fused apical plate 2′–4′ and intercalary plate equivalents (Fig. 2E).

Phylogenetic analysis. The alignment consisting of three different molecular loci covered 2,572 bp in total length, whereas 830 positions were parsimony informative (32.3%, 18.9 per terminal taxon). The ITS region comprised 743 bp and 428 informative sites (57.7%, 9.7 per terminal taxon), the first two domains of the LSU exhibited 785 bp and 251 informative positions (32%; 5.7 per terminal taxon), and the alignment of *cob* sequences were 1,044 bp long, with 151 informative positions (14.5%; 3.4 per terminal taxon). Separate analyses of the three partitions did not render conflicting and highly supported tree topologies, indicating that concatenated analyses were not perturbed by divergent locus evolution.

Figure 4 shows the best-scoring ML tree (–ln = 21,101.519651) with *Scrippsiella s.l.* retrieved as monophyletic (100LBS, 1.00BPP). *Scrippsiella s.l.* segregated into a number of lineages, including *Pernambugia tuberosa*, *S. lachrymosa* Lewis, *Calciodinellum,* and its relatives (i.e., the CAL clade: 65LBS, 1.00BPP), *S. precaria* Montresor & Zingone and its relatives (i.e., the PRE clade: 100LBS, 1.00BPP), as well as the *Scrippsiella trochoidea* (F.Stein) A.R.Loebl. species complex (STR-SC; 50LBS). Major clades of the STR-SC were STR1 (100LBS, 1.00BPP), STR2 (100LBS, 1.00BPP), and STR3 (100LBS, 1.00BPP). The two new species *Scrippsiella bicarinata* and *S. kirschiae* sampled with multiple strains were each monophyletic (and maximally supported). Together (albeit with low statistical support), they were closely related to the STR3 clade (59LBS) and constituted a monophyletic group (100LBS, 1.00BPP) also including the STR2 clade.

DISCUSSION

The diversity of extant Thoracosphaeraceae is known to a limited extent only. A series of taxa firstly discovered in the fossil record has been later shown to have stratigraphic occurrences into the late Pleistocene, or are today even known from recent sediments (Wall and Dale 1968, Versteegh 1993, Montresor et al. 1994). Many of such ''living fossils'' (Wall and Dale 1966), however, have not been established in culture so far for contemporary morphological and molecular investigations. Despite numerous studies that investigated the diversity of calcareous dinophytes in the Mediterranean Sea (Montresor et al. 1994, 1998, Meier et al. 2003), only one of the species described here as new has been probably illustrated in Satta et al. (2010: pl. 2 h), but the authors do not provide a scientific name. The discovery of two new species in one of the best-studied regions in the world underlines that a hidden diversity of still unknown calcareous dinophytes exists.

General morphologies of the motile cells and thecal plate patterns of the new species described here do not differ from other species that have been described under *Scrippsiella s.l.* They can be distinguished from other peridinoid dinophytes (such as *Pentapharsodinium*, *Peridinium* Ehrenb., *Protoperidinium* Bergh, and others) based on the presence of six cingular plates, thus showing two cingular sutures in mid-dorsal view of the motile cells (Fine and Loeblich 1976, Dale 1977, 1978). The globose shapes of the thecate cells in the new species rather correspond to those of, for example, *Calciodinellum operosum* and *Scrippsiella rotunda* Lewis than to the more conical epitheca of *Scrippsiella trochoidea* (Lewis

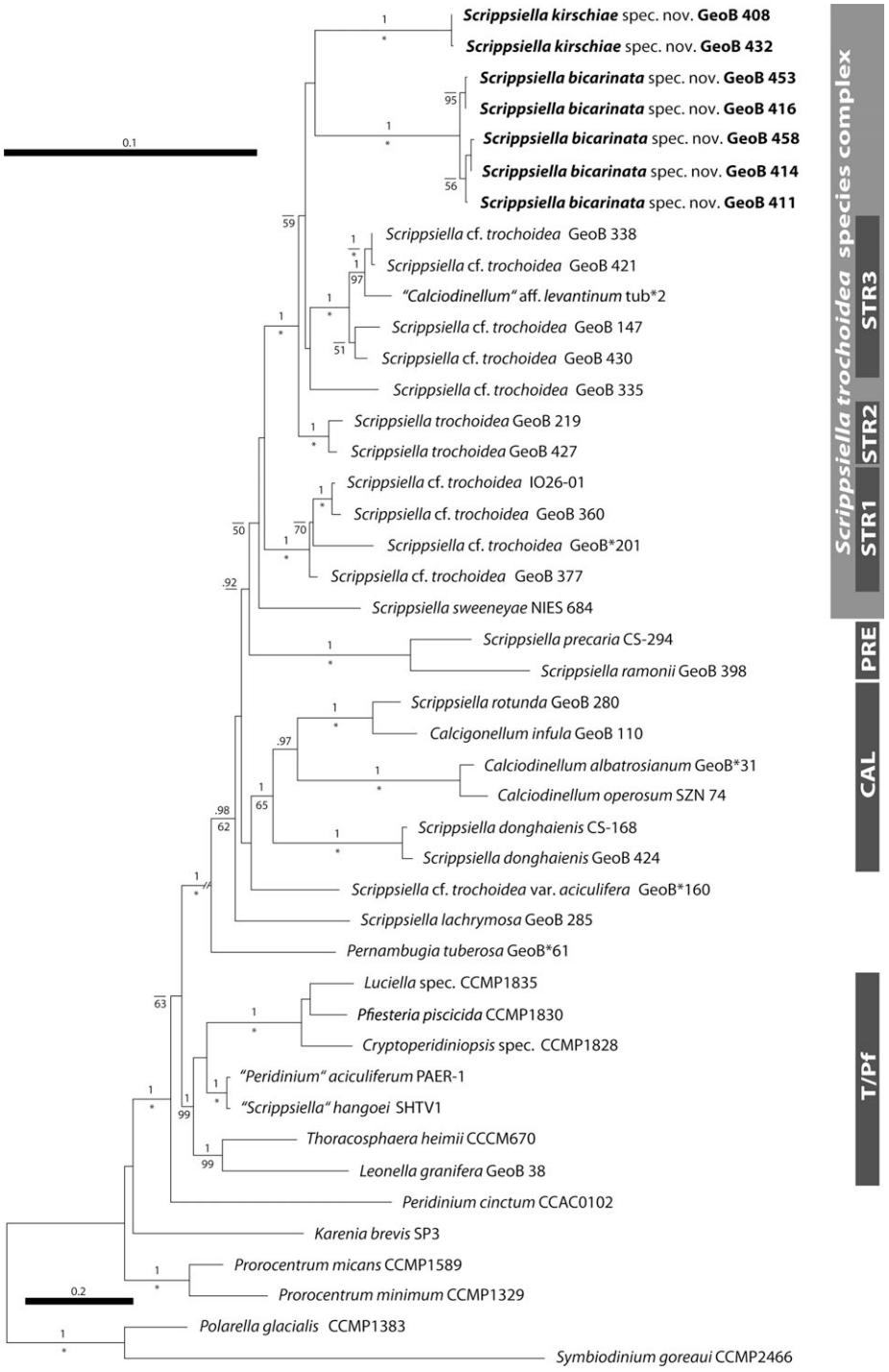

FIG. 4. *Scrippsiella bicarinata* and *Scrippsiella kirschiae* as distinct species within the *Scrippsiella trochoidea* species complex. Maximum likelihood (ML) tree (–ln = 21,101.519651) of 44 dinophyte strains as inferred from a MAFFT generated nucleotide alignment, comprising the complete ITS region, the LSU domains 1 + 2 and *cob* (in total 830 parsimony-informative positions). Major clades are indicated, and new species are highlighted in bold. Branch lengths are drawn on scale, with the scale bar indicating the number of substitutions per site. Numbers on branches are statistical support values (above: Bayesian posterior probabilities, values under .90 are not shown; below: ML bootstrap support values, values under 50 are not shown) and maximal support values are indicated by asterisks. The tree is rooted with seven members of the T/Pf-clade (Thoracosphaeraceae) as well as six dinophyte species belonging to the Gymnodiniales, Peridiniales, and Prorocentrales.

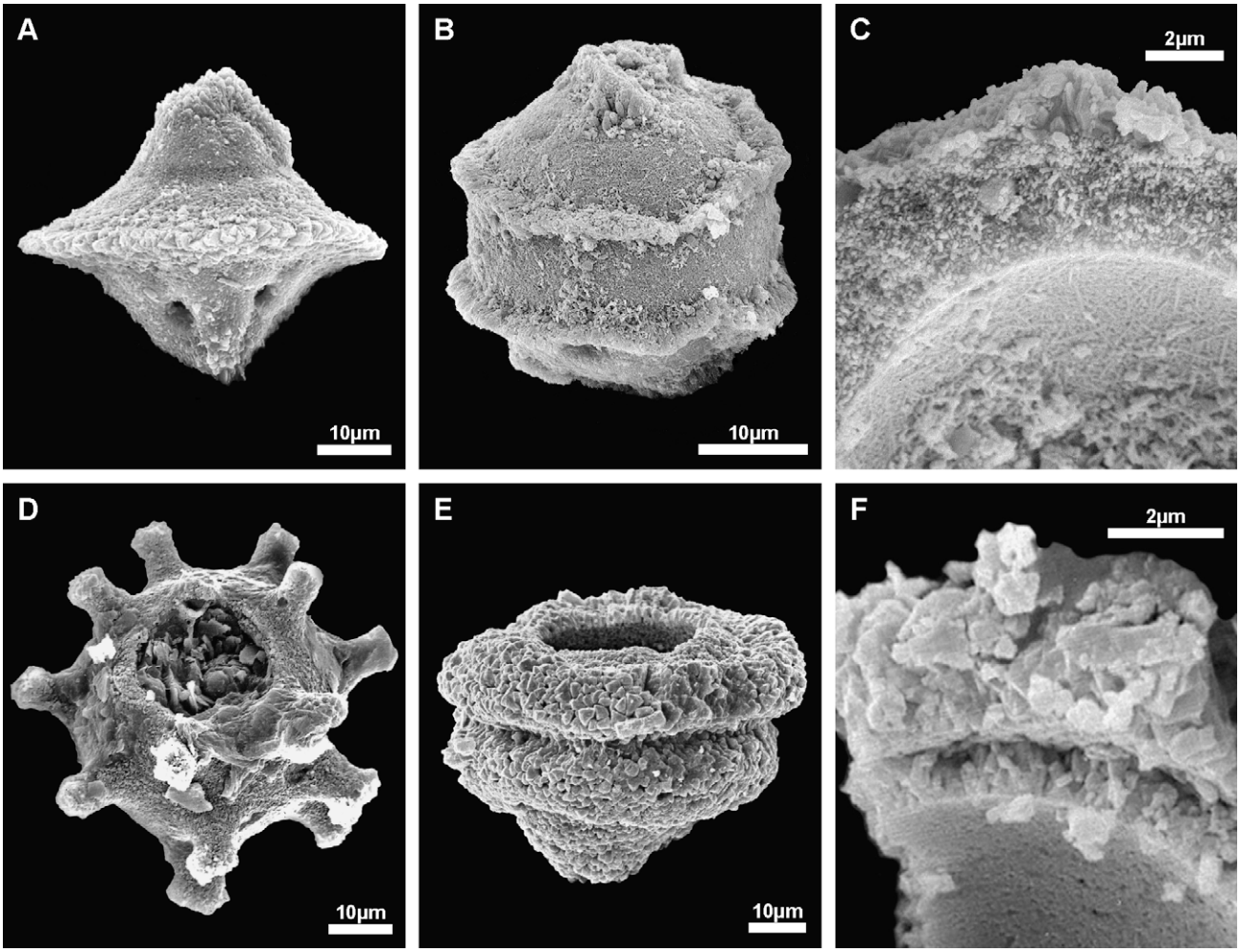

FIG. 5. The two new species do not fit in the circumscription of any known calcareous dinophyte. A, coccoid cell of *Carinellum parasole* Keupp (Lutetian, from Deflandre's original material) as example of a monocarinate form. B–C, *Bicarinellum castaninum* Deflandre, 1949 (Lutetian, from Deflandre's original material), with a single microgranular calcareous layer and additional coarser crystals inducing the tabulation (presumably corresponding to a second vestigial layer). B, Coccoid cell. C, Detail of the shell ultrastructure. D. *Alasphaera tuberculata* (Pflaumann & Krasheninnikov) Keupp (Hauterivium) as example with intratabulate tabulation. E. *Bitorus turbiformis* Keupp (Berriasian/Valanginian boundary interval) as bicarinate example with 3A archeopyle. F. Shell ultrastructure of *Bicarinellum jurassicum* (Deflandre) Keupp (Oxfordian, from Deflandre's original material) showing two distinct calcareous layers.

1991, Montresor et al. 1997, Zinssmeister et al. 2011). Intraspecific variability and occasional deviations from the regular plate formula has been previously shown for some *Scrippsiella* strains in cultivation (D'Onofrio et al. 1999, Gottschling et al. 2005b).

It is particularly the morphology of the coccoid stages that exhibits diagnostic characters for species delimitation within calcareous dinophytes. At a first glance, both the two new species are similar to those of *Bicarinellum* from the Mesozoic and Paleogene. In its current circumscription, *Bicarinellum* is considered extinct since 50 Ma (Willems 1988), and not only the gap in the fossil record intercedes for the distinctiveness of the new species from any known member of the Thoracosphaeraceae. The two new species belong to the relatively few calcareous dinophytes that exhibit more than the archeopyle as tabulation in their coccoid cells. They can be easily delimited from more or less holotabulate forms such as *Calciodinellum operosum* (Keupp 1984, Montresor et al. 1997) because of the absence of a complete tabulation pattern.

If ridges, reflecting the cingulum only, represent the tabulation (the ''cingulotabulate'' state in a broad sense: Sarjeant 1982), then their number is consistently either one or two within a particular species. As both new species always exhibit two ridges, the distinctiveness to tricarinate (i.e., species of *Posoniella* Streng, Banasová, Reháková & H.Willems, in which the equatorial ridge represents the cingulum: Streng et al. 2009) and such monocarinate forms as *Calcipterellum* Keupp (Keupp 1984), *Carinasphaera* (Kohring 1993b), *Carinellum* (Keupp 1981,

1984, Fig. 5A), and *Dimorphosphaera* Keupp (Keupp 1979) is, therefore, likewise evident. The only extant cingulotabulate species described so far is *Pirumella irregularis* (Akselman & Keupp) G.L.Williams, Lentin & Fensome (=*Scrippsiella patagonica* Akselman & Keupp: Akselman and Keupp 1990). Later light microscopic re-investigations, however, have shown that coccoid cells are probably not calcareous (high optical refraction of the cells indicates starch rather than calcium carbonate; unpublished data). Moreover, some protologue figures of the supposed coccoid cells show flagella that are never present in immotile stages. *Pirumella irregularis* thus may represent thecate cells of weak preparation, and the species is therefore not further considered for diagnostic purposes here.

Species with two cingular ridges have so far been described in *Bicarinellum* (Fig. 5B–C, F) and *Bitorus* (Fig. 5E), and the two ridges are considered to originate from the fusion of the corresponding pre- and postcingular plate equivalents (Keupp 1984, Willems 1988, Kienel 1994, Hildebrand-Habel and Willems 1999). They are, therefore, not cingulotabulate in a strict sense (Sarjeant 1982), and some degree of transition to an intratabulate type (as present in, e.g., *Alasphaera*: Keupp 1981, Fig. 5D) has occasionally been reported (in, e.g., *Bicarinellum jurassicum*: Keupp 1984). In the new species *S. bicarinata*, such transitions are clearly in the range of an intraspecific variability (even within a single cultivated strain), and this observation might also be applicable for fossil species. Both new species, however, can be further delimitated from all other bicarinate and/or intratabulate calcareous dinophytes based on additional character traits (as far as such traits are preserved in the fossils).

Operculum morphology appears consistent within species (Keupp and Versteegh 1989, Streng et al. 2004), and the bicarinate and/or intratabulate species described so far have apical archeopyles, corresponding either to a single plate equivalent (in species of *Alasphaera*: Fig. 5D and *Bicarinellum*) or to three articulating plate equivalents of the apical series (in species of *Bitorus*; Keupp 1992, Streng et al. 2004). To the contrary, both the new species have mesoepicystal combination opercula. This type is today found in species of *Calciodinellum* and *Scrippsiella* (Montresor et al. 1997, Streng et al. 2004, Zinssmeister et al. 2011), which in turn do not include bicarinate coccoid stages as known so far.

The ultrastructure of the shell has importance for species delimitation and phylogenetic reconstructions (Keupp 1981, Kohring et al. 2005, Meier et al. 2009). Forms with two calcareous layers (Fig. 5F) predominate in the Mesozoic, whereas single-layered species (Fig. 5C) are most frequent since the Paleogene. In such terms, the new species fit well in this evolutionary scenario. Moreover, the bicarinate and/or intratabulate species described so far either show irregularly (*Alasphaera*, *Bicarinellum*) or regularly arranged crystals (*Bitorus*) constituting the calcareous shells, whereas optical crystallography has not been worked out for those species yet. *Bitorus* may exhibit the ''regularly radial'' type, which would be distinct from both new species. They are, thus, the only bicarinate representatives of the Thoracosphaeraceae known so far evidentially with the ''regularly tangential'' type, as it is today found in such taxa as *Calciodinellum* and *Scrippsiella* (Montresor et al. 1997, Janofske 2000, Hildebrand-Habel 2002). The systematic investigation particularly of more fossil species from, for example, *Bicarinellum* and *Bitorus* would allow for a better conclusion about the diagnostic relevance of this character trait (Meier et al. 2009).

Stratigraphic occurrences may also be indicative for species delimitation. In the fossil record, species of *Bicarinellum* and *Bitorus* (furthest resembling the two new species morphologically) are firstly abundant in the Upper Jurassic and Lower Cretaceous (e.g., *Bicarinellum jurassicum*, *Bitorus turbiformis*). However, *Scrippsiella s.l.*, including the two new species, has come into existence in the Late Cretaceous as inferred from a dating study (Gottschling et al. 2008). There is, moreover, a gap in the fossil record of more than 40 Ma (Willems 1988) to bicarinate species known since the Paleogene (e.g., *Bicarinellum castaninum*, *Bitorus bulbjergensis* Kienel). It is, therefore, highly unlikely that *Scrippsiella bicarinata* and *S. kirschiae* are associated with the Mesozoic bicarinate forms. The youngest bicarinate fossils date back to the Priabonian (Hildebrand-Habel and Willems 1999), still leaving a record gap of approximately 35Ma to the extant species described here as new. It is again unlikely that *S. bicarinata* and *S. kirschiae*, or putative relatives, have been overlooked in the numerous taxonomic studies about Neogene calcareous dinophytes and that they are direct descendents of known and already described fossil forms.

Today, phylogenetic relationships and systematic positions can be inferred from the comparison of molecular sequence data. The evolution of the Dinophyceae is generally difficult to reconstruct, and analyses of multi-loci alignments have been proposed to improve phylogenetic trees (Zhang et al. 2007, Hoppenrath and Leander 2010, Gottschling et al. 2012, Tillmann et al. in press). The existence of the *Scrippsiella s.l.* clade, however, has been repeatedly shown in molecular phylogenies (Montresor et al. 2003, Gottschling et al. 2005b, Gu et al. 2011), and our three loci-approach for phylogenetic inference provides slightly improved supports for a number of nodes. The monophyly of *Scrippsiella s.l.* correlates with the presence of a mesoepicystal compound archeopyle that is thus considered the most striking morphological apomorphy of the clade (Streng et al. 2004, Gottschling et al. 2008). This character trait is also present in *S. bicarinata* and *S. kirschiae*, accounting for their correct systematic

position within *Scrippsiella s.l.* Both the new species appear closely related as inferred from molecular data and are nested within the core clade of STR-SC including STR2 and STR3. From an evolutionary perspective, the bicarinate species of *Scrippsiella s.l.* may thus derive from such forms with spiny coccoid cells as *Scrippsiella trochoidea* (Zinssmeister et al. 2011).

In conclusion, *S. bicarinata* and *S. kirschiae* are distinct from all known members of the Thoracosphaeraceae as inferred from morphological, molecular, and stratigraphical data. The two new species are, moreover, distinct also from each other. *Scrippsiella bicarinata* is the only species with the combination of the characters (i) tabulation in the coccoid cells with fusion of pre- and postcingular plate equivalents, (ii) mesoepicystal combination archeopyle, and (iii) single calcareous layer constituting the coccoid shell. *Scrippsiella kirschiae* is the only species with the combination of the characters (i) cingulotabulation in the coccoid cells, (ii) mesoepicystal combination archeopyle, and (iii) single calcareous layer constituting the coccoid shell. Homoplasy of character traits appears as major issue in calcareous dinophytes, and complex studies are necessary for reliable conclusions. Discovering the morphological and molecular diversity of the Thoracosphaeraceae, and inferring their evolutionary history, thus remain a tantalizing field in contemporary phycology.

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Supporting Information

The following supporting information is available for this article:

Table S1. Primer list. Abbreviations: fw, forward; rev, reverse.

Table S2. Species list. DNA-numbers follow our internal numbering code (abbreviations: GB, GenBank number; n.i., not indicated).

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PAPER 2 (MANUSCRIPT)

WAKING THE DEAD: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF EXTANT *POSONIELLA TRICARINELLOIDES* (THORACOSPHAERACEAE, DINOPHYCEAE)

Gu, H., Kirsch, M., Zinssmeister, C., **Soehner, S.**, Meier, K.J.S., Liu, T. & Gottschling, M. (in press) Protist

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Abstract: The Thoracosphaeraceae are dinophytes that produce calcareous shells during their life history, whose optical crystallography has been the basis for the division into subfamilies. To investigate the validity of the classification mainly applied by paleontologists, living material of phylogenetical key species is necessary albeit frequently difficult to access for contemporary morphological and molecular analyses. We isolated and established five living strains of the rare and fossil-based species †Posoniella tricarinelloides from different sediment samples collected in the South China Sea, Yellow Sea and in the Mediterranean Sea (west coast off Italy). Here we provide detailed descriptions of its morphology and conducted phylogenetic analyses based on hundreds of accessions and thousands of informative sites implemented in concatenate sequences of the ribosomal RNA region. Within the monophyletic Peridiniales, †P. tricarinelloides was reliably nested in the Thoracosphaeraceae and exhibited two distinct morphological types of coccoid cells. The two morphs of coccoid cells would have been assigned to different taxa at the subfamily level if found separately in fossil samples. Our results thus challenge previous classification concepts within the dinophytes and underline the importance of comparative morphological and molecular studies to better understand the complex biology of unicellular organisms such as †P. tricarinelloides.

Waking the dead: Morphological and molecular characterization of extant †*Posoniella tricarinelloides* (Thoracosphaeraceae, Dinophyceae)

(Original paper)

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Abstract

The Thoracosphaeraceae are dinophytes that produce calcareous shells during their life history, whose optical crystallography has been the basis for the division into subfamilies. To investigate the validity of the classification mainly applied by paleontologists, living material of phylogenetical key species is necessary albeit frequently difficult to access for contemporary morphological and molecular analyses. We isolated and established five living strains of the rare and fossil-based species †*Posoniella tricarinelloides* from different sediment samples collected in the South China Sea, Yellow Sea and in the Mediterranean Sea (west coast off Italy). Here we provide detailed descriptions of its morphology and conducted phylogenetic analyses based on hundreds of accessions and thousands of informative sites implemented in concatenate sequences of the ribosomal RNA region. Within the monophyletic Peridiniales, †*P. tricarinelloides* was reliably nested in the Thoracosphaeraceae and exhibited two distinct morphological types of coccoid cells. The two morphs of coccoid cells would have been assigned to different taxa at the subfamily level if found separately in fossil samples. Our results thus challenge previous classification concepts within the dinophytes and underline the importance of comparative morphological and molecular studies to better understand the complex biology of unicellular organisms such as †*P. tricarinelloides*.

Keywords: calcareous dinoflagellates, cyst, distribution, molecular systematics, theca, ultrastructure.

Introduction

The unicellular dinophytes have great ecological importance and are one of the major groups of the phytoplankton occurring worldwide in nearly all marine and freshwater habitats (Fensome et al. 1993; Steidinger and Tangen 1996). They exhibit many types of life styles and nutrition modes and encounter 2,000 extant and 2,500 fossil-based species (Taylor et al. 2008). Molecular phylogenies of dinophytes are still not satisfying because of various problems including the limited taxon sample (less than a quarter of dinophytes at the generic level are currently known with respect to genetic sequence data), insufficient genetic data (abundant single locus analyses), simple phylogenetic methodology (such as parsimony and neighbor-joining) and strong rate heterogeneity. Concatenation of molecular sequences (particularly those of the intensely-studied rRNA region comprising the small subunit: SSU, the 5.8S rRNA including the internal transcribed spacers: ITSs and the large subunit: LSU), results in improved phylogenetic trees (Gottschling et al. 2012; Gottschling and McLean in press; Hoppenrath and Leander 2010; Orr et al. 2012; Tillmann et al. 2012; Zhang et al. 2007) that may serve as a taxonomic backdrop for the precise systematic placement of particular dinophytes. Some of the morphologically well recognizable groups, including the Suessiales, Dinophysiales and Gonyaulacales, have been long-standing and are monophyletic based on molecular data (Daugbjerg et al. 2000; Saldarriaga et al. 2004). Such groups as the Peridiniales and Prorocentrales are more problematic: They are morphologically well circumscribed, but are –if at all– monophyletic in concatenated sequence analyses only (Tillmann et al. 2012; Zhang et al. 2007).

The availability of dinophytes for contemporary investigations on morphology, molecular phylogenetics, and life history is limited because of the necessity to cultivate strains for corresponding studies. This is also true for the Thoracosphaeraceae (Peridiniales), which are dinophytes that produce calcareous shells during their life history. They have a high potential

to fossilize in marine sediments and are therefore abundant in the fossil record, playing an important role for paleo-environmental reconstructions (Hildebrand-Habel and Streng 2003; Meier et al. 2004; Richter et al. 2007; Versteegh 1997; Masure and Vrielynck 2009; Zonneveld et al. 2005). A number of fossil-based Thoracosphaeraceae have stratigraphic occurrences into the late Pleistocene or are even known from Recent sediments (Elbrächter et al. 2008; Montresor et al. 1994, 1998; Versteegh 1993), but few of them have been brought into cultivation so far. Such extant, but taxonomically fossil-based taxa include currently such unavailable though phylogenetically striking species as †*Caracomia arctica* (M.W.Gilbert & D.L.Clark) Streng, Hildebrand-Habel & H.Willems, †*Melodomuncula berlinensis* G.Versteegh and †*Posoniella tricarinelloides* (G.Versteegh) Streng, Banasová, D.Reháková & H.Willems.

Most calcareous dinophytes develop two different stages during their life history, namely a motile thecate cell and a non-motile calcareous coccoid cell (usually described as 'cyst': Elbrächter et al. 2008; Pfiester and Anderson 1987; von Stosch 1973). Coccoid cells are considered the diploid life history stage for the majority of calcareous dinophytes [e.g., *Calciodinellum levantinum* (Kamptner) Janofske & Karwath: Meier et al. 2007], while thecate cells are presumably haploid and frequently reproduce vegetatively (Chambouvet et al. 2011; D´Onofrio et al. 1999; Montresor et al. 1998). Thecate cells exhibit a more or less taxon specific pattern of cellulose plates (that are designated based on the Kofoid system: Taylor 1980) and have therefore great systematic importance for dinophytes (Fensome et al. 1993). The thecate tabulation can be imprinted also in the coccoid stage in form of distinct ridges or sutures.

The potential to produce calcareous structures is restricted to (i.e., has been considered apomorphic for) the Thoracosphaeraceae (Elbrächter et al. 2008; Janofske 1992; Wall and Dale 1968b). In molecular trees, the Thoracosphaeraceae are monophyletic (including also

secondarily non-calcareous taxa) and segregate into three lineages (Gottschling et al. 2005a, 2012), namely the E/Pe-clade (including *Ensiculifera* Balech and *Pentapharsodinium* Indel. & A.R.Loebl.), the T/Pf-clade (including *Thoracopsphaera* Kamptner and *Pfiesteria* Steid. & J.M.Burkh.) and *Scrippsiella* Balech ex A.R.Loebl. *sensu lato* (*s.l.*). The morphological circumscription of calcareous dinophytes is based on size and shape of the coccoid cell (including –if present– tabulations and spines), archeopyle morphology and shell ultrastructure. For classification, the orientation of the crystallographic c-axis of the calcitic crystals constituting the shell has been considered to be of key importance (Elbrächter et al. 2008; Janofske 1996; Keupp 1991; Kohring et al. 2005; Meier et al. 2009; Streng et al. 2004). Three different types of shell ultrastructure are readily distinguished (and assigned to corresponding subfamilies), namely a type with irregularly arranged ('oblique') crystals (e.g., †*Calcicarpinum tetramurum* Kienel: Hildebrand-Habel 2002), a regular type with radial c-axis orientations (e.g., †*Caracomia stella* Streng, Hildebrand-Habel & H.Willems: Streng et al. 2002) and a regular type with tangential c-axis orientations (e.g., species of †*Calciodinellum* Defandre and *Scrippsiella*: Janofske 1996, 2000; Zinssmeister et al. 2012).

Linking motile and coccoid stages of the same species goes back to the pioneering work of Wall and Dale (1966, 1968a), who have performed the first cultivation experiments with calcareous dinophytes from environmental sediment samples. Since then, a small number of fossil-based taxa has been maintained in cultivation, including †*Calcicarpinum bivalvum* G.Versteegh [=*Pentapharsodinium tyrrhenicum* (Balech) Montresor, Zingone & D.Marino: Montresor et al. 1993], †*Calciodinellum operosum* Deflandre (Montresor et al. 1997), †*Calcigonellum infula* Deflandre (D´Onofrio et al. 1999), †*Leonella granifera* (Fütterer) Janofske & Karwath and †*Pernambugia tuberosa* (Kamptner) Janofske & Karwath (Janofske & Karwath in Karwath 2000). Coastal habitats are rather rarely investigated with respect to the presence of calcareous dinophytes (Soehner et al. 2012), and more fossil-based calcareous

dinophytes are to be expected specifically in such environments (Elbrächter et al. 2008; Montresor et al. 1998; Zinssmeister et al. 2012).

Geologically, †*P. tricarinelloides* is known from the Miocene and late Pliocene of Cyprus and the Vienna Basin (Bison et al. 2007; Streng et al. 2009; Versteegh 1993). The species is also reported from Recent sediments of the West Atlantic (Wall and Dale 1968a), the Mediterranean Sea (Meier et al. 2009; Montresor et al. 1994, 1998; Rubino et al. 2010) and the (deep) South China Sea (Gu et al. 2011). †*Posoniella* Streng, Banasová, Reháková & H.Willems is assigned to the forms with an irregular orientation of the shell-forming crystals in the paleontological classification of calcareous dinophytes (Meier et al. 2009; Versteegh 1993), whose extant members are imperfectly known at present. Germination experiments of †*Posoniella* have not been successful until now, and we here firstly report from five strains identified as †*P. tricarinelloides* and isolated from different sediment samples collected in the South China Sea, the Yellow Sea and the Mediterranean Sea. We provide descriptions of the motile and coccoid cells using light and scanning electron microscopy (SEM) and use concatenated ribosomal RNA sequences to clarify the phylogenetic position of this species.

Results

Morphology

Five different strains of †*P. tricarinelloides* each comprising thecate cells as well as coccoid cells of two distinct types were established, namely GeoB 410 and GeoB 413 from the coast off Formia, Tyrrhenian Sea (41°15'18.90"N, 13°36'29.30"E; April 2009), GeoB 429 from the coast off Gallipoli, Ionian Sea (40°3'25.62"N, 17°58'57.42"E; April 2009), PTFC01 from the South China Sea off Fangchenggang (21°29'58.38"N, 108°13'52.02"E; May 2011) and PTLY01 from the Yellow Sea off Lianyungang (34°48'45.77"N, 119°31'37.64"E; May 2011). Mediterranean strains are currently held in the culture collections at the Institute of Historical Geology / Palaeontology (University of Bremen, Germany) and the Institute of Systematic Botany and Mycology (University of Munich, Germany), while Chinese strains are maintained at the Third Institute of Oceanography, State Oceanic Administration (Xiamen, China), and are available upon request.

Material of strain PTLY01 from China was representative for the other strains established, and corresponding material was investigated in detail. Thecate cells of †*P. tricarinelloides* (Figs 1A–F,J, 2) divided vegetatively, and the process lasted approximately 12 min. They were 20.0–25.5 µm long (mean=21.80 ± 1.52 µm, n=50) and 15.5–20.5 µm wide (mean=18.26 ± 1.43 µm, n=50), with a median length:width ratio of approximately 1.2. Cells were compressed slightly in dorso-ventral view. The epitheca was hemispherical, and the hypotheca was conical. The Kofoidean plate formula was Po, X, 4′, 1a, 7″, 6C, 5S, 5‴, 2⁗. In light microscopy (Fig. 1A), chloroplasts were visible in the periphery of the cell, with several stalked pyrenoids. The nucleus was elongated and located in the central part of the cell.

The apical pore complex (Po) consisted of a round apical pore plate and a long canal plate (X) (Fig. 1B). The pore plate was raised to form an apical horn in some cells. The first apical plate (1′) was one-third in wide compared to its length and slightly asymmetrical (Figs 1J, 2A). The equatorially located cingulum was deep and wide and displaced approximately half of the cingulum width. The cingulum was composed of six plates of unequal size. The first one is rather narrow and was a transitional plate (1C) (Figs 1C,J, 2C). The second, third and sixth cingular plates were similar in size and narrower than the other two cingular plates (Figs 1D,F, 2B).

A single intercalary plate was present on the dorsal part of the epitheca. It was pentagonal and narrow (Figs 1D, 2A–B), but occasionally rectangular in shape (Fig. 1F). The intercalary plate was usually located between the plates 3' and 4' (in 16 out of 20 cells investigated; Figs 1F, 2A), but occasionally observed between the plates 2' and 3' (in 4 out of 20 cells). The sulcus consisted of an anterior sulcal plate (Sa), a median sulcal plate (Sm), a right sulcal plate (Sd), a left sulcal plate (Ss) and a posterior sulcal plate (Sp) (Fig. 1C). The plate Sp did not contact the cingulum directly. The two antapical plates were pentagonal and were of equal size (Figs 1E, 2D).

Two distinct types of coccoid cells (Figs 1G–H,K–L, 3) were observed in the strains of GeoB 410, GeoB 413, GeoB 429 and PTLY01 (Fig. 1H). To exclude the possibility of contaminations, 60 coccoid cells of GeoB 429 with the distinct morphology consistent with the type of †*P. tricarinelloides* were isolated and subsequently cultivated separately. Twenty-four of those isolated cells germinated and produced initially only thecate cells. After three weeks through three months, the monoclonal strains started to develop the two distinct types again.

Morphologically, the first type was represented by cells dark-brown in light microscopy (Fig. 1G) and with a *Posoniella*-like morphology (Fig. 3B–E). The cells were 19.5–50.1 μm long

(median: 39.9 ± 4.6 µm, n=43) and 33.6–52.3 µm wide (median: 44.0µm ± 4.7 µm, n=78) and showed a red accumulation body. Two transversal ridges of variable thickness were present on the anterior part of the cell (Fig. 3B–C), whereas other sutural ridges on the hypocyst delimited two antapical paraplates and the parasulcal area (Fig. 3D). A small, apical archeopyle was present (Fig. 3C). The calcareous shell consisted of needle like crystals that showed an 'oblique' arrangement of the shell forming crystals (Figs 1K, 3E). The innermost crystals were oriented regularly (Fig. 3E), with a tangential orientation of the crystallographic c-axes of the crystals (Fig. 1K). The second type of coccoid cells (Figs 1H, 3F) showed a red accumulation body and was about the same size as the first type. The cells were spherical and lighter in light microscopy than the first type. Cells had an outer diameter of 27.6–39.3 µm (median: 34.1 ± 2.5µm, n=99). The surface was smooth without any ornamentation such as ridges or sutures, and a small, apical archeopyle was likewise present (Fig. 3F). The crystals forming the shell were regularly arranged. However, the optical crystallography was different from the *Posoniella*-like cells and exhibited a radial orientation of the crystallites under polarized light (Fig. 1L).

Molecular phylogenetics

The Dinophyceae alignment was 5,499 bp long and comprised 3,089 parsimony informative sites (56%, 9.56 per terminal taxon). Tree topologies were largely similar, independently whether the Bayesian or the ML algorithm was applied. Many nodes showed high if not maximal statistical support values. Figure S1 shows the best-scoring ML tree (–ln= 15,5585.26), with the Dinophyceae retrieved as monophyletic (100LBS: ML bootstrap support, 1.00BPP: Bayesian posterior probabilities). They segregate in a number of more or less established taxonomic units such as the Dinophysiales (100LBS, .97BPP), Gonyaulacales (59LBS), Prorocentrales and Suessiales (99LBS, .97BPP; the "Gymnodiniales" were not

monophyletic). The Peridiniales were likewise retrieved as monophyletic group (although with low support values) and were the taxonomic focus of the second analysis.

The SSU+ITS+LSU alignment focusing on the Peridiniales was 4,568 bp long and comprised 1,381 parsimony informative sites (30%, 18.41 per terminal taxon). Tree topologies were largely congruent, independently whether the Bayesian or the ML algorithm was applied. Many nodes showed high if not maximal statistical support values. Figure 4 shows the best-scoring Bayesian tree, in which the Peridiniales were monophyletic (100LBS, 1.00BPP) and segregated into a number of lineages such as *Heterocapsa s.l.* (100LBS, 1.00BPP), *Peridiniopsis s.l.* (99LBS, 1.00BPP) and *Peridinium s.str.* (100LBS, 1.00BPP). The Thoracosphaeraceae were likewise monophyletic (although with low statistical support) and consisted of the clades E/Pe, T/Pf (96LBS, 1.00BPP) and *Scrippsiella s.l.* (89LBS, .96BPP), whereas the latter two clades showed a close relationship (100LBS, 1.00BPP). The different strains of †*Posoniella* established from Mediterranean and Chinese localities were almost identical in their rRNA sequences. As inferred from the phylogenetic analyses, †*Posoniella* doubtlessly belonged to the T/Pf-clade comprising calcareous (*Leonella*, *Thoracosphaera*) and non-calcareous taxa (e.g., *Cryptoperidiniopsis*, *Luciella*, *Pfiesteria*) as well, whereas the latter group was monophyletic (96LBS, 1.00BPP).

Discussion

Molecular phylogenetics have been challenging for dinophytes because of multiple reasons such as a limited available taxon sample, lateral gene transfers and divergent substitution rates (Hackett et al. 2004; Morden and Sherwood 2002; Shalchian-Tabrizi et al. 2006; Tillmann et al. 2012; Waller et al. 2006). In the past few years, several promising approaches have been applied in order to improve the dinophyte molecular tree, with a focus on the concatenation of molecular sequence data (Hoppenrath and Leander 2010; Orr et al. 2012). Molecular phylogenetic trees should be interpreted with caution, however, when mitochondrial loci (because of extensive mRNA editing: Waller and Jackson 2009; Zhang et al. 2008) and chloroplast loci (because of multiple endosymbiosis events: Howe et al. 2008; Shalchian-Tabrizi et al. 2006; Takishita et al. 2004; Yoon et al. 2005) are investigated.

Here, we follow the strategy to exploit concatenate sequences from the rich pool of rRNA loci (Gottschling and McLean in press; Saldarriaga et al. 2004; Tillmann et al. 2012). In the attachment, we provide the most comprehensive dinophyte molecular tree available at present in terms of both the taxon sample (more than 300 accessions) and the amount of genetic data (more than 3,000 informative alignment positions). The resulting trees are remarkably stable and plausible from a morphological perspective and show once more the monophyly of such systematic units as the Dinophysiales, the Gonyaulacales and the Suessiales. Given that taxon sampling has repeatedly been shown to have importance for the improved resolution of phylogenetic trees (Dunn et al. 2008; Gottschling et al. 2012; Heath et al. 2008; Sanderson 2008), the investigation of all strains, from which SSU and LSU sequences have been generated, might have had the greatest impact on the identification even of the Peridiniales and the Prorocentrales as monophyletic groups. This might be helpful as systematic backdrop for future phylogenetic analyses.

Our molecular trees reliably indicate the phylogenetic placement of †*Posoniella* within the T/Pf-clade of the Thoracosphaeraceae. Based on its distinct morphology this result comes as a great surprise, as †*Posoniella* has been considered a member of the forms with irregularly arranged and aggregated crystals constituting the shell of the coccoid cell (Meier et al. 2009; Versteegh 1993) such as †*C. bivalvum* of the E/Pe-clade. However, †*Posoniella* is spectacular not only because it is the sole representative of calcareous dinophytes known at present with two distinct morphs of coccoid cells, but also because the two morphs of coccoid cells would have been assigned to different taxa at the subfamily level if found separately in fossil samples. Another member of the Thoracosphaeraceae with two distinct types of coccoid cells is "*Scrippsiella*" *hangoei*, which belongs to the T/Pf-clade as well. However, the types in *S. hangoei* do not differ in their morphology, but in their ploidy level (Kremp and Parrow 2006), a trait that would be tempting to investigate also for †*Posoniella*. Anyhow, it remains to be determined, if other members of the calcareous dinophytes exhibit similar differentiated cells like †*P. tricarinelloides* and which precise functions those cells have in the algal life history.

Our findings more than challenge the (paleontological) classification concept of Keupp (1991) and subsequent authors (Janofske 1996; Hildebrand-Habel and Streng 2003; Kohring et al. 2005), who have put much attention to the crystallographic c-axis orientation of the calcitic crystals constituting the shell. Another striking result of our study is that the other type of coccoid cells present in †*Posoniella* shows the radial orientation of c-axes under polarized light. This character state of †*Posoniella* is shared with †*Leonella* (Janofske and Karwath in Karwath 2000), again a member of the T/Pf-clade. Optical crystallography therefore appears to remain an important character trait to circumscribe species (groups), but not on high taxonomic level (of subfamilies). The E/Pe-clade (irregular type) and *Scrippsiella*

s.l. (regular type with tangential orientation) are consistent in this respect, while the T/Pf-clade is heterogeneous and comprise all three types that are currently distinguished.

Archeopyle and operculum morphology has great importance to indicate relationships within calcareous dinophytes (Keupp and Versteegh 1989; Streng et al. 2004). The different types of archeopyles that are currently distinguished (Streng et al. 2004) correlate with molecular phylogenies of calcareous dinophytes (Gottschling et al. 2005a, 2012), as it is also indicated by this study. A simple apical operculum, corresponding to the 3' plate equivalent, is considered the ancestral condition and is today found in the two, only distantly related clades E/Pe and T/Pf. The more complex (mesoepicystal and epitractal) compound opercula include a greater number of plate equivalents and are considered the derived character state found today in *Scrippsiella s.l.* Both types of coccoid cells exhibit a simple apical archeopyle in †*Posoniella*, which is in agreement with its systematic placement within the T/Pf-clade.

The thecal tabulation patterns of †*P. tricarinelloides* deserves also discussion. In all three clades of the Thoracosphaeraceae, members with three intercalary plates are found (Gu et al. 2008; Hansen and Flaim 2007; Indelicato and Loeblich III 1986; Larsen et al. 1995; Montresor et al. 1993), although the shape and configuration of the 2a plate might show some variation (Attaran-Fariman and Bolch 2007; Gottschling et al. 2005b; Montresor and Zingone 1988). Three intercalary plates must be therefore considered the ancestral condition with the Thoracosphaeraceae, and species (groups) with reduced numbers such as *Luciella* P.L.Mason, Jeong, Litaker, Reece & Steid. (with two) and †*Posoniella* (with only one) occur exclusively in the T/Pf-clade (Calado et al. 2009; Jeong et al. 2005). Moreover, seven precingular and six cingular plates are found in †*P. tricarinelloides*, respectively. Both character states may represent the ancestral condition within the Thoracosphaeraceae, as they are found in *Scrippsiella s.l.* and also some (basal) members of the T/Pf-clade such as †*Leonella* (Janofske and Karwath in Karwath 2000) and "*Peridinium*" *aciculiferum* (Logares et al. 2007). To the

contrary, the number of both precingular and cingular plates is reduced particularly in *Pfiesteria* and related taxa (Litaker et al. 2005; Mason et al. 2007), but this evolution within the T/Pf-clade has taken place after the divergence from †*Posoniella* showing the ancestral conditions.

In conclusion, †*Posoniella* is the first calcareous dinophyte known at present exhibiting two distinct types of coccoid cells. Moreover, it is one of the now three known extant members of the Thoracosphaeraceae with the radial orientation of crystallographic c-axes in the shell forming crystals (the other two are †*Leonella* and †*Caracomia*). Our findings underline the complex biology of †*Posoniella* that is better to understand in the mutual comparison to molecular phylogenies based on both a large taxon sample and extensive sequence data. The biodiversity assessment is not completed at present and is particularly challenging for the work on unicellular organisms. Exploiting the rich pool of available rRNA sequences in order to improve molecular phylogenetic trees is not only promising for the dinophytes alone, but may have also importance for other protist lineages.

Material and Methods

Cultivation

Sediment and plankton samples were collected as described in detail previously (Soehner et al. 2012) in the Mediterranean Sea off Italy as well as in the Yellow Sea and the South China Sea off China. Each environmental sample was washed through stainless steel sieves (net size 100µm, 75 µm and 20 µm: Zefa; Munich, Germany) with 35 psu artificial seawater (hw marinemix professional: Wiegandt; Krefeld, Germany) at pH 8.0–8.2. Coccoid cells were isolated and initially cultivated together for each locality in 6-well micro plates (Zefa) by using K-Medium without silicate (Keller et al. 1987) in 35 psu artificial seawater at pH 8.0–8.2. Afterwards, monoclonal strains were established based on coccoid and thecate cells to exclude contaminations. The cultivation took place in a climate chamber Percival I-36VL (CLF PlantClimatics; Emersacker, Germany) at 23°C, 80 µmol photons m^{-2} s^{-1} and a 12:12 h light:dark photoperiod as described previously (Zinssmeister et al. 2011).

Morphology

Cells of all types were observed in a CKX41 inverse microscope (Olympus; Hamburg, Germany). The thecal plate pattern was determined by examining cells stained with calcofluor white M2R (Sigma-Aldrich; Munich, Germany), following the method of Fritz and Triemer (1985) and using an Axio Imager microscope (Zeiss; Göttingen, Germany) equipped with both differential interference illumination and epifluorescence. Light micrographs were obtained using an Axiocam HRc digital camera (Zeiss). The Kofoidean system (Fensome et al. 1993; Taylor 1980) was used for the designation of the thecal plate formula.

For thin sections, the samples were embedded in a synthetic resin (Spurr 1969) using the Embedding Medi Kit (Science Services; Munich, Germany) and following standard protocols. A 1:1 mixture of acetone and resin was used in a first embedding step for better infiltration of

the resin into the coccoid cells. After 1 h, the mixture was replaced by pure Spurr's resin and hardened for 48 h at 70°C. A Reichert-Jung 2055 Autocut microtome (Leica; Nussloch, Germany) was used to cut 3 µm ultra-thin sections. The method for identifying the crystallographic orientation of the calcite crystals based on standard methods (Bloss 1999) in thin sections was described in detail by Janofske (1996, 2000) and Montresor et al. (1997). The orientation of the c-axis was perpendicular to the cell surface, if the quadrants I and III of a conoscopic image showed yellow interference colors and quadrants II and IV showed blue interference colors ('radial' type). Conversely, the orientation of the c-axis would be tangential to the cell surface, if the quadrants II and IV showed yellow interference colors and quadrants I and III showed blue interference colors.

For SEM, cells were collected and processed based on standard protocols that were described in detail previously (Gottschling et al. 2012). Thecate cells were briefly dehydrated in graded acetone p.a. (Roth) series and critical point dried (K850 Critical Point Dryer, Quorum/Emitech; West Sussex, UK). Coccoid cells were demineralized in bi-distillate water and air-dried at a glass slide fixed on SEM stubs. Samples were sputter-coated with platinum and were documented using a LEO 1530 Gemini SEM (Zeiss/LEO; Oberkochen, Germany).

Molecular sequence analyses

Single thecate cells of †*P. tricarinelloides* (Chinese strains) were isolated and washed three times with sterilized bi-distillate water and were transferred into a 200 µl PCR tube. The cells were lysed by treatment in liquid nitrogen for 20 min and brought to room temperature for another 20 min. Alternatively, genomic DNA was extracted from fresh material using the Nucleo Spin Plant II Kit (Machery-Nagel, Düren, Germany). Complete SSU, both ITSs (including the 5.8S rRNA region), the first two domains of the LSU, and cytochrome b (cob) were amplified by PCR using the primer listed in Table S1 (see supplementary material)

following standard protocols (Gottschling and Plötner 2004; Gu et al. 2011; Zhang, Bhattacharya, Lin 2005). PCR products were sequenced directly in both directions using the ABI Big-Dye dye-terminator technique (Applied Biosystems, Foster City, CA, USA), according to the manufacturers' recommendations.

We compiled all sequences available (own data and downloads from GenBank) from the Dinophyceae that comprised the SSU and the first two domains of the LSU. Where available, we appended longer LSU sequences of particular dinophyte strains in this data matrix. In total, 319 accessions (plus 4 outgroup representatives from the Apicomplexa) were investigated by sequence comparison (supplementary material Tab. S2). In an additional approach, we investigated all sequences available from the Peridiniales that comprised the SSU, the ITSs and the first two domains of the LSU and appended cob sequences where available. The sequences were separately aligned by using 'MAFFT' v6.624b (Katoh et al. 2005; freely available at http://align.bmr.kyushuu.ac.jp/mafft/software/) and were concatenated afterwards. All data matrices are available via *.nex file by MG upon request.

Phylogenetic analyses were carried out by using Maximum-Likelihood (ML) and Bayesian approaches, as described in detail by Gottschling et al. (2012). We used the resources of the SGI system (Zuse Institute Berlin, ZIB) being one half of the North German High Performance Computer (HLRN). The Bayesian analysis was performed by using 'MrBayes' v3.1.2 (Ronquist and Huelsenbeck 2003; freely available at http://mrbayes.csit.fsu.edu/download.php) under the GTR+Γ substitution model and the random-addition-sequence method with 10 replicates. We ran two independent analyses of four chains (one cold and three heated) with 20,000,000 cycles, sampled every 1,000th cycle, with an appropriate burn-in (10%) as inferred from the evaluation of the trace files using Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). For the ML calculation, the MPI version of 'RAxML' v7.0.4 (Stamatakis 2006; freely available at

http://www.kramer.in.tum.de/exelixis/software.html) was applied by using the GTR+Γ substitution model. To determine best fitted ML tree, we executed 10-tree searches from distinct random stepwise addition sequence Maximum Parsimony starting trees and performed 1,000 non-parametric bootstrap replicates. Statistical support values (LBS: ML bootstrap support, BPP: Bayesian posterior probabilities) were drawn on the resulting, best-scoring trees.

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Figure Legends

Figure 1: Thecate cells of †*Posoniella tricarinelloides* and two distinct types of coccoid cells. A–F,J: thecate cells; A: light microscopy showing chloroplast, nucleus and pyrenoid; B: apical view; C: sulcal view; D–E: calcofluor white staining in light microscopy; F: dorsal view; J: ventral-lateral view; G–H,K–L: coccoid cells; G: light microscopy of *Posoniella*-like morphology; H: light microscopy of additional type; K–L: thin sections under polarized light; K: *Posoniella*-like coccoid cell showing the oblique type of crystal orientation; L: radial type (A,D–E,G–H,KL: light microscopy; B–C,F,J: SEM images; A–C,E–G,J: strain PTLY01; D: strain GeoB 413; H strain GeoB 429; K–L: strain GeoB 410; scale bars: A,D–L: 10 µm, B–C: 5 µm; figure abbreviations: ci, cingulum; cp, chloroplast; et, epitheca; ht, hypotheca; nu, nucleus; py, pyrenoid).

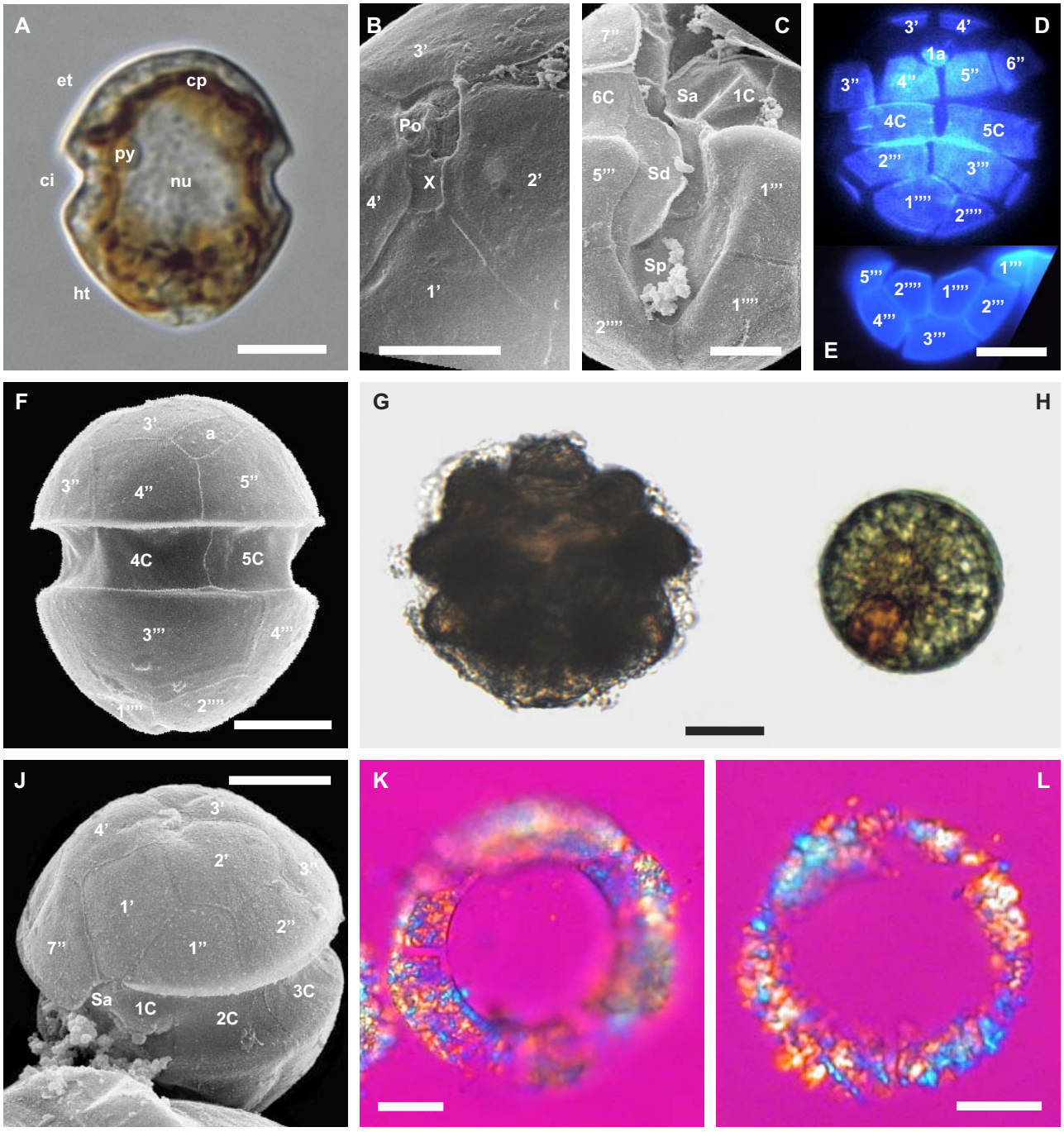
Figure 2: Thecal plate pattern of †*Posoniella tricarinelloides*. A: apical view; B: dorsal view; C: ventral view; D: antapical view (plate labeling based on the Kofoidean system (Taylor 1980) (Fensome et al. 1993).

Figure 3: †*Posoniella tricarinelloides* with two distinct types of coccoid cells. A: overview showing the two types of coccoid cells together; B: apical view of closed cell with *Posoniella*-like morphology; C: apical view of germinated *Posoniella*-like cell with small archeopyle; D: antapical view of cell with *Posoniella*-like morphology; E: cross section of *Posoniella*-like cell showing the ultrastructure of the shell; F: apical view of germinated cell representing the second type with small archeopyle (SEM images; strain GeoB 413; scale bars: 10 µm; figure abbreviations: ap, archeopyle; op, operculum).

Figure 4: †*Posoniella* as member of a clade including *Pfiesteria* and *Thoracosphaera*. Bayesian tree of 67 members of the Peridiniales as inferred from a MAFFT generated rRNA

nucleotide alignment spanning the complete SSU, ITS and LSU domains 1 through 2 (1,381 parsimony-informative positions). Major clades are indicated, and branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values for the clusters to the right of them (above: Bayesian posterior probabilities, values under .90 are not shown; below: ML bootstrap support values, values under 50 are not shown), and asterisks indicate maximal support values. The tree is rooted with eight sequences of the Amphidomataceae.

Figure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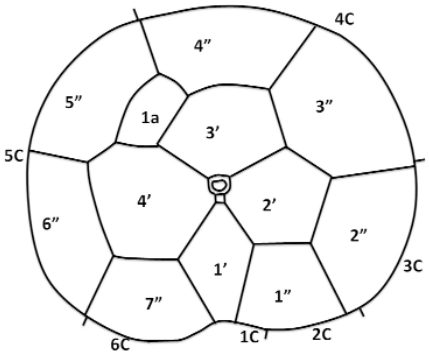
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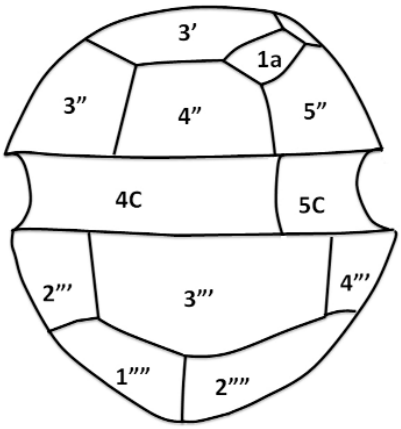
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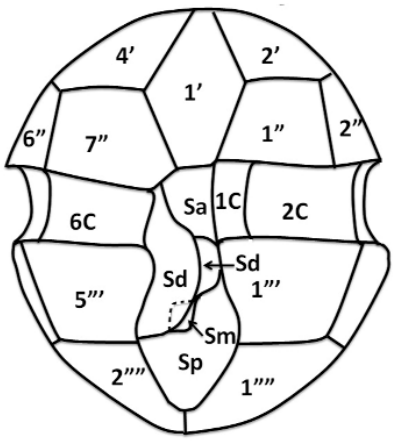
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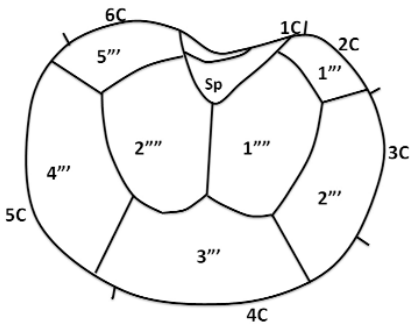
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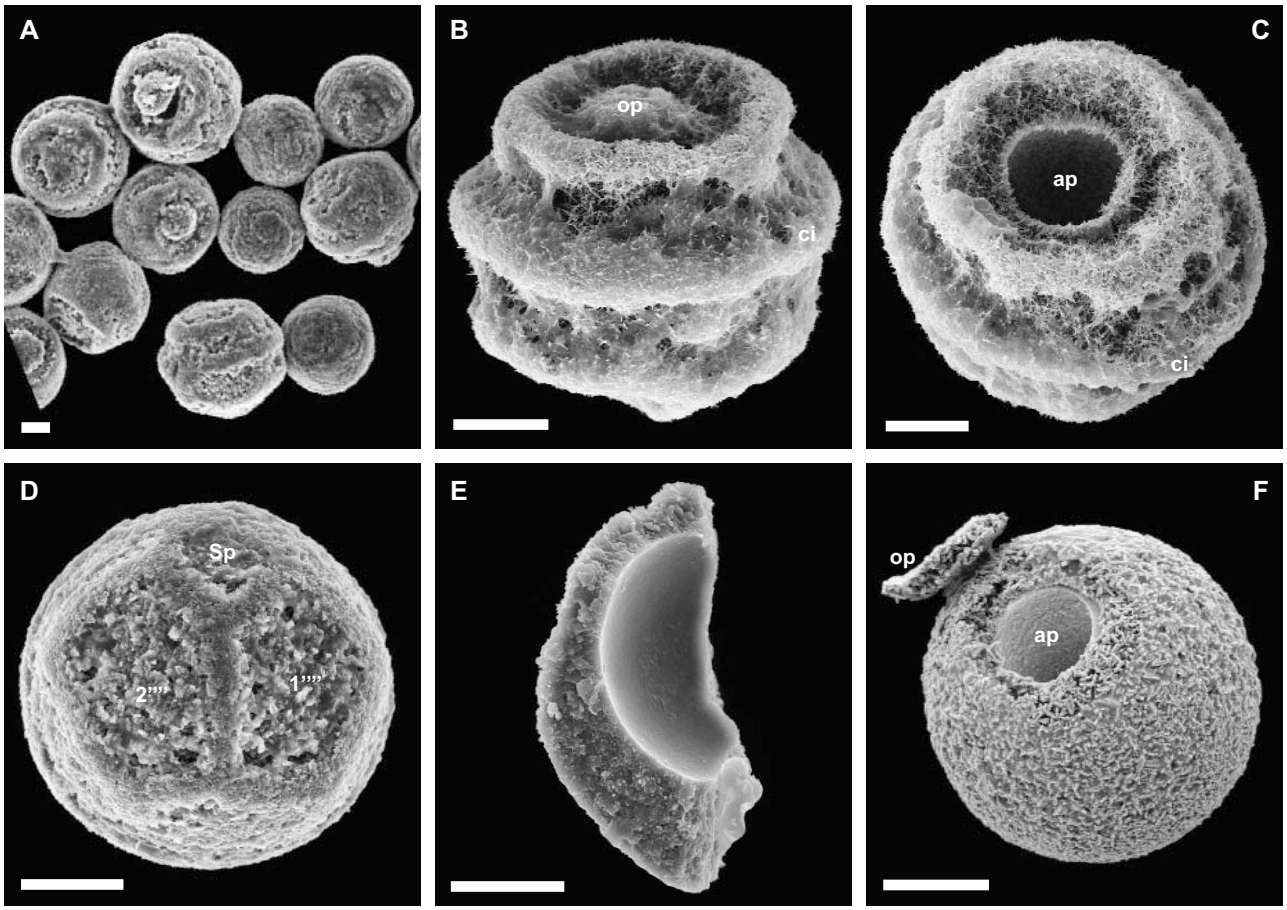
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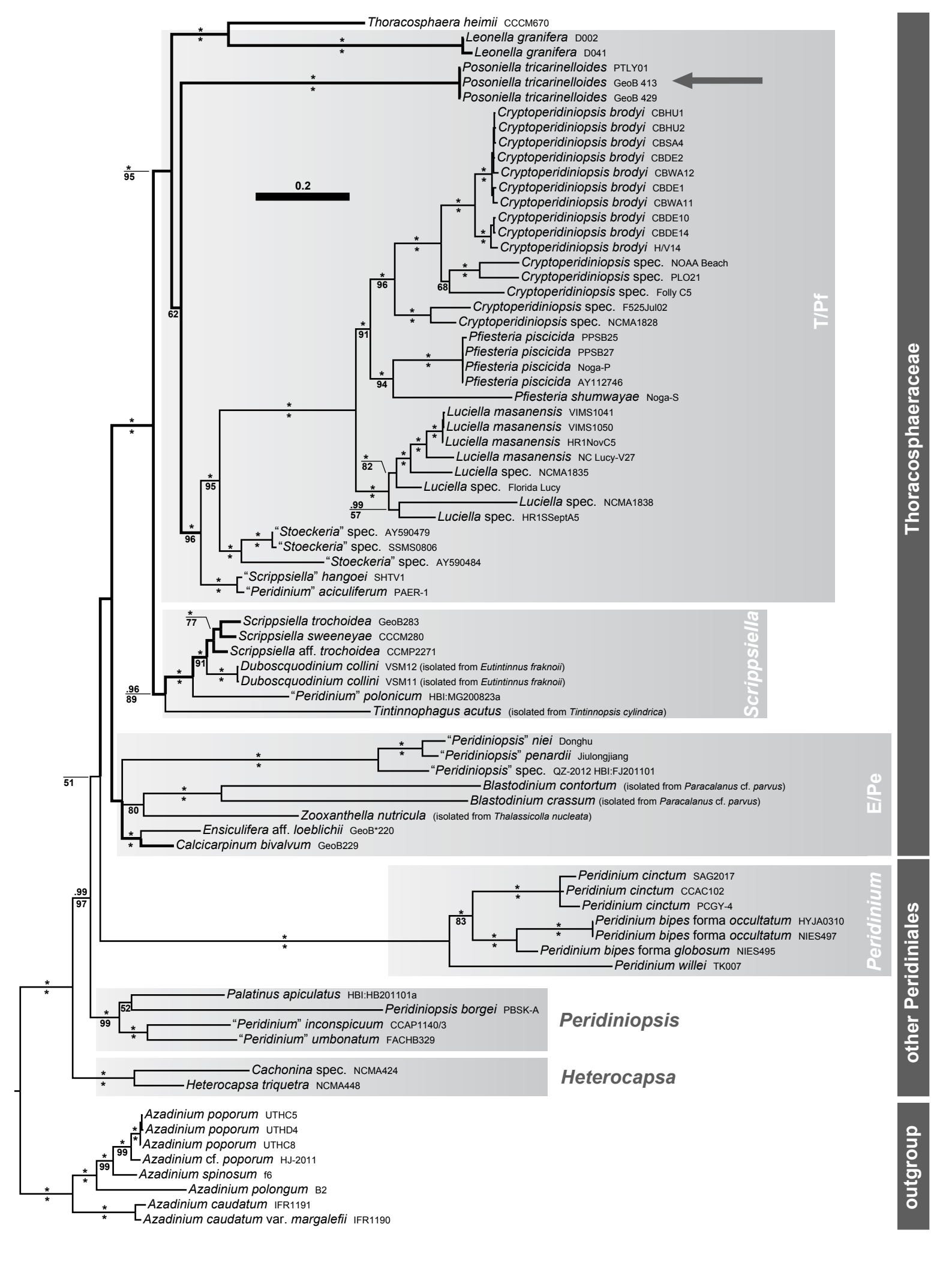

Thoracosphaera heimii CCCM670
Leonella granifera D002
Leonella granifera D041
Posoniella tricarinelloides PTLY01
Posoniella tricarinelloides GeoB 413
Posoniella tricarinelloides GeoB 429
Cryptoperidiniopsis brodyi CBHU1
Cryptoperidiniopsis brodyi CBHU2
Cryptoperidiniopsis brodyi CBSA4
Cryptoperidiniopsis brodyi CBDE2
Cryptoperidiniopsis brodyi CBWA12
Cryptoperidiniopsis brodyi CBDE1
Cryptoperidiniopsis brodyi CBWA11
Cryptoperidiniopsis brodyi CBDE10
Cryptoperidiniopsis brodyi CBDE14
Cryptoperidiniopsis brodyi H/V14
Cryptoperidiniopsis spec. NOAA Beach
Cryptoperidiniopsis spec. PLO21
Cryptoperidiniopsis spec. Folly C5
Cryptoperidiniopsis spec. F525Jul02
Cryptoperidiniopsis spec. NCMA1828
Pfiesteria piscicida PPSB25
Pfiesteria piscicida PPSB27
Pfiesteria piscicida Noga-P
Pfiesteria piscicida AY112746
Pfiesteria shumwayae Noga-S
Luciella masanensis VIMS1041
Luciella masanensis VIMS1050
Luciella masanensis HR1NovC5
Luciella masanensis NC Lucy-V27
Luciella spec. NCMA1835
Luciella spec. Florida Lucy
Luciella spec. NCMA1838
Luciella spec. HR1SSeptA5
"Stoeckeria" spec. AY590479
"Stoeckeria" spec. SSMS0806
"Stoeckeria" spec. AY590484
"Scrippsiella" hangoei SHTV1
"Peridinium" aciculiferum PAER-1
T/Pf
0.2
Scrippsiella trochoidea GeoB283
Scrippsiella sweeneyae CCCM280
Scrippsiella aff. trochoidea CCMP2271
Duboscquodinium collini VSM12 (isolated from Eutintinnus fraknoii)
Duboscquodinium collini VSM11 (isolated from Eutintinnus fraknoii)
"Peridinium" polonicum HBI:MG200823a
Tintinnophagus acutus (isolated from Tintinnopsis cylindrica)
Scrippsiella
"Peridiniopsis" niei Donghu
"Peridiniopsis" penardii Jiulongjiang
"Peridiniopsis" spec. QZ-2012 HBI:FJ201101
Blastodinium contortum (isolated from Paracalanus cf. parvus)
Blastodinium crassum (isolated from Paracalanus cf. parvus)
Zooxanthella nutricula (isolated from Thalassicolla nucleata)
Ensiculifera aff. loeblichii GeoB*220
Calcicarpinum bivalvum GeoB229
E/Pe
Peridinium cinctum SAG2017
Peridinium cinctum CCAC102
Peridinium cinctum PCGY-4
Peridinium bipes forma occultatum HYJA0310
Peridinium bipes forma occultatum NIES497
Peridinium bipes forma globosum NIES495
Peridinium willei TK007
Peridinium
Palatinus apiculatus HBI:HB201101a
Peridiniopsis borgei PBSK-A
"Peridinium" inconspicuum CCAP1140/3
"Peridinium" umbonatum FACHB329
Peridiniopsis
Cachonina spec. NCMA424
Heterocapsa triquetra NCMA448
Heterocapsa
Azadinium poporum UTHC5
Azadinium poporum UTHD4
Azadinium poporum UTHC8
Azadinium cf. poporum HJ-2011
Azadinium spinosum f6
Azadinium polongum B2
Azadinium caudatum IFR1191
Azadinium caudatum var. margalefii IFR1190
Thoracosphaeraceae
other Peridiniales
outgroup

Waking the dead: Morphological and molecular characterisation of the 'living fossil' *Posoniella tricarinelloides* (G.Versteegh) Streng, Banasová, Reháková & H.Willems (Thoracosphaeraceae, Dinophyceae)

Haifeng Gu, Monika Kirsch, Carmen Zinssmeister, Sylvia Soehner, K. J. Sebastian Meier, Tingting Liu, Marc Gottschling

SUPPLEMENTARY MATERIAL

TABLES

Table S1: Primer list. Abbreviations: fw, forward; rev, reverse.

Table S2: Voucher list. For dinophytes, all names are given under the rules of the ICBN, the author standard forms follow {Brummitt, 1992 #18289}. Abbreviations: ALV: Alveolates other than dinophytes (i.e., outgroups); AMP: Amphidomataceae; DIN: Dinophysiales; DPH: Dinophyceae of uncertain systematic placement; GON: Gonyaulacales; GYM: different lineages of the "Gymnodiniales"; PER: Peridiniales; PRO: Prorocentrales; SUE: Suessiales; SYN: Syndiniales.

SYN	*Eduboscquella cachoni* (Coats) Coats [isolated from *Eutintinnus tenuis* (Kof.& A.S.Campbell, 1929)]	SERC1	USA–MD: Rhode River (38°53'N, 76°33W)	JN934988
SYN	*Eduboscquella cachoni* (Coats) Coats [isolated from *Eutintinnus tenuis* (Kof. & A.S.Campbell, 1929)]	SERC12	USA–MD: Rhode River (38°53'N, 76°33W)	JN934987
SYN	*Eduboscquella crenulata* Coats, Bachvaroff & Delwiche (isolated from *Favella panamensis* Kof. & A.S.Campbell, 1929)	USNM1156917	USA–MD: Ocean City, Assawoman Bay (38°20'N, 75°06W)	JN606065
SYN	*Eduboscquella* spec. (isolated from *Favella panamensis* Kof. & A.S.Campbell, 1929)	MSNB4	South Korea: Masan Bay (35°12'N, 128°35'E)	JN934986
SYN	*Eduboscquella* spec. [isolated from *Favella arcuata* (K.Brandt, 1906)]	OC20	USA–MD: Ocean City, Assawoman Bay (38°20'N, 75°06W)	JN934989

SYN	*Eduboscquella* spec. (isolated from *Tintinnopsis* cf. *subacuta* Jörgensen, 1899)	SERC39	USA–MD: Rhode River (38°53'N, 76°33W)	JN934992
SYN	*Eduboscquella* spec. (isolated from *Tintinnopsis* cf. *subacuta* Jörgensen, 1899)	SERC62	USA–MD: Rhode River (38°53'N, 76°33W)	JN934990
SYN	*Eduboscquella* spec. [isolated from *Favella markusovszkyi* (Daday, 1887)]	VSM13	France: Villefranche-sur-Mer (43°42'N, 7°19'E)	JN934985
SYN	*Eduboscquella* spec. [isolated from *Tintinnopsis* spec.]	VSM21_VSM22	France: Villefranche-sur-Mer (43°42'N, 7°19'E)	JN934984
DPH	*Amoebophrya* spec. [isolated from *Gymnodinium instriatum* (Freudenthal & Lee, 1963)]	n.ind.	USA–MD: Chesapeake Bay	HM483394
DPH	*Amoebophrya* spec. [isolated from *Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup]	n.ind.	USA–MD: Chesapeake Bay	HM483395
DPH	*Amphidinium carterae* Hulburt	CMSTA010	n.ind.	EU046334, EU046327
DPH	*Amphidinium carterae* Hulburt	NCMA121	Caribbean Sea (15°00'N, 72°00'W)	EF057407, AY460585
DPH	*Amphidinium carterae* Hulburt	NCMA1314	USA–MA: Falmouth, Falmouth Great Pond (41°34'N, 70°35'W)	AF274251, EU165301
DPH	*Amphidinium klebsii* N.Carter	CMSTAC018	n.ind.	EU046335, EU046328
DPH	*Amphidinium massartii* Biecheler	NCMA1342	USA–FL: Knight Key (24°42'N, 81°08'W)	AY443011, EU165302
DPH	*Amphidinium operculatum* Clap. & J.Lachm.	NCMA123	USA–FL: Florida Keys, Tea Table Channel (25°00'N, 80°24'W)	EF057406, FJ939574
DPH	*Amphidinium* spec.	D1-CMSTAC020	n.ind.	EU046336, EU046329
DPH	*Amphidinium* spec.	D2-CMSTAC021	n.ind.	EU046337, EU046330
DPH	*Amphidinium* spec.	FA1-CMSTAC022	n.ind.	EU046338, EU046331

DPH	*Amphidinium* spec.	FC2-CMSTAC023	n.ind.	EU046339, EU046332
DPH	*Amphidinium* spec.	S1-CMSTAC025	n.ind.	EU046340, EU046333
DPH	*Amphidinium* spec. (isolated from *Waminoa* spec.)	THK-2011	n.ind.	AB626895, AB626894
DPH	*Ankistrodinium semilunatum* (Herdman) Hoppenrath, Sh.Murray, Sparmann & B.S.Leander	clone 4	USA–WA: Boundary Bay	JQ179860, JQ179863
DPH	*Apicoporus glaber* (Hoppenrath & Okolodkov) Sparmann, B.S.Leander & Hoppenrath	n.ind.	Germany: Sylt	EU293235, JQ179867
DPH	*Bysmatrum subsalsum* (Ostenf.) M.A.Faust & Steid.	KC32CCAUTH	off Greece, North Aegean Sea: Thessaloniki, Porto-Lagos (40°58'N, 25°07'E)	HQ845326
DPH	*Esoptrodinium* spec.	CCP1	USA–NC: Pond near Cashiers (35°08'N, 83°05'W)	JQ439938
DPH	*Esoptrodinium* spec.	CCP2	USA–NC: Pond near Cashiers (35°08'N, 83°05'W)	JQ439940
DPH	*Esoptrodinium* spec.	HP	USA–NC: Highlands, Harris Pond (35°03'N, 83°12'W)	JQ439943
DPH	*Esoptrodinium* spec.	PTP	USA–NC: Pond near Panthertown Valley (35°10'N, 83°03'W)	JQ439941
DPH	*Esoptrodinium* spec.	RP	USA–NC: Raleigh, Fish hatchery drainage pond (35°44'N, 78°41'W)	JQ439944
DPH	*Esoptrodinium* spec.	UNCCP	USA–NC: Charlotte, pond on UNC Charlotte's campus (35°18'N, 80°45'W)	JQ439942
DPH	*Glenodiniopsis steinii* (Lemmerm.) Wołosz.	NIES463	Japan: Iwate, Shizukuishi	AF274257, EF058255
DPH	"*Gymnodinium*" *impatiens* Skuja	CCAC0025	Germany: Brandenburg, near Neuglobsow	EF058239, EF058259

DPH	*Gyrodinium dominans* Hulburt	GDMS0704YD	off South Korea: Masan	FN669510
DPH	"*Gyrodinium*" *instriatum* Freud. & J.J.Lee	NCMA431	Portugal: near Santiago do Cacem, Santo Andre lagoon (38°03'N, 8°48'W)	AY443015, JQ972685, EF205007
DPH	*Gyrodinium moestrupii* E.Y.Yoon, N.S.Kang & H.J.Jeong	HJ-2011	South Korea: Saemankeum Bay (35°94′N, 126°54′E)	HE611580
DPH	*Hemidinium nasutum* F.Stein	NIES471	Japan: Ibaraki, Tsuchiura	AY443016, EF058260
DPH	*Jadwigia applanata* Moestrup, K.Lindb. & Daugbjerg	CCAC0021	Germany: Hessen, Biebergemünd, near Lochmühle	EF058240, AY950447
DPH	*Noctiluca scintillans* (Macartney) Kof. & Swezy	n.ind.	off Hong Kong: Clear Water Bay (22°20'N, 114°16'E)	GQ380592
DPH	"*Woloszynskia*" spec.	HBI:SC201101a	n.ind.	JQ639766, JQ639756
AMP	*Azadinium caudatum* var. *caudatum*Tillmann & Elbr.	IFR1191	North Atlantic (France)(47°49.93'N, 03°56.72'W)	JQ247701, JQ247702
AMP	*Azadinium caudatum* var. *margalefii* Tillmann & Elbr.	IFR1190	North Atlantic (France)(47°49.93'N, 03°56.72'W)	JQ247707, JQ247708
AMP	*Azadinium obesum* Tillmann & Elbr.	2E10	North Sea (Scotland) (57°3.9'N, 2°30.2'W)	GQ914935, GQ914936
AMP	*Azadinium polongum* Tillmann & Elbr.	B2	Northern North Sea (Shetland Islands)(60°12.73'N, 0°59.90'W)	JX559886
AMP	*Azadinium* cf. *poporum* Tillmann & Elbr.	HJ-2011	Pacific (Korea) (37°18'N, 126°36'E)	FR877580
AMP	*Azadinium poporum* Tillmann & Elbr.	UTHC5	North Sea (Danmark) (56°14.52'N, 07°27.54'E)	HQ324897, HQ324893
AMP	*Azadinium poporum* Tillmann & Elbr.	UTHC8	North Sea (Danmark) (56°14.52'N, 07°27.54'E)	HQ324898, HQ324894

AMP	*Azadinium poporum* Tillmann & Elbr.	UTHD4	North Sea (Danmark) (56°14.52'N, 07°27.54'E)	HQ324899, HQ324895
AMP	*Azadinium spinosum* Elbr. & Tillmann	SHETF6	Northern North Sea (Shetland Islands) (60°12.73'N, 0°59.90'W)	JX559885
AMP	*Azadinium spinosum* Elbr. & Tillmann	SM2	North Atlantic (Ireland) (51°39'4.70"N, 9°35'11.00"W)	JN680857, JN165101
GYM1	*Gymnodinium aureolum* (Hulburt) Gert Hansen	GASMK0803	South Korea: Saemankeum	FN392226
GYM1	*Gymnodinium aureolum* (Hulburt) Gert Hansen	SWA16	Benguela Current off Namibia	AY999082
GYM1	*Gymnodinium catenatum* H.W.Graham	GCCW991	South Korea: Jindong	DQ779989
GYM1	*Gymnodinium catenatum* H.W.Graham	GnCt01	South Korea: Nanpo, Jinhae Bay	DQ785882
GYM1	*Gymnodinium catenatum* H.W.Graham	NCMA414	Spain: Ria de Vigo	DQ779990
GYM1	*Gymnodinium catenatum* H.W.Graham	NCMA1940	Spain: Ria de Vigo	DQ785883
GYM1	*Gymnodinium dorsalisulcum* (E.M.Hulbert, J.A.McLaughlin & Zahl) Sh.Murray, M.Salas & Hallegr.	KDAAD	Australia: Darwin, Fannie Bay (12°26′S, 130°51′E)	DQ837534, DQ837533
GYM1	*Gymnodinium eucyaneum* H.J.Hu	HBI:HB201009a	n.ind.	JQ639760, JQ639750
GYM1	*Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert Hansen & Moestrup	Gi-1cp	South Korea: Yosu	DQ779992
GYM1	*Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert Hansen & Moestrup	GrIp02	South Korea: Hase	DQ779993
GYM1	*Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert Hansen & Moestrup	NCMA1678	Australia: East Victoria, Gippsland Lakes (38°00’S, 147°00’E)	DQ785884
GYM1	*Gymnodinium* spec.	HBI:HB201110a	n.ind.	JQ639761, JQ639751
GYM1	*Gyrodiniellum shiwhaense* N.S.Kang, H.J.Jeong & Moestrup	n.ind.	South Korea: Shiwha Bay (37°07’N, 126°08’E)	FR720082

GYM1	*Lepidodinium chlorophorum* (Elbr. & Schnepf) Gert Hansen, Botes & Salas	DIN3	France	AY331681
GYM1	*Lepidodinium chlorophorum* (Elbr. & Schnepf) Gert Hansen, Botes & Salas	K-0539	Germany: Sylt	AB686253, AB367942
GYM1	*Lepidodinium chlorophorum* (Elbr. & Schnepf) Gert Hansen, Botes & Salas	NIES1867	n.ind.	AB686253, AB367942
GYM1	*Lepidodinium viride* M.Watan., S.Suda, I.Inouye, Sawaguchi & Chihara	n.ind.	South Africa	DQ499645
GYM1	*Spiniferodinium galeiforme* T.Horig. & Chihara	n.ind.	n.ind.	GU295203, GU295206
"GYM2"	*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup	AY001	off Japan: Hokkaido	AB232670
"GYM2"	*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup	GnSg02	off South Korea: Jangmok	AY831410
"GYM2"	*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup	GnSg03	off South Korea: Jangmok	AY831411
"GYM2"	*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup	NCMA1321	off USA–NY: Great South Bay (40°23'N, 73°09'W)	AY831412
"GYM2"	*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup	NCMA1593	off USA–RI: Narragansett Bay (41°36'N, 71°24'W)	DQ779987
"GYM2"	*Akashiwo sanguinea* (K.Hirasaka) Gert Hansen & Moestrup	NCMA1837	UK, the Bermudas: Harington Sound off Rabbitt Island (32°20'N, 64°44'W)	DQ779988
"GYM3"	"*Amphidinium*" *mootonorum* Sh.Murray & D.J.Patt.	n.ind.	n.ind.	GU295202, GU295205
"GYM2"	*Cochlodinium polykrikoides* Margalef	CcPk02	off South Korea: Tongyoung	DQ779984
"GYM2"	*Cochlodinium polykrikoides* Margalef	CcPk03	off South Korea: Narodo	DQ779985
"GYM2"	*Cochlodinium polykrikoides* Margalef	CcPk05	off South Korea: Hakdong	DQ779986

“GYM2”	*Cochlodinium polykrikoides* Margalef	CcPk06	off South Korea	AY347309
“GYM2”	*Cochlodinium polykrikoides* Margalef	CPPV-1	off South Korea: Tongyoung	JQ616826, JQ616831
“GYM3”	“*Gymnodinium*” *galatheanum* Braarud	KT-77B, K-0522	n.ind.	AF172712, AF200675
“GYM3”	*Karenia brevis* (C.C.Davis) Gert Hansen & Moestrup	NCMA718	USA–FL (27°42’N, 82°48W)	AF274259, AF200677
“GYM3”	*Karenia brevis* (C.C.Davis) Gert Hansen & Moestrup	NCMA2229	USA–FL: off Manasota Key, Station VS05 (27°01’N, 82°29’W)	FJ587219, EU165309
“GYM3”	*Karenia brevis* (C.C.Davis) Gert Hansen & Moestrup	SP3	Gulf of Mexico, off USA–TX: Corpus Christi	AF352820, AY355456
“GYM3”	*Karenia mikimotoi* (Miyake & Komin. ex M.Oda) Gert Hansen & Moestrup	CAWD05	New Zealand: North Island, Hawke’s Bay	AM184120, AF009131, HM807311
“GYM3”	*Karenia mikimotoi* (Miyake & Komin. ex M.Oda) Gert Hansen & Moestrup	NCMA429	UK: England, Devon, Plymouth, Sutton Harbour (50°22’N, 4°10’W)	FJ587220, AF200678
“GYM3”	*Karenia* spec.	GrAr01	South Korea: Chilchondo	DQ779991
“GYM3”	*Karlodinium veneficum* (D.Ballant.) J.Larsen	NCMA1975	USA–MD: Princess Anne, Hyrock Farms (38°10’N, 75°44’W)	EF036540
“GYM3”	*Karlodinium veneficum* (D.Ballant.) J.Larsen	Pim05JulC4	USA–FL: St. John’s River	AY245692
“GYM3”	*Karlodinium* spec.	KAMS0708	South Korea: Masan Bay	FN357291
SUE	*Baldinia anauniensis* Gert Hansen & Daugbjerg	greenGS	Italy: Lake Tovel	EF052682, EF052683
SUE	*Biecheleria baltica* Moestrup, K.Lindb. & Daugbjerg	WHTV	off Finland (Baltic Sea): Tvärminne	EF058252, AY628430
SUE	*Biecheleria cincta* (Siano, Montresor & Zingone) Siano	MALINA FT56.6 PG2	Beaufort Sea, station 110 (71°42’N 126°29’W)	JF794059, JQ413373
SUE	*Biecheleria cincta* (Siano, Montresor & Zingone) Siano	Nam Seon Kang	South Korea: Shiwha Bay	FR690459

SUE	*Polarella glacialis* Montresor, Procaccini & Stoecker	NCMA1383	Ross Sea, off Antarctica: McMurdo Sound (77°50'S, 163°00'E)	EF417317, AY036080
SUE	*Protodinium simplex* Lohmann	NCMA419	Costa Rica Dome (9°48'N, 89°15'W)	DQ388466, FJ823590, EF205015
SUE	*Symbiodinium goreaui* Trench & R.J.Blank [isolated from *Discosoma sanctithomae* (Duchass. & Michelotti, 1860)]	NCMA2466	off Jamaica (18°00'N, 77°00'W)	EF036539, FJ939581
SUE	*Symbiodinium microadriaticum* Freud. [isolated from *Aiptasia pallida* (Agassiz in Verrill, 1864)]	NCMA830	off UK, the Bermudas (Sargossa Sea): Bermuda Biological Station (32°23'N 64°41'W)	AY456111, AY684265
SUE	*Symbiodinium* spec. [isolated from *Hippopus hippopus* (Linnaeus, 1758)]	NCMA832	off Australia (Coral Sea): Tridacna Reef (20°S, 149°E)	AY456113, AF060898
SUE	*Symbiodinium* spec. (isolated from *Haliclona koremella* de Laubenfels, 1954)	PSP1-05	off Palau (Western Pacific Ocean): Carp Island	AB016578, AJ308899
SUE	*Symbiodinium* spec.	SMFL1, free-living isolate	South Korea: Jeju Island	HE653238
SUE	*Symbiodinium* spec.	SMFL1, coral isolate	South Korea: Jeju Island	HE653239
SUE	"*Woloszynskia*" *pascheri* (Suchl.) Stosch	CCAC75	Germany: Lower Saxony, Göttingen, Botanical Garden of the university	EF058253, EF058276
DIN	*Dinophysis caudata* Kent	FTL69	USA–FL: gulf stream off Ft. Lauderdale (26°05'N, 80°03'W)	EU780644
DIN	*Dinophysis miles* Cleve	n.ind.	South China Sea: off Sanya (18°12'N, 119°27'E) (latitude, longitude) near in the	JN982970

DIN	*Histioneis* spec.	FTL62	USA–FL: gulf stream off Ft. Lauderdale (26°05'N, 80°03'W)	EU780646
DIN	*Ornithocercus magnificus* F.Stein	CBC4L7	USA–VA: shelf break off lower Chesapeake Bay (36°20'N 74°44'W)	EU780649
DIN	*Phalacroma rapa* E.Jørgensen	CBC4L5	USA–VA: shelf break off lower Chesapeake Bay (36°20'N 74°44'W)	EU780655
DIN	*Phalacroma rapa* E.Jørgensen	CBC4L201	USA–VA: shelf break off lower Chesapeake Bay (36°20'N 74°44'W)	FJ477082
DIN	*Phalacroma* cf. *rotundatum* (Clap. & J.Lachm.) Kof. & J.R.Michener	FTL121	USA–FL: gulf stream off Ft. Lauderdale (26°05'N, 80°03'W)	EU780657
DIN	*Phalacroma* spec.	CBC4L128	USA–VA: shelf break off lower Chesapeake Bay (36°20'N 74°44'W)	FJ477084
PRO	*Prorocentrum belizeanum* M.A.Faust	PBHV-1	Cuba	JQ638934, JQ638946
PRO	*Prorocentrum belizeanum* M.A.Faust	n.ind.	off Belize: Carrie Bow Cay (16°48'N, 88°05'W)	DQ238042
PRO	*Prorocentrum cassubicum* (Wołosz.) J.D.Dodge	LB1596	USA–CA: La Jolla	DQ388460, EU165314
PRO	*Prorocentrum compressum* (J.W.Bailey) T.H.Abé ex J.D.Dodge	PCPA01	Australia: Tasmania	EU196417, AY259169
PRO	*Prorocentrum dentatum* F.Stein	NCMA1517	South Pacific	AY803742, AY833515
PRO	*Prorocentrum donghaiense* D.D.Lu	n.ind.	East China Sea	DQ336054, AY465116, AY822610
PRO	*Prorocentrum emarginatum* Fukuyo	SM35	Fiji: Kadavu Island (19°00'S, 178°15'E)	EU196418, DQ336192

PRO	*Prorocentrum fukuyoi* Sh.Murray & Y.Nagahama	SM39	Japan: Hiroshima, Suzu (37°25'N, 17°17'E)	EU196420, EU196416
PRO	*Prorocentrum levis* M.A.Faust, Kibler, Vandersea, P.A.Tester & Litaker	IRTA001	off Spain: Catalonia	FJ160588, FJ489621, FJ160589
PRO	*Prorocentrum levis* M.A.Faust, Kibler, Vandersea, P.A.Tester & Litaker	VGO880	off Spain: Catalonia	FJ489617, FJ489615, FJ489616
PRO	*Prorocentrum levis* M.A.Faust, Kibler, Vandersea, P.A.Tester & Litaker	n.ind.	off Belize: Twin Cays (16º50'N, 88º06'W)	DQ238043
PRO	*Prorocentrum maculosum* M.A.Faust	PMHV-1	Cuba	JQ638940, JQ638945
PRO	*Prorocentrum mexicanum* Osorio-Tafall	NCMA687	USA–FL: off Knight Key (24°42'N, 81°08'W)	EU287485, DQ336183
PRO	*Prorocentrum micans* Ehrenb.	NCMA1589	USA–RI: Narragansett Bay (41°36'N, 71°24'W)	EU780638
PRO	*Prorocentrum minimum* (Pavill.) J.Schiller	NCMA1329	USA–NY: Long Island, Great South Bay (40°40'N, 73°15'W)	DQ336060, EU927539, EU532479
PRO	*Prorocentrum minimum* (Pavill.) J.Schiller	PIPV-1	Mexico	JQ616823, JQ616845
PRO	*Prorocentrum minimum* (Pavill.) J.Schiller	PMDH01	East China Sea	DQ028763
PRO	*Prorocentrum minimum* (Pavill.) J.Schiller	SERC	n.ind.	EU780639
PRO	*Prorocentrum rhathymum* A.R.Loebl., Sherley & R.J.Schmidt	PXPV-1	Mexico	JQ616822, JQ616832
PRO	*Prorocentrum* spec.	IRTA002	off Spain: Catalonia	FJ160591, FJ160593, FJ160592
GON	*Alexandrium affine* (H.Inoue & Fukuyo) Balech	JHW0210	South Korea: Jinhae Bay	AY775286, AY438015
GON	*Alexandrium affine* (H.Inoue & Fukuyo) Balech	NCMA112	Spain: Ria de Vigo (42°14'N, 8°48'W)	AY831409
GON	*Alexandrium andersoni* Balech	NCMA1597	USA–MA: Eastham, Town Cove (41°49'N, 69°58'W)	JF521619

GON	*Alexandrium catenella* (Whedon & Kof.) Balech	AxCt_K01	South Korea	AY347308
GON	*Alexandrium catenella* (Whedon & Kof.) Balech	Axsp-K01	South Korea: Nanpo, Jinhae Bay	DQ785885
GON	*Alexandrium catenella* (Whedon & Kof.) Balech	Axsp-K03	South Korea: Jangmok	DQ785886
GON	*Alexandrium catenella* (Whedon & Kof.) Balech	Axsp-K05	off southern South Korea	DQ785887
GON	*Alexandrium cohorticula* (Balech) Balech	ACMS01	Malaysia	AF113935, AF174614
GON	*Alexandrium fundyense* Balech	CCAP1119/9	Ireland: Belfast Lough	DQ785891
GON	*Alexandrium fundyense* Balech	NCMA1719	USA–NH: Portsmouth (43°06'N, 70°47'W)	DQ444290
GON	*Alexandrium hiranoi* T.Kita & Fukuyo	NIES612	Japan: Kanagawa, Kawasaki	AY641564, AY438018
GON	*Alexandrium insuetum* Balech	NCMA2082, NIES678	Japan: Kanagawa, Uchiumi Bay (34°01'N, 131°02'E)	JF521630
GON	*Alexandrium leei* Balech	JHW0006-2	n.ind.	AY641565, AY438019
GON	*Alexandrium minutum* Halim	AMAD16, CS324/16	Australia: Adelaide, Port River	JF521633
GON	*Alexandrium minutum* Halim	NCMA113	Spain: Ria de Vigo (42°14'N, 8°48'W)	AY831408
GON	*Alexandrium minutum* Halim	SZN30	Italy	AJ535380, EU707487, EU707548
GON	*Alexandrium ostenfeldii* (Paulsen) Balech & Tangen	AOFUN0801	Japan: Hokkaido, Funka Bay	AB538439
GON	*Alexandrium satoanum* K.Yuki & Fukuyo	JHW0007-9	n.ind.	AY641566, AY438020
GON	*Alexandrium tamarense* (M.Lebour) Balech	ATNWB01	Australia: Tasmania, North West Bay	JQ991015, EF178148
GON	*Alexandrium tamarense* (M.Lebour) Balech	AX-03	Japan	DQ785890
GON	*Alexandrium tamarense* (M.Lebour) Balech	CCAP1119/5	USA–MA: Gloucester, Ipswich Bay	AY831407
GON	*Alexandrium tamarense* (M.Lebour) Balech	HAT4	Japan	AB088295, AB088246
GON	*Alexandrium tamarense* (M.Lebour) Balech	HY970328M	South Korea: Masan Bay	AY831406

GON	*Alexandrium tamarense* (M.Lebour) Balech	HY97403M	South Korea: Masan Bay	DQ785888
GON	*Alexandrium tamarense* (M.Lebour) Balech	HY981028M	South Korea: Masan Bay	DQ785889
GON	*Alexandrium tamarense* (M.Lebour) Balech	NCMA1493	China: Guangdong, Daya Bay (22°18'N, 114°18'E)	JF521641
GON	*Alexandrium tamarense* (M.Lebour) Balech	SZN01	Italy	AJ535387, AJ535368
GON	*Alexandrium tamarense* (M.Lebour) Balech	SZN08	Italy	JN626282, AJ535369
GON	*Alexandrium tamarense* (M.Lebour) Balech	SZN19	Italy	AJ535386, AJ535370
GON	*Alexandrium tamarense* (M.Lebour) Balech	SZN21	Italy	JN626281, AJ535374
GON	*Alexandrium tamarense* (M.Lebour) Balech	ULW9903, type A	South Korea:Ulsan	AB088327, AB088270
GON	*Alexandrium tamutum* Montresor, Beran & U.John	SZN28	Italy	AJ535379, AJ535373
GON	*Alexandrium tamutum* Montresor, Beran & U.John	SZN29	Italy	AJ535378, AJ535372
GON	*Alexandrium* spec.	ACC01 clone 3	n.ind.	JN098280, AY268597
GON	*Alexandrium* spec.	ACC01 clone 7	n.ind.	JN098272, AY268597
GON	*Alexandrium* spec.	ACPP01	Australia: Victoria: Port Phillip Bay	JN626275, EF178141, AY916543
GON	*Alexandrium* spec.	ACQH01 clone 3	n.ind.	JN098283, AY056823
GON	*Alexandrium* spec.	ACQH01 clone 12	n.ind.	JN098292, AY056823
GON	*Alexandrium* spec.	NCMA1771	UK: England, Plymouth, Tamar Estuary (50°22'N, 4°09'W)	DQ785892
GON	*Alexandrium* spec.	NCMA1846	USA–MA: Gloucester, Ipswich Bay (42°38'N, 70°41'W)	JF521625
GON	*Alexandrium* spec.	NCMA1911 clone 5	USA–WA: Sequim Bay (48°06'N, 123°02'W)	JN098328, HQ260956

GON	*Alexandrium* spec.	NCMA1911 clone 7	USA–WA: Sequim Bay (48°06'N, 123°02'W)	JN098330, HQ260956
GON	*Ceratium furcoides* (Levander) Langhans	HBI:SC201002a	n.ind.	JQ639757, JQ639748
GON	*Ceratium longipes* (Bailey) Gran	NCMA1770	USA–ME: West Boothbay Harbor, Bigelow Laboratory dock (43°51'N, 69°38'W)	DQ388462, EU165305
GON	*Coolia canariensis* S.Fraga	CMJJ1	South Korea: Jeju	FR846195, FR846193
GON	*Coolia canariensis* S.Fraga	NQAIF252	Australia: Great Barrier Reef	HQ897282, HQ897278
GON	*Coolia malayensis* Leaw, P.T.Lim & Usup	NQAIF35	Australia: Great Barrier Reef	HQ897279, HQ897274
GON	*Coolia monotis* Meunier	CMJJ1	South Korea: Jeju	FR847217, FR847218
GON	*Coolia monotis* Meunier	CMJJ2	South Korea: Jeju	FR847220, FR847221
GON	*Coolia monotis* Meunier	NCMA1345	Caribbean Sea off USA–FL: Knight Key (24°42'N, 81°06'W)	EF492487, AM902743
GON	*Coolia* spec.	NQAIF90	Australia: Great Barrier Reef	HQ897280, HQ897276
GON	*Coolia* spec.	NQAIF103	Australia: Great Barrier Reef	HQ897281, HQ897277
GON	*Gambierdiscus caribaeus* Vandersea, Litaker, M.A.Faust, Kibler, W.C.Holland & Tester	GCJJ1	South Korea: Jeju	HE775087
GON	*Gambierdiscus* spec.	Go3_2f	Japan: Okinawa, Onna, Cape Maeda	AB548851, AB548855
GON	*Gambierdiscus* spec.	Go3_2r	Japan: Okinawa, Onna, Cape Maeda	AB548851, AB548856
GON	*Gambierdiscus* spec.	Go3C2	Japan: Okinawa, Onna, Cape Maeda	AB548853
GON	*Gambierdiscus* spec.	Go3C3	Japan: Okinawa, Onna, Cape Maeda	AB548854
GON	*Gambierdiscus* spec.	GoFD1	Japan: Okinawa, Onna, Cape Maeda	AB548851, AB548857
GON	*Gambierdiscus* spec.	GoFD3	Japan: Okinawa, Onna, Cape Maeda	AB548851, AB548859
GON	*Gambierdiscus* spec.	GoRB1	Japan: Okinawa, Onna, Cape Maeda	AB548851, AB548858
GON	*Gonyaulax cochlea* Meunier	NCMA1592	USA–RI: Narragansett Bay 41°36'N, 71°24'W)	AF274258, FJ939579

GON	*Gonyaulax spinifera* (Clap. & J.Lachm.) Diesing	GSA0602	off Italy: Emilia-Romagna, Cesenatico coasts	EU805590, EU805591
GON	*Gonyaulax spinifera* (Clap. & J.Lachm.) Diesing	GSCQ-1	Mexico	JQ638933, JQ638936
GON	*Gonyaulax spinifera* (Clap. & J.Lachm.) Diesing	NCMA409	USA–ME: West Boothbay Harbor, Bigelow Laboratory dock (43°51'N, 69°38'W)	AF022155, EU532478
GON	*Lingulodinium polyedrum* (F.Stein) J.D.Dodge	LPCQ1	Mexico	JQ616824, JQ616830
GON	*Lingulodinium polyedrum* (F.Stein) J.D.Dodge	NCMA1738	off USA–TX (27°48'N, 97°08'W)	EF492507, EU165313
GON	*Lingulodinium polyedrum* (F.Stein) J.D.Dodge	n.ind.	n.ind.	AF377944
GON	*Protoceratium reticulatum* (Clap. & J.Lachm.) Buetschli	NCMA1721	USA–FL: Miami, Biscayne Bay (25°48'N, 80°20'W)	AF274273, EU165321
GON	*Protoceratium reticulatum* (Clap. & J.Lachm.) Buetschli	n.ind.	Canada: British Columbia, Saanich Inlet	AB727656
GON	*Protoceratium reticulatum* (Clap. & J.Lachm.) Buetschli	n.ind.	Japan: Hokkaido, Lake Saroma	AB727654
GON	*Protoceratium reticulatum* (Clap. & J.Lachm.) Buetschli	n.ind.	Sweden: Kattegat coast	AB727655
GON	*Pyrocystis noctiluca* J.Murray & Haeckel	NCMA732	USA–CA: Santa Barbara Channel (34°15'N, 119°43'W)	AF022156, FJ939576
GON	*Thecadinium inclinatum* Balech	NCMA1890	Canada: British Columbia, Boundary Bay (49°00'N, 123°00'W)	AY238477, GU295209
GON	*Thecadinium Kofoidii* (Herdman) J.Larsen	n.ind.	n.ind.	GU295204, GU295207
PER	*Blastodinium contortum* Chatton [isolated from *Paracalanus* cf. *parvus* (Claus, 1863)]	n.ind.	USA–CA: Gulf of California, station 3 (24°14'N, 110°20'W)	FJ228701
PER	*Blastodinium crassum* Chatton [isolated from *Paracalanus* cf. *parvus* (Claus, 1863)]	n.ind.	USA–CA: Gulf of California, station 2 (24°13'N, 110°20'W)	FJ228702

PER	*Cachonina hallii* (Freud. & J.J.Lee) J.D.Dodge	n.ind.	n.ind.	AF033865, AF033867
PER	*Cachonina* spec.	NCMA424	Australia: New South Wales, Cronulla, CSIRO (34°05'S 151°10'E)	EF492492, JQ991005, EF205005
PER	*Calcicarpinum bivalvum* G.Versteegh [= "*Pentapharsodinium*" *tyrrhenicum* (Balech) Montresor, Zingone & D.Marino]	GeoB229	off Italy: Gulf of Taranto	HQ845329
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE1	Australia: Tasmania, Derwent River, Sullivans Cove	DQ991372
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE2	Australia: Tasmania, Derwent River, Sullivans Cove	DQ991373
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE10	Australia: Tasmania, Derwent River, Sandy Bay	DQ991374
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE14	Australia: Tasmania, Derwent River, Sandy Bay	DQ991375
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBHU1	Australia: Tasmania, Huon River	DQ991376
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBHU2	Australia: Tasmania, Huon River	DQ991377
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBSA4	off Australia: South Australia, Port Lincoln	DQ991378
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBWA11	Australia: Western Australia, Brunswick River	DQ991379
PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBWA12	Australia: Western Australia, Brunswick River	DQ991380

PER	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	H/V14; VIMS V14	USA–NC: Neuse River; USA–VA: Great Wicomico River	AY245690
PER	*Cryptoperidiniopsis* spec.	F525Jul02	USA–FL: St. John's River	AY590480
PER	*Cryptoperidiniopsis* spec.	Folly C5	USA–SC: Folly Beach	AY590481
PER	*Cryptoperidiniopsis* spec.	NCMA1828	USA–MD: King's Creek (38°24'N, 75°51'W)	AY590476, AY456119
PER	*Cryptoperidiniopsis* spec.	NOAA Beach	USA–NC: Piver's Island	AY590486
PER	*Cryptoperidiniopsis* spec. (isolated from gut of mullet)	PLO21	USA–FL: Bird Island Archipelago, St. Lucie River	AY245691
PER	*Duboscquodinium collini* Grassé [isolated from *Eutintinnus fraknoii* (Daday, 1887)]	VSM11	off France: Bay of Villefranche-sur-Mer	HM483399
PER	*Duboscquodinium collini* Grassé [isolated from *Eutintinnus fraknoii* (Daday, 1887)]	VSM12	off France: Bay of Villefranche-sur-Mer	HM483398
PER	*Durinskia baltica* (Levander) Carty & El.R.Cox	n.ind.	China: Xiantao	GU999528, GU999529
PER	*Ensiculifera* aff. *loeblichii* El.R.Cox & H.J.Arn.	GeoB*220	off Senegal	HQ845328
PER	*Glenodinium inaequale* Chodat	ASW12003	Greenland: Jakobshaven	EF058237, EF058257
PER	*Heterocapsa triquetra* (Ehrenb.) F.Stein	GSW206-2	South Korea: Gosung	AY421787, EF613355
PER	*Heterocapsa triquetra* (Ehrenb.) F.Stein	NCMA448	USA–MA: Falmouth, Perch Pond (41°32'N, 70°37'W)	GU594638, EU165307
PER	*Leonella granifera* (Fütterer) Janofske & Karwath	GeoB 38		JN982370, JN982407
PER	*Leonella granifera* (Fütterer) Janofske & Karwath	GeoB 132		AY499516, ###, ###
PER	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	HR1NovC5	USA–FL: St. Lucie River	AY590482
PER	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	NC Lucy-V27	USA–NC: New River	AY590485

PER	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	VIMS1041	USA–VA: James River	EU048552
PER	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	VIMS1050	USA–VA: James River	EU048553
PER	*Luciella* spec.	Florida Lucy	USA–FL: Indian River System	AY245689
PER	*Luciella* spec.	HR1SSeptA5	USA–FL: St. Lucie River	AY590483
PER	*Luciella* spec.	NCMA1835	USA–VA: Pokomoke Sound, station DHA (37°58'N, 75°42'W)	AY590477, AY456120
PER	*Luciella* spec.	NCMA1838	USA–NC: Neuse River	AY590478
PER	*Palatinus apiculatus* (Ehrenb.) Craveiro, Calado, Daugbjerg & Moestrup	HBI:HB201101a	n.ind.	JQ639763, JQ639771, JQ639753
PER	*Pentapharsodinium dalei* Indel. & A.R.Loebl.	SCCAP K-1100	Sweden: Koljö Fjord (58°14'N, 11°34E)	JX262492, JX262496, JX262498
PER	*Peridiniopsis borgei* Lemmerm.	PBSK-A	Sweden: Skåne, St. Kalkbrottsdammen	EF058241, EU445295, EF058261, EF417339
PER	"*Peridiniopsis*" *niei* G.X.Liu & Z.Y.Hu	Donghu	China: Hubei, Wuhan, Donghu Lake (30°33'N, 114°23'E)	HM596542, HM596550, HM596555, JQ281999
PER	"*Peridiniopsis*" *penardii* (Lemmerm.) Bourr.	Jiulongjiang	China: Fujian, Zhangzhou, Jiulongjiang River (24°35'N, 117°41'E)	HM596543, HM596551, HM596556, JQ282001
PER	"*Peridiniopsis*" spec.	QZ-2012 HBI:FJ201101	n.ind.	JQ639767, JQ639770, JQ639752
PER	"*Peridinium*" *aciculiferum* Lemmerm.	PAER-1	Sweden: Lake Erken	AY970653, AY970649, AY970652, DQ094825
PER	*Peridinium bipes* forma *globosum* Er.Lindem.	NIES495	Japan: Fukushima, Lake Onogawa	GU046392

PER	*Peridinium bipes* forma *occultatum* Er.Lindem.	HYJA0310	South Korea: Juam Reservoir	GU046390
PER	*Peridinium bipes* forma *occultatum* Er.Lindem.	NIES497	Japan: Nagano, Omachi, Taira, Lake Kizaki	GU046391
PER	"*Peridinium*" *centenniale* (Playfair) M.Lefèvre	CCAC2	UK–England: Cornwall, near St. Just	EF058236, EF058254
PER	*Peridinium cinctum* (O.F.Müll.) Ehrenb.	CCAC102	Germany: Lower Saxony, Spiekeroog	EF058244, EF058264
PER	*Peridinium cinctum* (O.F.Müll.) Ehrenb.	PCGY-4	Sweden: Skåne, Lake Gyllebo	EF058245, EF058265
PER	*Peridinium cinctum* (O.F.Müll.) Ehrenb.	SAG2017	Germany, Hessen, Marburg, Cappeler Weiher	EF058243, EF058263
PER	*Peridinium gatunense* Nygaard	PGDA-1	Sweden: Skåne, Lake Dagstorpsjön	EF058246, EF058267
PER	"*Peridinium*" *inconspicuum* Lemmerm.	CCAP1140/3	Germany: Schleswig-Holstein, Ostholstein, Kleiner Ukleisee	FR865631
PER	"*Peridinium*" *polonicum* Wołosz.	HBI:MG200823a	n.ind.	JQ639764, JQ639772, JQ639754
PER	"*Peridinium*" *umbonatum* F.Stein.	FACHB329	[Chinese text]	GU001636, GU001637, GU001638, GU001639
PER	*Peridinium volzii* Lemmerm.	NIES-501	Japan: Ibaraki, Tsuchiura	EF058248, EF058270
PER	*Peridinium willei* Huitf.-Kaas	NIES-304	Japan: Gunma, Tsukiyono	AF274272, EF058271
PER	*Peridinium willei* Huitf.-Kaas	NIES-365	Japan: Iwate, Ajiro	AF274280, EF058272
PER	*Peridinium willei* Huitf.-Kaas	NIES-366	Japan: Ibaraki, Tsuchiura	EF058249, EF058273
PER	*Peridinium willei* Huitf.-Kaas	PWCA-1	Canada: Alberta, Calgary, Glenmore Reservoir	DQ166211, EF058274
PER	*Peridinium willei* Huitf.-Kaas	TK007	off Japan: Hokkaido	AB232669
PER	*Pfiesteria piscicida* Steid. & J.M.Burkh.	n.ind.	USA–MD: Chicamacomico River	AY112746

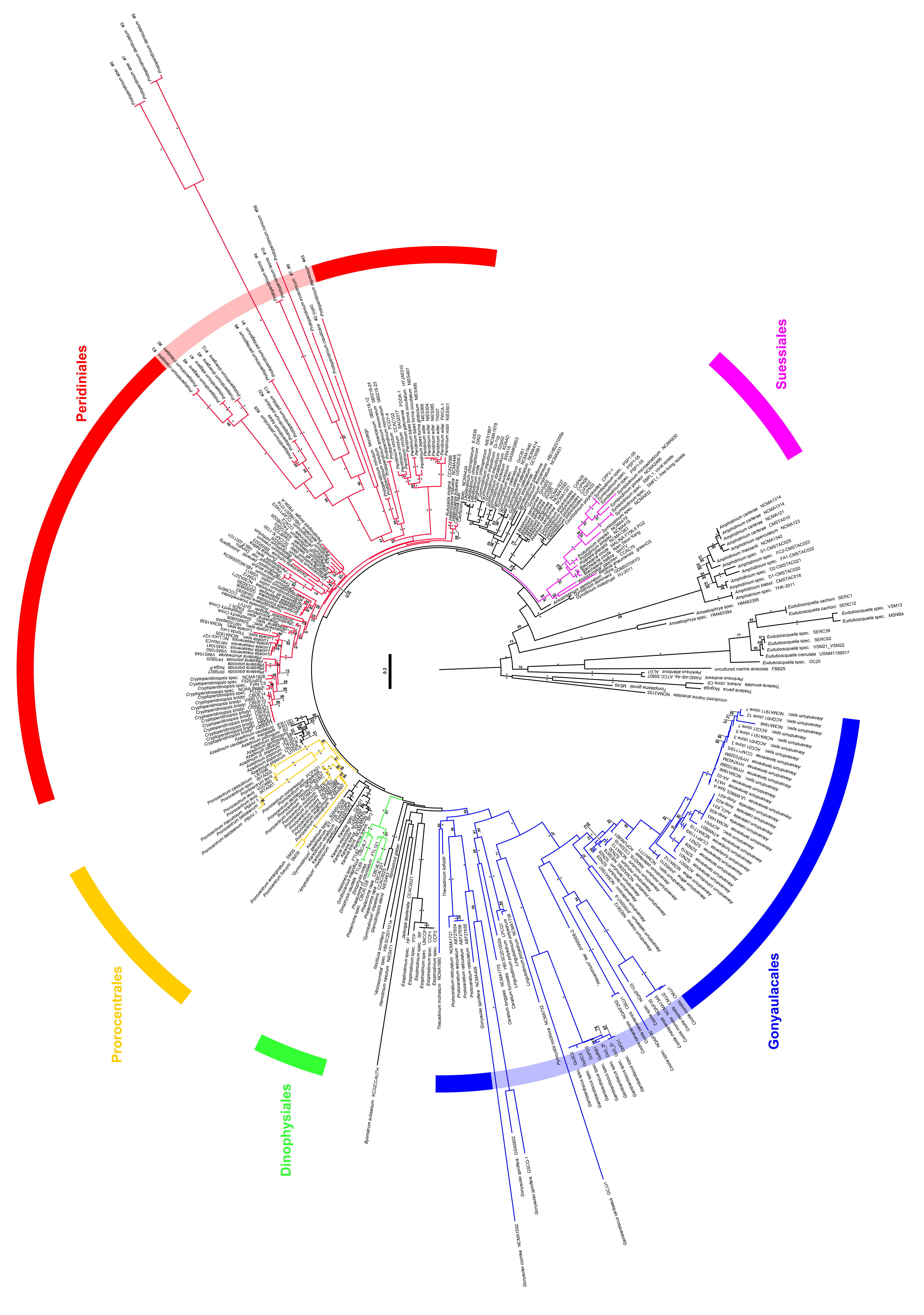
PER	*Pfiesteria piscicida* Steid. & J.M.Burkh.	Noga-P	USA–NC: Pamlico River	AY245693
PER	*Pfiesteria piscicida* Steid. & J.M.Burkh.	PPSB25	Indonesia: Surabaya (ballast water)	DQ991381
PER	*Pfiesteria piscicida* Steid. & J.M.Burkh.	PPSB27	Indonesia: Surabaya (ballast water)	DQ991382
PER	*Pfiesteria shumwayae* Glasgow & J.M.Burkh.	Noga-S; VIMS1049	USA–NC: Pamlico River	AY245694
PER	*Posoniella tricarinelloides* (G.Versteegh) Streng, Banasová, Reháková & H.Willems	PTLY01		###
PER	*Posoniella tricarinelloides* (G.Versteegh) Streng, Banasová, Reháková & H.Willems	GeoB 413		###, ###
PER	*Posoniella tricarinelloides* (G.Versteegh) Streng, Banasová, Reháková & H.Willems	GeoB 429		###
PER	*Protoperidinium abei* (Paulsen) Balech	#7	Japan: Hokkaidō, Shiribeshi, Otaru	AB181881, AB255839
PER	*Protoperidinium abei* (Paulsen) Balech	#9	Japan: Hokkaidō, Shiribeshi, Otaru	AB181882, AB255839
PER	*Protoperidinium americanum* (T.H.Abé) Balech	080218-12	Japan: Nagasaki, Sasebo harbor	AB716911, AB716925
PER	*Protoperidinium bipes* (Paulsen) Balech	n.ind.	Japan: Hokkaidō, Ishikari	AB284159, AB284160
PER	*Protoperidinium claudicans* (Paulsen) Balech	#2 (cyst)	Japan: Hokkaidō, Ishikari	AB255833, AB255842
PER	*Protoperidinium conicum* (Gran) Balech	#56	Japan: Hokkaidō, Shiribeshi, Otaru	AB181886, AB255844
PER	*Protoperidinium crassipes* (Kof.) Balech	#0	Japan: Hokkaidō, Shiribeshi, Otaru	AB181888, AB255845
PER	*Protoperidinium crassipes* (Kof.) Balech	#3	Japan: Hokkaidō, Shiribeshi, Otaru	AB181889, AB255846
PER	*Protoperidinium denticulatum* (Gran & Braarud) Balech	#3	Japan: Hokkaidō, Shiribeshi, Otaru	AB181890, AB255848
PER	*Protoperidinium denticulatum* (Gran & Braarud) Balech	#9	Japan: Hokkaidō, Shiribeshi, Otaru	AB181891, AB255849
PER	*Protoperidinium depressum* (Bailey) Balech	#45	Japan: Hokkaidō, Ishikari	AB255834, AB255850
PER	*Protoperidinium divergens* (Ehrenb.) Balech	#5	Japan: Hokkaidō, Shiribeshi, Otaru	AB181892, AB255851

PER	*Protoperidinium divergens* (Ehrenb.) Balech	#12	Japan: Hokkaidō, Tokachi, Kamikawa, Shimizu	AB181893, AB255852
PER	*Protoperidinium elegans* (Cleve) Balech	#1	offshore Pacific (28°57'N, 130°10'E)	AB255835, AB255853
PER	*Protoperidinium elegans* (Cleve) Balech	#8	offshore Pacific (29°17'N, 130°08'E)	AB255836, AB255854
PER	*Protoperidinium excentricum* (Paulsen) Balech	#7, #8	Japan: Tokyo, Shiooka Park	AB275355, AB255855
PER	*Protoperidinium leonis* (Pavill.) Balech	#4	Japan: Hokkaidō, Ishikari	AB181894, AB255856
PER	*Protoperidinium leonis* (Pavill.) Balech	#10	Japan: Hokkaidō, Ishikari	AB181898, AB255856
PER	*Protoperidinium monovelum* (T.H.Abé) Balech	080218-23	Japan: Nagasaki, Sasebo harbor	AB716913, AB716927
PER	*Protoperidinium monovelum* (T.H.Abé) Balech	080218-24	Japan: Nagasaki, Sasebo harbor	AB716914, AB716928
PER	*Protoperidinium pallidum* (Ostenf.) Balech	#13	Japan: Hokkaidō, Shiribeshi, Otaru	AB181899, AB255859
PER	*Protoperidinium pallidum* (Ostenf.) Balech	#22	Japan: Hokkaidō, Shiribeshi, Otaru	AB181901, AB255861
PER	*Protoperidinium pellucidum* Bergh	#28	Japan: Hokkaidō, Shiribeshi, Otaru	AB181903, AB255862
PER	*Protoperidinium pentagonum* (Gran) Balech	#1	Japan: Hiroshima, Takehara	AB255837, AB255864
PER	*Protoperidinium pentagonum* (Gran) Balech	#9	Japan: Hiroshima, Takehara	AB255838, AB255865
PER	*Archaeperidinium minutum* Jörgensen	Mondego	Portugal: Mondego estuary	GQ227501, GQ227502
PER	*Archaeperidinium minutum* Jörgensen	n.ind.	Canada: British Colombia, Victoria	AB564308, AB564310
PER	*Rufusiella insignis* (Hassall) A.R.Loebl.	CCAC0066	Germany: Hessen, Marburg, Nordeck	EF058238, EF058258
PER	"*Scrippsiella*" *hangoei* (J.Schiller) J.Larsen	SHTV1	off Finland (Baltic Sea): Tvärminne	AY970662, AY970654, AY970658, JN982417
PER	*Scrippsiella sweeneyae* Balech ex A.R.Loebl.	CCCM280	n.ind.	HQ845331, EU840175

PER	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	GeoB283	off Norway: Sør-Trøndelag, Snillfjord commune, Åstfjorden, Mjønos (harbour)	HQ845330, ###
PER	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	GSW9808	off Korea	AY421792, EF613366
PER	*Scrippsiella* aff. *trochoidea* (F.Stein) A.R.Loebl.	NCMA2271	USA–ND: Jim Lake	HM483396
PER	"*Stoeckeria*" spec., name not validly published (ICBN Art. 36.2)	Shepherd's Crook	USA–FL: Trout River	AY590479
PER	"*Stoeckeria*" spec., name not validly published (ICBN Art. 36.2)	Shepherd's Crook	USA–FL: St. Lucie River	AY590484
PER	"*Stoeckeria*" spec., name not validly published (ICBN Art. 36.2)	SSMS0806	South Korea: Masan	FN557541
PER	*Thoracosphaera heimii* (Lohmann) Kamptner	CCCM670	Gulf of Mexico	HQ845327, JN982437
PER	*Tintinnophagus acutus* Coats (isolated from *Tintinnopsis cylindrica* Daday, 1887)	–	USA–MD: Chesapeake Bay	HM483397
PER	*Zooxanthella nutricula* K.Brandt (isolated from *Thalassicolla nucleata* Huxley, 1851)	BBSR323	off UK, the Bermudas (Sargossa Sea): 3-5 miles SE of Bermuda	U52356, ###, ###

Figure Legend

Figure S1: Congruence between a molecular phylogeny and morphologically established dinophyte groups. Maximum Likelihood tree of 319 members of the Dinophyta as inferred from a MAFFT generated rRNA nucleotide alignment spanning SSU and LSU (3,089 parsimony-informative positions). Major clades are indicated, and branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values for the clusters to the right of them (above: ML bootstrap support values, values under 50 are not shown; below: Bayesian posterior probabilities, values under .90 are not shown), and asterisks indicate maximal support values. The tree is rooted with four sequences of the Apicomplexa.


Peridiniales
Suessiales
Gonyaulacales
Dinophysiales
Prorocentrales

PAPER 3 (PUBLICATION)

FIRST RECORD OF THE GENUS *AZADINIUM* (DINOPHYCEAE) FROM THE SHETLAND ISLANDS, INCLUDING THE DESCRIPTION OF *AZADINIUM POLONGUM* SP. NOV.

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First record of the genus *Azadinium* (Dinophyceae) from the Shetland Islands, including the description of *Azadinium polongum* sp. nov.

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ABSTRACT

Azadinium is a dinophycean genus capable of producing azaspiracids (AZAs), a recently discovered group of lipophilic phycotoxins causing human intoxication via mussel consumption. Although initially described from the North Sea, the genus currently consisting of four described species is probably distributed worldwide. Here we report on *Azadinium* from the Shetland Islands, which are located in the northernmost part of the North Sea and are largely influenced by the Atlantic Ocean. Two strains of *Azadinium* were isolated from a single water sample. One strain was identified as *Azadinium spinosum* based on morphology and sequence data and had an AZA cell quota of about 20 fg per cell, similar to all other described strains of the species. The toxin profile consisted of AZA-1 and AZA-2 in a 2.3:1 ratio and a yet undescribed AZA of 715 Da. The other strain represents a new species and is here described as *Azadinium polongum* sp. nov. Like *A. spinosum*, but different to *Azadinium obesum* and *Azadinium poporum*, *A. polongum* has an antapical spine. *A. polongum* differs from *A. spinosum* by an elongated shape of the pore plate (Po), and X-plate, the location of the ventral pore, and the absence of a distinct pyrenoid with starch sheath. Molecular analysis based on SSU, LSU, and ITS sequencing supported separation of *A. polongum* at the species level. Detailed LC–MS analysis showed that *A. polongum* does not produce any known AZAs in measureable amounts.

1. Introduction

Public health impairment through consumption of contaminated shellfish is a major problem caused by harmful algal blooms. Among the known responsible compounds, azaspiracids (AZAs) are the most recently discovered group of lipophilic polyether toxins of microalgal origin. After a first poisoning incident in the Netherlands in 1995, azaspiracid toxins were isolated and chemically characterized from Irish shellfish (Satake et al., 1998; Ofuji et al., 1999). Since then, AZA contamination of mussels has been a recurrent and serious problem in Ireland (Salas et al., 2011). In addition, toxins have been observed in samples from Europe, Morocco, Chile, and Japan (Brana Magdalena et al., 2003; Taleb et al., 2006; Amzil et al., 2008; Ueoka et al., 2009; Alvarez et al., 2010; Furey et al., 2010). Although chemistry and toxicity of AZA were intensively studied (Twiner et al., 2008), it took 12 years to discover a planktonic source of the toxin, a small dinophyte (Krock et al., 2009) identified as *Azadinium spinosum* Elbrächter et Tillmann (Tillmann et al., 2009).

The toxigenic type of this newly erected genus, *A. spinosum*, was initially isolated off the Scottish east coast, but was subsequently observed and isolated from Denmark (Tillmann et al., 2011) and Irish coastal waters (Salas et al., 2011). The description of *A. spinosum* was soon followed by both the discovery of new species and the new records of *Azadinium* in natural samples from various areas. First, *Azadinium obesum* Tillmann et Elbrächter was isolated and described from the same water sample as *A. spinosum* off Scotland (Tillmann et al., 2010), indicating co-occurrence with the type. Likewise, *Azadinium poporum* Tillmann et Elbrächter, the third species, was isolated from the same sample as an Danish *A. spinosum* isolate (Tillmann et al., 2011). Next, *Amphidoma caudata* Halldal, a species described with the same basic plate pattern as *Azadinium* (Dodge and Saunders, 1985), was revised both by morphological and molecular data and transferred to the genus as *Azadinium caudatum* (Haldahl) Nézan et Chomérat (Nézan et al., 2012), with two distinct varieties, var. *caudatum* and var. *margalefii*. Surprisingly, however, even "true" *Amphidoma* species and *Azadinium* turned out to be closely related, despite marked differences in epithecal plate pattern. This was recently shown by morphology and molecular phylogeny for the new species *Amphidoma languida* Tillmann, Salas et Elbrächter (Tillmann et al., 2012), which was isolated together with an Irish *A. spinosum*

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isolate (Salas et al., 2011) and is very similar in general size and shape to species of *Azadinium*. Both genera *Azadinium* and *Amphidoma* are now integrated in the family Amphidomataceae Sournia (Tillmann et al., 2012).

The three available strains of *A. spinosum* consistently produce AZA with a reported toxin profile consisting of AZA-1 and AZA-2, indicating that the production and profile of known AZAs is a stable characteristic of the species. Other species of *Azadinium* have initially been reported as non-toxigenic in terms of known AZAs. However, AZA production within Amphidomataceae probably is much more complex and diverse, as recent evidence indicates the presence of new AZAs with a modified substitution pattern in *A. poporum* and *A. languida* (Krock et al., 2012), but toxicity of these compounds still needs to tested.

Although mainly recorded from the North Sea and adjacent areas, species of *Azadinium* probably have a much wider distribution. Recently, a strain assigned to the genus *Azadinium* was isolated from coastal waters of Korea (Potvin et al., 2012), which is the first published report of *Azadinium* from the Pacific Ocean. In terms of morphology, the strain designated as *A.* cf. *poporum* by Potvin et al. (2012) is almost identical to the European *A. poporum*, but differs significantly in terms of sequence data. Recent reports of *Azadinium* from field samples include blooms in Argentina (Akselman and Negri, 2012), a record from Mexico (Hernandez-Becerril et al., 2010) and an entry in the check list of Black Sea phytoplankton (http://phyto.bss.ibss.org.ua/wiki/Azadinium_spinosum). In addition to these coastal records, specimens of *Azadinium* were detected in samples collected from the open Indian Ocean (Carbonell-Moore, pers. commun.). These records, taken together with the widespread records of AZA toxins, indicate a global distribution of *Azadinium* in general and of the toxigenic *A. spinosum* in particular. Here we report *Azadinium* from the Shetland Island, expanding the known distribution of the genus to the northernmost part of the North Sea, an area heavily influenced by the Atlantic Ocean. Two cultures were established, one representing the toxin-producing type, *A. spinosum*, and the other representing a new species, *A. polongum* sp. nov.

2. Material and methods

2.1. Isolation and culture

Two isolates of *Azadinium*, designated as isolate SHETF6 and SHETB2, were established from a water sample collected adjacent to the Shetland Islands (60°12.73′N and 0°59.90′W) during a cruise aboard the research vessel "Heincke" in May 2011. A 1-L Niskin bottle sample (10 m depth) was pre-screened (20 μm Nitex gauze), gently concentrated by gravity filtration using a 3-μm polycarbonate filter, and examined using an inverted microscope (Axiovert 200M, Zeiss, Germany). Cells of *Azadinium* were rare in the sample and were visually pre-identified at high magnification (640×) based on general cell size and shape. Pre-identified cells were isolated by micro-capillary into wells of 96-well plates filled with 0.2 mL filtered seawater. By this transfer technique, the inclusion of non-target cells is unavoidable. Therefore, each primary well of isolation was subsequently partitioned in 10-μL quantities distributed into 20 new wells pre-filled with 0.2 mL filtered seawater. Plates were incubated at 15 °C under a photon flux density of ca. 50 μmol m^{-2} s^{-1} on a 16:8 h light:dark photocycle in a controlled environment growth chamber. From these preparations, clonal cultures were established by isolation of single cells by micro-capillary. Established cultures were routinely held at 10 °C and 15 °C (SHETB2) or at 15 °C and 20 °C (SHETF6).

Cultures for toxin analysis were grown in plastic culture flasks at 10 °C (*A. polongum*) or 20 °C (*A. spinosum*) under a photon flux density of 25 μmol m^{-2} s^{-1} on a 16:8 h light:dark photocycle. For *A. spinosum* SHETF6, 200 mL of a dense culture (68,000 cell mL^{-1}, cell concentration determined by counting >800 cells under an optical microscope) were harvested in 4× 50 mL Falcon tubes by centrifugation (Eppendorf 5810R, Hamburg, Germany) at 3220 × *g* for 10 min. For *A. polongum* SHETB2, cultures were grown in parallel in 270-mL culture flasks to a mean cell density of 2700 cells mL^{-1}, with 500 mL then harvested by centrifugation of 10 × 50 mL. All cell pellets from one strain were combined in an Eppendorf microtube and again centrifuged (Eppendorf 5415, 16,000 × *g*, 5 min). The final pellet of *A. spinosum* and *A. polongum* were each subsequently suspended in 500 μL methanol and transferred into a FastPrep tube containing 0.9 g of lysing matrix D (Thermo Savant, Illkirch, France). Sample were homogenized by reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France) and then centrifuged (Eppendorf 5415 R, Hamburg, Germany) at 16,100 × *g* at 4 °C for 15 min. Each supernatant (400 μL) was transferred to a 0.45-μm pore-size spin-filter (Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 800 × *g*, with the resulting filtrate transferred into an LC autosampler vial for LC–MS/MS analysis.

2.2. Light microscopy (LM)

Observation of living cells was carried out with a stereomicroscope (Olympus SZH-ILLD) and with an inverted microscope (Axiovert 200M, Zeiss, Germany) equipped with epifluorescence and differential interference contrast optics. Light microscopic examination of the thecal plate tabulation was performed on formalin-fixed cells (1% final concentration) stained with calcofluor white (Fritz and Triemer, 1985). The shape and location of the nucleus was determined after staining of formalin-fixed cells for 10 min with 4′-6-diamidino-2-phenylindole (DAPI, 0.1 μg mL^{-1} final concentration). Photographs were taken with a digital camera (Axiocam MRc5, Zeiss, Germany) connected to the inverted microscope.

Cell length and width were measured at 1000× microscopic magnification using Zeiss Axiovision software (Zeiss, Germany) and freshly fixed cells (formalin final concentration 1%) of a culture growing at 10 °C.

2.3. Scanning electron microscopy (SEM)

For SEM examination of thecal plates, cells from growing cultures were fixed, prepared, and collected on 3-μm polycarbonate filters (Millipore) as described by Tillmann et al. (2010), with the following modification: after the 60% ethanol treatment, cells were fixed in a 60:40 mixture of deionized water and seawater containing 2% formalin for 3 h at 4 °C before dehydration. Filters were mounted on stubs, sputter-coated (Emscope SC500, Ashford, UK) with gold-palladium and viewed under a scanning electron microscope (FEI Quanta FEG 200, Eindhoven, Netherlands). Some SEM micrographs were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA).

2.4. Morphometric measurements

SEM photographs were used to measure pore plate dimensions of *A. polongum* and *A. spinosum* strains SHETF6, UTHE2, and 3D9, the latter two being archived SEM pictures. The software package Statistica (StatSoft) was used to compare pore-plate measurements (Student's *t*-test) for *A. polongum* with pooled measurements for the three *A. spinosum* strains and to plot values including 95% confidence ellipses.

2.5. Chemical analysis for azaspiracids

For both new isolates, an intensive analysis for the presence of AZAs was conducted. Samples were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) according to the methods described in detail by Tillmann et al. (2009). Selected reaction monitoring (SRM) experiments were carried out in positive ion mode by selecting the following transitions (precursor ion > fragment ion): (1) AZA-1 and AZA-6: m/z 842 > 824 collision energy (CE): 40 V and m/z 842 > 672 CE: 70 V; (2) AZA-2: m/z 856 > 838 CE: 40 V and m/z 856 > 672 CE: 70 V; (3) AZA-3: m/z 828 > 810 CE: 40 V and m/z 828 > 658 CE: 70 V; (4) AZA-4 and AZA-5: m/z 844 > 826 CE: 40 V (5) AZA-7, AZA-8, AZA-9 and AZA-10: m/z 858 > 840 CE: 40 V, and 6) AZA-11 and AZA-12: m/z 872 > 854 CE: 40 V. The following additional mass transitions were used for new AZAs: m/z 816 > 798, 830 > 812, 846 > 828, 868 > 806, 870 > 852 CE: 40 V and m/z 816 > 348, 830 > 348, 846 > 348, 858 > 348, 868 > 362 CE: 70 V.

2.5.1. Precursor ion experiments

Precursors of the fragments m/z 348 and m/z 362 were scanned in the positive ion mode from m/z 400 to 950 under the following conditions: curtain gas: 10 psi; CAD: medium; ion spray voltage: 5500 V; temperature: ambient; nebulizer gas: 10 psi; auxiliary gas: off; interface heater: on; declustering potential: 100 V; entrance potential: 10 V; collision energy: 70 V; exit potential: 12 V.

2.6. Molecular phylogenetic analysis

The molecular analysis was conducted by the cooperation of laboratories at the LMU Munich, Germany and the IFREMER Concarneau, France. Fresh material of the strains SHETB2 and SHETF6 was sent to both of the laboratories.

In Munich, genomic DNA was extracted using the Nucleo Spin Plant II Kit (Macherey-Nagel, Düren, Germany) according to manufactures instructions. The complete 18S rDNA, the first two domains of the 28S rDNA (D1/D2 region) and the internal transcribed spacer (ITS), including the 5.8S rDNA were amplified using the primers listed in Table S1 (provided as Supplementary Material) and sequenced following standard protocols (Gottschling et al., 2012).

In Concarneau, two optional methods were used to obtain genomic DNA: DNA extraction from an exponentially growing culture of *Azadinium* isolate prior to DNA amplification or direct PCR amplification from 1 to 4 cells isolated from Lugol-fixed cultures. For the first approach, cells from approximately 20 mL of isolate SHETB2 were harvested by centrifugation (4000 rpm, 20 min). The genomic DNA was extracted using the CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide) method (Doyle and Doyle, 1987). For the second approach, cells of isolates SHETB2 or SHETF6 were respectively deposited on a glass slide, using a micropipette under the Olympus IMT2 inverted light microscope. Subsequently, the cells were placed in a drop of a sodium thiosulfate solution to decrease the inhibiting effect of the fixative on the PCR (Auinger et al., 2008), rinsed twice in double distilled water (ddH_2O) before transfer to a 0.2-mL PCR tube containing 3 μL of ddH_2O and stored at −20 °C until the direct PCR. Afterward, the 18S rDNA, 28S rDNA (D1/D2 region), and the internal transcribed spacer (ITS) including the 5.8S rDNA and COI were amplified using the primers listed in Nézan et al. (2012). Genomic DNA was amplified in 25-μL PCR reaction containing either 1 μL of extracted DNA or isolated cells, 6.5 μL of ultrapure water, 2.5 μL of each primer (10 μM), and 12.5 μL of PCR Master Mix 1× (Promega, Madison, WI, USA) which includes the Taq polymerase, dNTPs, $MgCl_2$, and reaction buffers. The PCRs were performed in a Mastercycler Personal (Eppendorf, Hamburg, Germany) as follows: one initial denaturation step at 94 °C for 2 min, followed by 45 cycles each consisting of 94 °C for 30 s, 52 °C for 1 min, and 72 °C for 4 min, and a final elongation at 72 °C for 5 min. The PCR products were visualized, purified and sequenced following standard protocols (Nézan et al., 2012).

A dataset was compiled of all available *Amphidomataceae* sequences and a systematically representative set of dinophytes downloaded from GenBank (Table S2, provided as Supplementary Material). To avoid the effect of "long branch attractions," only taxa of similar branch length were chosen as outgroups. "MAFFT" v6.624b (Katoh and Toh, 2008; freely available at http://mafft.cbrc.jp/alignment/software/index.html) was used to align the sequences automatically. The alignment is available via nexus file upon request. Phylogenetic analyses were carried out using Maximum-Likelihood (ML) and Bayesian approaches, as described in detail previously (Gottschling et al., 2012). The Bayesian analysis was performed using "MrBayes" v3.1.2 (Ronquist and Huelsenbeck, 2003; freely available at http://mrbayes.sourceforge.net/download.php) under the GTR+C substitution model and the random-addition-sequence method with 10 replicates. We ran two independent analyses of four chains (one cold and three heated) with 20,000,000 cycles, sampled every 1000th cycle, with an appropriate burn-in (10%, after checking convergence). For the ML calculation, "RaxML" v7.2.6 (Stamatakis, 2006; freely available at http://www.kramer.in.tum.de/exelixis/software.html) was applied using the GTR+CAT substitution model to search for the best-scoring ML tree and a rapid bootstrap analysis of 500 non-parametric replicates. Statistical support values (LBS: ML bootstrap support, BPP: Bayesian posterior probabilities) were drawn on the resulting, best-scoring ML tree. The calculation of the pairwise genetic distance p was conducted using Mega version 5.0 (Tamura et al., 2011).

3. Results

Specimens of *Azadinium* were observed in concentrated whole water samples at four out of six stations along the west coast of the Shetlands and from one of these stations, two clonal cultures could be established. Cells of both cultures showed rather slow swimming speed interrupted by sudden jumps in various directions, behavioral traits previously described for members of the genus (Tillmann et al., 2009). From the onset, however, the two isolates displayed marked physiological differences, indicating that they might represent different species: Strain SHETF6 exhibited rapid growth at 15 °C and reached high cell densities at stationary phase (about 100,000 cells mL^{-1}), whereas strain SHETB2 grew much more slowly, reaching maximal cell densities of <3000 cells mL^{-1}. The cultures seem to have different temperature requirements, with cell densities of SHETB2, but not SHETF6, rapidly declining when cultures were grown at 20 °C. In addition, cysts were regularly observed in cultures of SHETB2, but not SHETF6.

Morphological attributes of strain SHETF6 supported identification of this isolate as *Azadinium spinosum* (Fig. 1). Concordant with previous descriptions of the species, the cells are slender and elongated (Fig. 1B), with LM revealing an antapical spine and one conspicuous pyrenoid located in the epicone (Fig. 1A). Plate pattern, plate size and arrangement (Fig. 1D and E), as well as presence and location of the ventral pore (Fig. 1C) are in total agreement with previous description of the type material for *A. spinosum* (Tillmann et al., 2009). Toxin analysis and rDNA sequence also reinforced identification of strain SHETF6 as *A. spinosum* (see below).

Examination of cell morphology and rDNA sequence (see below) supported placement of isolate SHETB2 in the genus *Azadinium*, as a new species.

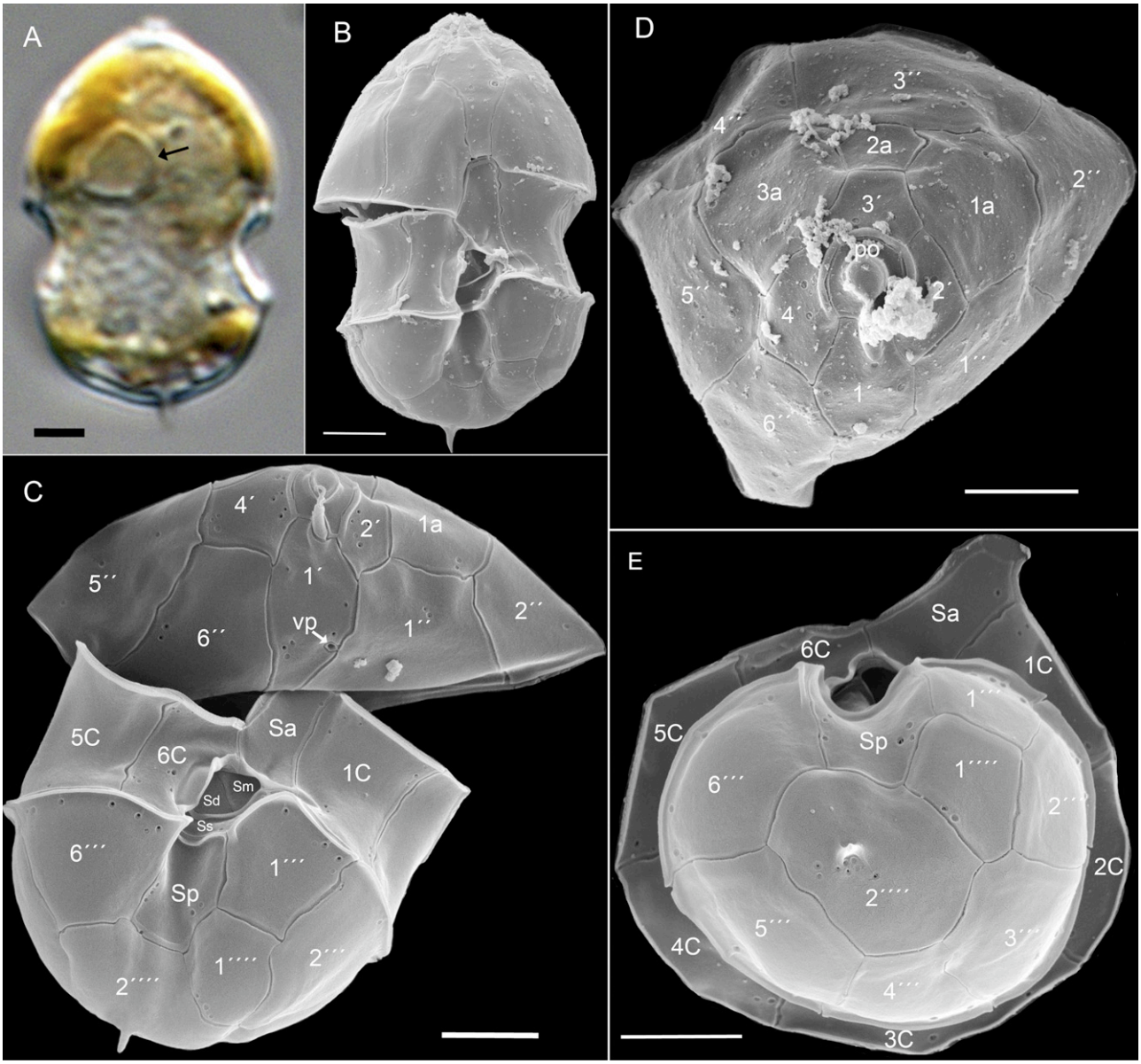

Fig. 1. *Azadinium spinosum* SHETF6, LM (A) and SEM (B–E). (A) Formalin fixed cell showing the antapical spine and one large pyrenoid (arrow). (B and C) Whole cell ventral view, with plate labels. (D) Plate pattern apical view and (E) plate pattern in antapical view. Scale bars = 2 μm.

3.1. Azadinium polongum *sp. nov. Tillmann*

3.1.1. Diagnosis

Differs from *Azadinium spinosum* in the elongated shape of the pore plate Po and the X-plate, the smaller size of Plate 2a, the location of the ventral pore and the absence of a distinct pyrenoid with starch sheath. Distinguished from both *A. obesum* and *A. poporum* by the presence of an antapical spine. The Kofoidian plate tabulation is: Po, cp, X, 4′, 3a, 6″, 6C, 5?S, 6‴, 2⁗. Cell length 10–17 μm, cell width 7–14 μm (Figs. 2–7).

Holotype: A SEM-stub (strain SHETB2, Stub designation CEDiT2012H20), and a formalin fixed sample (strain SHETB2, designation CEDiT2012I21) have been deposited at the Senckenberg Research Institute and Natural History Museum, Center of Excellence for Dinophyte Taxonomy, Germany. Fig. 4 has been chosen to represent the type in accordance to fulfil article 39.1 of the International Code of Botanical Nomenclature (ICBN).

Type locality: 60°12.73′N, 0°59.90′W, Shetland Islands

Habitat: marine plankton

Etymology: the epithet is inspired by the conspicuously elongated shape of the pore plate ('Po′ = designation for pore plate; longus (Latin) = long). The epithet is indeclinable.

3.1.2. Cell morphology

Cells of *A. polongum* are ovoid and, if at all, only slightly dorsoventrally compressed. The episome ends with a conspicuous apical pore complex (APC) and is slightly larger than the hyposome (Fig. 2). The cingulum is deep and wide. Cells are generally small but quite variable in size, ranging from 10.1 to 17.4 μm in length and 7.4 to 13.6 μm in width (median length: 13.0 μm, median width 9.7 μm, $n = 107$), with a median length/width ratio of 1.3. The large nucleus is spherical to slightly elongated and is located in the central part of the cell (Fig. 2E). The presumably single chloroplast is parietally arranged, lobed, and extends into the epi- and hyposome (Fig. 2). Light-microscopy did not indicate the presence of pyrenoid(s) with starch sheath. However, cells may have a number of large starch grains as indicated by positive Lugol staining (Fig. 2D).

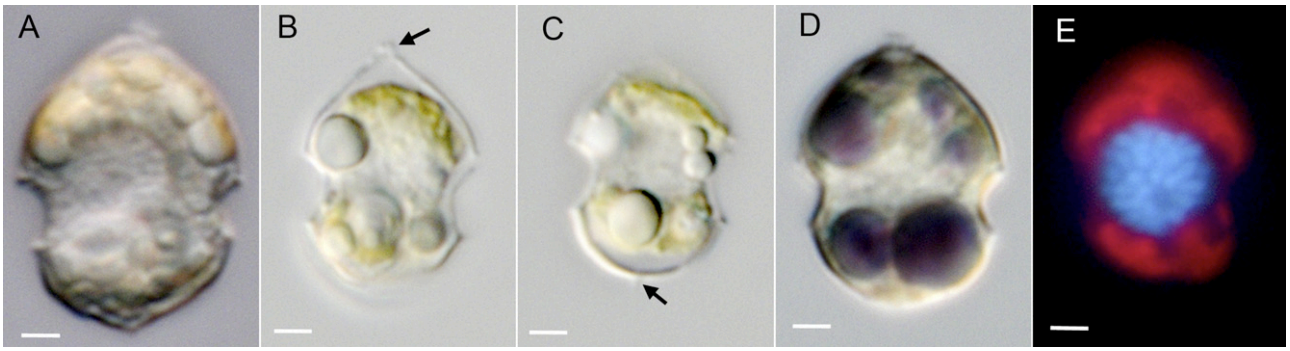

Fig. 2. *Azadinium polongum*, LM. (A) Live cell. (B and C) Formalin fixed cell in two focal planes showing the apical pore complex (arrow in B) and the antapical spine (arrow in C). (D) Lugol-fixed cell with large grains of stained material. (E) Formalin fixed cell stained with DAPI as viewed using UV excitation showing the round central nucleus. Scale bars = 2 μm.

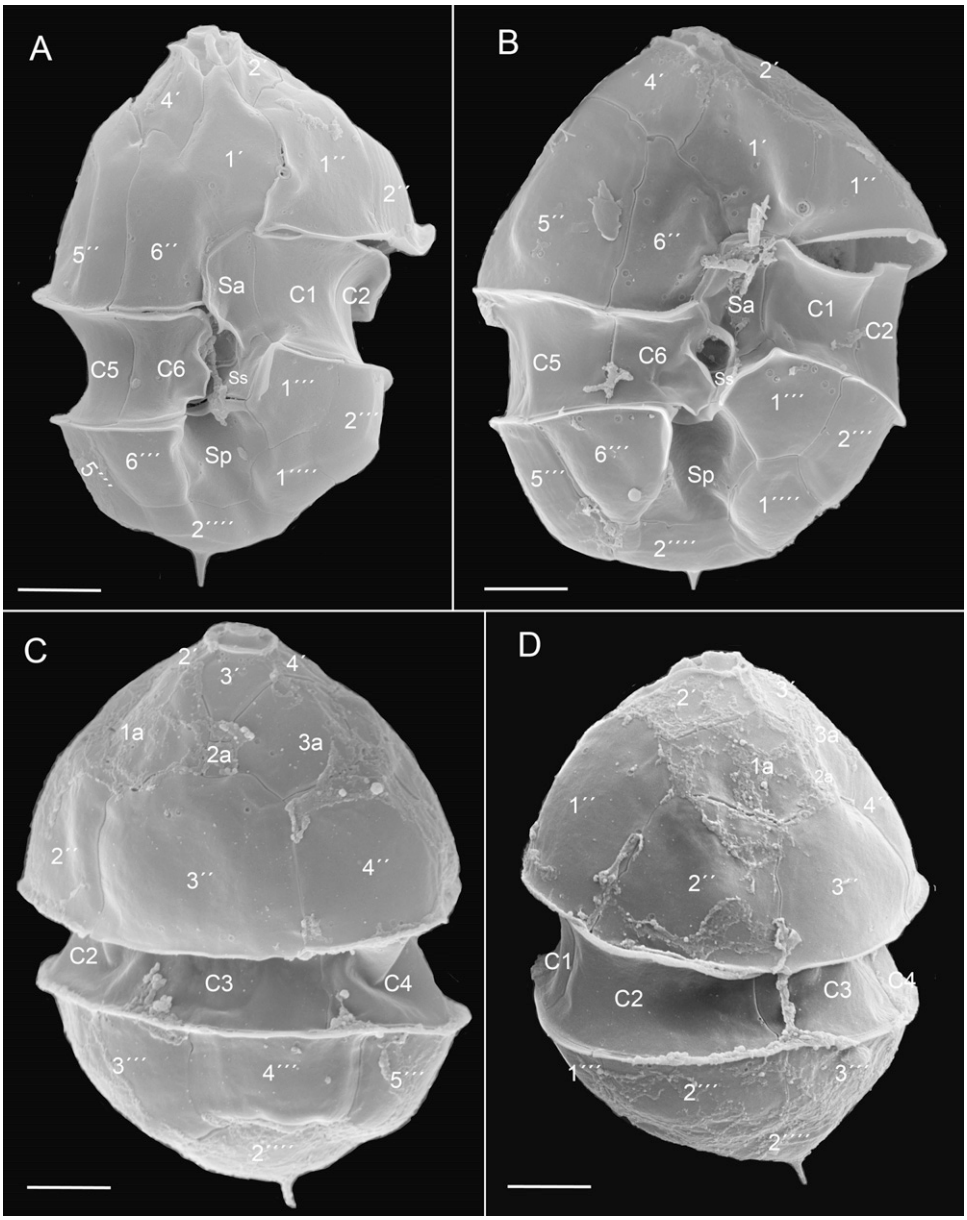

Fig. 3. *Azadinium polongum*. SEM micrographs of thecae of different cells. (A and B) Ventral view. (C) Dorsal view. (D) Left-lateral view. Scale bars = 2 μm.

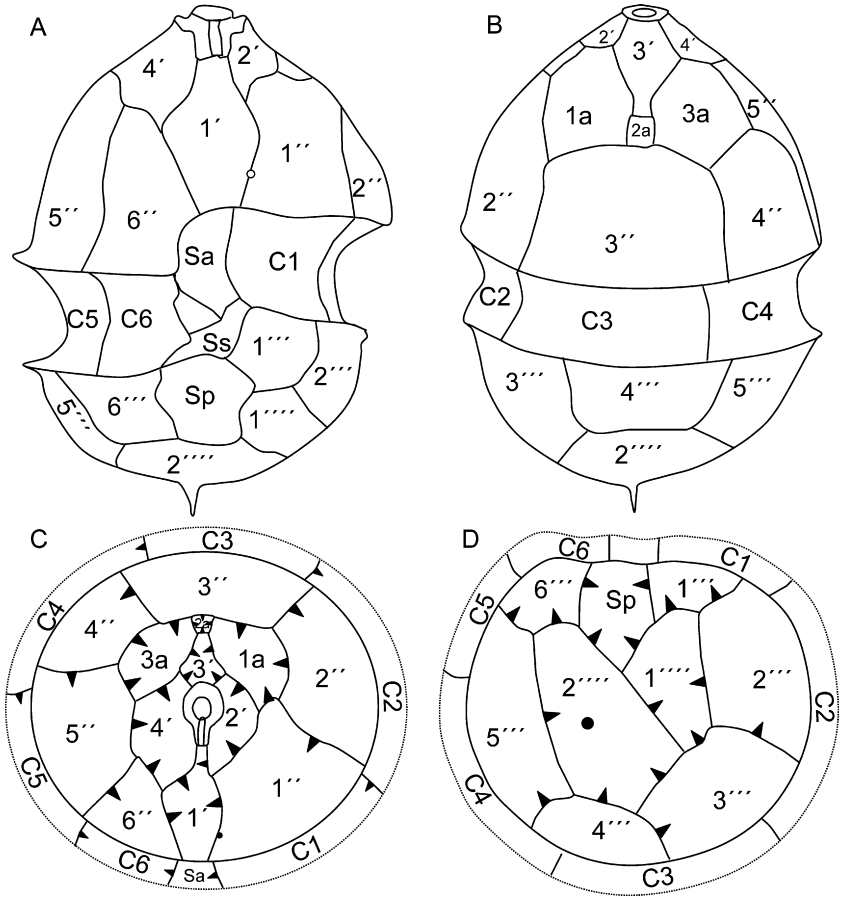

Fig. 4. *Azadinium polongum*. Diagrammatic illustration of thecal plates. (A) Ventral view. (B) Dorsal view. (C) Apical view. (D) Antapical view. *Abbreviations*: Sa, Sd, Sm, Sp, Ss: sulcal plates as detailed in Fig. 6. Arrowheads in C and D indicate plate overlap pattern.

Cells of *A. polongum* possess delicate thecal plates, which can be readily seen in the light microscope (Fig. 2) and stained with calcofluor white (not shown). However, the plate pattern was most easily resolved by SEM (Figs. 3, 5–7). Generally, the surface of the plates is smooth but a few pores of different sizes are irregularly scattered over the plates. The basic thecal plate arrangement is: Po, cp, X, 4′, 3a, 6″, 6C, 5?S, 6‴, 2⁗, as drawn in Fig. 4.

The apical series is composed of four plates (Fig. 5). Plate 1′ has an ortho pattern, but slightly asymmetric, with the suture joining Plate 6″ being shorter than that joining Plate 1″ (Fig. 5C). In its anterior part, Plate 1′ is progressively narrowed, ending in a slender tip abutting the pore plate. A ventral pore located at the border of Plate 1′ and 1″ is present at the lower third level of the epitheca (Figs. 5C and 7G and H). This pore has a distinct outer rim that measure 0.35 ± 0.02 μm and 0.21 ± 0.01 μm (n = 10) for outer and inner diameter, respectively. Comparing the lateral apical Plates 2′ and 4′, the left Plate 4′ is slightly larger. Both plates clearly invade the ventral area, with their tapering posterior ends pointing toward the sulcus. Dorsal apical Plate 3′ is 6-sided, small, and elongated posteriorly, ending in a narrow tip that abuts the small intercalary Plate 2a. Three intercalary plates are arranged more or less symmetrically on the dorsal side of the epitheca. The intercalary plates are very different in size, with the four-sided Plate 2a being distinctively smaller than the two other intercalary plates (Fig. 5A, B and D).

Within the series of six precingular plates, Plate 6″ is the smallest. Plate 1″ is in contact with an intercalary plate (1a) and thus contacts four epithecal plates, whereas Plate 6″ is narrower and only contacts three epithecal plates (Fig. 5).

The hypotheca consists of six postcingular and two antapical plates (Fig. 6A and B). The ventrally located Plates 1‴ and 6‴ are the smallest and the four-sided Plate 5‴ is the widest in the postcingular series. Plate 3‴ is in contact with both antapical plates (Fig. 6A and B). The two antapical plates are of markedly different size, with the smaller Plate 1⁗ slightly displaced to the left. The larger antapical Plate 2⁗ is bearing a small antapical spine that is usually accompanied by a small cluster of pores (Fig. 7F). The position of the spine is slightly variable, ranging from an axial position (e.g. Fig. 6C) to being slightly shifted to cells right side (e.g. Fig. 3A). Occasionally, the spine arise from a small bump (Fig. 7F).

The cingulum is wide (e.g. 2.6–2.9 μm; Figs. 3A and B and 6C), descending, and displaced by about half of its width. Narrow cingular lists are present. The cingulum is composed of six comparably sized plates, but plate C6 is more slender than the others (Fig. 6C and D). Furthermore, plate C6 is asymmetric in shape, with a conspicuous S-shaped extension partly covering the sulcal area and the flagellar pore region (Figs. 3A and B and 6C). The deeply concave sulcus (Fig. 6C and D) consists of a large anterior sulcal plate (Sa) that partly invades the epitheca, and a large posterior sulcal plate (Sp) that extends two-thirds of the way from the cingulum to the antapex. A left sulcal plate, Ss, is located anterior to Sp and abuts Plates 1‴, C1, Sa, Sd, Sm, Sp and C6. The right sulcal plate Sd abuts sulcal plates Ss and Sm, as well as cingular plate C6. The median sulcal plate Sm contacts sulcal plates Sa, Ss and Sd (Fig. 6C and D). As in other species of *Azadinium*, these plates have an apparently complicated three-dimensional morphology, with large flanges invading into the hypotheca (see Fig. 6D).

The apical pore complex (Fig. 7A–E) is distinctly elongated, with the apical pore being round or slightly ellipsoid (Fig. 7A and B) and shielded by a cover plate. The pore is located in the dorsal part of an elongated pore plate, the latter having a roundish dorsal part that is

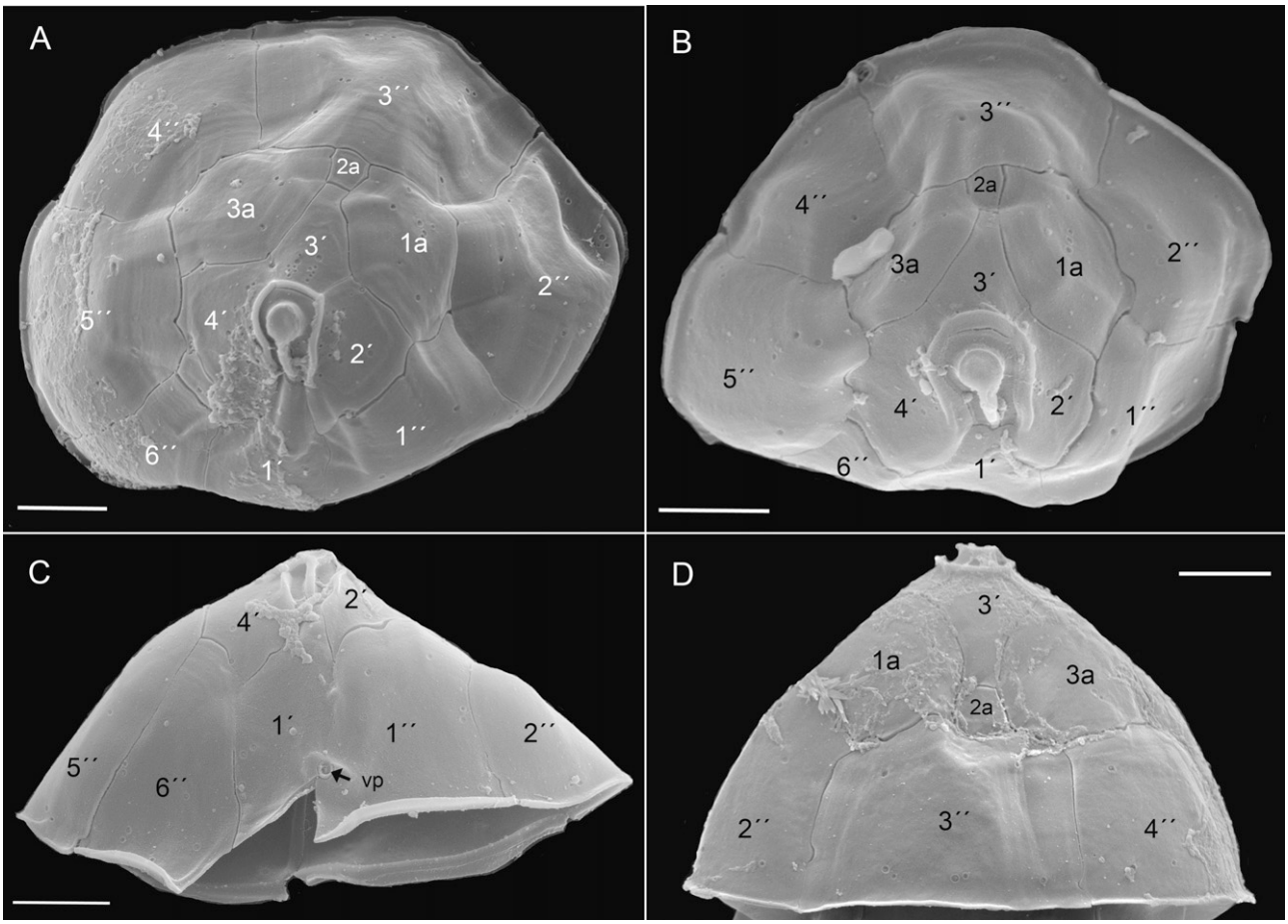

Fig. 5. *Azadinium polongum*. SEM micrographs of different cells. (A and B) Apical view showing the whole series of epithecal plates. (C and D) Epitheca in ventral (C) and dorsal (D) view. Scale bars = 2 μm.

considerable prolonged ventrally. A conspicuous rim borders the dorsal and lateral margins of the pore plate adjacent to apical Plates 2′, 3′, 4′, but it is lacking ventrally where the pore plate abuts the first apical plate and the X-plate. When viewed from inside the cell, the X-plate is obviously slender and elongated (Fig. 7C–E). The X-plate has a mean length of 0.75 μm (0.65–0.83, $n = 7$) and penetrates most of the elongated part of the pore plate. Ventrally, the X-plate abuts but does not invade the first apical plate. Occasionally, the X-plate appears to be slightly displaced into the pore plate, but is still connected to the 1′ plate by a narrow slit (Fig. 7B and D). The X-plate has a very characteristic three-dimensional structure, with finger-like protrusions contacting the cover plate (Fig. 7A and B).

Growth bands are visible as faint striated rows (Fig. 5A) present at overlapping plate margins. The plate overlap pattern was elucidated mainly from internal thecal views (see Fig. S1, provided as Supplementary Material and Fig. 7E) and is schematized in Fig. 4C and D.

A number of deviations from the typical plate pattern shown in Fig. 4 were observed. Although not explicitly quantified, these variations primarily consist of extra sutures between the epithecal plates, either of the precingular or apicals Plate 2′ or 4′. In addition, cells lacking the intercalary Plate 2a or with two small intercalary plates have been recorded (Fig. S2, provided as Supplementary Material).

Round cysts-like cells ranging in size from about 10 to 16 μm in diameter were regularly observed in cultures of *A. polongum* (Fig. 8). While cyst formation was not followed closely, the cells depicted in Fig. 8A–C appear to represent early cysts stages. These "early cysts," as observed in LM, are completely round, golden-brown in color and densely filled with large droplets, presumably representing reserve material. Epifluorescence microscopy indicates the presence of an intact chloroplast at this stage of cyst development (Fig. 8C). The majority of cysts, however, are almost colorless with pale white inclusions in LM; however, fluorescence microscopy revealed different stages of pigment reduction (Fig. 8D and E). The outer cyst layer appears thick and smooth in LM, although very fine radiating fibers seem to be present (Fig. 8F). In SEM, cysts are sometimes partly covered by thecal plates (Fig. 8G) or are covered by a dense fibrous mesh of filaments (Fig. 8H and I).

3.1.3. Morphometric analysis

The pore plates of *A. polongum* (strain SHETB2) and *A. spinosum* (strains 3D9, UTHE2 and SHETF6) showed no significant difference in width (largest left-to-right distance for the dorsal part of the Po) (Fig. 9A), ranging from 1 to 1.5 μm in both species. However, the length of the pore plate of *A. polongum* (2.1 ± 0.2, mean ± STD, $n = 65$) was significantly different from that of *A. spinosum* (1.5 ± 0.1, mean ± STD, $n = 57$) (*t*-test, $p < 0.001$). A scatter plot of pore plate length vs. length–width ratio (Fig. 9B) clearly separates the data points on both axes thereby underlining the difference in plate morphometry between the two species.

3.1.4. Toxin analysis

Using the selected reaction monitoring mode (SRM), *A. spinosum* SHETF6 exhibited a toxin profile of known AZAs consisting of AZA-1, and AZA-2, identical to previously isolated

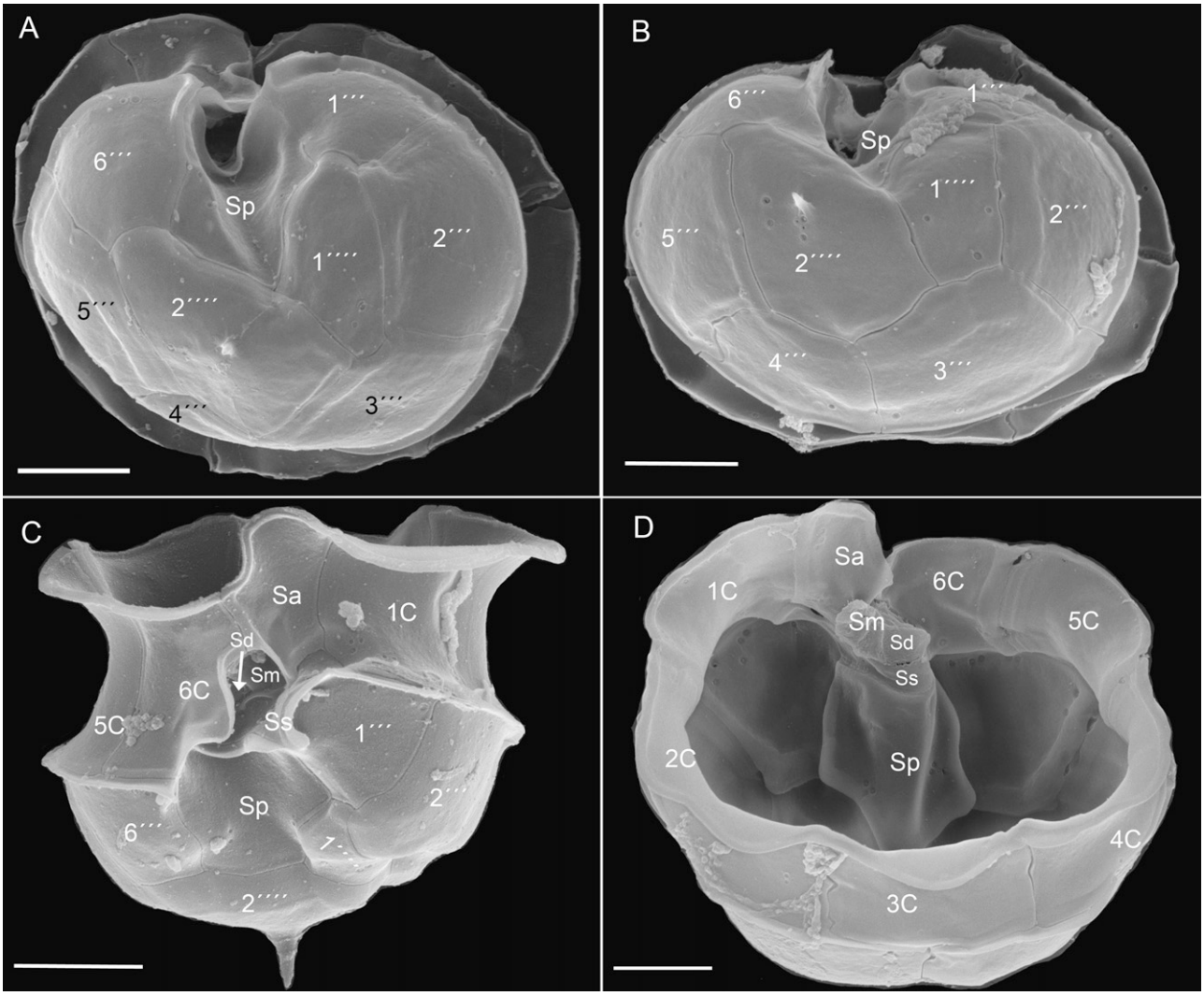

Fig. 6. *Azadinium polongum*. SEM micrographs of different cells. (A and B) Antapical view of hypothecal plates. (C) Ventral view of cingulum and hypotheca. (D) Apical view of the hypotheca showing the series of cingular plates with an internal view of the sulcal plates (Sa: anterior sulcal plate; Sp: posterior sulcal plate; Ss: left sulcal plate; Sm: median sulcal plate; Sd: right sulcal plate). Scale bars = 2 μm.

strains of the species. Combined cell quotas of AZA-1 and -2 from two different cultures of strain SHETF6 ranged from 19 to 22 fg per cell, with an AZA1/AZA2 ratio ranging from 1.9 to 2.8. The presence of other AZAs of known molecular mass can be excluded with a detection limit of 1.1 pg on column, corresponding to per cell detection limit of 0.008 fg. Using SRM, none of previously described AZAs were found in *A. polongum* at a detection limit of 1.1 pg on column (due to lower sample biomass corresponding to a per cell detection limit of 0.08 fg).

In addition, precursor ion experiments for detecting putative precursor masses of the characteristic CID-fragments *m*/*z* 348 and *m*/*z* 362 of AZAs revealed *A. spinosum* SHETF6 to have a previously undescribed AZA of the *m*/*z* 362 fragment type with a molecular mass of 715 Da. Peak area of this AZA accounted for 30% of the AZA-1 peak, indicating that this compound was a quantitatively important component of the *A. spinosum* total AZA profile. Precursor ion scans did not give any further signals for either SHETB2 (*A. polongum*) or SHETF6 (*A. spinosum*), indicating that neither strain produced other unknown AZA variants in larger amounts. However, the precursor ion mode is approximately a hundred times less sensitive than the SRM mode and strictly speaking does not allow for exact quantitative measurement. Considering a conservatively determined "detection limit" of 0.2 ng on column, this represents a cellular detection limit of unknown AZA variants of 1 fg cell^{-1} (*A. spinosum* SHETF6) or 10 fg cell^{-1} (*A. polongum* SHET B2).

3.1.5. Sequence data and phylogeny

The total length of the rDNA alignment for 33 taxa in total, including 15 ingroup and 18 outgroup taxa having comparable branch length, was 3351 bases long with 907 sites being parsimony informative (pi; 27%, 27.5 per terminal taxon). The SSU covered 1822 bases with 145 pi sites (8%), the ITS region 718 bases with 435 pi sites (61%) and the first two domains of the LSU covers 811 bases with 327 pi sites (40%). COI sequences showed zero to one nucleotide difference between different *Azadinium* species (data not shown). Tree topologies inferred from Bayesian and ML approaches were largely congruent. The best scoring ML tree is shown in Fig. 10. Many nodes had high or maximum support. Established taxonomic units such as Thoracosphaeraceae (95LBS, 0.83BPP), Gymnodinales 1 (100LBS, 1.00BPP), Gymnodinales 2 (96LBS, 1.00BPP) and Prorocentrales (100LBS, 1.00BPP) were formed. The Amphidomataceae were monophyletic (99LBS, 1.00BPP), with *Amphidoma* being most basal and *Azadinium* monophyletic with maximum support (100LBS, 1.00BPP). Within *Azadinium*, five species (*A. polongum* sp. nov. being one of them)

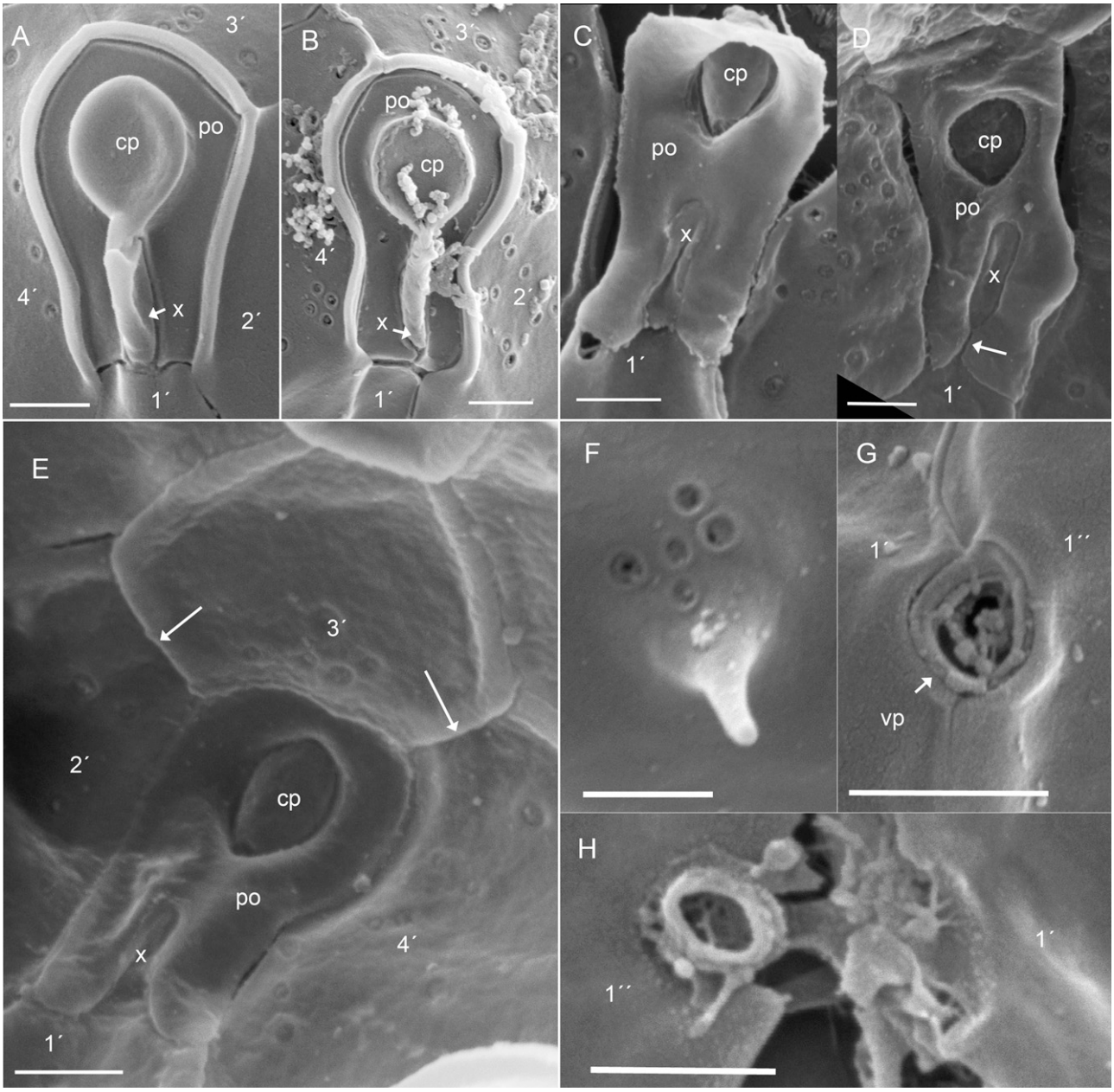

Fig. 7. *Azadinium polongum*. SEM micrographs of different cells. (A–D) Details of the apical pore complex (APC). (A and B) APC in apical view. (C and D) APC viewed from inside the cell. Note that the X-plate in B and D is slightly displaced into the pore plate but still is connected to the 1′ plate by a narrow slit. (E) Internal view of APC and apical plates. Note that Plate 3′ is overlapped by the adjacent apical Plates 2′ and 4′ (arrows). (F) Detailed view of an antapical spine with a cluster of pores emerging from a small bump. (G and H) Detailed external (G) and internal (H) view of the ventral pore located between Plates 1′ and 1′′. Po = pore plate; cp = cover plate; x = X-plate; vp = ventral pore. Scale bars = 0.5 μm.

were clearly separated and distinguishable. The results of the genetic distance analysis among Amphidomatacea are given in Table 1. The variation of the ITS region covering ITS1, 5.8S rRNA and ITS2 between *Azadinium* species, varieties, or different strains (in the case of *A. poporum* from Europe and *A.* cf. *poporum* from Korea) varied between 0.023 and 0.247. Other available strains of the same species, which are not listed in Table 1, exhibited the same genetic distances as the listed strain.

4. Discussion

The occurrence of *Azadinium* along the Scottish coast (North Sea) (Tillmann et al., 2009, 2010) and the Irish Atlantic coast (Salas et al., 2011), as well as a report of AZA in mussels from the north coast of Norway (Torgersen et al., 2008), suggest a distribution of the genus into more northern North Sea/Atlantic waters, an hypothesis confirmed by the present record from the Shetland Islands.

The Shetland Islands form the border between North Sea and Atlantic Ocean (International-Hydrographic-Organisation, 1953) with eastern Shetland Islands located in the North Sea and western Islands belonging to the Atlantic Ocean. According to that definition, the locality of our *Azadinium* record is situated in the North Sea. However, this area is heavily influenced by the East Shetland Atlantic Inflow, with Atlantic water therefore found on both sides of the Shetlands (Maravelias and Reid, 1997). Thus, occurrence of *Azadinium* in the North Atlantic is quite likely. During our cruise we failed to detect *Azadinium* and azaspiracids at a few stations adjacent to the more northerly located Faroe Islands (unpublished), but that negative observation may have been due to

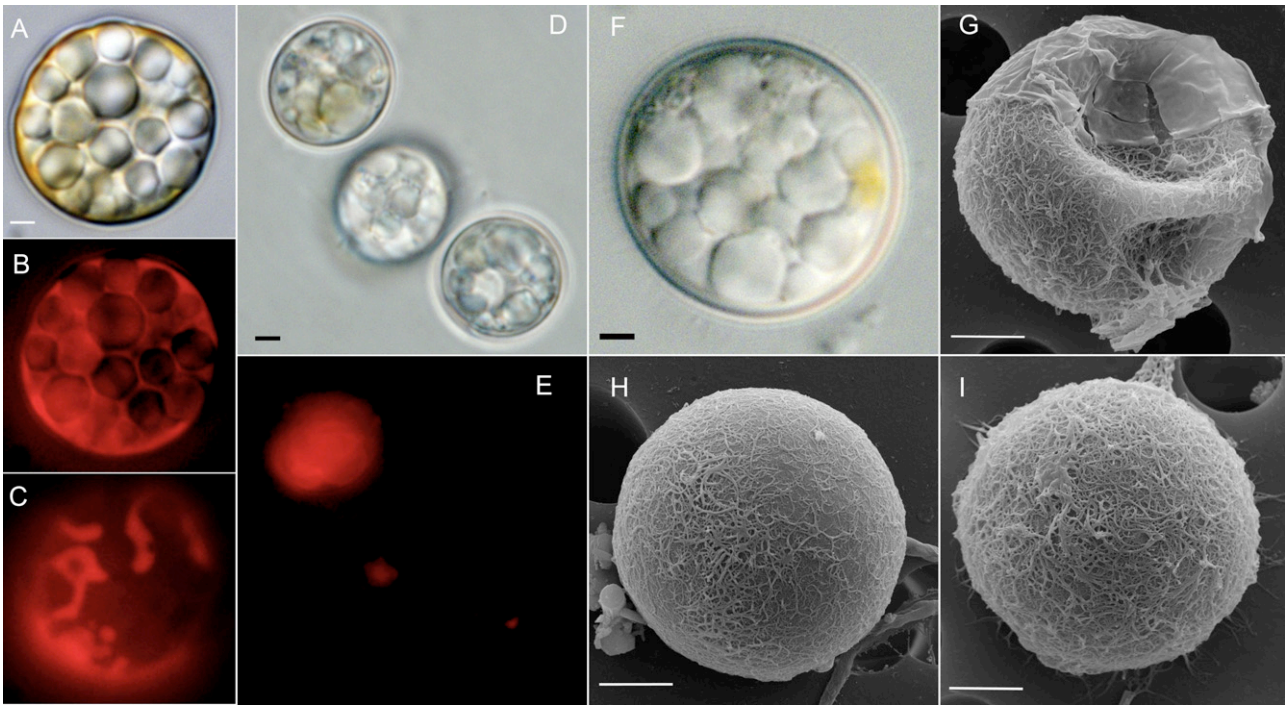

Fig. 8. *Azadinium polongum*. LM (A–F) and SEM (G–I) of cysts. (A–C) Presumably young cyst in brightfield (A) and in two different focal planes under blue-light-excitation (B and C) showing chlorophyll fluorescence. (D and E) Group of cysts in brightfield (D) and blue-light excitation (E). (G) Cyst with remains of the thecal plates attached. (H and I) Cysts covered by a dense mesh of fibrous material. Scale bars = 2 μm.

extremely low algal densities probably caused by high abundances of copepods.

Surface water temperature around the Shetlands is quite stable annually, rarely exceeding 12 °C during summer (Becker and Pauly, 1996). During our cruise, sea surface temperature was around 10 °C. Although not studied in detail, lab cultures of *A. spinosum* seemed to grow over a broad range of temperatures, however, best growth occurred at higher temperature (20 °C). In contrast, *A. polongum* grew well at 10 °C, but died rapidly at higher temperatures, indicating that this species is more stringently adapted and thus more restricted to lower temperatures.

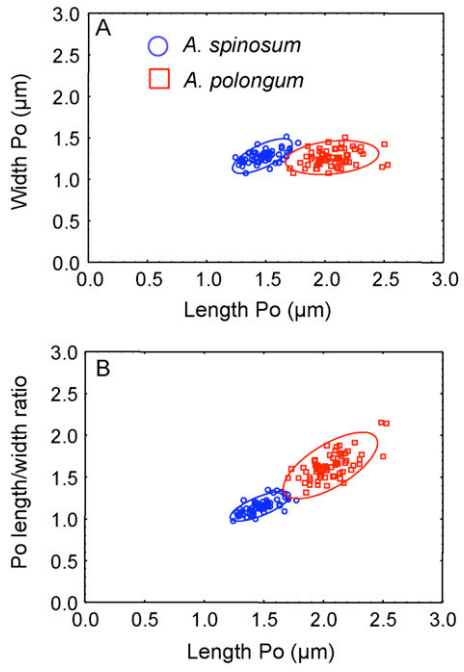

Fig. 9. Scatter plots of morphometric parameters of APC for *A. polongum* (red squares) and *A. spinosum* (blue circles). (A) Width versus length of the pore plate (Po). (B) Ratio of width/length versus length of the pore plate (Po). Circles represent 95% confidence ellipses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We observed specimens of *Azadinium* spp. at four out of six stations along the west coast of the Shetlands, but their abundances were generally very low. As indicated by qualitative inspection of net tows and whole water samples, the plankton was generally characterized as an early post-spring bloom community, with high abundance of copepods, diatoms present in varying numbers, and occurrence of different dinophytes (e.g. *Prorocentrum minimum* (Pavillard) Schiller, various species of *Protoperidinium*). In agreement with the *A. spinosum* record from the Shetlands, AZA-1 was detected at two of the Shetland west coast stations (including the station, where strain SHETF6 was isolated), however, only in low amounts ranging up to 0.02 ng L^{-1} (unpublished).

One of our two *Azadinium* isolates, strain SHETB2, was identified as a new species. While the characteristic swimming pattern of SHETB2 strongly indicates its affiliation with *Azadinium*, it is the genus characteristic Kofoidian thecal plate tabulation (Po, cp, X, 4′, 3a, 6″, 6C, 5?S, 6‴, 2⁗) that places this new taxon in the dinophyte genus *Azadinium* (Tillmann et al., 2009). Furthermore, discrimination of this taxon at the species level is justified by a number of distinctive morphological features. The most obvious is the shape of the pore plate that allows a clear separation of *A. polongum* (elongated pore plate) from other *Azadinium* species (round to ellipsoid pore plate, see Fig. 11). The difference in shape of the X-plate (elongated in *A. polongum*; round to ellipsoidal in other *Azadinium* species) might be related to the elongated shape of the pore plate of *A. polongum*. The X-plate also shows differences in arrangement across species. It invades the first apical plate in *A. spinosum*, *A. obesum* and *A. poporum*, but abuts the first apical plate in *A. polongum* and *A. caudatum* (Nézan et al., 2012). The shape and size of the dorsal intercalary Plate 2a was variable in our

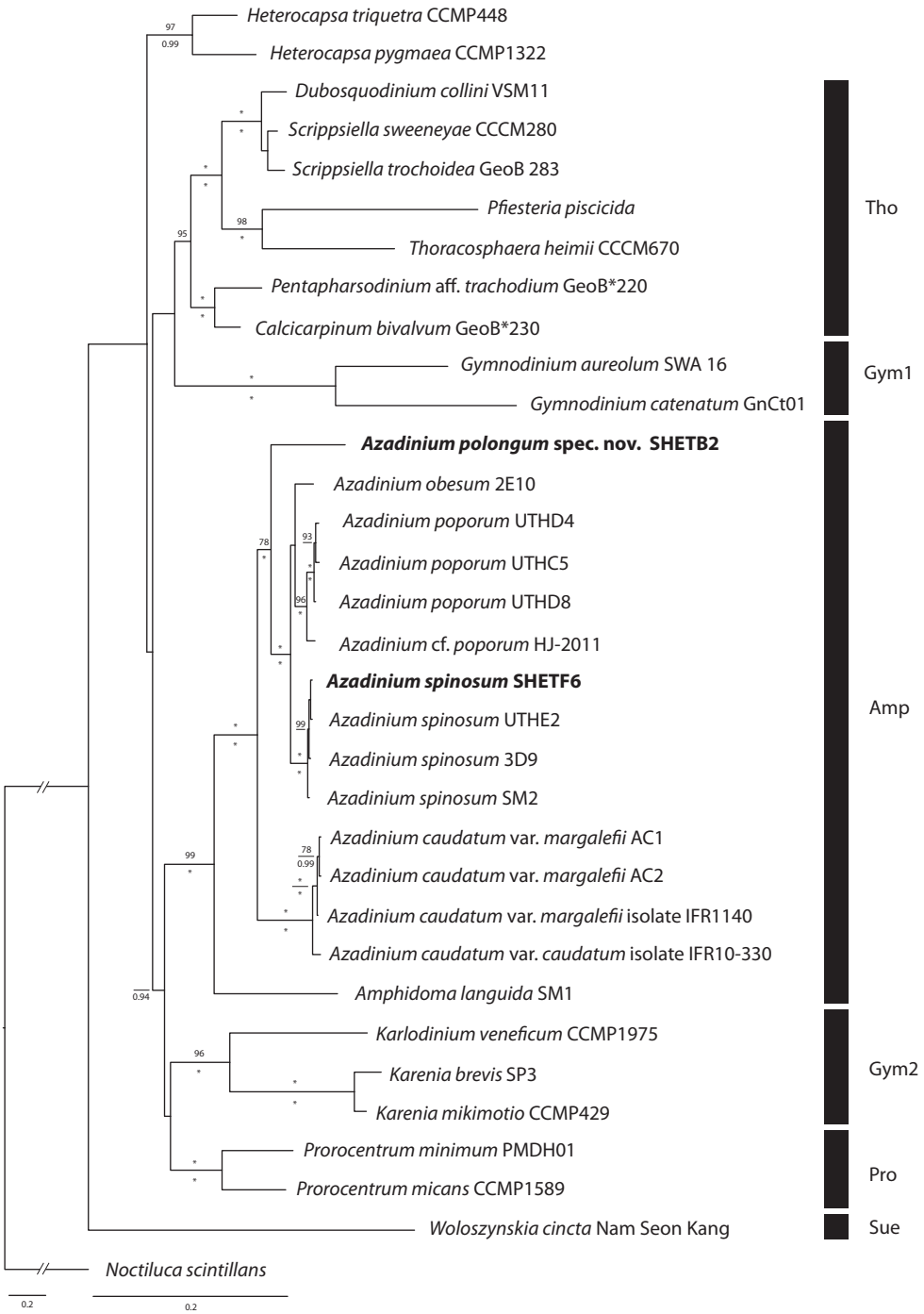

Fig. 10. Maximum likelihood (ML) tree of 33 taxa consisting 15 ingroups and 18 outgroups as inferred from a MAFFT generated alignment covering the complete SSU, complete ITS and the first two domains of the LSU region (907 parsimony informative sites). New strains are indicated in bold. Branch lengths are drawn to scale with a scale bar indicating the number of substitutions per site. Numbers on branches are support values (above: ML bootstrap support values, <60 not shown; below: Bayesian posterior probabilities, <0.90 not shown; maximal support is indicated by an asterisk). *Abbreviations*: Tho: Thoracosphaeraceae; Gym: Gymnodiniales; Amp: Amphidomataceae; Pro: Prorocentrales; Sue: Suessiales.

culture material of *A. polongum* (see Fig. S2, provided as Supplementary Material). Nevertheless, in its normal condition, Plate 2a of *A. polongum* is distinctly smaller than that of other small *Azadinium* species and is anteriorly linked to the elongated antapical portion of Plate 3′. These characteristic features are not found in *A. spinosum*, *A. obesum* and *A. poporum* (Tillmann et al., 2009, 2010, 2011) and more closely resemble the configuration of *A. caudatum* (Nézan et al., 2012).

The ventral pore, which is a characteristic feature of all *Azadinium* species, is more posteriorly positioned in *A. polongum* than in other species, being located in the lower third epitheca between Plate 1′ and 1″. Moreover, for *A. polongum*, the ventral pore is clearly located on the suture and is embedded in a cavity of Plate 1′ (see Fig. 7G and H) whereas for *A. spinosum* it is located within the 1′ plate and is connected to the suture of 1′ and 1″ by a narrow slit (Tillmann et al., 2009). Generally, the location of the ventral pore seems to be variable in *Azadinium* species, either on the left margin of Plate 1′ (*A. spinosum*, *A. obesum*) or on the left side of the Po (*A. poporum*) (Tillmann et al., 2009, 2010, 2011). In *A. caudatum* var. *margalefii*, this pore is located on the right margin of

Table 1
Estimated genetic distances (*P*-values) between species of Amphidomataceae, based on combined ITS region sequences.

Species	Strain no.	*A. polongum*	*A. obesum*	*A. spinosum*	*A. poporum*	*A.* cf. *poporum*	*A. caudatum* var. *margalefii*	*A. caudatum* var. *caudatum*	*A. languida*
Azadinium polongum	SHETB2								
Azadinium obesum	2E10	0.180							
Azadinium spinosum	SHETF6	0.187	0.075						
Azadinium poporum	UTHC5	0.184	0.052	0.101					
Azadinium cf. *poporum*	HI2011	0.185	0.057	0.105	0.023				
Azadinium caudatum var. *margalefii*	IFR1140	0.243	0.172	0.201	0.189	0.189			
Azadinium caudatum var. *caudatum*	IFR10-330	0.247	0.180	0.201	0.191	0.193	0.025		
Amphidoma languida	SM1	0.323	0.321	0.321	0.315	0.315	0.323	0.325	

the Po whereas for the second variety, *A. caudatum* var. *caudatum*, a similar pore is situated near the posterior right margin of Plate 1′ (Nézan et al., 2012). In the closely related *Amphidoma* species, Kofoid and Michener (1911) reported this pore on right edge of 1′ (*A. elongata* Kofoid et Sweezy) or at the midventral posterior tip of 1′ (*A. laticincta* Kofoid et Sweezy) while in *A. languida*, it is located on the anterior right margin of 1′ (Tillmann et al., 2012). Very rarely, the position of the ventral pore has been observed to vary even within a culture. In one specimen of *A. languida*, the ventral pore was located in the right side of the pore plate (Tillmann et al., 2012), as in *A. caudatum* var. *margalefii*, and, in one specimen of *A. poporum* isolated from Korea, it was located on the left side of Plate 1′ (Potvin et al., 2012). As the function (if any) of these pores is completely unknown, we cannot speculate on the potential consequences of the apparent variability in pore location among the Amphidomataceae.

The potential affinity of a few other described Dinophycean species (*Gonyaulax parva* Ramsjfell, *G. gracilis* Schiller) to *Azadinium* has been discussed before (Tillmann et al., 2011). *G. parva* clearly differs from *A. polongum* by the intercalary plates having the same size. The taxonomy of *G. gracilis* and specimens depicted under this name (see Tillmann et al., 2011) generally needs careful revision. At least one specimen depicted as *G. gracilis* by Bérard-Therriault et al. (1999) probably is a species of *Azadinium*. It has an antapical spine but other details are not visible, and we thus cannot exclude the possibility of that specimen being *A. polongum*.

In terms of plate overlap pattern, the new species *A. polongum* exactly resembles the type *A. spinosum* as described by Tillmann and Elbrächter (2010). Peculiarities in plate overlap of *A. spinosum*, including 3′ overlapped by the adjacent apical Plates 2′ and 4′ (see Fig. 7E), Plate 2a overlapped by all adjacent plates, and Sa overlapping plate C6, are also present in *A. polongum* (see Fig. S1, provided as Supplementary Material), thus indicating a conservative plate overlap pattern for the genus. However, *A. caudatum* has been found to exhibit a slightly different overlap pattern of the ventral apical plates in that Plate 1′ overlaps the adjacent apical Plates 2′ and 4′ (Nézan et al., 2012).

Differences in morphology between SHETB2 and SHETF6 are additionally reflected by differences in the biology/autecology of both isolates. The different growth behavior in terms of temperature requirement has been addressed above. Strikingly, final cell yield of *A. spinosum* SHETF6 was much higher compared to *A. polongum* indicating different nutrient or carbon requirements and/or pH tolerance (Hansen et al., 2007). Moreover, *A. polongum* produced cysts in culture, a feature not yet observed for other *Azadinium* species. However, successful isolation of *A. poporum* by incubating sediment samples (Potvin et al., 2012) make the presence of cysts quite likely for *A. poporum*. We currently know little about the nature of *A. polongum* cysts. SEM failed to detect any external cyst structures like paratabulation and/or archeopyle, and hatching was not observed. The reduced chlorophyll fluorescence of cysts (Fig. 8D and E) and their long persistence in an apparently unaltered state indicate that *A. polongum* cysts might allow long-term survival (hypnocysts), rather than serving as temporary cysts. If true, these hypnocysts might be part of the vegetative cycle (as has been observed *Scripsiella hangoei* (J. Schiller) J. Larsen, see Kremp and Parrow, 2006), or part of a sexual life cycle. Clearly, more data and observations are needed to clarify the whole life cycle of *Azadinium*.

Separate dinophyte lineages and different sites of rDNA have contrasting evolutionary rates (Hoppenrath and Leander, 2010). To avoid the disadvantages of single site phylogenies and to balance different rates of evolution, we combined slow sites like the SSU with quickly evolving sites like the ITSs. Various ratios of

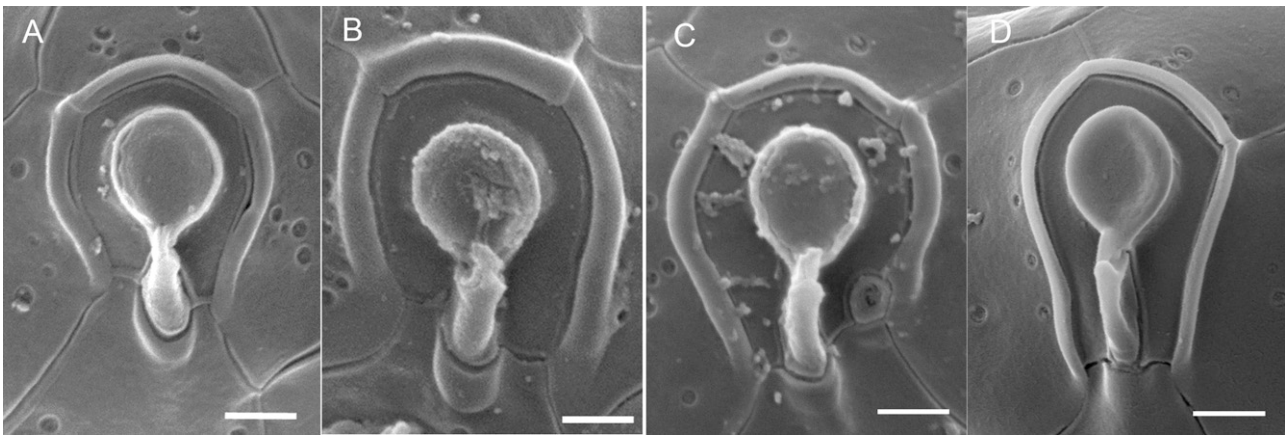

Fig. 11. *Azadinium* spp. SEM micrographs of the apical pore complex APC. (A) *A. spinosum*, (B) *A. obesum*, (C) *A. poporum*, (D) *A. polongum*. Scale bars = 0.5 μm.

parsimony information for the regions have been demonstrated before (Gottschling et al., 2012) and were confirmed here. With 61% of the positions as parsimony informative, the ITS region holds the main portion of phylogenetic information. With only 8%, the SSU seems to be much less informative. Nevertheless, for higher taxonomic analysis and the inclusion of many taxa, such slowly evolving sites are valuable to stabilize the analysis and to support the resolution of the basal nodes. In our analysis, the basal nodes and phylogenetic relationships were not resolved with high support; however, well established taxonomic units such as the Thoracosphaeraceae (95LBS, 0.83BPP), the Gymnodinales 1 (100LBS, 1.00BPP), the Gymnodinales 2 (96LBS, 1.00BPP), and the Prorocentrales (100LBS, 1.00BPP) were distinguished.

Together with *A. languida* the monophyletic and maximum supported *Azadinium* (100LBS, 1.00BPP) forms the highly supported *Amphidomataceae* (99LBS, 1.00BPP) (Tillmann et al., 2012). In total, five species are now clearly distinguishable within *Azadinium* (Tillmann et al., 2009, 2010, 2011; Nézan et al., 2012), and the relatively high genetic distances of the ITS region (Table 1) support the species delimitations (Litaker et al., 2007). The genetic distance of the new species *Azadinium polongum* (SHETB2) to the other small *Azadinium* species (*A. spinosum*, *A. obesum*, *A. poporum*, $p = 0.180–0.187$) is distinctly larger than the distance among these other small species ($p = 0.052–0.105$). SHETF6 could be added as a new strain of *A. spinosum* clustering to the other *A. spinosum* not only by morphology and toxins, but also by molecular evidence.

Toxin analysis of both Shetland-strains for known AZAs verified AZA-1 and -2 production solely for *A. spinosum* at a cell quota comparable to other isolates (Tillmann et al., 2009, 2011; Salas et al., 2011; Jauffrais et al., 2012), indicating the toxin content and profile as a stable characteristic in this species. In addition to AZA-1 and -2, a yet undescribed AZA of the *m*/*z* 362-fragment type with a molecular mass of 715 Da is here reported for *A. spinosum* for the first time. This compound also has been found in significant amounts in all other *A. spinosum* strains, and a manuscript including NMR structural elucidation is in preparation (Kilcoyne et al., manuscript in preparation). Production of AZAs within the genus was initially known only for *A. spinosum*, but is apparently more common within the Amphidomataceae. *A. languida* and *A. poporum* are now known to produce a new type of azaspiracid characterized by a modified fragment of *m*/*z* 348 compared to the fragment of *m*/*z* 362 characteristic for the previously known AZAs (Krock et al., 2012). We tested both Shetland-strains for the presence of known AZAs of both fragment types and detected only AZA-1 and-2 in *A. spinosum*. Although, we cannot exclude the presence of other yet unknown AZAs (in addition to the new 715 Da AZA), as the precursor ion scan method is much less sensitive than the single reaction mode (limit of detection of our measurement estimated ca. 1 and 10 fg per cell for SHETF6 and SHETB2, respectively). Patently, more analyses using larger culture volumes are needed. Nevertheless, *A. polongum* clearly is not toxigenic for known AZAs and thus represents another case of coexisting toxigenic and non-toxigenic species of *Azadinium*, as previously described for *A. obesum* (Tillmann et al., 2010).

Considering the short interval since the first identification of *Azadinium*, the diversity of the genus has increased rapidly, with five species now described and additional new species expected. The presence of an antapical spine in small *Azadinium* species was hitherto restricted to *A. spinosum*. With *A. polongum* also exhibiting an antapical spine, the identification of the toxigenic species *A. spinosum* only by light microscopy is unfortunately no longer convenient. *A. caudatum* also has a spine, however of distinctly different size and shape. Fortunately, a molecular approach that can be routinely applied to a larger number of field samples has been developed to identify *A. spinosum* and related taxa (Töbe et al., in press). This approach may thus be used to unambiguously confirm microscopic species assignments, like the assured presence of *A. spinosum* in the Black Sea (http://phyto.bss.ibss.org.ua/wiki/Azadinium_spinosum) and the presence of *A.* cf. *spinosum* in Argentinean coastal waters (Akselman and Negri, 2012).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2012.10.001.

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PAPER 4 (PUBLICATION)

CATCH ME IF YOU CAN: THE TAXONOMIC IDENTITY OF *SCRIPPSIELLA TROCHOIDEA* (F.STEIN) A.R.LOEBL. (THORACOSPHAERACEAE, DINOPHYCEAE)

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Research Article

Catch me if you can: the taxonomic identity of *Scrippsiella trochoidea* (F.STEIN) A.R.LOEBL. (Thoracosphaeraceae, Dinophyceae)

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The species concept is challenged for the unicellular dinophytes, exhibiting both high intraspecific variability (in terms of morphology) and cryptic speciation (as inferred from molecular data). As one of the most abundant species assigned to calcareous dinophytes (Thoracosphaeraceae, Dinophyceae), *Scrippsiella trochoidea* is cosmopolitan in distribution, but its taxonomic identity is presently unclear. We collected, isolated and cultivated *Scrippsiella trochoidea* (strain GeoB*185) from the type locality in the Kiel Fjord (Baltic Sea, Germany). We barcoded the species of the Thoracosphaeraceae based on ITS sequences (including 22 new sequences) and investigated the morphology of strain GeoB*185 by using light, fluorescence and electron microscopy. Numerous distinct lineages that had previously been determined as *Scrippsiella trochoidea* constituted a species complex rather than a single species. This species complex subsequently comprised three primary clades, for which the strain GeoB*185 was assigned to one of them. We designate an epitype for *Scrippsiella trochoidea*, which has been prepared from the culture collected in the Kiel Fjord. The unambiguous links between a scientific species name, its protologue, genetic characterization and spatial distribution bear particular importance for character-poor, unicellular organisms such as the dinophytes.

Key words: calcareous dinoflagellates, coccoid stage, cryptic speciation, distribution, epitypification, morphology, Peridiniales, phylogeny, thecate cell

Introduction

The unambiguity of scientific names is the necessary prerequisite for proper identification of species. A clearly defined designation is, furthermore, paramount in comparing their distribution patterns across geographic regions and generally for the reproducibility of scientific results. This is all the more the case as we are experiencing an exponential increase of our knowledge about species diversity. The links associated with a species name, its protologue, genetic characterization, and spatial distribution bear particular significance for character-poor, unicellular organisms such as the dinophytes. As a widely distributed species, *Scrippsiella trochoidea* (F.Stein) A.R.Loebl. (Thoracosphaeraceae, Dinophyceae) has been subjected to many biological, palaeo-climatological, and palaeo-environmental studies. This species belongs to the phototrophic dinophytes, which produce calcareous coccoid stages during life history (D'Onofrio *et al.*, 1999; Gottschling *et al.*, 2005*b*; McQuoid, 2005; Wang *et al.*, 2007). It belongs to *Scrippsiella* Balech ex A.R.Loebl., which comprises approximately 20 extant species that are abundant in marine waters of all climatic zones, polar as well as tropical habitat realms (Zonneveld *et al.*, 1999; Vink, 2004). *Scrippsiella* can be distinguished from other peridinoid dinophytes (such as *Pentapharsodinium* Indel. & A.R.Loebl., *Peridinium* Ehrenb., *Protoperidinium* Bergh and others) based on the presence of six cingular plates, thus showing two cingular sutures in mid-dorsal view of the motile cells (Fine & Loeblich III., 1976; Dale, 1977, 1978).

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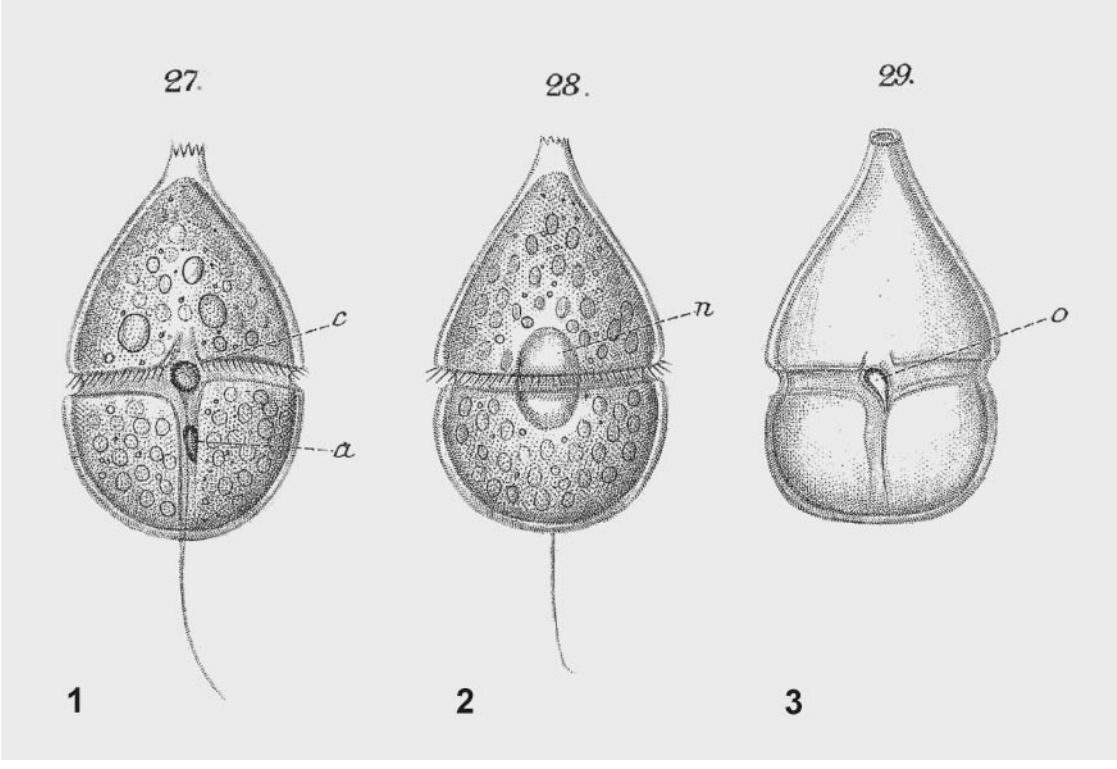
As in many other dinophytes, the life history of *Scrippsiella* includes (at least) two different stages, namely a motile vegetative cell ('thecate cell') and a non-motile coccoid stage (usually coined 'cyst'). Subsequently, two parallel and originally independent taxonomic systems have been developed for dinophytes: a 'neontological' system mainly based on the morphology of the thecate cell and a 'palaeontological' (para-)system based on characters of the coccoid stage. Particular focus emphasizing a single biological system of dinophytes (Fensome *et al.*, 1993; Elbrächter *et al.*, 2008) has enabled much progress in clarifying cyst–theca relationships amidst the various species of *Scrippsiella* within the past two decades. They are morphologically diverse with respect to the coccoid stages, while the tabulation pattern of the motile stage is rather homogeneous (Lewis, 1991; D'Onofrio *et al.*, 1999; Meier *et al.*, 2002; Gottschling *et al.*, 2005*b*; Gu *et al.*, 2008). The coccoid stages of *S. trochoidea* are characteristic because of the ovoid shape with the numerous triangular spines (in the centres of irregular base plates), which comprise the cell surface (Janofske, 2000), and have been separately described as *Rhabdothorax* Kamptner ex Gaarder & Heimdal (following the micropalaeontological taxonomic system). It has been found that the coccoid stages of *S. trochoidea* are abundant in coastal marine habitats and may function as short mandatory dormancy stages (Binder & Anderson, 1987; Montresor *et al.*, 1998).

Seen as such, the presence of clear taxonomical information about *Scrippsiella trochoidea* is important for a wide array of researchers. The basionym, *Glenodinium trochoideum* F.Stein, 1883 provides the oldest epithet for current species assigned to *Scrippsiella*. The name is based on a dinophyte collected at the Kiel Fjord in Northern Germany (Baltic Sea) at an unknown date between 1879 and 1883. If any original material was preserved, it has not been located in the course of this study. Moreover, plate III 27–29 in von Stein (1883) illustrating three motile stages (Figs 1–3) is thus the type of *G. trochoideum*. Later, Lemmermann (1910*a*, 1910*b*) also reported *S. trochoidea* from the Kiel Fjord, but later records of this species in the Baltic Sea are rare (Nehring, 1994, 1997; Hällfors, 2004).

Taxonomic ambiguity within the name *S. trochoidea* has arisen by sequence comparison of the Internal Transcribed Spacer (ITS) and other genetic loci. Molecular data indicate a large genetic heterogeneity of ribotypes among numerous different species, which all demonstrate morphology of the vegetative stage consistent with the protologue of *S. trochoidea* sharing the same tabulation pattern ('cryptic species': Montresor *et al.*, 2003*a*; Gottschling *et al.*, 2005*b*; Gu *et al.*, 2008). Within the *Scrippsiella trochoidea* species



Figs 1–3. Type of *Scrippsiella trochoidea* (≡ *Glenodinium trochoideum*), reproduction of plate III 27–29 (von Stein, 1883). Abbreviations (as noted in von Stein, 1883): a, 'eyespot' (i.e. red accumulation body); c, contractile vessel (i.e. exterior sulcal region with the flagellar pores); n, nucleus; o, 'mouth' (i.e. interior sulcal region with the flagellar pores).

complex (STR-SC), three major clades have been phylogenetically identified, each of which includes strains from global localities in temperate seas. However, strains from specimens collected from the type locality of *S. trochoidea* have not been included in the phylogenetic analyses, leaving the taxonomic and genetic circumscription of this species unclear.

In this study, we clarify the taxonomic identity of *Glenodinium trochoideum*, the basionym of *Scrippsiella trochoidea*. We have collected phytoplankton net samples at the type locality in the Baltic Sea and have established the strain GeoB*185, which exhibits a morphology consistent with the protologue of *G. trochoideum*. We also provide a molecular phylogeny of *Scrippsiella* species. This includes an ITS sequence of the new strain, from which we have prepared the epitype now deposited at the Centre of Excellence for Dinophyte Taxonomy (CEDiT; Wilhelmshaven, Germany). We thus aim to contribute to the disentanglement of the complex taxonomy afflicting (calcareous) dinophytes, while emphasizing a fundamental prerequisite for all further investigation and application regarding such unicellular algae.

Materials and methods

Morphology

The monoclonal strain GeoB*185 was established by isolation of a single thecate cell from a phytoplankton sample collected in the Kiel Fjord (Baltic Sea, Germany; GPS coordinates: 54°26.33′N, 10°12.70′E) during a cruise of the research ship Alkor in April 2000. Available upon request, the strain is currently held in the culture collections at the Institute of Historical Geology/Palaeontology (University of Bremen, Germany) and the Institute of Systematic Botany and Mycology (University of Munich). The strain GeoB*185 was cultivated in a climate chamber Percival I-36VL (CLF PlantClimatics; Emersacker, Germany) at 18 °C, 80 μmol photons m^{-2} s^{-1} and a 12:12 h light:dark photoperiod by using K-Medium without silicate (Keller *et al.*, 1987) and 35 psu artificial seawater (hw marinemix professional: Wiegandt; Krefeld, Germany) at pH 8.0–8.2. For germination experiments, single coccoid stages were isolated from the stem culture and were transferred to a new cultivation plate under the standard conditions described above. Incidentally, sub-cultures were grown under slightly differing conditions with respect to salinity (35 psu and higher) and temperature (up to 23 °C) to explore possible variations in morphology. Herein, strain GeoB*185 suffered contamination via a chrysophycean-like flagellate (as inferred from sequence comparison, as can be seen below).

For the preparation of the epitype, cells of strain GeoB*185 were centrifuged at a maximum speed of 200 g for 1 h. Spare water was removed, and 120 μl 3% glutaraldehyde (Plano; Wetzler, Germany) in 35 psu artificial seawater were added to the 30 μl remnant with a distinct pellet. Double-staining was performed by using 0.5% (water-based) astra blue in 2% tartaric acid (Fluka; Buchs, Switzerland followed by two cleaning steps in 120 μl 30 psu artificial seawater for 15 min and in 15 psu artificial seawater for 15 min) and 0.1% (ethanol-based) eosin (Merck; Darmstadt, Germany) during a graded ethanol (Roth; Karlsruhe, Germany) series. Ethanol-based Technovit 7100 (Heraeus; Wehrheim, Germany) was used for embedding, following the manufacturer's instructions. For the final preparation, 40 μl aliquots of the Technovit mixture including the embedded samples were transferred to four glass slides. The epitype is deposited at the Centre of Excellence for Dinophyte Taxonomy (CEDiT; Wilhelmshaven, Germany). Copies are held in the herbaria of Berlin, Bremen and Munich.

The techniques of light (LM) and scanning electron microscopy (SEM) followed standard protocols (Janofske, 2000) and were basically the same as described in Gottschling *et al.* (in press). Briefly, SEM samples were either air-dried or dehydrated in a graded acetone series and critical-point-dried, followed by sputter coating with platinum. The thecal plate pattern was obtained by examining the culture stained with calcofluor white M2R (Sigma-Aldrich, Munich, Germany) in epifluorescence microscopy (Fritz & Triemer, 1985). The Kofoidean system (Taylor, 1980; Fensome *et al.*, 1993) was used to designate the plate formula. Measurements were obtained for 6–20 thecate cells and calcareous coccoid stages (see below).

Molecular analyses

Genomic DNA was extracted from fresh material by using the Nucleo Spin Plant II Kit (Machery-Nagel, Düren, Germany). Both ITSs including the 5.8S rRNA region were amplified by using the primer pair ITS1 5′-GGTGAACCTGAGGAAGGAT-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ following standard protocols (Gottschling & Plötner, 2004). If gel electrophoresis yielded more than a single band, they were excised, purified and sequenced separately.

Ninety-three sequences of dinophytes were investigated (Table 1), while the taxon sample comprised the currently known diversity of *Scrippsiella* ribotypes found in different regions of the global oceans. The data matrix was assembled from previously published sequences (D'Onofrio *et al.*, 1999; Montresor *et al.*, 2003*b*; Gottschling *et al.*, 2005*a*, 2005*b*; Attaran-Fariman & Bolch, 2007; Gu *et al.*, 2008) and included 22 new additional sequences from strains out of our own culture collection (see Table 1 for details). The sequences were aligned by using 'MAFFT' v6.624b (Katoh *et al.*, 2005; Katoh & Toh, 2008; freely available at http://align.bmr.kyushuu.ac.jp/mafft/software/). The alignment is available via nexus file upon request.

Table 1. Voucher list. Abbreviation: n.i., not indicated. Para- and/or polyphyletic taxa are indicated by quotation marks.

DNA No.	Strain No.	Species name with author	Collector	Locality	Lat.	Long.	GenBank No.
D142	GeoB 229	*Calcicarpinum bivalvum* G.Versteegh [= '*Pentapharsodinium*' *tyrrhenicum* (Balech) Montresor, Zingone & D.Marino]	n.i.	Gulf of Taranto (Italy)	40°07′N	17°19′E	AY499512
D129	GeoB 110	*Calcigonellum infula* Deflandre, 1949 [≡ *Scrippsiella infula* (Deflandre) Montresor]	n.i.	Mediterranean Sea (Spain)	41°21′N	3°01′E	AY499523
D004	GeoB*31	*Calciodinellum albatrosianum* (Kamptner) Janofske & Karwath	n.i.	Atlantic (Cape Verde Islands)	11°29′N	21°01′W	AY499522
D009	GeoB*120	*Calciodinellum* cf. *albatrosianum* (Kamptner) Janofske & Karwath	n.i.	Equatorial Atlantic (Gabon)	3°44′S	9°47′E	HQ729482
D051	GeoB 149	*Calciodinellum albatrosianum* (Kamptner) Janofske & Karwath	n.i.	Western South Atlantic (Brazil)	7°45′S	28°15′W	AY676143
D012	M34–*26/4	*Calciodinellum albatrosianum* (Kamptner) Janofske & Karwath	n.i.	Western North Atlantic (Barbados)	12°16′N	58°20′W	AY676145
D052	GeoB 122	'*Calciodinellum*' *levantinum* S.Meier, Janofske & H.Willems	n.i.	Equatorial Atlantic (Togo)	1°55′N	3°13′E	AY676146
D1011	GeoB*165	'*Calciodinellum*' *levantinum* S.Meier, Janofske & H.Willems	n.i.	Mediterranean Sea (Egypt)	32°43′N	34°10′E	AY676147
D127	GeoB 34	*Calciodinellum* cf. *operosum* Deflandre, 1949 [≡ *Scrippsiella* cf. *operosa* (Deflandre) Montresor]	n.i.	North Atlantic	8°30′N	32°27′W	HQ729486
D006	SZN 74	*Calciodinellum operosum* Deflandre, 1949 [≡ *Scrippsiella operosa* (Deflandre) Montresor]	Montresor	Gulf of Naples (Italy)	40°43′N	14°10′E	AY327462
D061	GeoB 111	'*Calciodinellum*' spec.	n.i.	North Atlantic (Azores)	37°32′N	20°39′W	AY800132
D107	GeoB 199	'*Calciodinellum*' spec.	n.i.	Western North Atlantic (Lesser Antilles)	14°54′N	55°44′W	AY676149
D114	GeoB*205	'*Calciodinellum*' spec.	n.i.	Mediterranean Sea (Greece)	36°47′N	26°21′E	HQ729485
D001	tub*2	'*Calciodinellum*' spec.	n.i.	Eastern South Pacific (Chile)	28°15′N	78°00′W	AY499532
–	–	*Duboscquodinium collini* Grassé, 1952 from *Eutintinnus fraknoii* (Daday, 1887)	VSM11	Mediterranean Sea (France)	43°41′N	7°19′E	HM483399
D208	GeoB 284	*Ensiculifera* aff. *imariensis* S.Kobayashi & Matsuoka	Gottschling & Petersen	Atlantic (Norway)	63°28′N	9°25′E	AY728076
–	NIES 7	*Heterocapsa triquetra* F.Stein	n.i.	Osaka Bay (Japan)	34°N	135°W	AB084101
–	SZN 19	*Pentapharsodinium dalei* Indel. & A.R.Loebl.	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527817
D124	Rengefors Lab s.n.	'*Peridinium*' *aciculiferum* Lemmerm.	n.i.	Lake Erken (Sweden)	59°51′N	18°35′E	AY499514
D005	GeoB 61	*Pernambugia tuberosa* (Kamptner) Janofske & Karwath	n.i.	South Atlantic (Brazil)	11°32′S	28°35′W	AY499519
D132	CS–168	*Scrippsiella donghaienis* H.Gu	Stauber	Southern Ocean (Australia)	33°S	138°E	AY499533
D238	GeoB 305	*Scrippsiella donghaienis* H.Gu	Gottschling & Petersen	Eastern North Atlantic (Sweden)	58°54′N	11°12′E	AY788357
D315	GeoB 356	*Scrippsiella donghaienis* H.Gu	Häusermann	South Pacific (Chile)	42°22′S	72°24′W	HQ729492
D380	GeoB 424	*Scrippsiella donghaienis* H.Gu	Greif	Western South Atlantic (Uruguay)	34°47′N	53°28′W	HQ729502
–	SSDH01	*Scrippsiella donghaienis* H.Gu	Wenling & Chao	Eastern Chinese Sea (China)	29°00′N	122°30′E	AY685008
D057	SHTV1	'*Scrippsiella*' *hangoei* (J.Schiller) J.Larsen	Kremp	Baltic Sea (Finland)	59°50′N	23°12′E	AY499515
–	SCBC17	*Scrippsiella irregularis* Attaran-Fariman & Bolch	Khodami	Gulf of Oman (Iran)	25°11′N	61°34′E	EF584460
–	SPXM01	*Scrippsiella irregularis* Attaran-Fariman & Bolch	Wenling & Chao	South China Sea (China)	24°25′N	118°5′E	EU325948
D192	GeoB 259	*Scrippsiella lachrymosa* Lewis	Gottschling & Petersen	Eastern North Atlantic (Norway)	63°23′N	9°30′E	AY728078

D209	GeoB 285	*Scrippsiella lachrymosa* Lewis	Gottschling & Petersen	Eastern North Atlantic (Norway)	63°40′N	8°18′E	AY788354
D303	GeoB 341	*Scrippsiella lachrymosa* Lewis	Meier	North Atlantic (Canada)	48°7′N	69°40′W	HQ729487
D174	IO25–01	*Scrippsiella lachrymosa* Lewis	Amorim	Eastern North Atlantic (Portugal)	40°38′N	8°46′W	AY676150
–	SZN75	*Scrippsiella lachrymosa* Lewis	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527819
D134	CS–294	*Scrippsiella precaria* Montresor & Zingone	Bolch	Ballast water (Australia)			AY499518
D374	GeoB 378	*Scrippsiella precaria* Montresor & Zingone	Gottschling, Zinssmeister, Soehner	Mediterranean Sea (Italy)	40°40′N	14°46′E	HQ729500
D351	GeoB 398	*Scrippsiella ramonii* Montresor	Gottschling, Zinssmeister, Soehner	Mediterranean Sea (Italy)	40°40′N	14°46′E	HQ729497
D232	GeoB 280	*Scrippsiella rotunda* Lewis	Gottschling & Petersen	Eastern North Atlantic (Norway)	63°23′N	9°30′E	AY788355
–	SSND11	*Scrippsiella rotunda* Lewis	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325952
–	SZN 66	*Scrippsiella rotunda* Lewis	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527821
D066	GeoB*161	*Scrippsiella* spec.	n.i.	Red Sea (Saudi Arabia)	27°44′N	35°03′E	AY499527
D104	GeoB*195	*Scrippsiella* spec.	n.i.	North Atlantic (Bermuda)	25°03′N	58°04′E	AY676153
D229	GeoB 277	*Scrippsiella* spec.	Gottschling & Petersen	Eastern North Atlantic (Norway)	63°41′N	9°51′E	AY788356
–	SSND04	*Scrippsiella* spec.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325944
–	SSND07	*Scrippsiella* spec.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325945
–	SSND12	*Scrippsiella* spec.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325946
–	SSND14	*Scrippsiella* spec.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325947
D069	CCCM 280	*Scrippsiella sweeneyae* Balech ex A.R.Loebl.	Chan	not indicated			AY499528
D161	NIES 684	*Scrippsiella sweeneyae* Balech ex A.R.Loebl.	Yoshimatsu	Seto Inland Sea (Japan)	34°25′N	134°00′E	AY499520
D1008	GeoB*109	*Scrippsiella trifida* Lewis	n.i.	Mediterranean Sea (Spain)	42°26′N	3°41′E	HQ729484
D382	GeoB 434	*Scrippsiella trifida* Lewis	Greif	Western South Atlantic (Uruguay)	34°47′N	53°28′W	HQ729503
D321	1008B	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Hargraves	Atlantic (Florida, USA)	27°32′N	80°21′W	HQ729494
–	CCMP 2271	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Jim Lake (USA)	47°10′N	98°48′W	HM483396
D054	GeoB 138	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Atlantic (Mauritania)	21°16′N	20°42′W	AY499525
D049	GeoB 140	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Eastern North Atlantic (Mauritania)	21°16′N	20°42′W	AY676152
D050	GeoB 147	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	South Atlantic			HQ729483
D319	GeoB*185	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl. (epitype)	Meier	Baltic Sea (Germany: type locality)	54°26′N	10°13′E	HQ729493
D099	GeoB 188	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Gottschling	Mediterranean Sea (France)	42°28′N	3°07′E	AY499524
D1016	GeoB*200	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Western North Atlantic	25°03′N	58°04′E	AY676157
D109	GeoB*201	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Mediterranean Sea (Greece)	36°14′N	26°03′E	AY676158
D117	GeoB 210	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	n.i.	Mediterranean Sea (Greece)	39°59′N	26°04′E	AY676159
D1006	GeoB*216	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	North Sea (Germany)	53°45′N	8°34′E	AY728079
D152	GeoB 219	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	n.i.	Eastern South Atlantic (Namibia)	29°51′S	13°25′E	AY676154
D147	GeoB*241	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Gottschling	Gulf of Mexico (USA)	30°25′N	88°17′W	AY676156
D185	GeoB 251	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	Gottschling & Petersen	Eastern North Atlantic (Sweden)	58°45′N	11°11′E	AY788358
D306	GeoB 331	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Zonneveld	Gulf of Taranto (Italy)	40°00′N	17°50′E	HQ729488
D307	GeoB 335	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Zonneveld	Gulf of Taranto (Italy)	39°51′N	17°55′E	HQ729489
D308	GeoB 338	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Zonneveld	Gulf of Taranto (Italy)	40°00′N	17°50′E	HQ729490
D312	GeoB 352	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	Häusermann	South Pacific (Chile)	42°22′S	72°24′W	HQ729491
D325	GeoB 360	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Zinßmeister	North Sea (Doggerbank)	53° 49′N	7°53′E	HQ729495
D326	GeoB*362	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Zinßmeister	North Sea (Doggerbank)	55°27′N	4°08′E	HQ729496
D352	GeoB 339	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Gottschling, Zinssmeister, Soehner	Mediterranean Sea (Italy)	40°40′N	14°46′E	HQ729498
D357	GeoB 377	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Gottschling, Zinssmeister, Soehner	Mediterranean Sea (Italy)	40°40′N	14°46′E	HQ729499

(*Continued on next page*)

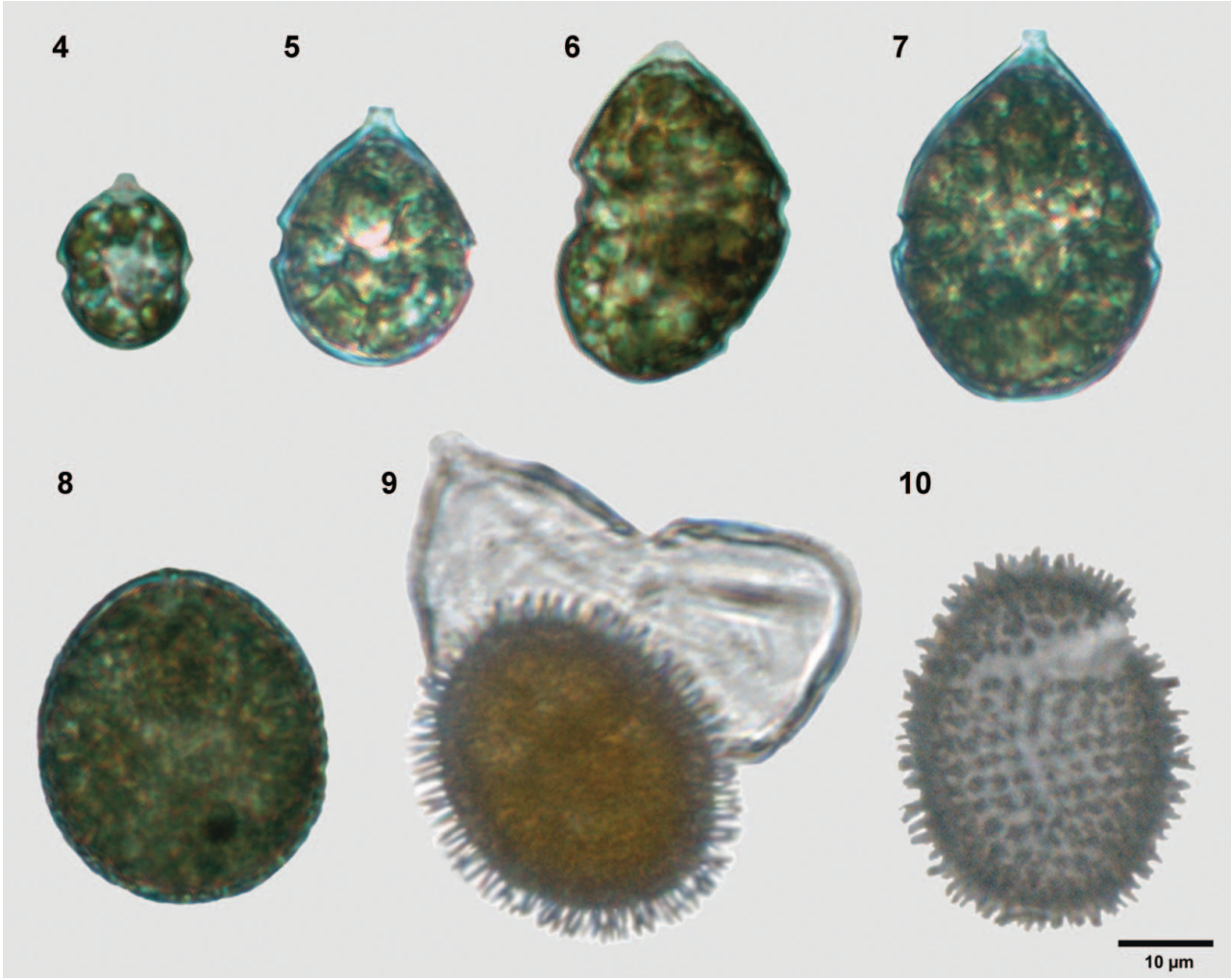
Table 1. Voucher list. Abbreviation: n.i., not indicated. Para- and/or polyphyletic taxa are indicated by quotation marks. (*Continued*)

DNA No.	Strain No.	Species name with author	Collector	Locality	Lat.	Long.	GenBank No.
D376	GeoB 421	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Greif	Western South Atlantic (Uruguay)	34°47′N	53°28′W	HQ729501
D169	IO14–01	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	Amorim	Eastern North Atlantic (Portugal)	37°57′N	8°55′W	AY676162
D201	IO24–01	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	Amorim	Eastern North Atlantic (Portugal)	38°42′N	09°26′W	AY728080
D175	IO26–01	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Amorim	Eastern North Atlantic (Portugal)	37°57′N	8°53′W	AY676163
D024	M34–*25/5	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Atlantic (Guyana)	11°54′N	57°49′W	AY499531
D118	NIES 369	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	Sawaguchi	Pacific (Japan)	40°30′N	141°30′E	AY499530
–	SSND01	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325954
–	SSDH02	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Wenling & Chao	Eastern Chinese Sea (China)	29°00′N	122°30′E	EU325953
–	STGX01	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Wenling & Chao	South China Sea (China)	21°30′N	109°5′E	EU325959
–	STND01	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325957
–	STND02	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Wenling & Chao	Eastern Chinese Sea (China)	27°12′N	121°26′E	EU325958
–	STXM01	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	Wenling & Chao	South China Sea (China)	24°25′N	118°5′E	EU325956
–	SZN 33	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527070
–	SZN 61	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527075
–	SZN 64	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527079
–	SZN 77	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527096
–	SZN 82	*Scrippsiella* '*trochoidea*' (F.Stein) A.R.Loebl.	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527101
D131	GeoB*160	*Scrippsiella* '*trochoidea*' var. *aciculifera* Montresor	n.i.	Red Sea (Saudi Arabia)	27°44′N	35°03′E	AY499526
D120	GeoB*213	*Scrippsiella* '*trochoidea*' var. *aciculifera* Montresor	n.i.	North Sea (Germany)	53°45′N	8°34′E	AY676164
–	SCCAP K–0499	*Scrippsiella* '*trochoidea*' var. *aciculifera* Montresor	n.i.	Skåle Fjorden (Faroe Islands)	62°N	7°W	AF527065
–	SZN 60	*Scrippsiella* '*trochoidea*' var. *aciculifera* Montresor	n.i.	Gulf of Naples (Italy)	40°43′N	14°10′E	AF527071

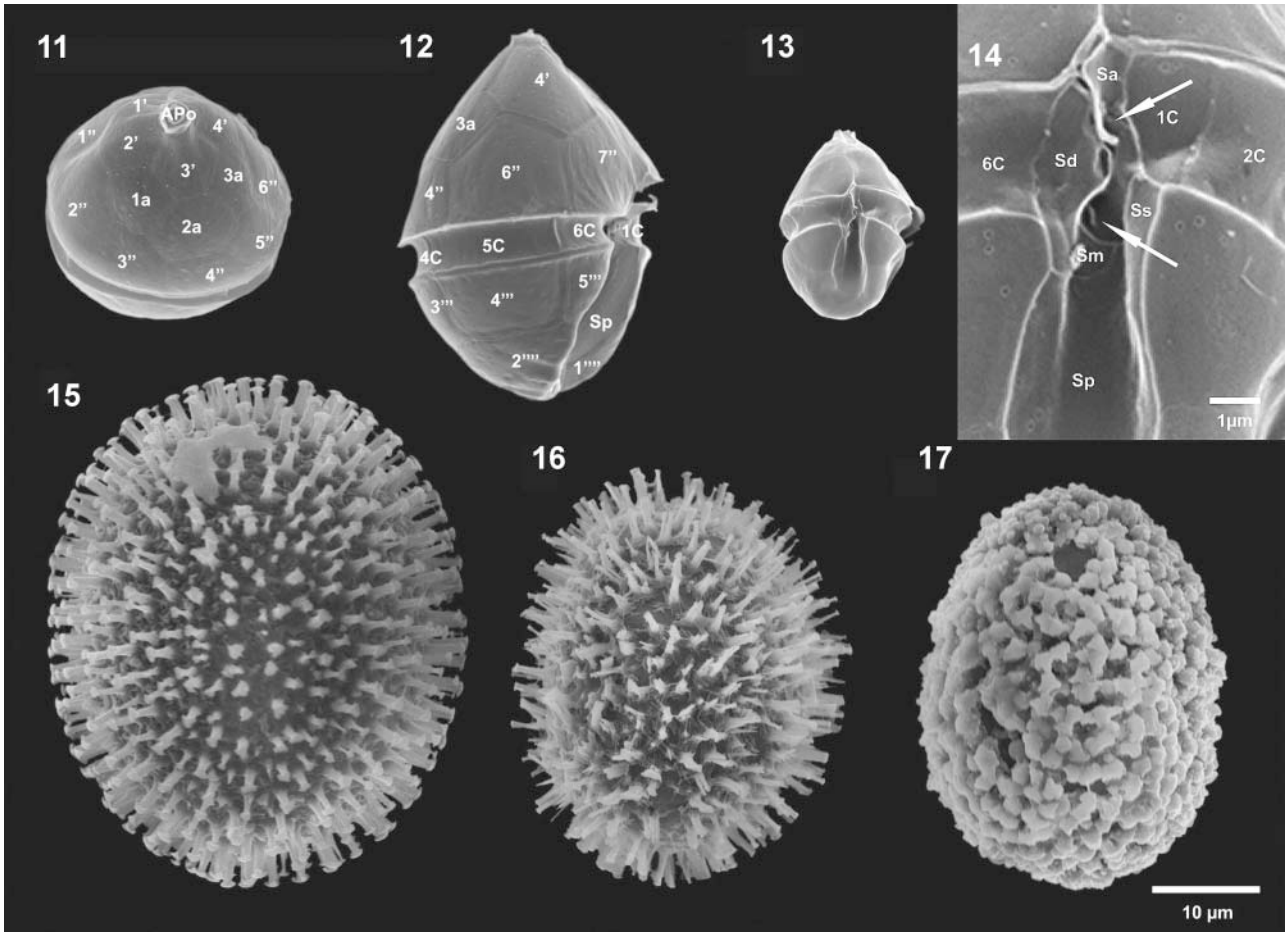
Phylogenetic analyses were carried out by using Maximum-Likelihood (ML) and Bayesian approaches, as described in detail by Gottschling *et al.* (in press). Calculations were carried out by using the resources of the Leibniz Rechenzentrum (LRZ, Munich; linux cluster HLRB-II) and of the SGI system (Zuse Institute Berlin, ZIB) being one half of the North German High Performance Computer (HLRN). The Bayesian analysis was performed by using 'MrBayes' v3.1.2 (Ronquist & Huelsenbeck, 2003; freely available at http://mrbayes.csit.fsu.edu/download.php) under the GTR+Γ substitution model and the random-addition-sequence method with 10 replicates. We ran two independent analyses of four chains (one cold and three heated) with 20,000,000 cycles, sampled every 1000th cycle, with an appropriate burn-in (10%, after checking convergence). For the ML calculation, 'RAxML' v7.2.6 (Stamatakis, 2006; freely available at http://wwwkramer.in.tum.de/exelixis/software.html) was applied by using the GTR+CAT substitution model to search for the best-scoring ML tree and a rapid bootstrap analysis of 1000 non-parametric replicates. Statistical support values (LBS: ML bootstrap support, BPP: Bayesian posterior probabilities) were drawn on the resulting, best-scoring ML tree.

Results

Strain GeoB*185 includes two principal stages: the phototropic motile cells, covered by a theca constituted by cellulosic plates (Figs 4–8, 11–14), and non-motile coccoid cells (Figs 9–10, 15–17). The phototrophic cells are golden-brown and exhibit a conical epitheca, with an



Figs 4–10. Life history stages of *Scrippsiella trochoidea*, strain GeoB*185 (light microscopy, all in the same scale). **4**, thecate cell, small morphotype; **5**, thecate cell, medium-sized morphotype; **6**, dividing thecate cell (the new thecate cell originates at the right side of the cingulum; **7**, thecate cell, large morphotype ('planozygote'); **8**, encysting stage of a large morphotype; **9**, coccoid stage, apically with thecal remnant; **10**, empty coccoid stage, with mesoepicystal combination archaeopyle.



Figs 11–17. Life history stages of *Scrippsiella trochoidea*, strain GeoB*185 (scanning electron microscopy, with the exception of 14 all in the same scale). **11**, medium-sized thecate cell, dorso-apical view; **12**, medium-sized thecate cell, lateral view; **13**, small thecate cell, ventral view; **14**, small thecate cell, sulcal region with the origins of the flagella indicated by arrows; **15**, coccoid stage, with spines flattened at the tips (cultivated at 18 °C and 35 psu salinity); **16**, coccoid stage, with thinner and irregular spines (cultivated at 18 °C and 35 psu salinity); **17**, coccoid stage, with thick and short spines (cultivated at 23 °C and 35 psu salinity); thecal plates are indicated where appropriate. Abbreviations: APo, apical pore plate; n′, apical plate; n″, precingular plate; n‴, postcingular plate, n⁗, antapical plate; na, anterior intercalary plate; nC, cingular plate; Sa, apical sulcal plate; Sd, right sulcal plate; Sm, median sulcal plate; Sp, posterior sulcal plate; Ss, left sulcal plate.

acuminate apex, and a hemispheric hypotheca. The cingular girdle is wide and deeply excavate. The cell surface is smooth and does not show any ornamentation. Small circular openings of trichocysts are rare and – if present – either irregularly arranged on the thecal plates or sometimes linearly arranged near the cingular plates.

Thecate cells of GeoB*185 show morphotypes of three distinct size classes, ranging from 19–42 μm in length (small cells: 19–21 μm, medium-sized cells: 25–32 μm, large cells: 36–42 μm) and 15–37 μm in width (small cells: 15–17 μm, medium-sized cells: 20–24 μm, large cells: 25–37 μm). The medium-sized cells (Figs 5–6, 11–12) represent the predominant morphotype and frequently reproduce themselves vegetatively. At the end of the division process, the daughter cell is still attached with the apex at the cingulum of the mother cell (Fig. 6). The smaller morphotype (Figs 4, 13) swims faster than all other cells, frequently near the medium surface. The third morphotype (Figs 7–8) is as large as the coccoid stage (see below). The apex is less acuminate and somewhat rounded. With increasing size, the cell becomes more spherical in shape and darker in colour, develops a red accumulation body, and slows down when swimming.

The thecal plate formula is APo, x, 4′, 3a, 7″, 6c, 5s, 5‴, 2⁗ and is consistent among all morphotypes. Two flagella originate from the sulcal region (Fig. 14), which is composed of five plates, with slightly varying arrangements among individuals. The Sd plate largely covers the smaller plates Sa, Ss and Sm. The transverse flagellum originates on the right-hand side between the plates Sa and Ss that are

both in contact with plate 1C. Frequently, the Sa plate has a small elongation towards the ventral axis, accompanying the apical part of the Sd plate. The longitudinal flagellum originates at the junction between the plates Sd, Sm and Ss and is functionally constrained by the groove of the Sp plate.

The large morphotype (Figs 7–8) as well as encystment has mostly been observed during night time and in the early morning (i.e. in the dark, before the light of the climate chamber turns on). Before encystment, the large thecate morphotype sinks down to the substrate or the bottom of the culture plate and, after completing its transformation into a coccoid stage, always adheres to it by an unknown mechanism (Figs 9–10, 9–10, 15–17). The coccoid stage of GeoB*185 is generally developed within the theca (Fig. 9) and frequently shows remnants of the shed epi- or hypotheca. At an early coccoid stage, it is hyaline and changes to a brownish colour after approximately 20 min. The coccoid cells are spherical to mostly ovoid, 37–46 μm in length and 29–38 μm in width.

The wall of the coccoid stage consists of an inner organic and an outer calcareous layer with numerous spines, each of which possesses an irregularly undulating base at maturity. In immature coccoid stages, the bases of the spines are initially not interlocked. The spines are triradiate in cross-section, and the tips are pointed, blunt or cleft (varying among individuals). Shape, width and length of the spines vary depending on experimental conditions (Figs 15–17): The spines are thicker at higher salinity (35 psu and higher) and temperature (23 °C). The simple operculum consists of the articulating thecal plate equivalents 1' through 4' and 1a through 3a (Fig. 10), and the coccoid stage thus had a mesoepicystal combination archaeopyle. Under standard culture conditions, excystment and production of a new generation of thecate cells takes place after days or several months. The direct observation of a medium-sized thecate cell emerging form on coccoid stage has been possible only once.

The alignment is 636 bp long and comprised 313 parsimony informative sites (49%, 3.4 per terminal taxon). Figure 18 shows the best-scoring ML tree (–ln = 8041.97), with *Scrippsiella sensu lato* (*s.l.*) – including coccoid stage-based taxa such as *Calcigonellum* Deflandre, 1949, *Calciodinellum* Deflandre, 1947, and *Pernambugia* Janofske & Karwath as well as the parasitic *Duboscquodinium* Grassé, 1952 – retrieved as monophyletic (93LBS, 1.00BPP). *Scrippsiella s.l.* segregates into eight major lineages, namely *Pernambugia tuberosa* Janofske & Karwath, *Calciodinellum* and its relatives (i.e. the CAL clade: 67LBS, .99BPP), *S. lachrymosa* Lewis (i.e. the LAC clade: 100LBS, 1.00BPP), and *S. precaria* Montresor & Zingone and its relatives (i.e. the PRE clade: 100LBS, 1.00BPP) as well as four lineages that can be subsumed under the STR-SC. The internal phylogeny of the STR-SC is only partly resolved and supported by high statistical values. However, three major assemblages can be identified, namely STR1 (95LBS, 1.00BPP), STR2 (98LBS, 1.00BPP) and STR3 (72LBS, .96BPP). The STR2 clade includes one of the two sequences obtained from GeoB*185 collected at the type locality of *S. trochoidea* in the Kiel Fjord (the other, i.e. contamination, sequence shows great similarity to a chrysophycean-like flagellate as inferred from a NCBI Blast Search: EF577176). Taxa exhibiting spiny coccoid stages are found in the PRE clade as well as in the STR-SC.

Discussion

The taxonomy of *S. trochoidea* is challenging (Elbrächter *et al.*, 2008) in five main respects: (1) the type material of *S. trochoidea* consists of a single illustration (von Stein, 1883); (2) *S. trochoidea* is not a single species, but rather a species complex (D'Onofrio *et al.*, 1999; Montresor *et al.*, 2003*b*; Gottschling *et al.*, 2005*b*); (3) the species are genetically distinct, but are indistinguishable in gross morphology ('cryptic species' with the same tabulation patterns and similar spiny coccoid stages: Montresor *et al.*, 2003*b*; Gottschling *et al.*, 2005*b*); (4) strains of the same ribotype show in turn a remarkable morphological variability in detail (D'Onofrio *et al.*, 1999; Gottschling *et al.*, 2005*b*); and (5) strains of the same ribotype are widely distributed (Gottschling *et al.*, 2005*b*; Gu *et al.*, 2008). For all these reasons, it is paramount to clarify the taxonomic identity of *S. trochoidea*. To collect living material from the type locality appears the most sensible approach for an adequate, state of the art morphological and molecular re-investigation.

To the best of our knowledge, a single scrippsielloid species predominates in the Kiel Fjord. Its occurrence has been continuously documented over the past century (von Stein, 1883; Lemmermann, 1910*a*, 1910*b*; Wasmund *et al.*, 2008). *Scrippsiella lachrymosa* has also been reported sporadically from this locality (Nehring, 1994, 1997), but this species can be easily distinguished from the *S. trochoidea*-like species based on the size of the thecate cell as well as from the morphology of the coccoid stage (Lewis, 1991). Morphologically, cells of strain GeoB*185 are consistent with the protologue of *G. trochoideum* including the illustration, although the original interpretations of von Stein (1883) must be seen in a historical context: The 'eye spot' is the red accumulation body, the transversal flagellum has been described as the 'ciliate girdle' of the cingulum, and the 'mouth' rather represents the sulcal region with the flagellar pores.

Morphotype variability found within GeoB*185 comprising thecate cells of distinct sizes have already been reported for (cultivated) *S. trochoidea*-like species (Lemmermann, 1910*a*; Braarud, 1958; Fine & Loeblich III., 1974, 1976). At present, it is, however, still not certain which are the morphotypes involved in conjugation, karyogamy and meiosis. Since the predominant (smaller) thecate cells of pelagic '*Calciodinellum*' *levantinum* S.Meier, Janofske &

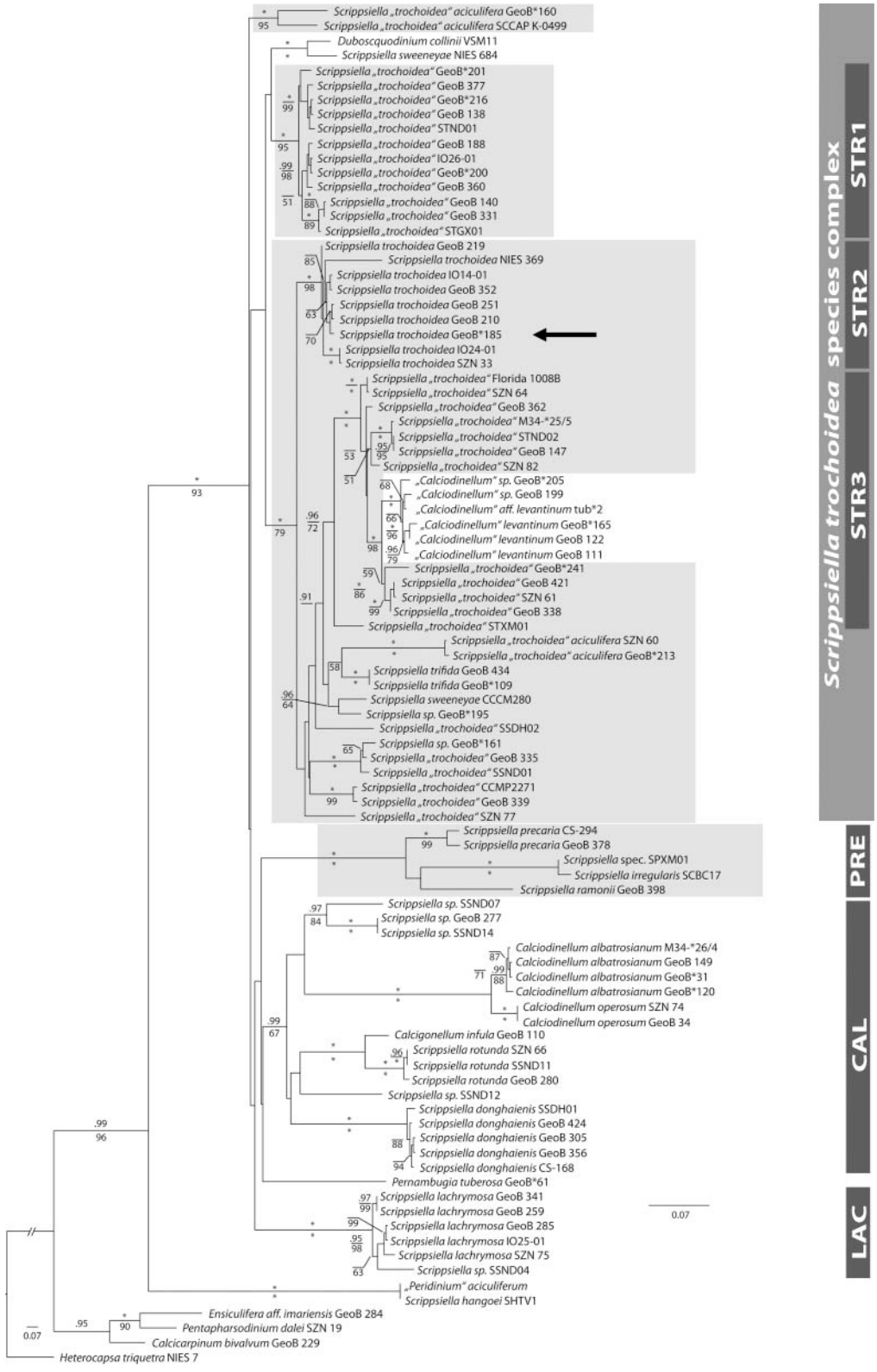


Fig. 18. Maximum likelihood (ML) tree (–ln = 8041.97) of 93 taxa of the Thoracosphaeraceae and outgroups, derived from the sequence comparison comprising the Internal Transcribed Spacer 1, 5.8S ribosomal RNA and the Internal Transcribed Spacer 2. Major clades are indicated, and taxa with spiny coccoid stages are shaded in grey. Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. The numbers on the branches are statistical support values (above: Bayesian posterior probabilities, values <0.90 are not shown; below: ML bootstrap values, values <50 are not shown). Asterisks indicate maximal support.

H.Willems are haploid (Meier *et al.*, 2007), it appears plausible that the predominant (medium-sized) thecate cells are haploid in *S. trochoidea* as well. These medium-sized cells are usually considered to fuse, originating planozygotes with two longitudinal flagella (Gao *et al.*, 1989*a*, 1989*b*; Gu *et al.*, 2008). The coccoid stages, to which the planozygotes develop, would subsequently be diploid (shown for '*C.*' *levantinum*: Meier *et al.*, 2007). The function of the small, fast-swimming thecate stages of *S. trochoidea* remains to be established. Ploidy levels of dinophyte cells are still largely unknown, and their determination will be a major task for future research by using, for example, fluorescence *in situ* hybridization and probes of single-copy genes.

Our molecular phylogeny including 22 new sequences confirms evidence of the existence of cryptic species, an aspect addressed previously by various authors (D'Onofrio *et al.*, 1999; Montresor *et al.*, 2003*a*; Gottschling *et al.*, 2005*b*; Gu *et al.*, 2008). The strain GeoB*185, from which cells have been prepared for epitypification, has been collected in the Kiel Fjord, and its ITS sequence clusters within the STR2 clade. As inferred from sequence comparison, this clade comprises strains, which have been sampled at various localities in the Baltic Sea, Mediterranean Sea, eastern South Atlantic as well as the eastern South and western North Pacific. Therefore, the strains grouped in this clade seem to have a broad distribution pattern (Gottschling *et al.*, 2005*b*).

It presently remains unclear, whether the STR2 clade includes one, few or several species of *Scrippsiella*. The region downstream of helix II found in the secondary structure model of the ITS1 (Gottschling & Plötner, 2004) is very divergent in its primary nucleotide sequence among species. However, it might be intraspecifically invariant (Gottschling & Kirsch, 2009), comprising classes of sequence motifs that do not show intermediates between lineages (as has been demonstrated for members of the STR2 clade: unpublished data). This region has the potential to serve as a species-specific DNA barcode for dinophytes (Litaker *et al.*, 2007) and thus might help to determine cryptic species as proposed previously (Gottschling *et al.*, 2005*b*; Gottschling & Kirsch, 2009). However, breeding experiments by using monoclonal cultures are also necessary to verify the status of isolated reproductive units.

The clarification of the systematic position of the original material of *S. trochoidea* has taxonomic consequences. Irrespective of whether the STR2 clade represents one or more species, its distinctiveness from dinophytes of the clades STR1 and STR3 that are morphologically similar to *S. trochoidea* is evident. Several species have been synonymized with, or considered closely related to, *S. trochoidea* in the past. Collecting material from the type locality of the various basionyms – *Peridinium ovaliforme* Kisselev (Barents Sea), *Rhabdosphaera erinaceus* Kamptner (Adriatic Sea), *Scrippsiella faeroensis* (Paulsen) Balech & L.O.Soares (North Atlantic off Faroe Islands), *S. regalis* (Gaarder) Janofske (Sargasso Sea), and last but not least *S. sweeneyae* Balech ex A.R.Loebl., the type of *Scrippsiella* (East Pacific off California) – and providing a morphological and molecular characterization might be a good starting point to check the relevance of these species names. Since the vast majority of the 'cryptic species', is not yet properly described; it remains a major task to work out the morphologically diagnostic and the ecological differences between them.

Taxonomic conclusions

Scrippsiella trochoidea (F.Stein) A.R.Loebl., *Journal of Protozoology* **23**: 25 (1976), basionym: *Glenodinium trochoideum* F.Stein, *Der Organismus der Arthrodelen Flagellaten nach eigenen Forschungen in systematischer Reihenfolge bearbeitet* **2**: pl. III 27–29 (1883). ≡ *Peridinium trochoideum* (F.Stein) Lemmerm., *Archiv für Hydrobiologie und Planktonkunde* **5**: 336–338, figs 33–36 (1910).–Type: Baltic Sea, off Federal Republic of Germany. Schleswig-Holstein, Kiel Fjord, s.d. [extant]: S.F.N.R. von Stein s.n. [holotype: *Der Organismus der Arthrodelen Flagellaten nach eigenen Forschungen in systematischer Reihenfolge bearbeitet* **2** (1883): pl. III 27–29!]; Baltic Sea, off Federal Republic of Germany. Schleswig-Holstein, Kiel Fjord, Apr 2000 [extant]: K.J.S. Meier s.n. [GeoB*185] (epitype, designated here: CEDiT-2011E13!, copies: B-400040745! BREM! M-156524!).

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PAPER 5 (PUBLICATION)

WHO AM I – AND IF SO, HOW MANY? SPECIES DIVERSITY OF CALCAREOUS DINOPHYTES (THORACOSPHAERACEAE, PERIDINIALES) IN THE MEDITERRANEAN SEA

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ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Who am I — and if so, how many? Species diversity of calcareous dinophytes (Thoracosphaeraceae, Peridiniales) in the Mediterranean Sea

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Abstract The diversity of extant calcareous dinophytes (Thoracosphaeraceae, Dinophyceae) is not fully recorded at present. The establishment of algal strains collected at multiple localities is necessary for a rigorous study of taxonomy, morphology and evolution in these unicellular organisms. We collected sediment and water tow samples from more than 60 localities in coastal areas of the eastern Mediterranean Sea and documented 15 morphospecies of calcareous dinophytes. Internal transcribed spacer (ITS) barcoding identified numerous species of the *Scrippsiella trochoidea* species complex that were genetically distinct, but indistinguishable in gross morphology (i.e. with the same tabulation patterns of the motile theca and similar spiny coccoid stages). We assessed a possible minimal number of cryptic species using ITS ribotype networks that indicated the existence of at least 21 species within the *Scrippsiella trochoidea* species complex. Species diversity of calcareous dinophytes appears higher in the Mediterranean Sea than in other parts of the world's oceans such as the North Sea. Our data underline the importance of field work to record the diversity of calcareous dinophytes and other unicellular life forms.

Keywords Calcareous dinophytes · ITS · Ribotype · Cryptic species

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Introduction

Dinophytes are distributed in marine and freshwater environments worldwide from arctic regions through tropical seas and constitute a considerable fraction of the plankton. Being primary producers as well as predators make the dinophytes an important component of the global aquatic ecosystem with an impact on carbon fixation. Together with the Ciliata and Apicomplexa (= Sporozoa), the Dinophyceae belong to the Alveolata and are a well-supported monophyletic group based on both molecular data and many apomorphies. Morphologically, the dinophytes exhibit unique traits, such as the coiled transverse flagellum, associated with a transverse groove termed the 'cingulum' (Taylor 1980; Fensome et al. 1999; Rizzo 2003; Leander and Keeling 2004; Harper et al. 2005). The Thoracosphaeraceae (Peridiniales) include all dinophytes that produce calcareous coccoid stages during their life history [important representative taxa are *Pentapharsodinium* Indel. & A.R.Loebl., *Scrippsiella* Balech ex A.R.Loebl and *Thoracosphaera* Kamptner] as well as some (presumably secondary) non-calcareous relatives such as *Ensiculifera* Balech, 1967 and *Pfiesteria* Steid. & J.M.Burkh. (Elbrächter et al. 2008). Approximately 35 extant species of calcareous dinophytes have been described currently based on morphology (Zonneveld et al. 2005), plus about 260 fossil species (Fensome and Williams 2004; Streng et al. 2004).

The Thoracosphaeraceae are considered a monophyletic group based on both morphological and molecular data (Wall and Dale 1968; Janofske 1992; Gottschling et al. 2005a, 2012). They segregate into three lineages, namely the E/Pe-clade (*Ensiculifera*/*Pentapharsodinium*-clade: marine environments), the T/Pf-clade (*Thoracosphaera*/*Pfiesteria-clade*: marine, brackish and fresh water environments), and *Scrippsiella s.l.* (marine and brackish environments), whereas the latter two clades show a close relationship. *Scrippsiella s.l.* segregates, in turn, into a number of lineages, basically corresponding to established taxonomic units (Gottschling et al. 2005b), and include *Pernambugia tuberosa* Janofske & Karwath (Karwath 2000), the CAL clade [with *Claciodinellum operosum* Deflandre, 1947 (Deflandre 1947)], the LAC clade [with *Scrippsiella lachrymosa* Lewis (Lewis 1991)], and the PRE clade [with *S. precaria* Montresor & Zingone (Montresor and Zingone 1988)] as well as the *S. trochoidea* (F.Stein) A.R.Loebl. [Loeblich 1976, basionym: *Glenodinium trochoideum* F.Stein (Stein 1883)] species complex (STR-SC; Montresor et al. 2003; Gottschling et al. 2005b; Gu et al. 2008; Zinssmeister et al. 2011). Phylogeny of the STR-SC is only partly resolved, but three major assemblages are currently identified, namely STR1, STR2 and STR3 (i.e. *S. trochoidea* cluster 1 through 3). STR3 includes the "*Calciodinellum*" *levantinum* S.Meier, Janofske & H.Willems (Meier et al. 2002) species group that is not closely related to the type species of *Calciodinellum*, *C. operosum*.

For manifold reasons, any species concept is challenged for the unicellular and character-poor dinophytes in general and the Thoracsophaeraceae in particular (Gottschling et al. 2005b; Elbrächter et al. 2008). The life history of Thoracosphaeraceae usually includes at least two different stages, namely the motile theca and an immotile coccoid stage (described frequently as 'cyst'). In dinophytes in general, and in calcareous dinophytes in particular, the morphology of the coccoid stages is diverse, while the thecate tabulation pattern of cellulose plates is rather homogeneous (D´Onofrio et al. 1999; Meier et al. 2002; Gottschling et al. 2005b; Gu et al. 2008). However, many ecological and checklist studies consider the morphology of the theca only, although a reliable species determination is not possible using this approach. The identification of species (fossil and extant) based on morphometrics is thus problematic as coccoid stages can show high intraspecific variability. For example, it has been shown that a single strain of *S. trochoidea* reveals morphological differences of coccoid cells under different cultivation conditions (Zinssmeister et al. 2011). Moreover, molecular sequence data have shown the existence of a large genetic heterogeneity of ribotypes among numerous different strains with the same gross morphology ('cryptic species', found primarily in the STR-SC: Montresor et al. 2003; Gottschling et al. 2005b; Gu et al. 2008).

Ribotyping is a fingerprint method analogous to phenotyping, genotyping or haplotyping. It uses DNA encoding ribosomal RNA from organisms or cells to define a specific sequence. A bifurcate gene tree is not always sufficient to illustrate all the phylogenetic information present in a molecular data set (Posada and Crandall 2001), since evidence for recombination and homoplasy is forced into non-reticulating tree topologies. Haplo- or ribotype networks consider such information by allowing loops and including missing intermediate mutational steps in the graphical illustration. The analysis of networks has been applied successfully to the investigation of intraspecific variability and population genetics. Cryptic species and speciation processes in plants and animals can also be inferred from network analyses of mitochondrial (Daniels and Ruhberg 2010), chloroplast (Lo et al. 2010), and nuclear (Peng et al. 2010) sequence data. The ribosomal internal transcribed spacer (ITS) region has been proposed to serve as a species-specific DNA barcode for dinophytes (Litaker et al. 2007; Genovesi et al. 2011; Stern et al. 2012) and thus might help to identify cryptic species as proposed previously (Gottschling et al. 2005b; Gottschling and Kirsch 2009). However, it is unclear at present whether a specific ribotype corresponds to several species, is unique to a single species or is a polymorphism within a species. If ITS ribotypes belong to a single reproductive unit (i.e. biological species), then a continuum between such ribotypes in terms of similarity is to be expected because of intraspecific variability. This hypothesis would be rejected by distinct classes of similarity or groups of ribotypes within a network.

With respect to taxonomy and evolution, the investigation of unicellular algae such as the dinophytes is laborious. It includes the collection of the organisms in the field and the establishment of (preferably monoclonal) strains that are held in culture collections (and which should be at other researchers disposal). Moreover, the investigated material must be preserved in form of isolates in a DNA bank as well as microscopic slides, since cultivation is frequently not possible over long periods of time. A considerable number of species assigned to the Thoracosphaeraceae are based on fossil types and have further been found in recent sediments (summarised in Elbrächter et al. 2008). From some of them [such as *C. operosum* and *Calcicarpinum bivalvum* G.Versteegh (Versteegh 1993) = "*Pentapharsodinium*" *tyrrhenicum* (Balech) Montresor, Zingione & D.Marino (Montresor et al. 1993)] strains could be established, and they have been investigated morphologically and / or molecularly (Montresor et al. 1993, 1997; D´Onofrio et al. 1999). However, many such 'living fossils' have not been brought into culture yet, despite their importance for understanding the evolution of the entire group (Elbrächter et al. 2008).

In this study, we summarise our extensive field trips to the eastern Mediterranean Sea (Italy, Greece and Crete),

following the pioneering work of Wall and Dale (1966, 1968) and Montresor et al. (1994). We provide species records assigned to the Thoracosphaeraceae based on morphology and — where possible — ITS barcoding of established strains for the more than 60 localities. We compare our results with those from a pilot field trip to Scandinavia (Gottschling and Kirsch 2009) to explore whether species diversity differs between ecologically distinct areas. Using ribotype networks, we quantify species number, which may have importance especially for the STR-SC containing many cryptic species (Montresor et al. 2003; Gottschling et al. 2005b; Gu et al. 2008).

Materials and methods

We collected sediment and water tow samples at 22 localities in Italy (April 2009), 31 localities in Greece (March 2010) and 11 localities on Crete (May 2010; Table S1 in the electronic supplementary material). Vertical water tow samples from the ground to the water surface were taken with a plankton net (mesh size 20 μm). In order to collect many samples in a short period of time, we used a self-manufactured, rocket-like bore probe (described in detail in Gottschling and Kirsch 2009).

With respect to the establishment of cultures from the samples, we focussed on species that could be assigned to the Thoracosphaeraceae. The grain size fraction of 20 μm – 75 μm of the sediment samples was supplied with K-Medium without silicate (Keller et al. 1987) and 35‰ artificial seawater (HW Marinemix Professional: Wiegandt; Krefeld, Germany) at pH 8.0 – 8.2. Six-well microplates (Zefa, Munich, Germany) were stored in a climate chamber Percival I-36VL (CLF PlantClimatics; Emersacker, Germany) at 18 °C, 80 μmol photons $m^{-2}s^{-1}$ and a 12:12 h light:dark photoperiod. Coccoid stages as well as motile thecas (generated from the sediment samples as well as from the water tow samples) were isolated and were grown under the conditions specified above. The established strains are currently held in the culture collections at the Institute of Historical Geology / Palaeontology (University of Bremen, Germany) and at the Institute of Systematic Botany and Mycology (University of Munich), and are available upon request.

The techniques of light (LM) and scanning electron microscopy (SEM) were used to identify the strains taxonomically. We followed standard protocols (Janofske 2000) that were basically the same as described in Gottschling et al. (2012). Briefly, SEM samples were either air-dried or dehydrated in a graded acetone series and critical point dried, followed by sputter-coating with platinum. The Kofoidean system (Taylor 1980; Fensome et al. 1993) was used for thecate plate designation.

Genomic DNA was extracted from fresh material using the Nucleo Spin Plant II Kit (Macherey-Nagel, Düren, Germany). Both ITS regions including the 5.8S rRNA were amplified using the primer pair ITS1 5′-GGTGAA CCTGAGGAAGGAT-3′ (Gottschling et al. 2005a) and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990) and were sequenced directly following standard protocols. The obtained sequences of cultivated and morphologically determined strains were compared to available NCBI GenBank entries using Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For ribotype network analyses, TCS v12.2.0 (Clement et al. 2000) was used following the developers' instructions to assess a possible minimal number of calcareous dinophyte species in specific clades (i.e. STR1, STR2, STR3 and others). TCS is a software program (Clement et al. 2000) to estimate gene genealogies including multifurcations and/or reticulations (i.e. networks). Indels were AC-coded.

Results

Within 15 sampling days total, we collected sediment and water tow samples densely at 64 localities in Italy, Greece and Crete (Fig. 1; only the samples of Italy have been investigated exhaustively in terms of morphology and sequencing so far). In total, 63 strains of dinophytes were established from the collected material, 54 of which were identified morphologically as belonging to 17 distinct morphospecies of the Thoracosphaeraceae (Table S1, Fig. 2). Thirty-five strains were sequenced and the morphological identifications were confirmed as *Calcicarpinum bivalvum* [= "*Pentapharsodinium*" *tyrrhenicum* (Balech) Montresor, Zingone & D.Marino], *Calcigonellum infula* Deflandre, 1949 (Deflandre 1949), *Calciodinellum operosum*, *Scrippsiella bicarinata* Zinssmeister, S.Soehner, S.Meier & Gottschling (Zinssmeister et al. in press), *S. kirschiae* Zinssmeister, S.Soehner, S.Meier & Gottschling (Zinssmeister et al. in press), *S. lachrymosa* Lewis, *S. precaria* Montresor & Zingone, *S. ramonii* Montresor (Montresor 1995), *S. rotunda* Lewis (Lewis 1991) and *S. trochoidea*, respectively (Table S1). This diversity in the samples included also empty coccoid stages of *Follisdinellum* G.Versteegh (Versteegh 1993) and *Calciperidinium* G.Versteegh (Versteegh 1993), but it has not yet been possible to establish strains from them.

Forty new sequences from the Mediterranean Sea and other oceans were submitted to the NCBI database: JQ422480-JQ422519 (Table S2).

Figure 3 shows the molecular sequence variation within four major clades of *Scrippsiella* illustrated as TCS ribotype networks. For the PRE clade, three morphospecies were

Fig. 1 Samples collected at 64 localities pictured on an outline map of Italy and Greece

included, and a single ribotype was identified for *S. ramonii*, with three sequences all derived from Italian strains. For *S. precaria*, two different ribotypes from Italy, Greece and Australia were identified. The samples from Italy and Greece shared the same ribotype, whereas the Australian ribotype was different in 13 sites of the sequence. Six different ribotypes from Iran and China were present among eight sequences of *S. irregularis* Attaran-Fariman & Bolch (Attaran-Fariman and Bolch 2007). There were a total of 63 and 76 mutational steps between the three species, respectively. Thirteen strains of the morphospecies *S. lachrymosa* (LAC clade) from China, Canada, Norway, Portugal, Scotland, Greece and Germany were included, whereas a total of 47 mutational steps were found between the six distinct ribotypes. Three of the six ribotypes were found in samples from Norwegian coastal waters, and two different ribotypes in samples from the Shetland Islands, Scotland.

From the recent Mediterranean samples, eight different ribotypes were assigned to the STR-SC. All available sequences clustering within the three distinct clades of the STR-SC (i.e. STR1, STR2 and STR3) were included in the analysis and the clades were each analysed separately. In the STR1 clade, four groups of nine different ribotypes in total were identified (seven newly sequenced strains from Italy and Greece were included in the analysis). In the STR2 clade (including the true *S. trochoidea*), five different ribotypes with a total of 14 mutational steps were found. Sequences of "*C.*" *levantinum* and related taxa belonging to the STR3 clade comprised 22 different ribotypes from strains sampled worldwide. Six of these ribotypes were assigned to *"Calciodinellum"*, 12 mutational steps apart from *S. trochoidea*-like sequences. The remaining 18 ribotypes, with up to 51 mutational steps in between, showed the morphology of *S. trochoidea*, which was divisible into roughly seven ribotype groups.

Discussion

In recent years, much effort has been devoted to the documentation of marine biodiversity (Beaugrand et al. 2010; Tittensor et al. 2010; Williams et al. 2010; http://www.coml.org); however, exact species numbers and correct scientific names are still needed for many marine organisms. This is particularly true for such unicellular life forms as the (calcareous) dinophytes, which have importance for the reconstruction of ancient circulation and productivity of the world's oceans and thus provide basic data for the impact of the global climate change as paleoenvironmental tools (Zonneveld et al. 1999; Esper et al. 2004; Meier et al. 2004; Vink 2004). Extant calcareous dinophytes have been collected frequently in pelagic environments during field trips using scientific research vessels, and relatively few studies have examined samples from coastal waters (Montresor et al. 1998; Godhe et al. 2001; Gottschling and Kirsch 2009).

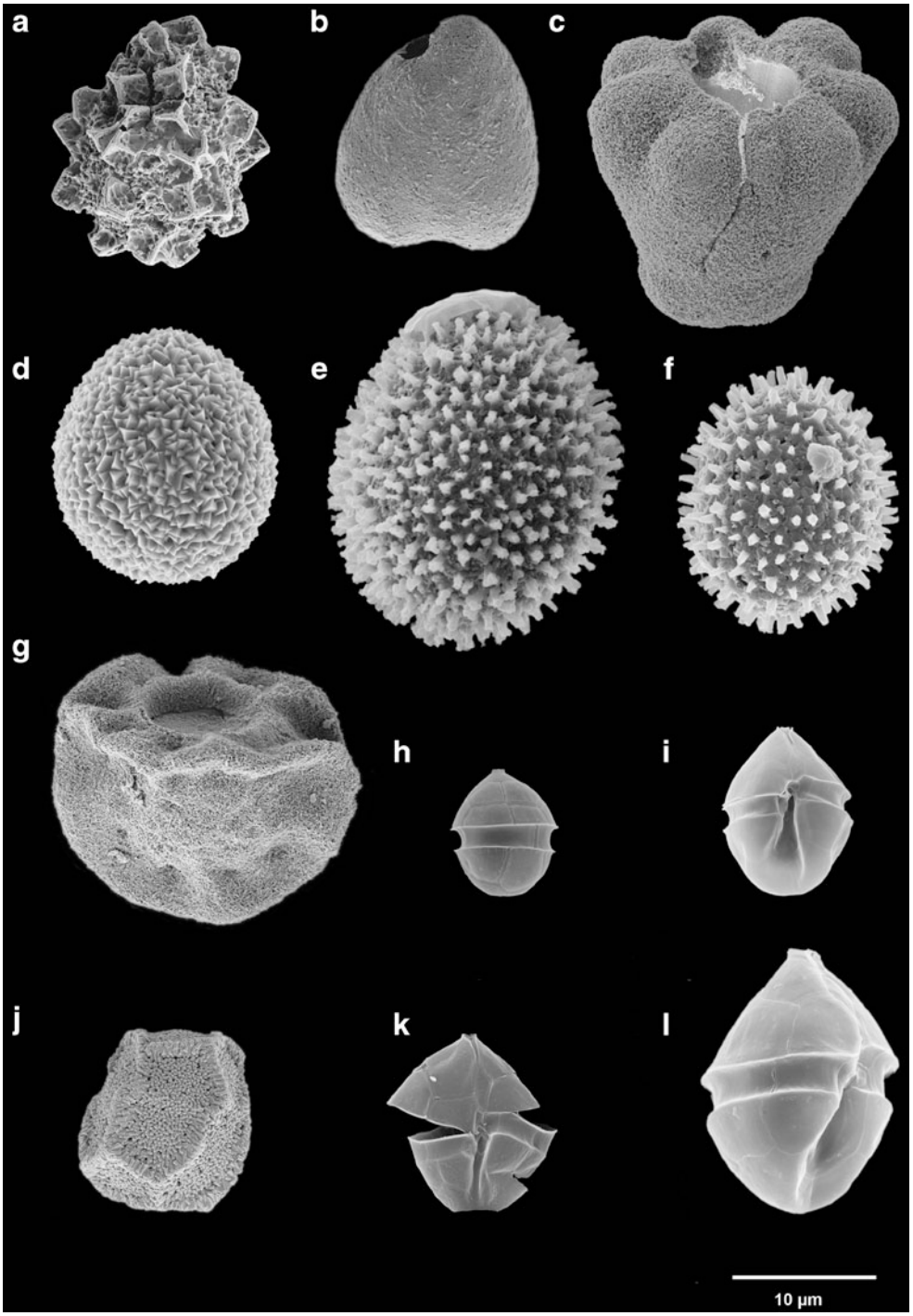

Fig. 2 a–l. Morphological diversity of calcareous dinophytes as found in the Mediterranean Sea, strain number is given, if no strain number is available the provenance is given (scanning electron microscopy of coocoid stage **a-g, j** and theca **h, i** and **k, l;** all at the same scale) **a** *Scrippsiella trifida* (GeoB 433); **b** *Calciperidinium asymmetricum* (Gallipoli, Italy); **c** *Follisdinellum* spec. (Salerno, Italy); **d–f** coccoid stages, morphotypes of *Scrippsiella trochoidea* (GeoB 283, GeoB*185, GeoM 5137); **g** *Calcicarpinum bivalvum* (Salerno, Italy); **h** small theca of *Calcicarpinum bivalvum* (GeoB 230); **i** small theca of *Scrippsiella trifida* (GeoB 401); **j** *Calciodinellum* spec. (Salerno, Italy); **k** small theca of *Scrippsiella trochoidea* (GeoB 376); **l** mid-sized theca of *Scrippsiella trochoidea* (GeoB*185)

The sediment-collecting tool described in Gottschling and Kirsch (2009) has enabled us to collect many samples within a short period of time. When compared to other oceans, the Mediterranean Sea is rather well sampled and investigated in terms of biodiversity assessment. The Gulf of Naples has been a primary research area for calcareous dinophytes, whereas other parts of the Mediterranean Sea, such as Greek coastal sites, have scarcely been sampled so far. We have identified morphologically 17 species of the Thoracosphaeraceae (Table S1), representing about two-thirds of the species known from the Mediterranean Sea, where approximately 27 morphospecies are distinguished currently (Montresor et al. 1998; Meier et al. 2002; Gómez 2003; Satta et al. 2010; Zinssmeister et al. 2011). Nevertheless, species diversity in the Mediterranean Sea appears much higher in comparison to other marine environments such as the North Sea, from which fewer than ten morphospecies of calcareous dinophytes have been

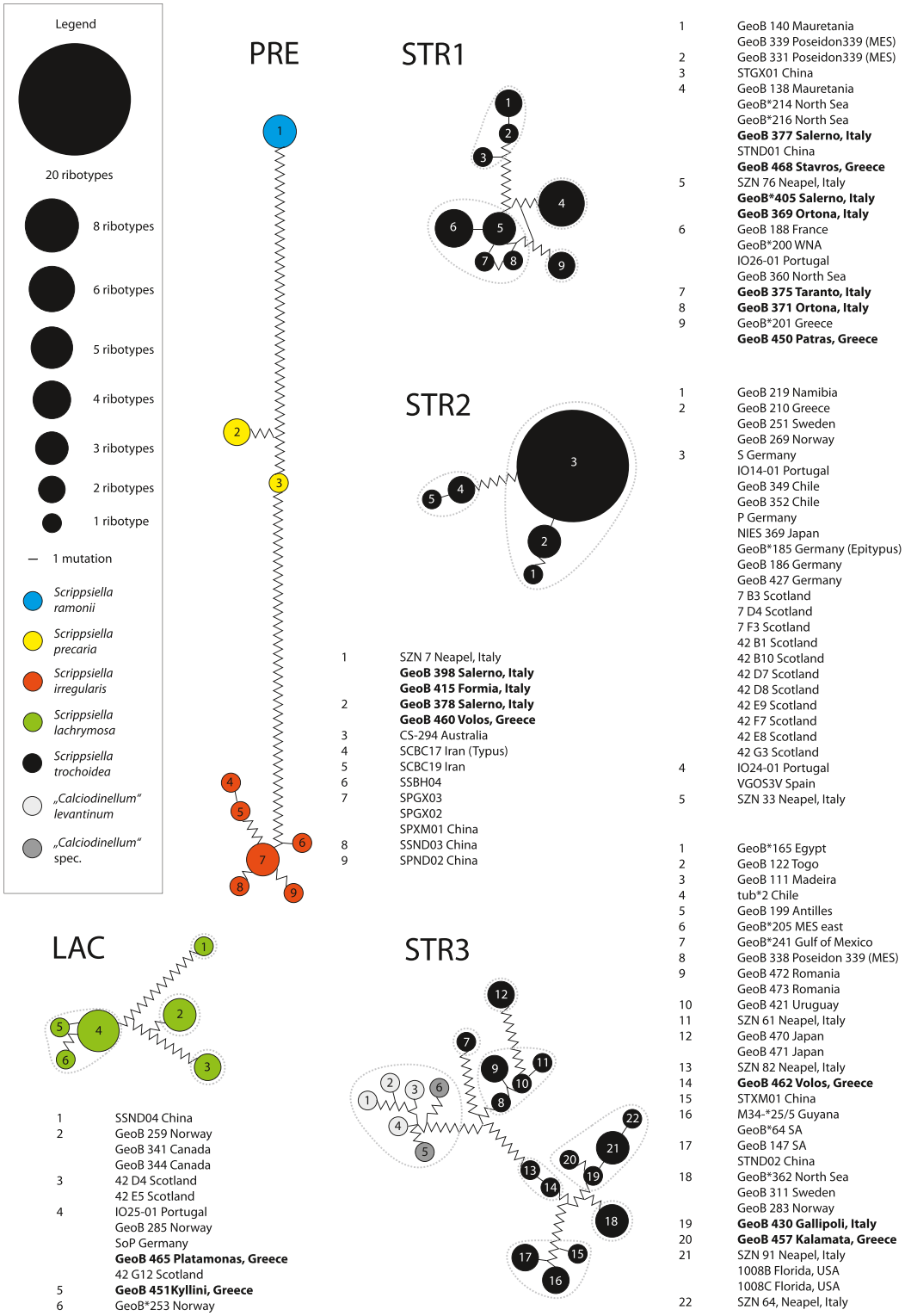

Fig. 3 Molecular diversity of ITS ribotypes within different clades of the Thoracosphaeracea (created with TCS). Number of similar ribotypes indicated by *circle size*, presumable cryptic species indicated by *dashed grey line*, newly added sequences from the Mediterranean Sea indicated in *bold*. The different morphospecies are *colour-coded* (see legend). GenBank accession numbers of used sequence data are listed in Table S1. *LAC* Clade including *Scrippsiella lachrymosa*, *PRE* clade includung *S. precaria*, *S. ramonii* and *S. irregularis*, STR1, STR2 and STR3 are major assemblages of the *S. trochoidea* species complex

documented so far (Persson et al. 2000; Godhe et al. 2001; Gottschling and Kirsch 2009). The species found in the samples from Italy, Greece and Crete comprise not only frequently encountered members of the Thoracopshaeraceae (including *S. trochoidea*), but also a number of taxa such as *Calciperidinium* and *Follisdinellum* that are known primarily from the fossil record, and which have been documented from recent sediments only rarely (Montresor et al. 1998; Tommasa et al. 2004). Unfortunately, it was not possible to establish strains until now, and it remains to be determined whether sampling at alternative dates during the course of a year could solve this problem.

Our ribotype networks show clearly distinct classes of sequence similarity within the clades PRE, LAC, STR1, STR2, and STR3. This supports the assumption that such clades represent more than a single reproductive unit (i.e. biological species). The STR3 clade in particular might have relavence to assess the minimal absolute number of species, since it includes morphologically and ecologically distinct forms (Meier and Willems 2003; Gottschling et al. 2005b; Meier et al. 2007): *Scrippsiella trochoidea* is characterised by benthic coccoid cells developing numerous spines, while "*C.*" *levantinum* is a pelagic species with smooth coccoid stages; both are doubtlessly isolated from another reproductively. Under the assumption that "*C.*" *levantinum* represents a single species, seven additional, molecularly distinct groups of ribotypes (all of which corresponding morphologically to *S. trochoidea*-like species) can be estimated for the STR3 clade. The same approach leads to the differentiation of four species in the STR1 clade, two species in the STR2 clade (including the true *S. trochoidea*: Zinssmeister et al. 2011), and four *S. lachrymosa*-like species as minimal numbers. In total, the six morphospecies included in the four TCS network analyses might segregate into the considerably high number of 21 species circumscribed molecularly, but crossing experiments using monoclonal strains are needed to verify the status of isolated reproductive units.

Especially in unicellular organisms such as (calcareous) dinophytes, species determination based on morphology is highly time- and cost-consuming and frequently subject to error. Moreover, morphologically plasticity (Zinssmeister et al. 2011) and cryptic species (Montresor et al. 2003; Gottschling and Kirsch 2009; Gottschling et al. 2005b) necessitate rapid and accurate tools for the reliable identification of species.

DNA barcoding (Hebert et al. 2003; Tautz et al. 2003; http://www.barcodinglife.com) has become a comparatively reasonable and fast methodology for determination of species, including animals (Hebert et al. 2003, 2004; Ward et al. 2005), plants (Kress et al. 2005; CBOL Plant Working Group 2009) and fungi (Feau et al. 2009). For dinophytes, the mitochondrial genes cytochrome *b* oxidase and cytochrome oxidase I have been proposed as general barcoding markers (Lin et al. 2009; Stern et al. 2010). However, resolution down to species level has not been satisfactory. Such loci might instead be useful for taxonomically broad investigations. As in fungi (Horton and Bruns 2001) the nuclear ITS has been recommended repeatly as an appropriate barcoding region for dinophytes at the species level (Gottschling et al. 2005b; Litaker et al. 2007; Gottschling and Kirsch 2009; Genovesi et al. 2011; Stern et al. 2012). Moreover, enormous numbers of ITS sequences have been accumulated in GenBank over the last decade, tendering for taxonomic comparison.

Our own sequencing efforts, with emphasis on the Thoracosphaeraceae, have confirmed that the ribosomal ITS region is suitable as a species-specific DNA barcode (Table S1). We have identified ten described morphospecies and one variety of calcareous dinophytes by sequence comparison. However, sequence data are available only for 13 of the Thoracosphearaceae species present in the Mediterranean Sea (D´Onofrio et al. 1999; Montresor et al. 2003; Gottschling et al. 2005a; Penna et al. 2010; Zinssmeister et al. 2011), and the completion of our studies has importance also for future taxonomic work. For example, *S. precaria* has been described from the Gulf of Naples (Montresor and Zingone 1988), but sequences of this species from the Mediterranean Sea have been not published so far. The establishment of a new strain collected close to the type locality and its subsequent molecular characterisation as presented here might contribute to disentangle the complex alpha-taxonomy of calcareous dinophytes. Moreover, two new *Scrippsiella* species have been described morphologically and included in a molecular phylogeny (Zinssmeister et al. in press).

In conclusion, there is no unambiguous criterion for species delimitation in unicellular organisms such as the dinophytes. Determination has been particularly challenging in calcareous dinophytes, since species such as *S. trochoidea* show enormous genetic variation and distinct groupings, but are indistinguishable in gross morphology ('cryptic species': Montresor et al. 2003; Gottschling et al. 2005b; Gottschling and Kirsch 2009). Occasionally, closely related species occur at the same locality, as has been shown previously also for different strains assigned to the calcareous morphospecies *S. lachrymosa* (Gottschling and Kirsch 2009), but also for other dinophytes such as *Alexandrium tamarense* (Lilly et al. 2007; Genovesi et al. 2011). If closely related species really occur sympatrically, then a driving force other than spatial isolation must be ascertained for speciation in calcareous dinophytes. More research is necessary to fully understand the diversification of calcareous dinophytes and the mechanisms causing it.

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PAPER 6 (PUBLICATION)

DELIMITATION OF THE THORACOSPHAERACEAE (DINOPHYCEAE), INCLUDING THE CALCAREOUS DINOFLAGELLATES, BASED ON LARGE AMOUNTS OF RIBOSOMAL RNA SEQUENCE DATA

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ORIGINAL PAPER

Delimitation of the Thoracosphaeraceae (Dinophyceae), Including the Calcareous Dinoflagellates, Based on Large Amounts of Ribosomal RNA Sequence Data

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The phylogenetic relationships of the Dinophyceae (Alveolata) are not sufficiently resolved at present. The Thoracosphaeraceae (Peridiniales) are the only group of the Alveolata that include members with calcareous coccoid stages; this trait is considered apomorphic. Although the coccoid stage apparently is not calcareous, *Bysmatrum* has been assigned to the Thoracosphaeraceae based on thecal morphology. We tested the monophyly of the Thoracosphaeraceae using large sets of ribosomal RNA sequence data of the Alveolata including the Dinophyceae. Phylogenetic analyses were performed using Maximum Likelihood and Bayesian approaches. The Thoracosphaeraceae were monophyletic, but included also a number of non-calcareous dinophytes (such as *Pentapharsodinium* and *Pfiesteria*) and even parasites (such as *Duboscquodinium* and *Tintinnophagus*). *Bysmatrum* had an isolated and uncertain phylogenetic position outside the Thoracosphaeraceae. The phylogenetic relationships among calcareous dinophytes appear complex, and the assumption of the single origin of the potential to produce calcareous structures is challenged. The application of concatenated ribosomal RNA sequence data may prove promising for phylogenetic reconstructions of the Dinophyceae in future.

Key words: Calcareous dinoflagellates; ITS; LSU; molecular systematics; morphology; rRNA; SSU.

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Introduction

Genes and spacers of the ribosomal RNA (rRNA) operon are among the most widely used genetic loci to reconstruct the entire Tree of Life as well as phylogenies of many particular organismal groups. Among unicellular eukaryotic life forms, molecular phylogenies using different rRNA sequences are particularly numerous among the alveolates, including the Dinophyceae (Daugbjerg et al. 2000; Gottschling et al. 2005a; John et al. 2003; Kremp et al. 2005; Saldarriaga et al. 2004), with more than 2,000 extant species described. Being as well primary producers and predators in marine and fresh water environments makes the Dinophyceae with their impact on carbon fixation an important part of the global aquatic ecosystem.

Dinophyceae exhibit many types of life style and nutrition, beside the phototroph and mixotroph forms. Some species are endosymbionts of marine animals and protozoa and contribute to the formation of coral reefs, while approximately 10% of the known species are parasitic. Together with the Ciliata and Apicomplexa (= Sporozoa), the Dinophyceae belong to the Alveolata and are a well-supported monophyletic group based on both molecular data and many apomorphies. Compared to all other eukaryotes, the genome of the Dinophyceae is highly unusual with respect to structure and regulation (reviewed by Moreno Díaz de la Espina et al. 2005). The nucleus contains chromosomes that are permanently condensed throughout the cell cycle except during DNA replication (Dodge 1966), displaying a liquid crystalline state (Rill et al. 1989). Morphologically, the Dinophyceae exhibit unique traits such as the coiled transverse flagellum associated with a transverse groove termed the 'cingulum' (Fensome et al. 1999; Harper et al. 2005; Leander and Keeling 2004; Rizzo 2003; Taylor 1980).

Using molecular data, the phylogeny of the Dinophyceae is difficult to reconstruct because of multiple endosymbiosis events, lateral gene transfers, and divergent substitution rates (Bhattacharya and Nosenko 2008; Howe et al. 2008; Minge et al. 2010; Moore et al. 2008; Morden and Sherwood 2002; Saldarriaga et al. 2004; Shalchian-Tabrizi et al. 2006; Yoon et al. 2005). A considerable fraction of published Dinophyceae molecular phylogenies relies exclusively on sequences of the small subunit rRNA (SSU; app. 1,800 bp in length), although the power of this locus for evolutionary reconstructions is limited (Taylor 2004). Phylogenetic trees as inferred from nuclear ribosomal sequences show polytomies in many crucial nodes, and the application of additional genetic markers is therefore highly recommended. Multi-gene approaches (Hoppenrath and Leander 2010; Yoon et al. 2005; Zhang et al. 2007, 2008), comprising sequences not only from the nucleus but also from mitochondria and chloroplasts, provide somewhat better resolution, and this is promising for future studies of phylogeny.

The branch lengths in the phylogenetic trees of the Dinophyceae are highly unbalanced. Many sequences of groups such as the Peridiniales render rather short branches, while some Dinophyceae including *Nocticula* and *Oxyrrhis* have very long branches and an unresolved phylogenetic position. Moreover, only few groups such as the Gonyaulacales, Suessiales, and Dinophysiales constitute monophyletic groups in molecular trees, while other traditional taxonomic units including the Peridiniales and Gymnodiniales appear highly para- and polyphyletic (Kremp et al. 2005; Saldarriaga et al. 2004; Zhang et al. 2007). The Thoracosphaeraceae (Peridiniales) include all Dinophyceae that produce calcareous coccoid stages during their development (important taxa are *Calcicarpinum*, *Scrippsiella*, and *Thoracosphaera*) as well as some (presumably secondarily) non-calcareous relatives such as *Pentapharsodinium* and *Pfiesteria* (Elbrächter et al. 2008). The monophyly of the Thoracosphaeraceae has not been shown in all previous phylogenetic studies, but this might be primarily because of the generally poor resolution of molecular trees in the Dinophyceae. They appear, however, to constitute a natural group in some studies, despite either limited molecular data (only sequences of the Internal Transcribed Spacer, ITS: Gottschling et al. 2005a) and/or a limited taxon sample (Tillmann et al. 2009; Zhang et al. 2007). The hypothesis that the Thoracosphaeraceae are monophyletic remains thus to be rigorously tested.

Currently comprising five species, *Bysmatrum* has been previously assigned to the Thoracosphaeraceae based on thecal morphology. The name has been introduced for benthic scrippsielloid algae (Faust and Steidinger 1998), since most of the motile stages of *Scrippsiella* share planktonic life forms. Moreover, both taxa differ in their morphologies: In *Bysmatrum*, plate 3′ separates the intercalary plates 2a and 3a and has a variously vermiculate to reticulate theca (Faust and Steidinger 1998; Murray et al. 2006; Ten-Hage et al. 2001). In contrast, plates 2a and 3a do always contact in *Scrippsiella*, and the theca is smooth without any ornamental structures (D'Onofrio et al. 1999; Gottschling et al. 2005b; Montresor et al. 2003).

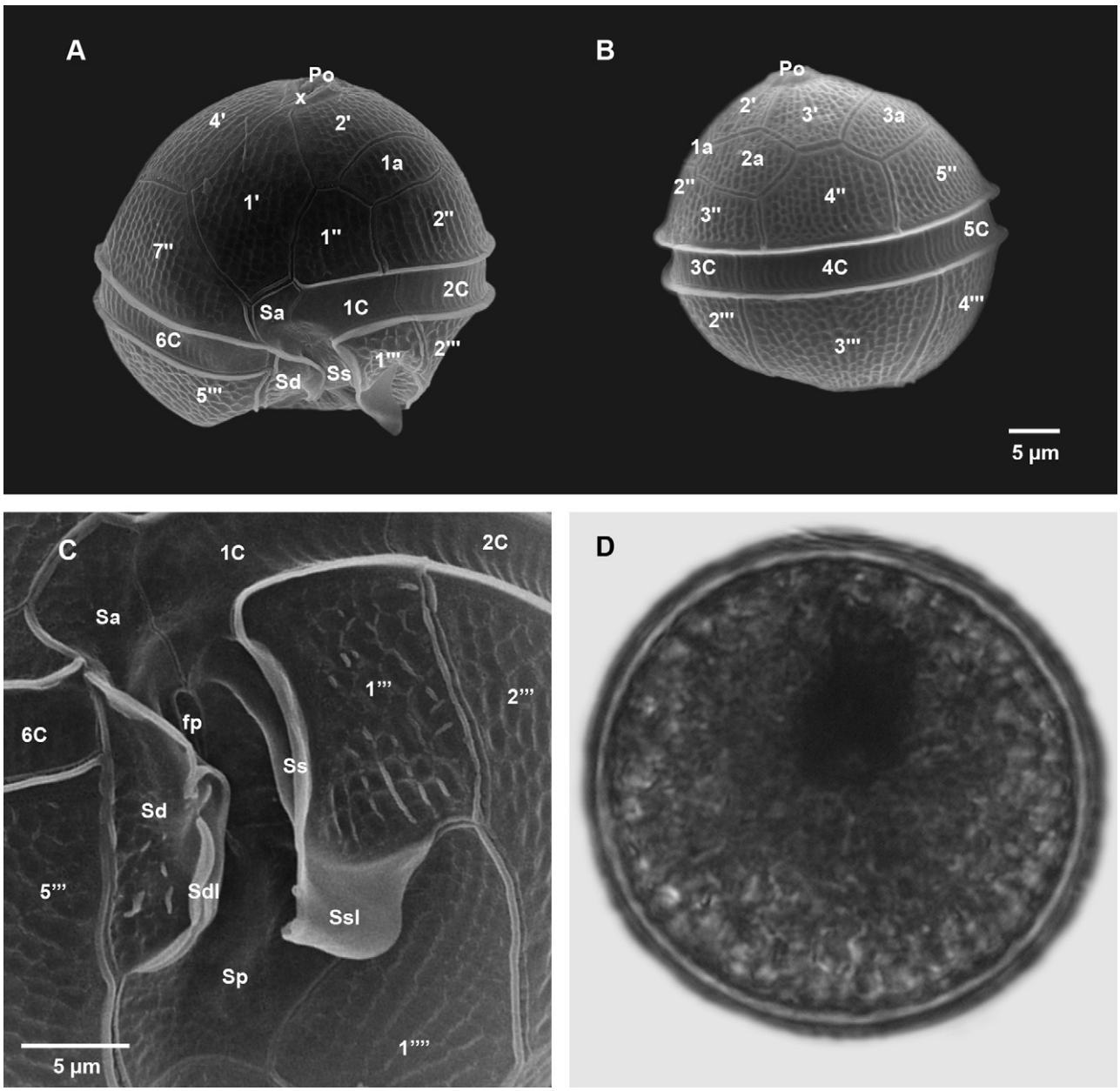
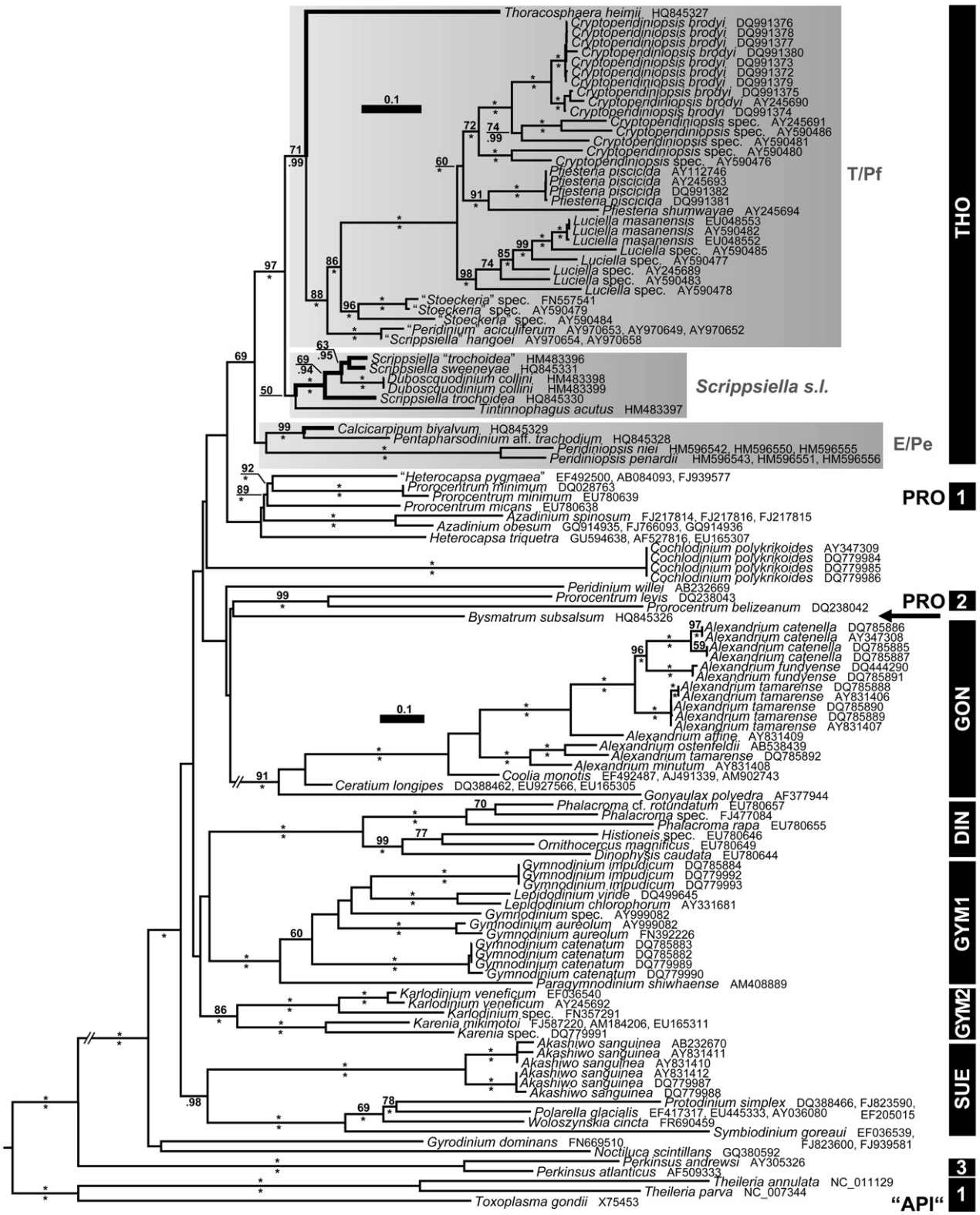


Figure 1. *Bysmatrum* had a peridinean tabulation pattern. Scanning electron microscope (SEM; Fig. 1A–C) and light microscope images (Fig. 1D) of *Bysmatrum*. **A**: Ventral view of epitheca, cingulum, and parts of hypotheca and sulcal region. **B**: Dorsal view of the thecate cell, the intercalary plates 2a and 3a were separated by plates 3′ and 4″. **C**: Detail of the sulcal region with 4 sulcal plates and well developed lists (Sdl and Ssl) at the plates Sd and 1‴. **D**: Non-calcified coccoid stage (without scale bar, image taken at x400). Abbreviations: nC, cingular plates; fp, flagellar pore (anterior or posterior flagellar pore); n', apical plates; n", precingular plates; n"', postcingular plates; n"", antapical plates; na, anterior intercalary plates; P_o, apical pore plate; Sa, apical sulcal plate; Sd, right sulcal plate; Sdl, right sulcal list at plate Sd; Sp, posterior sulcal plate; Ss, left sulcal plate; Ssl, left sulcal list at plate 1"'; x, channel plate.



In this study, we test the hypothesis whether the Thoracosphaeraceae are monophyletic and intend to determine the phylogenetic position of *Bysmatrum subsalsum*, the type of *Bysmatrum*. To address both reliably, large molecular data sets are necessary, and we use sequences comprising the complete SSU, the 5.8S rRNA (including the ITSs), and partial sequences of the large rRNA subunit (LSU). We therefore investigate the largest taxon sample possible at present, including –to the best of our knowledge– all available Alveolata sequences spanning this genetic region. We thus also aim at a better internal resolution of Dinophyceae molecular trees as a backbone for future phylogenetic studies.

Results

Morphology

Bysmatrum subsalsum exhibited photosynthetic, armored, pentagonal through round thecate cells, 21–45 μm long and 23–47 μm wide (Fig. 1A–B). The colour was golden-brown, and red-orange accumulation bodies were present in larger thecate cells. The epitheca had a hemispherical shape, and the hypotheca was round through pentagonal, showing an emargination of the sulcus together with the antapical plates. The cingulum was wide and deep. The plate ornamentation was generally reticulate, the plates Sd and 1‴ were reticulate and striate, and the cingulum was transversely striate.

Thecal plate morphology of *B. subsalsum* (Fig. 1A–C) corresponded to the typical peridinean pattern, consisting of 7 precingular plates, 4 sulcal plates, 5 postcingular plates, and 2 antapical plates (specific Kofoid formula: P_0, ACP, X, 4′, 3a, 7″, 6c, 4 s, 5‴, 2⁗). All major plates had more or less the same size, and the anterior intercalary plates 2a and 3a were separated from each other by the apical plates 3' and 4″. The shape of plate 1a was pentagonal, of plate 2a hexagonal, and of plate 3a pentagonal. The apical plate 1' was asymmetric and pentagonal. It connected the canal plate X and the anterior sulcal plate (Sa). Plate 1' was displaced to the right side between the apex and the sulcus and did not contact both in a direct line. The apical closing plate was located within the pore plate and delineated the plasma from the surrounding medium. There were four emarginated sulcal plates (Fig. 1C). The right sulcal plate (Sd) had an extensive list and almost covered the flagellar pore. Plate 1‴ also showed a list antapically.

The coccoid stage of *B. subsalsum* (Fig. 1D) was not calcified, and cells were spherical through ovoid, 41–51 μm in diamater. The colour was golden-brown, and a red-orange accumulation body was present.

Molecular Phylogenies

Tree topologies derived from the Alveolata alignments (Figs S1–S2, S4 in the Supplementary Material) were largely congruent, independently whether the Bayesian or the ML algorithm was applied. Many nodes showed high if not maximal statistical support values (LBS: ML support values; BPP: Bayesian posterior probabilities). Using the Ciliata as monophyletic outgroup, members of the Apicomplexa were paraphyletic, consisting of three lineages (Fig. S1 in the Supplementary Material): *Cryptosporidium* (100LBS, 1.00BPP), *Perkinsus* including an unspecified marine alveolate (99LBS, 1.00BPP), and a third large and diverse main clade (1.00BPP). *Cryptosporidium* was the sister group of all other Apicomplexa+Dinophyceae (although support below 50LBS and .90BPP, respectively) as well as *Perkinsus* of the Dinophyceae (100 LBS, 1.00BPP). The Dinophyceae were monophyletic (100LBS, 1.00BPP) and segregated in a number of lineages. One of these lineages were the Thoracosphaeraceae (including the important species of *Calcicarpinum*, *Scrippsiella*, *Thoracosphaera*, and *Pfiesteria*; 90LBS, .99BPP). *Bysmatrum* had a

Figure 2. The Thoracosphaeraceae were monophyletic and included both calcareous and non-calcareous forms. Maximum likelihood (ML) tree (–ln = 87,808) of 113 members of the Dinophyceae (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MUSCLE generated rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 2 (2,286 parsimony-informative positions). Major clades are indicated; members of the Thoracophaeraceae with known calcareous coccoid stages are highlighted by bold branches. Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (above: ML bootstrap support values, values under 50 are not shown; below: Bayesian posterior probabilities, values under .90 are not shown); maximal support values are indicated by asterisks. The tree is rooted with five sequences of the Apicomplexa. Abbreviations: API1, API3: different clades of Apicomplexa; DIN: Dinophysiales; GON: Gonyaulacales; GYM1, GYM2: different clades of Gymnodiniales; PRO1, PRO2: different clades of Prorocentrales; SUE: Suessiales; THO: Thoracosphaeraceae.

phylogenetic position outside the Thoracosphaeraceae and exhibited a close relationship on a long branch to the Gonyaulacales (.98BPP).

Tree topologies derived from the Dinophyceae alignment (Fig. 2 and Figs S3, S5–S6 in the Supplementary Material) were also largely congruent, independently whether the Bayesian or the ML algorithm was applied. Many nodes exhibited high support values, but the phylogenetic backbone and the basal nodes were only weakly resolved. The Dinophyceae were monophyletic (Fig. 2; 100LBS, 1.00BPP), and the Dinophysiales (100LBS, 1.00BPP) and Gonyaulacales (100LBS, 1.00BPP) corresponded to established systematic units among their major lineages. Several other clades and lineages of the Gymnodiniales and Prorocentrales, however, did not constitute monophyletic groups. The Peridiniales were likewise not monophyletic, and the monophyly of the Thoracophaeraceae (69LBS) was not as clearly supported as inferred from the Alveolata alignment. Internally, the Thoracosphaeraceae segregated into three lineages, namely the E/Pe-clade (marine and possibly also freshwater environments), the T/Pf-clade (71LBS, .99BPP; marine, brackish, and fresh water environments), and *Scrippsiella* s.l. (50LBS; marine environments), whereas the latter two clades showed a close relationship (97LBS, 1.00BPP). *Bysmatrum* did not nest with the Thoracosphaeraceae, and its closest relative could not be determined reliably.

Species with calcareous coccoid stages known did not constitute a monophyletic group and were scattered throughout the three clades of the Thoracosphaeraceae. In the E/Pe-clade, calcareous *Calcicarpinum bivalvum* and non-calcareous *Pentapharsodinium* aff. *trachodium* were closely related (99LB, 1.00BPP) and constituted the sister group of non-calcareous species assigned to *Peridiniopsis*. Non-calcareous and parasitic *Duboscquodinium* was nested within calcareous *Scrippsiella*, and together (100LBS, 1.00BPP) they constituted the sister group of non-calcareous and parasitic *Tintinnophagus*. Finally, the non-calcareous pfiesterians (i.e., *Cryptoperidiniopsis*, *Luciella*, *Pfiesteria*, and "*Stoeckeria*") plus "*Peridinium*" *aciculifera* and "*Scrippsiella*" *hangoei* constituted the sister group (88LBS, 1.00BPP) of calcareous *Thoracosphaera* in the T/Pf-clade (71LBS, .99BPP).

Discussion

Despite the extensive comparison of rRNA sequences, the phylogenetic relationships of the Dinophyceae are not sufficiently resolved at present. Several strategies have been pursued to overcome this problem. The consideration of additional loci such as nuclear and mitochondrial coding genes in concatenated phylogenetic analyses has improved the resolution of molecular trees in the Dinophyceae (Hoppenrath and Leander 2010; Zhang et al. 2007, 2008), but the taxon sampling as well as the amount of genetic information is currently still limited. Moreover, chloroplast genes have been sequenced to infer the phylogenetic relationships, with unsatisfying results mainly caused by multiple endosymbiosis events in the Dinophyceae (Bhattacharya and Nosenko 2008; Howe et al. 2008; Minge et al. 2010). Another strategy to improve molecular trees is the compilation of the comprehensive rRNA sequence data presently available. A number of particular strains have been independently sequenced for the SSU, the ITS region, and / or the LSU, but they have not been brought together in a concatenated alignment yet. In this study, we have compiled all rRNA sequences of the Alveolata that span the entire SSU, the ITS region, and the first three domains of the LSU to explore the utility of this commonly used marker in phylogenetic studies. We thus present data matrices with more informative sites than any previous phylogenetic analysis of the Dinophyceae.

To test the monophyly of the Thoracosphaeraceae based on large molecular data sets has been one major goal of this study, and our results confirm and improve previous trees of calcareous dinophytes with smaller amounts of sequence data (Gottschling et al. 2005a) and / or a limited taxon sample (Tillmann et al. 2009; Zhang et al. 2007). The assumption that the Thoracosphaerales (i.e., *Thoracosphaera*) and the Calciodinelloideae (i.e., *Scrippsiella* and relatives) have to be assigned to different taxonomic units (Fensome et al. 1993; Tangen et al. 1982), implying that they are not closely related, is clearly rejected by the data presented here. The monophyly of the Thoracosphaeraceae remains, however, somewhat ambiguous, since the support is only moderate as inferred from the alignment comprising more diverse but shorter rRNA sequences. This is particularly because of the weak association of the E/Pe-clade (with calcareous *Calcicarpinum bivalvum*) to the other calcareous dinophytes. Species currently assigned to *Peridiniopsis* might also belong to this clade as it has been assumed previously based on morphology, but the extant diversity of the E/Pe-clade is otherwise highly fragmentarily investigated at present (Elbrächter et al. 2008). It remains to be determined whether

an improved taxon sampling and future molecular studies will better enlighten the precise relationships of and within the E/Pe-clade. The vast majority of the Thoracosphaeraceae (i.e., *Scrippsiella s.l.* and the T/Pf-clade), however, clearly constitute a monophyletic group. The acceptance of the Pfiesteriaceae as a distinct systematic unit (Steidinger et al. 1996) would, anyhow, leave the remainders of the Thoracosphaeraceae paraphyletic.

Within the impressive diversity of the Alveolata, the potential to produce calcareous structures is restricted to (i.e., has been considered apomorphic for) the Thoracosphaeraceae, arguing for the monophyly of this group (Janofske 1992; Kohring et al. 2005; Wall and Dale 1968). Previous molecular studies have revealed, however, that a number of species with no calcareous coccoid stages known (i.e., primarily *Pfiesteria* and its relatives) are nested within the Thoracosphaeraceae (Gottschling et al. 2005a; Kremp et al. 2005; Tillmann et al. 2009; Zhang et al. 2007). From an evolutionary perspective, the close relationships between scrippsielloid algae and the parasites *Duboscquodinium* and *Tintinnophagus* (Coats et al. 2010) are now particularly surprising. The assumption that the potential to produce calcareous structures has arisen only once in the Dinophyceae is therefore challenged by the phylogenetic results presented here as well as by the recent observation of different calcification modes during encystment of such algae (Meier et al. 2007). It is also possible, however, that the relationships within the Thoracosphaeraceae appear still complex because of our limited knowledge about the diversity of developmental stages among (calcareous) dinophytes. More research is necessary to validate, for example, that a parasitic life style is integral part of the development of (calcareous) *Calcicarpinum bivalvum* (= "*Pentapharsodinium*" *tyrrhenicum*: Smith et al. 2007).

Another goal of our study has been the determination of the systematic position of *Bysmatrum*. The thecal plate arrangements of the strain under investigation is consistent with previous descriptions (Faust and Steidinger 1998; Murray et al. 2006; Steidinger and Balech 1977) and correspond to the typical peridinean pattern (Fensome et al. 1993; Taylor 1980). As inferred from the molecular trees, *Bysmatrum* does most probably not belong to the Thoracosphaeraceae as previously assumed (Steidinger and Balech 1977), but must be considered an unusual member of the Dinophyceae of uncertain systematic placement at present, presumably close to the Gonyaulacales. Our results support the assumption that the peridinean plate pattern is widespread and present in different lineages of the Dinophyceae (Taylor 2004). Therefore, it cannot be considered an apomorphic trait of the Peridiniales that seem to be a paraphyletic group, from which other lineages of the Dinophyceae have been probably derived.

The monophyly of some established systematic units such as the Dinophysiales and the Gonyaulacales are clearly supported by the molecular data presented here. The repeatedly shown molecular polyphyly of the Prorocentrales in rRNA trees (Grzebyk et al. 1998; Hoppenrath and Leander 2008), however, remains a mystery, since the group is clearly monophyletic based on morphological apomorphic traits such as a cluster of very small platelets around two pores and the lack of a girdle and sulcus. A multi-gene approach as well as a *cox*1 phylogeny render the Prorocentrales monophyletic (Murray et al. 2009; Zhang et al. 2007), and the polyphyly of the Prorocentrales in rRNA trees has been explained by intrinsic inadequacies of the molecules used to resolve the phylogeny (Taylor 2004). In our molecular tree of the Alveolata, two unequal copies of rRNA genes, present on different chromosomes of *Plasmodium vivax* of the same individuals, illustrate this problem. Intragenomic polymorphisms of ribosomal genes have been identified in various eukaryotic lineages (Griffiths-Jones 2007; Le Blancq et al. 1997; Simon and Weiß 2008; Thornhill et al. 2007; Torres-Machorro et al. 2010), with putatively fatal implications for reconstructions of phylogenetic relationships. Thus, the consideration of non-orthologous sequences might explain the molecular polyphyly of the Prorocentrales, and it remains an open question, how many rRNA sequences are additionally affected in the Dinophyceae.

In conclusion, the application of long rRNA sequences helps to test hypotheses on relationships in the Dinophyceae more rigorously. *Bysmatrum* clearly belongs to the Dinophyceae (although the precise systematic placement cannot be determined at present), and the Thoracosphaeraceae including both calcareous and non-calcareous forms most probably constitute a monophyletic group. From a morphological perspective, putatively close relatives of the Thoracosphaeraceae such as some freshwater species of *Peridinium* (but not the type species *P. cinctum*: Calado et al. 2009; Gottschling et al. 2005a; Logares et al. 2007) and the heterotrophic *Protoperidinium* (Elbrächter et al. 2008) should be included in future molecular studies using long rRNA sequences. The phylogenetic trees provided

in this study may prove helpful to revise the systematics of the Dinophyceae in general and of the Peridiniales in particular. Sequences from genes and spacers of the rRNA operon are available from less than 25% of the currently described taxa of the Dinophyceae at the generic level, and more research is necessary to improve the knowledge about their systematics and phylogenetic relationships.

Methods

Light and electron microscopy: *Bysmatrum subsalsum* was collected in Greece (Supplementary Material Table S1) and is currently cultivated at the universities of Thessaloniki (Department of Botany, School of Biology), Bremen (Historical Geology and Paleontology department), and Munich (Systematic Botany and Mycology department of the LMU). It grows in sterile filtrated K-Medium, specifically in 35‰ artificial seawater (hw marinemix professional, Wiegandt, Krefeld, Germany) without silicate (Keller et al. 1987) at pH 8.0–8.2, and is stored in a Percival I-36VL climate chamber (CLF PlantClimatics, Emersacker, Germany) at 23 °C, 80 μmol photons m^{-2} s^{-1}, and 12:12-h light:dark photoperiod. Cells were observed in a CKX41 inverse microscope (Olympus, Hamburg, Germany).

For scanning electron microscope (SEM) studies, cells were fixed with 2,5% glutaraldehyde (Plano, Wetzler, Germany) in 0.2 M cacodylate buffer (Roth, Karlsruhe, Germany), with 0.4 M NaCl (Roth), pH 8.0 for 1 h, transferred in a Swinnex filter holder (Schubert & Weiss Omnilab, München, Germany) equipped with a polycarbonate membrane with 5 μm pores. Liquids were changed with a plastic syringe connected to the filter holder. The cells were washed in 75 mM cacodylate buffer (Roth), 2 mM $MgCl_2$ (Roth), 0.4 M NaCl (Roth), pH 8.0 and distillated water, dehydrated in a graded acetone p.a. series (Roth), and critical point dried. The filters were placed on SEM stubs, and samples were sputter-coated with platinum and documented with a LEO 438 VP SEM. The Kofoid system (Fensome et al. 1993; Taylor 1980) was used for thecal plate designation.

Molecular work and phylogenetic analyses: Sequences of those Alveolata that comprised the SSU, 5.8S rRNA (including the ITSs), and the first three domains of the LSU were downloaded from GenBank. Fresh material (clonal cultures, mostly cultivated at the University of Bremen, Germany) was used for sequencing of five species of the Thoracosphaeraceae plus *Bysmatrum*. To exclude the possibility of contaminations, DNA isolation and sequencing were independently performed in the labs of MG, UJ, JP, and MS, following standard protocols that are described in detail in Gottschling and Plötner (2004). The specific primers for amplification used in this study are listed in Table S2. In total, 160 terminal taxa were investigated in this study (Table S1).

The consideration particularly of the highly divergent ITS sequences over a broad taxonomic range such as the dinophytes should be treated with caution, and we explored the possible negative effects for our phylogenetic reconstructions by RY-coding, excluding phylogenetically ambiguous positions, using different alignment programs, and applying an infinite mixture model to the data (see the Supplementary Materials for details). For the main part of our study, sequences of two different taxon samples were aligned using 'MUSCLE' v3.6 (Edgar 2004; http://www.drive5.com/MUSCLE/downloads.htm), with the default settings: The first taxon sample included all sequences of the Alveolata available comprising the complete SSU, the complete ITS region (including the 5.8S rRNA), and the first three domains of the LSU; the other data matrix used shorter LSU sequences in order to include a broader taxon sample of the Dinophyceae. The alignments were partitioned into three parts (for details see Tables S3–S4 in the Supplementary Material, and all final data matrices are available under doi:10.5061/dryad.d1vg6 or from MG upon request).

Phylogenetic analyses were run using distinct models / data partitions, with individual per partition branch length optimisation. Calculations were carried out by using the resources of the Leibniz Rechenzentrum (LRZ, Munich; linux cluster HLRB-II) and of the SGI system (Zuse Institute Berlin, ZIB) being one half of the North German High Performance Computer (HLRN). Maximum Likelihood-based analyses were conducted using the PTHREADS version of 'RAxML' VII (Stamatakis 2006; Stamatakis et al. 2008; http://www.phylo.org/portal/Home.do) and applying the GTR substitution matrix. To determine best fitted ML-trees, we executed 10-tree searches from distinct random stepwise addition sequence Maximum Parsimony starting trees and 10,000 non-parametric bootstrap replicates. Bayesian analyses were performed with 'MrBayes' v3.1.2 (Huelsenbeck and Ronquist 2001; http://www.mrbayes.csit.fsu.edu/) under the GTR+Γ substitution model using the random-addition-sequence method with 10 replicates. We ran two independent analyses of four chains (one cold and three heated) with 20,000,000 cycles, sampled every 1,000th cycle, with an appropriate burn-in (10%) as inferred from the evaluation of the trace files using Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). The statistical support values were drawn on the best scoring ML-trees.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.06.003.

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Delimitation of the Thoracosphaeraceae (Dinophyceae), including the calcareous dinoflagellates, based on large amounts of ribosomal RNA sequence data

(Research Article)

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SUPPLEMENTARY MATERIAL

EXTENDED MATERIALS AND METHODS

The data matrices could not be processed manually because of high sequence variation (particularly of the ITS regions), and we examined the effects of different alignment programs on the phylogenetic reconstructions. Sequences of the two different taxon samples (Alveolata, Dinophyceae) were aligned using 'MAFFT' v6.523 (Katoh et al. 2005; http://align.bmr.kyushu-u.ac.jp/MAFFT/software/), 'MUSCLE' v3.6 (Edgar 2004; http://www.drive5.com/MUSCLE/downloads.htm), and 'PRANK' v100311 (Löytynoja and Goldman 2008; http://www.ebi.ac.uk/goldman-srv/PRANK/src/PRANK/), each with the default settings. Moreover, MAFFT provides an additional alignment approach that considers the secondary structure of the rRNA molecules, and we computed a fourth alignment using the 'QINSI' option. For all resulting alignments, jModelTest (Posada 2008; http://darwin.uvigo.es/software/jmodeltest.html) was applied (excluding the option for 'proportion of invariable sites' to avoid over-parametrisation), and the AIC criterion was used to determine the best-fitting model (that was consistently the GTR+Γ as also implemented in RAxML, our preference software program for phylogenetic analyses).

We estimated whether a compositional bias was present in our data sets using the chi-square test implemented in PAUP* v4.0b10 (Swofford 2002). Since sequence homogeneity was rejected for both taxon samples ($p \leq 0.05$), we transferred the alignments to RY-coded data matrices (Phillips & Penny 2003). Subsequently, only transversions were considered in those phylogenetic analyses, and potential GC-biases were removed, decreasing the potential for systematic error. Reducing the level of heterogeneity was also done by removal of a fraction of fast evolving characters using the software program 'Gblocks' v0.91b (Castresana 2002; http://molevol.cmima.csic.es/castresana/Gblocks.html). Different block parameter settings were applied: All gap positions were excluded if no gaps were allowed and if at least half of the sequences had gaps, respectively. The minimum length of the nucleotide blocks after gap exclusion was set to five and ten, respectively. Otherwise, the default settings were used.

As described in the main text of the study, all phylogenetic analyses were performed using 'RAxML' VII (Stamatakis 2006; Stamatakis et al. 2008; http://www.phylo.org/portal/Home.do) and 'MrBayes' v3.1.2 (Huelsenbeck and Ronquist 2001; http://www.mrbayes.csit.fsu.edu/) as standard software programs. To explore more complex models than GTR+Γ, we applied PhyloBayes (Lartillot et al. 2009) using the 'qmm' option (i.e., without partition of the data). We run four independent chains of 2,500 cycles, compared the chains, checked for convergence, and used an appropriate burn-in (20%). All final data matrices and trees are available under doi:10.5061/dryad.d1vg6 or from MG upon request.

EXTENDED RESULTS AND DISCUSSION

Figure S1: *Bysmatrum* showed a distant relationship to the otherwise monophyletic Thoracosphaeraceae in the Alveolata data set. Maximum likelihood (ML) tree (–ln = 93,419) of 88 members of the Alveolata (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MUSCLE generated rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 3 (3,238 parsimony-informative positions). Clades with relevance for this study are indicated, and members of the Thoracosphaeraceae with known calcareous coccoid stages are highlighted by bold branches. Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (above: ML bootstrap support values, values under 50 are not shown; below: Bayesian posterior probabilities, values under .90 are not shown), and maximal support values are indicated by asterisks. The tree is rooted with the members of the Ciliata. Abbreviations: API1, API2, API3: different clades of Apicomplexa; CIL: Ciliata; DIN: Dinophyceae.

The trees derived from RY-coded sequences (Figs. S2–S3 as examples from the MUSCLE alignment) were largely congruent to those obtained in the analyses using sequences with four character states (Figs 1 in the main text, S1). The decrease of bootstrap support values for the monophyly of the Thoracosphaeraceae, and the slightly differing internal topologies, can be explained by a reduction of informative sites in the data sets (3,238→2,604 in the Alveolata alignment and 2,286→1,825 in the Dinophyceae alignment, both constructed by MUSCLE). Likewise, the systematic position of *Bysmatrum* remained basically unresolved as in the trees inferred from sequences with four character states. Thus, RY-coding of the rRNA sequences under investigation here does not lead to conflicting tree topologies, and the impact of sequence heterogeneity can be considered rather minor for the phylogenetic reconstructions.

The different alignment approaches yielded data matrices of greatly different lengths (Tab. S3). Particularly, the PRANK alignments were more than twice as long as those generated by MAFFT or MUSCLE. Moreover, different performances of PRANK rendered alignments of different lengths (i.e., the results were not reproducible), but this was not observed for MAFFT or MUSCLE. The amount of constant or uninformative positions, however, was more or less proportional to the lengths of the alignments, leaving a comparable total number of informative sites under the different approaches. All programs identified the principle organization of the rRNA operon, consisting of SSU, ITS1, 5.8S rRNA, ITS2, and LSU. The number of informative sites per terminal taxon was largely similar for the different partitions of the Alveolata alignments, with the SSU exhibiting the fewest (ranging from 9.58 through 10.05) and the ITSs and the LSU the most informative sites (ranging from 9.32 through 15.20 and 12.24 through 13.83, respectively). In the Dinophyceae alignments, such numbers were likewise similar, with the ITS region exhibiting the largest amounts of informative sites per terminal taxon (ranging from 6.90 through 10.06).

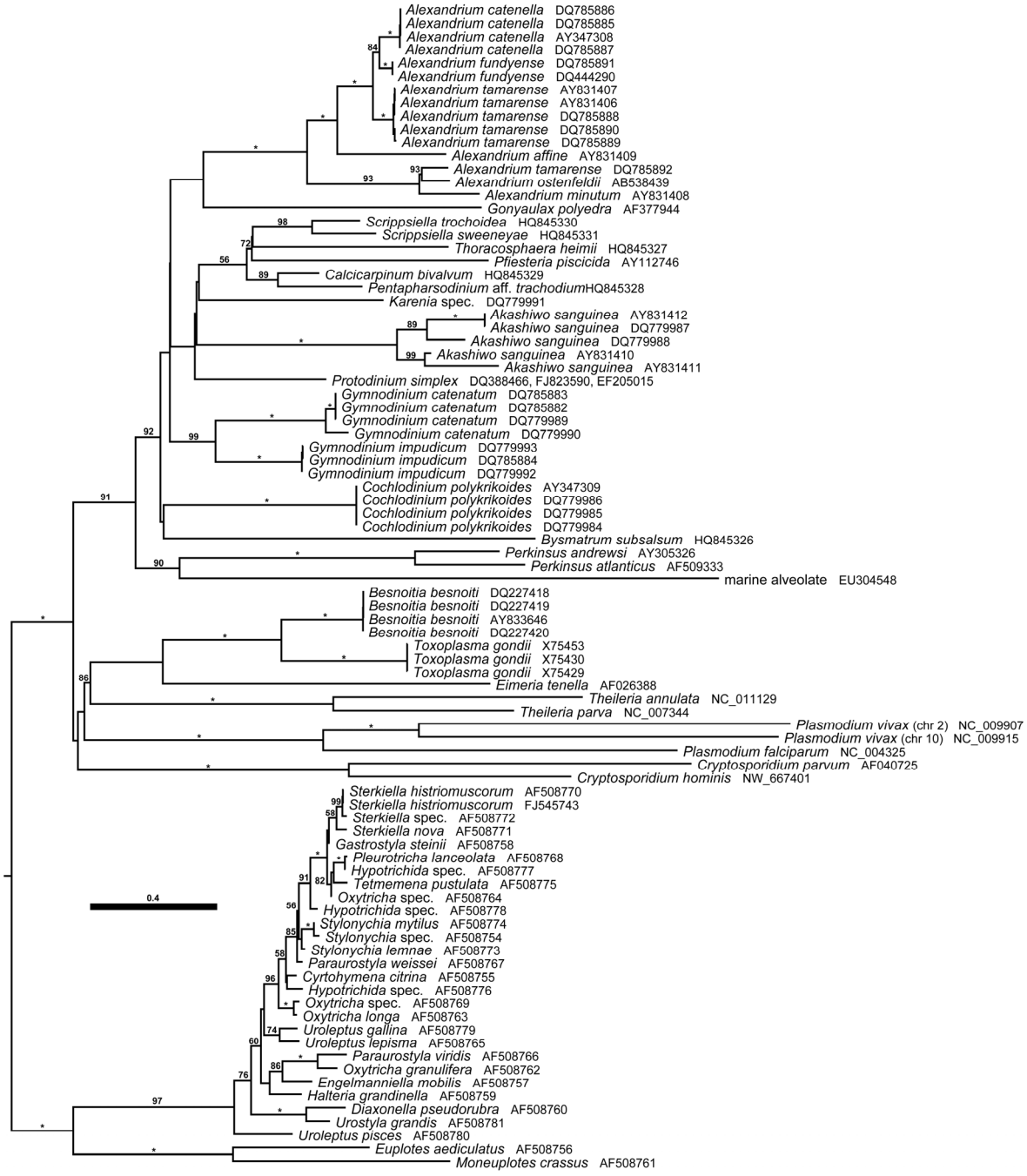

Figure S2: RY-coding did not affect tree topology with respect to the Thoracospaheraceae derived from the Alveolata data set. RAxML tree (–ln = 41,952) of 88 members of the Alveolata (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MUSCLE generated and RY-coded rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 3 (2,604 parsimony-informative positions). Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (values under 50 are not shown), and maximal support values are indicated by asterisks. Note that the Thoracosphaeraceae were monophyletic (although with lower support as in Fig. 2 in the main text: 90LBS→56LBS), and the systematic position of *Bysmatrum* remained unresolved (here: close to *Cochlodinium*). Note, furthermore, that *Cryptosporidium* is closely related to the apicomplexan main clade, which is more plausible than the topology shown in Figure S1.

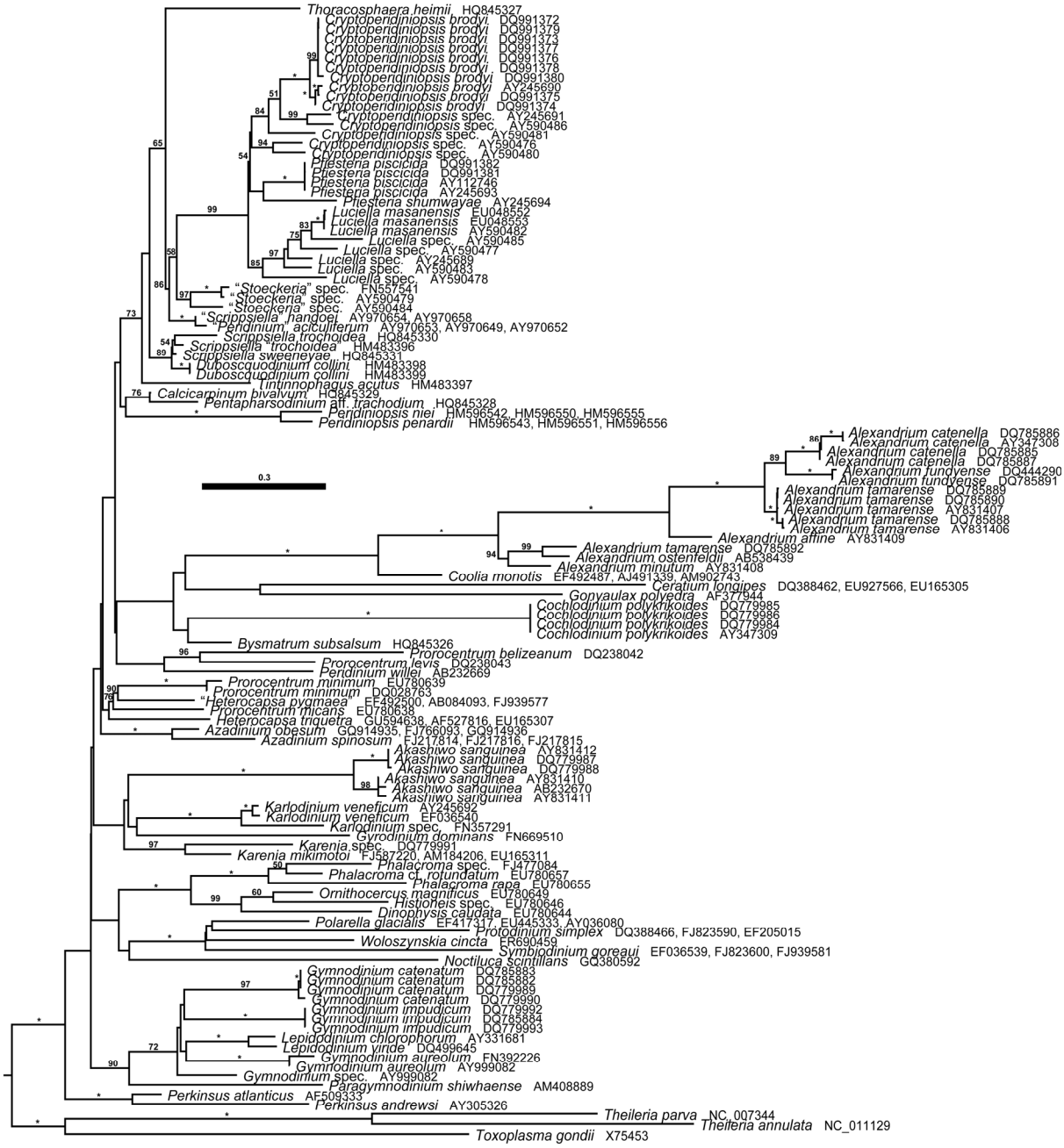

Figure S3: RY-coding did not affect tree topology with respect to the Thoracospaheraceae derived from the Dinophyceae data set. RAxML tree (–ln = 37,827) of 113 members of the Dinophyceae (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MUSCLE generated and RY-coded rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 2 (1,825 parsimony-informative positions). Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (values under 50 are not shown), and maximal support values are indicated by asterisks. Note that the Thoracosphaeraceae were monophyletic (although with lower support as in Fig. 2 in the main text: 69LBS→<50LBS), and the systematic position of *Bysmatrum* remained unresolved (here: close to *Cochlodinium*). The close relationship, however, of *Scrippsiella s.l.* with the T/Pf-clade was retrieved moderately supported (73LBS), as in Figure 2 in the main text.

Tree topologies derived from the Alveolata and the Dinophyceae data matrices were largely similar, independently of the alignment approach used and whether the Bayesian or the ML algorithm was applied. The Thoracosphaeraceae (as retrieved in Figs 1 in the main text and S1) were monophyletic in the majority of the analyses, partly with high bootstrap support. If the Thoracosphaeraceae were not monophyletic (as inferred from, e.g., the MAFFT Alveolata and PRANK Dinophyceae alignments), they were collapsed and did not show highly supported alternative relationships as shown in Figures 1 in the main text and S1 (as examples derived from the MUSCLE alignments). Missing retrieval of the Thoracosphaeraceae always referred to the E/Pe-clade with calcareous *Calcicarpinum bivalvum*, while the close relationship between *Scrippsiella s.l.* and the T/Pf-clade was supported in all analyses (LBS<90, BPP<.95), independently of the alignment approach used and whether the Bayesian or the ML algorithm was applied.

Applying the software program Gblocks to the data matrices (to reduce the number of phylogenetically ambiguous alignment positions) did not lead to either better resolved trees in general or to better supported nodes in particular. In the contrary, Bayesian posterior probabilities as well as ML bootstrap support were frequently reduced in comparison to trees using the complete rRNA sequences (compare Figs S4–S5 to Figs 1 in the main text and S1). The decrease in support values can be explained by the decrease of informative sites in the alignments (Tab. S4), indicating that the phylogenetic information implemented in rRNA sequences is far from being saturated. The Thoracosphaeraceae (as retrieved in Figs 1 in the main text and S1), however, were monophyletic in the majority of the analyses, partly with high bootstrap support. If the Thoracosphaeraceae were not monophyletic (as inferred from, e.g., the MAFFT alignments), they were collapsed and did not show highly supported alternative relationships as shown in Figures S4–S5 (as examples derived from the MAFFT-QINSI alignments). Missing retrieval of the Thoracosphaeraceae always referred to the E/Pe-clade with calcareous *Calcicarpinum bivalvum*, while the close relationship between *Scrippsiella s.l.* and the T/Pf-clade was supported in all analyses (LBS<90, BPP<.95), independently of the alignment approach used and whether the Bayesian or the ML algorithm was applied.

Figure S4: Neither an alternative alignment approach nor Gblocks processing did affect tree topology with respect to the Thoracospaheraceae derived from the Alveolata data set.

RAxML tree (–ln = 59,499) of 88 members of the Alveolata (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MAFFT-QINSI generated and Gblocks processed rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 3 (2,101 parsimony-informative positions; see Tab. S4 for details). Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (values under 50 are not shown), and maximal support values are indicated by asterisks. Note that the Thoracosphaeraceae were monophyletic (88LBS), and the systematic position of *Bysmatrum* remained unresolved (here: close to the Gonyaulacales), as it is also shown in Figure S1.

Figure S5: Gblocks processing decreased the resolution of the trees derived from the Dinophyceae data set. RAxML tree (–ln = 61,095) of 113 members of the Dinophyceae (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MAFFT-QINSI generated and Gblocks processed rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 2 (1,608 parsimony-informative positions; see Tab. S4 for details). Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (values under 50 are not shown), and maximal support values are indicated by asterisks. Note that the Thoracosphaeraceae were collapsed (and include *Peridinium willei* on a long branch), and the systematic position of *Bysmatrum* remained likewise unresolved. The close relationship, however, of *Scrippsiella s.l.* with the T/Pf-clade was highly supported (96LBS), as in Figure 2 in the main text.

Tests of the different alignments always yielded GTR+Γ as the best-fitting model for the data sets (in combination as well as for each of the three partitions separately). To explore a more complex model and the effect of using a single partition, we finally calculated trees using PhyloBayes. The corresponding topologies did basically not differ from those derived in the previous analyses. The Thoracosphaeraceae (as retrieved in Figs 1 in the main text and S1), however, were not fully resolved, and they constituted a moderately supported phylogenetic group together with *Bysmatrum* and *Peridinium willei* using the MAFFT-QINSI, Gblocks processed alignment of the Dinophyceae (Fig. S6). The phylogenetic position of *Bysmatrum* appears particularly implausible and might be a result rather of a long-branch attraction. The close relationship between *Scrippsiella s.l.* and the T/Pf-clade, however, was again highly supported (1.00BPP).

In summary, we have explored the possible negative effects of heterogeneous rRNA sequences (particularly of the ITS region) for our phylogenetic reconstructions by RY-coding, excluding phylogenetically ambiguous positions, using different alignment programs, and applying an infinite mixture model to the data. The resulting trees are not entirely congruent, but they do not lead to conflicting and highly supported tree topologies. We conclude that our molecular trees (Figs 1 in the main text and S1) are thus reliable results of our phylogenetic analyses. The Thoracosphaeraceae are probably a monophyletic group comprising calcareous (such as *Scrippsiella* and *Thoracosphaera*) as well as non-calcareous (such as *Ensiculifera* and *Pfiesteria*) dinophytes. In future, it is highly desirable to include more genetic data for phylogenetic reconstructions, as the present rRNA sequences do not provide fully resolved trees. As long as no more genetic data are available for the reconstruction of dinophyte evolution, however, we should make use of as much sequence data as possible by, for example, compiling the existing rRNA sequences in cases where clonal cultures have been investigated.

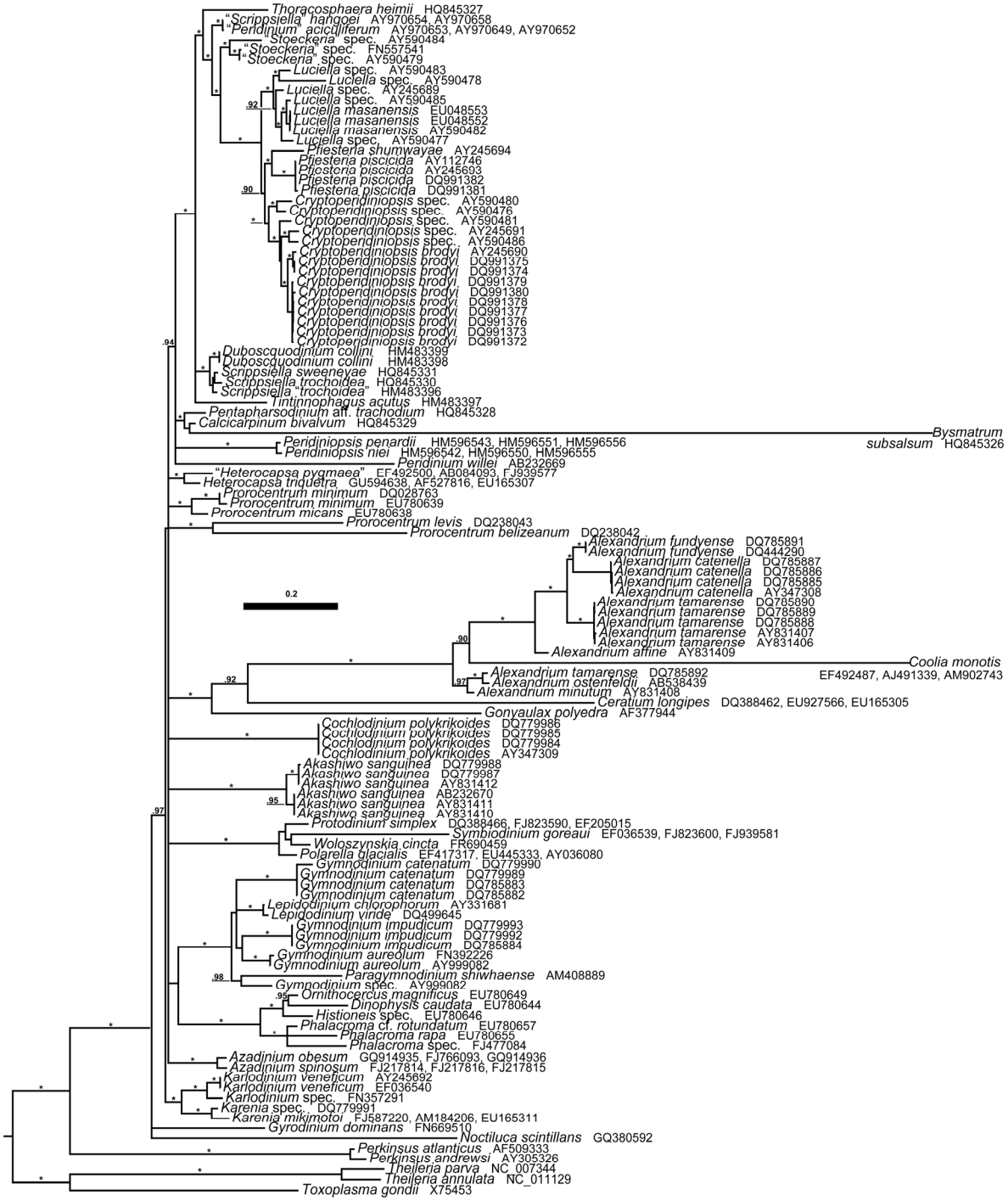

Figure S6: The application of an infinite mixture model did not lead to a better resolved tree derived from the Dinophyceae data set. PhyloBayes tree (–ln = 54,145) of 113 members of the Dinophyceae (including five new sequences of the Thoracosphaeraceae plus *Bysmatrum*) as inferred from a MAFFT-QINSI generated and Gblocks processed rRNA nucleotide alignment spanning the complete SSU, ITS region, and LSU domains 1 through 2 (1,608 parsimony-informative positions; see Tab. S4 for details). Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (values under .90 are not shown), and maximal support values are indicated by asterisks. Note that the Thoracosphaeraceae were collapsed (and include *Bysmatrum* on a long branch), but the close relationship of *Scrippsiella s.l.* with the T/Pf-clade was highly supported (1.00BPP), as in Figure 2 in the main text.

TABLES

Table S1: Voucher list. Abbreviations: API1, API2, API3: different clades of Apicomplexa; CIL: Ciliata; DIN: Dinophysiales; DPH: Dinophyceae of uncertain systematic placement; GON: Gonyaulacales; GYM1, GYM2: different clades of "Gymnodiniales"; n.ind.: not indicated; PER: "Peridiniales" other than Thoracosphaeraceae; PRO1, PRO2: different clades of Prorocentrales; SUE: Suessiales; THO: Thoracosphaeraceae.

Taxonomy	Species name with author	Strain No.	Locality	GenBankNo(s)
CIL	*Cyrtohymena citrina* (Helm.Berger & W.Foissner, 1987)	n.ind.	USA–CO: Aspen, Maroon Creek	AF508755
CIL	*Diaxonella pseudorubra* (Kaltenbach, 1960)	n.ind.	USA–CO: Boulder County, pond on University of Colorado campus	AF508760
CIL	*Engelmanniella mobilis* (Engelm., 1862)	n.ind.	n.ind.	AF508757
CIL	*Euplotes aediculatus* Pierson, 1943	n.ind.	USA–CO: Boulder County, Teller Lake	AF508756
CIL	*Gastrostyla steinii* Engelm., 1862	n.ind.	n.ind.	AF508758
CIL	*Halteria grandinella* (O.F.Müll., 1773)	n.ind.	USA–CO: Boulder County, pond on University of Colorado campus	AF508759
CIL	*Hypotrichida* spec.	Florida	USA–FL: Sarasota, Misty Creek Pond	AF508778
CIL	*Hypotrichida* spec.	KEC2002	USA–CO: Roaring Fork River	AF508776
CIL	*Hypotrichida* spec.	OrrK1999	USA–CO: Aspen, Ten Mile Creek	AF508777
CIL	*Moneuplotes crassus* (Dujard., 1841)	n.ind.	n.ind.	AF508761
CIL	*Oxytricha granulifera* W.Foissner & H.Adam, 1983	n.ind.	USA–CO: Aspen, Roaring Fork River	AF508762

CIL	*Oxytricha longa* Gelei & M.Szabados, 1950	n.ind.	USA–CO: Aspen, Ten Mile Creek	AF508763
CIL	*Oxytricha* spec.	Steamboat Hot Springs	USA–CO: Steamboat Springs	AF508769
CIL	*Oxytricha* spec.	Misty	USA–FL: Sarasota, Misty Creek Pond	AF508764
CIL	*Paraurostyla viridis* (F.Stein, 1859)	n.ind.	USA–FL: Sarasota, Misty Creek Pond	AF508766
CIL	*Paraurostyla weissei* (F.Stein, 1859)	n.ind.	USA–CO: Boulder County, Teller Lake	AF508767
CIL	*Pleurotricha lanceolata* (Ehrenb., 1838)	n.ind.	USA–CO: Aspen, Ten Mile Creek	AF508768
CIL	*Sterkiella histriomuscorum* (W.Foissner, Blatterer, Helm.Berger and Kohmann, 1991)	n.ind.	USA–IN: Bloomington, Jordan River	AF508770
CIL	*Sterkiella histriomuscorum* (W.Foissner, Blatterer, Helm.Berger & Kohmann, 1991)	n.ind.	n.ind.	FJ545743
CIL	*Sterkiella nova* (W.Foissner & Helm.Berger, 1999)	n.ind.	USA–NC: Burlington	AF508771
CIL	*Sterkiella* spec.	n.ind.	USA–CO: Aspen, Roaring Fork River	AF508772
CIL	*Stylonychia lemnae* Ammermann & Schlegel, 1983	n.ind.	USA–CO: Boulder County, Teller Lake	AF508773
CIL	*Stylonychia mytilus* (O.F.Müll., 1773)	n.ind.	China: Harbin	AF508774
CIL	*Stylonychia* spec.	n.ind.	USA–CO: Aspen, Ten Mile Creek	AF508754
CIL	*Tetmemena pustulata* (O.F.Müll., 1786)	n.ind.	USA–CO: Boulder County, pond on University of Colorado campus	AF508775
CIL	*Uroleptus lepisma* (Wenzel, 1953)	n.ind.	USA–CO: Aspen, Ten Mile Creek	AF508765
CIL	*Uroleptus pisces* (O.F.Müll., 1773)	n.ind.	USA–CO: Boulder County, Teller Lake	AF508780

CIL	*Uroleptus gallina* (O.F.Müll., 1786)	n.ind.	USA–CO: Boulder County, Teller Lake	AF508779
CIL	*Urostyla grandis* Ehrenb., 1830	n.ind.	USA–CO: Boulder County, pond on University of Colorado campus	AF508781
API1	*Besnoitia besnoiti* (Marotel, 1912) (isolated from *Bos taurus* Linnaeus, 1758)	n.ind.	Portugal	AY833646
API1	*Besnoitia besnoiti* (Marotel, 1912) (isolated from *Bos taurus* Linnaeus, 1758)	n.ind.	Spain: Burgos	DQ227418
API1	*Besnoitia besnoiti* (Marotel, 1912) (isolated from *Bos taurus* Linnaeus, 1758)	n.ind.	Spain: Catalonia	DQ227419
API1	*Besnoitia besnoiti* (Marotel, 1912) (isolated from *Bos taurus* Linnaeus, 1758)	n.ind.	Israel	DQ227420
API1	*Eimeria tenella* Railliet & Lucy, 1891 [isolated from *Gallus gallus* (Linnaeus, 1758)]	Houghton	n.ind.	AF026388
API1	*Plasmodium falciparum* W.Welch, 1897 (isolated from *Homo sapiens* Linnaeus, 1758)	3D7	The Netherlands: Amsterdam, Schipol Airport	NC_004325 (AL844501)
API1	*Plasmodium vivax* Grassi & Feletti, 1890 (chromosome 2) (isolated from *Homo sapiens* Linnaeus, 1758)	Salvador I	El Salvador	NC_009907 (CM000443)
API1	*Plasmodium vivax* Grassi & Feletti, 1890 (chromosome 10) (isolated from *Homo sapiens* Linnaeus, 1758)	Salvador I	El Salvador	NC_009915 (CM000451)
API1	*Theileria annulata* (Dschunkowsky & Luhs, 1904) (isolated from *Bos taurus* Linnaeus, 1758)	Ankara, clone C9	Turkey	NC_011129
API1	*Theileria parva* (Theiler, 1904) (isolated from *Bos taurus* Linnaeus, 1758)	Muguga	Kenya	NC_007344 (AAGK01000001)
API1	*Toxoplasma gondii* (Nicolle & Manceaux, 1908)	P	n.ind.	X75453
API1	*Toxoplasma gondii* (Nicolle & Manceaux, 1908)	RH	n.ind.	X75429

API1	*Toxoplasma gondii* (Nicolle & Manceaux, 1908)	Sailie	n.ind.	X75430
API2	*Cryptosporidium hominis* Morgan-Ryan, A.Fall, L.A.Ward, Hijjawi, I.Sulaiman, Fayer, R.C.Thomps., M.Olson, A.Lal & L.Xiao, 2002 (isolated from *Homo sapiens* Linnaeus, 1758)	TU502	Uganda	NW_667401
API2	*Cryptosporidium parvum* Tyzzer, 1912	KSU-1	n.ind.	AF040725
API3	*Perkinsus andrewsi* Coss, J.Robledo, G.Ruiz & Vasta, 2001 [isolated from *Macoma balthica* (Linnaeus, 1758)]	PAND-A8–4a, ATCC 50807	USA–MD: Rhode River	AY305326
API3	*Perkinsus atlanticus* C.Azevedo, 1989 (isolated from *Venerupis decussata* Linnaeus, 1758)	ALG1	Portugal	AF509333
API3	uncultured marine alveolate	FBB25	USA–MA: Boston, Blanes Bay Microbial Observatory	EU304548
DPH	*Azadinium obesum* Tillmann & Elbr.	2E10	off Scotland (UK), North Sea: east coast (57°04'N, 2°30'W)	GQ914935, FJ766093, GQ914936
DPH	*Azadinium spinosum* Elbr. & Tillmann	3D9	off Scotland (UK), North Sea: east coast (57°04'N, 2°30'W)	FJ217814, FJ217816, FJ217815
DPH	*Bysmatrum subsalsum* (Ostenf.) M.A.Faust & Steid.	KC32CCAUTH	off Greece, North Aegean Sea: Thessaloniki, Porto-Lagos (40°58'N, 25°07'E)	HQ845326
DPH	*Cochlodinium polykrikoides* Margalef	CcPk02	off South Korea: Tongyoung	DQ779984
DPH	*Cochlodinium polykrikoides* Margalef	CcPk03	off South Korea: Narodo	DQ779985
DPH	*Cochlodinium polykrikoides* Margalef	CcPk05	off South Korea: Hakdong	DQ779986
DPH	*Cochlodinium polykrikoides* Margalef	CcPk06	off South Korea	AY347309
DPH	*Gyrodinium dominans* Hulburt	GDMS0704YD	off South Korea: Masan	FN669510
DPH	*Noctiluca scintillans* (Macartney, 1810)	n.ind.	off Hong Kong: Clear Water Bay (22°20'N, 114°16'E)	GQ380592

GYM1	*Gymnodinium aureolum* (Hulburt) Gert.Hansen	GASMK0803	South Korea: Saemankeum	FN392226
GYM1	*Gymnodinium aureolum* (Hulburt) Gert.Hansen	SWA 16	Benguela Current off Namibia	AY999082
GYM1	*Gymnodinium catenatum* H.W.Graham, 1943	CCMP414	Spain: Ria de Vigo	DQ779990
GYM1	*Gymnodinium catenatum* H.W.Graham, 1943	CCMP1940	Spain: Ria de Vigo	DQ785883
GYM1	*Gymnodinium catenatum* H.W.Graham, 1943	GCCW991	South Korea: Jindong	DQ779989
GYM1	*Gymnodinium catenatum* H.W.Graham, 1943	GnCt01	South Korea: Nanpo, Jinhae Bay	DQ785882
GYM1	*Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert.Hansen & Moestrup	CCMP1678	Australia: East Victoria, Gippsland Lakes (38°00'S, 147°00'E)	DQ785884
GYM1	*Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert.Hansen & Moestrup	Gi-1cp	South Korea: Yosu	DQ779992
GYM1	*Gymnodinium impudicum* (S.Fraga & I.Bravo) Gert.Hansen & Moestrup	GrIp02	South Korea: Hase	DQ779993
GYM1	*Gymnodinium* spec.	n.ind.	South Korea: Shiwha Bay	FR720082
GYM1	*Lepidodinium chlorophorum* (Elbr. & Schnepf) Gert.Hansen, Botes & Salas	DIN3	France	AY331681
GYM1	*Lepidodinium viride* M.Watan., S.Suda, I.Inouye, Sawaguchi & Chihara	n.ind.	South Africa	DQ499645
GYM1	*Paragymnodinium shiwhaense* N.S.Kang, H.J.Jeong, Moestrup & W.Shin	Jeong2006-1	South Korea: Shiwha (37°18'N, 126°36'E)	AM408889
GYM2	*Karenia mikimotoi* (Miyake & Komin. ex M.Oda) Gert.Hansen & Moestrup	CCMP429	UK: England, Devon, Plymouth, Sutton Harbour (50°22'N, 4°10'W)	FJ587220, AM184206, EU165311
GYM2	*Karenia* spec. [as *Gymnodinium aureolum* (Hulburt) Gert.Hansen]	GrAr01	South Korea: Chilchondo	DQ779991
GYM2	*Karlodinium* spec.	KAMS0708	South Korea: Masan Bay	FN357291
GYM2	*Karlodinium veneficum* (D.Ballant.) J.Larsen	CCMP1975	USA–MD: Princess Anne, Hyrock Farms (38°10'N, 75°44'W)	EF036540
GYM2	*Karlodinium veneficum* (D.Ballant.) J.Larsen	Pim05JulC4	USA–FL: St. John's River	AY245692

SUE	*Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup	AY001	off Japan: Hokkaido	AB232670
SUE	*Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup	CCMP1321	off USA–NY: Great South Bay	AY831412
SUE	*Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup	CCMP1593	off USA–RI: Narragansett Bay (41°36'N, 71°24'W)	DQ779987
SUE	*Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup	CCMP1837	UK, the Bermudas: Harington Sound off Rabbitt Island (32°20'N, 64°44'W)	DQ779988
SUE	*Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup	GnSg02	off South Korea: Jangmok	AY831410
SUE	*Akashiwo sanguinea* (K.Hirasaka) Gert.Hansen & Moestrup	GnSg03	off South Korea: Jangmok	AY831411
SUE	*Polarella glacialis* Montresor, Procaccini & Stoecker	CCMP1383	Ross Sea, off Antarctica: McMurdo Sound (77°50'S, 163°00'E)	EF417317, EU445333, AY036080
SUE	*Protodinium simplex* Lohmann	CCMP419	Costa Rica Dome (9°48'N, 89°15'W)	DQ388466, FJ823590, EF205015
SUE	*Symbiodinium goreaui* Trench & R.J.Blank [isolated from *Discosoma sanctithomae* (Duchass. & Michelotti, 1860)]	CCMP2466	off Jamaica (18°00'N, 77°00'W)	EF036539, FJ823600, FJ939581
SUE	*Woloszynskia cincta* Siano, Montresor & Zingone	Nam Seon Kang	South Korea: Shiwha Bay	FR690459
DIN	*Dinophysis caudata* Kent	FTL69	USA–FL: gulf stream off Ft. Lauderdale (26°05'N, 80°03'W)	EU780644
DIN	*Histioneis* spec.	FTL62	USA–FL: gulf stream off Ft. Lauderdale (26°05'N, 80°03'W)	EU780646

DIN	*Ornithocercus magnificus* F.Stein, 1883	CBC4L7	USA–VA: shelf break off lower Chesapeake Bay (36°10'N, 74°20'W)	EU780649
DIN	*Phalacroma rapa* E.Jørgensen	CBC4L5	USA–VA: shelf break off lower Chesapeake Bay (36°10'N, 74°20'W)	EU780655
DIN	*Phalacroma* cf. *rotundatum* (Clap. & J.Lachm., 1859)	FTL121	USA–FL: gulf stream off Ft. Lauderdale (26°05'N, 80°03'W)	EU780657
DIN	*Phalacroma* spec.	CBC4L128	USA–VA: shelf break off lower Chesapeake Bay (36°10'N, 74°20'W)	FJ477084
PRO1	"*Heterocapsa pygmaea*" A.R.Loebl., R.J.Schmidt & Sherley	CCMP1322	off USA–TX: Galveston Channel (29°23'N, 94°53'W)	EF492500, AB084093, FJ939577
PRO1	*Prorocentrum micans* Ehrenb.	CCMP1589	USA–RI: Narragansett Bay (41°36'N, 71°24'W)	EU780638
PRO1	*Prorocentrum minimum* (Pavill.) J.Schiller	PMDH01	East China Sea	DQ028763
PRO1	*Prorocentrum minimum* (Pavill.) J.Schiller	SERC	n.ind.	EU780639
PRO2	*Prorocentrum belizeanum* M.A.Faust	n.ind.	off Belize: Carrie Bow Cay (16°48'N, 88°05'W)	DQ238042
PRO2	*Prorocentrum levis* M.A.Faust, Kibler, Vandersea, P.A.Tester & Litaker	n.ind.	off Belize: Twin Cays (16º50'N, 88º06'W)	DQ238043
PER	*Heterocapsa triquetra* (Ehrenb., 1840)	CCMP448	USA–MA: Falmouth, Perch Pond (41°32'N, 70°37'W)	GU594638, AF527816, EU165307
PER	*Peridiniosis niei* G.X.Liu & Z.Y.Hu	Donghu	China	HM596542, HM596550, HM596555

PER	*Peridiniopsis penardii* (Lemmerm.) Bourr.	Jiulongjiang	China	HM596543, HM596551, HM596556
PER	*Peridinium willei* Huitf.-Kaas	TK007	off Japan: Hokkaido	AB232669
THO	*Calcicarpinum bivalvum* G.Versteegh [= "*Pentapharsodinium*" *tyrrhenicum* (Balech) Montresor, Zingone & D.Marino]	GeoB 229	off Italy: Gulf of Taranto	HQ845329
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE1	Australia: Tasmania, Derwent River, Sullivans Cove	DQ991372
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE2	Australia: Tasmania, Derwent River, Sullivans Cove	DQ991373
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE10	Australia: Tasmania, Derwent River, Sandy Bay	DQ991374
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBDE14	Australia: Tasmania, Derwent River, Sandy Bay	DQ991375
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBHU1	Australia: Tasmania, Huon River	DQ991376
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBHU2	Australia: Tasmania, Huon River	DQ991377
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBSA4	off Australia: South Australia, Port Lincoln	DQ991378
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBWA11	Australia: Western Australia, Brunswick River	DQ991379
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	CBWA12	Australia: Western Australia, Brunswick River	DQ991380
THO	*Cryptoperidiniopsis brodyi* Steid., Landsberg, P.L.Mason, Vogelbein, P.A.Tester & Litaker	H/V14; VIMS V14	USA–NC: Neuse River; USA–VA: Great Wicomico River	AY245690

THO	*Cryptoperidiniopsis* spec.	CCMP1828	USA–MD: King's Creek (38°24'N, 75°51'W)	AY590476
THO	*Cryptoperidiniopsis* spec.	F525Jul02	USA–FL: St. John's River	AY590480
THO	*Cryptoperidiniopsis* spec.	Folly C5	USA–SC: Folly Beach	AY590481
THO	*Cryptoperidiniopsis* spec.	NOAA Beach	USA–NC: Piver's Island	AY590486
THO	*Cryptoperidiniopsis* spec. (isolated from gut of mullet)	PLO21	USA–FL: Bird Island Archipelago, St. Lucie River	AY245691
THO	*Duboscquodinium collini* Grassé, 1952 [isolated from *Eutintinnus fraknoii* (Daday, 1887)]	VSM11	off France: Bay of Villefranche-sur-Mer	HM483399
THO	*Duboscquodinium collini* Grassé, 1952 [isolated from *Eutintinnus fraknoii* (Daday, 1887)]	VSM12	off France: Bay of Villefranche-sur-Mer	HM483398
THO	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	HR1NovC5	USA–FL: St. Lucie River	AY590482
THO	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	VIMS 1041	USA–VA: James River	EU048552
THO	*Luciella masanensis* P.L.Mason, H.J.Jeong, Litaker, Reece & Steid.	VIMS 1050	USA–VA: James River	EU048553
THO	*Luciella* spec.	CCMP1835	USA–VA: Pokomoke Sound, station DHA (37°58'N, 75°42'W)	AY590477
THO	*Luciella* spec.	CCMP1838	USA–NC: Neuse River	AY590478
THO	*Luciella* spec.	Florida Lucy	USA–FL: Indian River System	AY245689
THO	*Luciella* spec.	HR1SSeptA5	USA–FL: St. Lucie River	AY590483
THO	*Luciella* spec.	NC Lucy-V27	USA–NC: New River	AY590485
THO	*Pentapharsodinium* aff. *trachodium* Indel. & A.R.Loebl. (≡ *Ensiculifera* aff. *loeblichii* El.R.Cox & H.J.Arn.)	GeoB*220	off Senegal	HQ845328

THO	"*Peridinium*" *aciculiferum* Lemmerm.	PAER-1	Sweden: Lake Erken	AY970653, AY970649, AY970652
THO	*Pfiesteria piscicida* Steid. & J.M.Burkh.	n.ind.	USA–MD: Chicamacomico River	AY112746
THO	*Pfiesteria piscicida* Steid. & J.M.Burkh.	Noga-P	USA–NC: Pamlico River	AY245693
THO	*Pfiesteria piscicida* Steid. & J.M.Burkh.	PPSB25	Indonesia: Surabaya (ballast water)	DQ991381
THO	*Pfiesteria piscicida* Steid. & J.M.Burkh.	PPSB27	Indonesia: Surabaya (ballast water)	DQ991382
THO	*Pfiesteria shumwayae* Glasgow & J.M.Burkh.	Noga-S; VIMS 1049	USA–NC: Pamlico River	AY245694
THO	"*Scrippsiella*" *hangoei* (J.Schiller) J.Larsen	SHTV1	off Finland (Baltic Sea): Tvärminne	AY970654, AY970658
THO	*Scrippsiella sweeneyae* Balech ex A.R.Loebl.	CCCM 280	n.ind.	HQ845331
THO	*Scrippsiella* "*trochoidea*" (F.Stein) A.R.Loebl.	CCMP 2271	USA–ND: Jim Lake	HM483396
THO	*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.	GeoB 283	off Norway: Sør-Trøndelag, Snillfjord commune, Åstfjorden, Mjønos (harbour)	HQ845330
THO	"*Stoeckeria*" spec., name not validly published (ICBN Art. 36.2)	Shepherd's Crook	USA–FL: Trout River	AY590479
THO	"*Stoeckeria*" spec., name not validly published (ICBN Art. 36.2)	Shepherd's Crook	USA–FL: St. Lucie River	AY590484
THO	"*Stoeckeria*" spec., name not validly published (ICBN Art. 36.2)	SSMS0806	South Korea: Masan	FN557541
THO	*Thoracosphaera heimii* (Lohmann) Kamptner	CCCM670	Gulf of Mexico	HQ845327
THO	*Tintinnophagus acutus* Coats, 2010 isolated from *Tintinnopsis cylindrica* Daday, 1887)	–	USA–MD: Chesapeake Bay	HM483397
GON	*Alexandrium affine* (H.Inoue & Fukuyo, 1985)	CCMP112	Spain: Ria de Vigo (42°14'N, 8°48'W)	AY831409
GON	*Alexandrium catenella* (Whedon & Kof., 1936)	AxCt_K01	South Korea	AY347308
GON	*Alexandrium catenella* (Whedon & Kof., 1936)	Axsp-K01	South Korea: Nanpo, Jinhae Bay	DQ785885

GON	*Alexandrium catenella* (Whedon & Kof., 1936)	Axsp-K03	South Korea: Jangmok	DQ785886
GON	*Alexandrium catenella* (Whedon & Kof., 1936)	Axsp-K05	off southern South Korea	DQ785887
GON	*Alexandrium fundyense* Balech, 1985	CCMP1719	USA–NH: Portsmouth (43°06'N, 70°47'W)	DQ444290
GON	*Alexandrium fundyense* Balech, 1985	CCAP 1119/9	Ireland: Belfast Lough	DQ785891
GON	*Alexandrium minutum* Halim	CCMP113	Spain: Ria de Vigo (42°14'N, 8°48'W)	AY831408
GON	*Alexandrium ostenfeldii* (Paulsen) Balech & Tangen	AOFUN0801	Japan: Hokkaido, Funka Bay	AB538439
GON	*Alexandrium tamarense* (M.Lebour, 1925)	AX-03	Japan	DQ785890
GON	*Alexandrium tamarense* (M.Lebour, 1925)	CCAP 1119/5	USA–MA: Gloucester, Ipswich Bay	AY831407
GON	*Alexandrium tamarense* (M.Lebour, 1925)	CCMP1771	UK: England, Plymouth, Tamar Estuary (50°22'N, 4°09'W)	DQ785892
GON	*Alexandrium tamarense* (M.Lebour, 1925)	HY970328M	South Korea: Masan Bay	AY831406
GON	*Alexandrium tamarense* (M.Lebour, 1925)	HY97403M	South Korea: Masan Bay	DQ785888
GON	*Alexandrium tamarense* (M.Lebour, 1925)	HY981028M	South Korea: Masan Bay	DQ785889
GON	*Ceratium longipes* (Bailey) Gran	CCMP1770	USA–ME: West Boothbay Harbor, Bigelow Laboratory dock (43°51'N, 69°38'W)	DQ388462, EU927566, EU165305
GON	*Coolia monotis* Meunier	CCMP1345	Caribbean Sea off USA–FL: Knight Key (24°42'N, 81°06'W)	EF492487, AJ491339, AM902743
GON	*Gonyaulax polyedra* F.Stein, 1883	n.ind.	n.ind.	AF377944