Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften

an der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

# Diversity and morphology of calcareous dinophytes (Thoracosphaeraceae, Peridiniales)

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München, Februar 2013

## Erklärung

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

## Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

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Dissertation eingereicht am: 11.02.2013
Mündliche Prüfung am: 26.04.2013

## Note

This doctoral research project has been carried out under the supervision of PD Dr. Marc Gottschling at the Ludwig-Maximilians-University Munich from February 2009 until now. The project was funded by Deutsche Forschungsgemeinschaft (DFG) in cooperation with Prof. Dr. Helmut Keupp, geological science; department paleontology at the FU Berlin and Prof. Dr. Helmut Willems, historical geology and palaontology at the University of Bremen.

This thesis is based on and includes the following articles / manuscripts:

**Chapter 1**

**Who am I – and if so, how many? Species diversity of calcareous dinophytes (Thoracosphaeraceae, Dinophyceae) in the Mediterranean Sea.**

Soehner S., **C. Zinssmeister**, M. Kirsch, M. Gottschling.

Org Divers Evol **12**(4): 339-348

Own contribution: Field work (40%); strain isolation and cultivation (50%); morphological analysis (incl. images: 100%); manuscript preparation (20%).

**Chapter 2**

**Same but different: Two novel bicarinate species of extant calcareous dinophytes (Thoracosphaeraceae, Dinophyceae) from the Mediterranean Sea.**

**Zinssmeister, C.**, S. Soehner, M. Kirsch, E. Facher, K. J. S. Meier, H. Keupp, M. Gottschling

J Phycol. **48**(5): 1107-1118

Own contribution: Field work (40%); cultivation (50%); species descriptions and images (90%); manuscript preparation (30%).

**Chapter 3**

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

**Zinssmeister, C.**, S. Soehner, E. Facher, M. Kirsch, K. J. S. Meier, M. Gottschling (2011).

Syst Biodivers **9**(2): 145-157.

Own contribution: Field work (40%); strain isolation and cultivation (60%); morphological analysis (incl. images: 90%); manuscript preparation (50%).

**Chapter 4**

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

Gottschling, M., S. Soehner, **C. Zinssmeister**, U. John, J. Plötner, M. Schweikert, K. Aligizaki, M. Elbrächter (2012).

Protist **163**(1): 15-24.

Own contribution: cultivation (25%); morphological analysis and images (80%); manuscript preparation – morphological part (80%).

**Chapter 5**

**Ultrastructure of calcareous dinophytes (Thoracosphaeraceae, Peridiniales) with a focus on vacuolar crystal-like particles.**

**Zinssmeister, C.**, H. Keupp, G.Tischendorf, F. Kaulbars, M. Gottschling (2013).

PlosONE **8**(1): e54038.

Own contribution: cultivation (50%); morphological, anatomical analysis and images (80%); manuscript preparation (40%).

## Summary

Dinophytes are unicellular eukaryotic algae that, together with their closest relatives, ciliates and apicomplexans, belong to the superphylum of alveolates. Some of them, namely calcareous dinophytes (Thoracosphaeraceae, Peridiniales), develop an immotile calcareous cell during their life history. They accumulate in the oceans' sediments analogously to terrestrial seed banks. Although the diversity of calcareous dinophytes was investigated in several studies, only a few of them provide data from coastal waters and sediments. The main goal of this thesis was to record the diversity of calcareous dinophytes from marine environments using morphological, anatomical, taxonomical and evolutionary approaches. An essential part of the project was to establish living dinophyte cultures, assuring constant access to fresh material for morphological and molecular analysis (**chapters 1-5**). The morphological diversity of extant species as well as those described from the fossil record was documented (**chapter 1**), and the conflict between molecular and the morphological data was presented (**chapter 1-5**). Furthermore, detailed morphological descriptions of two new species, *Scrippsiella bicarinata* und *S. kirschiae* (**chapter 2**) and morphological analysis of *Bysmatrum sp.*, a species of doubtful phylogenetic position (**chapter 4**), were provided. *Scrippsiella trochoidea* (basionym: *Glenodinium trochoideum*), a species with a previously ambiguous description, has been redescribed and epitypified by myself based on material collected from the type locality to assure a reliable determination of this species (**Chapter 3**). Comparative ultrastructure investigations using light and electron microscopic techniques at various stages of the life cycle showed that the anatomical structure during the biomineralization processes differs within subgroups of calcareous dinophytes (**chapter 5**) and could be used as a useful phylogenetic trait.

## Zusammenfassung

Dinophyten sind einzellige Eukaryoten und bilden zusammen mit ihren nächsten Verwandten, den Ciliaten und Apicomplexa, das Taxon der Alveolaten. Innerhalb der Dinophyten besitzen Vertreter der kalkigen Dinophyten (Thoracosphaeraceae) die Fähigkeit, Kalkstrukturen während ihres Lebenszyklus‘ auszubilden, die sich in marinen Sedimenten anreichern können. Obwohl sich mehrere Studien mit der Erfassung der Diversität bei kalkigen Dinophyten beschäftigen, waren küstennahe Bereiche bisher wenig erforscht.

Ziel dieser Arbeit ist die geographisch engmaschige Aufsammlung von Proben mithilfe eines Schwerelots, das dies in kürzester Zeit ermöglicht. Im Rahmen der vorliegenden Dissertation wurde die Diversität kalkiger Dinophyten morphologisch und anatomisch mithilfe von licht- und elektronenmikroskopischen Methoden untersucht. Die Grundlage hierfür bildete die Etablierung einer Lebendsammlung von Dinophyten, die den Zugriff auf frisches Material für morphologische und molekulare Analysemethoden gewährleistete (**Kapitel 1-5**). Hierdurch gelang es eine Vielzahl rezenter und auf fossilen Beschreibungen basierender Arten (**Kapitel 1**) kalkiger Dinophyten zu kultivieren, deren morphologische Vielfalt darzustellen und diese molekular-phylogenetischen Daten gegenüber zu stellen (**Kapitel 1-4**). Dies umfasste außerdem die detaillierte morphologische Beschreibung zweier neuer Arten, *Scrippsiella bicarinata* und *S. kirschiae,* (**Kapitel 2**), morphologische Analysen zur Klärung der systematisch problematischen Stellung von *Bysmatrum sp.* (**Kapitel 4**) und die Kultivierung und Untersuchung von kryptischen (morphologisch nicht abgrenzbaren) *Scrippsiella cf. trochoidea* Stämmen (**Kapitel 1, 3**). Aufgrund von unzureichend fixiertem, dokumentiertem oder nicht vorhandenem Typusmaterial stellte eine präzise Artidentifizierung mitunter eine Herausforderung dar. Durch Epitypisierung gelang es, den wissenschaftlichen Namen *Scrippsiella trochoidea* (Basionym: *Glenodinium trochoideum*) taxonomisch zu klären und in Zukunft eine verlässliche Bestimmung dieser Art zu ermöglichen. (**Kapitel 3**). Des Weiteren zeigten vergleichende Ergebnisse von ultrastrukturellen Untersuchungen während der Biomineralisation von kalkigen Dinophyten, dass sich die Kalzifizierungs-Prozesse innerhalb der Subgruppen unterscheiden (**Kapitel 5**) und teilweise als phylogenetisches Merkmal genutzt werden können.

## Content

# Introduction

## Biodiversity and taxonomic research

In these days of accelerated growth of human population and limited natural resources it is a priority to protect biological diversity and use it with responsibility (Wilson, 1992). “Biodiversity” or “biological diversity” is defined as the variability among organisms from all sources, the diversity of ecosystems, the diversity between species and the genetic diversity within species (Art. 2 of the Convention on Biological Diversity). For an understanding of biodiversity, it is necessary to inventory species, to investigate their phylogeny and function in their ecosystems and in an ecological global context.

Since the start of Linnaean taxonomy 250 years ago, approximately 1.9 million species have been named and cataloged (http://eol.org/info/about_biodiversity, April 2012) (Stuart et al., 2010). The number of living species on earth is uncertain and has been estimated from 3 to over 100 million (May, 2010, 2011; Wilson, 2004) (http://www.cbd.int, October 2012). Current studies have calculated the number of eukaryotic species to be 8.7 million, ± 1.3 million (May, 2011; Mora et al., 2011; Strain, 2011). This includes 2.2 millions of marine organisms, of which 91% are yet to be discovered and described. The number of algae has been estimated between 30,000 and over one million species (Guiry, 2012). Our knowledge about the majority of them is incomplete, and proper naming is a challenge due to synonym. For example, the statistics of “World Register of Marine Species” provide data containing 215,016 accepted species names in October 2012, of which 196,933 were nomenclaturally checked, plus 368,516 species names that were considered synonyms (Appeltans et al., 2012).

The first step to understanding species diversity is fieldwork. Depending on the taxon, it can be challenging to find and collect particular species, especially those that are difficult to access in remote habitats, such as polar regions (Grant et al., 2011), deep sea (Brandt et al., 2007a; Brandt et al., 2007b; Van Dover et al., 2002) or tropical rain forests (Kier et al., 2005). Moreover, many invertebrates or unicellular species are poorly documented compared to vertebrates or vascular plants. Several reasons are conceivable, for example homoplasy, a cryptic appearance and small to microscopic size, such as in bacteria, protozoa, and algae.

The further work of recording the diversity and describing new species is still a challenge for alpha-taxonomists, as the characterization of a species can be confusing based on different species concepts (de Queiroz, 2005; Guiry, 2012; Hey, 2006; Torretti, 2010). Organisms are classified within higher taxonomic levels and compared with already identified

species. The requisites to describe a species new to science are described in the International Code of Nomenclature for algae, fungi, and plants (ICN, formerly ICBN), or in the International Code for Zoological Nomenclature (ICZN) for animals. At the beginning of taxonomical research, descriptions of species were based on the morphology and anatomy alone and only later were linked to physical type material.

A new era in taxonomic research began in the 1990s, when molecular techniques such as DNA sequencing were introduced. These molecular approaches are helpful in identifying species using a short DNA sequence, which is called DNA barcoding. Moreover, they are so far the only possibility for detecting morphologically indistinguishable but genetically distinct populations, so called “cryptic species.” Other taxonomic problems arise when species names are not validly published, when type material is lost or absent, is inadequate for proper morphological identification, or insufficient for DNA extraction. In 1994, the ICBN introduced the tool of designating an epitype in such uncertain cases as described in Article 9.7 (McNeill et al., 2006).

Most unicellular species are poor in characteristic traits. Calcareous dinophytes develop at least two different stages during their life history, a motile thecate cell and a calcareous immotile coccoid cell, both of vital importance in the context of taxonomy. Misinterpretations of those two stages as being different species occurred quite often before their link in the dinophyte life cycle was recognized in cultivation experiments in the 1960s (Elbrächter et al., 2008). Therefore, enough material of both stages is needed for proper identification of a taxon using molecular and morphological approaches, including scanning electron microscopy (SEM). A viable alternative is to culturivate those organisms under laboratory conditions and establish (preferably monoclonal) strains. It is time-consuming and challenging to isolate a single cell to serve as the progenitor of the monoclonal culture, to grow it without contamination, and finally to achieve a cell-rich culture. Its undisputed advantage is that material is always available for molecular and morphological analyses, as well as for investigation of dinophyte life history traits and their response to different simulated environmental conditions.

## Life style and ecology of calcareous dinophytes (Thoracosphaeraceae)

Dinophytes are a group of unicellular algae distributed in all marine and freshwater habitats from the Arctic region to tropical areas. They exhibit many types of life styles and nutrition modes and include 2,000 extant and 2,500 fossil-based species (Taylor et al., 2008). Approximately half of the dinophytes are phototrophic, while the remaining species are heterotrophic or mixotrophic (Costas and Goyanes, 2005). They are, after the diatoms, the second largest group of phytoplankton, which plays an important role by generating half of our planets primary production (Boyce et al., 2010; Hallegraeff, 2010; Klais et al., 2011; von Dassow and Montresor, 2011). Some phototrophic dinophytes are symbionts with various groups of protists and metazoans (Esteban et al., 2010; Hackett et al., 2004; Venn et al., 2008) and play an important role as symbiotic partners in the growth and formation of coral reefs (Yamashita et al., 2010). Ten percent of the dinophytes have developed a parasitic life style as ecto- or endoparasites (Coats, 1999; Coats et al., 2010; Levy et al., 2007; Shields, 1994; Skovgaard and Daugbjerg, 2008).

Calcareous dinophytes (Thoracosphaeraceae) are a phototrophic subgroup of the Peridiniales and have the potential to produce an immotile calcareous shell varying in size from about 10 µm to 120 µm during their life history (Streng et al., 2009). 250 fossil-based calcareous dinophytes and approximately 35 extant (morpho-)species have been described (Elbrächter et al., 2008). They are found in sub-Arctic to tropical environments, mostly in neritic areas of marine and brackish water. Only a few species such as *Thoracosphaera heimii* (Lohmann) Kamptner have also been found in the open sea (Vink et al., 2000).

Some species, such as *Scrippsiella trochoidea* (F.Stein) A.R.Loebl., are known to accumulate in masses (Tang and Gobler, 2012). This natural phenomenon has been known since biblical ages (Ehrenberg, 1838), and the term “red tide” is often used as a synonym for harmful algae blooms (HABs) caused by toxin-producing species. Toxins in HABs accumulate in the food chain through organisms that feed on phytoplankton and affect organisms at higher trophic levels (Hackett et al., 2004; Hallegraeff, 2010). Nontoxic algae blooms have a significant impact on marine organisms and may cause economic damage to fish farms by their sheer biomass leading to anoxic conditions, especially in lakes or shallow bays without extensive water exchange.

Immotile coccoid cells are able to survive over decades in sediments (Lundholm et al., 2011) and generate an analogue to a terrestrial “seed bank”, which can contribute to seasonally returning blooming events under good growth conditions. Their high potential to

fossilize in marine sediments makes calcareous dinophytes an important tool of (paleo-) environmental reconstructions (Esper et al., 2000; Marino et al., 2011; Masure and Vrielynck, 2009; Montresor et al., 1998; Richter et al., 2007; Versteegh, 1997; Zonneveld et al., 2005). The diversity of calcareous dinophytes has been well documented by marine field work using research vessels (Vink, 2004), but only a few studies provide data on their diversity in coastal waters and sediments. Particularly harbors and bays may function as a sediment trap accumulating much higher calcareous dinophyte concentrations than found anywhere else. Additionally, those dinophytes known from the fossil record are rarely found and documented in recent coastal sediments, such as *Calciperidinium* G.Versteegh and *Follisdinellum* G.Versteegh (Montresor et al., 1998; Tommasa et al., 2004).

## Phylogeny and classification of calcareous dinophytes

The Dinophyta belong to the Alveolata, together with the Ciliata and Apicomplexa (= Sporozoa). Alveolates are a well-supported monophyletic group based on molecular and morphological data, such as the presence of amphiesmal vesicles (Bachvaroff et al., 2011; Fensome et al., 1999; Harper et al., 2005; Leander and Keeling, 2004). Recent multigene molecular clock analyses of eukaryotes dated the rise of Alveolata in a range from 1445–1236 Ma to 1206–1020 Ma (Parfrey et al., 2011). The possible divergence of the three main alveolate groups was estimated to 900–1317 Ma (Taylor, 2004). The split of dinophytes and apicomplexans took place roughly 800–900 Ma (Wisecaver and Hackett, 2011).

The monophyly of dinophytes is well-supported by molecular and morphological data (Costas and Goyanes, 2005; De Schepper et al., 2004; Fensome et al., 1997; Fensome et al., 1999; Leander and Keeling, 2004). The Dinophyta segregate into six subgroups based on the motile cell morphology (Fensome et al., 1993). However, the phylogenetic relationships within the dinophytes are not sufficiently clarified at present. A recent molecular study confirmed the monophyly of Dinophysiales, Gonyaulacales, and Suessiales with high statistical support and also for Peridiniales and Prorocentrales with lower support values (Tillmann et al., 2012). In this study, the “Gymnodiniales” was shown to be paraphyletic and split into three clades (Tillmann et al., 2012).

The Peridiniales includes the monophyletic group of calcareous dinophytes, Thoracosphaeraceae (Gottschling et al., 2005a; Gottschling et al., 2005b; Gottschling et al., 2012; Tillmann et al., 2012). Based on molecular data, the Thoracosphaeraceae consists of three main lineages (fig. 1**)**: the E/Pe-clade (for *Ensiculifera* Balech and *Pentapharsodinium*

Indel. & A.R.Loebl.), the T/Pf-clade (for *Thoracosphaera* Kamptner and *Pfiesteria* Steid. & J.M.Burkh.), and *Scrippsiella* sensu lato (s.l.). Species with non-calcareous stages are included within the calcareous dinophytes such as *Ensiculifera* and *Pfiesteria*. The ability to produce calcareous structures is assumed to be secondarily reduced, for example in pfiesterian species, or has not yet been observed. The *Scrippsiella* s.l.clade including *Pernambugia tuberose* Janofske & Karwath is divided into at least six lineages at high taxonomic level: the CAL-clade including *Calciodinellum opserosum* Deflandre, 1947, the LAC-clade comprising *Scrippsiella lachrymosa* Lewis, the PRE-clade comprising *Scrippsiella precaria* Montresor & Zingone, and three distinct clades named STR1, STR2 and STR3 (*Scrippsiella trochoidea* cluster 1 to 3) belonging to the *Scrippsiella trochoidea* (F.Stein) A.R.Loebl. species complex (STR-SC). Those three clades probably represent cryptic species of *Scrippsiella trochoidea*, which are morphologically indistinguishable. (Gottschling et al., 2005b; Montresor et al., 2003).

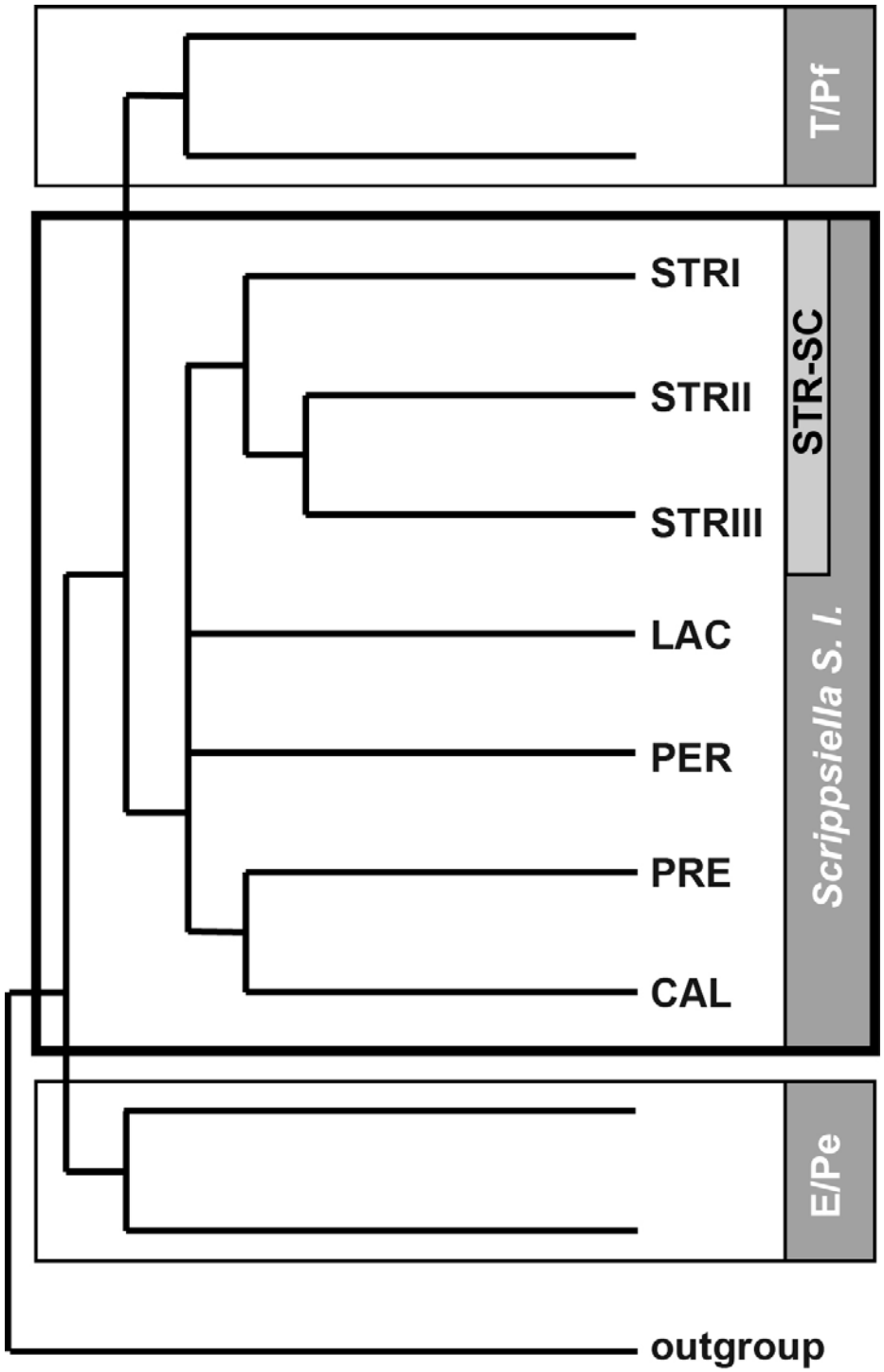


**Fig. 1:** The cladogram represents the phylogeny of Thoracosphaeraceae, which split in three main clades: the E/Pe-clade (*Ensiculifera/Pentapharsodinium*-clade), the T/Pf-clade (*Thoracosphaera/Pfiesteria*-clade) and the *Scrippsiella* s.l. clade. The *Scrippsiella* s.l. clade splits in the CAL-clade including *Calciodinellum opserosum*, the PRE-clade including *S. precaria*, the PER-branch with *Pernambugia tuberosa*, the LAC-clade including *Scrippsiella lachrymosa*, and at least three clades STR1, STR2, and STR3 (*Scrippsiella trochoidea* clades 1 to 3, within the *S. trochoidea* species complex (STR-SC) (concluding figure from several publications (Gottschling et al., 2005b; Soehner et al., 2012; Zinssmeister et al., 2011).

## Morphology and anatomy of calcareous dinophytes

### Morphology of the motile theca

The shape of the motile thecate cell of calcareous dinophytes, which is the planktonic stage, is basically globular. The theca consists of an upper hemisphere called the epitheca, which indicates the direction of movement, and a lower hemisphere, called the hypotheca. Between these, and usually equatorially, there is a cavity called the cingulum (figs 2 and 4). The top of the epitheca is terminated by the apical pore, and its shape is species-specific, ranging from round-ovoid, as in *Calciodinellum operosum* Deflandre, to conical, as in *Scrippsiella trochoidea*. The hypotheca is round and exhibits the sulcus, which defines the so-called ventral side.

Dinophytes are dinokont flagellates with two morphologically differentiated flagella (fig. 2). The flagella usually originate within the sulcus from two pores, which are often covered by the right sulcal plate (fig. 3). The first is a coiled or ribbon-like transverse flagellum for locomotion, which originates in the sulcus and is always embedded within the cingular groove in an anticlockwise direction. The second, longitudinal flagellum follows within the sulcus to the antapical part of the cell and is responsible for forward movement. The nucleus of dinophytes exhibits an extraordinary organization of the DNA (fig. 2), and is thus called “dinokaryon”, in which the chromosomes are always condensed, even during the cell division. The dinophyte nucleus contains a very high amount of DNA, approximately 3,000–215,000 Mbp (the haploid human genome copy is 3,180 Mbp) (Hackett et al., 2004).

Chloroplasts of dinophytes (fig. 2) are surrounded by three or four membranes, in comparison to usually two membranes in land plants and green algae. Plastids are acquired secondarily from red algae, a phenomenon also known from haptophytes, cryptomonads, stramenopiles, ciliates, and apicomplexans (Agrawal and Striepen, 2010; Dorrell and Smith, 2011; Hackett et al., 2004; Janouskovec et al., 2010). However, based on molecular investigations, dinophytes lost or acquired plastids by replacement of various tertiary or serial secondary endosymbioses from, for example, diatoms, haptophytes, cryptophytes and green algae (Dorrell and Smith, 2011; Keeling, 2010). Plastids of dinophytes are therefore not considered homologous to those of other eukaryotes (Green, 2011)

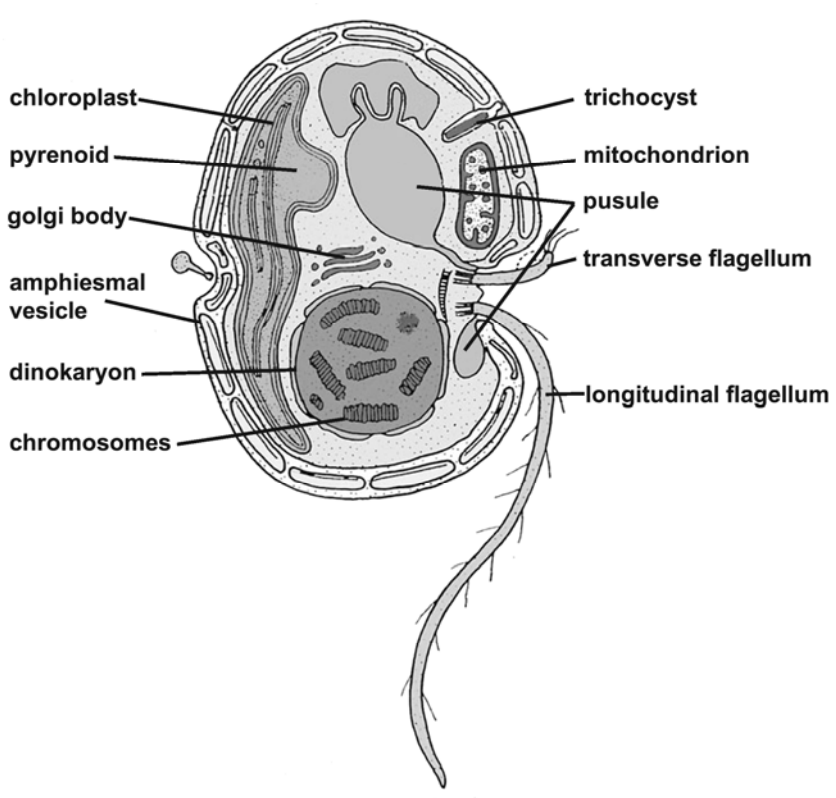


**Figure 2**. Generalized longitudinal section of a dinophyte (modified from (Taylor, 1980), page 68, fig. 1 and M. Hoppenrath, http://tolweb.org/Dinoflagellates/2445 (October 2012)).

Dinophytes develop cellulose plates inside amphiesmal vesicles (fig. 2). These theca plates can be plain or ornamented, and trichocyst pores are sometimes well-developed at the plate surface, as in *Calicarpinum bivalvum* (Balech) Montresor, Zingone & D. Marino. The connection (suture) between those plates is often conspicuous, and their arrangement is an important character in taxonomic identification. Therefore, tabulation systems have been developed by homologizing specific theca plates. The Kofoidean system (Taylor, 1999) is usually used for the taxa of the Peridiniales (Fensome et al., 1993; Taylor, 1980), including calcareous dinophytes (fig. 3). The epitheca is characterized by apical plates (labeled n') involving the apical pore (Po) and (x) channel plate. The precingular series (n") is apically adjacent to the cingulum and anterior intercalary plates (na). The cingular plate series are labeled (nC), of which the first cingular plate (1C), that is considered to be a part of the sulcus, was also named transitional plate (t). The hypotheca comprises two plate series, the postcingular plates (n'"), antapically adjacent to the cingular plates, and the antapical plates (n"") (fig. 2). The sulcal plates comprise the apical sulcal plate (Sa), the posterior sulcal plate (Sp) and the left sulcal plate (Ss). The right sulcal plate (Sd) usually covers the flagella pore aperture. The theca plate formula of a species can be written similar to the floral formula of an angiosperm (e.g.,Po, x, 4', 3a, 7'', 6c, 5s, 5''', 2'''' for *Scrippsiella trochoidea*).

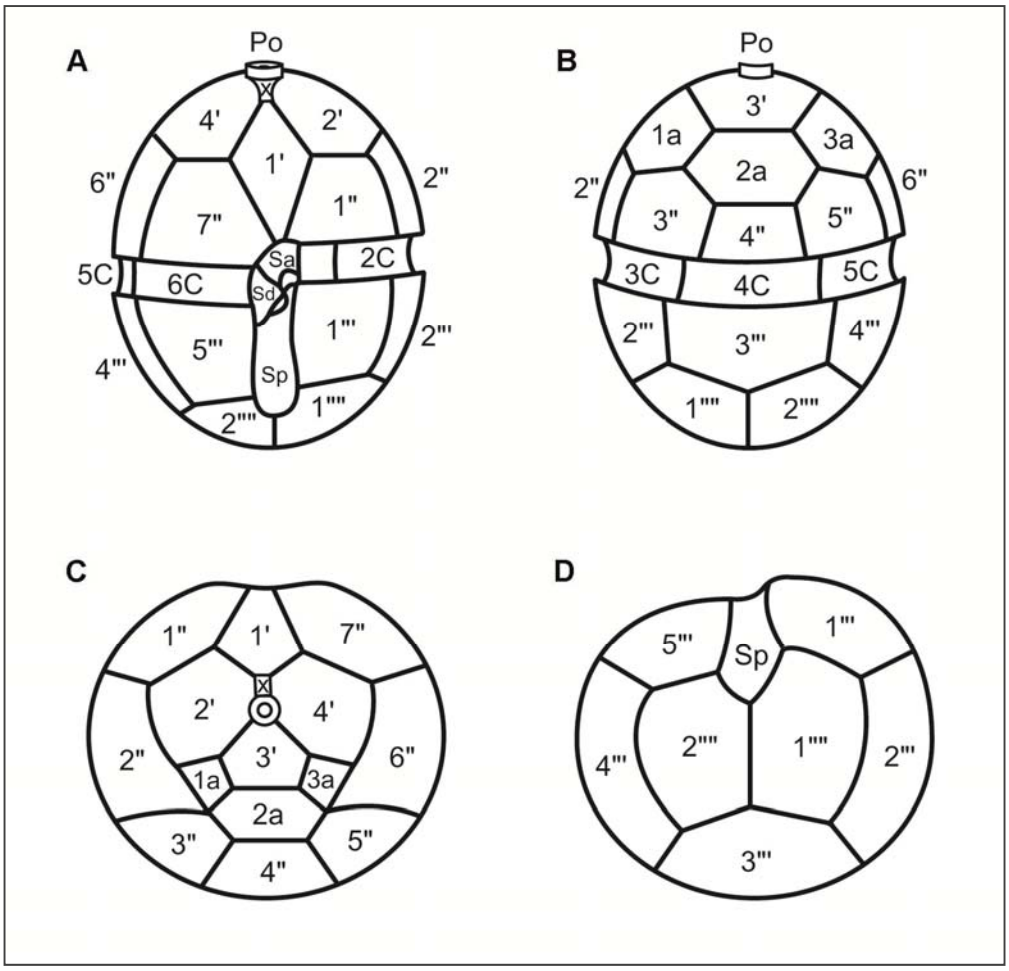


**Fig. 3**. Generalized theca plate pattern following the Kofoidian system for *Scrippsiella* s.l.. (modified graphic from (Gottschling et al., 2005b) A: Ventral view of the theca with sulcal plates B: Dorsal view of the theca. C: Apical view of the theca. D: Antapical view of the theca.
Abbreviations: Po, apical pore plate; x, channel plate; n', apical plates; n'', precingular plates; n''', postcingular plates; n'''', antapical plate; na, anterior intercalary plates; nC, cingular plates; Sa, anterior sulcal plate; Sd, right sulcal plate; Sm, median sulcal plate (not visible here); Sp, posterior sulcal plate; Ss, left sulcal plate (not visible here).

Besides the number of particular plates, the shape is another diagnostic feature of theca cells. Plates can develop different numbers of sutures, angles, and sometimes additional structures, such as the spine at the 1C plate in *Ensiculifera*. The number of cingular plates is an important character for identification within calcareous dinophytes. Species of the E/Pe-clade (marked in Fig. 1) such as *Pentapharsodinum* can be distinguished by their five cingular plates; species of the *Scrippsiella* s.l. clade possess six cingular plates. Theca plates of species within the T/Pf-clade, such as *Leonella granifera*, are thin and possess six cingular plates (Janofske and Karwath, 2000).

**Morphology of the calcareous coccoid cell**

The morphological description of (fossil) calcareous dinophytes is based on the size and shape of the coccoid cell. This also includes the structures of spines, ornamentation, and tabulation (a calcareous structure imprinted from the cellulose plate pattern of the motile cells), the archaeopyle/operculum morphology (the achaeopyle is the aperture in the coccoid cell left by the hatched dinophyte, the lid is called the operculum), the wall thickness, and ultrastructure of the calcite orientation of the wall and their crystallographic c-axes (Kohring et al., 2005; Meier et al., 2009; Streng et al., 2004).

The composition of the operculum is taxonomically important and named after a system introduced by Evitt in 1967 (Kohring et al., 2005; Streng et al., 2004). Figure 4 summarizes several archeopyle types comprising different thecal plate equivalents (see fig. 3). The operculum can be composed of only one plate: the 3'-plate equivalent that is common and probably ancestral for the T/Pf-clade, or of an intercalary plate 2a equivalent, as in *Calciperidinium asymmetricum* (Kohring et al., 2005; Streng et al., 2004; Zonneveld et al., 2005). A combination operculum with at least three or more plate equivalents is interpreted as a derived state (Kohring et al., 2005; Streng et al., 2004), and has been described for *Scrippsiella* Balech ex A.R.Loebl. and its relatives. Plate combinations up to an epitractal archaeopyle, which includes the complete upper part of the cell, as shown in fig. 4 are possible.

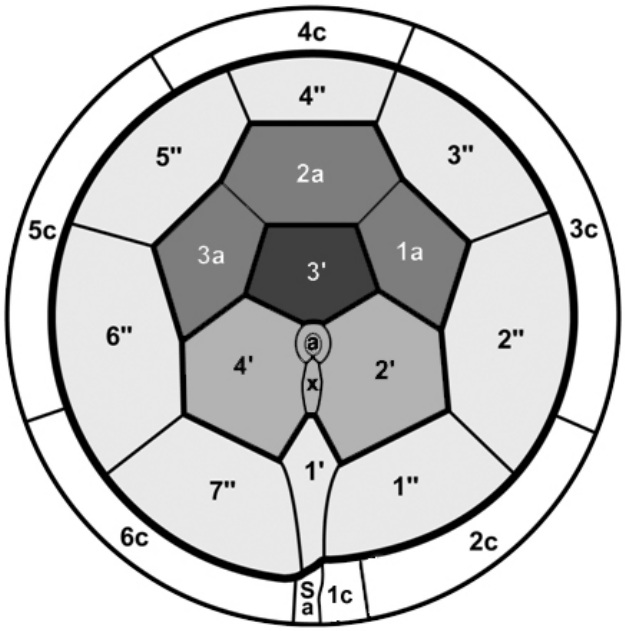


**Fig. 4**: Schematic diagram of archeopyle tabulation-like pattern types found in calcareous dinophytes following the Kofoidean system. Dark grey: apical archeopyle of 3'-plate equivalent; grey: mesoepicystal archeopyle incorporation of plates 2'-3'-4'; medium grey: mesoepicystal archeopyle with combined plates 2‘-3‘-4‘-2a-3a-4a; light grey: epitractal archeopyle includes all plates within the thick black line, 2‘-3‘-4‘-2a-3a-4a and 1’’ to 7’’, the whole upper part of the cell (modified graphic from (Kohring et al., 2005), page 87, fig. 6)

The ultrastructure of the calcareous shell in cross section is used as a character trait for identification. The arrangement of calcareous crystals may exhibit different positions in their c-axis orientation and can be visualized under polarized light (Meier et al., 2009). Among extant calcareous dinophytes, three types of wall ultrastructure and c-axis orientations are distinguished (fig. 5), namely an irregular oblique type, e.g. *Calcicarpinum bivalvum* Versteegh (Gottschling et al., 2005a; Streng et al., 2004), a regular radial type with radial c-axis orientations e.g. *Caracomia stella* Streng, Hildebrand-Habel & H.Willems (Gottschling et al., 2005a; Hildebrand-Habel and Streng, 2003; Streng et al., 2002), and a regular type with tangential c-axis orientations that represents the major type of extant calcareous dinophytes, for example †*Calciodinellum* Defandre 1947 and *Scrippsiella* (Gottschling et al., 2005a; Janofske, 2000).

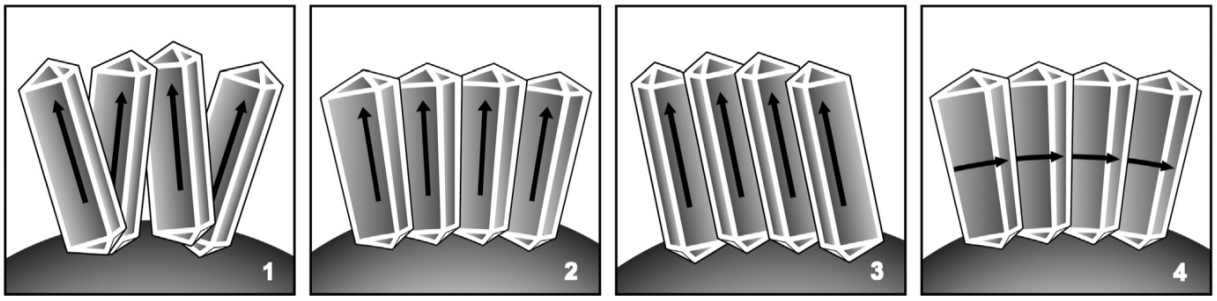


**Fig. 5:** "Schematic drawings of calcareous shell walls. Fig. 1: Form with irregularly arranged crystals Fig. 2: Form with regularly arranged crystals and c-axes that are orientated radially. Fig. 3: Form with regularly arranged crystals and oblique c-axes (pithonelloids, extinct). Fig. 4: Forms with regularly arranged crystals and tangential c-axes." (Gottschling et al., 2005a), page 445, fig. 1-4.

**Biomineralization within calcareous dinophytes**

The surface morphology of the coccoid stage has been studied extensively, and the ultrastructure of the shells has been described for several species of calcareous dinophytes (Meier et al., 2009). However, the process of biomineralization from thecate cells through calcareous coccoid cells is still poorly understood. Four different hypotheses of possible calcification processes have been suggested for calcareous dinophytes (Elbrächter et al., 2008): (1) externally at the cell surface, (2) replacement of cellulose theca plates within amphiesmal vesicles with the calcareous structure, (3) development of calcareous structure inside cellulose-free amphiesmal vesicles, or (4) development of calcareous structure between the outer and middle membrane associated with Golgi derived vesicles.

Light microscopic observations of cultivated *Scrippsiella* s.l. strains and others reject the first two possibilities. The calcareous coccoid cell always hatches outside of a thecate cell, after removal of the cellulose theca, which is attached to the surface immediately after hatching (Gao et al., 1989; Zinssmeister et al., 2011). Gao, (Gao et al., 1989) described cells of *Scrippsiella sp.* in the phase of mineralization, which are surrounded by two continuous matrices limited by an outer, a middle, and an inner unit membrane. The outer matrix could be the location where the mineralization takes place first in form of visible protrusions. Coccoid cells of *Thoracosphaera heimii*, develop a single matrix surrounded by an outer and inner unit membrane (Inouye and Pienaar, 1983; Tangen et al., 1982). Crystals are found in mature coccoid cells within the matrix. Furthermore, large cytoplasmatic vacuoles containing crystal-like bodies have been discovered. It remains to be determined whether those vesicles are derived from the Golgi apparatus.

## Aims of the thesis

(i) Inventory of calcareous dinophytes in marine environments. (ii) Cultivation of calcareous dinophyte strains for molecular and morphological investigation. (iii) Detailed morphological study of collected dinophytes that can potentially serve as the basis for the description of new species and for phylogenetic analyses. (iv) Analysis of the correct application of several taxon names and epitypification if necessary. (v) Detailed anatomical investigation of calcifying cells to better understand the mechanisms of biomineralization in calcareous dinophytes.

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# Chapter 1

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

S. Soehner, **C. Zinssmeister**, M. Kirsch & M. Gottschling (2012)
*Organisms Diversity & Evolution* **12**:339-348

Org Divers Evol (2012) 12:339–348
DOI 10.1007/s13127-012-0109-z

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

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

**Sylvia Soehner · Carmen Zinssmeister · Monika Kirsch · Marc Gottschling**

Received: 28 February 2011 / Accepted: 27 August 2012 / Published online: 5 October 2012


**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

**Electronic supplementary material** The online version of this article (doi:10.1007/s13127-012-0109-z) contains supplementary material, which is available to authorized users.

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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).

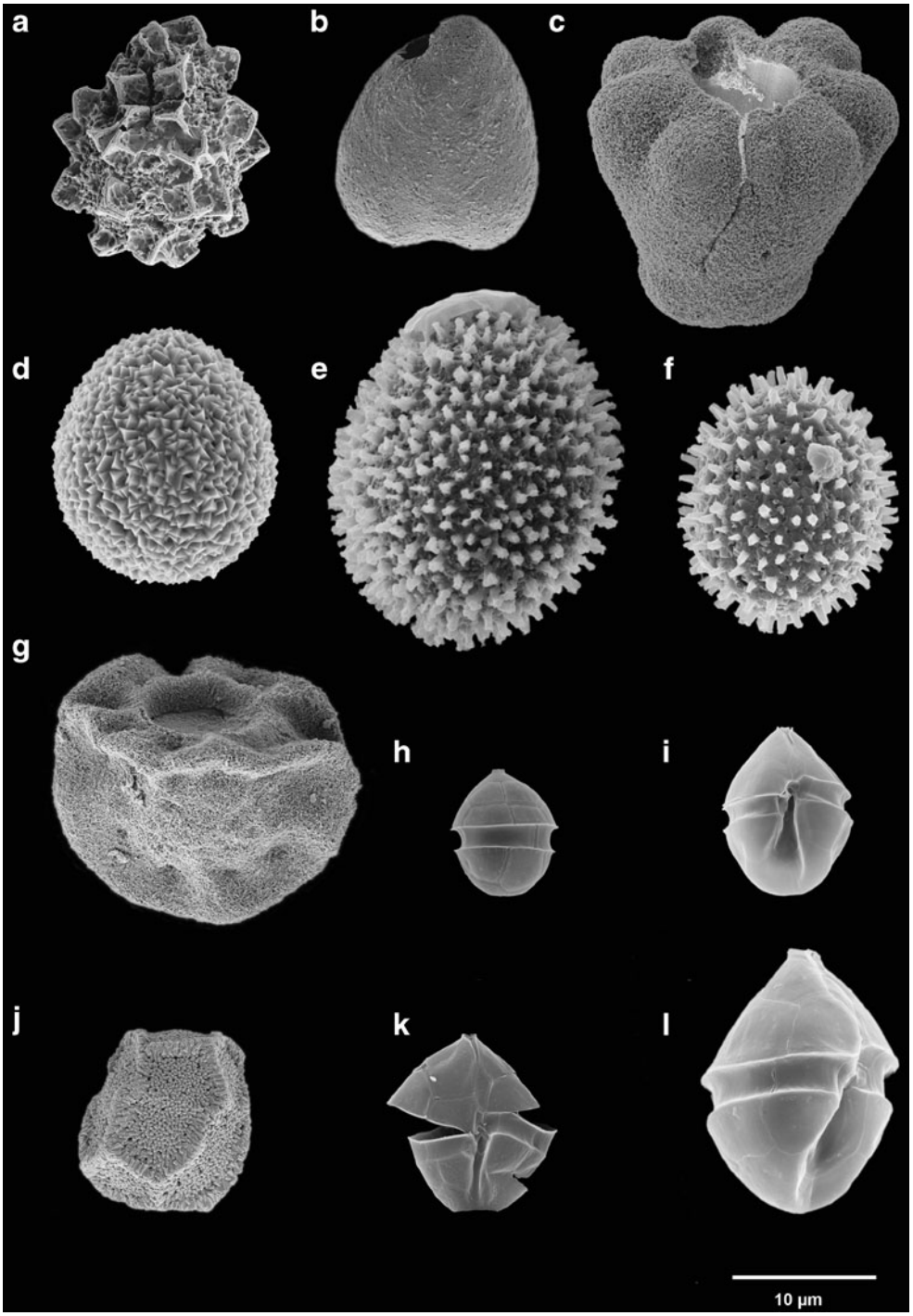


**Fig. 2 a–l.** Morphological diversity of calcareous dinophytes as found in the Mediterranean Sea, strain number is given, if no strain number is available the provenance is given (scanning electron microscopy of coocoid stage **a-g, j** and theca **h, i** and **k, l;** all at the same scale) **a** *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

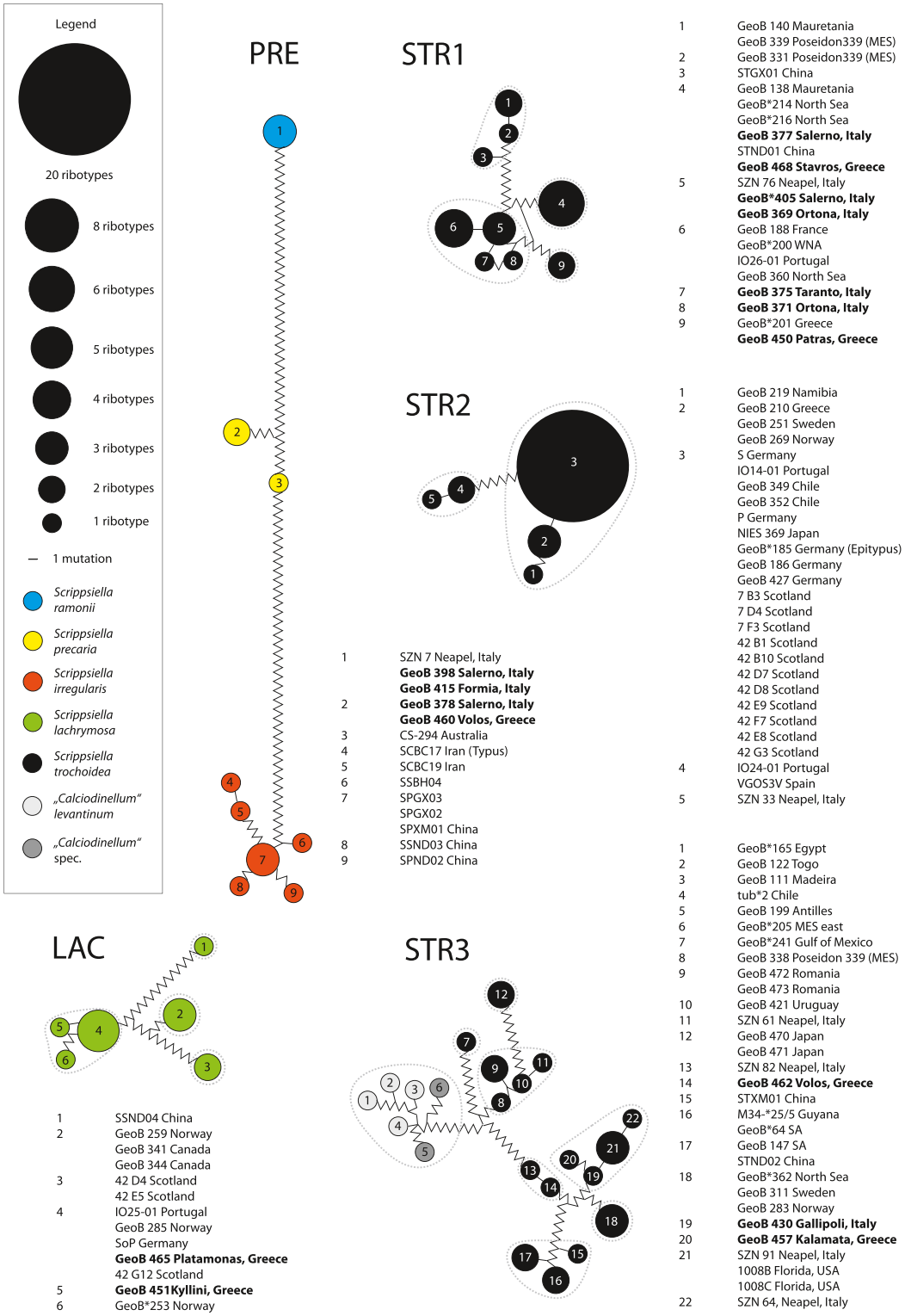


**Fig. 3** Molecular diversity of ITS ribotypes within different clades of the Thoracosphaeracea (created with TCS). Number of similar ribotypes indicated by *circle size*, presumable cryptic species indicated by *dashed grey line*, newly added sequences from the Mediterranean Sea indicated in *bold*. 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.

**Acknowledgements** We thank Julia Daum, Patricia Silva Flores and Martina Rom-Roeske for their assistance during cultivation of the strains. Mona Hoppenrath (Bremerhaven) and Michael Schweikert (Stuttgart) gave valuable advices in methodologies. We thank two anonymous reviewers for constructive and motivating reviews of our manuscript. Financial support was provided by the Deutsche Forschungsgemeinschaft (grants KE 322/36, RI 1738/5, and WI 725/25), and the Münchener Universitätsgesellschaft, which is grateful acknowledged here.

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# Chapter 2

## Same but different: Two novel bicarinate species of extant calcareous dinophytes (Thoracosphaeraceae, Peridiniales) from the Mediterranean Sea

**Zinssmeister, C.**, S. Soehner, M. Kirsch, E. Facher, K. J. S. Meier, H. Keupp & M. Gottschling (2012)
*Journal of Phycology* **48**:1107–1118.

*J. Phycol.* **48,** 1107–1118 (2012)

DOI: 10.1111/j.1529-8817.2012.01182.x

# 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

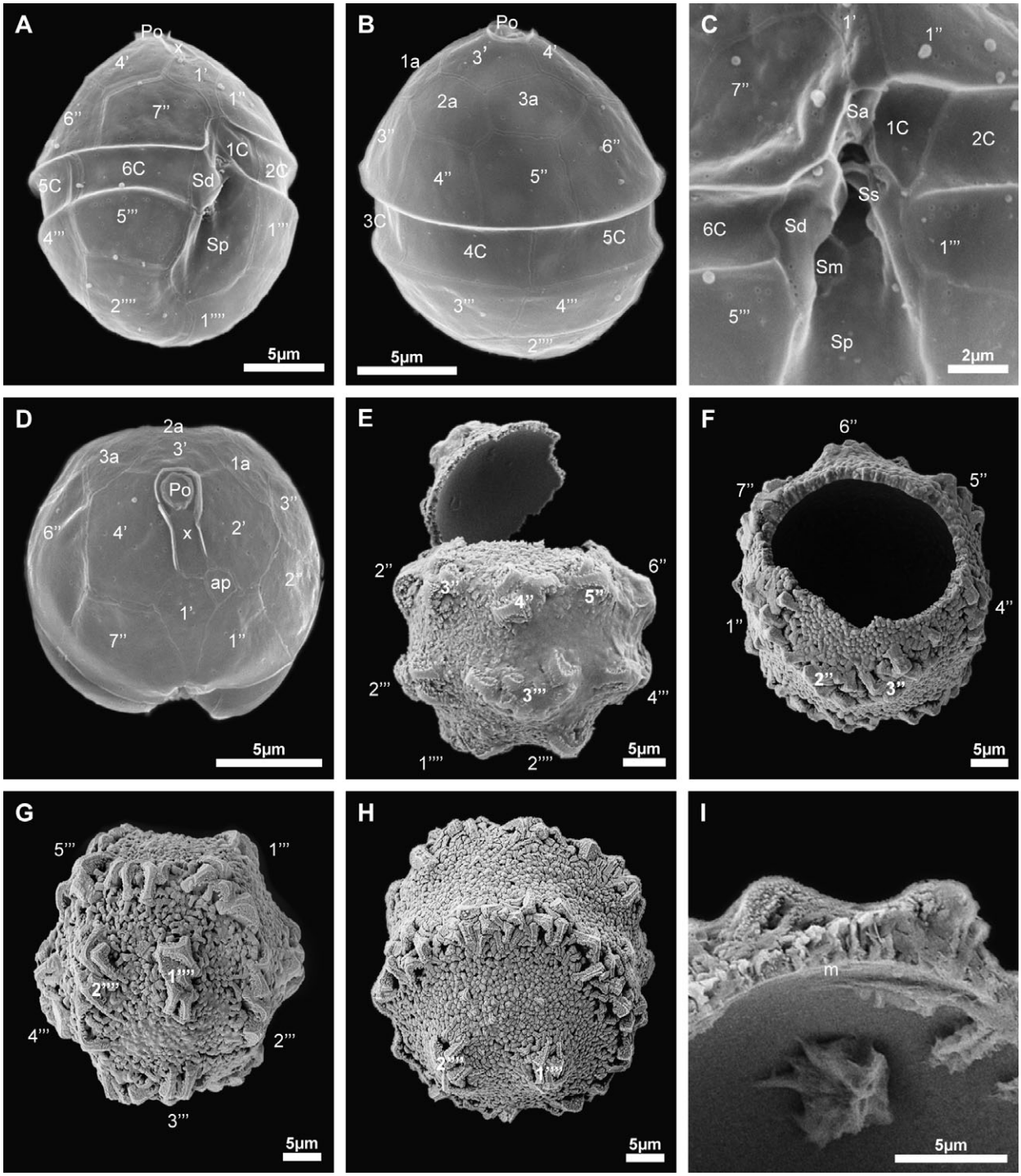


FIG. 1. *Scrippsiella bicarinata* spec. nov. showing an intratabulate tabulation in the coccoid stage as reflection of pre- and postcingular plates (SEM images; A–G: strain GeoB 416, H–I: strain GeoB 411). A, ventro-lateral view of the thecate cell, with epitheca, cingulum, and hypotheca. B, dorsal view 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.

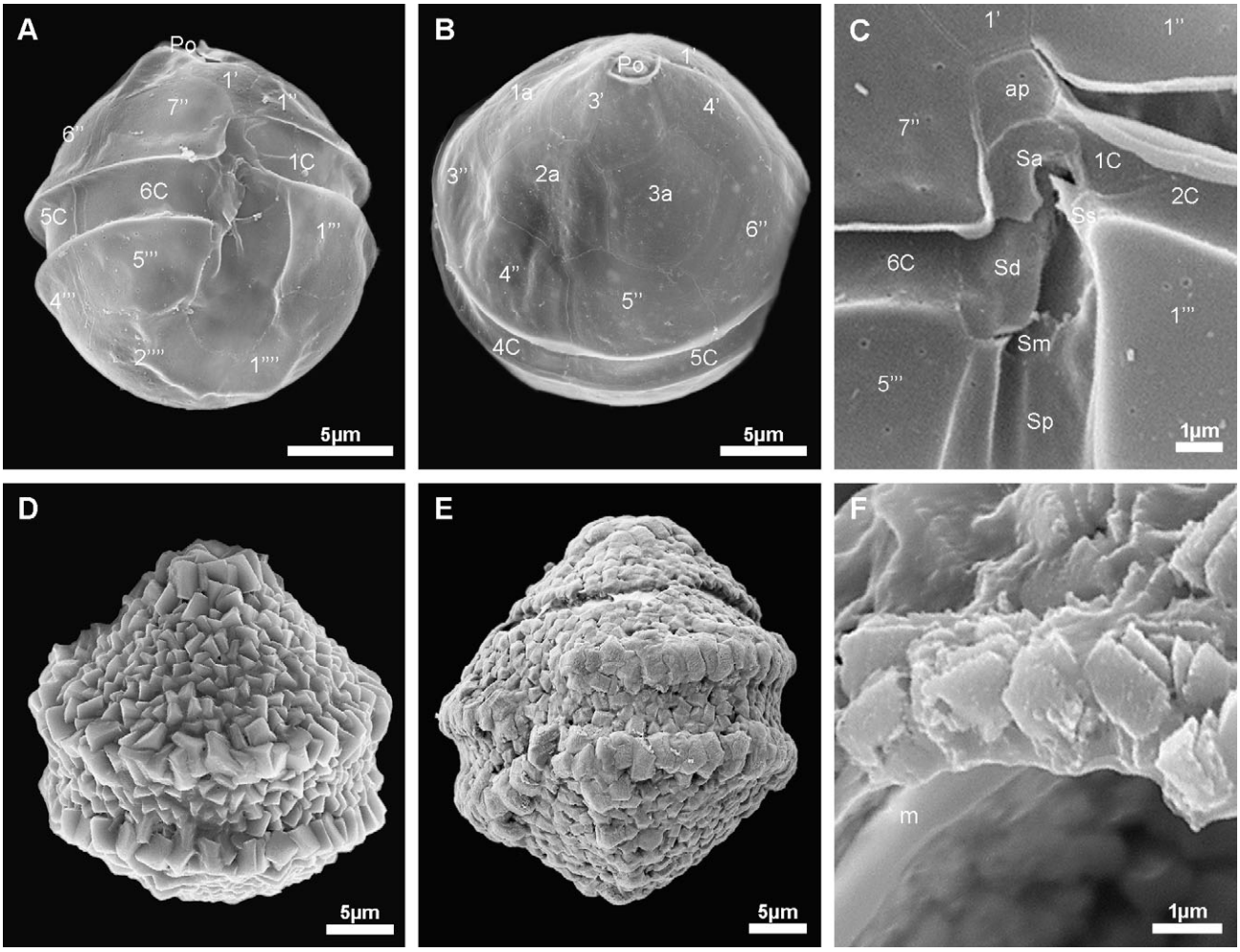


FIG. 2. *Scrippsiella kirschiae* spec. nov. showing a cingulotabulate tabulation in the coccoid stage (SEM images; strain GeoB 408). A, ventral view of thecate cell, with epitheca, cingulum, and hypotheca. B, latero-apical view of thecate cell. C, ventral detail of thecate cell with the sulcal region 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.

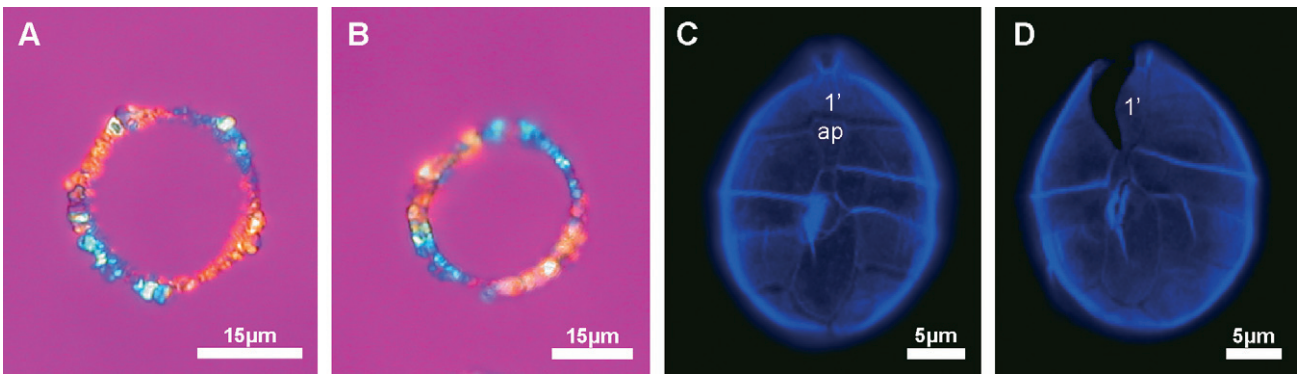


FIG. 3. Cells in polarized and fluorescent light microscopy exhibiting more traits. A–B, optical crystallography of the two new species showing the ''regularly tangential'' ultrastructure type (light microscopy with employed gypsum plate under polarized light, 400× magnification). A, *Scrippsiella bicarinata*. 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

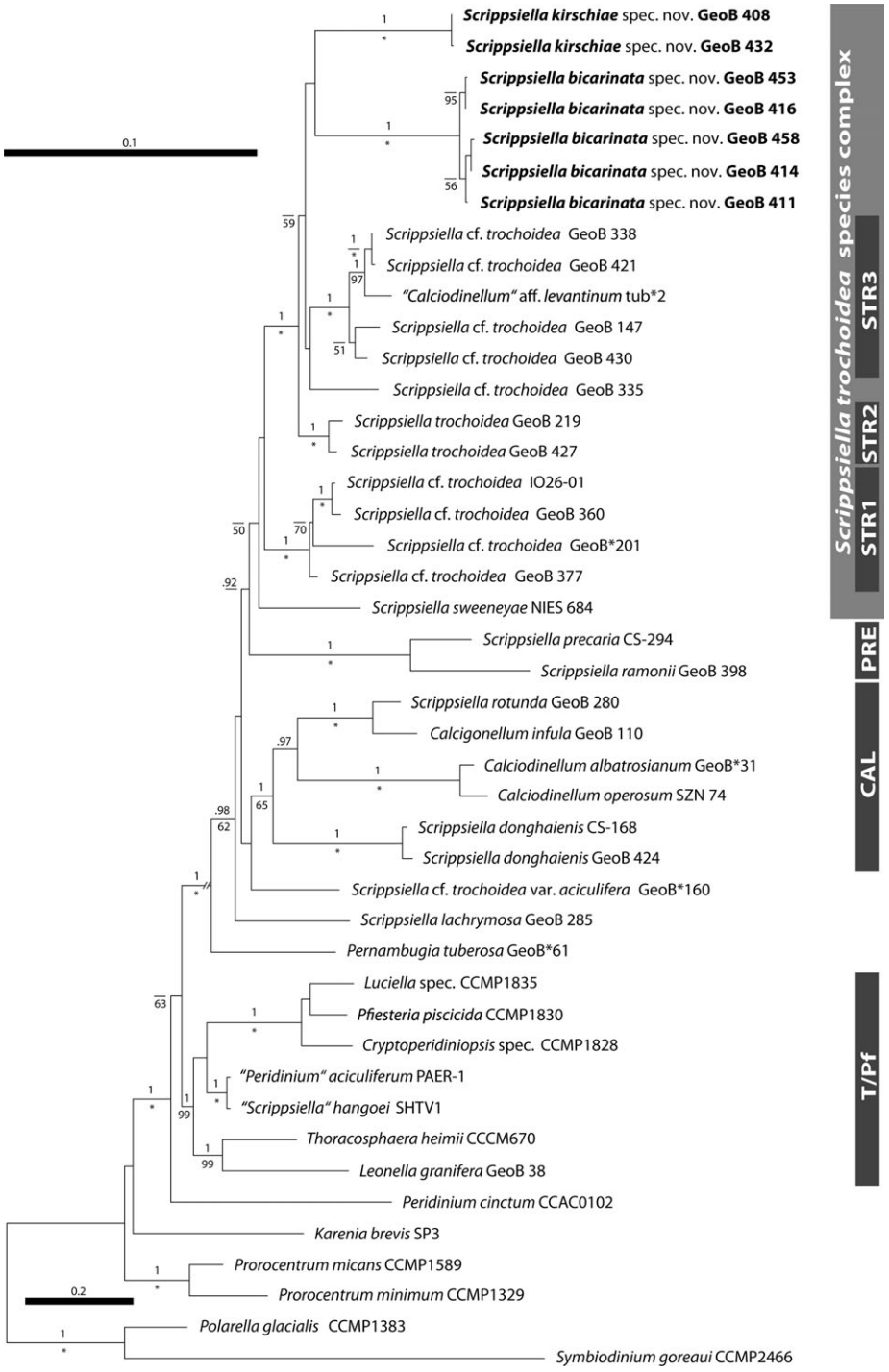


FIG. 4. *Scrippsiella bicarinata* and *Scrippsiella kirschiae* as distinct species within the *Scrippsiella trochoidea* species complex. Maximum likelihood (ML) tree (–ln = 21,101.519651) of 44 dinophyte strains as inferred from a MAFFT generated nucleotide alignment, comprising the complete ITS region, 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.

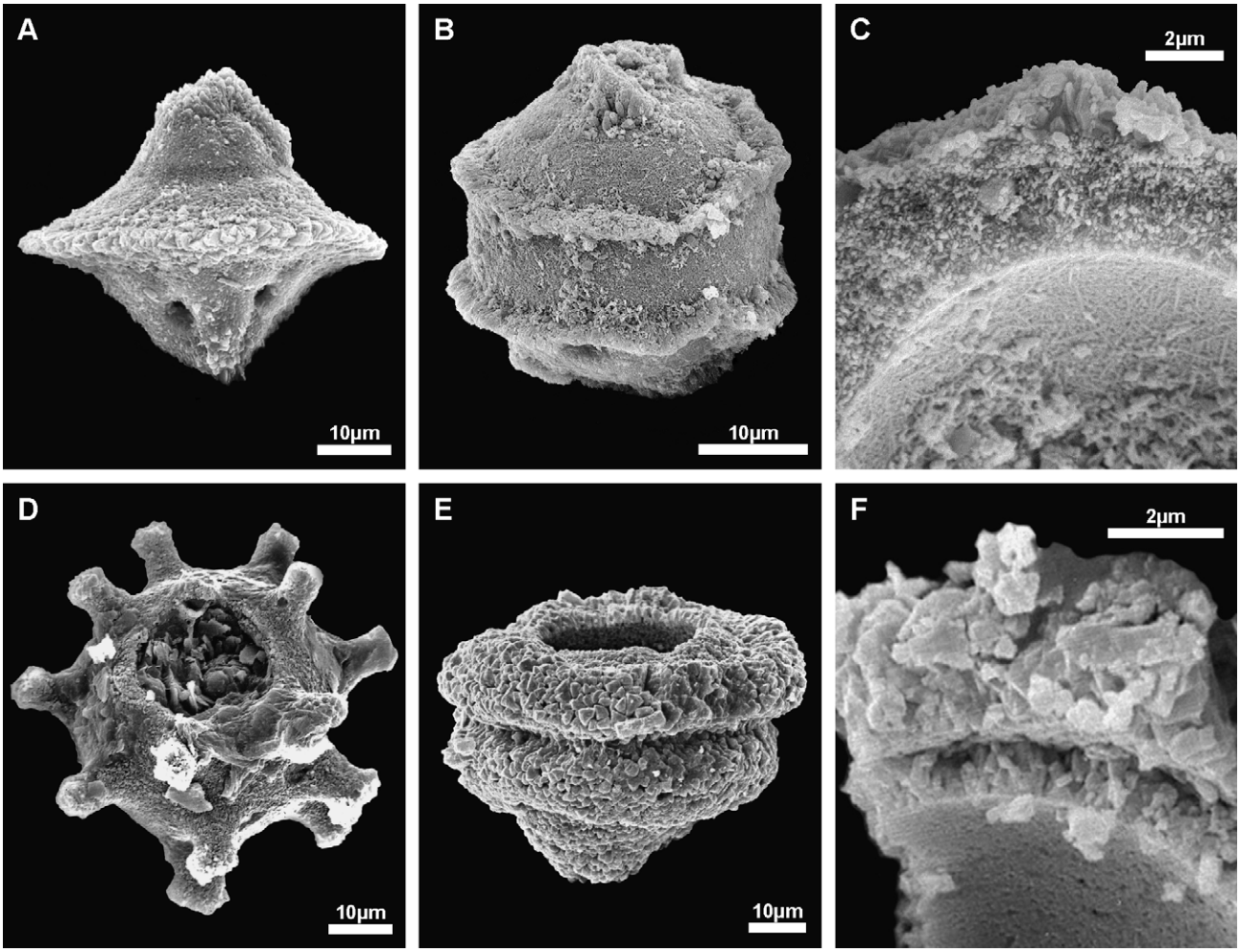


FIG. 5. The two new species do not fit in the circumscription of any known calcareous dinophyte. A, coccoid cell of *Carinellum parasole* Keupp (Lutetian, from Deflandre's original material) as example of a monocarinate form. B–C, *Bicarinellum castaninum* Deflandre, 1949 (Lutetian, from Deflandre's 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.

We are grateful to T. Uhle (Berlin) for improvement of the Latin descriptions and to three anonymous reviewers for their critical reading of the manuscript. We thank the Deutsche Forschungsgemeinschaft (grants KE 322/36, RI 1738/5, and WI 725/25) for financial support.

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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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# Chapter 3

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

**Zinssmeister, C.**, S. Soehner, E. Facher,
M. Kirsch, K.J.S. Meier, & M. Gottschling (2011)
*Systematics and Biodiversity* **9**:145-157.

*Systematics and Biodiversity* (2011), **9(2)**: 145–157

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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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(Received 4 February 2011; revised 2 May 2011; accepted 3 May 2011; printed 30 June 2011)

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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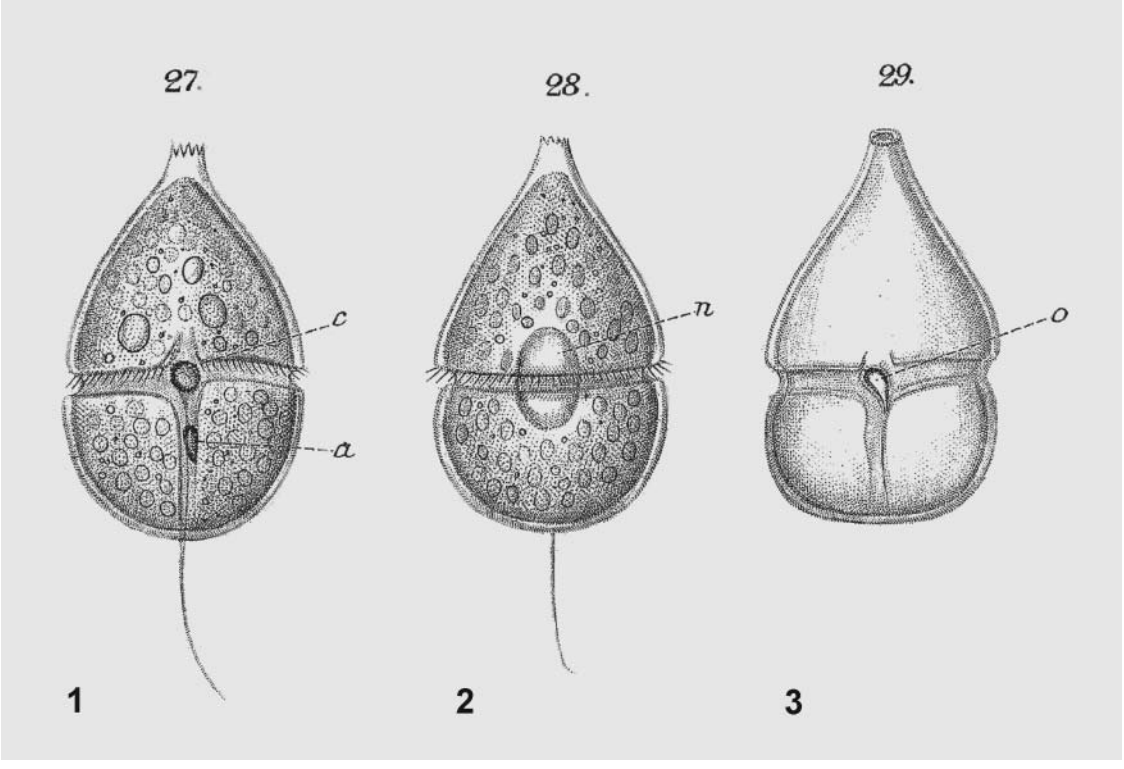
ISSN 1477-2000 print / 1478-0933 online

DOI: 10.1080/14772000.2011.586071

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 $\mu$mol 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 $\mu$l 3% glutaraldehyde (Plano; Wetzler, Germany) in 35 psu artificial seawater were added to the 30 $\mu$l 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 $\mu$l 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 $\mu$l 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*)

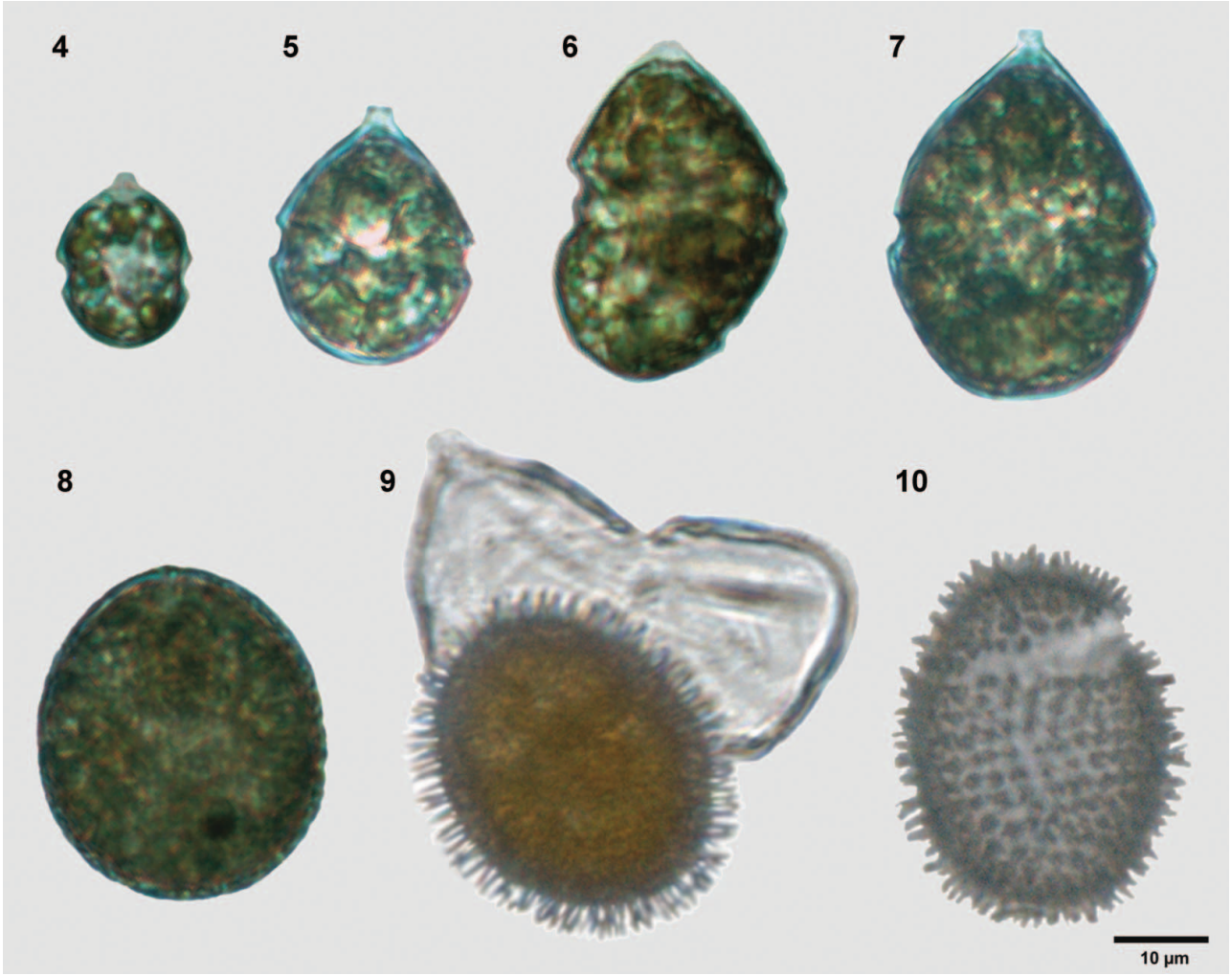
**Table 1.** Voucher list. Abbreviation: n.i., not indicated. Para- and/or polyphyletic taxa are indicated by quotation marks. (*Continued*)

| 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 |

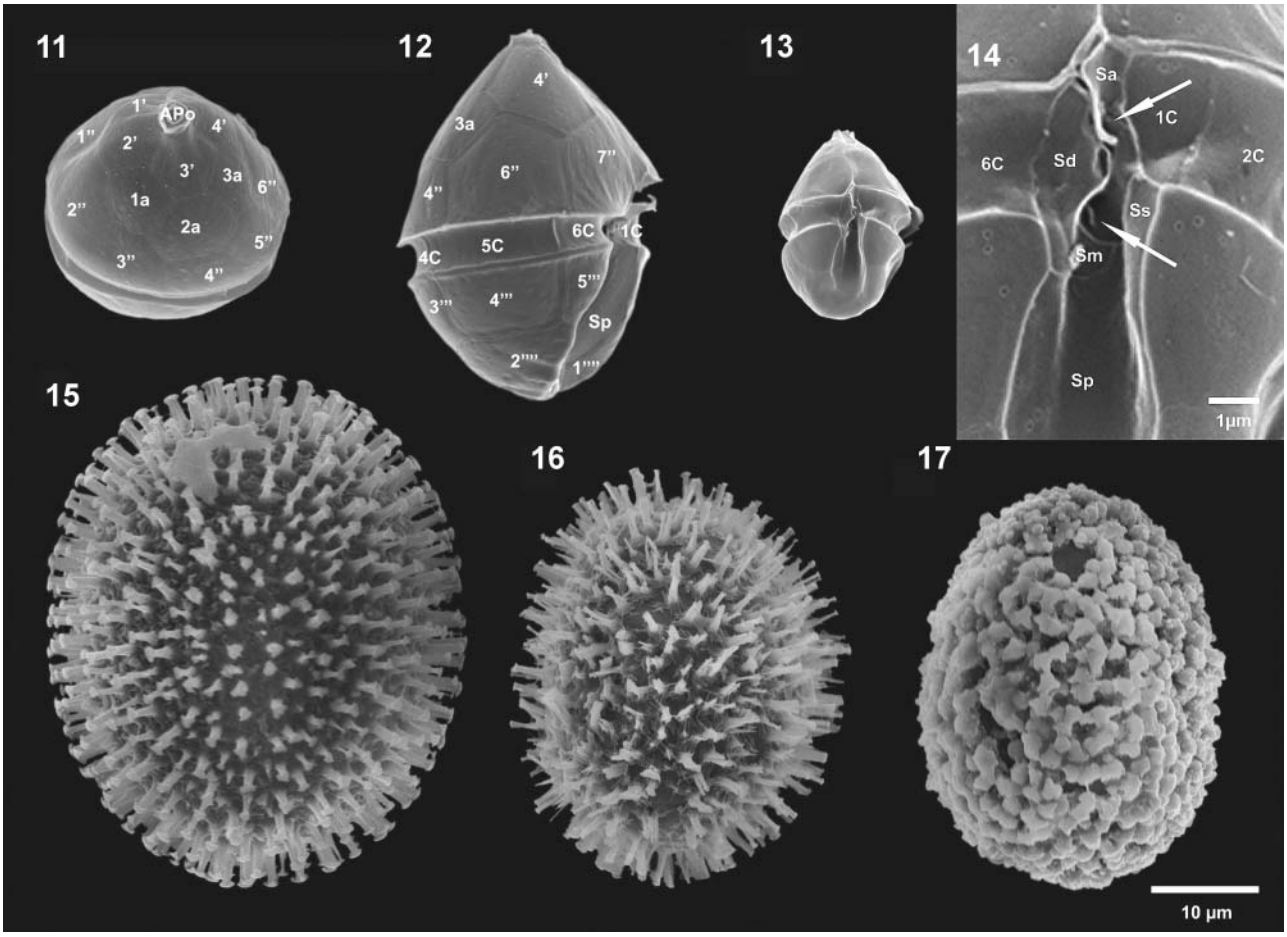
Phylogenetic analyses were carried out by using Maximum-Likelihood (ML) and Bayesian approaches, as described in detail by Gottschling *et al.* (in press). Calculations were carried out by using the resources of the Leibniz Rechenzentrum (LRZ, Munich; linux cluster HLRB-II) and of the SGI system (Zuse 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 $\mu$m in length (small cells: 19–21 $\mu$m, medium-sized cells: 25–32 $\mu$m, large cells: 36–42 $\mu$m) and 15–37 $\mu$m in width (small cells: 15–17 $\mu$m, medium-sized cells: 20–24 $\mu$m, large cells: 25–37 $\mu$m). 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 $\mu$m in length and 29–38 $\mu$m 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 &

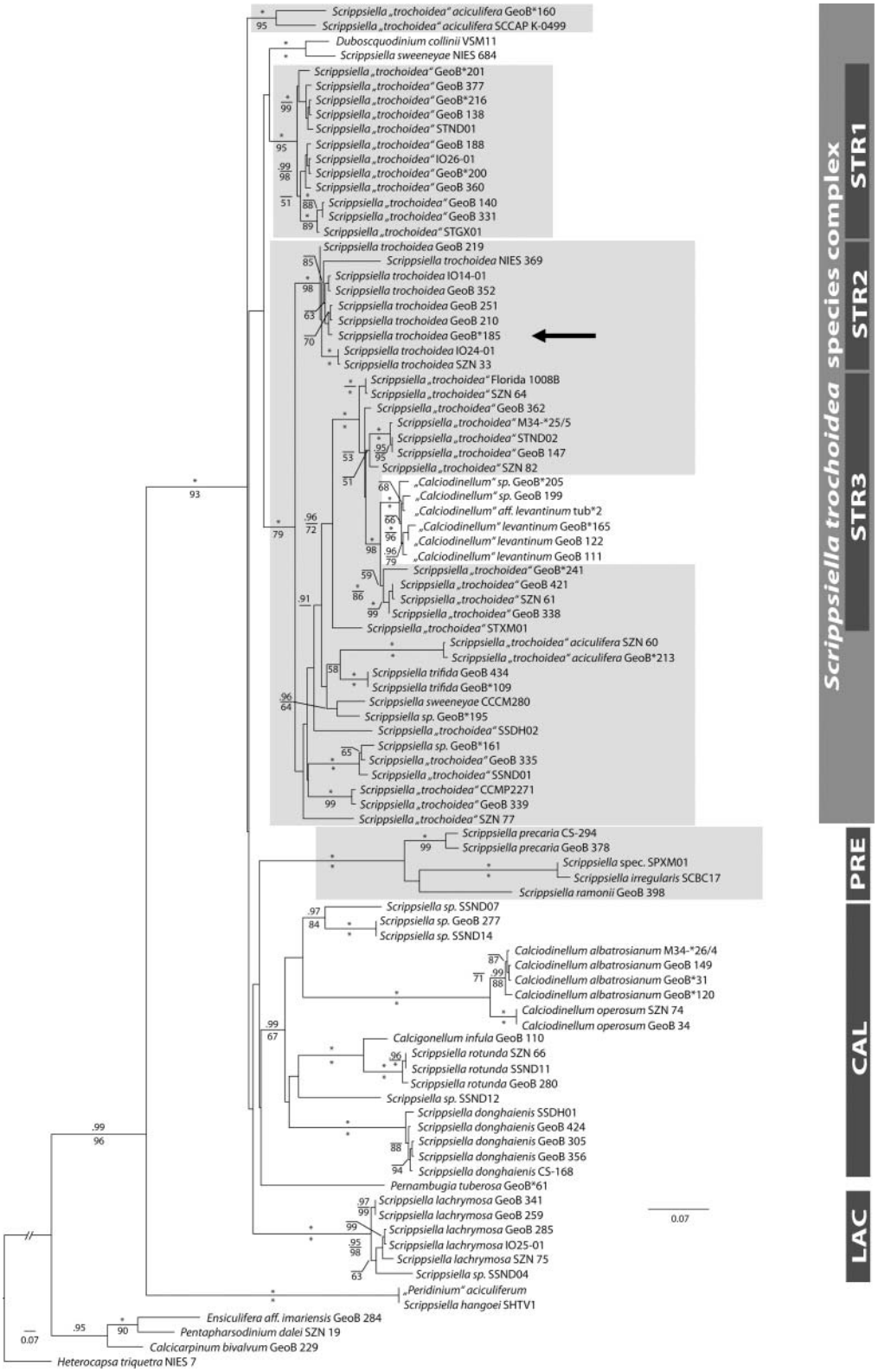



**Fig. 18.** Maximum likelihood (ML) tree (–ln = 8041.97) of 93 taxa of the Thoracosphaeraceae and outgroups, derived from the sequence comparison comprising the Internal Transcribed Spacer 1, 5.8S ribosomal RNA and the Internal Transcribed Spacer 2. Major clades are indicated, and taxa with spiny coccoid 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!).

## Acknowledgements

We thank Julia Daum, Patricia Silva Flores and Martina Rom-Roeske (all Munich) for their key assistance during the cultivation of the strains. Mona Hoppenrath (Bremerhaven) and Michael Schweikert (Stuttgart) gave valuable advice regarding methodology. We further thank two anonymous reviewers, who helped to improve our manuscript. Anne Beck (Berlin) and Adrienne Jochum (Frankfurt) kindly provided editorial insights in reviewing this manuscript. Financial support by the Deutsche Forschungsgemeinschaft (grants KE 322/36, RI 1738/5, and WI 725/25) and the Münchener Universitätsgesellschaft is gratefully acknowledged here.

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**Associate Editor: Elliot Shubert**

# Chapter 4

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

Gottschling, M., S. Soehner, **C. Zinssmeister**, U. John, J. Plötner,
M. Schweikert, K. Aligizaki & M. Elbrächter (2012)
*Protist* **163**:15-24

Protist, Vol. 163, 15–24, January 2012
http://www.elsevier.de/protis
Published online date 8 July 2011

Protist

**ORIGINAL PAPER**

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

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Submitted November 4, 2010; Accepted May 21, 2011
Monitoring Editor: Hervé Philippe

**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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doi:10.1016/j.protis.2011.06.003

## 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).

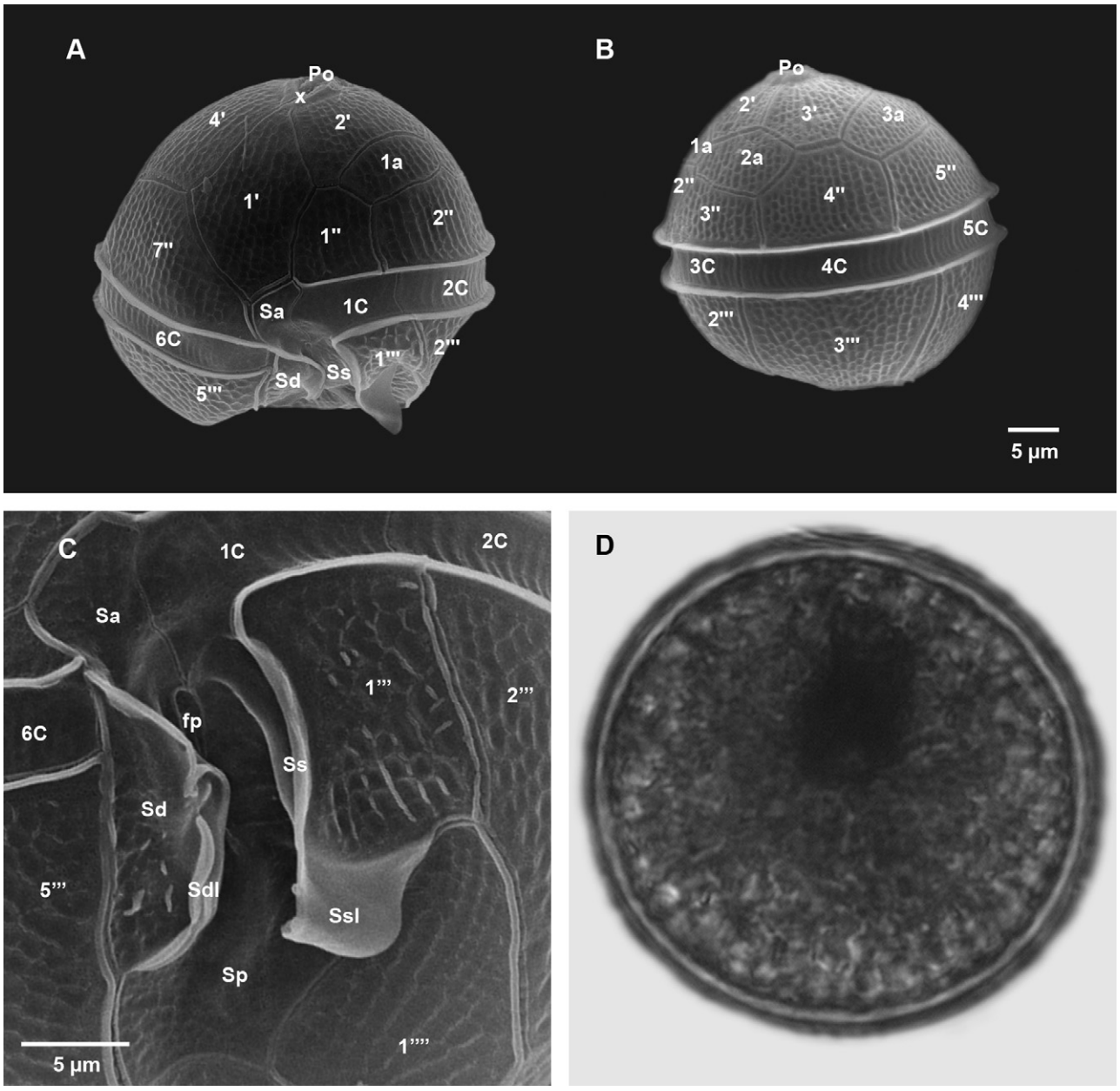
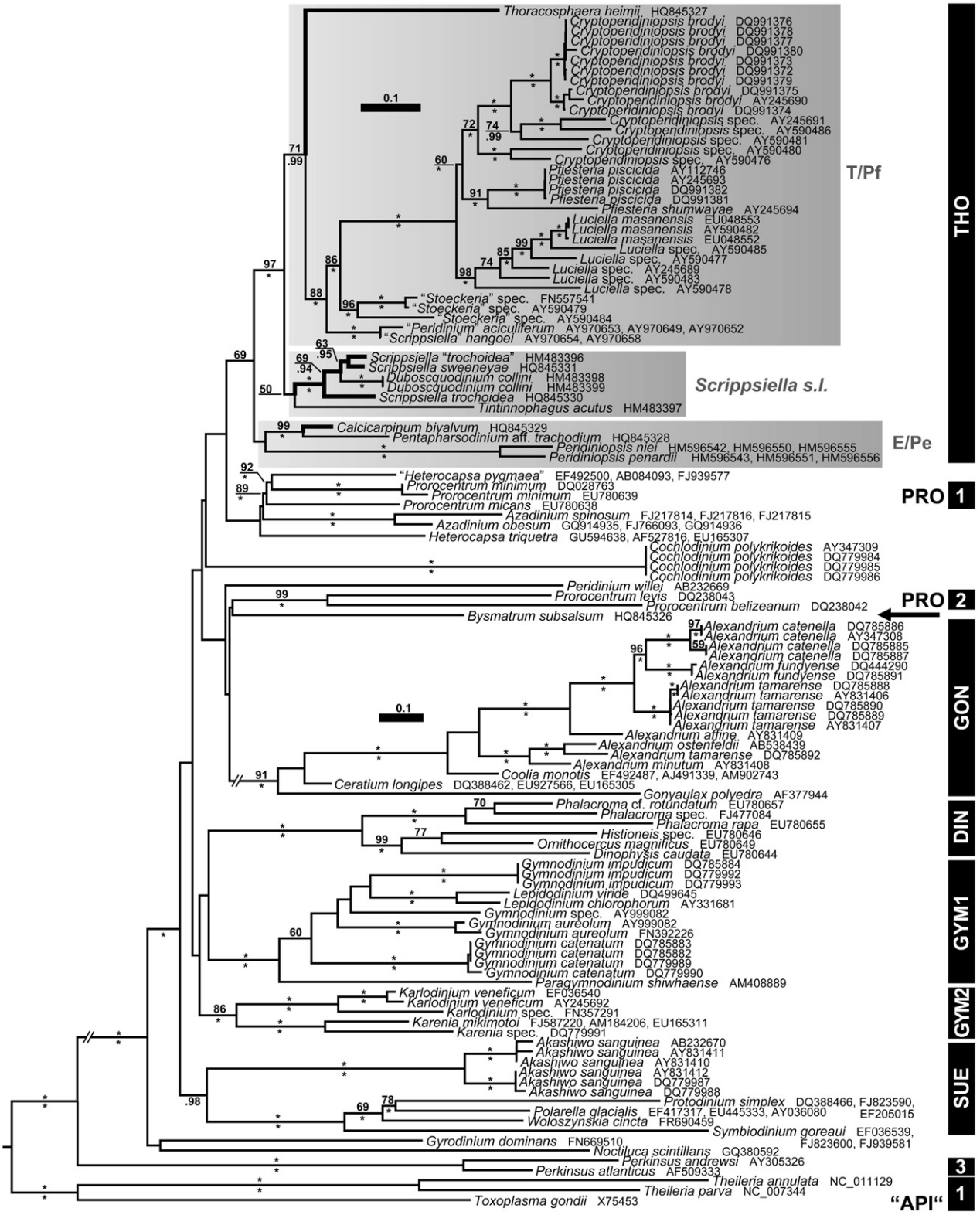



**Figure 1.** *Bysmatrum* had a peridinean tabulation pattern. Scanning electron microscope (SEM; Fig. 1A–C) and light microscope images (Fig. 1D) of *Bysmatrum*. **A**: Ventral view of epitheca, cingulum, and parts of hypotheca and sulcal region. **B**: Dorsal view of the thecate cell, the intercalary 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.


Thoracosphaera heimii HQ845327
Cryptoperidiniopsis brodyi DQ991376
Cryptoperidiniopsis brodyi DQ991378
Cryptoperidiniopsis brodyi DQ991377
Cryptoperidiniopsis brodyi DQ991380
Cryptoperidiniopsis brodyi DQ991373
Cryptoperidiniopsis brodyi DQ991372
Cryptoperidiniopsis brodyi DQ991379
Cryptoperidiniopsis brodyi DQ991375
Cryptoperidiniopsis brodyi AY245690
Cryptoperidiniopsis brodyi DQ991374
Cryptoperidiniopsis spec. AY245691
Cryptoperidiniopsis spec. AY590486
Cryptoperidiniopsis spec. AY590481
Cryptoperidiniopsis spec. AY590480
Cryptoperidiniopsis spec. AY590476
Pfiesteria piscicida AY112746
Pfiesteria piscicida AY245693
Pfiesteria piscicida DQ991382
Pfiesteria piscicida DQ991381
Pfiesteria shumwayae AY245694
T/Pf
Luciella masanensis EU048553
Luciella masanensis AY590482
Luciella masanensis EU048552
Luciella spec. AY590485
Luciella spec. AY590477
Luciella spec. AY245689
Luciella spec. AY590483
Luciella spec. AY590478
"Stoeckeria" spec. FN557541
"Stoeckeria" spec. AY590479
"Stoeckeria" spec. AY590484
"Peridinium" aciculiferum AY970653, AY970649, AY970652
"Scrippsiella" hangoei AY970654, AY970658
Scrippsiella "trochoidea" HM483396
Scrippsiella sweeneyae HQ845331
Duboscquodinium collini HM483398
Duboscquodinium collini HM483399
Scrippsiella trochoidea HQ845330
Tintinnophagus acutus HM483397
Scrippsiella s.l.
Calcicarpinum bivalvum HQ845329
Pentapharsodinium aff. trachodium HQ845328
Peridiniopsis niei HM596542, HM596550, HM596555
Peridiniopsis penardii HM596543, HM596551, HM596556
E/Pe
THO
"Heterocapsa pygmaea" EF492500, AB084093, FJ939577
Prorocentrum minimum DQ028763
Prorocentrum minimum EU780639
Prorocentrum micans EU780638
Azadinium spinosum FJ217814, FJ217816, FJ217815
Azadinium obesum GQ914935, FJ766093, GQ914936
Heterocapsa triquetra GU594638, AF527816, EU165307
PRO 1
Cochlodinium polykrikoides AY347309
Cochlodinium polykrikoides DQ779984
Cochlodinium polykrikoides DQ779985
Cochlodinium polykrikoides DQ779986
Peridinium willei AB232669
Prorocentrum levis DQ238043
Prorocentrum belizeanum DQ238042
Bysmatrum subsalsum HQ845326
PRO 2
Alexandrium catenella DQ785886
Alexandrium catenella AY347308
Alexandrium catenella DQ785885
Alexandrium catenella DQ785887
Alexandrium fundyense DQ444290
Alexandrium fundyense DQ785891
Alexandrium tamarense DQ785888
Alexandrium tamarense AY831406
Alexandrium tamarense DQ785890
Alexandrium tamarense DQ785889
Alexandrium tamarense AY831407
Alexandrium affine AY831409
Alexandrium ostenfeldii AB538439
Alexandrium tamarense DQ785892
Alexandrium minutum AY831408
Coolia monotis EF492487, AJ491339, AM902743
Ceratium longipes DQ388462, EU927566, EU165305
GON
Gonyaulax polyedra AF377944
Phalacroma cf. rotundatum EU780657
Phalacroma spec. FJ477084
Phalacroma rapa EU780655
Histioneis spec. EU780646
Ornithocercus magnificus EU780649
Dinophysis caudata EU780644
DIN
Gymnodinium impudicum DQ785884
Gymnodinium impudicum DQ779992
Gymnodinium impudicum DQ779993
Lepidodinium viride DQ499645
Lepidodinium chlorophorum AY331681
Gymnodinium spec. AY999082
Gymnodinium aureolum AY999082
Gymnodinium aureolum FN392226
Gymnodinium catenatum DQ785883
Gymnodinium catenatum DQ785882
Gymnodinium catenatum DQ779989
Gymnodinium catenatum DQ779990
Paragymnodinium shiwhaense AM408889
GYM1
Karlodinium veneficum EF036540
Karlodinium veneficum AY245692
Karlodinium spec. FN357291
Karenia mikimotoi FJ587220, AM184206, EU165311
Karenia spec. DQ779991
GYM2
Akashiwo sanguinea AB232670
Akashiwo sanguinea AY831411
Akashiwo sanguinea AY831410
Akashiwo sanguinea AY831412
Akashiwo sanguinea DQ779987
Akashiwo sanguinea DQ779988
Protodinium simplex DQ388466, FJ823590,
Polarella glacialis EF417317, EU445333, AY036080 EF205015
Woloszynskia cincta FR690459
Symbiodinium goreaui EF036539,
Gyrodinium dominans FN669510 FJ823600, FJ939581
Noctiluca scintillans GQ380592
Perkinsus andrewsi AY305326
Perkinsus atlanticus AF509333
Theileria annulata NC_011129
Theileria parva NC_007344
Toxoplasma gondii X75453
SUE
3
1
"API"
0.1
0.1

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 *Dubosquodinium* 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.

## Acknowledgements

This study was performed together with Georgios Nikolaidis (Department of Botany, School of Biology, Aristotle University of Thessaloniki), who died during the course of the project. We are deeply saddened by the loss of our good friend and colleague. We would like to express our gratitude to Michael Ott (Munich) for help with using the HPC servers of the LMU. Hervé Philippe (Montreal) and two anonymous reviewers provided valuable suggestions to improve a previous draft of the manuscript which is acknowledged here. We thank the Deutsche Forschungsgemeinschaft (grants KE 322/36, PL 213/4, RI 1738/5, and WI 725/25) and the Münchener Universitätsgesellschaft for financial support.

## 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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# Chapter 5

## Ultrastructure of calcareous dinophytes (Thoracosphaeraceae, Peridiniales) with a focus on vacuolar crystal-like particles

**Zinssmeister, C.**, H. Keupp, G. Tischendorf, F. Kaulbars & M. Gottschling (2013) *PLoS One* **8**(1):e54038.

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# Ultrastructure of Calcareous Dinophytes (*Thoracosphaeraceae*, *Peridiniales*) with a Focus on Vacuolar Crystal-Like Particles

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## Abstract

Biomineralization in calcareous dinophytes (Thoracosphaeracaea, Peridiniales) takes place in coccoid cells and is presently poorly understood. Vacuolar crystal-like particles as well as collection sites within the prospective calcareous shell may play a crucial role during this process at the ultrastructural level. Using transmission electron microscopy, we investigated the ultrastructure of coccoid cells at an early developmental stage in fourteen calcareous dinophyte strains (corresponding to at least ten species of *Calciodinellum*, *Calcigonellum*, *Leonella*, *Pernambugia*, *Scrippsiella*, and *Thoracosphaera*). The shell of the coccoid cells consisted either of one (*Leonella*, *Thoracosphaera*) or two matrices (*Scrippsiella* and its relatives) of unknown element composition, whereas calcite is deposited in the only or the outer layer, respectively. We observed crystal-like particles in cytoplasmic vacuoles in cells of nine of the strains investigated and assume that they are widespread among calcareous dinophytes. However, similar structures are also found outside the Thoracosphaeraceae, and we postulate an evolutionarily old physiological pathway (possibly involved in detoxification) that later was specialized for calcification. We aim to contribute to a deeper knowledge of the biomineralization process in calcareous dinophytes.

**Citation:** Zinssmeister C, Keupp H, Tischendorf G, Kaulbars F, Gottschling M (2013) Ultrastructure of Calcareous Dinophytes (*Thoracosphaeraceae*, *Peridiniales*) with a Focus on Vacuolar Crystal-Like Particles. PLoS ONE 8(1): e54038. doi:10.1371/journal.pone.0054038

**Editor:** Vipul Bansal, RMIT University, Australia

**Received** September 6, 2012; **Accepted** December 7, 2012; **Published** January 8, 2013



**Funding:** Financial support was provided by the Deutsche Forschungsgemeinschaft (grants KE 322/30, KE 322/36, WI 725/18 and WI 725/25), and the Münchener Universitätsgesellschaft for equipment support. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Biomineralization is defined as the fundamental biological process by which living organisms produce minerals, often to harden or stiffen existing tissues or subcellular organic matrices. Mineralized structures have evolved multiple times independently and are taxonomically widely distributed over the tree of life. Subsequently, many similar cellular steps take place in distantly related lineages [1–4], and the resulting, occasionally complex crystal architectures may have multifunctional properties. The biomineralization process and its structural basis are well understood in metazoans including mollusks, corals, and vertebrates [5–7], but also in such protists as foraminifers and coccolithophores [8–10]. Mineralized structures are likewise found in some taxa of the unicellular Dinophyceae (Alveolata), where the mechanisms of crystal formation are largely elusive.

Many dinophytes develop at least two principally different stages during their life history: a phototrophic, motile cell (i.e., the theca, composed of cellulose plates that are formed in amphiesmal vesicles) and an immotile coccoid cell (commonly termed a 'cyst'). In the calcareous dinophytes (Thoracosphaeraceae, Peridiniales), it is particularly the shell of the coccoid cells that is mineralized by calcitic crystals [11–12]. Shell morphology and ultrastructure is diverse among calcareous dinophytes [13], and many species have been described, particularly from the fossil record [14–16]. As the potential to form calcareous structures is unique within the entire alveolates, it has been considered an apomorphic character trait supporting the monophyly of the Thoracosphaeraceae [17]. This assumption has gained some corroboration from molecular sequence data, although a number of (presumably secondarily) non-calcareous taxa might be also included in this group [18–20]. Molecular phylogenies segregate calcareous dinophytes into three main lineages, namely the E/Pe-clade (for *Ensiculifera* Balech, 1967 and *Pentapharsodinium* Indel. & A.R.Loebl.), the T/Pf-clade (for *Thoracosphaera* Kamptner and *Pfiesteria* Steid. & J.M.Burkh.), and *Scrippsiella* Balech ex A.R.Loebl. *sensu lato* (*s.l.*).

Since the early studies of dinophyte anatomy using transmission electron microscopy (TEM) [21–23], much progress has been made in understanding their complex and diverse organizations at the subcellular level. Much attention has been given to the 'apical furrow' system [24–25] and the flagellar apparatus [26–29], but the biomineralization process in calcareous dinophytes has not been the focus of such studies. Ultrastructure investigations into the subcellular components involved in biological processes such as the encystment of cells expand the basic data necessary for robust phylogenetic reconstructions.

Immature coccoid cells of *Scrippsiella minima* X.Gao & J.D.Dodge, in which the initial phase of mineralization takes place, are surrounded by two continuous matrices of unknown compounds that are delineated by an outer, middle, and inner unit

**Table 1.** Species list of TEM investigations (abbreviations: n.i., not indicated).

| Strain No. | Taxonomy | Species name with author | Locality | Lat. | Long. | Collector |
|---|---|---|---|---|---|---|
| GeoB 110 | *Scrippsiella* | *Calcigonellum infula* Deflandre, 1949 | Mediterranean Sea (Spain) | 41°21′N | 3°01′E | n.i. |
| SZN#74 | *Scrippsiella* | *Calciodinellum operosum* Deflandre, 1947 | Mediterranean Sea (Italy) | 40°43′N | 14°10′E | Montresor |
| GeoB 34 | *Scrippsiella* | *Calciodinellum* aff. *Operosum* Deflandre, 1947 | Middle Atlantic | 08°30′N | 32°27′W | n.i. |
| tub*2 | *Scrippsiella* | "*Calciodinellum*" spec. | Eastern South Pacific (Chile) | 28°15′S | 78°00′W | n.i. |
| GeoB 38 | T/Pf | *Leonella granifera* (D.Fütterer) Janofske & Karwath | Western Atlantic (Brazil) | 06°57′N | 47°54′W | n.i. |
| GeoB*61 | *Scrippsiella* | *Pernambugia tuberosa* (Kamptner) Janofske & Karwath | South Atlantic (Brazil) | 11°32′S | 28°35′W | n.i. |
| GeoB 411 | *Scrippsiella* | *Scrippsiella bicarinata* Zinssmeister, S.Soehner, S.Meier & Gottschling | Mediterranean Sea (Italy) | 41°15′N | 13°36′E | Gottschling, Zinßmeister & Söhner |
| GeoB*185 | *Scrippsiella* | *Scrippsiella trochoidea* (F.Stein) A.R.Loebl. | Baltic Sea (Germany) | 54°22′N | 10°09′E | Meier |
| GeoB 188 | *Scrippsiella* | *Scrippsiella* aff. *Trochoidea* (F.Stein) A.R.Loebl. | Mediterranean Sea (France) | 42°28′53′′N | 3°08′E | Gottschling |
| GeoB 283 | *Scrippsiella* | *Scrippsiella* aff. *trochoidea* (F.Stein) A.R.Loebl. | North Sea (Norway) | 63°28′N | 9°25′E | Gottschling & Petersen |
| GeoB 377 | *Scrippsiella* | *Scrippsiella* aff. *trochoidea* (F.Stein) A.R.Loebl. | Mediterranean Sea (Italy) | 40°40′N | 14°46′E | Gottschling, Zinßmeister & Söhner |
| M34*25/5 | *Scrippsiella* | *Scrippsiella* aff. *trochoidea* (F.Stein) A.R.Loebl. | South Atlantic (Guyana) | 11°54′N | 57°48′90′′W | n.i. |
| GeoB 228 | *Scrippsiella* | *Scrippsiella trochoidea* var. *aciculifera* Montresor | Mediterranean Sea (Italy) | 40°07′N | 17°19′E | n.i. |
| GeoB 211 | T/Pf | *Thoracosphaera heimii* (Lohmann) Kamptner | Eastern Mediterranean Sea | – | – | n.i. |

doi:10.1371/journal.pone.0054038.t001

membrane [30]. It has been assumed that calcification proceeds within the outer matrix, starting at protrusions composed of fibrous material. In coccoid cells of *Thoracosphaera heimii* (Lohmann) Kamptner, a single matrix develops, delineated by an outer and inner unit membrane [31–32]. The matrix is filled with numerous large, regularly arranged crystals in mature coccoid cells, while small, cylindrical seed crystals are found in immature coccoid cells. Inouye and Pienaar [31] have studied the crystallization process in *Th. heimii* in more detail and have discovered small and large cytoplasmic vesicles (or vacuoles) likewise containing cylindrical crystals. Such vacuoles may derive from the Golgi apparatus and actively transport seed crystals from the center towards the periphery of the cell. 'Crystal-like bodies' similar in appearance have been reported outside the calcareous dinophytes, including other Peridiniales [33–35] and Suessiales [24,36].

In this study, we investigate the ultrastructure of several calcareous dinophytes to document the subcellular structures that may play a role in biomineralization. Elbrächter and colleagues [37] have identified this field as one of the most serious gaps in knowledge in their Agenda Calcareous Dinophytes. We focus on immature coccoid cells because they are the likely stages, in which such structures can be observed. The study of (even immature) coccoid cells is challenging, as fixatives frequently do not penetrate the shell [30,38]. Nevertheless, we have found vacuoles containing crystal-like particles comparable to *Thoracosphaera* [31] also in other species. We aim to contribute to a more complete understanding of the biomineralization process in calcareous dinophytes.

## Materials and Methods

### Morphology

Fourteen calcareous dinophyte strains were collected and isolated from environmental samples (Table 1). They were cultivated in a climate chamber Percival I-36VL (CLF PlantClimatics; Emersacker, Germany) at 18°C or 23°C, 80 µmol photons $m^{-2}$ $s^{-1}$, and a 12:12 h light: dark photoperiod by using K-Medium without silicate [39] and 35 or 30 psu artificial seawater (hw marinemix professional: Wiegandt; Krefeld, Germany) at pH 8.2. 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.

Cultivated living cells were observed using an Olympus CKX41 inverse microscope, equipped with a Kappa camera DX 20H-FW (supplied with Calypso software). 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 (details are given in [40]). Samples were sputter-coated with platinum and documented with a LEO 438 VP (Zeiss) SEM.

### Transmission electron microscopy (TEM)

For TEM standard protocols, large thecate and coccoid cells (with a focus on early stages of the encystment) were fixed with 2.5% glutaraldehyde (Plano; Wetzler, Germany) in 0.2 M cacodylate buffer (Roth; Karlsruhe, Germany) and 0.25 M saccharose (Roth) at pH 8.0 for 1.5 h. An alternative protocol fixed the cells directly in cultivation media that were afterwards transferred to 0.2 M cacodylate buffer and 0.25 M saccharose at pH 8.0. After fixation, the cells were washed in 0.2 M $CaCO_3$

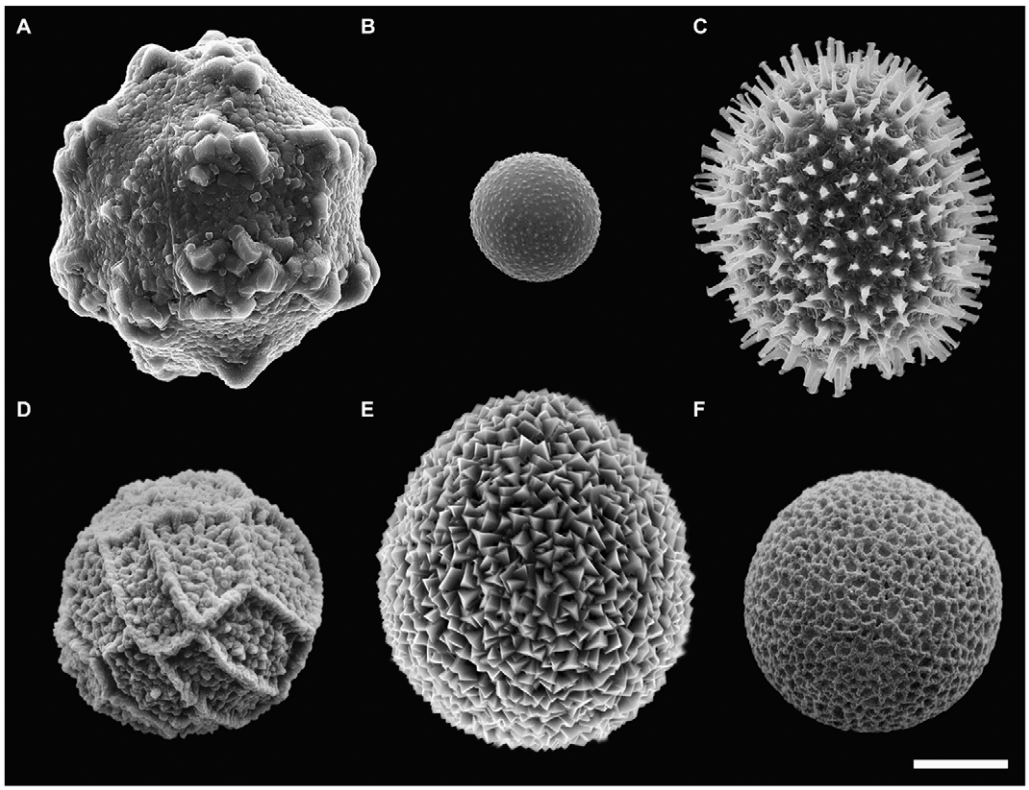


**Figure 1. Morphological diversity of calcareous coccoid cells.** (SEM). A: *Scrippsiella bicarinata* (GeoB 411, note the bicarinate tabulation resulting from the fusion of pre- and postcingular plate equivalents). B: *Thoracosphaera heimii* (CCCM 670, note the small size). C: *Scrippsiella trochoidea* (GeoB*185, note the spiny surface). D: *Calciodinellum operosum* (SZN#74, note the holotabulate tabulation). E: *Scrippsiella* aff. *trochoidea* (GeoB 283, note the spiny surface). F: *Calciodinellum* aff. *operosum* (GeoB 34, note the smooth surface). Scale bar: 10 μm.
doi:10.1371/journal.pone.0054038.g001

buffer at pH .0 in a graded saccharose series (0.125 M, 0.05 M, 0.025 M, 0.01 M, without) each for 15 min and post-fixed with 1% osmiumtetroxide (Science Services; Munich, Germany) in 0.2 M cacodylate buffer. Following the instructions of the Embedding Medi Kit (Science Services), samples were dehydrated in a graded ethanol (Roth) or acetone (Roth) series (30%, 50%, 70%, 90%, 100%, 100%, 100%), and gradually infiltrated and embedded in Spurr's resin [41].

The largest thecae and cells with a roundish form even as large as coccoid stages were selected for sectioning. Ultrathin sections were prepared with an ultra microtome (Leica EM UC6 Ultramikrotom). Sections were spread with 99% chloroform (Roth) and collected on copper 200 square mesh and 200 single bar grids (Plano) covered with collodium. Grids were stained with 1% aqueous uranylacetate (Plano) for 2 min and lead citrate (Plano) for 4 min [42]. TEM observations were done using a FEI Morgagni or a Zeiss EM 912.

## Results

### Cell morphology and life history

*Leonella granifera* (Fütterer) Janofske & Karwath and *Th. heimii* (both members of the T/Pf clade) produced mainly coccoid cells dividing vegetatively (thecate cells were rarely found under cultivation conditions). All investigated species of *Scrippsiella s.l.* (i.e., including also those of *Calcigonellum* Deflandre, 1949, *Calciodinellum* Deflandre, 1947, and *Pernambugia* Janofske & Karwath) developed golden brown, photosynthetically active thecate cells, which were always abundant under cultivation conditions because of vegetative division. The shape of the thecate cells was spherical to ovoid, with a rounded to conical apex. Early coccoid cells developed after shed of the theca by ecdysis and were darker than the thecate cells from the beginning. A red accumulation body was often already visible at this stage. Coccoid cells appeared first hyaline and darkened towards the brownish-opaque color at maturity after a few minutes.

Figure 1 shows a SEM image selection of the calcareous coccoid cell diversity investigated here with respect to their ultrastructure. Mature coccoid cells varied in size across species and were spherical to ovoid. The shell surface likewise differed between taxa, ranging from smooth without ornamental structures (*Th. heimii*: Fig. 1B) to reticulate (*Calciodinellum* aff. *operosum* Deflandre, 1947: Fig. 1F), spiny [*Scrippsiella trochoidea* (F.Stein) A.R.Loebl.: Fig. 1C,E9, imperfectly intratabulate (*Scrippsiella bicarinata* Zinssmeister, S.Soehner, S.Meier & Gottschling: Fig. 1A) to holotabulate (*C. operosum*: Fig. 1D). In some cases, remnants of an outer membrane covering the coccoid cell were present, and this membrane was always entirely intact in *Th. heimii* (Fig. 1B) and *L. granifera*.

### Comparative ultrastructure

Cell ultrastructure was largely similar in organization among different calcareous dinophyte species (Figs. 2–4). Thecate cells were always smaller than coccoid cells within particular strains. Thecal plates were surrounded by a unit membrane (Figs. 2A, 4A,E), which was visible particularly at their boundaries (Figs. 2C, 4E). Chloroplasts, mitochondria, and other compartments such as trichocysts were likewise present in all of the cells examined.

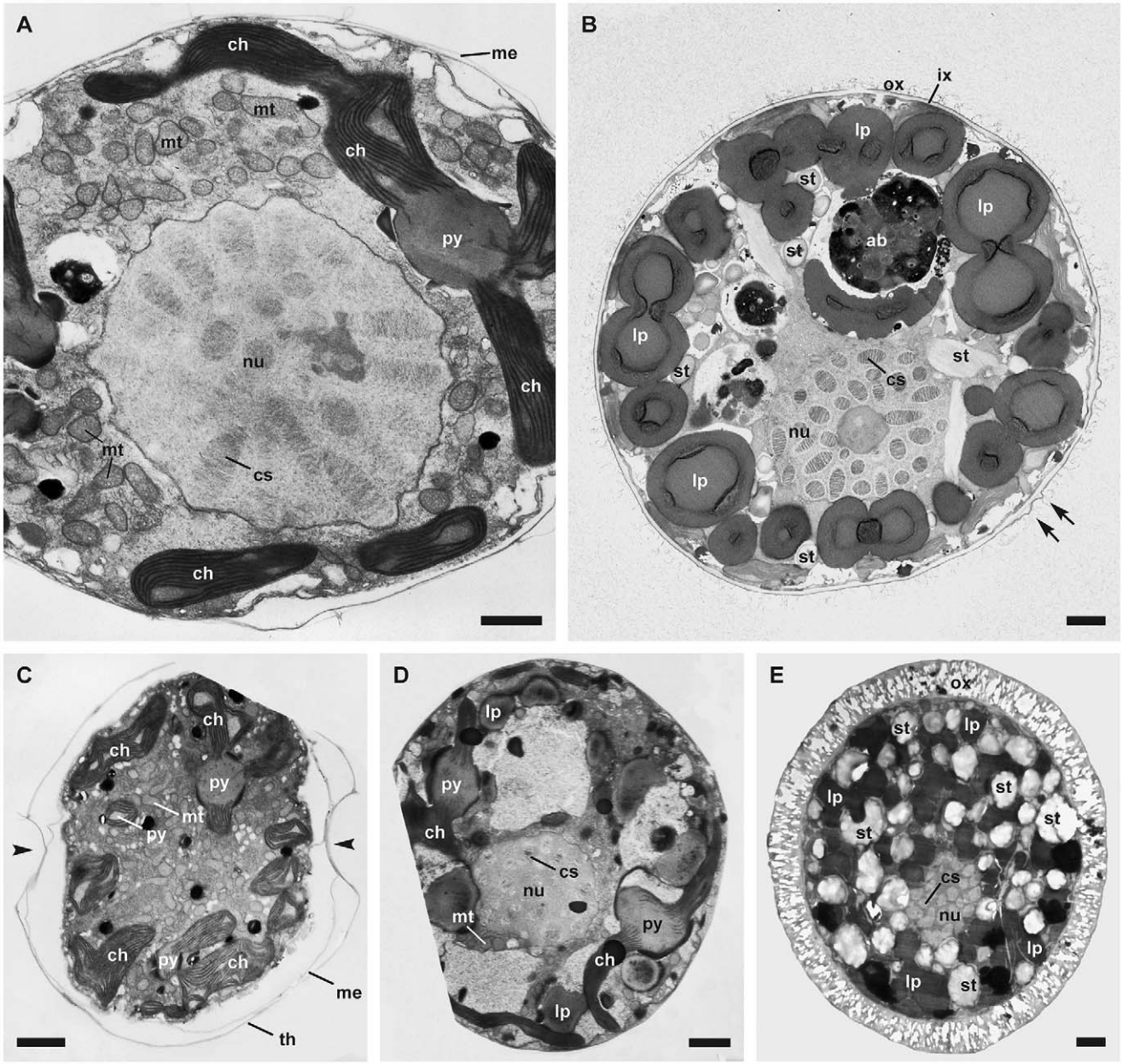


**Figure 2. General ultrastructure.** (TEM). A. Large thecate cell of *Scrippsiella bicarinata* (GeoB 411, note the peripheral chloroplast connected to a multiply-stalked pyrenoid). B. Early coccoid cell of *Pernambugia tuberosa* (GeoB*61, note the numerous peripheral lipid droplets, the two matrices surrounding the cell and the protrusions highlighted by arrows). C. Large thecate cell (longisection) of *Scrippsiella bicarinata* (GeoB 411, note the multiply-stalked pyrenoids and the numerous mitochondria in the center of the cell). D. Early coccoid cell of *Scrippsiella* aff. *trochoidea* (GeoB 283, note the chloroplasts with stalked pyrenoids and the lipid droplets). E. Mature coccoid cell of *Leonella granifera* (GeoB 38, note the numerous starch grains and lipid droplets and that the cell is surrounded by a single layer containing large, regularly arranged calcareous crystals). Abbreviations: ab, accumulation body; ch, chloroplast; cs, chromosomes; ix, inner matrix; lp, lipid droplet; nu, nucleus; mt, mitochondrion; ox, outer matrix; py, pyrenoid; st, starch grain. Scale bars: 2 μm.
doi:10.1371/journal.pone.0054038.g002

In all of the thecate cells investigated (Fig. 2), many large chloroplasts were present in peripheral positions. Moreover, different types of pyrenoids were found within those cells that showed structural associations with the chloroplasts. Some large chloroplasts constituted a network, as they were connected by multiply-stalked pyrenoids (Figs. 2A,C–D, 3A–B). Additionally, starch grains adjacent to pyrenoids were present (Fig. 3A). Smaller chloroplasts showed internally fusiform, interlamellar pyrenoids (Figs. 2A,C, 3B). Some chloroplasts were neither attached to pyrenoids nor to starch grains and were particularly small in size. (Figs. 3A, 5E). The thylakoid lamellae were more or less parallel to each other, an arrangement that was occasionally perturbed by the presence of pyrenoids (in which case the lamella fibers led into the pyrenoids). The thylakoids consisted of two to four lamellae (Figs. 2A, 3C, 4G).

Oval to elongated mitochondria with tubular cristae and surrounded by two unit membranes were visible (Fig. 3D). They were numerous and distributed all over the cytoplasm of thecate

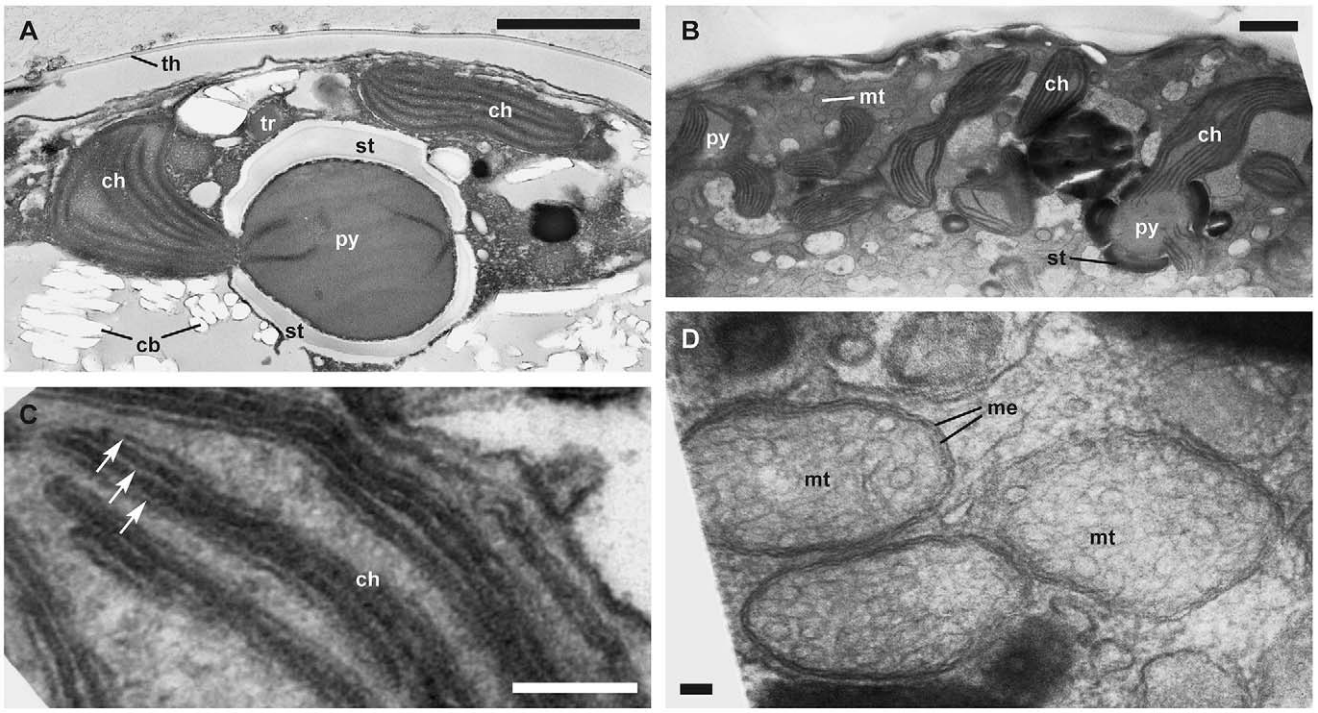


**Figure 3. Ultrastructural traits in detail.** (TEM). A. Multiply-stalked pyrenoid covered by a starch shed of *Calciodinellum* aff. *operosum* (GeoB 34, note the large, vacuolar crystal-like particles). B. Different chloroplast types of *Scrippsiella bicarinata* (GeoB 411, note that chloroplasts could be connected to multiply-stalked pyrenoids covered by a starch shed, or have interlamellar pyrenoids, with thylakoid lamellae leading through the pyrenoid. C. Two to four thylakoid lamellae (arrows) of *Scrippsiella bicarinata* (GeoB 411). D. Mitochondria with tubular cristae of *Scrippsiella* aff. *trochoidea* (GeoB 283). Abbreviations: ch, chloroplast; cb, crystal-like particle; me, membrane; mt, mitochondrion; py, pyrenoid; st, starch grain; tr, trichocyst. Scale bars: A and B 1 μm, C and D 0.1 μm.
doi:10.1371/journal.pone.0054038.g003

cells (Fig. 2A,C). The globose dinokaryon surrounded by two unit membranes was located close to the center of the cell and always showed condensed, rod-shaped chromosomes (Figs. 2A–B,D,E). The Golgi apparatus was located in the center of the cell close to the dinokaryon and consisted of a stack of few, flattened cisterns of the dictyosome. Golgi-derived vesicles were likewise visible near the dictyosome (Fig. 4G). A roundish accumulation body (Fig. 2B) was often present in large thecate cells and was always developed in coccoid cells.

In *S.* aff. *trochoidea*, a remarkably high number of lysozymes (Fig. 6C,D) were scattered throughout the cytoplasm and were even found within vesicles. The pusular system was formed by tubules of about 50 to 100 nm (Figs. 4A,H), and vesicles leading close to the flagellar base. Under the plasmalemma, cellulose plates were present that overlapped at their boundaries (Figs. 4A–E). The apical furrow system consisted of the apical pore plates, and the pore itself was covered by one such plate (Fig. 4A). The sulcal region consisted of few overlapping sulcal plates (Fig. 4C). Under the thecal plates, a few unit membranes were sometimes visible (Figs. 4, 6C,D). Microtubular fibers were located near the cell periphery, either as part of the cytoskeleton (Fig. 4A) or functioning as anchorage for the flagellar apparatus (Fig. 4D).

Before encystment and particularly in cells of the coccoid stage, size and number of chloroplasts decreased. Moreover, the number of starch grains and lipid droplets (both with a storage function) increased during encystment (Figs 2B,D) and in coccoid cells (Fig. 2E). At early stages of encystment, the species of the *Scrippsiella s.l.* lineage exhibited two layers, an outer and an inner organic matrix (Figs. 2B, 5A,C–D,F, 6C,D) of unknown composition. Between these two layers, small protrusions could occasionally be detected (Figs. 2B, 5F). By contrast, early coccoid cells of *L. granifera* (Fig. 2E) and *Th. heimii* (both belonging to the T/Pf-clade) were surrounded by a single organic matrix. Directly under this layer, protrusions similar in appearance to those found in *Scrippsiella s.l.* were present (Fig. 2B). Mature coccoid cells were usually not suitable for TEM, except those of *L. granifera* (Fig. 2E). The calcitic crystals deposited in the single matrix surrounding the cell were not preserved after the treatment with uranylacetate and lead citrate and were therefore recognized as empty space in the thin sections.

## Crystal-like particles and biomineralization

In nine of fourteen strains (corresponding to at least eight species), crystal-like particles were detected in encysting cells. They were found within cytoplasmic vacuoles, which were peripherally located in the cell body. Such vacuoles were variable in size and were particularly large in *Calcigonellum infula* Deflandre, 1949 (Fig. 5C), *C.* aff. *operosum* (Fig. 5F), and *S.* aff. *trochoidea* (Fig. 6C–D). The crystal-like particles in the vacuoles were between 50 to 380 nm in length and 24 to 86 nm in width. They had an elongated and cylindrical shape and were irregularly scattered with sometimes large gaps between each other. Only *C. operosum* (Fig. 5G) showed large, densely arranged crystal-like particles of varying size (330 to 1200 nm long and 70 to 470 nm wide) and shape, ranging from rod-like to cylindrical to square-cut. Crystal-like particles of *Pernambugia tuberosa* (Kamptner) Janofske & Karwath (Fig. 5D), *S. bicarinata* (Fig. 5E), *S. trochoidea* (Fig. 6A–B), *S. trochoidea* var. *aciculifera* Montresor (Fig. 5A), and *Th. heimii* (Fig. 5B) had an ovoid to barrel-like shape with blunt edges. In *C.*

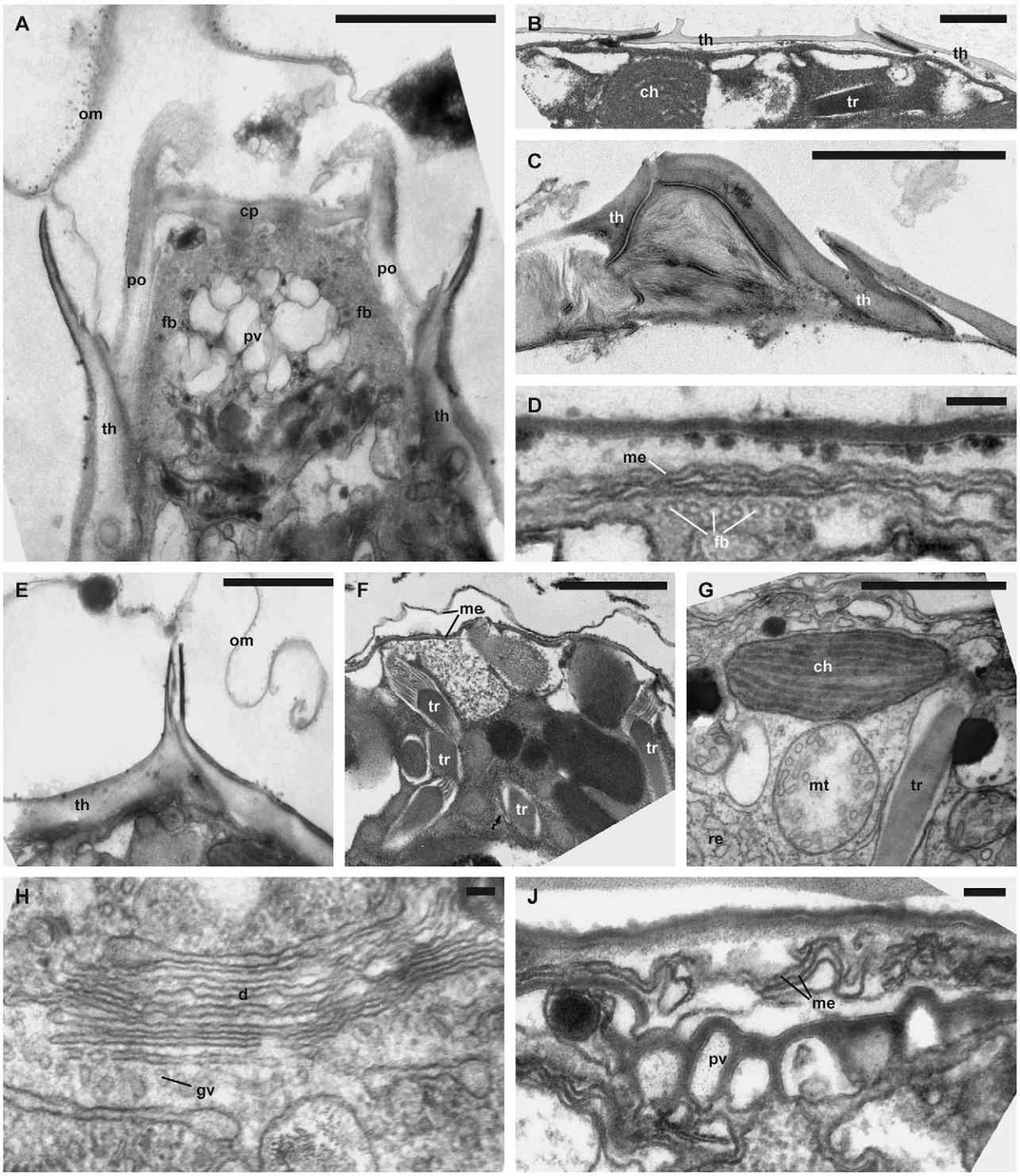


**Figure 4. Ultrastructural traits in detail.** (TEM). A. 'Apical furrow' system of *Scripsiella trochoidea* (GeoB 377, note the numerous vesicles under the cell surface). B. Overlapping thecal plates of *Scripsiella trochoidea* (GeoB 188, note the outer protrusions of overlapping theca plates). C. Overlapping theca plates in the sulcal region of ''*Calciodinellum*'' spec. (tub*2). D. Strand of peripheral microtubules in *Scrippsiella bicarinata* (GeoB 411, note the multiple membranes under the cell surface). E. Thecal plate boundary of *Scripsiella trochoidea* (GeoB 377, note the detached outer unit membrane). F. Trichocysts of *Scrippsiella trochoidea* var. *aciculifera* (GeoB 228). G. Subcellular organization of *Scrippsiella bicarinata* (GeoB 411, note the longisection of a trichocyst and the rough endoplasmatic reticulum indicated by an arrow). H. Golgi apparatus of *Scrippsiella trochoidea* (M34*25/5). J. Pusule of *Scrippsiella bicarinata* (GeoB 411, note the multiple membranes under the cell surface). Abbreviations: cb, crystal-like particle; ch, chloroplast; cp, cover plate; d, dictyosome, fb, microtubular fiber; gv, Golgi-derived vesicle; me, unit membrane; mt, mitochondrion; po, pore plate; pv, pusular vesicle; py, pyrenoid; re, rough endoplasmatic reticulum; st, starch grain; tr, trichocyst; th, thecal plate. Scale bars: A to C and E to G 1 μm, D, H, J 0.1 μm.

doi:10.1371/journal.pone.0054038.g004

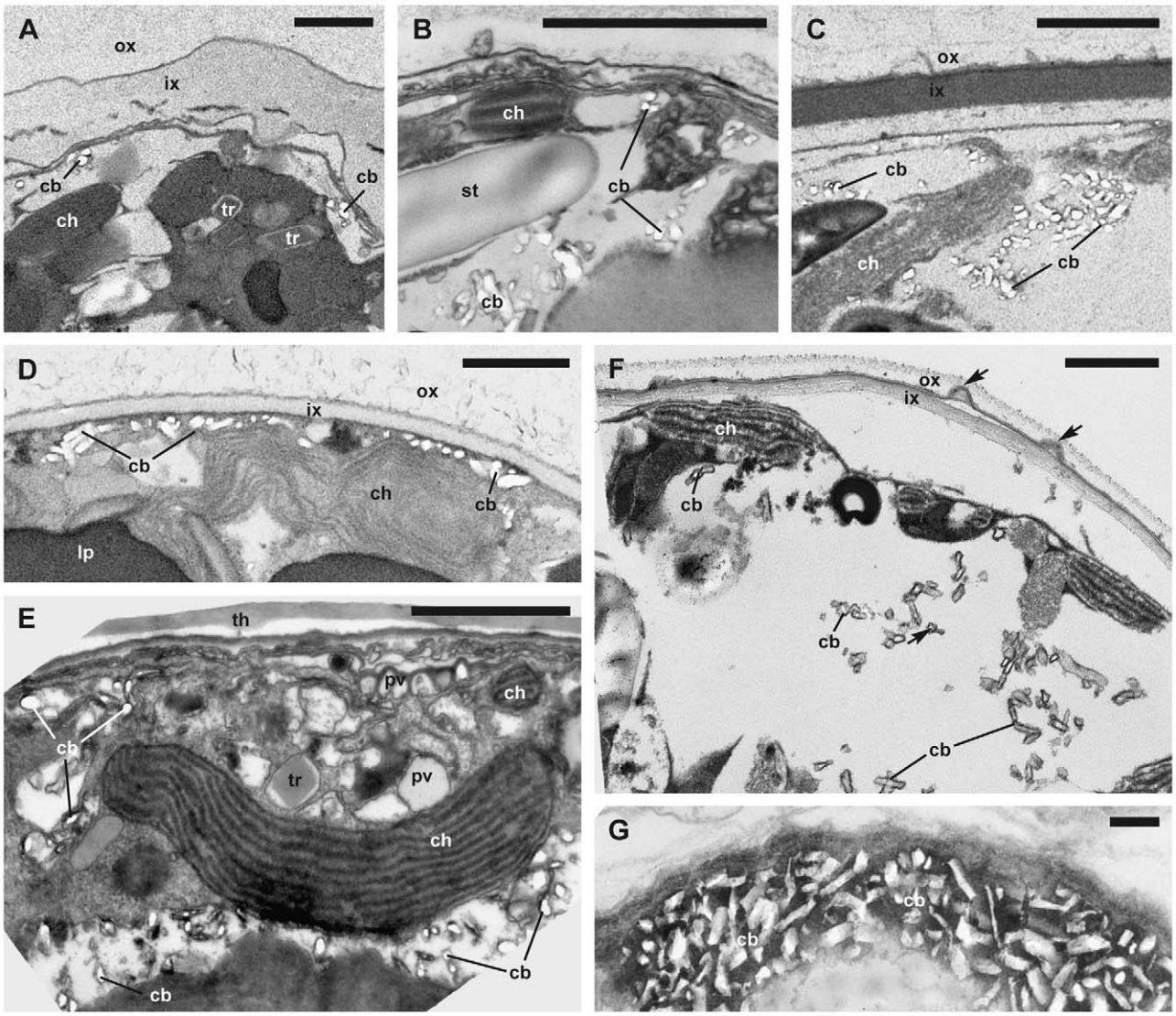


**Figure 5. Vacuolar crystal-like particles.** (TEM). A. *Scrippsiella trochoidea* var. *aciculifera* (GeoB 228, note the two matrices surrounding the cell, the middle unit membrane is partly disbanded). B. *Thoracosphaera heimii* (GeoB 211, note the single matrix that will calcify). C. *Calcigonellum infula* (GeoB*110, note the two matrices surrounding the cell). D. *Pernambugia tuberosa* (GeoB*61, note the two matrices surrounding the cell). E. *Scrippsiella bicarinata* (GeoB 411, early coccoid cell, the theca is still attached). F. *Calciodinellum* aff. *operosum* (GeoB 34, note the two matrices surrounding the cell and the protrusions between the outer and inner matrix indicated by arrows). G. *Calciodinellum operosum* (SZN#74, note the large crystal-like particles). Abbreviations: cb, crystal-like particle; ch, chloroplast; ix, inner matrix; ox, outer matrix; pv, pusular vesicle; py, pyrenoid; st, starch grain; tr, trichocyst; th, thecal plate. Scale bars: 1 μm.
doi:10.1371/journal.pone.0054038.g005

*infula* (Fig. 5C), *C.* aff. *operosum* (Fig. 5F), and *S.* aff. *trochoidea* (Fig. 6C–D), the shape of the crystal-like particles was square-cut.

The crystal-like particles showed some structural association to the vacuolar membrane (in, e.g., *P. tuberosa*: Fig. 5D and *S. trochoidea*: Fig. 6A–B) and/or internal membranes of the vacuoles (in, e.g., *S. bicarinata*: Fig. 5E and *Th. heimii*: Fig. 5B). In *S.* aff. *trochoidea* (Fig. 6C–D) and *C. operosum* (Fig. 5G), the crystals seemed to be present in higher number and surrounded by dense material of unknown origin, possibly unit membranes. A connection between membranes and crystal-like particles was not detected in *C. infula* (Fig. 5C) and *C.* aff. *operosum* (Fig. 5F), but it was not clear whether this was a distinctive character.

## Discussion

### Ultrastructure

Ultrastructure studies of coccoid dinophyte cells are still rare because of many methodological problems [30,38]. Nevertheless, they have great importance in better understanding the precise biological function of this specific developmental stage and gaining more basic data for phylogenetic reconstructions. Ecological stress such as temperature and light [43] and reduced availability of iron or nutrients may interfere with the interpretation of cellular ultrastructure [44–45]. We have aimed to avoid such bias by investigating cells held under constant culture conditions. Most of the cells studied here have shown subcellular details such as a reduced number of chloroplasts and an increased number of starch grains and lipid drops. These features have been interpreted

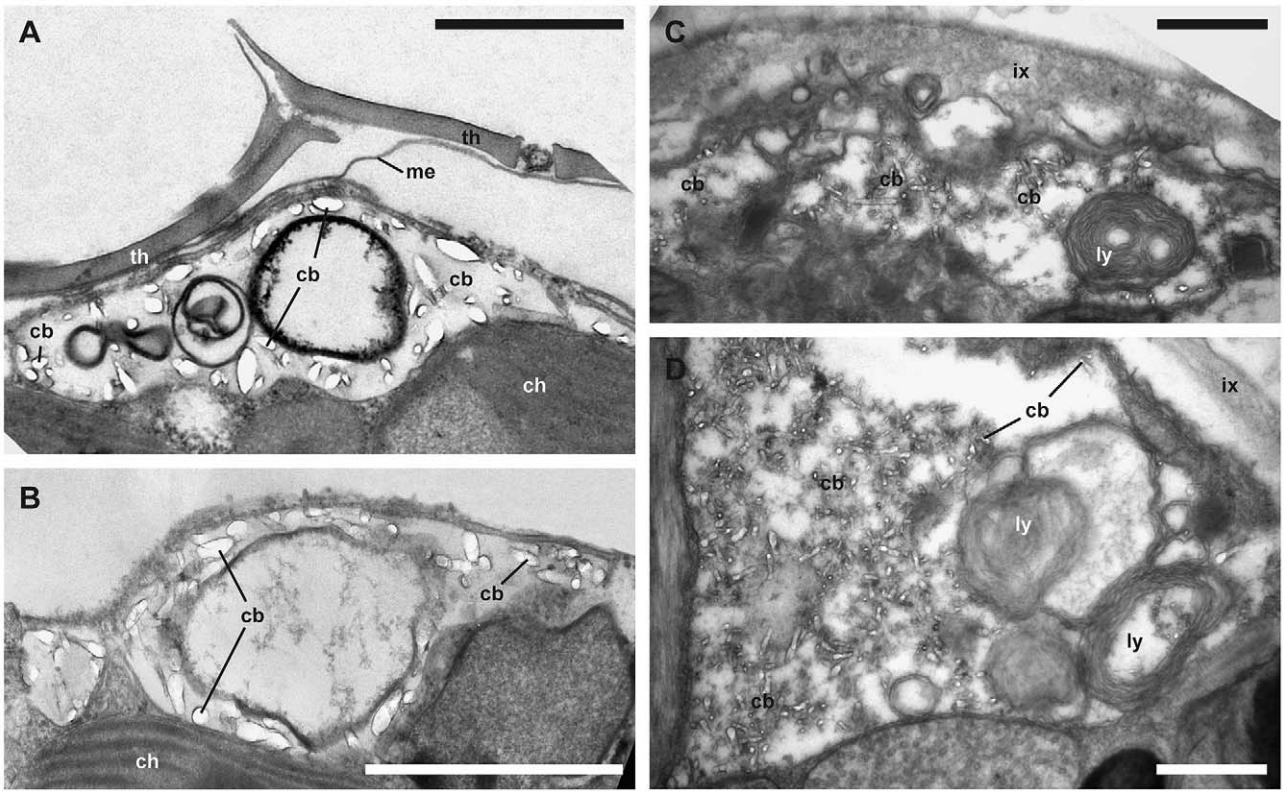


**Figure 6. Vacuolar crystal-like particles.** (TEM). A: *Scrippsiella trochoidea* (GeoB*185, early coccoid cell, the theca is still attached; note the intravacuolar vesicles). B. *Scrippsiella trochoidea* (GeoB*185). C. *Scrippsiella* aff. *trochoidea* (GeoB 283, note the lysozymes as intravacuolar membrane whorls). D. *Scrippsiella* aff. *trochoidea* (GeoB 283, note the lysozymes as intravacuolar membrane whorls). Abbreviations: cb, crystal-like particle; ch, chloroplast; ix, inner matrix; ly, lysosme; me, unit membrane; th, thecal plate. Scale bars: 1 μm.
doi:10.1371/journal.pone.0054038.g006

as typical indicators for encysting cells or (early) coccoid cells, respectively [27,30,38,46].

The number of layers constituting the shell of coccoid cells might have some phylogenetic significance [47]. *Thoracosphaera* [31–32] and *Leonella* Janofske & Karwath have a single matrix surrounding the cell (delineated by two unit membranes), while all extant species of *Scrippsiella* (including *S. minima* [30]) and its putative relatives such as *Calciodinellum* and *Pernambugia* share two layers (delineated by three unit membranes). These two types of shell architecture may correlate with molecular phylogenies, in which *Scrippsiella s.l.* and the T/Pf-clade represent two distinct lineages of the Thoracosphaeraceae [19–20]. As most peridinioid dinophytes exhibit the two-layer type [48–49], it is most plausible to assume that the one-layer type is apomorphic and today restricted to the T/Pf-clade. For future research, it is tempting to investigate the number of layers in coccoid cells of other members of the T/Pf-clade such as *Pfiesteria*.

For *S. minima*, Gao and co-workers [30] concentrated on mucofibrous material between the inner and the middle unit membranes surrounding the coccoid cell. This material is raised to form protrusions that are calcified in later stages of development. We have found structures similar in appearance in, for example, *Calciodinellum* and *Pernambugia*. They have also been documented (but neither described nor discussed) for *Thoracosphaera* [31] directly under the single matrix constituting the shell. Based on the relative position of such protrusions it is plausible to assume that the outer matrix in *Scrippsiella s.l.* is homologous to the single layer in the T/Pf-clade. To the best of our knowledge, protrusions of mucofibrous material as prerequisite for calcification [30] have not been reported outside the Thoracosphaeraceae, and it is therefore likely that they are apomorphic and play an important role during the biomineralization process.

In *Thoracosphaera*, calcification starts with the deposition of seed crystals in the single matrix present [31–32], while it is the outer matrix of *S. minima*, in which mineralization takes place [30]. This underlines once more the probable homology of both structures and is in accordance with the results presented here, as we never have observed crystal-like structures in an inner matrix. However, there are many fossil calcareous dinophytes known, particularly from the Mesozoic, with two distinct calcified layers that can be structurally differentiated [16,50]. A direct comparison to the extant species is impossible, since all these forms have become extinct.

The presence of vacuoles including crystal-like particles has been previously reported from *Scrippsiella sweeneyae* Balech ex A.R.Loebl. [51] and *Th. heimii* [31], and the latter authors have assumed a crucial role of those structures during biomineralization. A striking observation of the present study is that vacuolar crystal-like particles are abundant among calcareous dinophytes (records for seven more species). They have also been documented in *Tyrannodinium edax* (A.J.Schill.) Calado (= *Peridinium berolinense* Lemmerm. [35], a non-calcareous member of the T/Pf-clade [52]). However, vacuolar crystal-like particles have been sporadically reported (albeit under different names) from dinophytes (and also in thecate cells), but never have they been the focus of ultrastructural studies in a comparative approach. They have been found in the Gonyaulacales [53], Peridiniales [33–34], and

Suessiales [24,36,38,54], but their variation in size, shape, and subcellular distribution makes an overall homology unlikely.

## Chemistry and Function

Analytical chemistry of the crystal-like particles might likewise be indicative of their independent evolutionary origin, although the precise molecular composition based on ultimate analyses is rarely investigated. In the Suessiales, the vacuolar crystals with a characteristic rectangular shape are composed of calcium oxalate [55], while the bi-rhombohedral particles found in the Gonyaulacales contain guanine and other as yet unidentified components [56]. Conversely, the mature shell of calcareous dinophytes is composed of calcite elements, as it has been determined for *S. trochoidea* [12] and *Th. heimii* [57]. Inouye and Pienaar [31] have shown that the vacuolar crystal-like particles are sensitive to acid, and there is no reason to assume that they are not calcitic. For future research, the precise molecular composition of such particles found outside the Thoracosphaeraceae (in, e.g., *Galeidinium* Tam. & T.Horig. [34] and *Peridiniopsis* Lemmerm. [33]) is essential to reliably determine whether they are homologous across the Peridiniales.

Multiple functions of vacuolar crystal-like particles have been discussed. In the Suessiales, the mature elements are characteristically brick-like [24,36,58–59] and are associated with an eyespot in a regular arrangement of one to several rows [60–61]. Eyespots effectively absorb and reflect blue-green laser light [62] and are structurally connected to the flagellar apparatus. The support of these structures in locomotion has been therefore suggested [63]. More generally, the vacuoles containing crystal-like particles have been variously interpreted to be involved in the detoxification of the dinophyte cell [38,53,64]. Many calcifying organisms have access to corresponding physiological pathways to compose their aragonite and calcite structures [65–66]. Calcareous dinophytes may have thus modified the potential for detoxification to create calcitic shells for protection and/or as weight for sinking [67]. This specific function is particularly evident in such cavate coccoid cells of, for example, *Calcicarpinum* Deflandre, 1949 and *Posoniella* Streng, Banasová, Reháková & H.Willems. Such calcareous dinophytes are primarily found in surface sediments at coastal sites as the establishment of a dormancy seedbank [68–69].

## Conclusion

Compared to calcareous dinophytes, biomineralization in other unicellular organisms, such as the foraminifers and coccolithophores, has been more thoroughly investigated [9–10]. Foraminifers show principle differences in this process, as needle-like seed crystals are formed in vacuoles prior to the calcification of the shell (miliolid species), or not (hyaline species) [8]. At the ultrastructure level, the miliolid type somewhat resembles what is demonstrated for (calcareous) dinophytes in this and other studies. In coccolithophores, the crystallization process leading to the mature coccoliths takes place in Golgi-derived vesicles [10] moving from the cell center to the periphery.

The assumption that calcareous dinophytes have a similar calcification mechanism as coccolithophores has been postulated by Tangen and colleagues [32]. It is generally accepted that biomineralization in calcareous dinophytes also takes place under strong control at the cellular level [13,70–71]. Tabulation patterns that are reflected in the shell of the coccoid cells in at least some members of the Thoracosphaeraceae indicate that biomineralization is linked to amphiesmal vesicles constituting the thecal plates. In coccoid cells at an early developmental stage, calcitic seed crystals are formed in vesicles that probably derive from the Golgi apparatus. Such vesicles are transported to the cell periphery, and the seed crystals are deposited in the outer (or only) matrix surrounding the coccoid cell. They may accumulate at collection sites, where they are visible as protrusions of mucofibrous material (functioning as 'skeletons': [30]). However, it remains unclear at present how the seed crystals pass through the inner matrix in *Scrippsiella* and its relatives. More research on the life history, ultrastructure, and physiology is necessary, and high-spatial resolution analyses such as NanoSIMS, Raman spectroscopy, soft X-ray microscopy, and atomic force microscopy may be promising approaches in developing a comprehensive scenario for biomineralization in calcareous dinophytes.

## Acknowledgments

We thank Michael Schweikert (Stuttgart) for his valuable methodological advice, Silvia Dobler (Wanner lab, Munich) especially for her technical advice and the possibility to use facilities, and Heidemarie Gassler (Haszprunar lab, Munich) for her technical advice using the ultramicrotome and TEM. We further thank Julia Daum, Fernanda Fadel, Patricia Silva Flores, Caroline Müller (all Munich), Martina Rom-Roeske (Cologne) and Monika Kirsch (Bremen) for their assistance during cultivation of the strains. We are grateful for the help of Jared Lockwood (Munich), who thoroughly improved the English text.

## Author Contributions

Conceived and designed the experiments: CZ MG. Performed the experiments: CZ GT FK. Analyzed the data: CZ HK GT. Contributed reagents/materials/analysis tools: CZ HK GT FK MG. Wrote the paper: CZ MG.

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# General Discussion

## Recording the diversity of calcareous dinophytes

Broad attention has been drawn to biodiversity studies in recent years (Alexander et al., 2011; Beaugrand et al., 2010; Bianchi and Morri, 2000; Tittensor et al., 2010; Williams et al., 2010). Various approaches estimated the total number of species between three and 100 million (May, 2010; Mora et al., 2011; Strain, 2011). Data from many taxa are incomplete, and accepted scientific species names adjusted for synonymy has been missing, as shown on the homepage of the "World Register of Marine Species" (Appeltans et al., 2012). The number of described dinophytes is about 2,000 extant species and ca. 2,500 species based on the fossil record (Taylor et al., 2008). However, the diversity of dinophytes seems to be higher as new species are still being found (**Chapter 2**). This could only be investigated through field trips, preferably together with investigations of ecology and life history.

Generally, the diversity of marine dinophytes is particularly known from field trips using research vessels (Meier and Willems, 2003; Richter et al., 2007; Vink, 2004). So far, mostly pelagic habitats have been investigated and only a few studies have provided data from coastal waters and sediments. Sediment traps, which are found in harbors, have shown the presence of high calcareous dinophyte diversity (Gottschling and Kirsch, 2009). Therefore, a fast method using a self-made rocket-like bore probe for collecting sediment samples directly from coastal sites (Gottschling and Kirsch, 2009) has been applied in this project. This enabled a dense sampling in relatively short time with special focus on the Mediterranean Sea (**Chapter 1**).

The Mediterranean Sea has been one of the best-studied regions in the world in terms of biodiversity assessment, with approximately 25 (morpho-) species of calcareous dinophytes known from the region (Gomez, 2003; Meier et al., 2002; Montresor et al., 1998; Satta et al., 2010). Compared to the Gulf of Naples, where calcareous dinophyte diversity research has primarily taken place, other regions of the Mediterranean Sea have been under-sampled, such as the coast of Greece. Over half of the species recently described from the Mediterranean Sea and taxa known from the fossil record (**Chapter 1**) (such as *Calcigonellum infula* Deflandre, *Calciodinellum albatrosianum* (Kamptner) Janofske & Karwath, *C. levantinum* S.Meier, Janofske & H.Willems, and *C. operosum* Deflandre), as well as the genetically diverse *Scrippsiella* species (**Chapter 3**) were recovered in samples collected in my field work.

Additionally, two new species have been found (**Chapter 2**), as well as species that are rarely documented in sediments and known only from fossil records, such as *Calciperidinium bivalvum* G.Versteegh, *Follisdinellum* G.Versteegh (Montresor et al., 1998; Tommasa et al., 2004; Versteegh, 1993).

## Scientific, economic and public importance of dinophyte living collections

The cultivation of microorganisms, such as *Saccharomyces cerevisiae* for the production of beer, bread and wine, has been of economic importance and an essential tradition for centuries *(Legras et al., 2007)*. Aquacultures of unicellular algae have become an important source for bio-products such as bio-fuel *(Blackburn and Volkman, 2012)*. Herbaria that serve as repositories of land plant diversity cannot store algae in the same way. Constant access to fresh material is crucial for morphological and molecular investigations. This is of key importance for the identification and investigation of calcareous dinophytes isolated from sediments and plankton samples (**Chapter 1-4**). Therefore, the establishment of a culture collection that continuously provides fresh material has been one of the main goals of my project.

The isolation and establishment of algal strains from environmental samples is time-consuming and challenging. Reliable species identification is not always possible using a light microscope because of the fast movement of thecate cells and missing knowledge about their coccoid stage. Proper identification through successful proliferation and the establishment of a strain, or, ideally, a monoclonal culture without contamination, is the most reasonable way. However, thecate cells and living coccoid cells isolated from environmental samples have not always grown and developed, as with *Follisdinellum sp.*, a living fossil from the Mediterranean Sea (**Chapter 1**). Therefore, further research is needed to improve and optimize cultivation strategies for some sensitive and fossil-based species.

Calcareous dinophytes in cultivation have continuously developed both stages, the motile thecate cell and the calcareous coccoid cell. Only *Calcicarpinum bivalvum* stopped developing coccoid cells after a few months and started proliferating exclusively through thecate cells. Although some strains had been kept in cultivation over several years, they still showed seasonal adaptation with decreased growth and higher mortality during wintertime and an increased growth rate during spring and summer. Overall, I was successful in cultivating over 200 strains collected mostly by myself during field trips. These strains

consisted of species whose names are based on fossils, recently described species, and two new ones (**Chapter 1-5**). They provided the material for morphological investigations, with light, scanning- (**Chapter 1-4**) and transmission-electron microscopes (**Chapter 5**), life history observations (**Chapter 2 and 3**), and molecular analyses (**Chapter 1-4**).

## Morphological studies increase the number of characters in phylogenetic analyses and contribute to the description of new species

### Two new species

During the course of my project, two new species have been found and described, namely *Scrippsiella bicarinata* and *S. kirschiae* (**Chapter 2**). The general morphology of the motile cells and thecal plate patterns of *S. bicarinata* and *S. kirschiae* have been similar to those described for other species in the *Scrippsiella* s.l. clade. However, the coccoid cells showed unique shell characters, which enabled diagnosis and description of these two new species (**Chapter 2**). Both have been found in different localities on the Greek and Italian coasts. The Italian coast belongs to one of the best-studied regions of calcareous dinophytes diversity research. A reason why these species had not been recognized before could be that observations of phytoplankton diversity were based on open water, but not sediment samples. Water samples usually contain only thecate cells, which could have been misidentified as the already known *Scrippsiella* species. This is another indication that dinophyte diversity is likely to be much higher than observed by previous researchers, and that knowledge of the different life history stages by means of cultivation is necessary for proper identification.

### Phylogenetic position of *Bysmatrum sp.*

The systematic position of the benthic marine dinophyte *Bysmatrum* has been unclear (**Chapter 4**). *Bysmatrum* comprises five species that develop characteristic uncalcified coccoid cells. Previously, species of *Bysmatrum* have been assigned to *Scrippsiella* based on the tabulation patterns of its thecate cell (Faust and Steidinger, 1998). However, morphological investigations of character traits from motile stages also showed differences between *Scrippsiella*-like species and *Bysmatrum.* As described in my thesis, the surface of the cell is distinguished by an ornamented structure, for example reticulate in *Bysmatrum subsalsum,* the type of the genus, but not smooth as in *Scrippsiella*. The number of the theca plate patterns is similar to that in *Scrippsiella,* but the intercalary plates attach to each other and are separated through the 3’ plate. Additionally, *Bysmatrum* shows extended parts at some plates in the sulcal area.

The molecular phylogeny of dinophytes has confirmed the morphological distinction of *Bysmatrum sp.* from calcareous dinophytes such as *Scrippsiella* and allowed for its exclusion from the Thoracosphaeraceae (Gottschling et al., 2012; Jeong et al., 2012). The exact phylogenetic position remains unclear, but a close relationship to Gonyaulacales has been suggested (**Chapter 4**). Molecular results have placed *Bysmatrum* in an isolated phylogenetic position within the Dinophyta between Gonyaulacales and Peridiniales. Morphologically, the plate number and the specific formation of intercalary plates of *Bysmatrum* have suggested its placement within Peridiniales rather than in Gonyaulacales (Fensome et al., 1993). As it has been shown for *Bysmatrum*, tabulation patterns do not provide good morphological data for a perfect solution to characterize subgroups of the Dinophyta, and in those cases additional data are needed for an accurate classification.

**Species within *Scrippsiella* s.l.**

Species determination in dinophytes has been challenging, especially in the STR-SC clade (*Scrippsiella trochoidea* species complex), a subclade of *Scrippsiella* s.l. The STR-SC clade comprises genetically distinct, but morphologically indistinguishable cryptic species (Gottschling and Kirsch, 2009; Gottschling et al., 2005b; Montresor et al., 2003). All of them have shown character traits of *S. trochoidea* with the same tabulation patterns of the motile thecate cells and similar spiny and calcareous coccoid cells (**Chapter 1 and 3**). Thus, a molecular approach seems to be the only way to determine cryptic species within the STR-SC clade.

The STR-SC clade comprises seven distinct ribotypes of strains that represent cryptic species, all collected in the Mediterranean Sea (**Chapter 1**). Within the STR-SC clade, three main lineages of *S. trochoidea* have been distinguished, that have been labeled STR1, STR2, and STR3 in figure 1 (page 7). From those three, STR2 includes strain from a wide range of geographical localities, namely the Baltic Sea, the Mediterranean Sea, the eastern South Atlantic, the eastern South and the western North Pacific Ocean (Gottschling et al., 2005b). The geographic ranges of the strains in STR1 and STR3 partially overlap with STR2 (**Chapter 1**), which suggests that geographical correlations of these “cryptic species” are not possible.

Species delimitation has been a topic of constant debate among scientists, and various species concepts have been developed (de Queiroz, 2005; Hausdorf, 2011; Lee, 2003). An important approach to resolve species status within the STR-SC clade might be mating experiments, which have not been possible in the context and time frame of this project. Therefore, at this stage of dinophytes studies the most common biological species concept by Ernst Mayr (1904–2005) could be used for calcareous dinophytes. Mayr's definition of a species is a community of potentially reproductively non-isolated individuals within an ecological niche (Mayr, 1982, 1996; Sobel et al., 2010).

The question remains how speciation of closely related cryptic species within the STR-SC clade could have taken place resulting in their sympatric occurrence (**Chapter 1 and 3**). Differences in vegetative and sexual phases of their blooming in their natural habitats, for example as a result of temporal shift, could not be excluded. These phases have not been investigated within the STR-SC clades until now and could explain sympatric speciation based on differences in their sexual reproduction or blooming time. Such temporally shifted phases within STR-SC could not have been proved under different cultivation conditions, since strains produced all life history stages continuously. To answer the question of temporal shift in the life history of cryptic species in the future, samples of motile thecae of different subclades have to be collected from one location throughout the year. For proper identification of the subclades, molecular investigation of single cells or of monoclonal strains via barcoding is crucial.

Another explanation for the occurrence of molecularly different cryptic species at one locality is allopatric speciation followed by secondary contact. Such contact could have been possible through the transport of ships' ballast water, which has been already shown in several studies (Hallegraeff and Bolch, 1991, 1992; Morozova et al., 2011; Pertola et al., 2006; Ribeiro et al., 2012). This could also be a plausible scenario for dinophytes ensuring their rapid dispersal independently from sea currents. As shown in cultivation experiments, calcareous dinophytes can relatively easily adapt to different environmental conditions, such as temperature and salinity. Therefore, colonization of new marine habitats through ships' ballast water is possible.

## The importance of epitypification for unicellular organisms such as dinophytes.

Following the rules of the ICN, species names are based on a valid description and the link to type material. The physical types of unicellular organisms such as dinophytes consist mostly of an illustration or of glass slide preparations, for example as found in the Ehrenberg collection in Berlin (MfN), Germany. This should enable proper identification of a species, but in many cases type material has been lost or is in poor condition, which makes species identification problematic or impossible. In 1994, the ICBN has introduced the tool of designating an epitype in such cases as described in article 9.7 ICBN (Vienna code): *"An epitype is 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 of a taxon(…)"*(McNeill et al., 2006)

Although the option of epitypification has been a useful tool for replacement of lost or insufficient type material, relatively few studies have employed this method. Epitypification has been uncommon in most groups, with the majority of epitypified organisms being fungi (Hyde and Zhang, 2008; Voglmayr and Jaklitsch, 2011). It has been used only once before in dinophytes (Gonyaulacales) (Litaker et al., 2009), which shows a need for epitypification to clarify the correct application of species names within dinophytes.

One important candidate for epitypification within calcareous dinophytes has been *Scrippsiella trochoidea* (= *Glenodinium trochoideum*) (**Chapter 3**). The type material consists of a single illustration (von Stein, 1883), and physical type material was not located during my project. Furthermore, the name *Scrippsiella trochoidea* has been used for several cryptic species within the STR-SC clade (see above). For morphological and molecular re-investigation, living material was collected from the type locality, namely the Kiel Fjord (Germany) (**Chapter 3**). Cells of the established monoclonal strain GeoB*185 from the type locality were morphologically consistent with the protologue of *G. trochoideum*. The molecular phylogeny shows that *Scrippsiella trochoidea* is nested within the STR2 clade together with the strains originating from various geographical localities (see "Species within *Scrippsiella* s.l." chapter), suggesting that it is a geographically widespread taxon (**Chapter 3**).

Living cultures from the strain used for epitypification have been deposited in algae collections. This enables the continuous access to fresh material for further molecular, morphological or cultivation experiments. Following this example of the epitypification for *Scrippsiella trochoidea*, other problematic species names within the calcareous dinophytes such as *S. sweeneyae* Balech ex A.R.Loebl., the type species of *Scrippsiella* could be clarified in the future.

## Biomineralization process in calcareous dinophytes

Two main life history stages are morphologically recognizable in most calcareous dinophytes, namely the motile thecate cell and the immotile coccoid cell. However, ultrastructure investigations of the biomineralization process and development of the calcareous coccoid stage have been rare. Biomineralization is common in organisms (Raven and Giordano, 2009), and the process is well studied in other unicellular organisms such as foraminifers (de Nooijer et al., 2009) and coccolithophores (Young et al., 1999; Young and Henriksen, 2003). Within coccolithophores, the crystallization process takes place within Golgi-derived vesicles, with an increase of $Ca^{2+}$ ions (Mackinder et al., 2010). The calcareous crystals develop and grow within these vesicles until they are carried to their extracellular destination (Young and Henriksen, 2003).

The main reason for the lack of knowledge of biomineralization within calcareous dinophytes is connected to methodological problems (Bibby and Dodge, 1972; Gao et al., 1989). For example, in this thesis investigated *Scrippsiella* s.l. species, which have a coccoid stage where fixatives cannot not be injected without damaging the cell ultrastructure. Therefore, cells during encystment or in early coccoid stage without fully developed calcareous shells were investigated and fixed directly from cultivated strain material to avoid additional stress (**Chapter 5**). These cells showed characters considered typical for coccoid cells such as increased starch grains, lipid droplets and a decreased number of plastids (Calado et al., 2006; Gao et al., 1989). To understand the entire process, cells should be studied in all life history stages. Additionally, the physical properties of the calcareous shell have precluded scientists so far from observing its ultrastructure.

Despite the methodological difficulties, I was able to obtain and investigate ultrathin sections of cells from 14 calcareous dinophyte strains (representing 12 species of *Scrippsiella* s.l. and two of T/Pf-clade (**Chapter 5**). Early encystment stages of species belonging to T/Pf-clade such as *Thoracosphaera* and *Leonella* have shown a single layer surrounding the cell delineated by two unit membranes, while all investigated species of *Scrippsiella* s.l. (including *S. minima*: (Gao et al., 1989)) share two layers delineated by three unit membranes. The number of layers surrounding cells of the early encystment stage may have phylogenetic significance (Cox, 1971), as they correlate with the T/Pf-clade and *Scrippsiella* s.l. clade in molecular phylogenies (Gottschling et al., 2005a; Gottschling et al., 2012).

Vacuoles including crystal-like particles, which have been assumed to play a main role in the biomineralisation process, have been previously reported from *Scrippsiella sweeneyae* (Bibby and Dodge, 1974) and *T. heimii* (Inouye and Pienaar, 1983). Vacuoles containing particles of other chemical compounds such as oxalate and guanine are common and have been also documented from non-calcareous dinophytes (Wedemayer and Wilcox, 1984) and other non-peridinean dinophytes (Bibby and Dodge, 1972; Calado and Moestrup, 2002; Craveiro et al., 2010; Kremp et al., 2005; Lewis and Burton, 1988; Moestrup et al., 2009; Tamura et al., 2005). However, the sensitivity to acidic treatment of the crystal-like particles within calcareous dinophytes proved that they and the calcareous shell are composed of calcium carbonate. For a precise chemical determination of the crystal-like particles, element composition analyses could be used to prove if these crystal-like particles are calcitic and could be found in other dinophyte taxa also outside the calcareous dinophytes.

The function of vacuolar crystal-like particles seems to be diverse within the other groups of dinophytes. Characteristic brick-like crystals in Suessiales (Craveiro et al., 2010; Horiguchi and Pienaar, 1994; Kremp et al., 2005; Siano et al., 2010) have been associated with an eyespot function (Calado and Moestrup, 2005; Moestrup and Daugbjerg, 2007) connected to the flagellar apparatus and supporting the locomotion (Dodge, 1983). Other authors have interpreted this structure as being involved in detoxification of the dinophyte cell (Bibby and Dodge, 1972; Lewis and Burton, 1988; Pokorny and Gold, 1973). The change of the physiological pathways during their evolution, as described for other calcifying organisms (Carney et al., 2007; Simkiss, 1977), may have been likewise modified in calcareous dinophytes for protection and as sinking weight (Montresor et al., 1998).

Knowledge of the calcification process within calcareous dinophytes will be an important step in understanding the influence of changing climatic conditions. The effect of ocean acidification to various calcifying organisms such as molluscs, corals, foraminifers and coccolithophores has been investigated (Hofmann et al., 2010), but no studies on calcareous dinophytes have been conducted so far. In most investigated cases, organisms show a decreased potential to constitute their calcareous structure and subsequently a depressed growth (Hofmann et al., 2010). Whether calcareous dinophytes could adapt to changing pH conditions and whether their diversity can be maintained under ocean acidification remains unclear.

Until now, the effect of ocean acidification on host and parasitic relationships has been insufficiently investigated (MacLeod and Poulin, 2012). Increasing infection and diseases have been suggested to be connected with climatic changes (MacLeod and Poulin, 2012). Hosts weakened by ocean acidification are more susceptible, which leads to an increased parasitic growth and a serious threat to the host population. If the parasites are more vulnerable to changing conditions, natural regulation of host populations would be missing (MacLeod and Poulin, 2012). Ten percent of all dinophytes have developed a parasitic life style; some of which are calcareous dinophytes, and may be intolerant to ocean acidification. A possible adaptation could be to a vegetative life history with focus on the motile theca connected to a parasitic life style, as it is known from *Pentapharsodinium sp.* (Smith et al., 2007; Smith, 2011). Little is known about the influence of parasitic dinophytes on ecosystems and biodiversity and this should be the focus of further investigation in the context of climatic change.

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## Acknowledgements

An erster Stelle möchte ich meinem Doktorvater PD Dr. Marc Gottschling für die Betreuung meiner Doktorarbeit, seine immerwährende Bereitschaft Fragen zu beantworten, seine konstruktive Kritik, die schöne und produktive Zeit, sowohl während den Feldreisen als auch in der Systematischen Botanik und Mykologie, danken.

Ich möchte mich bei meinen Kollegen aus der AG Agerer, AG Heubl und AG Renner für die tolle gemeinsame Zeit im Institut beruflich als auch privat bedanken.

Ein Dank für die gemeinsame Zusammenarbeit gehört auch meinen Kollegen der AG Gottschling, Sylvia, Caro, Juliane, Julia, Patricia und Martina angefangen bei der Dinophyten Betreuung bis zu fachlichen Diskussionen.

Gleichzeitig möchte ich mich auch bei den kalkigen Dinophyten für ihr gutes Wachstum und ihre (unfreiwillige) Kooperationsbereitschaft bedanken, obwohl es mit ihnen nicht immer leicht war und so mancher Versuch nur ein Versuch blieb.

Für die tolle Zusammenarbeit, für Rat und Tat möchte ich Monika Kirsch, Dr. Eva Facher, PD Dr. Mike Schweikert, Dr. Malte Elbrächter, Prof. Dr. Helmut Keupp, Prof. Dr. Antonio Calado und Dr. Sandra Craveiro und den Co-Autoren einen Dank aussprechen.

Für ihre Unterstützung, konstruktive Kritik und vor allem ihre Freundschaft möchte ich besonders Jörg Bachmayr, Dr. Natalia Filipowicz (declare to nothing), Dr. Norbert Holstein (ohne dich ist die Mittagspause einfach nix!), Jens Chr. A. Köhler (best Admin forever!), Dr. Katharina Händeler (wir gehen zusammen tauchen, versprochen!), Steffanie Eggemann (der Berg ruft!), Müppi (echte Fründe…), Rossi, Robär; Yaron, Coco und für ihre Hilfe Jared Lockwood und Dr. Eva Facher herzlich danken.

Ein besonderer Dank für ihre Geduld und ihren Beistand geht an Nadja Welz, meine Familie, natürlich inklusive Iwan Bolis (Feigenplantage).

Für die Finanzierung meiner Doktorarbeit möchte ich der DFG und der LMU München danken.

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## Talks

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12/11: **Prize for best talk at 5. NOBIS-Austria Tagung Salzburg**: “The need for clarifying taxonomic identities in calcareous dinophytes (Thoracosphaeraceae, Peridiniales) such as *Scrippsiella trochoidea* (F. Stein) A.R. Loebl.”

02/11: **BioSystematics Berlin,** Germany: The taxonomic identity of *Scrippsiella trochoidea* (Thoracosphaeraceae, Dinophyceae), an ecologically important species of the marine phytoplankton

07/10: **Invited Seminar**: Systematic Botany and Mycology, LMU Munich: “With the rocket to the past - a long way to a phylogeny of calcareous dinoflagellates”

09/09: **Special course: Taxonomy of Recent Dinophyceae**, Dr. M. Elbrächter, List/Sylt, Germany: “Calcareous dinoflagellates (Thoracosphaeraceae, Dinophyta)”

02/07: **I. Simposio Biodiversidad en el occidente de Panama**, Universidad Autónoma de Chiriquí (UNACHI), Panama:“Diversity of Acochlidia (Mollusca, Gastropoda) in Panamá”

## Publications


Dinapoli, A., **Zinssmeister, C.** Klussmann-Kolb, A. (2011). "New insights into the phylogeny of the Pyramidellidae (Gastropoda)." *J Mollus Stud* **77**: 1–7.

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