

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

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

of calcareous dinophytes appears higher in the Mediterranean Sea than in other parts of the world's oceans such as the North Sea. Our data underline the importance of field work to record the diversity of calcareous dinophytes and other unicellular life forms.

Keywords Calcareous dinophytes · ITS · Ribotype · Cryptic species

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 (Peridinales) include all dinophytes that produce calcareous coccoid stages during their life history [important representative taxa are *Pentaparsodinium* Indel. & A.R.Loeb., *Scrippsiella* Balech ex A.R.Loeb. 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).

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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 *Claciadinellum 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.Loebli. [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 Thoracosphaeraceae 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, Zingone & 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ 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 Kofoidian 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.

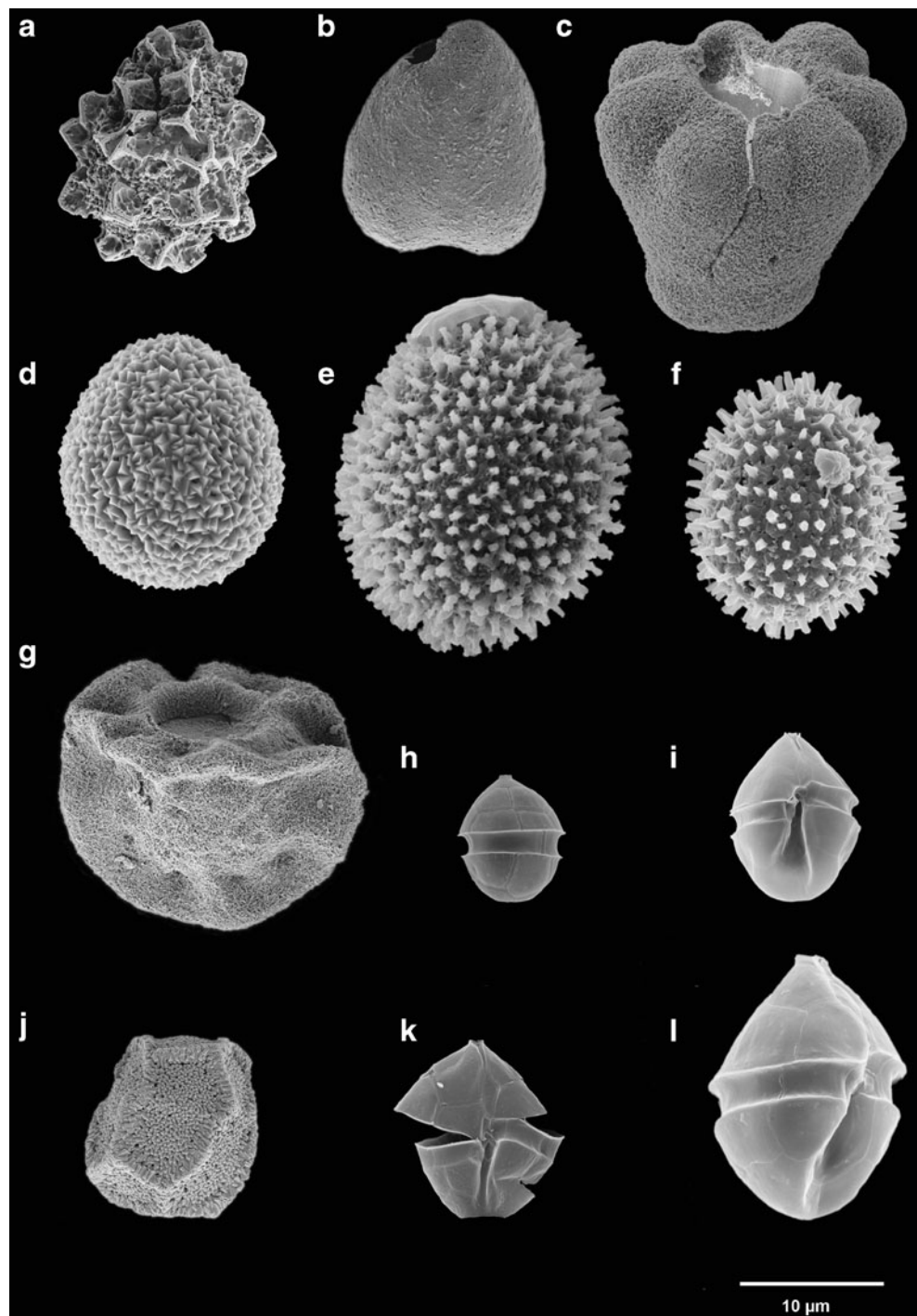
From the recent Mediterranean samples, eight different ribotypes were assigned to the STR-SC. All available sequences clustering within the three distinct clades of the STR-SC (i.e. STR1, STR2 and STR3) were included in the analysis and the clades were each analysed separately. In the STR1 clade, four 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

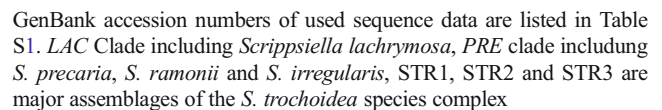
In recent years, much effort has been devoted to the documentation of marine biodiversity (Beaugrand et al. 2010; Tittensor et al. 2010; Williams et al. 2010; <http://www.coml.org>); however, exact species numbers and correct scientific names are still needed for many marine organisms. This is particularly 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 paleo-environmental 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** coocoid 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



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 Thoracosphaeraceae (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 relevance 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, morphological 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 repeatedly 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 Thoracosphaeraceae species present in the Mediterranean Sea (D’Onofrio et al. 1999; Montresor et al. 2003; Gottschling et al. 2005a; Penna et al. 2010; Zinssmeister et al. 2011), and the completion of our studies has importance also for future taxonomic work. For example, *S. precaria* has been described from the Gulf of Naples (Montresor and Zingone 1988), but sequences of this species from the Mediterranean Sea have been not published so far. The establishment of a new strain collected close to the type locality and its subsequent molecular characterisation as presented here might contribute to disentangle the complex alpha-taxonomy of calcareous dinophytes. Moreover, two new *Scrippsiella* species have been described morphologically and included in a molecular phylogeny (Zinssmeister et al. *in press*).

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

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References

- Attaran-Fariman, G., & Bolch, C. J. S. (2007). *Scrippsiella irregularis* sp. nov. (Dinophyceae), a new dinoflagellate from the southeast coast of Iran. *Phycologia*, 46, 572–582.
- Beaugrand, G., Edwards, M., & Legendre, L. (2010). Marine biodiversity, ecosystem functioning, and carbon cycles. *Proceedings of the National Academy of Sciences*, 107, 10120–10124.
- CBOL Plant Working Group. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences*, 106(31), 12794–12797.
- Clement, M. J., Posada, D., & Crandall, K. A. (2000). TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659.
- D'Onofrio, G., Marino, D., Bianco, L., Busico, E., & Montresor, M. (1999). Toward an assessment on the taxonomy of dinoflagellates that produce calcareous cysts (Calciodinelloideae, Dinophyceae): A morphological and molecular approach. *Journal of Phycology*, 35, 1063–1078.
- Daniels, S. R., & Ruhberg, H. (2010). Molecular and morphological variation in a South African velvet worm *Peripatopsis moseleyi* (Onychophora, Peripatopsidae): Evidence for cryptic speciation. *Journal of Zoology*, 282, 171–179.
- Deflandre, G. (1947). *Calciodinellum* nov. gen., premier représentant d'une famille nouvelle de Dinoflagellés fossiles à theque calcaire. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences*, 224, 1781–1782.
- Deflandre, G. (1949). Les Calciodinellidés. Dinoflagellatés fossiles à thèque calcaire. *Le Botaniste*, 34, 191–219.
- Elbrächter, M., Gottschling, M., Hildebrand-Habel, T., Keupp, H., Kohring, R., Lewis, J., et al. (2008). Establishing an agenda for calcareous dinoflagellate research (Thoracosphaeraceae, Dinophyceae) including a nomenclatural synopsis of generic names. *Taxon*, 57, 1289–1303.
- Esper, O., Versteegh, G. J. M., Zonneveld, K. A. F., & Willems, H. (2004). A palynological reconstruction of the Agulhas Retroflection (South Atlantic Ocean) during the Late Quaternary. *Global and Planetary Change*, 41, 31–62.
- Feau, N., Vialle, A., Allaire, M., Tanguay, P., Joly, D. L., Frey, P., et al. (2009). Fungal pathogen (mis-)identifications: A case study with DNA barcodes on *Melampsora* rusts of aspen and white poplar. *Mycological Research*, 113, 713–724.
- Fensome, R. A., & Williams, G. L. (2004). *The Lentin and Williams index of fossil dinoflagellates*. College Park: American Association of Stratigraphic Palynologists.
- Fensome, R. A., Taylor, F. J. R., Norris, G., Sarjeant, W. A. S., Wharton, D. I., & Williams, G. L. (1993). A classification of living and fossil dinoflagellates. *Micropaleontology Special Publication Number*, 7, 1–245.
- Fensome, R. A., Saldarriaga, J. F., & Taylor, F. J. R. (1999). Dinoflagellate phylogeny revisited: Reconciling morphological and molecular based phylogenies. *Grana*, 38, 66–80.
- Genovesi, B., Shin-Grzerbyk, M., Grzerbyk, D., Laabir, M., Gagnaire, P., Vaquer, A., et al. (2011). Assessment of cryptic species diversity within blooms and cyst bank of the *Alexandrium tamarense* complex (Dinophyceae) in a Mediterranean lagoon facilitated by semi-multiplex PCR. *Journal of Plankton Research*, 33, 405–414.
- Godhe, A., Norén, F., Kuylensstierna, M., Ekberg, C., & Karlson, B. (2001). Relationships between planktonic dinoflagellate abundance, cysts recovered in sediment traps and environmental factors in the Gullmar Fjord, Sweden. *Journal of Plankton Research*, 23, 923–938.
- Gómez, F. (2003). Checklist of Mediterranean free-living dinoflagellates. *Botanica Marina*, 46, 215–242.
- Gottschling, M., & Kirsch, M. (2009). Annotated list of Scandinavian calcareous dinoflagellates collected in fall 2003. *Berliner Paläobiologische Abhandlungen*, 10, 193–198.
- Gottschling, M., Keupp, H., Plötner, J., Knop, R., Willems, H., & Kirsch, M. (2005a). Phylogeny of calcareous dinoflagellates as inferred from ITS and ribosomal sequence data. *Molecular Phylogenetics and Evolution*, 36, 444–455.
- Gottschling, M., Knop, R., Plötner, J., Kirsch, M., Willems, H., & Keupp, H. (2005b). A molecular phylogeny of *Scrippsiella sensu lato* (Calciodinellaceae, Dinophyta) with interpretations on morphology and distribution. *European Journal of Phycology*, 40, 207–220.
- Gottschling, M., Soehner, S., Zinssmeister, C., John, U., Plötner, J., Schweikert, M., et al. (2012). Delimitation of the Thoracosphaeraceae (Dinophyceae), including the calcareous dinoflagellates, based on large amounts of ribosomal RNA sequence data. *Protist*, 163, 15–24.
- Gu, H.-F., Sun, J., Kooistra, W. H. C. F., & Zeng, R. (2008). Phylogenetic position and morphology of thecae and cysts of *Scrippsiella* (Dinophyceae) species in the East China Sea. *Journal of Phycology*, 44, 478–494.
- Harper, J. T., Waanders, E., & Keeling, P. J. (2005). On the monophyly of chromalveolates using a six-protein phylogeny of eukaryotes. *International Journal of Systematic and Evolutionary Microbiology*, 55, 487–496.
- Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Science*, 270, 313–321.
- Hebert, P. D. N., Stoeckle, M. Y., & Zemlak, C. M. F. (2004). Identification of birds through DNA barcodes. *PLoS Biology*, 2(10), e312.
- Horton, T. R., & Bruns, T. D. (2001). The molecular revolution in ectomycorrhizal ecology: Peeking into the black-box. *Molecular Ecology*, 10, 1855–1871.
- Janofske, D. (1992). Kalkiges Nannoplankton, insbesondere Kalkige Dinoflagellaten-Zysten der alpinen Ober-Trias: Taxonomie, Biostratigraphie und Bedeutung für die Phylogenie der Peridinales. *Berliner Geowissenschaftliche Abhandlungen (E)*, 4, 1–53.
- Janofske, D. (2000). *Scrippsiella trochoidea* and *Scrippsiella regalis*, nov. comb. (Peridinales, Dinophyceae): A comparison. *Journal of Phycology*, 36, 178–189.
- Karwath, B. (2000). Ecological studies on living and fossil calcareous dinoflagellate of the equatorial and tropical Atlantic Ocean. *Berichte, Fachbereich Geowissenschaften, Universität Bremen*, 152, 1–175.
- Keller, M. D., Selvin, R. C., Claus, W., & Guillard, R. R. L. (1987). Media for the culture of oceanic ultraphytoplankton. *Journal of Phycology*, 23, 633–638.
- Kress, W. J., Wurdack, K. J., Zimmer, E., Weight, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences*, 102, 8369–8374.
- Leander, B. S., & Keeling, P. J. (2004). Early evolutionary history of dinoflagellates and apicomplexans (Alveolata) as inferred from hsp90 and actin phylogenies. *Journal of Phycology*, 40, 341–350.

- Lewis, J. (1991). Cyst-theca relationships in *Scrippsiella* (Dinophyceae) and related orthoperidinioid genera. *Botanica Marina*, 34, 91–106.
- Lilly, E. L., Halanaych, K. M., & Anderson, D. M. (2007). Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). *Journal of Phycology*, 43, 1329–1338.
- Lin, S., Zhang, H., Hou, Y., Zhuang, Y., & Miranda, L. (2009). High-level diversity of dinoflagellates in the natural environment, revealed by assessment of mitochondrial *cox1* and *cob* genes for dinoflagellate DNA barcoding. *Applied and Environmental Microbiology*, 75(12), 1279–1290.
- Litaker, R. W., Vandersea, M. W., Kibler, S. R., Reece, K. S., Stokes, N. A., Lutzoni, F. M., et al. (2007). Recognizing dinoflagellate species using ITS rDNA sequences. *Journal of Phycology*, 43, 344–355.
- Lo, E. Y. Y., Stefanovic, S., & Dickinson, T. A. (2010). Reconstructing reticulation history in a phylogenetic framework and the potential of allopatric speciation driven by polyploidy in an agamic complex in *Crataegus* (Rosaceae). *Evolution*, 64, 3593–3608.
- Loeblich, A. R. III (1976). Dinoflagellate evolution: Speculation and evidence. *Journal of Protozoology*, 23(1), 13–28.
- Meier, K. J. S., & Willems, H. (2003). Calcareous dinoflagellate cysts in surface sediments from the Mediterranean Sea: Distribution patterns and influence of main environmental gradients. *Marine Micropaleontology*, 48, 321–354.
- Meier, K. J. S., Janofske, D., & Willems, H. (2002). New calcareous dinoflagellates (Calciodinelloideae) from the Mediterranean Sea. *Journal of Phycology*, 38, 602–615.
- Meier, K. J. S., Höll, C., & Willems, H. (2004). Effect of temperature on culture growth and cyst production in the calcareous dinoflagellates *Calciodinellum albatrosianum*, *Leonella granifera* and *Pernambugia tuberosa*. *Micropaleontology*, 50, 93–106.
- Meier, K. J. S., Young, J. R., Kirsch, M., & Feist-Burkhardt, S. (2007). Evolution of different life-cycle strategies in oceanic calcareous dinoflagellates. *European Journal of Phycology*, 42, 81–89.
- Montresor, M. (1995). *Scrippsiella ramonii* sp. nov. (Peridinales, Dinophyceae), a marine dinoflagellate producing a calcareous resting cyst. *Phycologia*, 34(1), 87–91.
- Montresor, M., & Zingone, A. (1988). *Scrippsiella precaria* spec. nov. (Dinophyceae), a marine dinoflagellate from the Gulf of Naples. *Phycologia*, 27, 387–394.
- Montresor, M., Zingone, A., & Marino, D. (1993). The calcareous resting cyst of *Pentaparsodinium tyrrhenicum* comb. nov. (Dinophyceae). *Journal of Phycology*, 29, 223–230.
- Montresor, M., Montesarchio, E., Marino, D., & Zingone, A. (1994). Calcareous dinoflagellate cysts in marine sediments of the Gulf of Naples (Mediterranean Sea). *Review of Palaeobotany and Palynology*, 84, 45–56.
- Montresor, M., Janofske, D., & Willems, H. (1997). The cyst-theca relationship in *Calciodinellum operosum* emend. (Peridinales, Dinophyceae) and a new approach for the study of calcareous cysts. *Journal of Phycology*, 33, 122–131.
- Montresor, M., Zingone, A., & Sarno, D. (1998). Dinoflagellate cyst production at a coastal Mediterranean site. *Journal of Plankton Research*, 20, 2291–2312.
- Montresor, M., Sgroso, S., Procaccini, G., & Kooistra, W. H. C. F. (2003). Intraspecific diversity in *Scrippsiella trochoidea* (Dinophyceae): Evidence for cryptic species. *Phycologia*, 42, 56–70.
- Peng, Y. Y., Baum, B. R., Ren, C. Z., Jiang, Q. T., Chen, G. Y., Zheng, Y. L., et al. (2010). The evolution pattern of rDNA ITS in *Avena* and phylogenetic relationship of the *Avena* species (Poaceae: Aveneae). *Hereditas*, 147, 183–204.
- Penna, A., Battocchi, C., Garcés, E., Anglès, S., Cucchiari, E., Totti, C., et al. (2010). Detection of microalgal resting cysts in European coastal sediments using a PCR-based assay. *Deep Sea Research Part II: Topical Studies in Oceanography*, 57, 288–300.
- Persson, A., Godhe, A., & Karlson, B. (2000). Dinoflagellate cysts in recent sediments from the west coast of Sweden. *Botanica Marina*, 43, 69–79.
- Posada, D., & Crandall, K. A. (2001). Intraspecific gene genealogies: Trees grafting into networks. *Trends in Ecology & Evolution*, 16, 37–45.
- Rizzo, P. J. (2003). Those amazing dinoflagellate chromosomes. *Cell Research*, 13, 215–217.
- Satta, C. T., Anglès, S., Garcés, E., Lugliè, A., Padedda, B. M., & Sechi, N. (2010). Dinoflagellate cysts in recent sediments from two semi-enclosed areas of the Western Mediterranean Sea subject to high human impact. *Deep Sea Research Part II: Topical Studies in Oceanography*, 57, 256–267.
- Stein, F. (1883). Die Naturgeschichte der arthrodelen Flagellaten. Der Organismus der Infusionstiere. III. Pt. 2., 1–30.
- Stern, R. F., Horak, A., Andrew, R. L., Coffroth, M.-A., Andersen, R. A., Küpper, F. C., et al. (2010). Environmental barcoding reveals massive dinoflagellate diversity in marine environments. *PLoS One*, 5(11), e13991.
- Stern, R. F., Andersen, R. A., Jameson, I., Küpper, F. C., Coffroth, M.-A., Vaulot, D., et al. (2012). Evaluating the ribosomal internal transcribed spacer (ITS) as candidate dinoflagellate barcode marker. *PLoS One*, 7(8), e42780.
- Streng, M., Hildebrand-Habel, T., & Willems, H. (2004). A proposed classification of archeopyle types in calcareous dinoflagellate cysts. *Journal of Paleontology*, 78, 456–483.
- Tautz, D., Arctander, P., Minelli, A., Thomas, R. H., & Vogler, A. P. (2003). A plea for DNA taxonomy. *Trends in Ecology & Evolution*, 18(2), 70–74.
- Taylor, F. J. R. (1980). On dinoflagellate evolution. *Biosystems*, 13, 65–108.
- Tittensor, D. P., Mora, C., Jetz, W., Lotze, H. K., Ricard, D., Vanden Bergh, E., et al. (2010). Global patterns and predictors of marine biodiversity across taxa. *Nature*, 466, 1098–1101.
- Tommasa, L. D., Danovaro, R., Belmonte, G., & Boero, F. (2004). Resting stage abundance in the biogenic fraction of surface sediments from the deep Mediterranean Sea. *Scientia Marina*, 68, 103–111.
- Versteegh, G. (1993). New Pliocene and Pleistocene calcareous dinoflagellate cysts from southern Italy and Crete. *Review of Palaeobotany and Palynology*, 78, 353–380.
- Vink, A. (2004). Calcareous dinoflagellate cysts in South and equatorial Atlantic surface sediments: diversity, distribution, ecology and potential for palaeoenvironmental reconstruction. *Marine Micropaleontology*, 50, 43–88.
- Wall, D., & Dale, B. (1966). "Living fossils" in Western Atlantic plankton. *Nature*, 211, 1025–1026.
- Wall, D., & Dale, B. (1968). Quaternary calcareous dinoflagellates (Calciodinelloideae) and their natural affinities. *Journal of Paleontology*, 42, 1395–1408.
- Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Science*, 360, 1847–1857.
- White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols: A guide to methods and amplifications* (pp. 315–322). New York: Academic.
- Williams, M. J., Ausubel, J., Poiner, I., Garcia, S. M., Baker, D. J., Clark, M. R., et al. (2010). Making marine life count: a new baseline for policy. *PLoS Biology*, 8(11), e1000531.
- Zinssmeister, C., Soehner, S., Facher, E., Kirsch, M., Meier, K. J. S., & Gottschling, M. (2011). Catch me if you can: The

- taxonomic identity of *Scrippsiella trochoidea* (F.Stein) A.R.Loeb. (Thoracosphaeraceae, Dinophyceae). *Systematics and Biodiversity*, 9, 145–157.
- Zinssmeister, C., Soehner, S., Kirsch, M., Facher, E., Meier, K. J. S., Keupp, H. & Gottschling, M. (in press). Same but different: Two novel bicarinate species of extant calcareous dinophytes (Thoracosphaeraceae, Peridinales) from the Mediterranean Sea. *Journal of Phycology*, 47. doi:[10.1111/j.1529-8817.2012.01182.x](https://doi.org/10.1111/j.1529-8817.2012.01182.x)
- Zonneveld, K. A. F., Höll, C., Janofske, D., Karwath, B., Kerntopf, B., Rühlemann, C., et al. (1999). Calcareous dinoflagellate cysts as paleo-environmental tools. In G. Fischer & G. Wefer (Eds.), *Use of proxies in paleoceanography: Examples from the South Atlantic* (pp. 145–164). Berlin: Springer.
- Zonneveld, K. A. F., Meier, K. J. S., Esper, O., Siggelkow, D., Wendler, I., & Willems, H. (2005). The (palaeo-)environmental significance of modern calcareous dinoflagellate cysts: A review. *Paläontologische Zeitschrift*, 79, 61–77.