

# TIMING DEEP DIVERGENCE EVENTS IN CALCAREOUS DINOFLAGELLATES<sup>1</sup>

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Based on morphological and molecular data, calcareous dinoflagellates (Thoracosphaeraceae, Peridinales) are a monophyletic group comprising the three major clades *Enciculifera/Pentapharsodinium*, *Thoracosphaera/Pfiesteria*, and *Scrippsiella sensu lato*. We used stratigraphically well-documented first occurrences of particular archeopyle types to constrain relaxed Bayesian molecular clocks applied to nuclear rRNA sequences of 18 representatives of the three main clades. By comparing divergence estimates obtained in differently calibrated clocks with first stratigraphic occurrences of taxa not themselves used as constraints, we identified plausible divergence times for several subclades of calcareous dinoflagellates. The initial diversification of extant calcareous dinoflagellates probably took place in the Late Jurassic, with the three main clades all established by the Cretaceous. The two mesoepicystal operculum types observed in calcareous dinoflagellates probably evolved independently from simple apical archeopyles. Based on our taxon sample, the K/T boundary had relatively little effect on the diversity of the group, with several lineages dating to before 65 mya (million years ago). The first stratigraphic occurrences of key taxa, such as *Thoracosphaera* and *Calciodinellum* (not themselves used as constraints), are in agreement with the molecular time estimates. Conflicts that involve “*Calciodinellum levantinum*, *Leonella*, *Pentapharsodinium*,

*Pernambugia*, and the *Scrippsiella trochoidea* species complex may be due to inaccurate assignment of fossils because of high morphological homoplasy and insufficient knowledge of the extant diversity of calcareous dinoflagellates.

**Key index words:** calcareous nannoplankton; dinoflagellates; K/T boundary; micropaleontology; molecular clock; time estimate

**Abbreviation:** K/T boundary, Cretaceous/Tertiary boundary

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Calcareous dinoflagellates (Thoracosphaeraceae [=Calciodinellaceae], Peridinales) are unicellular algae and comprise ~30 extant and >200 extinct species (Streng et al. 2004a, Elbrächter et al. in press). Because of their excellent fossil preservation, calcareous dinoflagellates play an important role in climate and environmental reconstruction (Versteegh 1994, Vink et al. 2001, Esper et al. 2004, Meier et al. 2004, Zonneveld et al. 2005). Two parallel, initially independent classification systems have been developed for dinoflagellates in general and also for calcareous dinoflagellates, a “neontological” system mainly based on the morphology of the theca and a “palaeontological” system based on characters of the coccoid stage (Fensome et al. 1993, Elbrächter et al. in press).

The ability to form calcareous cell walls in coccoid stages is considered apomorphic within dinoflagellates and argues for the monophyly of the Thoracosphaeraceae (Wall and Dale 1968, Janofské 1992, Kohring et al. 2005, Elbrächter et al. in press).

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Morphological studies have suggested that calcareous dinoflagellates (as Calciodinelloideae, i.e., excluding *Thoracosphaera*) may be related to extinct taxa, such as †Palaeoperidinioideae, †Deflandreioideae, and †Ovoidinioideae, which have similar tabulation patterns (crosses indicate species based on fossils; Fensome et al. 1993). Among extant forms, calcareous dinoflagellates may constitute a lineage with the heterotrophic Protoperidiniaceae, the freshwater species of *Peridinium*, and *Peridiniopsis* (Elbrächter et al. in press).

Using molecular data, the phylogeny of dinoflagellates has been difficult to reconstruct because of multiple endosymbiosis events, lateral gene transfers, and divergent substitution rates (Saldarriaga et al. 2001, 2004, Morden and Sherwood 2002, Yoon et al. 2002, Hackett et al. 2004, Taylor 2004, Shalchian-Tabrizi et al. 2006). However, molecular investigations focusing on the internal relationships in Thoracosphaeraceae show the existence of three well-supported clades that also include several (secondarily) noncalcareous taxa (Gottschling et al. 2005a): (i) an E/Pe clade, comprising noncalcareous *Ensiculifera* and partly calcareous *Pentaparthodinium* (including *P. tyrrhenicum* = *Calcicarpinum bivalvum*); (ii) a T/Pf clade that includes calcareous forms, such as *Thoracosphaera* and *Leonella*, as well as the noncalcareous *Pfiesteria*; and (iii) the almost completely calcareous *Scrippsiella* sensu lato (s.l.) clade. *Scrippsiella* s.l., in turn, segregates into four groups, basically corresponding to established taxonomic units (Gottschling et al. 2005b), namely, (a) *Scrippsiella precaria* and *Scrippsiella ramonii*; (b) *Pernambugia*; (c) the CAL clade, which includes *Calciodinellum* and *Calcigonellum*; and (d) *Scrippsiella* sensu stricto. The latter consists mainly of the *S. trochoidea* species complex, which includes the “*C.*” *levantinum* species group (not closely related to the type species of *Calciodinellum*, *C. operosum*) and several cryptic taxa (Montresor et al. 2003, Gottschling et al. 2005b). The high amount of genetic variation in modern species is in contrast to the long stratigraphic ranges of some calcareous dinoflagellate “species” or at least the apparently continuous occurrence of morphological characters that are considered to characterize species.

Stratigraphic data and phylogenetic analysis suggest an evolutionary direction of archeopyle evolution in calcareous dinoflagellates (Streng et al. 2004a). The archeopyle (Fig. 1) is formed by a suture that separates a lid (called operculum), made up from a variable number of theca plate equivalents, from the rest of the cyst (Evitt 1967). Early calcareous dinoflagellates are characterized by a simple apical archeopyle with an operculum presumably comprising a single (3′) plate equivalent (Streng et al. 2004a; Fig. 1, this study). This feature is considered to be the ancestral condition and today present in the E/Pe and T/Pf clades (Gottschling et al. 2005a). A combination archeopyle with at

least three plate equivalents (Fig. 1) is interpreted as the derived character state. Within mesoepicystal combination archeopyles, Streng et al. (2004a) distinguish two operculum types: a simple operculum comprising multiple plate equivalents and a compound operculum that disarticulates into a variable number of monoplacoid pieces. A compound mesoepicystal operculum, termed 3AtI, is only known from the Middle Aptian to Early Cenomanian (~118 to 98 mya ago) in †*Orthopithonella gustafsonii*. A simple mesoepicystal operculum, termed (3AtI)—the parentheses in the annotation indicating that the plate equivalents included are not disarticulating—is today restricted to *Scrippsiella* s.l. and appeared ~70 mya ago in the Early Maastrichtian (Streng et al. 2004a). So far, it has not been worked out whether these operculum types are homologous or convergent developments.

Although calcareous dinoflagellates have left an impressive record of fossilized coccoid stages, which are predominantly interpreted as hypnozygotes or resting cysts (Keupp 1981, 1987, 1991, Fensome et al. 1993, 1999, Zügel 1994, Zonneveld et al. 1999, 2005, Hildebrand-Habel and Willems 2000, Meier et al. 2004, 2007, Streng et al. 2004b, Head et al. 2006), the temporal origin of their major clades is poorly understood. Thoracosphaeraceae may have arisen in the Late Triassic (Janofske 1992), but the affinities of the earliest fossils to extant calcareous dinoflagellates are doubtful for two reasons: they do not show dinoflagellate characteristics, such as an archeopyle and paratabulation, and there is a gap of ~50 mya between the isolated Triassic forms and the uninterrupted fossil record of undoubted Thoracosphaeraceae starting in the Late Middle Jurassic (Fig. 1). The oldest unequivocal paratabulated calcareous cysts have been described from the Early Oxfordian of France (~160 mya; Keupp 1984, Keupp and Ilg 1989), and undoubted Thoracosphaeraceae are highly diverse during the Cretaceous and Cenozoic (Keupp 1981, 1987, 1991, Kohring 1993, 1997, Willems 1994, Hildebrand-Habel et al. 1999, Hildebrand-Habel and Streng 2003).

Over the past 10 years, estimates of clade divergence times from molecular clocks have provided new insights into the evolution and diversification of organisms (Sanderson 1998, Bromham and Penny 2003, Douzery et al. 2004, Sanderson et al. 2004, Renner 2005). Molecular clocks are calibrated or constrained with independently derived absolute dates from fossils, geological events, or other well-dated changes that affected the taxa under investigation. So far, there have been few such studies for dinoflagellates despite their high-quality fossil record (Fensome et al. 1993) and the availability of large molecular data sets (Saldarriaga et al. 2001, 2004, Santos et al. 2002, Yoon et al. 2002, Logares et al. 2007, Zhang et al. 2007). John et al. (2003) estimated the divergence time of the toxic *Alexandrium tamarense* species complex and determined it

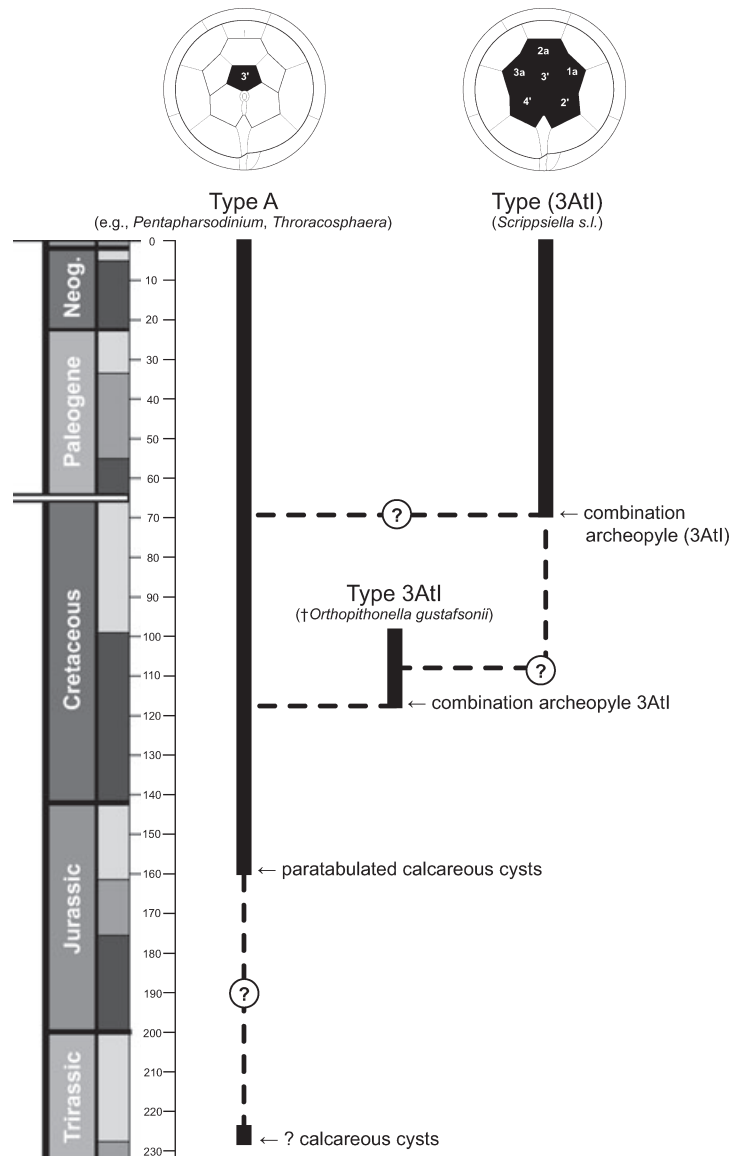


FIG. 1. Stratigraphic ranges of key characters in calcareous dinoflagellates. Hatched lines indicate alternative hypotheses about the origin of the operculum types 3AtI and (3AtI) as either homologous or convergent. Terminology of operculum types and thecal plate equivalents follows Streng et al. (2004a) and Kohring et al. (2005), respectively.

fell early in the Neogene; LaJeunesse (2004) estimated the ages of various species radiations of endosymbiotic dinoflagellates in the Atlantic and Indo-Pacific and found them to fall in the Miocene and Pliocene; and Pochon et al. (2006) used a fossil-constrained relaxed molecular clock to infer the divergence time of the crown group of *Symbiodinium*, which falls in the mid-Miocene.

In this study, we used a Bayesian relaxed clock approach (Thorne et al. 1998, Thorne and Kishino 2002) and different fossil-based constraints to infer the origin of the major lineages of calcareous dinoflagellates. Table 1 summarizes the earliest geological occurrences of calcareous dinoflagellates, with approximate absolute dates, character traits, and sub-

ordinate taxa. These data constitute the stratigraphic backdrop of this study. The sequence data come from the conserved regions of the first internal transcribed spacer (ITS1, as inferred from its secondary structure: Gottschling and Plötner 2004), the 5.8S ribosomal RNA (rRNA), and domains D1 and D2 of the large subunit (LSU) of rRNA. Studies that have compared Bayesian approaches with other molecular-dating algorithms, such as strict clocks, nonparametric rate smoothing, penalized likelihood (Sanderson 2002), and local clocks (Yoder and Yang 2000), have determined that local clocks and Bayesian approaches outperform other methods (Pérez-Losada et al. 2004, Ho et al. 2005). Relaxing the clock assumption (which is that sister groups have

TABLE 1. Stratigraphic ranges and approximate absolute geological dates as used in this study.

No.	Dated (groups of organisms and/or apomorphic traits)	Nodes in Figure 3	Geological age	Age (mya) <sup>a</sup>	Reference
1	Calcareous cysts	32	Carnian (Triassic)	228	Janofske (1992)
2	Paratabulated calcareous cysts	32	Early Oxfordian (Jurassic)	160	Keupp (1984), Keupp and Ilg (1989)
3	Spiny calcareous cysts ("Rhabdothorax" Kamptner ex Gaarder et Heimdal)	21	Barremian/Aptian (Cretaceous)	125	Keupp and Mutterlose (1994)
4	Combination archeopyle 3AtI (in † <i>Orthopithonella gustafsonii</i> ) (H. M. Bolli) Lentini et G. L. Williams	26	Middle Aptian (Cretaceous)	118	Streng et al. (2004a)
5	Combination archeopyle (3AtI) (in extant members of <i>Scrippsella</i> s.l.)	26	Early Maastrichtian (Cretaceous)	70	Streng et al. (2004a)
6	Smooth cysts of the "Calciadinellum" levantinum species group	19	Late Maastrichtian (Cretaceous)	69	Hildebrand-Habel et al. (1999), Hildebrand-Habel and Streng (2003)
7	<i>Calciadinellum albatrosianum</i> (Kamptner) Janofske et Karwath and CAL clade (putative apomorphy: cyst crystals arranged rosette-shaped)	24	Late Maastrichtian (Cretaceous)	67	Hildebrand-Habel and Streng (2003)
8	<i>Thoracosphaera</i>	27	Late Maastrichtian (Cretaceous)	67	Hildebrand-Habel et al. (1999)
9	Paratabulated cysts of <i>Calciadinellum operosum</i>	23	Late Middle Eocene (Cenozoic)	37.5	Hildebrand-Habel and Streng (2003)
10	<i>Cacigonellum infula</i> Deflandre	22	Late Middle Eocene (Cenozoic)	37	Hildebrand-Habel and Streng (2003)
11	<i>Pernambugia</i>	25	Early Oligocene (Cenozoic)	33	Streng et al. (2004a)
12	<i>Leonella</i>	27	Middle Oligocene (Cenozoic)	28.4	Keupp and Kohring (1994)
13	Cyst of <i>Pentapharsodinium tyrrhenicum</i> (= <i>Calciocarpinum bivalvum</i> )	30	Late Pleistocene (Cenozoic)	0.12	Versteegh (1993)

<sup>a</sup>Estimated as inferred from Gradstein et al. (2004).

the same substitution rate) will recover true rates and ages only when several calibration points are accurate, and even with accurate constraints, estimates will remain imprecise because of the errors associated with the dating of fossils and the necessarily loose association between genetic divergence events and morphological change. The divergence times for the major clades in Thoracosphaeraceae obtained in this study agree with other data on dinoflagellate diversification and provide a backdrop for more comprehensive reconstructions of their phylogeny.

#### MATERIALS AND METHODS

We sequenced the ITSs, 5.8S rRNA, and domains D1 and D2 of the LSU for 18 strains of calcareous dinoflagellates and a relevant outgroup. Seventeen new sequences have been submitted to GenBank (Table S1, see the supplementary material). Highly divergent regions of the ITS1 (including helix III of the secondary structure: Gottschling and Plötner 2004) and the entire ITS2 were excluded from analyses in order to avoid phylogenetic bias (as in Gottschling et al. 2005a). The taxa sampled represent the major lineages of calcareous dinoflagellates as observed in previous molecular-phylogenetic analyses that included larger taxon samples (d'Onofrio et al. 1999, Montresor et al. 2003, Gottschling et al. 2005a,b). Trees were rooted with *Woloszynskia halophila* (for taxonomic authors, see Table 1 and Table S1) based on the tree topology in Kremp et al. (2005). DNA extraction, PCR,

purification, and sequencing followed standard protocols (Gottschling and Plötner 2004, Kremp et al. 2005).

Sequences were aligned manually using Se-AL v2.0a72 (available at <http://tree.bio.ed.ac.uk/software/seal/>); the final matrix is available at <http://htcc.pt-dlr.de/dateien/GottschlingCalcDinosTimeEst.nex>. Maximum likelihood (ML)-based phylogenetic analyses were conducted using RAxML-VI-HPC (Stamatakis 2006; available at <http://icwww.epfl.ch/~stamatak>) following the manual's recommendations. To find the best-known ML tree, we executed 100 tree searches, starting from random stepwise-addition maximum-parsimony (MP) trees, using the general-time-reversible plus gamma (GTR +  $\Gamma$ ) model (with 25 rate categories). Nonparametric bootstrapping under ML was also carried out with RAxML, and the bootstrap values (from 1,000 replicates) were drawn on a majority-rule consensus tree using the consense tree program (version 3.66) of the Phylip package (Felsenstein 1989; available at <http://evolution.genetics.washington.edu/phylip/getme.html>). Gaps were treated as missing data in all analyses.

The topology used for time estimation reflects the morphological and molecular trees of d'Onofrio et al. (1999), Montresor et al. (2003), Gottschling et al. (2005a,b), and Kremp et al. (2005) and includes two polytomies to account for poorly supported parts of the tree. We used Thorne's Bayesian relaxed clock approach (Thorne et al. 1998, Thorne and Kishino 2002), implemented in the multidivtime software available from his Web page (<http://statgen.ncsu.edu/thorne/thorne.html>). Different from most other clock approaches, this program allows polytomies in the input tree. The only substitution model implemented in multidivtime is the F84 +  $\Gamma$

model, and parameter values under this model were estimated with PAML version 3.14 (<http://abacus.gene.ucl.ac.uk/software/paml.html>). Thorne's program *estbranches* was then used to calculate branch lengths and their variance, given the sequence data (18 rRNA sequences of a length of 874 nucleotides [nt]), the model parameter values from PAML, and the specified rooted topology. Branch lengths from *estbranches* became the priors for Markov chain Monte Carlo (mcmc) searches in multidivtime that sought to find the most likely model of rate change (with rate change assumed to be log-normally distributed), given the topology, time constraints on nodes (below), and a Brownian motion parameter ( $v$ ) that controls the magnitude of autocorrelation per million years (mya) along the descending branches of the tree. Prior distributions on parameters of the relaxed clock model were as follows: the mean and SD of the prior for the number of time units between the root and the tips were set to 1.5. The mean and SD of the prior for the ingroup root rate were set to 0.0027 substitutions/site/mya by dividing the median of the distances between the ingroup root and the tips by 200 mya. The prior and SD for  $v$  were set to 0.7, following Thorne's manual's recommendation that the time units between root and tips multiplied by  $v$  be  $\sim 1$ . Markov chains in multidivtime were run for 10 million generations, sampling every 100th generation for a total of 100,000 trees, with a burn-in of 10,000 generations before the first sampling of the Markov chain. Analyses were repeated at least once with different chain lengths to assure that chains were long enough to converge.

Different runs employed alternative constraints (Table 2) so as to explore the fit between the resulting estimates and lineages' first fossil appearance, in an attempt at cross validation. Thus, the root node 32 was either set to maximally Triassic (Carnian, 228 mya; Table 2, runs 1 and 2) or to Late Jurassic (Early Oxfordian, 160 mya; Table 2, runs 3 and 4). Also, since it is unclear whether the operculum types 3AtI (in †*O. gustafsonii*) and (3AtI) (in extant member of *Scrippsiella* s.l.) are homologous or evolved independently from simple apical archeopyles, we employed the first stratigraphic occurrence of each type as alternative calibration points: In runs 1 and 3, the polytomous node 26 (*Scrippsiella* s.l.) was constrained to minimally 118 mya based on the first stratigraphic occurrence of the 3AtI operculum type. In runs 2 and 4, node 26 was

instead constrained to minimally 70 mya, based on the first record of the simple mesoepicystal operculum type (3AtI).

## RESULTS

The aligned sequence data (helix I and helix II of the ITS1, the 5.8S rRNA, and the domains D1 and D2 of the LSU of rRNA) comprised 917 bases in length. Calcareous dinoflagellates were monophyletic and consisted of an E/Pe clade, a T/Pf clade, and *Scrippsiella* s.l. (Fig. 2). *Scrippsiella* s.l. and the T/Pf clade were well supported as sister groups, but the E/Pe clade received only moderate bootstrap support. The internal phylogeny of *Scrippsiella* s.l. is poorly resolved, with *S. precaria* forming a long branch nested in the *S. trochoidea* species complex (but bootstrap support value <50).

Assuming (in run 1) that the basic split in calcareous dinoflagellates was Triassic (i.e., constraining node 32 to maximally 228 mya) and accepting the single origin (i.e., homology) of the operculum types 3AtI and (3AtI) (i.e., constraining node 26 to minimally 118 mya) yielded 181 mya for the E/Pe clade and 122 mya for the T/Pf clade. Estimates changed dramatically in run 2, which assumed the operculum types to be convergent and the origin of *Scrippsiella* s.l. to date to the Late Cretaceous (i.e., constraining node 32 as in run 1 and node 26 to minimally 70 mya). With this combination of constraints, we obtained 165 mya for the E/Pe clade and 89 mya for the T/Pf clade.

Estimates that more closely fit first fossil occurrences were obtained in run 3, which assumed a Jurassic age of the basal split in extant calcareous dinoflagellates (maximal age for node 32 set to 160 mya) and homology of the operculum types

TABLE 2. Estimated divergence times and their 95% confidence intervals (in million years ago [mya]) inferred from four calibration schemes used in a relaxed molecular clock (for details see Materials and Methods). The root node (32 in Fig. 3) was constrained as maximally Triassic (runs 1 and 2) or Late Jurassic in age (runs 3 and 4). For runs 2 and 4, node 26 was constrained to minimally 70 mya based on the first occurrence of the operculum type (3AtI). Runs 1 and 3 explored the effect of instead constraining node 26 to minimally 118 mya based on the first stratigraphic occurrence of operculum type 3AtI. Estimates that fit with the first occurrences of taxa not themselves used as constraints are in bold.

Run	1	2	3	4
Constraints of nodes 26 and 32 in Figure 3	Min. 118 Max. 228	Min. 70 Max. 228	Min. 118 Max. 160	Min. 70 Max. 160
Node 19	(0-)13(-49)	(0-)9(-32)	(0-)15(-50)	(0-)8(-29)
Node 20	(44-)82(-116)	(27-)54(-91)	(40-)79(-109)	(27-)49(-73)
Node 21	(78-)104(-131)	(46-)70(-108)	(73-)100(-120)	(46-)63(-83)
Node 22	(12-)46(-87)	(7-)28(-62)	(10-)45(-86)	(7-)27(-53)
Node 23	(3-)21(-58)	(2-)12(-36)	(2-)22(-60)	(2-)12(-34)
Node 24	(68-)100(-131)	(40-)67(-106)	(63-)96(-121)	(40-)60(-83)
Node 25	(77-)109(-136)	(47-)74(-113)	(73-)104(-126)	(46-)66(-88)
Node 26	Constrained to min. 118	Constrained to min. 70	Constrained to min. 118	Constrained to min. 70
Node 27	(66-)103(-138)	(44-)75(-114)	(50-)84(-115)	(41-)64(-89)
Node 28	(90-)122(-156)	(58-)89(-131)	(67-)100(-128)	(54-)76(-101)
Node 29	(131-)151(-179)	(85-)114(-155)	(123-)135(-153)	(80-)95(-117)
Node 30	(49-)117(-189)	(45-)108(-180)	(32-)77(-132)	(33-)79(-133)
Node 31	(113-)181(-221)	(98-)165(-216)	(80-)130(-156)	(79-)125(-155)
Root 32	Constrained to max. 228	Constrained to max. 228	Constrained to max. 160	Constrained to max. 160

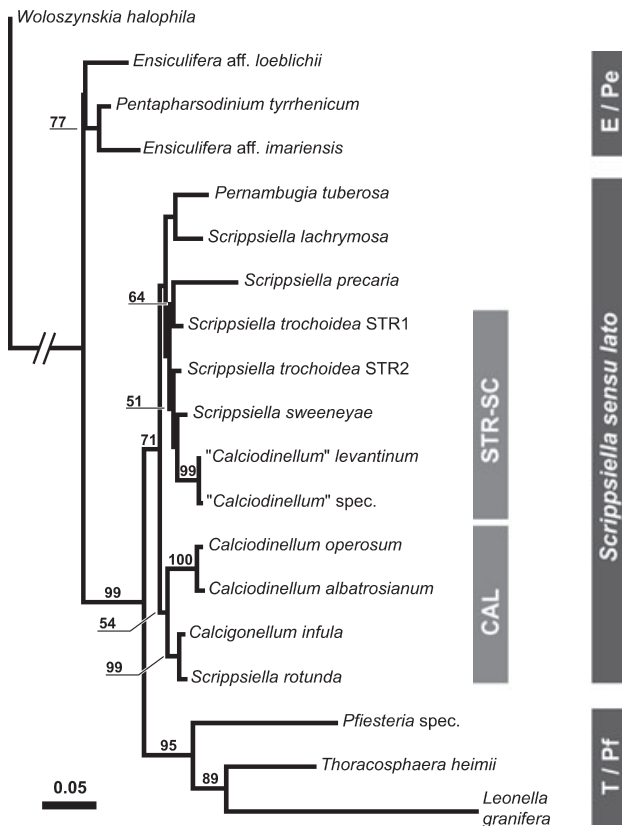


FIG. 2. Maximum-likelihood (ML) phylogeny for calcareous dinoflagellates based on sequences of the LSU rRNA domains D1 and D2, the 5.8S rRNA, and helices I and II of ITS1. Branch lengths are proportional to numbers of substitutions under the GTR +  $\Gamma$  model (a scale bar is in the lower left). Subordinate taxa include the CAL clade (comprising *Calciodinellum*, *Calcigonellum*, and others) and the STR-SC clade (comprising the *Scrippsiella trochoidea* species complex). Numbers above branches indicate ML bootstrap support from 1,000 replicates. GTR, general time reversible; ITS, internal transcribed spacer.

(minimal age for node 26, 118 mya). With this combination of constraints, the E/Pe clade was estimated as 130 mya, and the T/Pf clade as 100 mya. Under the assumptions in run 4 of a convergent development of the two operculum types (maximal age for node 32 of 160 mya, minimal age for node 26 of 70 mya), we obtained 125 mya for the E/Pe clade and 76 for the T/P clade (Table 2). The oldest known fossils of *Thoracosphaera* (node 27) date to the latest Cretaceous (Table 1), fitting with the age of 64 mya estimated for this node in run 4. For the CAL clade nested within *Scrippsiella* s.l., run 4 yielded an age of 60 mya, which is close to the first fossil occurrence of the CAL clade in the latest Cretaceous (Table 1). The 95% confidence intervals of all estimates, however, are large (see Table 2).

A few of the estimates contrasted starkly with the fossil record, regardless of the combination of temporal constraints employed. Thus, the species group that includes "*C.*" *levantinum* is considered to appear in the latest Cretaceous, but none of our

estimates for the respective node (node 19 in Fig. 3) exceeds 20 mya (Table 2). Likewise, *Pernambugia* is known since the early Oligocene, but its genetic separation from other species of *Scrippsiella* s.l. (node 25) appears to have occurred before the K/T boundary (Table 2). By far the largest discrepancy was found within the E/Pe clade, in which the split between calcareous *Pentapharsodinium tyrrhenicum* and *Ensiculifera* aff. *imariensis* (node 30) was dated to the Cretaceous (Table 2), while *Calcicarpinum bivalvum*, the fossil cyst of *P. tyrrhenicum*, dates only to the Late Pleistocene (Table 1).

## DISCUSSION

*Congruence between fossil-based dating and molecular time estimates.* Of our four experimental assignments of fossils to the root node and to the *Scrippsiella* s.l. node, we consider the combination used in run 4 the most plausible because it yielded divergence times that fit reasonably well with the first stratigraphic occurrences of key taxa, such as *Thoracosphaera* and *Calciodinellum*, which were not themselves used as constraints and hence provided independent corroboration. First, *Thoracosphaera* (node 27 in Fig. 3) is a common element of the modern phytoplankton in the pelagial (Karwath et al. 2000, Hernández-Becerril and Bravo-Sierra 2004, Tanimura and Shimada 2004, Vink 2004) and is found uninterruptedly from the Late Maastrichtian onward (Kohring 1993, 1997, Hildebrand-Habel et al. 1999, Streng et al. 2004b, Zonneveld et al. 2005). This finding matches well with the 64 mya age estimated for the split between *Thoracosphaera* and *Leonella* obtained in run 4 (Table 2; Fig. 3). By contrast, experimental runs that assumed the homology of the operculum types 3AtI and (3AtI) and therefore their single origin at ~118 mya (runs 1 and 3) yielded ages ~40 and 20 mya older than the first known fossils of *Thoracosphaera*.

Another corroboration of our time estimates comes from the CAL clade (containing *Calciodinellum* and *Calcigonellum*), whose extant species are mostly restricted to coastal localities, although *Calciodinellum albatrosianum* is also frequent in pelagic habitats (Meier et al. 2002, Tanimura and Shimada 2004, Vink 2004, Gottschling et al. 2005b). The earliest fossils of the CAL clade are dated to the Maastrichtian (Hildebrand-Habel and Streng 2003), matching well with our estimates of 60 mya (in run 4) or 67 mya (in run 2). A Late Cretaceous origin of the CAL clade implies the independent development of the operculum types 3AtI and (3AtI). However, the 95% confidence intervals (Table 2) around all our estimates are large, reflecting the limited amount of information in the relatively short rRNA sequences and the small number of nodes that could be constrained in any one run.

*Conflicts between the first fossil appearances and molecular time estimates.* While the time estimates from run

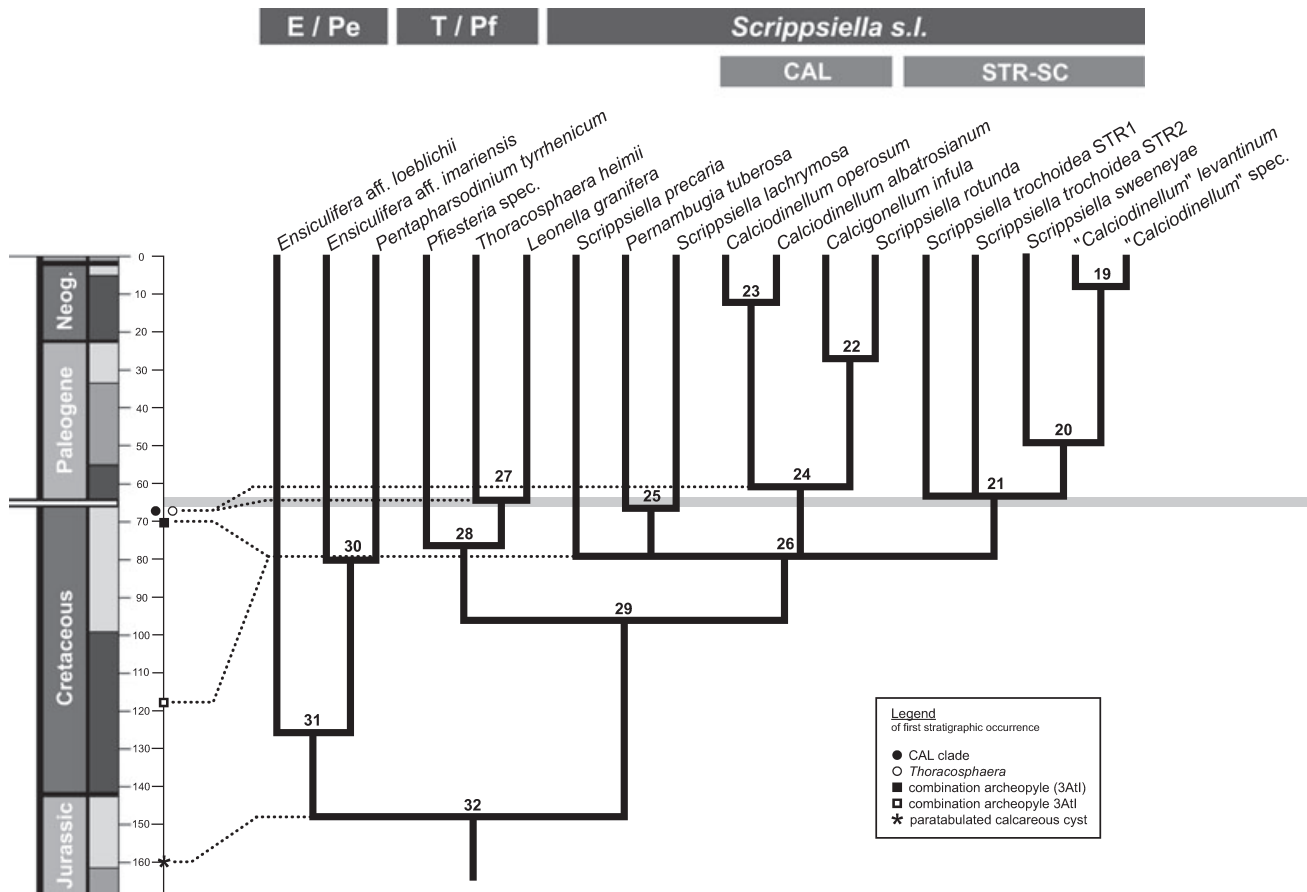


FIG. 3. Chronogram for the extant calcareous dinoflagellates as inferred from relaxed clock run 4, in which the root node 32 was constrained to maximally 160 million years ago (mya), and node 26 to minimally 70 mya. The midpoint estimates and 95% confidence intervals for each of the numbered nodes are shown in Table 2; clade names are as in Fig. 2. The symbols on the time scale mark the first stratigraphic occurrences of key morphological features (see legend inset and text for details). The three major clades, E/Pe, T/Pf, and *Scrippsiella* s.l., were established by the Cretaceous, and several lineages pass through the K/T boundary (shown as a grey bar at 65 mya). Subordinate taxa include the CAL clade (comprising *Calciodinellum*, *Calcigonellum*, and others) and the STR-SC clade (comprising the *Scrippsiella trochoidea* species complex).

4 (Fig. 3) may reflect the broad evolutionary history of calcareous dinoflagellates, conflicts with the fossil record still give pause. Meier et al. (2002) described "*C.*" *levantinum*, which today is widely distributed in the pelagial (Tanimura and Shimada 2004, Vink 2004), but molecular data show that the species does not belong to the CAL clade and is nested instead within the *S. trochoidea* species complex [Fig. 2; Gottschling et al. (2005a,b)]. The oldest fossils associated with the "*C.*" *levantinum* species group are from the Late Maastrichtian (Hildebrand-Habel et al. 1999, Hildebrand-Habel and Streng 2003), but none of our time estimates for node 19 is older than Miocene. Such conflicts may point to inaccurate determination of fossils due to the high degree of homoplasy in dinoflagellates.

Paleontologists have informally applied the name "*Rhabdothorax*" to fossils similar to the spinose cysts of extant *S. trochoidea*. The spinose cysts of the latter have been considered apomorphic for the *S. trochoidea* species complex, which comprises several cryptic taxa (Gottschling et al.

2005b; node 21 in Fig. 3), but none of the molecular midpoint time estimates for node 21 (Table 2) is close to the oldest fossils of "*Rhabdothorax*" from the Barremian/Aptian boundary (Keupp and Mutterlose 1994). Thus, extant members of the *S. trochoidea* species complex are probably not closely related to fossils from before the K/T boundary as assumed in morphological studies (Janofske 2000, Hildebrand-Habel and Streng 2003).

Presence or absence of paratabulation has been used to distinguish between *Calciodinellum operosum* and *C. albatrosianum*, although the extent of paratabulation has been shown to vary within clonal strains of these species (Montresor et al. 1997, d'Onofrio et al. 1999, Gottschling et al. 2005b). The first occurrence of paratabulated *C. operosum* is dated to the mid-Eocene (Hildebrand-Habel and Streng 2003), which is distinctly older than any of our molecular clock estimates for node 23 (Table 2). An explanation may be that the *Calciodinellum* lineage originated in the Late Maastrichtian

(Hildebrand-Habel and Streng 2003) as a smooth form, acquired paratabulation sometime in the Eocene, and that the two extant species then diverged from each other in the Miocene (Fig. 3).

Other conflicts involve the ages of *Leonella*, *Pentaparsodinium*, and *Pernambugia*, all estimated as older than their oldest known fossils (Tables 1 and 2; Fig. 3). *P. tyrrenicum* is estimated as having separated from *Enciculifera* aff. *imariensis* in the Late Cretaceous (Table 2; Fig. 3) but is known (as *Calciarpinum bivalvum*) only since the Late Pleistocene (Versteegh 1993). An explanation for this discrepancy may be insufficient knowledge about the diversity of the E/Pe clade, so that fossils cannot reliably be assigned to extant representatives (Elbrächter et al. in press). Some of the latter are even known as “living fossils” from the current benthos (Montresor et al. 1994, 1998, della Tommasa et al. 2004, Vink 2004). Putative close relatives of *P. tyrrenicum*, such as *Bicarinellum* and *Praealcaligonellum*, have not yet been brought into culture for investigations that would permit a fuller understanding of the evolutionary history of the E/Pe lineage.

*An evolutionary scenario for calcareous dinoflagellates.* Our molecular clock estimates address two important questions in the evolution of calcareous dinoflagellates. The initial diversification of extant Thoracosphaeraceae appears to have taken place in the Late Jurassic, rather than already in the Late Triassic (given that runs that assumed a Late Triassic root age yielded improbable divergence times for other nodes), and putative Triassic calcareous dinoflagellates (Janofske 1992) are therefore unlikely to represent crown group representatives. A Late Jurassic origin is in accordance with the dramatic increase in the abundance and diversity of dinoflagellates in the Jurassic and Cretaceous (Fensome et al. 1996, Hackett et al. 2004, Saldarriaga et al. 2004, Taylor 2004) and is also inferred in chemostratigraphic reconstructions (Moldowan et al. 1996, Moldowan and Talyzina 1998). Furthermore, our results point to a convergent evolution of the two mesoepicystal operculum types, 3AtI and (3AtI), which also fits with the 25 mya gap between the stratigraphic termination of †*O. gustafsonii* with a compound mesoepicystal operculum (i.e., the 3AtI type) and the first occurrence of the simple mesoepicystal operculum [i.e., the (3AtI) type; Streng et al. 2004a]. The three major clades of calcareous dinoflagellates, E/Pe, T/Pf, and *Scrippsiella* s.l., likely were established by the Cretaceous (Fig. 3), and the radiation of the modern forms represented by *Scrippsiella* s.l. may also have begun in the Late Cretaceous.

While the diversity and abundance of many marine planktonic organisms, especially foraminifers and calcareous nannoplankton, were greatly diminished at, and immediately following, the K/T boundary, calcareous dinoflagellates appear to have been little affected in diversity, even though faunal

assemblages changed considerably (Hildebrand-Habel and Streng 2003, Bown 2005). This phenomenon is suggested by our chronogram, with several lineages passing through the K/T boundary (Fig. 3) and would resemble the situation in organic-walled dinoflagellates, which were not much affected by the K/T extinction event either (Edwards 1993, MacRae et al. 1996). Based on fossils, calcareous dinoflagellates continued to increase in diversity during the early Paleocene, with at least one monospecific mass occurrence directly above the boundary (‘*Thoracosphaera*-bloom’: Hildebrand-Habel and Streng 2003). However, the relationships of many of the fossils from around the K/T boundary with extant species remain to be investigated based on more refined analyses of cyst morphology. More detailed reconstructions of evolutionary events, such as are already available for other protist phytoplankton groups (e.g., Bacillariophyta: Kooistra and Medlin 1996; Coccolithophorida: Sáez et al. 2003; *Symbiodinium*: Pochon et al. 2006), will require additional taxon and gene sampling.

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### Supplementary Material

The following supplementary material is available for this article:

**Table S1.** Sequenced material held at the University of Bremen (Germany), voucher specimens, localities, and GenBank accession numbers.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00479.x>.

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