

# They are young, and they are many: dating freshwater lineages in unicellular dinophytes

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## Summary

Dinophytes are one of few protist groups that have an extensive fossil record and are therefore appropriate for time estimations. However, insufficient sequence data and strong rate heterogeneity have been hindering to put dinophyte evolution into a time frame until now. Marine-to-freshwater transitions within this group are considered geologically old and evolutionarily exceptional due to strong physiological constraints that prevent such processes. Phylogenies based on concatenated rRNA sequences (including 19 new GenBank entries) of two major dinophyte lineages, Gymnodiniaceae and Peridiniales, were carried out using an uncorrelated molecular clock and five calibration points based on fossils. Contrarily to previous assumptions, marine-to-freshwater transitions are more frequent in dinophytes (i.e. five marine-freshwater transitions in Gymnodiniaceae, up to ten but seven strongly supported transitions in Peridiniales), and none of them occurred as early as 140 MYA. Furthermore, most marine-to-freshwater transitions, and the followed diversification, took place after the Cretaceous–Paleogene boundary. Not older than 40 MYA, the youngest transitions within Gymnodiniaceae and Peridiniales occurred under the influence of the Eocene climate shift. Our evolutionary scenario indicates a gradual diversification of dinophytes without noticeable impact of catastrophic events, and their freshwater lineages have

originated several times independently at different points in time.

## Introduction

The majority of the world's surface is covered with water (Simon *et al.*, 2015), but the difference between marine and fresh water bodies in terms of volume is immense. Oceans comprise around 71% of the globe's surface (Costanza, 1999), and not more than 3% of it is covered in freshwater (Gleick, 1999; Downing *et al.*, 2006). Marine and freshwater ecosystems differ greatly in their physicochemical characteristics, subsequently dictating physiological adaptations developed in the inhabiting organisms. Only a handful of organisms, such as migratory fish (Myers, 1949), have considerable salinity tolerance ranges, whereas most species show a remarkably high fidelity to their respective habitats. On a more general level, a number of eukaryotic lineages often group into distinct and species-rich marine or freshwater phylogenetic clusters, a phenomenon known not only from macroorganisms but also from the microbiome (Shalchian-Tabrizi *et al.*, 2008; Logares *et al.*, 2009, 2010; Heger *et al.*, 2010).

Some of the important questions in evolutionary biology are the origin, number and age of evolutionary transitions between freshwater and marine environments. Aquatic, especially marine environments lack apparent obstacles for pelagic organisms (Paulay and Meyer, 2002; Bråte *et al.*, 2010), thus reducing the impact of allopatric diversification. However, the fundamental osmotic difference between the oceans and diverse freshwater habitats acts as a strong physiological barrier (Pokorný, 2009). Only a few organisms are physiologically adapted to successfully overcome such a barrier and establish new populations. Subsequently, such marine-to-freshwater (or *vice versa*) transitions are considered to be exceptional and uncommon (100 transitions in 800 MYA for all the eukaryotes: Cavalier-Smith, 2009). Regardless, microorganisms form much larger populations, have higher reproductive rates (Dolan, 2005; Snoke *et al.*, 2006), and are significantly smaller than their macroscopic counterparts. Therefore, it is assumed that barrier-crossing between marine and

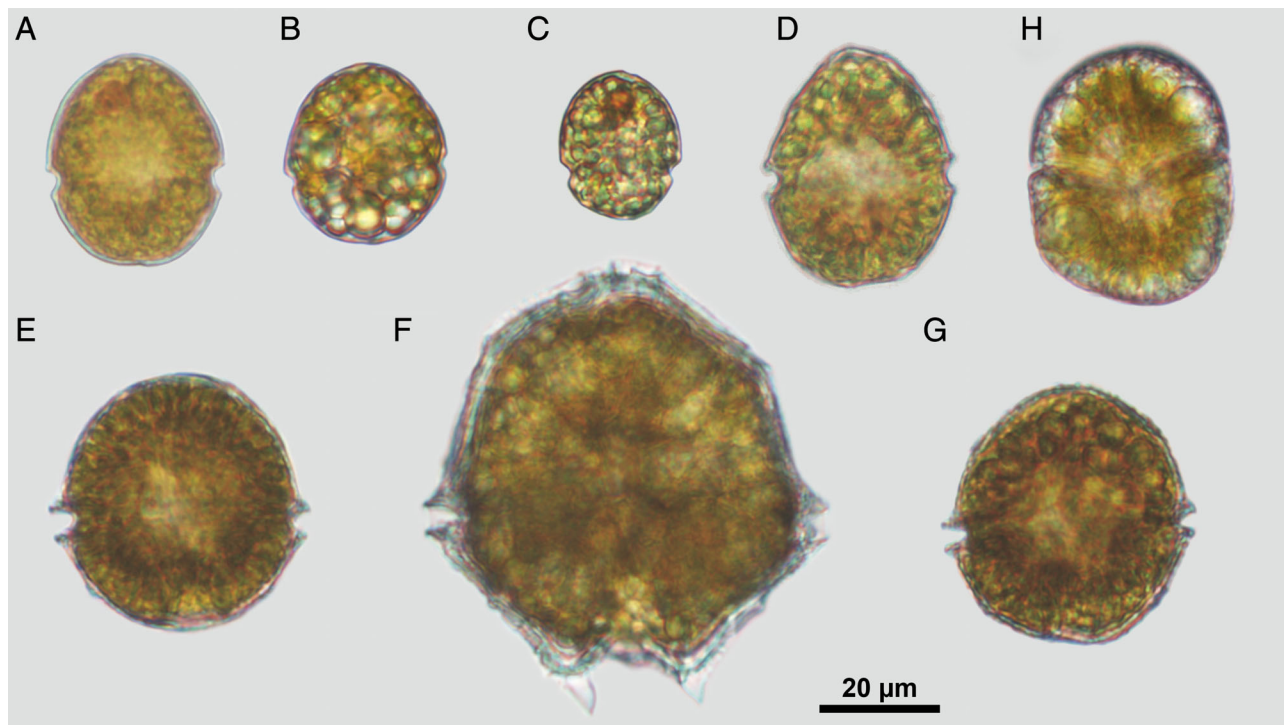
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freshwater environments takes place more often in the microbiome (Finlay *et al.*, 2006; Bass *et al.*, 2007). More specifically, several (albeit infrequent) transitions between marine and freshwater environments are inferred for microbial groups such as chlorophytes, cryptophytes, dinophytes and haptophytes (Lewis and McCourt, 2004; Logares *et al.*, 2007b; Shalchian-Tabrizi *et al.*, 2008, 2011; Leliaert *et al.*, 2012), indicating that some groups are indeed capable of crossing such a strong physiological barrier.

Dominating the freshwater and marine aquatic ecosystems, microbial eukaryotes play an important role in carbon fixation (Jardillier *et al.*, 2010) and climate regulation (Simó, 2001). Dinophytes are one of major phytoplankton groups and inhabit a continuum of water environments from open seas, shallow coastal waters, estuaries, rivers and lakes to puddles (Fensome *et al.*, 1993; Taylor *et al.*, 2008). The majority of dinophyte species live in marine environments, but approximately 350 species can be found in freshwater habitats (Mertens *et al.*, 2012; Moestrup and Calado, 2018; Fig. 1). Quantification and dating of marine-to-freshwater transitions is the basis toward understanding the past evolutionary processes

leading to the high biodiversity in dinophytes. Such studies are rare because of the limited taxon sample availability (compared with the total known diversity), insufficient sequence data and strong rate heterogeneity (particularly of rRNA sequences; Saldarriaga *et al.*, 2004; Murray *et al.*, 2005; Gu *et al.*, 2013). Nevertheless, preliminary data are available for some subordinate groups. Comprised of approximately 50 extant species (Elbrächter *et al.*, 2008), the Thoracosphaeraceae (Peridinales) can be found in either freshwater or marine habitats. The group clearly prefers the latter, but at least three independent freshwater lineages are recognized within this group (Moestrup and Daugbjerg, 2007; Logares *et al.*, 2007a; Craveiro *et al.*, 2013; Gottschling and Söhner, 2013). Similar dynamics is inferred from the Gymnodiniaceae, as at least five lineages have independently colonized freshwater habitats (Kretschmann *et al.*, 2015).

To deepen our knowledge of marine-to-freshwater transitions of all kinds of organisms, the time component placing transition events into a certain geological era plays an important role. Evidence based on the fossil record (Gray and Taylor, 1988; Batten, 1989) suggests that marine-to-freshwater transitions within dinophytes started to happen



**Fig. 1.** Diversity of freshwater dinophytes (all cells on the same scale).

A. *Chimonodinium lomnickii* var. *wierzejskii* (Thoracosphaeraceae).

B. *Durinskia oculata* (Kryptoperidiniaceae).

C. *Parvodinium umbonatum* (Peridiniopsidaceae).

D. *Palatinus apiculatus* (Peridiniopsidaceae).

E. *Peridinium cinctum* (Peridiniaceae).

F. *Peridinium bipes* (Peridiniaceae).

G. *Peridinium willei* (Peridiniaceae).

H. *Spiniferodinium limneticum* (Gymnodiniaceae). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

as early as 140 MYA. Molecular clock analyses shed light on certain previously unexplainable evolutionary events (Medlin, 2008). Based on the integrative fossil record and a relaxed molecular clock approach dinophytes diverged from their sister group Apicomplexa around 1100 MYA (Parfrey *et al.*, 2011; Feulner *et al.*, 2015), but did not diversify until the beginning of the Mesozoic (i.e., after the 'phytoplankton blackout': Riegel, 2008; Servais *et al.*, 2016; and when Pangea started to break apart ~250 MYA: Fensome *et al.*, 1996, 1999). The downfall resulted in an increase of continental shelf areas providing many new habitats for coastal and benthic dinophytes (Fensome *et al.*, 1996; Hoppenrath *et al.*, 2014). Time estimates focusing exclusively on dinophytes are only sporadically available and focus on rather lower taxonomic levels (*Alexandrium*: John *et al.*, 2003; *Symbiodinium*: LaJeunesse, 2004; LaJeunesse *et al.*, 2018; Pochon *et al.*, 2006; calcareous dinophytes: Gottschling *et al.*, 2008; Kryptoperidiniaceae: Žerdoner Čalasan *et al.*, 2018) yet to understand the marine-to-freshwater transitions to a greater extent, putting them into a time frame is of the essence.

A considerably higher coverage of genetic information and well-resolved phylogenetic relations between dinophyte taxa on a higher level (Gottschling *et al.*, 2012; Orr *et al.*, 2012; Gu *et al.*, 2013; Kretschmann *et al.*, 2015, 2018; Janoušková *et al.*, 2017; Price and Bhattacharya, 2017) are the first steps toward a better understanding of complex processes such as marine-to-freshwater transitions. Together with their rich high-quality fossil record (Fensome *et al.*, 1993, 1996), we can use an integrative approach to assign these transitions to geological events (Logares *et al.*, 2009). The aim of our study was to provide the number and the time frame of marine-to-freshwater transitions by carrying out a molecular clock analysis for the Gymnodiniaceae and the Peridinales (Fig. 1) – two groups with the highest number of marine-to-freshwater transitions known in dinophytes.

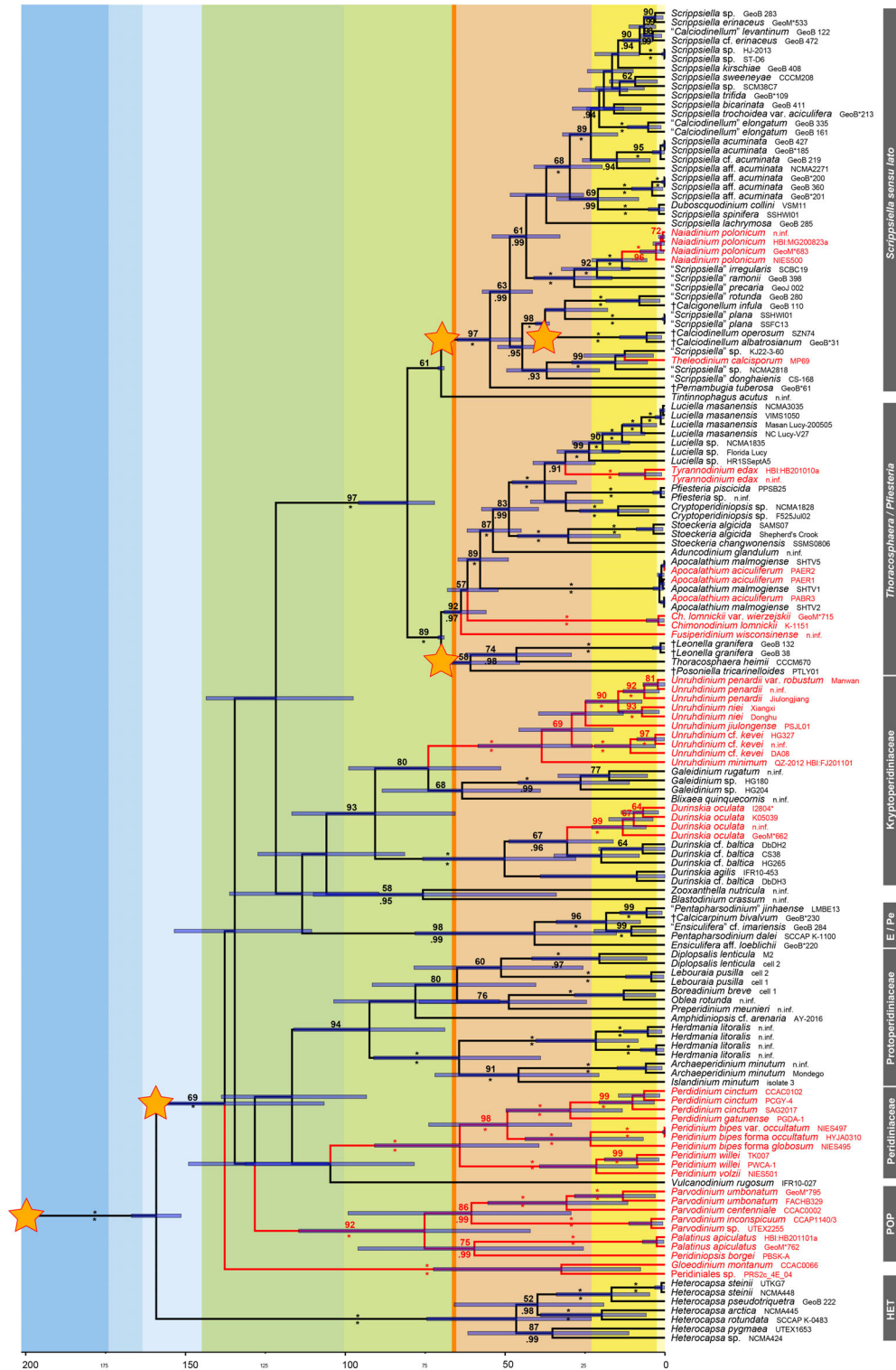
## Results

The alignment was comprised of 2 050 parsimony-informative positions (32%, mean of 9.2 per terminal taxon) and included small ribosomal subunit (SSU), internal transcribed spacer region (ITS1, 5.8S and ITS2) and large ribosomal subunit (LSU). Our data set provided a well-resolved backbone phylogeny using a representative taxon sample of marine and freshwater representatives of the Peridinales (Fig. 2) and the Gymnodiniaceae (Fig. 3; both groups were considered as the out groups for each other). Maximum Likelihood (likelihood bootstrap support; LBS) and Bayesian analyses (Bayesian posterior probabilities; BPP) recovered phylogenetic trees with highly congruent topologies. The Peridinales (100 LBS,

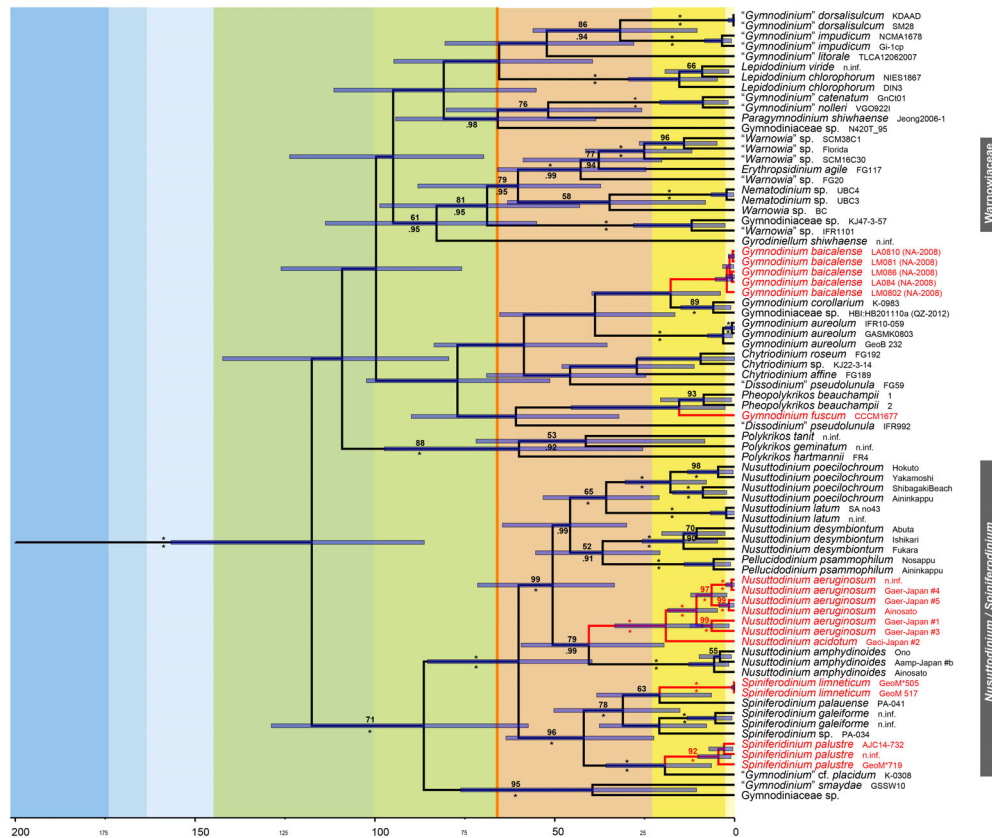
1.00 BPP) segregated into eight highly diverse monophyletic lineages, including *Scrippsiella sensu lato* (97 LBS, 1.00 BPP), a clade comprising *Thoracosphaera* and *Pfiesteria* with relatives (T/Pf clade; 89 LBS, 1.00 BPP), the Kryptoperidiniaceae (93 LBS), a clade comprising *Ensiculifera* and *Pentaparsodinium* with relatives (98 LBS, 0.99 BPP), the heterotrophic Protoperidiniaceae (94 LBS), the Peridiniaceae (100 LBS, 1.00 BPP), the Peridiniopsidaceae (92 LBS, 1.00 BPP) and the Heterocapsaceae (100 LBS, 1.00 BPP). The relationships among such lineages were not always clear, but some nodes were retrieved with high support, for example, the close relationship between *Scrippsiella s.l.* and the T/Pf clade (97 LBS, 1.00 BPP).

To assess the influence of highly variable regions, two separate alignments were used for the time estimation analysis. Due to a high variability of the ITS region, certain positions were namely aligned unreliably, which might cause artefacts in the topologies or time spans. Hence, to further check for the influence of potentially poorly aligned positions, we carried out the analyses once using the ITS region (Fig. 3, Supporting Information Fig. S2) and once excluding it from the alignment (Supporting Information Figs S1 and S3). No significant differences in topologies, time frames or in effective sample size (ESS) values were recovered, which underlines the solidity of our approach. Furthermore, two different tree priors (Speciation: Yule process; Figs 2 and 3, Supporting Information Fig. S1; and Coalescence: constant size; Supporting Information Figs S2 and S3) were used. For representative time estimation analysis, one should namely use Yule process, in cases of having one species per sample in the taxon set, or alternatively Coalescence Model, in cases of having more accessions per species included in the analysis (see BEAST tutorials 2012). However, we cannot be sure either of the criteria is fulfilled by our taxon sample due to the unresolved taxonomy hindering species delimitation in dinophytes. Hence, both tree priors were tested. Nevertheless, the final dated phylogeny in terms of time spans and topology again hardly differed (the greatest difference referred to the Peridiniaceae crown group that was dated to 64 and 70 MYA, respectively). The only topological change worth mentioning was the paraphyly (in Speciation: Yule process) or monophyly (in Coalescence: constant size) between *Chimonodinium* and *Fusiperidinium* from the Thoracosphaeraceae. Nevertheless, the statistical support of both scenarios was not well supported in either of the analyses (BPP < 0.90).

Based on highly supported sister group relationships between marine and freshwater clades, seven independent marine-to-freshwater transitions within the Peridinales were recovered. Three additional lineages (i.e., the Peridiniaceae, an unnamed clade that included *Gloeodinium* with a relative,



**Fig. 2.** Dated phylogeny of Peridinales. Dated phylogeny (ultrametric maximum clade credibility tree with node ages from the Bayesian uncorrelated log-normal analysis, under the Yule model) using concatenated rRNA sequences (i.e., SSU, ITS, LSU). Median rate is given in units of substitutions per million years (including 95% confidence intervals). Absolute ages are in million years, and epochs are indicated in the same colors as in Gradstein *et al.* (2012). The numbers on the branches are statistical support values (above: ML bootstrap values, values < 50 are not shown; below: Bayesian posterior probabilities, values < 0.90 are not shown; asterisks indicate maximal support). Note the presence of up to ten independent marine-to-freshwater transitions, six of which happened after the K-Pg boundary, depicted with an orange line. The calibration points are indicated with yellow stars. Freshwater taxa are colored in red. Abbreviations: E/Pe, clade including *Ensiculifera* and *Pentapharsodinium*; HET, Heterocapsaceae; POP, Peridiniopsidaceae. The used outgroup Gymnodiniaceae is shown separately in the Fig. 3. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Fig. 3.** Dated phylogeny of Gymnodiniaceae. Dated phylogeny (ultrametric maximum clade credibility tree with node ages from the Bayesian uncorrelated lognormal analysis, under the Yule model) using concatenated rRNA sequences (i.e., SSU, ITS, LSU). Median rate is given in units of substitutions per million years (including 95% confidence intervals). Absolute ages are in million years, and epochs are indicated in the same colors as in Gradstein *et al.* (2012). The numbers on the branches are statistical support values (above: ML bootstrap values, values < 50 are not shown; below: Bayesian posterior probabilities, values < 0.90 are not shown; asterisks indicate maximal support). Note the presence of five independent marine-to-freshwater transitions, all of which happened after the K-Pg boundary, depicted with an orange line. Freshwater taxa are colored in red. The used outgroup Peridiniaceae as well as the calibration points indicated with yellow stars are shown separately in Fig. 2. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

and the Peridiniopsidaceae) may have colonized the freshwater habitat independently as well, but statistical support for deep nodes was overall low. An alternative scenario would assume an initial marine-to-freshwater transition at the base of the Peridiniaceae, with at least one transition back into the marine environment and thus requiring at least two evolutionary steps. Four distantly related freshwater lineages – the Peridiniaceae, the *Gloeodinium* lineage, the Peridiniopsidaceae, and *Unruhodium* of the Kryptoperidiniaceae – originated prior to the Cretaceous-Paleogene (K-Pg) boundary (though switches to the freshwater environment could not be precisely dated). However, diversification of such lineages mostly took place after the K-Pg boundary (Supporting Information Fig. S4), except for the Peridiniopsidaceae, for which it may have started already in the late Cretaceous. Four freshwater lineages originated after the K-Pg boundary and were usually nested deeply in marine clades (e.g., *Durinskia* from the Kryptoperidiniaceae, *Naiadinium* and *Theleodinium* from *Scrippsiella s.l.*) and for the remaining two clades (*Chimonodinium* and *Fusiperidinium*), it is unclear whether the transition took place

after or before the K-Pg boundary. The T/Pf clade (except for the strictly marine *Leonella*, †*Posoniella* and *Thoracosphaera*) showed a general preference for habitats with reduced salinity. Within this clade, four lineages moved into the freshwater environment, namely *Apocalathium*, *Chimonodinium*, *Fusiperidinium* and *Tyrannodinium*. Stem ages, however, differed greatly.

Resolution within Gymnodiniaceae (100 LBS, 1.00 BPP) was poorer and only some groups at higher taxonomic levels could be identified, such as the *Nusuttodinium/Spiniferodinium*-clade (100 LBS, 1.00 BPP) and the warnowians (81 LBS, 0.95 BPP). Five transitions were still inferred, three alone in the *Nusuttodinium/Spiniferodinium*-clade (100 LBS, 1.00 BPP): Freshwater *Spiniferodinium palustre* constituted the sister species of marine “*Gymnodinium*” cf. *placidum* (100 LBS, 1.00 BPP), freshwater *Spiniferodinium limneticum* constituted the sister species of marine *Spiniferodinium palauense* (63 LBS) and freshwater and marine representatives of *Nusuttodinium* constituted sister groups as well (79 LBS, 0.99 BPP). The other two

transitions were represented by the isolated species *Gymnodinium fuscum* and in an undetermined gymnodiniacean clade isolated from freshwater sponges. All freshwater transitions and consequent radiations within Gymnodiniaceae occurred long after the K-Pg boundary (Supporting Information Fig. S1). All five lineages originated and diversified between late Miocene and early Oligocene (between 30 and 15 MYA), except for the *Nusuttodinium aeruginosum* species group, which possibly transitioned in mid-Miocene already (around 40 MYA).

## Discussion

There is a growing body of evidence that some protist lineages may have originated in freshwater (Logares *et al.*, 2009; Mulikjanian *et al.*, 2012; Dittami *et al.*, 2017; Sánchez-Baracaldo *et al.*, 2017) and that evolutionary transitions followed the direction from freshwater into marine habitats (Cavalier-Smith, 2009). In such studies, it is also explained that large and species-rich (sister) groups with fundamentally different ecological preferences to either the marine or the freshwater environment developed parallelly in many eukaryotic lineages. Dinophytes appear different in such aspects: The last common ancestor most likely lived in a marine environment, and freshwater dinophyte groups are relatively species-poor and deeply nested in primarily marine groups (Moestrup and Daugbjerg, 2007; Gottschling and Söhner, 2013; Kretschmann *et al.*, 2015, 2018). Before our study, marine-to-freshwater transitions have been considered infrequent in dinophytes (Logares *et al.*, 2007b), although terms such as 'infrequent' or 'rare' are not objectifiable. Nevertheless, we account for approximately five lineages within Gymnodiniaceae and up to ten lineages within Peridiniales, all of which independently and successfully colonized different freshwater habitats. More and more freshwater lineages with close relatives among marine representatives are known from other groups as well, such as Gonyaulacales, †Suessiales, and Tovelliaceae (Hansen *et al.*, 2007; Fawcett and Parrow, 2012), and their precise phylogenetic positions will be determined once we improve the molecular trees of dinophytes.

The present time estimates are in agreement with previous analyses investigating Peridiniales (Gottschling *et al.*, 2008; Žerdoner Čalasan *et al.*, 2018). Merely, the Kryptoperidiniaceae have been dated slightly older before (Late: between 100 MYA and 66 MYA, versus Upper: between 145 and 100 MYA, Cretaceous stem-group ages), but this can be easily explained by the inclusion of long-branching *Kryptoperidinium* that was omitted from the present analysis. The overall high number of freshwater lineages, even in subsets of dinophytes, is

indicative of a more dynamic biotic exchange between marine and freshwater environments. In the aspect of transition polarity, dinophytes are rather similar to haptophytes (Shalchian-Tabrizi *et al.*, 2011), diatoms (Alverson *et al.*, 2007) and cercozoan amoebae (Berney *et al.*, 2013), which presumably also originated in marine environments and migrated into freshwater habitats to a certain extent. Although marine-to-freshwater transitions are physiologically more challenging than the reverse process (Cavalier-Smith, 2009), not a single doubtless case of such a recolonization of the marine environment could be inferred for dinophytes.

In addition to the higher number of different freshwater lineages (albeit still lower in comparison to the marine lineages), we also present new insights about the geological age of the processes. Since the Cretaceous, marine-to-freshwater transitions occur continuously in a more gradual process and without noticeable impacts of catastrophic events. Most freshwater stem nodes are dated to after the K/Pg-boundary (< 65 MYA), and diversification within such lineages has mostly taken place during the last 10 million years. However, prior studies have posited that most marine-to-freshwater transitions in dinophytes took place prior to the K/Pg-boundary (Cavalier-Smith, 2009; Logares *et al.*, 2009). This conclusion seems to be corroborated with tree topology and with the presence of relatively long branches separating several freshwater lineages from marine groups (Logares *et al.*, 2007b), but it is not based on a time-calibrated phylogenetic analysis as presented here. The fossil record placing freshwater dinophytes into the Mesozoic (Gray and Taylor, 1988; Batten, 1989) also seems to support the idea of freshwater dinophyte lineages being consistently old, but the precise systematic affiliation of such fossils has not yet been determined. We cannot exclude that such Mesozoic fossils in fact correspond to (also extant) freshwater dinophyte lineages, but the data presented here rather suggest that they are representatives of a stem lineage or even of an extinct lineage. The fossil record of freshwater dinophytes is scarce (Fensome *et al.*, 1993), which means that it should be considered with caution. An incomplete or lost fossil record combined with often overlooked high rates of heterogeneity (not necessarily indicating high ages) could be the reason for the assumption of predominantly Mesozoic marine-to-freshwater transitions to have persisted until now.

Our analysis challenges previous evolutionary scenarios, as it shows that marine-to-freshwater transitions are not infrequent and are geologically rather young. Mostly not older than 40 MYA, the origin and diversification of gymnodiniacean and peridinialean freshwater dinophytes correlate with the Eocene climate dynamic changes. Approximately 37 MYA, the Earth started to cool down,

and generally warm and humid environments were re-placed by continental ice sheets and/or glaciers, which stretched as far as to the sea level (Barron and Baldauf, 1989; Miller *et al.*, 1991; Lear *et al.*, 2008). A general decline in the sea level caused by cooling resulted in the isolation of lagoons, which became isolated from the rest of the open sea (Matthews, 2013). If those lagoons were supplied with fresh water from the land, then the concentration of chloride and sodium ions would decline sharply, resulting in many new and initially uncolonized freshwater habitats. In such cases, the organisms trapped in such lagoons had to either adapt to new conditions or become extinct. The cooling of the Earth was already connected with the diversification processes in *Symbiodinium*, an endosymbiotic dinophyte group found in marine invertebrates and protists (Pochon *et al.*, 2006).

The origin and diversification of the few freshwater lineages older than the Eocene may also correlate with significant geological events within the Earth's history. Considering the age of marine-to-freshwater transitions, only three distantly related groups (namely the Peridiniaceae, the Peridiniopsidaceae, and the *Gloeodinium* lineage) could be older than the K-Pg boundary and of Cretaceous origin. For this era, major environmental perturbations and significant eustatic sea-level falls are assumed, which had immense effects on shallow marine shelf environments (Sims *et al.*, 2006; Tennant *et al.*, 2017). It appears that dinophytes were not greatly affected by catastrophic events at, for example, the K-Pg boundary, as numerous lineages passed through this massive extinction event (Gottschling *et al.*, 2008; Figs 2 and 3, Supporting Information Figs S1–S3). Because of large confidence intervals in this part of the dating analysis (spanning on average 75 MYA), such correlations should be considered with caution. However, most freshwater dinophyte diversification appears to have taken place during the Neogene (< 25 MYA; Supporting Information Fig. S4). The impact of the catastrophic flood of the Mediterranean after the Messinian salinity crisis (Bison *et al.*, 2007; Popescu *et al.*, 2009), or repeated Pleistocene glaciations (Pelzer, 1991; Lindner *et al.*, 2004), remains to be determined. For robust conclusions, a (nearly) complete taxon sample is required, but we are still missing parts in most dinophyte lineages.

The question arises, why would dinophytes cross the marine/freshwater barrier more frequently than other protist groups such as chlorophytes and rhodophytes. A general explanation is that this ecologically and physiologically diverse group copes much better with extreme temperatures and salinity ranges (Taylor *et al.*, 2008). As living models, species such as *Alexandrium ostenfeldii* and *Huia caspica* might be a key in this respect, as they are found in both marine and freshwater habitats (Kremp *et al.*, 2014; Gu *et al.*, 2016; Martens *et al.*, 2016).

Something similar can be said for pfiesterian dinophytes, a group with a reduced salinity preference (Litaker *et al.*, 2005; Calado *et al.*, 2009; Burkholder and Marshall, 2012). Another example is the phenotypically differentiated species pair, freshwater *Apocalathium aciculiferum* and brackish *Apocalathium malmogiense*, sharing (almost) identical rRNA sequences (Gottschling *et al.*, 2005; Logares *et al.*, 2007a). Thus, it is argued that the marine-to-freshwater transition in this species pair is ongoing (Logares *et al.*, 2008; Annenkova *et al.*, 2015). Finally, many dinophytes produce benthic resting stages (Fensome *et al.*, 1993; Mertens *et al.*, 2012; Gu *et al.*, 2016), which enable them to withstand long periods of unfavourable conditions. This particular ability could be of great evolutionary advantage if not a necessary prerequisite to surviving intermediate stages during such marine-to-freshwater transitions.

### Experimental procedures

The inference of dinophyte evolution has been improved using next-generation sequencing (NGS; Price and Bhattacharya, 2017; Janouškovec *et al.*, 2017), but a wide range of information of the taxon sample continues to be recruited from the rRNA operon sequences only. Due to restrictions such as limited taxon sample, insufficient genetic data, complex phylogenetic methods and rate heterogeneity, a representative taxon sample covering the currently known dinophyte diversity is not present at this moment in time (Gottschling *et al.*, 2012; Gu *et al.*, 2013). However, the phylogeny of the Peridinales (Gottschling *et al.*, 2017) and the Gymnodiniaceae (Kretschmann *et al.*, 2015), including many freshwater presenters, is relatively well-understood, which is why these two lineages were selected for the time estimation analysis.

For establishment of own monoclonal strains (Supporting Information Table S1), single motile cells were isolated and placed in 24-well microplates (Zefa; Munich, Germany) containing freshwater WC growth medium (Woods Hole Combo, modified after Guillard and Lorenzen, 1972) without silicate. The plates were stored in climate chambers at 12°C or 18°C and a 12:12 h L:D photoperiod. The established monoclonal strains are currently held in the culture collection at the Institute of Systematic Botany and Mycology (University of Munich) and are available upon request. Nineteen new nuclear rRNA sequences of dinophyte were obtained as described previously (Gottschling *et al.*, 2012; Kretschmann *et al.*, 2018).

For the phylogenetic analysis, separate loci-based SSU, ITS, and LSU matrices were constructed, aligned using 'MAFFT' version 6.502a (Kato and Standley, 2013), and concatenated with SeaView version 4.6.1 (Gouy *et al.*, 2010). We compiled a taxon sample that was

representative with respect to the known molecular sequence diversity of the Gymnodiniaceae and the Peri-diniales (functioning as mutual outgroups) and included at least one (or more if available) representatives for every freshwater lineage (see the taxon sample used in this study: Supporting Information Table S1). Though they are of paleontological importance (Fensome *et al.*, 1993, 1996), the exclusively marine Protoperidiniaceae were partially excluded because of high substitution rates resulting in long branches (such as *Protoperidinium abei* and *Protoperidinium denticulatum*: Gu *et al.*, 2013; as well as long-branching *Kryptoperidinium* from the Kryptoperidiniaceae: Kretschmann *et al.*, 2018). Not all Protoperidiniaceae are subjected to this phenomenon, and this lineage is nevertheless well represented in our taxon samples including only representatives exhibiting branches of similar length in comparison to the rest of taxa.

Phylogenetic analyses were carried out using both the Maximum Likelihood (ML) and the Bayesian inference approach (BI). Maximum Likelihood-based analyses were conducted using RAxML RAxML-HPC version 8.2.10 (Stamatakis, 2014) with applied GTR +  $\Gamma$  substitution matrix. To determine best-fitted ML-trees, we carried out 10-tree searches from distinct random stepwise addition sequence Maximum Parsimony starting trees and 10 000 non-parametric bootstrap replicates. Bayesian analyses were carried out using MrBayes version 3.2.6 (Ronquist *et al.*, 2012) under the GTR +  $\Gamma$  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, sampling every 1000th cycle, with an appropriate burn-in (10%) as inferred from the evaluation of the trace files using Tracer version 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>). The statistical support values were drawn on the presented chronograms. Marine-to-freshwater transitions were reliably inferred based on sister group relationships between marine and freshwater clades (or freshwater clades nesting within marine clades) with statistical support  $\geq 80$  LBS and/or  $\geq 0.90$  BPP respectively. However, pure topology (of, e.g., less well resolved deeper nodes) was taken into account as well. All phylogenetic inferences were carried out at the CIPRES Science Gateway computing facility (Miller *et al.*, 2010). The aligned matrices are available as \*.nex files upon request.

To estimate divergence times in the Gymnodiniaceae and the Peridinales, the aforementioned alignment was used and the phylogeny was dated using BEAST version 1.8.3 (Drummond *et al.*, 2012). We carried out several different analyses using different evolutionary models with corresponding parameters (e.g., Yule process versus Coalescence) and different alignments (e.g., including and excluding heterogeneous sites). The final decision on the analysis was made based on ESS

values, which had to exceed 200. The most fitting evolutionary model (GTR +  $\Gamma$  + i) and the rate heterogeneity were assessed with jModelTest (Darrriba *et al.*, 2012); the +i option was ignored, as it leads to over-parameterization (see RAxML manual). Unlike some other generally scarce dinophyte dating studies (John *et al.*, 2003; LaJeunesse, 2004; Pochon *et al.*, 2006), we used in-group fossils (the advantages for this strategy are discussed in Gottschling *et al.*, 2008).

For the presented analysis, a Yule branching process with lognormal priors and empirically defined base frequencies was adopted using the complete alignment and following five calibration points (minimal ages for stem groups, see Gottschling *et al.*, 2008): Peridinales were estimated at  $200 \pm 1$  MYA (Fensome *et al.*, 1996), Peridinales excluding Heterocapsaceae were estimated at  $160 \pm 4$  MYA based on the first fossil occurrence of (calcareous) coccooid cells with tabulation (Keupp, 1984; Keupp and Ilg, 1989), *Scrippsiella s.l.* was estimated at  $70 \pm 0.5$  MYA based on the combination archaeopyle (Streng *et al.*, 2004) as apomorphic trait, the *Thoracosphaera/Pfiesteria* clade at  $70 \pm 0.5$  MYA based on the first occurrence of *Thoracosphaera* in the fossil record (Fensome *et al.*, 1996), and †*Calciadinellum* at  $37.5 \pm 0.5$  MYA based on its tabulated coccooid cells (Hildebrand-Habel and Streng, 2003). For the GTR +  $\Gamma$  substitution model with four discrete categories, we applied an uncorrelated relaxed molecular clock with a diffuse gamma distribution of shape 0.001, scale 1 000.0 and offset 0.0 for the ucl.d.mean rate. The starting tree was constructed at random, and the final topology was estimated by combining 15 independent chains of 50 million generations each, sampling every 10 000th iteration. TRACER version 1.6 (<http://tree.bio.ed.ac.uk/software/tracer/>) was used to evaluate ESS values and to confirm adequate combining of the Markov chain Monte Carlo chains with an appropriate burn-in (10%). All dating analyses were again performed at the CIPRES Science Gateway computing facility (Miller *et al.*, 2010).

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## Supporting Information

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

## Appendix S1: Supporting Information