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Molecular data and the evolutionary history of dinoflagellates

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Abstract

We have sequenced small-subunit (SSU) ribosomal RNA (rRNA) genes from 16 dinoflagellates, produced phylogenetic trees of the group containing 105 taxa, and combined small- and partial large-subunit (LSU) rRNA data to produce new phylogenetic trees. We compare phylogenetic trees based on dinoflagellate rRNA and protein genes with established hypotheses of dinoflagellate evolution based on morphological data. Protein-gene trees have too few species for meaningful in-group phylogenetic analyses, but provide important insights on the phylogenetic position of dinoflagellates as a whole, on the identity of their close relatives, and on specific questions of evolutionary history. Phylogenetic trees obtained from dinoflagellate SSU rRNA genes are generally poorly resolved, but include by far the most species and some well-supported clades. Combined analyses of SSU and LSU somewhat improve support for several nodes, but are still weakly resolved. All analyses agree on the placement of dinoflagellates with ciliates and apicomplexans (=Sporozoa) in a well-supported clade, the alveolates. The closest relatives to dinokaryotic dinoflagellates appear to be apicomplexans, *Perkinsus*, *Parvilucifera*, syndinians and *Oxyrrhis*. The position of *Noctiluca scintillans* is unstable, while Blastodiniales as currently circumscribed seems polyphyletic. The same is true for Gymnodiniales: all phylogenetic trees examined (SSU and LSU-based) suggest that thecal plates have been lost repeatedly during dinoflagellate evolution. It is unclear whether any gymnodinialean clades originated before the theca. Peridiniales appear to be a paraphyletic group from which other dinoflagellate orders like Prorocentrales, Dinophysiales, most Gymnodiniales, and possibly also Gonyaulacales originated. Dinophysiales and Suessiales are strongly supported holophyletic groups, as is Gonyaulacales, although with more modest support. Prorocentrales is a monophyletic group only in some LSU-based trees. Within Gonyaulacales, molecular data broadly agree with classificatory schemes based on morphology. Implications of this taxonomic scheme for the evolution of selected dinoflagellate features (the nucleus, mitosis, flagella and photosynthesis) are discussed.

Keywords: Dinoflagellates; Ribosomal RNA; Chloroplast evolution; Molecular phylogeny; Theca; Photosynthesis

Introduction

The importance of dinoflagellates in aquatic communities is hard to overestimate. They are ubiquitous in marine and freshwater environments, where they constitute a large percentage of both the phytoplankton and

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the microzooplankton, and in benthic communities as interstitial flora and fauna or as symbionts in reef-building corals, other invertebrates and unicellular organisms (Taylor, 1987). Both ecto- and endoparasitic dinoflagellate species are also common, infecting hosts ranging from other protists like ciliates, radiolarians or even other dinoflagellates, to crustaceans, cnidarians, appendicularians, polychaetes, fish and many others (Cachon and Cachon, 1987). Many species of dinoflagellates are notorious for producing toxins that can cause human illness through shellfish or fish poisoning (Steidinger, 1993); dinoflagellates are the ultimate cause of diseases like diarrheic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP) and ciguatera. Some toxic dinoflagellates (as well as other protists) can also cause fish kills and mortality of other marine fauna (Steidinger, 1993).

One recent definition of dinoflagellates is found in Fensome et al. (1993, p. 3): they are ''eukaryotic, primarily single-celled organisms in which the motile cell possesses two dissimilar flagella: a ribbon-like flagellum with multiple waves which beats to the cell's left, and a more conventional flagellum with one or a few waves which beats posteriorly''. Taxonomic treatments of the group have traditionally been based on two sets of cytological characters. One is the presence of a dinokaryon, a uniquely modified nucleus that lacks nucleosomal histones and contains fibrillar chromosomes with a typical ultrastructure that remain condensed throughout the cell cycle, and that divides through a special type of closed mitosis with an extranuclear spindle (review in Dodge, 1987). Dinokarya are present in most dinoflagellates, but not in the parasitic order Syndiniales or in particular life stages of the Blastodiniales (also parasitic) and Noctilucales (Fensome et al., 1993). The other character, applied to dinokaryotic dinoflagellates, is the arrangement of cortical alveoli, flattened vesicles immediately underneath the plasma membrane that often contain cellulose thecal plates (in dinoflagellate literature cortical alveoli are generally referred to as amphiesmal vesicles, review in Netzel and Dürr (1984)). In thecate orders (Gonyaulacales, Peridiniales, Dinophysiales, Prorocentrales), the theca is contained in relatively few alveoli with a pattern that can be determined relatively easily (thecal plate tabulation). Athecate taxa, however, (notably the order Gymnodiniales, but also Syndiniales, Noctilucales, etc.) often contain hundreds of alveoli, making it difficult to determine homologies and locational relationships. As a consequence, thecate taxa are much easier to classify than athecate ones.

Thecal plate patterns are also easier to determine in species that are easily found as motile stages, the cell type that typically displays this feature. However, these motile stages are often very short phases of dinoflagellate life cycles; some species are most often found as cysts (Suessiales, Thoracosphaerales, Phytodiniales, a few Gonyaulacales), plasmodia (many Syndiniales) or as strongly modified trophonts that are not easily comparable to the typical dinoflagellate motile stages (Noctilucales or Blastodiniales). The tabulation of the motile stages is often reflected in cysts, a feature that has been used extensively to detect relationships between extant and fossil genera (Fensome et al., 1993; Fensome et al., 1999; most fossil dinoflagellates are cysts). Within some thecate orders, a (putative) radiation of forms can be followed remarkably well using extant species (e.g. in Dinophysiales, Gonyaulacales and Peridiniales, Taylor, 1980), even if the direction of the changes cannot. Nevertheless, this cannot be done between orders, for there appear to be few intermediate forms. As a consequence, except for some cases where informative intermediate fossil taxa have been found (Fensome et al., 1993), the mutual relationship of many dinoflagellate orders is still unclear. Also unclear is which groups of dinoflagellates are early or late diverging; different sets of characters support different hypotheses (discussion in Taylor, 1980; Fensome et al., 1993).

Early phylogenetic studies showed the monophyly of dinoflagellates (Maroteaux et al., 1985; Herzog and Maroteaux, 1986) and disproved notions that dinoflagellates are early branches of the eukaryote tree (the mesokaryotic theory, Dodge, 1965, 1966). A relationship between dinoflagellates and ciliates that had been postulated earlier (Corliss, 1975; Taylor, 1976) was also corroborated by these sequences, as was a newly discovered one to apicomplexans (Wolters, 1991; Gajadhar et al., 1991). In 1991 a new taxon, the Alveolata, was created encompasing ciliates, dinoflagellates, apicomplexans and their close relatives, the protalveolates (Cavalier-Smith, 1991), and numerous studies have repeatedly supported its validity (e.g. Cavalier-Smith, 1993; Van de Peer et al., 1996; Fast et al., 2002). The relationship of alveolates to other groups has been more difficult to resolve, but recent studies based on phylogenies of concatenated proteins and chloroplast-targeted genes (Baldauf et al., 2000; Fast et al., 2001) have supported the relationship between this group and chromists as predicted by the chromalveolate hypothesis (Cavalier-Smith, 1999, 2003) and by earlier taxonomic schemes (e.g. Taylor, 1976).

Within alveolates, dinoflagellates are more closely related to the apicomplexans than to the ciliates (Fast et al., 2002). Other close relatives of dinoflagellates include forms that share a number of features typical of all alveolates (e.g. cortical alveoli, mitochondria with tubular cristae, presence of trichocysts in diverse forms), but lack the synapomorphies that define ciliates, dinoflagellates or apicomplexans, the so-called protalveolates (Cavalier-Smith, 1991, 1993). The genus *Perkinsus*, for example, a parasite of oysters and other bivalves, and *Parvilucifera*, a parasite infecting

dinoflagellates, often form a clade closely related to dinoflagellates (Siddall et al., 1997; Norén et al., 1999; Saldarriaga et al., 2003a); the genus *Rastrimonas* (formerly *Cryptophagus*, Brugerolle, 2003), a parasite of cryptomonads, could be a third member of this group (Brugerolle, 2002b). Other protalveolate taxa that seem to have close links to the dinoflagellates are the free-living genus *Oxyrrhis*, recently excluded from the group (Fensome et al., 1993), and the ellobiopsids, a group of parasites of crustaceans that are either derived from or very closely related to dinoflagellates (J. Silbermann, personal communication). The genus *Colpodella*, however, appears to be a basal branch to the apicomplexans (Cavalier-Smith, 2000; Brugerolle, 2002a; Kuvardina et al., 2002; Leander and Keeling, 2003). Other protalveolates have not been characterized at the molecular level, and so it remains to be seen where the phylogenetic affiliation of *Colponema, Acrocoelus*, and others may lie.

Nearly all molecular phylogenetic studies of the in-group relationships of dinoflagellates have used rRNA, either partial sequences of the large-subunit (LSU) ribosomal RNA (rRNA) gene (LSU, e.g. Lenaers et al., 1991; Zardoya et al., 1995; Daugbjerg et al., 2000), or the small-subunit (SSU) rRNA gene (SSU, e.g. Saunders et al., 1997; Grzebyk et al., 1998; Gunderson et al., 1999; Saldarriaga et al., 2001). Phylogenies based on SSU are the only ones with data for phylogenetically important groups like the Syndiniales, Noctilucales or Blastodiniales. Relationships of orders to one-another are mostly unresolved (e.g. Saunders et al., 1997; Saldarriaga et al., 2001), but those at the base of the lineage are often well supported, as are some late-branching groups. Phylogenies based on the first two or three domains (D1–D3) of the LSU contain fewer taxa than SSU-based ones, but since the two molecules appear to evolve at different rates (Ben Ali et al., 2001; John et al., 2003) they have also proven very valuable since bootstrap support for certain groupings is greater. Protein-gene based phylogenies are still scarce, e.g. HSP90, actin, alpha- and beta-tubulin genes (Saldarriaga et al., 2003a; B. Leander, unpublished data), and plastid-encoded genes (e.g. psbA in Takishita and Uchida (1999), psaA in Yoon et al. (2002), Zhang et al. (2000)). None of these yet contain many taxa, and support for their in-group clades tends to be weak. Nevertheless, they have proven valuable for determining the position of some basal taxa (e.g. Saldarriaga et al., 2003a).

The objective of the present work is to clarify some key events in the evolutionary history of the dinoflagellates and their close relatives. We re-examine all available data, molecular, morphological, paleontological and biochemical, to produce a phylogenetic framework for the group, with special consideration given to rRNA trees. We explore the origin of the dinokaryon and of dinokaryotic dinoflagellates, the development of cortical alveoli in the group and the history of dinoflagellate photosynthesis. We also examine some smaller scale questions, e.g. the relationship of *Perkinsus*, *Oxyrrhis*, *Noctiluca*, Syndiniales and Blastodiniales to other dinoflagellates and the circumscription of the different groups of Gymnodiniales. Lastly, we consider whether the phylogeny of Gonyaulacales, generally better supported than other parts of the tree, is congruent with the proposals for dinoflagellate classification based on morphology put forward by Fensome et al. (1993).

Materials and methods

Organisms, DNA extraction, amplification and sequencing

Photosynthetic dinoflagellate species were obtained from non-axenic culture collections (Table 1) and cultured according to established protocols (e.g. Harrison et al., 1980). The heterotrophic *Protoperidinium* species were fed the diatom *Ditylum brightwellii* and maintained at 12°C in F/2 medium at 30 µmols photons $m^{-2} s^{-1}$ on a plankton wheel at 1 rpm. Cells were harvested by centrifugation. DNA was extracted using the DNeasy Plant DNA Purification Kit (Qiagen). Whenever possible, the 18S (nuclear SSU) rRNA gene was amplified as a single fragment using a polymerase chain reaction with two eukaryotic universal SSU primers (5′-CGAATTCAACCTGGTTGATCCTGCCAGT-3′ and 5′-CCGGATCCTGATCCTTCTGCAGGTTCACCTAC-3′). However, in many cases two overlapping fragments had to be produced using internal primers designed to match existing eukaryotic SSU sequences (4F: 5′-CGGAATTCCAGTC-3′ and 11R: 5′-GGATCACAGCTG-3′). PCR products were either sequenced directly or cloned into pCR-2.1 vector using the TOPO TA cloning kit (Invitrogen). Sequencing reactions were completed with both the original PCR primers as well as 2–3 additional primers in each direction. When using cloned fragments, 2–4 clones were sequenced to detect and clarify possible ambiguities.

Phylogenetic analysis

New sequences were added to the SSU alignment of Saldarriaga et al. (2001); they are now available from GenBank. The final multiple alignment contained 98 dinoflagellate species, plus *Perkinsus*, *Parvilucifera* and several ciliate and apicomplexan sequences that were used as outgroups. The sequence for *Oxyrrhis marina* was excluded from the analyses: previous experience showed that this species has an extremely derived SSU sequence that distorts the topologies of SSU trees (Saldarriaga et al., 2003a). We also included seven

Table 1. List of strains examined in this study and GenBank Accession Numbers for their nuclear SSU rRNA sequences

Species name	Strain	Genbank accession number
Amphidinium britannicum (Herdman) Lebour (as *Amphidinium asymmetricum* var. *compactum*)[a]	CCCM 081	AY443010
Amphidinium operculatum Claparède & Lachmann[a]	CCMP 1342	AY443011
Amphidinium rhynchocephalum Anissimowa[a]	UTEX LB 1946	AY443012
Amylax diacantha Meunier	None	AY443013
Ceratium hirundinella (O. F. Müller) Dujardin	None	AY443014
Gyrodinium instriatum Freudenthal & Lee	CCMP 431	AY443015
Hemidinium nasutum Stein	NIES 471	AY443016
Peridinium polonicum Woloszynska	NIES 500	AY443017
Peridinium wierzejskii Woloszynska	NIES 502	AY443018
Prorocentrum gracile Schütt	CCCM 765	AY443019
Protoperidinium conicum (Gran) Balech	None	AY443020
Protoperidinium excentricum (Paulsen) Balech	None	AY443021
Protoperidinium pellucidum Bergh	None	AY443022
Pyrophacus steinii (Schiller) Wall & Dale	NIES 321	AY443024
Symbiodinium sp. (symbiont of *Aiptasia pallida*) (="*Symbiodinium bermudense*")	None	AY443023
Woloszynskia leopoliensis (Woloszynska) Thompson	NIES 619	AY443025

Abbreviations: CCCM: Canadian Centre for the Culture of Microorganisms; CCMP: Provasoli-Guillard National Center for Culture of Marine Phytoplankton; NIES: National Institute for Environmental Studies, Japan; UTEX: Culture Collection of Algae at the University of Texas, Austin.

[a] A major revision of the genus *Amphidinium* is underway (N. Daugbjerg, personal communication). The names of all three *Amphidinium* species examined here are likely to change soon.

sequences obtained from environmental samples of marine picoplankton by López-García et al. (2001) (GenBank accessions AF290066, AF290068, AF290077 and AF290078) and Moon-van der Staay et al. (2001) (GenBank accessions AJ402326, AJ402330 and AJ402354). Only unambiguously aligned sections of the molecule (1479 characters) were used in the phylogenetic analyses. A second set of analyses of SSU data was performed excluding all ciliate and apicomplexan taxa (*Perkinsus* was used as the outgroup); by doing this we were able to align confidently a significantly larger portion of the SSU molecule (1649 sites).

SSU sequences were also concatenated with published sequences for sections of the LSU rRNA gene (LSU). Concatenated alignments that included SSU and domains D1–D3 of the LSU included 25 alveolate species (22 of them dinoflagellates) and 2418 nucleotides, while alignments with SSU and domains D1–D2 of the LSU included 34 species (31 of them dinoflagellates) and 2100 nucleotides. Phylogenetic trees based on LSU only were also calculated for comparison; in them the choice of sites was extremely conservative, only 447 sites for alignments of domains D1–D2, 718 sites for those of domains D1–D3.

Distances were calculated with PUZZLE 5.0. (Strimmer and von Haeseler, 1996) using the HKY substitution frequency matrix. Nucleotide frequencies and transition/transversion ratios were estimated from the data, and site-to-site variation was modeled by a gamma distribution with invariable sites plus 8 variable rate categories and the shape parameter alpha estimated from the data. Distance trees were constructed using BioNJ (Gascuel, 1997), Weighbor (Bruno et al., 2000) and Fitch-Margoliash (Felsenstein, 1993). One hundred bootstrap data sets were made using SEQBOOT and trees inferred as described for parsimony and corrected distances, where distances were calculated using puzzleboot (by M. Holder and A. Roger) with the alpha shape parameter, nucleotide frequencies and transition/transversion ratio from the initial tree enforced on the 100 replicates. Maximum likelihood trees were calculated for the concatenated SSU/LSU (D1–D2) datasets and for a heavily reduced alignment of SSU sequences (40 species; 35 dinoflagellates). They were inferred under an HKY model incorporating a discrete gamma distribution (invariable sites and 8 variable rate categories; shape parameter, nucleotide frequencies and transition/transversion ratio estimated from the data, 5 jumbles, PAUP 4.0, Swofford, 1999). Maximum likelihood trees were also calculated from the 100 bootstrap data sets in the case of the concatenated data.

Results

SSU rRNA phylogeny

It is unknown whether the sequences from the environmental samples from López-García et al. (2001)

and Moon-van der Staay et al. (2001) come from organisms that would be called dinoflagellates based on morphology, and for that reason it is very difficult to say whether the dinoflagellate clade as a whole was supported in our trees or not. Those environmental sequences, however, always grouped in two clades. One of them (group II in López-García et al., 2001) generally also included all known sequences of Syndiniales (*Hematodinium* and 3 species of *Amoebophrya*; in the Fitch tree *Hematodinium* was outside of the group). The other clade (group I in López-García et al., 2001) included only environmental sequences, and in the BioNJ (Fig. 1) and weighbour trees branched basal to all other dinoflagellates but not to the *Perkinsus*/*Parvilucifera* grouping (in the Fitch tree this clade branched after the Syndinians and *Noctiluca*). If one assumes that all these environmental sequences come from true dinoflagellates, then the dinoflagellate clade is supported in all trees by bootstraps of 60–65%.

Placement of *Noctiluca* in all trees was very unstable. In BioNJ and Weighbor trees, it branched with negligible support at the base of all established dinoflagellates (including syndinians but not the members of the group I clade). Interestingly, SSU trees including only dinoflagellates and *Perkinsus* that utilized more sites invariably placed *Noctiluca scintillans* within the GPP complex (Fig. 2). All other dinoflagellates, including two species considered members of the Blastodiniales in Fensome et al. (1993) (*Amyloodinium* sp. and *Haplozoon axiothellae*), form a single clade in all trees examined. A large part of that clade is composed of very short-branched members of the orders Gymnodiniales, Peridiniales, Prorocentrales and Dinophysiales, the so-called GPP complex (Saunders et al., 1997), along with *Thoracosphaera* (Thoracosphaerales), *Hemidinium* (Phytodiniales), *Amyloodinium*, *Haplozoon* and the parasitic genus *Pfiesteria*. Only the order Dinophysiales, represented only by the genus *Dinophysis*, groups strongly as a distinct clade within the GPP complex (Edvardsen et al., 2003). The Prorocentrales break into two groups, one containing benthic species (*Prorocentrum lima, P. concavum*), the other more planktonic species (*P. micans, P. gracile, P. minimum*, Grzebyk et al., 1998). The Gymnodiniales scatter throughout the tree, forming at least five major subgroups. One, composed of several (but not all) species of the genus *Amphidinium*, lacks the characteristic short branches of the GPP complex and generally groups close to Gonyaulacales. A second group of Gymnodiniales always groups strongly with the only two extant genera of the order Suessiales, *Symbiodinium* and *Polarella* (bootstrap supports 97–99%). The last three strongly supported gymnodinialean clades are bona fide members of the GPP complex: one includes the type species of *Gymnodinium* (*G. fuscum*) and close relatives (including *Lepidodinium viride*); the second, members of *Karenia* and *Karlodinium* but also *Amphidinium herdmanii*; and the third, three putative members of the genus *Gyrodinium* (*G. instriatum* and *G. dorsum* have identical SSU sequences that differ from that of *G. uncatenum* by only 3 nucleotides out of 1755). The sequences for *Amphidinium* cf. *operculatum*, *Amphidinium massartii* and "*Amphidinium rhynchocephalum*" are also identical, differing by 8 nucleotides (from a total of 1752) from that of *A. carterae*.

In some trees (e.g. Fitch), the majority of Peridiniales form a clade, albeit very weakly supported and interrupted by *Haplozoon axiothellae*. It includes all members of *Heterocapsa, Scrippsiella* and *Pentapharsodinium*, plus *Lessardia, Roscoffia, Peridiniopsis,* and two species of *Peridinium*, *P. umbonatum* and *P. wierzejskii* (in Weighbor and BioNJ trees this clade is interrupted by gymnodinialean and/or prorocentralean groups; *Thecadinium dragescoi* is probably a misnamed member of the peridinialean genus *Amphidiniopsis*, M. Hoppenrath, pers. comm.). Nevertheless, several peridinialean taxa never group with the bulk of the order. These include a well-supported clade of the three *Protoperidinium* species and a well-supported grouping of three *Peridinium* species (*Peridinium* sp., *P. willei* and *P. bipes*) that sometimes includes *Glenodiniopsis steinii*. The diatom-bearing genera *Kryptoperidinium* and *Durinskia* form a weakly supported clade in BioNJ trees, as do *Pfiesteria* and the putatively blastodinialean *Amyloodinium* in the Fitch and Weighbor trees. None of these groupings ever branch with the bulk of the Peridiniales.

The Gonyaulacales generally have longer branches than other dinoflagellates (only Syndiniales, *Haplozoon, Protoperidinium* and the *A. carterae* clade have comparably long branches). They tend to form a clade to the exclusion of almost all other dinoflagellates (e.g. in the Fitch and BioNJ trees), although it is never well supported. The phytodinialean genus *Halostylodinium* consistently branches within the clade. Within the Gonyaulacales (Table 2), several groupings appear consistently, e.g. one containing *Ostreopsis*, *Alexandrium*, *Fragilidium*, *Pyrophacus*, *Pyrodinium* and *Pyrocystis* (suborder Goniodominae, 50–60% bootstrap support) and another containing all *Ceratium* species (Ceratiineae, 75–90% bootstrap support). Members of the Gonyaulacineae (*Protoceratium*, *Lingulodinium*, *Gonyaulax*, *Amylax* and *Ceratocorys*) consistently branch at the base of the Gonyaulacales, as a paraphyletic group that gives rise to the Ceratiineae and Goniodominae and that also contains *Crypthecodinium* and *Halostylodinium*.

LSU rRNA phylogeny

Phylogenetic trees based on LSU data were similar to those based on SSU. As LSU sequences for *Perkinsus*,

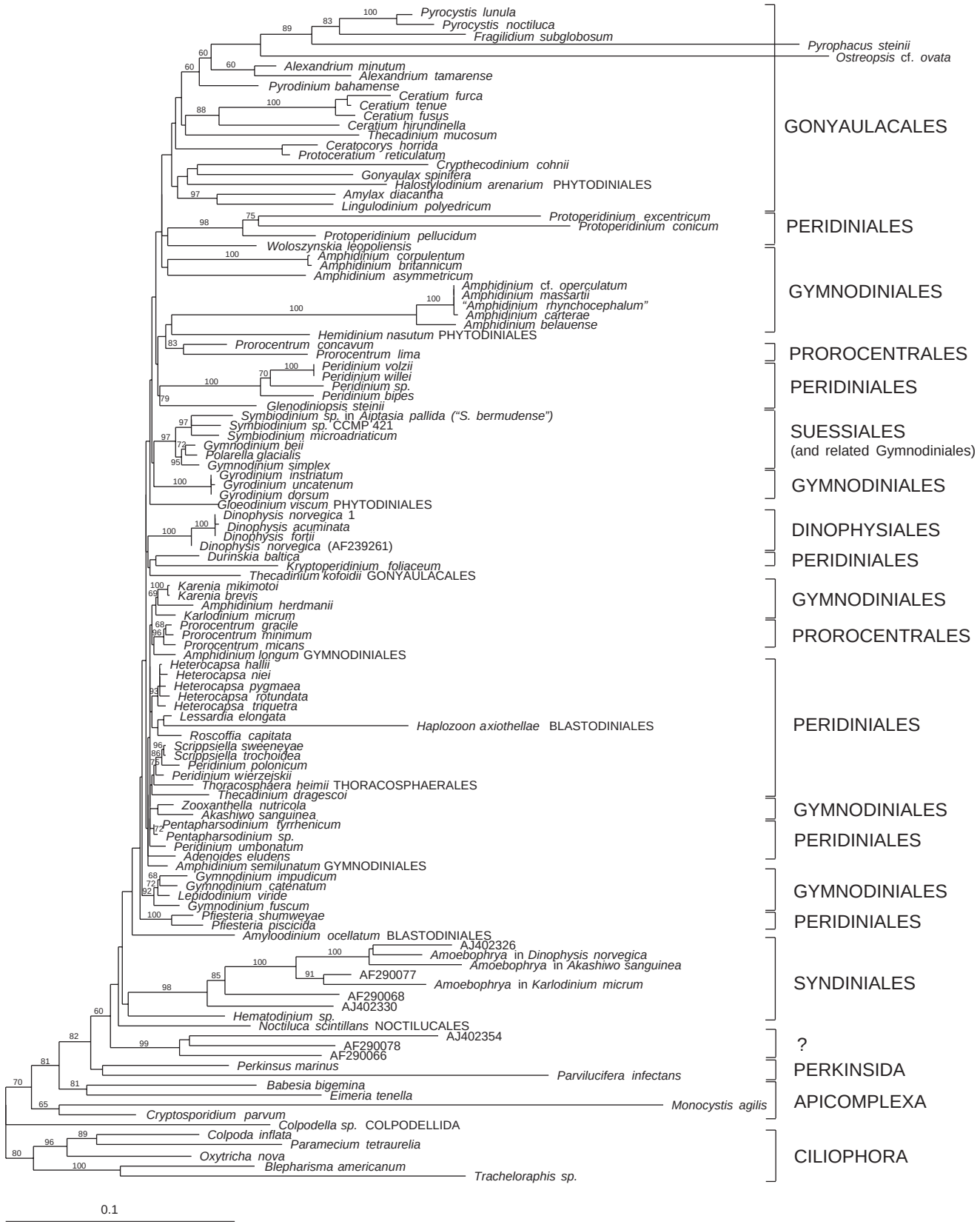

Fig. 1. Phylogenetic tree constructed with BioNJ from a gamma-corrected distance matrix of SSU rRNA sequences (1479 nucleotides) from 117 species of alveolates, including 98 dinoflagellates and 7 undescribed species from environmental samples identified by their GenBank accession numbers (López-García et al., 2001; Moon-van der Stay et al., 2001). Bootstrap values are shown when larger than 60%. Problematic names of taxa are given in quotation marks.

Fig. 2. Phylogenetic tree constructed with weighted neighbour joining from a gamma-corrected distance matrix of SSU rRNA sequences (1649 nucleotides) from 98 dinoflagellates, 7 undescribed species from environmental samples identified by their GenBank accession numbers, and *Perkinsus marinus*, used as the outgroup. Bootstrap values are shown when larger than 60%. Problematic names of taxa are given in quotation marks.

Table 2. Classification of the order Gonyaulacales according to Fensome et al. (1993), including only species for which SSU sequence data are available

Suborder	Family	Subfamily	Genus
Gonyaulacinae	Gonyaulacaceae	Cribroperidinioideae	*Protoceratium*
			Lingulodinium
		Gonyaulacoideae	*Gonyaulax*
			Amylax
	Ceratocoryaceae		*Ceratocorys*
Ceratiineae	Ceratiaceae		*Ceratium*
Goniodomineae	Goniodomaceae	Gambierdiscoideae	*Ostreopsis*
			Coolia
		Helgolandinioideae	*Alexandrium*
			Fragilidium
			Pyrophacus
		Pyrodinioideae	*Pyrodinium*
	Pyrocystaceae		*Pyrocystis*
Uncertain	Crypthecodiniaceae		*Crypthecodinium*
	Uncertain		*Thecadinium*
Order Phytodiniales in Horiguchi et al. (2000)			*Halostylodinium*

Oxyrrhis, Syndiniales, Noctilucales or Blastodiniales are unavailable, the trees consisted of a large, badly resolved group of very short-branched taxa (the GPP complex, Gymnodiniales, Peridiniales, Prorocentrales and Dinophysiales) and a monophyletic grouping of longer-branched members of the order Gonyaulacales (Fig. 3). Within the GPP complex, groupings well supported in SSU trees are also well supported here, e.g. the *Gymnodinium fuscum* group (henceforth *Gymnodinium* sensu stricto, Daugbjerg et al. (2000)), the *Karenia*/*Karlodinium* group, Suessiales (including several *Gymnodinium* species), and Dinophysiales. There are, however, several differences from SSU trees. In at least some LSU trees, all Prorocentrales do group together (e.g. in the Weighbor tree, Fig. 3), and while the *A. carterae* group still holds together with good support and a relatively long branch, it is not at the base of the Gonyaulacales (in Weighbor and Fitch trees it interrupts a badly supported clade of Peridiniales). The position of *Woloszynskia* is also different in SSU and LSU trees: while in LSU it branches with the Suessiales with 95–97% bootstrap support, in SSU its position is very unstable (the two alignments contain different species of the genus: *W. pseudopalustris* in LSU, *W. leopoliensis* in SSU).

Gonyaulacales also form a clade in most LSU trees, albeit with modest bootstrap support (in the ML tree, the genus *Ceratium* branches together with the Apicomplexan outgroup). The majority of the gonyaulacalean species with LSU data are members of the Goniodominae (*Alexandrium, Fragilidium, Coolia* and *Ostreopsis*), and they form a clade excluding all other taxa, with low bootstrap support (the sequence for *Ceratium furca* interrupts a very strongly supported clade of many *Alexandrium* species in all trees; we suspect this to be an error). The other *Ceratium* sequences, as well as those for *Protoceratium* and *Gonyaulax,* often make a paraphyletic group at the base of the Gonyaulacales that gives rise to the Goniodominae (not in the Fitch trees, where Goniodominae appear to give rise to Gonyaulacinae and *Ceratium*). *Protoceratium* and *Gonyaulax* were never sisters.

Combined rRNA phylogeny

Phylogenetic trees based on combined datasets (Fig. 4) generally show the basic structure discussed above: a badly supported backbone of short-branched taxa (the GPP complex) that includes some very well-supported subgroups, and the Gonyaulacales, longer-branched taxa that invariably form a clade, here very well supported (80–100% bootstrap support). The well-supported groups in the GPP complex are identical to those discussed above, but their relative order is variable. Prorocentrales never group together, forming the same two clades as in SSU trees. Within Gonyaulacales, the Gonyaulacinae (*Gonyaulax* and *Protoceratium*) generally branch as sisters to a group that contains *Ceratium* and the Goniodominae (in the Fitch and Weighbor trees based on SSU/D1/D2/D3 concatenations the *Gonyaulax*/*Protoceratium* clade is not retained). One major difference between the concatenated and single gene trees is that in all concatenated trees the two *Heterocapsa* species included are sisters to the bulk of the GPP complex with bootstrap support between 43% and 71%. In the

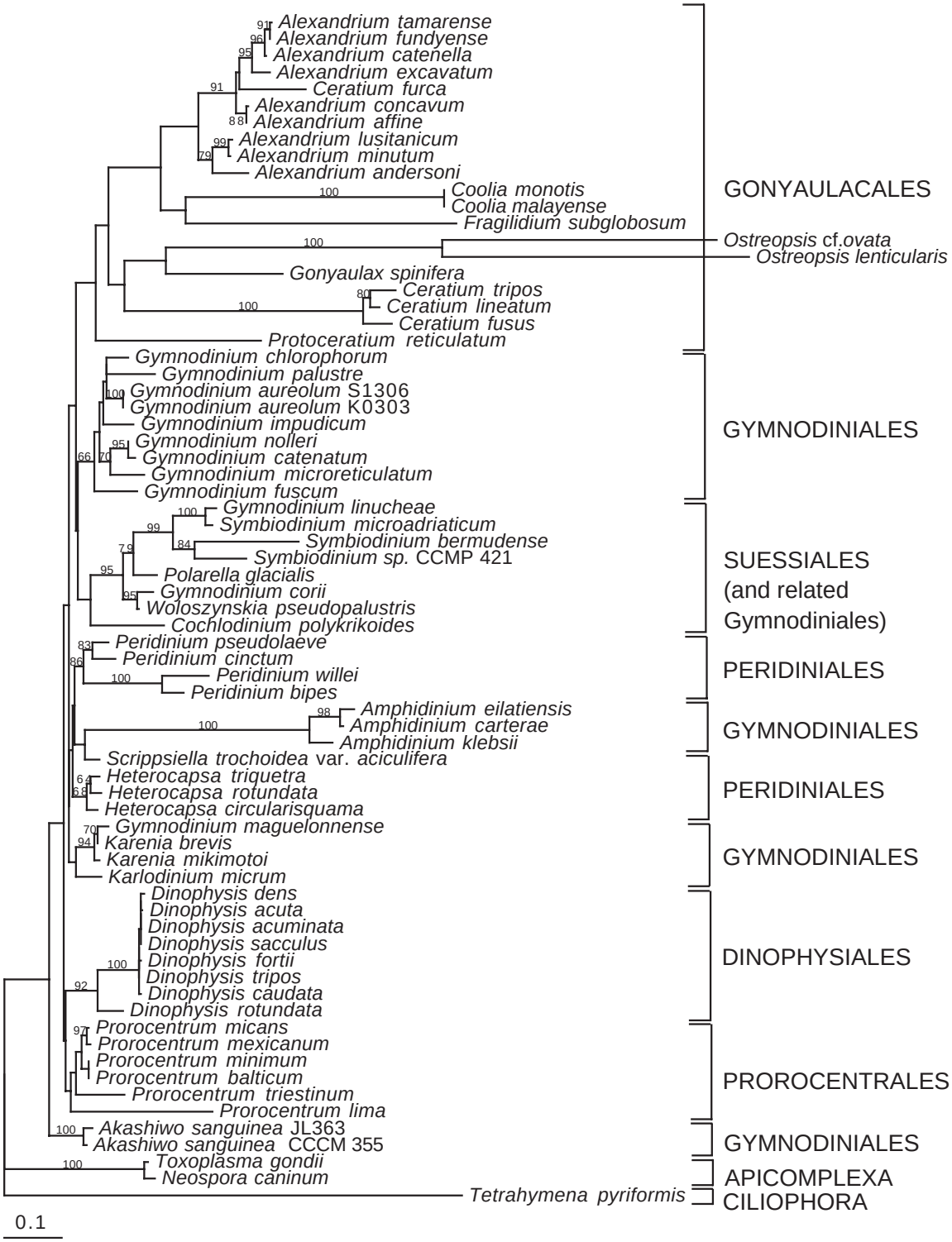

Fig. 3. Phylogenetic tree constructed with weighted neighbor joining from a gamma-corrected distance matrix of domains D1 and D2 of the LSU rRNA gene (447 nucleotides) from 71 alveolates, 68 of them dinoflagellates. Bootstrap values shown when larger than 60%.

Weighbor trees and in the Fitch and ML trees based on the SSU/D1/D2/D3 concatenation these two species make a clade, in the other trees they do not. Many nodes have better bootstrap support than in the single-gene trees. It is unclear whether this is a consequence of fewer taxa or the longer sequence.

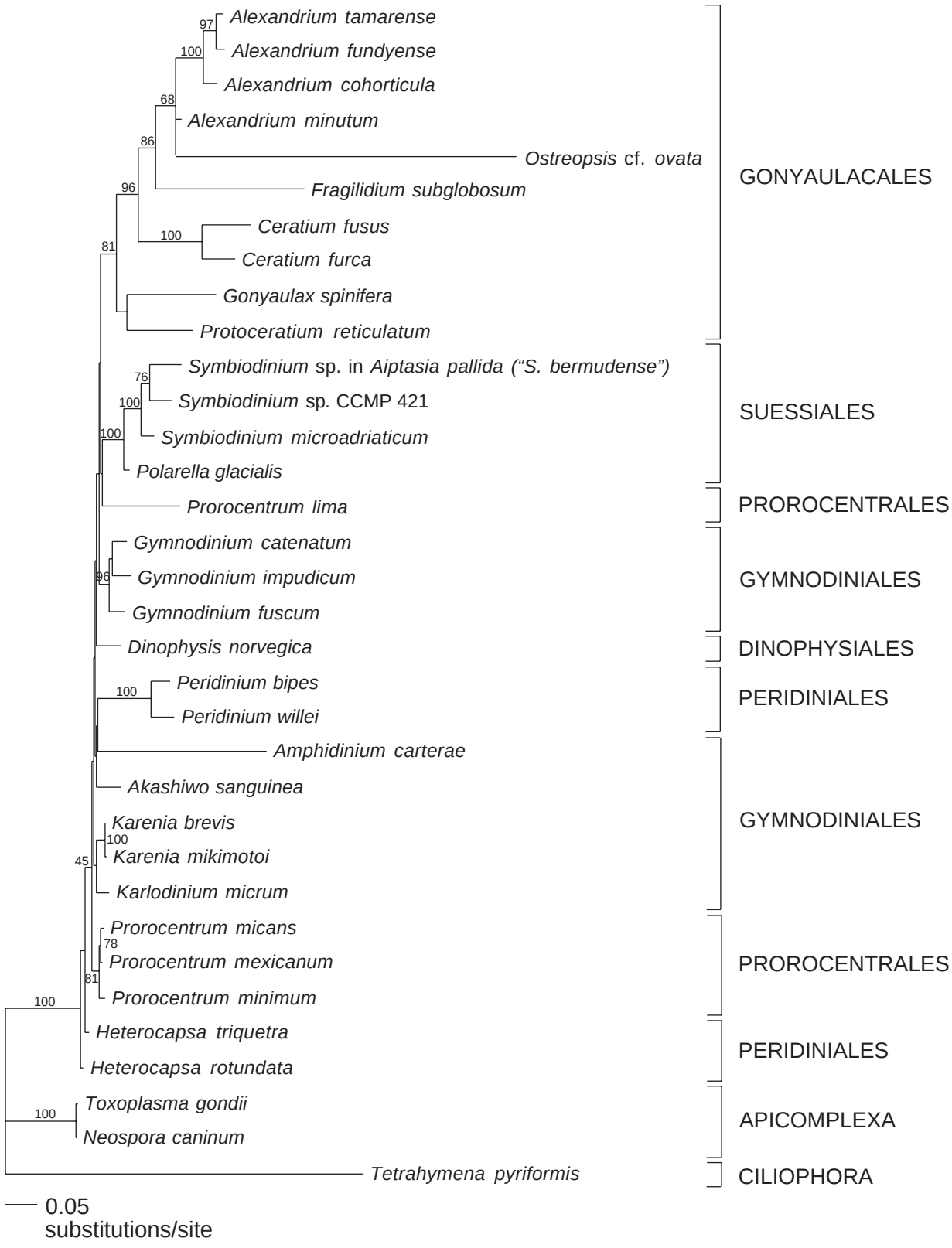

Fig. 4. Maximum likelihood phylogenetic tree constructed from concatenated LSU (domains D1 and D2) and SSU rRNA sequences (2100 nucleotides) from 34 alveolates, 31 of them dinoflagellates. Transition/transversion ratio 2.13; other trees found with slightly lower log likelihoods differed only in minor details. Bootstrap values are shown when higher than 60%.

Discussion

Rates of evolution, the structure of dinoflagellate phylogenetic trees and the mesozoic radiation of dinoflagellates

There is a striking asymmetry of evolutionary rates in the ribosomal genes of dinoflagellates, more pronounced in the SSU genes but also present in the domains of the LSU investigated here. As a consequence, both SSU- and LSU-based phylogenetic trees present a very characteristic structure: a large group of very short-branched GPP species, and a clade with medium- to very long branches. As far as these two groupings are concerned, the differences in evolutionary rate are certainly correlated with the phylogenetic history of the group: one clade of medium to long-branched species is composed exclusively of taxa classified in the order Gonyaulacales, and there are typically no Gonyaulacales elsewhere. Nevertheless, other species also have divergent sequences, including *Oxyrrhis*, *Haplozoon*, *Protoperidinium* and *Amoebophrya* in SSU trees and the *A. carterae* clade in both SSU and LSU. The fact that, with the exception of *Oxyrrhis* (Saldarriaga et al., 2003a) and in a few trees the *A. carterae* clade these long-branched taxa do not generally intrude into the Gonyaulacales is a sign that the grouping may reflect a real phylogenetic signal rather than long-branch attraction. It is interesting that no protein sequences known from the gonyaulacalean *Crypthecodinium cohnii* are particularly divergent; the asymmetry of evolutionary rates in ribosomal genes of dinoflagellates may not extend to protein genes.

The "backbone" of all dinoflagellate rRNA trees is very weakly supported. This is consistent with a rapid dinoflagellate radiation into the major forms we see today. The palaeontological record gives a very similar picture (Fensome et al., 1999): although putative dinoflagellate fossils in the form of acritarchs and biogeochemical traces exist from as early as the Cambrian (e.g. Moldowan and Talyzina, 1998, discussion in Fensome et al., 1999), undisputed dinoflagellates appear for the first time in the early Mesozoic, and by the mid-Jurassic practically all variations of at least gonyaulacalean and peridinialean forms were already present. Nevertheless, the fossil record consists almost exclusively of groups that produce fossilizable cysts (ca. 15% of extant species of dinoflagellates, Head, 1996), other groups are very badly represented, making it unclear whether the rapid increase in gonyaulacalean and peridinialean morphological types in the early Jurassic was caused by a radiation of the whole group. The congruence between the patterns suggested by the fossil record and the rRNA trees, which include non-cyst-formers, implies a general radiation that included athecate forms.

A phylogenetic framework for understanding dinoflagellate evolution

Fig. 5 shows a hypothesis on the evolutionary history of dinoflagellates and their close relatives. It is based on features of molecular trees that are well supported and/or congruent with one-another and on morphological and palaeontological information.

Perkinsus, *Oxyrrhis* and the Syndiniales

The relative positions of *Perkinsus*, the dinokaryotic dinoflagellates and the apicomplexans derived from molecular data are well supported by many different genes coding both for rRNAs and for proteins (e.g. Reece et al., 1997; Fast et al., 2001; Saldarriaga et al., 2003a). The relationship between *Colpodella* and the apicomplexans is based entirely on SSU rRNA phylogenies, it is recovered consistently and correlates well with morphological data (Kuvardina et al., 2002; Leander et al., 2003).

The phylogenetic position of *Oxyrrhis* has been more problematic. Phylogenies based on the SSU gene place it within the dinokaryotic dinoflagellates, with 76–81% bootstrap support for a clade of *Oxyrrhis* and *Gonyaulax spinifera* (Saldarriaga et al., 2003a). Protein-gene data give a very different result: all protein-gene phylogenies investigated to date (actin, alpha- and beta-tubulin in Saldarriaga et al., 2003a, also HSP90, B. Leander, personal communication) place *Oxyrrhis* at the base of the dinoflagellates. Because of the extreme divergence of the *Oxyrrhis* SSU sequence and the congruence amongst the protein-gene phylogenies (and also a short LSU fragment, Lenaers et al., 1991), it is likely that *Oxyrrhis* is a sister to the dinokaryotic dinoflagellates, but this will only be established by improved sampling of protein-coding genes from dinoflagellates. In any case, *Oxyrrhis* seems to have diverged later than *Perkinsus*; like dinokaryotes it lacks a curved ribbon at the apical end (regarded by some authors as a homologue to the apicomplexan conoid) that is typical of several protalveolates (e.g. *Colpodella*, *Perkinsus, Parvilucifera* and *Rastrimonas*) and the micronemes shared by them and apicomplexans.

Mitosis in *Perkinsus* is apparently similar to that in syndinians and dinokaryotic dinoflagellates in that channels containing an extranuclear spindle traverse the nucleus (Perkins, 1996), while *Oxyrrhis* has an intranuclear spindle (Triemer, 1982). Postulating that *Oxyrrhis* originated later than *Perkinsus* assumes a reversal in the organisation of the mitotic apparatus of *Oxyrrhis* (ciliates, like *Oxyrrhis*, have a closed mitosis with an internal spindle, apicomplexans a semiopen one). Nevertheless, other features strongly ally *Oxyrrhis* to the dinoflagellates, notably the lack of most real histones (Li, 1984) and several flagellar features (see

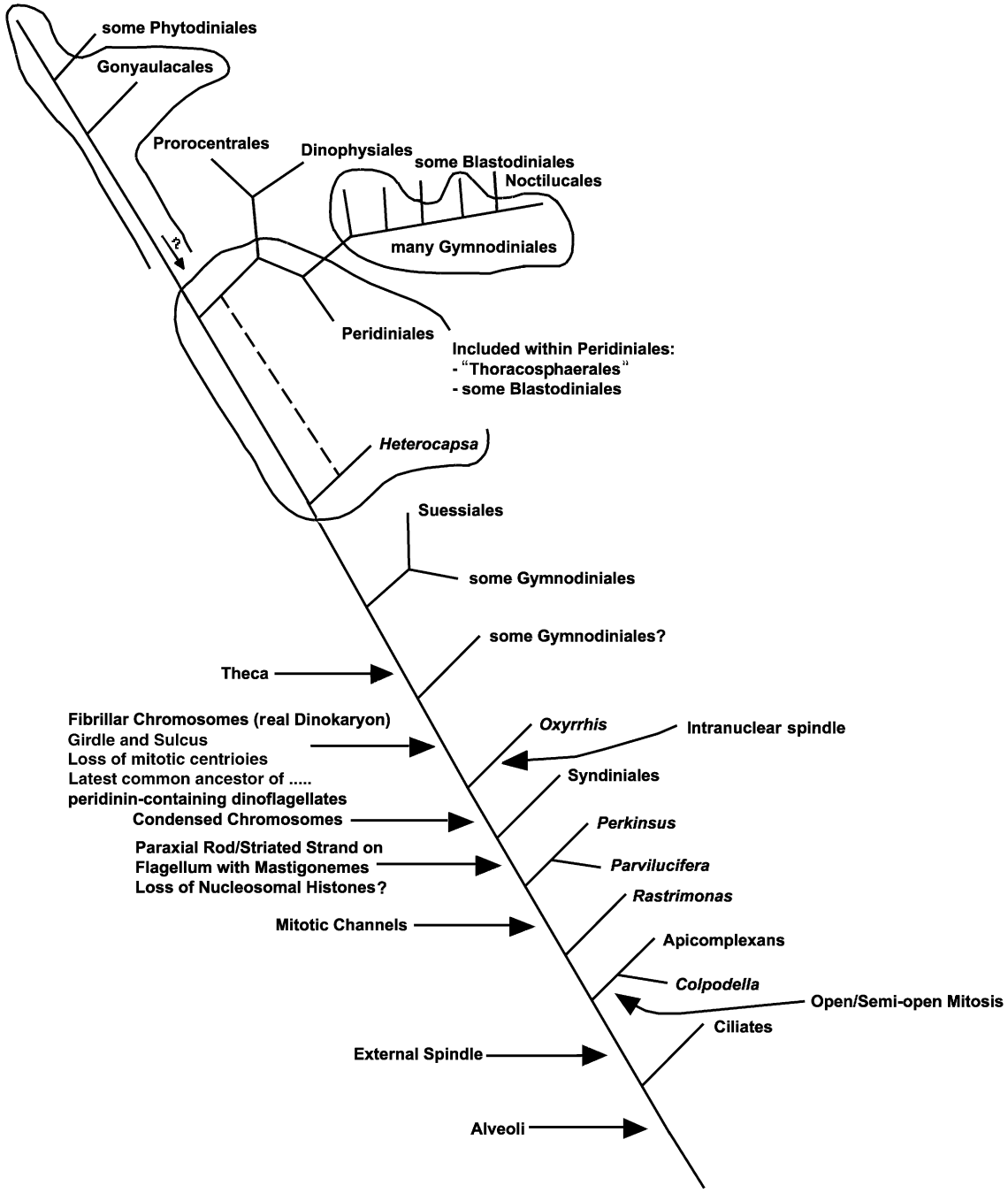

Fig. 5. Hypothesis on the evolutionary history of dinoflagellates and their close relatives based on the features of molecular trees that are well supported and/or congruent with one-another and on morphological and palaeontological information.

discussion below). It is not known whether *Perkinsus* has histones, but electron microscopy shows nuclei with decondensed chromatin during the majority of their life cycle (Perkins, 1996), very different from those of either *Oxyrrhis* or the dinokaryotic dinoflagellates and similar to those of most other eukaryotes. All in all, because of the strength of the molecular data and the unlikelihood that histones were lost more than once, it seems clear that *Oxyrrhis* branches between *Perkinsus* and the dinokaryotic dinoflagellates, but that assessment could change if *Perkinsus* unexpectedly proved to lack histones.

The order Syndiniales is very heterogeneous (e.g. Fensome et al., 1993). The only molecular data available for the group are SSU sequences from *Hematodinium* and *Amoebophrya* (different families), but no data are yet available for the morphologically most aberrant family of the order, the Duboscquellaceae. *Hematodinium* and

Amoebophrya generally form a clade in phylogenetic trees (always with weak bootstrap), but in a few analyses they separate. Interestingly, the diversity of the order might be underestimated: many SSU sequences obtained from picoplanctonic environmental samples cluster with very high bootstrap support (up to 99%) around *Amoebophrya*. When these sequences were first presented (Moon-van der Staay et al., 2001; López-García et al., 2001), it could not be stated categorically that those sequences were actually from syndinians. The addition of *Hematodinium* to the data set greatly strengthens that assumption, as this syndinian branches at the base of the clade that contains several strains of *Amoebophrya* (another syndinian) and the picoplanctonic taxa. The monophyly of the order Syndiniales is controversial, and so it will be interesting to see whether *Syndinium* and the Dubosquellaceae also branch in this clade. It is also interesting to speculate whether those sequences from picoplanktonic cells represent free-living organisms (there are no named free-living syndinians) or the infective stages of parasitic forms. A second group of environmental marine sequences forms a well-resolved clade that is not closely related to any named alveolates. Since there is no morphological information for it, it is impossible to say whether the sequences are syndinian (or indeed dinoflagellate) or not. They always branch after *Perkinsus*, and so we consider them members of the dinoflagellate lineage, but little can be inferred about dinoflagellate evolution from them until their morphology is characterized.

The relative positions of the syndinians and *Oxyrrhis* cannot be determined from the available molecular data alone: only SSU sequences are known for syndinians, and the *Oxyrrhis* sequence for that gene is misleading (Saldarriaga et al., 2003a). However, syndinians and *Perkinsus* share an invagination of the nuclear membrane in interphase that houses centrioles (Ris and Kubai, 1974; Perkins, 1996) that does not occur in dinokaryotic dinoflagellates or in *Oxyrrhis*. For this reason, we weakly favour a topology where syndinians are sisters to a clade comprising *Oxyrrhis* and the dinokaryotic dinoflagellates. Nevertheless, more data is needed to confirm this.

Noctilucales and Blastodiniales

In the most recent general classification of dinoflagellates (Fensome et al., 1993) Noctilucales and Blastodiniales are basal classes of their own within the subdivision of dinokaryotic dinoflagellates. This is because members of both orders have non-dinokaryotic life stages: the trophonts of *Noctiluca*, *Blastodinium*, *Amyloodinium* and many others have nuclei that lack the typical fibrillar chromosomes of dinokaryotic dinoflagellates and stain brightly with alkali fast green, a chemical reagent that colors basic proteins (histones of typical eukaryotic nuclei are easily stained by it, dinokarya are not). Nevertheless, these species have life stages with real dinokarya: at certain phases of their life cycle trophonts start a series of divisions that produce ever smaller nuclei with chromosomes that gradually condense to produce the typical dinokarya (Soyer, 1969, 1971, 1972). The dinokaryotic cells thus produced have the typical appearance of biflagellate dinoflagellates.

Molecular sequences (only SSU) exist for three taxa of either Noctilucales or Blastodiniales: *Noctiluca*, *Amyloodinium* and *Haplozoon*. *Noctiluca* branches basal to the dinokaryotic dinoflagellates (and usually also the syndinians) in many phylogenetic trees, but never with high bootstrap support (e.g. Fig. 1). However, in analyses with few outgroups and more aligned sites *Noctiluca* branches from within the GPP complex (Fig. 2). Moreover, *Noctiluca* chromatin may be more similar to that of dinokaryotes than to typical eukaryotes: electrophoretic gels of nuclear basic proteins extracted from the *Noctiluca* trophont produce a band pattern consistent with that of completely dinokaryotic dinoflagellates, not with eukaryotic, histone-containing nuclei (Li, 1984). In other words, *Noctiluca* may well lack typical core histones throughout its life cycle, suggesting that the alkali fast green stains other non-core-histone proteins in the trophont nucleus. As a consequence, the basal position of *Noctiluca* within the dinokaryotic dinoflagellates should be reexamined: the two main arguments for proposing such a basal position have been shown to be either very weak (SSU-based phylogenetic analyses), or probably wrong (the ostensible presence of histones in the nuclei of feeding stages). The fact that the vegetative stages (trophonts) of other Noctilucales, genera like *Leptodiscus*, *Craspedotella*, *Petalodinium*, *Kofoidinium* and *Spatulodinium*, appear to have typical dinokarya (M. Elbrächter, personal communication) further strengthens this view. Three morphological features of *Noctiluca* and other Noctilucales argue for a relationship of the order to at least some groups of gymnodinialean dinoflagellates: young trophonts and/or dinospores of several of the less morphologically derived noctilucalean taxa (e.g. *Kofoidinium* and *Spatulodinium*) are practically indistinguishable from a number of athecate dinoflagellate genera, especially *Amphidinium* (Cachon and Cachon, 1968). More importantly, *Noctiluca* shares with members of the genus *Gymnodinium* senso stricto (Daugbjerg et al., 2000) two rare morphological features. First, *Gymnodinium*, like the gametes of *Noctiluca*, lacks a transverse striated flagellar root, a feature typical of most dinoflagellates (Hansen et al., 2000). Furthermore, the nuclear envelope of many (but not all) *Gymnodinium* (as well as *Polykrikos*) and the trophont of *Noctiluca* have peculiar chambers (ampullae) in which the nuclear pores are situated (Afzelius, 1963; Soyer, 1969; Dodge and Crawford, 1969; Daugbjerg et al., 2000). These chambers disappear in *Noctiluca* as the dinospores are

formed (Soyer, 1972), and so they may not be homologous to the ones in *Gymnodinium*, but if they are homologues they would provide an important morphological connection between the two groups. It is unknown whether other Noctilucales have ampullae around the nucleus.

The order Blastodiniales is almost certainly polyphyletic (e.g. Chatton, 1920; Fensome et al., 1993). *Amyloodinium* and *Haplozoon* never branch together in our trees, although both are typically members of the GPP complex (in some trees *Amyloodinium* may branch at the base of the dinokaryotic dinoflagellates as a whole, e.g. Fig. 1, but see also Fig. 2). Furthermore, although several members of the order have, like *Noctiluca*, non-dinokaryotic nuclei in some life stages (e.g. *Blastodinium, Amyloodinium, Oodinium, Caryotoma* and *Crepidoodinium*: Soyer, 1971; Lom and Lawler, 1973; Cachon and Cachon, 1977; Hollande and Corbel, 1982; Lom et al., 1993), others do not: *Dissodinium* and *Protoodinium* are purely dinokaryotic (Cachon and Cachon, 1971; Drebes, 1981), as are probably *Piscinoodinium* and *Haplozoon* (trophonts in these two genera are dinokaryotic (Lom and Schubert, 1983; Siebert and West, 1974), and dinospores have never been shown to have anything other than a dinokaryon in dinokaryotic dinoflagellates). Other genera are understudied, e.g. *Apodinium*, *Cachonella*, *Sphaeripara*; the true phylogenetic affinities of these taxa are unclear. The derived position of *Amyloodinium* in SSU trees is strongly supported by morphology: *Amyloodinium* (and also *Pfiesteria*, its sister taxon in most trees) has dinospores with a thecal plate pattern like that of Peridiniales (Landsberg et al., 1994; Steidinger et al., 1996; Fensome et al., 1999). Could other Blastodiniales also be Peridiniales? The trophont of *Oodinium fritillariae* has thecal plates like *Amyloodinium*, but also has ampullae around the nucleus (Cachon and Cachon, 1977), the *Protoodinium* trophont even has peridinialean tabulation (Cachon and Cachon, 1971). Other Blastodiniales share more similarities with athecate dinoflagellates: *Haplozoon, Crepidoodinium* and probably also *Piscinoodinium* have many polygonal alveoli in surface view (Lom, 1981; Lom and Schubert, 1983; Lom et al., 1993; Leander et al., 2002). It is important to keep in mind, however, that many features known for Blastodiniales (e.g. the small, polygonal alveoli) have been observed in their trophonts, an often heavily modified life stage. The morphology of their dinospores will surely be much more helpful in determining their true phylogenetic affinities, as shown by the example of *Amyloodinium ocellatum*.

Gymnodiniales, Suessiales and the search for the first dinokaryotic dinoflagellates

The branching order of extant groups at the base of the dinokaryotic dinoflagellates is proving to be very difficult to determine using molecular methods: phylogenetic trees calculated through different algorithms and based on different genes place very different taxa at those basal positions, and bootstrap supports are never strong. Nevertheless, there are tendencies that warrant comparison with the morphological and paleontological data available.

Yoon et al. (2002), for example, propose *Karenia* and *Karlodinium* as sister taxa to the rest of the dinokaryotic dinoflagellates. They used three plastid-encoded genes (psaA, psbA and rbcL) to test the phylogenetic relationships between haptophytes and the plastids of peridinin- and 19-hexanoyloxyfucoxanthin-containing dinoflagellates (photosynthetic dinoflagellates contain different types of chloroplasts, the type that contains peridinin is by far most common, see below). They found, as expected, a strong phylogenetic relationship between haptophytes and the plastids of *Karenia* and *Karlodinium*, the two genera with 19-hexanoyloxyfucoxanthin-containing plastids (see also Tengs et al., 2000; Ishida and Green, 2002). Surprisingly, they also found that in psaA and psbA-based trees peridinin-containing dinoflagellates group either as a sister-taxon to *Karenia* and *Karlodinium*, or embedded within a clade with 19-hexanoyloxyfucoxanthin-containing ancestors. They proposed an early tertiary endosymbiosis event for the dinoflagellate lineage, and a later transformation of that plastid into the peridinin-type after the divergence of *Karenia* and *Karlodinium*.

The *Karenia*/*Karlodinium* clade is one of the groupings that does sometimes branch at the base of the dinokaryotic dinoflagellates in SSU-based phylogenetic trees (e.g. Fig. 2). Both of these genera are athecate taxa currently classified in the order Gymnodiniales, the grouping proposed by Fensome et al. (1993) as the most basal of the wholly dinokaryotic dinoflagellates. However, there are reasons to question this model for the origins of peridinin-containing plastids and the phylogenetic position of the 19-hexanoyloxyfucoxanthin-containing dinoflagellates. First, as Yoon et al. (2002) point out, the divergence rate of the dinoflagellate genes they examined is noticeably accelerated. Consequently, the dinoflagellate sequences may be attracted to one-another because they share long branches. The authors attempted to correct for this attraction, but nevertheless the concern remains. Second, an analogous study of the relationships between *Karenia*, the haptophytes and the peridinin-containing dinoflagellates using a nuclear-encoded but plastid-targeted gene (psbO, Ishida and Green, 2002) produced different results: the one sequence for a peridinin-containing dinoflagellate (*Heterocapsa triquetra*) was strongly excluded from a *Karenia*/haptophyte grouping. This finding is probably more reliable because the divergence rates in the nuclear-encoded dinoflagellate psbO genes appear to be much slower than the plastid-encoded genes.

The genus *Heterocapsa* occupies a basal position within Dinokaryota surprisingly often in phylogenetic trees, especially those based on LSU (maximum likelihood) and alpha-tubulin; in combined SSU/LSU trees *Heterocapsa* consistently occupied such a position, although with a low bootstrap support (40–50%). *Heterocapsa* also has a somewhat atypical sulcal tabulation that could be interpreted as primitive with respect to that of the rest of the Peridiniales and the Gonyaulacales (discussion in Fensome et al., 1993). It is thus not unreasonable that this group may have diverged before the split between those two orders. Paleontological data also agree with this hypothesis: the earliest fossils from the family Heterocapsaceae are early Jurassic (Wille, 1982), prior to the radiative explosion of all other peridininialean and gonyaulacalean forms in the Mesozoic (Fensome et al., 1999).

The phylogenetic history of gymnodinialean dinoflagellates is particularly difficult to discern for several reasons. First, although the order is well defined as a group in which the cellular cortex contains relatively numerous amphiesmal vesicles arranged non-serially (Fensome et al., 1993), several of the species that have historically been classified here have been shown to contain tabulations that make them obvious members of other orders (e.g. Hansen, 1995 for *Katodinium rotundatum*/*Heterocapsa rotundata*, Montresor et al., 1999 for *Polarella glacialis*, Saldarriaga et al., 2003b for *Gymnodinium elongatum*/*Lessardia elongata*). These tabulations are very difficult to discover using light microscopy alone, so it is a virtual certainty that several (perhaps many) taxa currently classified in Gymnodiniales are really members of other orders. This makes the evaluation of phylogenetic trees where putatively gymnodinialean clades intrude into thecate orders very difficult; a stringent evaluation of the tabulational patterns of putatively gymnodinialean taxa is needed before strong statements can be made about their phylogenetic history. Furthermore, small, non-serially arranged amphiesmal vesicles do not necessarily imply the absence of a theca (Netzel and Dürr, 1984), many gymnodinialean taxa have either a full-fledged theca (e.g. the genus *Woloszynskia*: Crawford et al., 1970; Crawford and Dodge, 1971), or an incipient one (several members of *Gymnodinium*, e.g. *G. fuscum* and *G. cryophilum*: Hansen et al., 2000; Wilcox et al., 1982); others have flocculent material or just liquid and no signs of a theca (e.g. *Karlodinium micrum*, Leadbeater and Dodge, 1966; *A. carterae*, Dodge and Crawford, 1968). These features can only be studied by electron microscopy, and because relatively few species have been investigated in such detail, the degree to which presence and type of intraalveolar material in Gymnodiniales is phylogenetically informative remains unknown.

Molecular data only exists for some gymnodinialean families, there are still no data available for *Polykrikaceae, Warnowiaceae, Actiniscaceae, Ptychodiscaceae* and others. Even so, molecular phylogenies always show a number of separate gymnodinialean clades originating from within the GPP complex, generally separated from thecate forms by very weak bootstrap supports and not necessarily basal to them. But are Gymnodiniales sensu stricto (i.e. dinoflagellates with numerous small alveoli arranged non-serially) polyphyletic or not? Or is the reason for the polyphyly of the Gymnodiniales sensu lato only the fact that it contains species with unrecognized non-gymnodinialean tabulations? Molecular data seem to suggest that even Gymnodiniales sensu stricto are polyphyletic: well-studied taxa with small alveoli (e.g. *A. carterae*, *Karenia brevis*, *G. fuscum*) never group together in phylogenetic trees.

The fact that in virtually all molecular trees gymnodinialean species arise from within the GPP complex, separated from thecate taxa by very weak bootstrap values, suggests that most, if not all, groups of Gymnodiniales had thecate ancestors; the different types of alveolar inclusions in the group, from thecae to flocculent material to nothing at all would therefore represent intermediate stages of thecal loss. The alternative would be that the Gymnodiniales (or at least some of their subgroups) are the sister group to the other dinokaryotic dinoflagellates. This view is supported by the fact that the small alveoli of Gymnodiniales are shared with more basal members of the dinoflagellate lineage, e.g. the syndinians (plasmodial life stage), *Oxyrrhis* and even *Colpodella*. Molecular data cannot distinguish between these possibilities at present; it cannot determine whether some Gymnodiniales are ancestral and others derived. Palaeontology is not very helpful in this regard either: gymnodinialean cysts are often very difficult to ally to identifiable motile stages, so fossil cysts of this type are particularly likely to be considered acritarchs, microfossils without known taxonomical affinities (Fensome et al., 1993); the earliest certain gymnodinialean fossils (skeletal elements from Actiniscaceae and Dicroerismaceae) are relatively recent, from Tertiary formations.

Palaeontological data suggest an early origin for another order of dinokaryotic dinoflagellates, the Suessiales. They comprise organisms with amphiesmal vesicles arranged in 7–10 latitudinal series, fewer than in typical athecate dinoflagellates and more than in thecate ones, a feature that suggests an interesting position for the order between thecate and athecate forms. Much more interesting, however, is the fact that suessialean fossils are known from the mid-Triassic, prior to the emergence of most (if not all) peridinialean and gonyaulacalean forms (the gonyaulacalean Shublikodiniaceae, like the earliest suessialean fossils, are from the mid-Triassic, Fensome et al., 1999; the identity of any Palaeozoic fossils as true dinoflagellates is still controversial). Molecular trees presently do not support any

particular position of the Suessiales: although the group rarely appears at the base of the dinokaryotic dinoflagellates (e.g. in Edvardsen et al., 2003), its position in other parts of the tree is never supported either. One additional feature of the Suessiales is becoming clearer as the SSU gene of more species of dinoflagellates is sequenced: the group is likely to be larger than previously assumed. Montresor et al. (1999) described the first extant member of the family Suessiaceae, a group until then known only from fossils, and since then many species of putatively athecate dinoflagellates have been shown to group in the same clade in both SSU- and LSU-based trees (e.g. *G. beii, G. simplex, G. corii* and *Woloszynskia pseudopalustris*). It will be interesting to see whether close morphological examination of more of these species will agree with the molecular data.

In summary, the problem of the earliest-branching dinokaryotic dinoflagellates is very far from being resolved: morphological and palaeontological data could be interpreted as pointing towards gymnodinialean and suessialean taxa for these positions, molecular data towards *Heterocapsa*. What is clear is that Gymnodiniales are a polyphyletic group; many gymnodinialean taxa are more closely related to thecate forms than to other gymnodinialeans and thecal loss in dinoflagellates seems to be common. Suessiales may form an intermediate stage between the primitively athecate gymnodiniales (if they exist) and thecate forms, but this is particularly uncertain as phylogenetic trees do not usually put them in a basal position. If dinokaryotic dinoflagellates indeed underwent an event of rapid evolutionary radiation early in their history, it will be very difficult to determine the phylogenetic order of the groups that originated in that explosion.

Phytodiniales

The order Phytodiniales (also Dinococcales, Dinocapsales or Dinamoebales, see Fensome et al. (1993) for a nomenclatural discussion) contains dinoflagellates in which the principal life stage is either a non-calcareous coccoid cell or a continuous-walled multicellular stage. It is a polyphyletic grouping of convenience used to contain species that are poorly understood; the only criterion for determining whether a species belongs to this order is a shift in life cycle that has also been seen in many dinoflagellate genera with well-known tabulations, e.g. in *Symbiodinium* (Suessiales), *Pyrocystis* (Gonyaulacales) and *Thoracosphaera* (probably Peridiniales). SSU rRNA sequences exist for three dinoflagellate species formally classified in the Phytodiniales: *Halostylodinium arenarium*, *Hemidinium nasutum*, and *Gloeodinium viscum. H. nasutum* and the type species of *Gloeodinium*, *G. montanum*, have extremely similar coccoid stages, the two species have even been proposed to be identical (Popovsky, 1971). A fourth species in our trees, *Glenodiniopsis steinii*, is currently classified in the Peridiniales, but has a coccoid life stage reminiscent of the *Gloeodinium*-like stage of *Hemidinium nasutum* (Popovsky and Pfiester, 1990).

Halostylodinium arenarium groups with gonyaulacalean taxa in all phylogenetic trees examined, a placement that is congruent with most (but not all) tabulational features of the species as interpreted by Horiguchi et al. (2000). *Glenodiniopsis*, *Hemidinium* and *Gloeodinium* on the other hand, consistently branch within the GPP complex, although only in the Weighbor trees do the three species weakly branch close to one-another (clades consisting of two of the three species are common in many trees). This placement is congruent with the peridinialean tabulation of the motile stage of *Glenodiniopsis*, and suggests that once the tabulations of *Hemidinium* and *Gloeodinium* are fully determined (only a partial tabulation is known for *Hemidinium nasutum*, no tabulational data exists for *G. viscum*) they will show peridinialean affinities. Molecular data do not strongly support a phylogenetic relationship between *Hemidinium nasutum* and *Gloeodinium viscum*, but do not disprove it either.

Peridiniales, Gonyaulacales, Dinophysiales and Prorocentrales

Members of the Peridiniales, Gonyaulacales, Dinophysiales and Prorocentrales are likely to have a common ancestor. The relative positions of the thecal plates in Peridiniales and Gonyaulacales are so similar that a close relationship between the two orders has never been doubted, and paleontological and morphological evidence points to a close relationship between Peridiniales, Dinophysiales and Prorocentrales. Palaeontological data yielded very strong evidence linking Dinophysiales to peridinialean ancestors: the fossil genus *Nannoceratopsis*, found as dinosporin cysts in marine strata of Jurassic origin, has distinctly dinophysialean features in its lateral compressed shape, hyposomal sagittal features and hyposomal pseudotabulation, but its epitheca has distinct peridinialean traits, very different from those of other Dinophysiales (Piel and Evitt, 1980; Fensome et al., 1993). Within Peridiniales, the groups with the most similarity to *Nannoceratopsis* are the fossil Comparodiniaceae and the extant Oxytoxaceae (Fensome et al., 1993). No molecular data exist for *Oxytoxum*, but a close relationship between Peridiniales and Dinophysiales is weakly apparent in molecular phylogenetic trees: in our trees Dinophysiales are always embedded in the GPP complex (alternative placings for the group also exist, e.g. Edvardsen et al., 2003). Prorocentrales also branch within the GPP complex, even if in SSU trees the order splits into 2 groups (or more: Grzebyk et al., 1998). Despite this split, we consider that this morphologically very cohesive order is monophyletic, as tabulation patterns within it are both radically derived and

homogenous. Interestingly, at least some LSU trees (notably Weighbor) show Prorocentrales as monophyletic. The phylogenetic origins of the group are more difficult to discern. The fact that Prorocentrales are members of the GPP complex in molecular trees weakly argues for a relationship to Peridiniales and Dinophysiales, as well as to many Gymnodiniales. Two large lateral plates (valves) that contact each other along a sagittal suture are a common feature of Prorocentrales and Dinophysiales; they may be closely related to each other (Taylor, 1980). Nevertheless, no intermediate fossil forms exist to shed light on this.

Thus, a relationship between Peridiniales and the Dinophysiales/Prorocentrales on the one hand, and Gonyaulacales on the other, seems likely. But which appeared first? The earliest dinoflagellate fossils (except for the controversial Palaeozoic forms already mentioned) are, in addition to the Suessiales, members of the gonyaulacalean Shublikodiniaceae (Fensome et al., 1999). This is an exclusively fossil family with very characteristic tabulation: more than five climactal plates and at least three fundital plates. Fensome et al. (1993) points out tabulational resemblances between this family and two other groups: early cladopyxiineans and living members of the genus *Glenodinium*. What is interesting is that whereas Shublikodiniaceae and Cladopyxiineae are early lineages within the Gonyaulacales (as classified by Fensome et al., 1993), Glenodiniaceae are undoubtedly peridinialean forms. Thus, the lines between the two orders blur at this level. Molecular data tend to give trees where a paraphyletic Peridiniales is ancestral to the holophyletic Gonyaulacales, although bootstrap support for this branching order is generally low. Nevertheless, molecular data do not exist for many putatively basal groups of either Gonyaulacales or Peridiniales, genera like *Cladopyxis, Acanthodinium, Amphidoma* or *Palaeophalacroma* (Gonyaulacales), or *Glenodinium* (Peridiniales). *Heterocapsa* (Peridiniales) is an exception to this, and as discussed above, it tends to take a basal position to other thecate dinoflagellates in many phylogenetic trees, particularly those based on combined data sets of small- and large subunit rRNA genes. The implication of this position would be that Peridiniales are indeed ancestral to Gonyaulacaleans, a thesis that runs contrary to palaeontological data: no true peridinialean fossils are known from before the appearance of the earliest gonyaulacaleans, the Shublikodiniaceae. Nevertheless, just as it is dangerous to give too much credence to the branching order of *Heterocapsa* in phylogenetic trees because of mediocre supports of the relevant nodes, the present lack of peridinialean fossils from the Triassic does not necessarily mean that the group was completely absent then.

One more dinoflagellate ''order'' appears to be very closely related to Peridiniales: the Thoracosphaerales. The principal life-stage of *Thoracosphaera*, the only genus in the order, is a coccoid cell surrounded by a calcareous wall, very similar to calcareous cysts of a subgroup (subfamily Calciodinelloideae of the Peridiniaceae) within the Peridiniales that includes *Scrippsiella, Ensiculifera*, etc. Nevertheless, the motile stage of *Thoracosphaera* is apparently athecate and the archeopyle of the cyst quite atypical, so a separate order was created for it (Tangen et al., 1982). Molecular data tends to support a relationship between *Thoracosphaera* and several genera of Peridiniales, including *Peridiniopsis* and, interestingly, *Scrippsiella*. This position in molecular trees (as well as the calcareous cyst wall) would predict a peridinialean tabulation of the motile stage. If this turns out to be the case, the order Thoracosphaerales should be abolished and *Thoracosphaera* made a member of the Peridiniales and of the Calciodinelloideae.

If the scenario presented above is correct, the Peridiniales would occupy a very important place for the phylogeny of the dinoflagellates as a whole. They would be a complexly paraphyletic group that gave rise not only to the other obviously thecate taxa (Dinophysiales and Prorocentrales, and perhaps also Gonyaulacales), but to many athecate and putatively athecate forms as well (many lineages of Gymnodiniales, Thoracosphaerales, as well as, perhaps, also Noctilucales and Blastodiniales).

Whereas branching orders within Peridiniales are not resolved in any of our trees, within Gonyaulacales the rate of evolution of both the large- and the SSU rRNA genes is faster, and as a consequence branches of the resulting phylogenetic trees are longer; lineages are also generally separated by better bootstrap supports. Felicitously, the Gonyaulacales are also a group with a very good fossil record, and the tabulational patterns of extant and fossil members are well known. For this reason, the group provides a good model to contrast taxonomic schemes based on morphology (i.e. tabulation) with molecular data.

The two sets of data correlate well within this group. Two of the three gonyaulacalean suborders for which there are SSU sequences are normally recovered in phylogenetic trees with decent bootstrap support: Goniodomineae (50–60%) and Ceratiineae (75–90%). The third suborder, Gonyaulacineae, usually forms a paraphyletic group that gives rise to both Goniodomineae and Ceratiineae, as well as to taxa of uncertain taxonomic position like *Thecadinium* and *Crypthecodinium* (and the formally phytodinialean genus *Halostylodinium*). Large subunit gene trees also generally support the monophyly of Goniodominae, although with weak bootstrap support and one important caveat: it is always intruded by the sequence from *Ceratium furca* (SSU and morphological data agree that this species is a Ceratiineae, the veracity of the identity of this LSU sequence should be reexamined). The other

Ceratiineae (i.e. the genus *Ceratium*) group strongly, and *Protoceratium* and *Gonyaulax*, the only Gonyaulacineae in the trees, make a paraphyletic group at the base of the order. One difference between molecular trees and taxonomic schemes based on morphology (i.e. Fensome et al., 1993) is the position of *Protoceratium*: SSU-based phylogenies never place it with *Lingulodinium, Gonyaulax* and *Amylax* in the family Gonyaulacaceae, but rather with *Ceratocorys* (Ceratocoryaceae, 100% bootstrap support).

Tracing morphological characters onto molecular trees: from protalveolates to dinoflagellates

Having proposed a putative framework for the phylogenetic history of dinoflagellates, we now examine the evolutionary history of certain morphological features in its light.

The nucleus

Although undoubtedly eukaryotic (e.g. Mínguez et al., 1994; Salamin Michel et al., 1996), dinokaryotic nuclei show important biochemical differences with respect to the nuclei of other eukaryotes: they lack histones (e.g. Rizzo, 1991), can contain very large amounts of DNA (2–200 pg DNA per haploid nucleus, nuclei of human cells have ca. 5.6 pg DNA per cell, Sigee, 1986) and up to 70% of the thymine in their DNA is replaced by 5-hydroxymethyluracil (Rae, 1976). They also divide through a type of mitosis characteristic for the group: the nuclear membrane remains intact, chromosomes remain attached to the nuclear membrane, and, during mitosis, channels are formed that contain the microtubules of the mitotic spindle; microtubules attach to the chromosomes only where they touch the nuclear membrane (references in Dodge, 1987). The scale of the ultrastructural and biochemical reorganization that occurred in the nuclei of the alveolates that became dinoflagellates is unparalleled in any other group of eukaryotes and the process that led to it is completely unknown. It is thus of interest to trace some of the features of this change down the dinoflagellate lineage, to determine when exactly the different characters of the dinokaryon originated.

The question of the presence or absence of histones in the dinoflagellate lineage has interested many researchers over the years. The paradigm on the absence of typical histones in the group is based on several facts: nucleosomes have not been detected in dinoflagellates using any method (e.g. electron-microscopical observation of chromatin spreads, digestion of internucleosomal DNA followed by electrophoresis, etc., Rizzo, 1991), the ratio of basic chromatin to DNA is much lower in dinokaryotic dinoflagellates than in any other eukaryote (Rizzo and Noodén, 1973), and electrophoresis of dinoflagellate nuclear basic proteins has consistently produced banding patterns that do not correspond to the ones formed by eukaryotic histones (e.g. Rizzo, 1981). Only recently have some of the nuclear basic proteins from dinoflagellates started to be sequenced (Sala-Rovira et al., 1991; Taroncher-Oldenburg and Anderson, 2000; Chudnovsky et al., 2002), and to date there are three sequences available, from *Crypthecodinium cohnii*, *Alexandrium fundyense* and *Lingulodinium polyedrum* (all Gonyaulacales). Homologies of these histone-like proteins (HLPs) of dinoflagellates to other proteins are not obvious, but Kasinsky et al. (2001) reported a 31% similarity in amino acid composition between the complete HCc2 of *Crypthecodinium cohnii* (a histone-like protein) and the C-terminus of the linker histone H1b of the sea urchin. Nucleosomal histones have never been detected in dinoflagellate nuclei.

The presence or absence of histone proteins in the nuclei of protalveolates and dinoflagellates is obviously a very important feature in the study of the phylogenetic questions that interest us. It is highly unlikely that nucleosomal histones in dinoflagellates were lost more than once. Historically, the determination of just which taxa (or in some cases life stages) of dinoflagellates have histones and which do not has been done by chemical staining of the basic proteins in their nuclei: dinokarya do not stain with alkaline fast green, while the nuclei of most eukaryotes, including syndinians, *Oxyrrhis* and the trophonts of taxa like *Noctiluca*, *Blastodinium* and *Oodinium* do (references in Table 3). The ultrastructure of those nuclei is also quite different from that of dinokarya, and so it was thought that they were profoundly different from them. Preliminary biochemical analyses of the nuclear basic proteins of *Oxyrrhis* and the *Noctiluca* trophont (Li, 1984) have shown however that the electrophoretic pattern of those proteins in SDS- and acidic urea gels resembles the ones of dinokaryotic HLPs, not the patterns of histone-containing organisms. If the basic proteins in the nuclei of *Noctiluca* and *Oxyrrhis* are not normal histones, then the change from histone-containing to histone-lacking nuclei in the dinoflagellate lineage occurred earlier than previously assumed. Where exactly is not easy to determine. No biochemical studies on syndinian nuclei exist, but Hollande (1974) did stain the nuclei of four species with alkali fast green. The nuclei of different syndinians stain differently: in *Syndinium* and *Solenodinium* the chromosomes are stained, while in *Amoebophrya* and *Duboscquella* only the nucleoli are. Unfortunately only *Amoebophrya* is represented in molecular based phylogenetic trees, so it is uncertain whether the order is really monophyletic; nevertheless, the staining pattern in *Amoebophrya* and *Duboscquella* is more consistent with the presence of histone-like proteins in those organisms rather than real histones. Ciliates and apicomplexans clearly have histones

Table 3. Nuclear features of the dinoflagellates and related groups

Taxon	Condensed chromosomes in interphase?	Alkali-staining, histones	Mitosis
Ciliates	No	Yes	Closed, intranuclear spindle (Raikov, 1994)
Apicomplexans	No	Yes	Coccidia, haemosporidia: semiopen Gregarines: open or semiopen (Raikov, 1994)
Colpodella	No (Brugerolle, 2002a)	?	Semiopen (Brugerolle, 2002a)
Acrocoelus	No (Fernández et al., 1999)	?	?
Perkinsus	No (Perkins, 1996)	?	Closed, with channels and external spindle (Perkins, 1996)
Parvilucifera	No. Has an outer layer of fibrils around the chromatin in the zoospore nucleus (Norén et al., 1999)	?	?
Rastrimonas	No (Brugerolle, 2002b)	?	Closed, external spindle and no channels (in anaphase the nuclear envelope disappears in the median zone, Brugerolle, 2002b)
Colponema	No (Mignot and Brugerolle, 1975)	?	?
Oxyrrhis	Yes, but not fibrillar as in typical dinokarya	Staining: Yes Histones: probably not (Li, 1984; Kato et al., 1997)	Closed, intranuclear spindle (Triemer, 1982)
Syndinium	No, chromatin masses that do not correspond in number to chromosomes (Ris and Kubai, 1974; Soyer, 1974)	Staining: yes (also in *Solenodinium*. In *Amoebophrya* and *Duboscquella* only the nucleoli stain, Hollande, 1974) Histones: ?	Closed, one channel
Noctiluca	*Trophont*: No *Zoospore*: Yes (Hardly any interphase during sporulation) (Soyer, 1969, 1972)	*Trophont*: Staining: yes Histones: probably not (Li, 1984) *Zoospore*: no (Soyer, 1969, 1972)	Closed, channels (also in trophonts producing trophonts) (Soyer, 1969)
Blastodinium	*Trophont*: No *Zoospore*: Yes (Hardly any interphase during sporulation) (Soyer, 1971)	*Trophont*: Staining: Yes Histones: ? *Zoospore*: No (Soyer, 1971)	Closed, channels (Soyer, 1971)
Oodinium	*Trophont*: No Zoospore: Yes (Cachon and Cachon, 1977)	Trophont: Staining: Yes (weak) Histones: ? *Zoospore*: No (Cachon and Cachon, 1977)	Closed, channels (Cachon and Cachon, 1971)
Dinokaryotic Dinoflagellates	Yes	No (Rizzo, 1981)	Closed, channels

(e.g. Creedon et al., 1992; Bernhard and Schlegel, 1998), so the change between histone-containing and histone-lacking organisms occurred after the divergence of the apicomplexans, probably before the divergence of the syndinians. No biochemical data exist regarding the nuclear composition of either *Perkinsus* or *Parvilucifera* (their nuclei look more eukaryote-like than dinoflagellate-like in ultrastructural studies), a gap that needs to be filled by future research.

It now appears that the lack of nucleosomal histones and the chromosomal reorganization that that implies is a feature that may be more widespread in the dinoflagellate lineage than previously assumed, present not only in all Dinokaryota (including Noctilucales and Blastodiniales) but in *Oxyrrhis* and possibly syndinians as well. If our phylogenetic framework turns out to be correct, this feature could be added to the definition of the dinoflagellate taxon.

Mitosis

Most chromists have an open (sometimes semi-open) mitosis, ciliates have a closed mitosis with an intranuclear spindle, and the majority of apicomplexans (and *Colpodella*, Brugerolle, 2002a) have a semi-open one (in a number of gregarines it is open). The dinoflagellate lineage is very consistent in this regard (Table 3): *Perkinsus*, the syndinians, *Oxyrrhis* and the dinokaryotic dinoflagellates all have a closed mitosis, and in most of the members of the group the spindle is external (Perkins, 1996; Triemer and Fritz, 1984). *Oxyrrhis,* however, has an internal spindle (Triemer, 1982). With the exception of *Oxyrrhis*, all members of the lineage form channels during mitosis, syndinians only one, dinokaryotic dinoflagellates more (the number of channels in *Perkinsus* is unclear, and mitosis in *Parvilucifera* is entirely unknown). The mitotic channels thus probably originated at the base of the dinoflagellate lineage, roughly at the same time as the histones were lost (a detailed study of these features in *Perkinsus* is needed to confirm this). The external spindle possibly originated before this, prior to the differentiation of the apicomplexan lineage. The only way to explain the state of these characters in *Oxyrrhis* while taking into account the molecular data on its position in the phylogenetic tree is to postulate an internalization of the mitotic spindle and the loss of all mitotic channels (deep, narrow nuclear membrane invaginations are common in *Oxyrrhis* during interphase (Triemer, 1982); they may or may not have any relationship to mitotic channels). Interestingly, *Rastrimonas* divides through a modified closed mitosis (in anaphase the nuclear envelope disappears in the median zone) with an external spindle, but it does not seem to form channels (Brugerolle, 2002b). It will be interesting to see where this genus falls in phylogenetic trees.

One other feature significant for understanding the evolution of these organisms is the nature of their centrosomes, the cell regions that act as microtubule organizing centers (MTOCs). In dinokaryotic dinoflagellates spindle microtubules originate in centriole-lacking centrosomes (also called archeoplasmic spheres) located outside the nucleus and connected to the basal bodies by a microtubular fibre (Perret et al., 1993; Ausseil et al., 2000). Centrosomes in *Perkinsus* and in syndinians, however, do contain centrioles (references in Table 3), while in *Oxyrrhis* the mitotic spindle originates in electron-dense plaques embedded in the nuclear envelope (Triemer, 1982). Similar electron-dense zones also exist in the nuclear envelope of syndinians (and in *Oodinium*, Cachon and Cachon, 1977), but whereas in *Oxyrrhis* the plaques act as MTOCs for microtubules that either cross the nucleus or attach to chromosomes (Triemer, 1982), in syndinians these are kinetochores, with chromosomes attached on the inner side of the membrane and microtubules on the outer side. Whether these structures in syndinians and *Oxyrrhis* are homologous structures is unknown. Interestingly, *Oxyrrhis* centrioles may also be involved in mitosis: they migrate towards the nuclear poles early in division, and remain there throughout mitosis (Triemer, 1982). However, microtubules were never observed between these centrioles and the nucleus, so their role is unclear.

Plastids and photosynthesis

Only roughly half of the species of dinoflagellates are known to be photosynthetic (Taylor, 1987), and the type of plastids that they contain can be extremely different from one-another (Schnepf and Elbrächter, 1999; Saldarriaga et al., 2001): although most photosynthetic dinoflagellates harbour peridinin-containing plastids surrounded by two to three membranes (here called peridinin plastids), other forms probably arose from haptophyte, prasinophyte, cryptomonad or diatom endosymbionts (Watanabe and Sasa, 1991; Chesnick et al., 1997; Tengs et al., 2000; Hackett et al., 2003). This promiscuity in the incorporation of endosymbionts is a feature unique to dinoflagellates; no other group of eukaryotes contains a comparable variety of plastid types.

Mixotrophy is unusually common in photosynthetic dinoflagellates (Schnepf and Elbrächter, 1992; Stoecker, 1999), so in the absence of other data it was originally postulated that the peridinin plastid was incorporated by a full-fledged dinoflagellate (e.g. Whatley et al., 1979; Gibbs, 1981), just as the other types of plastids in the lineage are still believed to have been. However, the incorporation of the ancestor of the peridinin plastid probably occurred much earlier (Cavalier-Smith, 1999, 2003; Fast et al., 2001; Harper and Keeling, 2003). The first clues to this arose when a plastid remnant was found in apicomplexans, the closest relatives to dinoflagellates (Wilson et al., 1991). Phylogenetic trees were at first produced based on genes from the plastid genomes of both dinoflagellates and apicomplexans, and these trees tended to cluster the two together. However, the plastid genomes of both groups are extremely derived, so long branch attractions could be excluded (Takishita and Uchida, 1999; Zhang et al., 2000). Fast et al. (2001) used nuclear encoded, plastid-targeted genes with more conclusive results: there appears to have been a gene duplication event in an ancestor of not only dinoflagellates and apicomplexans but also the rest of the alveolates and chromists. The product of that gene duplication (a plastid-targetted GAPDH of cytosolic ancestry) appears to exist in plastid-bearing alveolates as well as in cryptomonads, heterokonts and haptophytes (Fast et al., 2001; Harper and Keeling, 2003), implying that the ancestor of all of these groups contained a plastid.

Within the dinoflagellate lineage, photosynthetic forms only appear relatively late in the evolutionary

history of the group, early branching taxa (i.e. *Perkinsus*, *Parvilucifera*, the syndinians and *Oxyrrhis*) are all non-photosynthetic. Interestingly, however, two iron superoxide dismutases have been described in *Perkinsus* (Wright et al., 2002), and one of these encodes a substantial amino-terminal leader that is predicted to begin with a signal peptide. This is preliminary but suggestive evidence that *Perkinsus* might harbour a relict plastid, something that should become clear when more data from that organism are available.

That all peridinin-containing dinoflagellates must have a common ancestor is beyond doubt: peridinin probably originated only once (e.g. Saunders et al., 1997; Saldarriaga et al., 2001). The implication of this is that all the non-photosynthetic lineages that appear after the latest possible common ancestor of peridinin-containing dinoflagellates must represent instances of loss of plastids or at least of photosynthetic ability (non-photosynthetic plastids can be difficult to identify). Using the same logic, all lineages branching after that latest possible peridinin-containing ancestor that contain plastids different from the peridinin type, must be instances of plastid replacement (Saldarriaga et al., 2001). Plastid replacement differs fundamentally from secondary symbiogenesis in that it probably occurs by the recruitment of at least some of the pre-existing plastid-targeting machinery rather than the evolution of entirely novel systems (Cavalier-Smith, 2003). It seems to have been able to occur multiple times in dinoflagellates, but never in other chromalveolates, perhaps because they retained the ability to phagocytose (necessary to acquire foreign algae) and were able to effect such recruitment because they also retained the ancestral chromalveolate ability to target endomembrane vesicles to the outermost smooth (epiplastid) membrane surrounding the plastid (for details on the origins of chromalveolate plastid protein targeting, see Cavalier-Smith, 2003). Interestingly, the same characteristics are found in the chlorarachniophyte algae, which have recently been shown to have replaced many of their plastid genes with homologues from other algae (Archibald et al., 2003), but have not been demonstrated to have replaced their plastid.

The flagella and the definition of Dinoflagellates

The definition used by Fensome et al. (1993) in their treatment of dinoflagellates is based on flagellar characters. The transverse flagellum of dinoflagellates is very distinct in its ultrastructure: the flagellar axoneme is accompanied by a striated strand throughout its entire length and both structures are contained by a common plasmalemma that produces a ribbon-like structure. Simple mastigonemes arise in a row along the outer edge of the axoneme (Gaines and Taylor, 1985). The striated strand is always shorter than the axoneme, so the flagellum takes a "wavy" appearance. In addition to these ultrastructural features, the fact that both flagella insert laterally is characteristic of the group (the "apical" flagellar insertion in the Prorocentrales is not topologically different from that of the rest of the dinoflagellates, Taylor, 1980).

The flagella of apicomplexans and cilia of ciliates are generally smooth (in apicomplexans only the microgametes of some groups are flagellated), but most taxa in the dinoflagellate lineage, including *Perkinsus*, *Parvilucifera*, *Oxyrrhis* and at least some syndinians (e.g. *Amoebophrya*, W. Coats, personal communication) appear to have at least one flagellum that carries mastigonemes (Table 4, the syndinian genus *Hematodinium* apparently does not, Appleton and Vickerman, 1998). The same is true for at least some species of *Colpodella*, a sister taxon to the apicomplexans (Leander et al., 2003, but see also Brugerolle, 2002a). This fact combined with the presence of more complex mastigonemes in heterokonts and cryptomonads suggests that simple non-tubular mastigonemes may have been an early feature of alveolates, and that ciliates, apicomplexa and some syndinians lost them secondarily. This would only be true, however, if the mastigonemes in the dinoflagellate lineage are related to those of heterokonts; the two structures are not ultrastructurally identical, but the possibility remains that at least some proteins of which they are made are homologues and that their very marked ultrastructural differences arose by divergence from a common ancestral mastigoneme rather than de novo (see also Cavalier-Smith, 2004).

A paraxial rod in the transverse flagellum (here defined as the flagellum that carries mastigonemes in a lateral row) is, on the other hand, only present in *Oxyrrhis*, in the dinokaryotic dinoflagellates and in at least one syndinian (*Amoebophrya*); it is not present in the apicomplexan lineage, *Perkinsus* or *Parvilucifera*. The ultrastructures of the paraxial rod/striated strand of *Oxyrrhis* and the dinokaryotic dinoflagellates are different, however, but the exact nature of those differences is not understood (Gaines and Taylor, 1985; Dodge and Crawford, 1971).

The longitudinal flagellum of dinoflagellates rarely carries mastigonemes (never in a lateral row) and paraflagellar material, sometimes in the form of a paraxial rod (e.g. Leadbeater and Dodge, 1967; Maruyama, 1982) that can cause a characteristic "ribbon-like" appearance of the flagellum. This is found in several members of the dinoflagellate lineage, e.g. in *Parvilucifera* and in many dinokaryotic dinoflagellates, especially Gonyaulacales (Leadbeater and Dodge, 1967; Maruyama, 1982; Norén et al., 1999). Additional features of the flagellar apparatuses of dinoflagellates and their relatives (ultrastructure and arrangement of basal bodies, microtubular assemblages, fibrous roots, etc.) have been shown to be phylogenetically informative (Roberts, 1991; Perret et al., 1993), but data are still

Table 4. Flagella in the dinoflagellates and related lineages

Taxon	Flagellar insertion	Anterior/transversal flagellum	Posterior/longitudinal flagellum	Reference
Apicomplexans	Essentially apical, when present	Only in microgametes of some groups. No mastigonemes.	Only in microgametes of some groups. No mastigonemes.	Perkins et al. (2002) (see references)
Colpodella	Subapical	*C. vorax*: Paraxonemal structure in the proximal portion. No mastigonemes. *C. edax*: Mastigonemes present	Unremarkable. No mastigonemes.	Brugerolle (2002a), Leander et al. (2003)
Acrocoelus	Ventral	Both flagella are posteriorly directed. No mastigonemes.	Unremarkable. No mastigonemes.	Fernández et al. (1999)
Perkinsus	Ventral	Only in zoospores. Filamentous mastigonemes along one side, in groups. Spur at the base of each group.	Only in zoospores. Unremarkable, much shorter than anterior flagellum. No mastigonemes.	Perkins (1996)
Parvilucifera	Subapical	Only in zoospore. Short mastigonemes on one side, long, thin hairs on the other.	Only in zoospore. Much shorter than anterior flagellum Proximal part with a wing, distal part lacks the peripheral doublets of the axoneme on the side opposite to the wing after it terminates.	Norén et al. (1999)
Rastrimonas	Subapical	Anterior flagellum shorter. Mastigonemes "have not been satisfactorily demonstrated"	Longer. Terminates in a thin filament.	Brugerolle (2002b)
Colponema	Subapical	Anterior flagellum shorter. Filamentous mastigonemes along one side	Longer. With a very high wing in the median and distal sections.	Mignot and Brugerolle (1975)
Oxyrrhis	Ventral, emerge from the base of the tentacle	Single row of fine hairs. Paraxial rod present.	Mostly smooth, but has a paintbrush-like structure at the end	Dodge and Crawford (1971)
Amoebophrya	Ventral	Single row of mastigonemes. Paraxial rod present.	Unremarkable. No mastigonemes	W. Coats, personal communication
Dinokaryotes	Ventral	Single row of fine hairs, contains a striated strand	Smooth. A paraxial rod and/or diffuse paraflagellar material can be present	Leadbeater and Dodge (1967), Maruyama (1982)

scarce and comprehensive analyses of their evolutionary history seem premature.

Conclusions

The degree to which the current classification of the dinoflagellates corresponds with the phylogenetic history of the group is very unclear. On the one hand, the molecular data shown here does support many groups, notably Gonyaulacales, Dinophysiales and an extended Suessiales, as well as most gonyaulacalean suborders and families and many genera (*Alexandrium, Pyrocystis, Ceratium, Gonyaulax, Heterocapsa, Protoperidinium, Pentapharsodinium, Scrippsiella, Karenia, Pfiesteria, Dinophysis* and *Amoebophrya*). Data on key taxa of other groupings (notably Noctilucales and Syndiniales) are still missing, so their monophyly cannot be ascertained. But many taxa (Gymnodiniales, Blastodiniales, Phytodiniales, *Gymnodinium, Amphidinium, Gyrodinium* and *Peridinium*) appear polyphyletic, probably because of real polyphyletic origins of the subgroups contained in them. Peridiniales are apparently paraphyletic because of intruding cryptically thecate forms and the (putative) position of the order

at the base of the diversification of dinokaryotic dinoflagellates. Incorrect topologies caused by the unavoidably low resolution of the backbone of current phylogenetic trees, however, may be the cause of other apparent polyphylies, e.g. that of *Prorocentrum* and the order Prorocentrales. Whether this effect applies to other groups is both unknown and very important for determining the true phylogeny of dinoflagellates.

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