Molecular data and
the evolutionary history of dinoflagellates

by

Juan Fernando Saldarriaga Echavarría

Diplom, Ruprecht-Karls-Universität Heidelberg, 1993

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Botany

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

November 2003

© Juan Fernando Saldarriaga Echavarría, 2003

ABSTRACT

New sequences of ribosomal and protein genes were combined with available morphological and paleontological data to produce a phylogenetic framework for dinoflagellates. The evolutionary history of some of the major morphological features of the group was then investigated in the light of that framework. Phylogenetic trees of dinoflagellates based on the small subunit ribosomal RNA gene (SSU) are generally poorly resolved but include many well-supported clades, and while combined analyses of SSU and LSU (large subunit ribosomal RNA) improve the support for several nodes, they are still generally unsatisfactory. Protein-gene based trees lack the degree of species representation necessary for meaningful in-group phylogenetic analyses, but do provide important insights to the phylogenetic position of dinoflagellates as a whole and on the identity of their close relatives. Molecular data agree with paleontology in suggesting an early evolutionary radiation of the group, but whereas paleontological data include only taxa with fossilizable cysts, the new data examined here establish that this radiation event included all dinokaryotic lineages, including athecate forms. Plastids were lost and replaced many times in dinoflagellates, a situation entirely unique for this group. Histones could well have been lost earlier in the lineage than previously assumed. The closest relatives to the dinokaryotic dinoflagellates appear to be apicomplexans, *Perkinsus* and *Parvilucifera*, syndinians and *Oxyrrhis*. Gonyaulacales, Dinophysiales and an expanded Suessiales are all holophyletic orders, while Gymnodiniales, Blastodinales and Phytodinales as currently circumscribed are polyphyletic.

Peridinales is likely to be a paraphyletic taxon that probably gave rise to Dinophysiales and Prorocentrales, as well as to several groups of Gymnodinales and Blastodinales, and possibly also to Gonyaulacales.

TABLE OF CONTENTS

Abstract	ii
List of Tables	viii
List of Figures	viii
Acknowledgements	xii
Preface	xiv
 Chapter 1: Introduction to the Project and Ribosomal Gene Phylogenies	1
1.1. Introduction	1
1.2. Materials and Methods	8
1.2.1. On the choice of molecular phylogenetic markers	8
1.2.2. Organisms, Extraction, Amplification and Sequencing	9
1.2.3. Phylogenetic Analysis	17
1.3. Results	19
1.3.1. SSU rRNA Phylogeny	19
1.3.2. LSU rRNA Phylogeny	28
1.3.3. Combined rRNA Phylogeny	30
 Chapter 2: Molecular Data and the Phylogenetic Position of <i>Oxyrrhis marina</i> and <i>Perkinsus marinus</i>	33
2.1. Introduction	33
2.2. Materials and Methods	36
2.3. Results and Discussion	40

2.3.1. SSU rRNA phylogeny of <i>Oxyrrhis</i> and <i>Perkinsus</i>	40
2.3.2. Phylogenetic position of <i>Oxyrrhis</i> and <i>Perkinsus</i> based on actin, alpha-tubulin and beta-tubulin sequences	44
 Chapter 3: Proposing a Phylogenetic Framework for Understanding the Evolutionary History of Dinoflagellates	53
3.1. <i>Perkinsus</i> , <i>Parvilucifera</i> , <i>Oxyrrhis</i> and the Syndiniales	55
3.2. Noctilucales and Blastodinales.....	58
3.3. Gymnodinales, Suessiales and the search for the first dinokaryotic dinoflagellates	62
3.4. Phytodinales.....	68
3.5. Thecate dinoflagellates: Peridinales, Gonyaulacales, Dinophysiales and Prorocentrales.....	70
3.6. Rates of evolution, the structure of dinoflagellate phylogenetic trees and the Mesozoic radiation of dinoflagellates.....	76
 Chapter 4: Origin and Evolution of Dinoflagellate Features	79
4.1. The Nucleus	79
4.2. Mitosis.....	85
4.3. Plastids and Photosynthesis	87
4.3.1. The photosynthetic ancestry of dinoflagellates.....	87
4.3.2. Plastid loss and replacement.....	89

4.4. Flagella and the definition of dinoflagellates	95
Concluding Remarks	100
Appendix: <i>Lessardia elongata</i> and the Taxonomic Position of the Genus	
<i>Roscoffia</i>	102
A.1 Introduction	102
A.2 Materials and Methods.....	105
A.2.1. Organisms and Culture Conditions.....	105
A.2.2. Light Microscopy	105
A.2.3. Scanning Electron Microscopy	106
A.2.4. Transmission Electron Microscopy.....	106
A.2.5. Molecular Phylogenetic Analysis	107
A.3. Taxon Descriptions	107
A.3.1. Description of <i>Lessardia</i> Saldarriaga et Taylor	
.....	107
A.3.2. Description of <i>Lessardia elongata</i> Saldarriaga	
et Taylor	108
A.4. Results	110
A.4.1. Morphological Examination	110
A.4.2. Molecular Phylogenetic Analysis	117
A.5. Discussion.....	117
A.6. Note on the Authorship of the Material Presented in the	
Appendix	124

Literature Cited	125
------------------------	-----

LIST OF TABLES

Table 1.1	Source species for the SSU rRNA sequences obtained in the present work, including their strain designations and GenBank accession numbers.	12
Table 1.2.	Classification of the order Gonyaulacales according to Fensome et al. 1993, including only species for which SSU rRNA sequence data is available.....	27
Table 2.1.	GenBank accession numbers of the protein gene sequences obtained for the present work.	39
Table 4.1.	Nuclear features of dinoflagellates and closely related groups.....	83
Table 4.2.	Flagellar features of dinoflagellates and closely related groups.	97

LIST OF FIGURES

Figure 1.1.	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.....	23
Figure 1.2.	Phylogenetic tree constructed with Fitch 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.....	24
Figure 1.3.	Maximum likelihood phylogenetic tree constructed from 40 alveolate SSU rRNA sequences, 35 of them dinoflagellates, and corrected for rate heterogeneity.	25
Figure 1.4.	Phylogenetic tree constructed with weighted neighbor 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 <i>Perkinsus marinus</i> , used as the outgroup.....	26
Figure 1.5.	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, 69 of them dinoflagellates.....	29
Figure 1.6.	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.....	32
Figure 2.1.	Phylogenetic tree constructed with weighted neighbor joining from a gamma-corrected distance matrix of SSU rRNA sequences (1488 nucleotides) from 78 phylogenetically diverse eukaryotic species.	42

Figure 2.2.	Phylogenetic tree constructed with weighted neighbor joining from a gamma-corrected distance matrix of SSU rRNA sequences (1488 nucleotides) from 97 alveolate species, including 52 dinoflagellates and 27 undescribed species from environmental samples.	43
Figure 2.3.	Phylogenetic tree constructed with weighted neighbor joining from a gamma-corrected distance matrix of actin sequences (244 amino acids) from 85 phylogenetically diverse eukaryotic species.....	48
Figure 2.4.	Phylogenetic tree constructed with weighted neighbor joining from a gamma-corrected distance matrix of alpha-tubulin sequences (384 amino acids) from 50 phylogenetically diverse eukaryotic species.....	49
Figure 2.5.	Phylogenetic tree constructed with weighted neighbor joining from a gamma-corrected distance matrix of beta-tubulin sequences (395 amino acids) from 56 phylogenetically diverse eukaryotic species.....	50
Figure 2.6.	Gamma-corrected protein maximum likelihood phylogenetic tree based on actin sequences from dinoflagellates, apicomplexans, <i>Perkinsus</i> and <i>Oxyrrhis</i> . Heterokonts are used as the outgroup.	51
Figure 2.7.	Gamma-corrected protein maximum likelihood phylogenetic tree based on alpha-tubulin sequences from alveolates.	51
Figure 2.8.	Gamma-corrected protein maximum likelihood phylogenetic tree based on beta-tubulin sequences from alveolates.	52

Figure 2.9.	Gamma-corrected protein maximum likelihood phylogenetic tree based on concatenated actin, alpha- and beta-tubulin sequences from alveolates.	52
Figure 3.1.	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 paleontological information.....	54
Figure A.1.	General morphology of <i>Lessardia elongata</i>	113
Figure A.2.	Details in the morphology of <i>Lessardia elongata</i>	114
Figure A.3.	Scanning electron micrographs of the thecal plate pattern of <i>Lessardia elongata</i>	115
Figure A.4.	Line drawings of the thecal plate patterns of <i>Lessardia elongata</i>	116
Figure A.5.	Line drawings of the epithecae and hypothecae of <i>Roscoffia capitata</i> , <i>Lessardia elongata</i> and <i>Blepharocysta</i> sp.	123

ACKNOWLEDGMENTS

A project such as this cannot be achieved by one person alone, and I am greatly indebted to the many people who helped me along the way. First and foremost, I would like to thank my three supervisors, Max Taylor, Patrick Keeling and Tom Cavalier-Smith, for friendship, funding and counselling, psychological and otherwise. I especially appreciate the willingness of the three of them to spend many hours attempting to explain to me the intricacies of dinoflagellate biology, protistology and molecular biology; the knowledge that I (hopefully) gained during those sessions is one of the things that I value the most of my experience at UBC.

Ema Chao and Naomi Fast were lab fairies that somehow always managed to make things work. They had willing helpers in Maggie Beaton, Langtuo Deng, Ken-ichiro Ishida, Todd Harper, Matt Field and John Archibald. Brian Leander and Elaine Humphrey were of invaluable help in matters regarding microscopy, and Donna Dinh was a constantly good-natured source of culture material. Fellow students of the Taylor, Cavalier-Smith and Keeling labs, Tony Wagey, David Cassis, Qing Qian, Zhaoduo Zhang, Michelle McEwan, Matt Rogers and Audrey de Koning, were also great sources of support. However, in spite of my gratitude to all of them for their help in scientific matters, it is their friendship what I value most, thank you all very much for that. Margaret Taylor also deserves many, many thanks, she did more than she thinks to make me feel welcome in Vancouver.

Many thanks are also due to other members of the Department of Botany, especially the rest of my committee, Mary Berbee, Curtis Suttle, Brian Leander and Philippe Tortell, and the two departmental heads active "during my time", Iain Taylor and Carl Douglas. At the beginning of my stay at UBC I had to deal with serious medical problems, and I was pleasantly surprised by the degree of personal commitment displayed by many members of the department, for example the very first visit I received at hospital was of all four departmental secretaries, I was, and still am, extremely grateful to them for that (as well as for all the help that I got over the years).

Finally, I would like to thank family and friends for moral support in times of need, and for fun and games at other times; because of them my stay at UBC was not only fruitful, but also very pleasant. Thank you very much.

PREFACE

The findings presented in this thesis either have been or will be published in the following articles:

1. Saldarriaga JF, Taylor FJR, Keeling PJ and Cavalier-Smith T 2001:
Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple chloroplast losses and replacements. *J. Mol. Evol.* 53: 204-213.
2. Saldarriaga JF, McEwan ML, Fast NM, Taylor FJR and Keeling PJ 2003: Three protein gene phylogenies suggest that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. *Int. J. System. Evol. Microbiol.* 53: 355-365.
3. Saldarriaga JF, Taylor FJR, Cavalier-Smith T, Menden-Deuer S and Keeling PJ 2004: Molecular data and the evolutionary history of dinoflagellates. *Europ. J. Protistol.*, in press.
4. Saldarriaga JF, Leander BS, Taylor FJR and Keeling PJ 2003: *Lessardia elongata* gen. et sp. nov. (Dinoflagellata, Peridiniales, Podolampaceae) and the taxonomic position of the genus *Roscoffia*. *J. Phycol.* 39: 368-378.

CHAPTER 1. INTRODUCTION TO THE PROJECT AND RIBOSOMAL-GENE PHYLOGENIES

1.1. 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 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 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 diarrhetic 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 other marine fauna mortalities (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 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 characteristic ultrastructure that remain condensed throughout the cell cycle (cf. Dodge 1987). Dinokarya are present in most dinoflagellates, but not in the parasitic order Syndiniales or in particular life stages of the Blastodinales (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, cf. Netzel and Dürr 1984). In thecate orders (Gonyaulacales, Peridinales, 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 Gymnodinales, but also Syndiniales, Noctilucales, etc.) often contain hundreds of alveoli, making it difficult to determine homologies and positional 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 commonly found as motile stages, the cell type that typically displays this feature. However, these motile stages are often ephemeral phases of dinoflagellate life

cycles; some species are most often found as cysts (Suessiales, Thoracosphaerales, Phytodinales, a few Gonyaulacales), plasmodia (many Syndinales) or as strongly modified trophonts that are not easily comparable to the typical dinoflagellate motile stages (Noctilucales and Blastodinales). 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 dinoflagellate fossils 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 Peridinales, Taylor 1980, Hallegraeff and Lucas 1988), even if the polarity of the changes cannot. Nevertheless, this cannot be done between orders as there are few intermediate forms. As a consequence, except for some cases where informative intermediate fossil taxa have been found (Fensome et al. 1993), the relationships of many dinoflagellate orders to one-another are still unclear. Also unclear is which groups of dinoflagellates are early or late diverging, different sets of characters support different hypotheses (cf. Taylor 1980, Fensome et al. 1993).

As a whole, dinoflagellates appear to have an unusual ability to take in endosymbionts. Roughly half of the species in the group are photosynthetic (Taylor 1987). Typical dinoflagellate plastids are surrounded by three membranes and contain closely appressed thylakoids in groups of three, chlorophylls a and c₂, and a number of carotenoids of which the most important is peridinin (e.g. Schnepf and Elbrächter 1999). The genome of at least some of these peridinin-containing plastids exists as single-gene mini-circles, an organization unique to

dinoflagellates (Zhang et al. 1999). From the position of peridinin-containing dinoflagellates in published 18S rRNA trees, it appears that this type of plastid was acquired only once, relatively early in their evolutionary history (Saunders et al. 1997). Other, atypical plastids also exist in dinoflagellates. *Karenia brevis*, *Karenia mikimotoi* and *Karlodinium micrum* have 19'-hexanoyloxyfucoxanthin-containing plastids derived from haptophytes (Tengs et al. 2000), while *Lepidodinium viride* and *Gymnodinium chlorophorum* have plastids with prasinophyte pigments (Watanabe and Sasa 1991, Schnepf and Elbrächter 1999) and *Kryptoperidinium foliaceum* and *Durinskia baltica* (as *Peridinium foliaceum* and *P. balticum* in Chesnick et al. 1997) have fucoxanthin-containing diatoms. The order Dinophysiales includes colourless heterotrophic species as well as photosynthetic forms (Taylor 1980, Hallegraeff and Lucas 1988) that contain cryptomonad-like plastids (Schnepf and Elbrächter 1988, Hackett et al. 2003) with phycobilins in the thylakoid lumen. Photosynthetic (and non-photosynthetic) members of the Dinophysiales have been impossible to culture, and so the suspicion exists that their photosynthetic organelles may be kleptochloroplasts (functional but non-reproductive plastids that are regularly taken up from photosynthetic prey, an occasional occurrence in heterotrophic dinoflagellates, e.g. Stoecker 1999). However, the plastids of Dinophysiales are remarkably homogeneous in morphology, a feature that weakens the kleptochloroplast argument. A very different type of plastid appears to exist in *Dinophysis* (*Phalacroma*) *rapa* (Hallegraeff and Lucas 1988, Schnepf and Elbrächter 1999), but there is little information about it.

Early phylogenetic studies established 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 1976a) was also corroborated by these sequence analyses, as was a newly discovered affiliation to apicomplexans (Wolters 1991, Gajadhar et al. 1991). In 1991 a new taxon, the Alveolata, was established encompassing ciliates, dinoflagellates, apicomplexans and their close relatives (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 a relationship between this group and chromists as predicted by the chromalveolate hypothesis (Cavalier-Smith 1999, 2003) and by earlier taxonomic schemes (e.g. Taylor 1976a).

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); 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 close relative of the apicomplexans (Cavalier-Smith 2000, Brugerolle 2002a, Kuvardina et al. 2002, Leander et al. 2003, Leander and Keeling 2003). Other protalveolates have not been characterized at the molecular level, and so it remains to be determined where the phylogenetic affiliation of *Colponema*, *Acrocoelus*, the ebriids and others may lie.

Nearly all molecular phylogenetic studies of the in-group relationships of dinoflagellates have used ribosomal RNA genes (rRNA), either partial sequences of the large-subunit ribosomal RNA gene (LSU, e.g. Lenaers et al. 1991, Zardoya et al. 1995, Daugbjerg et al. 2000), or the small-subunit rRNA gene (SSU, e.g. Saunders et al. 1997, Grzebyk et al. 1998, Gunderson et al. 1999). Relationships of orders to one-another are mostly unresolved (e.g. Saunders et al. 1997), 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 for SSU, but since the two molecules appear to

evolve at different rates (Ben Ali 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, they are based on HSP90 (B. Leander, unpublished data), actin, alpha- and beta-tubulin genes, as well as some plastid-encoded genes (e.g. psbA in Takishita and Ushida 1999, psaA in Zhang et al. 2000, Yoon et al. 2002). Phylogenetic trees based on protein genes still contain few taxa, and support for their in-group clades tends to be weak.

The objective of the present work was to clarify some of the main events in the evolutionary history of the dinoflagellates and their close relatives. This is difficult, especially since at present there are substantial gaps in our understanding of the basic phylogeny of the group. In order to propose such a phylogeny, new molecular data were obtained and compared to existing morphological, paleontological and biochemical information. Most of the new molecular data were in the form of SSU ribosomal RNA gene sequences that were examined on their own and in combination with existing data for sections of the LSU rRNA gene. A number of phylogenetic trees based on new protein gene sequences (actin, alpha- and beta-tubulin) were also used.

This first chapter of the thesis introduces the project and outlines the methodology used for sequencing dinoflagellate ribosomal genes. It also presents the phylogenetic trees obtained from these data. This is followed by an examination of the phylogenetic positions of *Oxyrrhis marina* and *Perkinsus marinus*, two taxa that have proven to be important in the understanding of the

phylogeny of the group. Protein gene based phylogenies are most relevant for this second chapter of the work, and so they will be presented there. The third chapter of the thesis combines the data obtained from the molecular work with existing morphological, paleontological and biochemical data to propose a phylogenetic framework that illustrates the history of dinoflagellates and their close relatives. That phylogenetic framework is then used in the fourth chapter to trace some of the events in the evolutionary history of dinoflagellates, for example the loss of histones and the appearance of a dinokaryon, the history of photosynthesis in the group and the development of cortical alveoli and flagella.

1.2. MATERIALS AND METHODS

1.2.1. On the choice of molecular phylogenetic markers

Several factors have influenced the choice of genes to sequence. The small-subunit ribosomal RNA gene is highly conserved, and thus likely to be appropriate for the large-scale phylogenetic questions that will be addressed here. It is also repeated hundreds of times within a normal genome, a feature that makes it easier to amplify than protein genes (Hillis and Dixon 1991). Most important, however, is the fact that more sequences are available for the SSU ribosomal RNA gene than for any other, and for the purposes of the present work a good species representation within the dinoflagellates as well as among neighboring taxa is desirable. Protein genes, although more difficult to sequence,

represent an independent line of molecular evidence that could be helpful in resolving phylogenetic questions that are not solved with information from ribosomal genes. Actin and alpha- and beta-tubulin have proven to be reasonably conserved genes that have been helpful in resolving phylogenetic questions in other groups (e.g. Keeling 2001). No new sequences for the large subunit ribosomal RNA gene were produced in the present work, but many of those published in the literature were included in several phylogenetic analyses.

1.2.2. Organisms, Extraction, Amplification and Sequencing

The present work is wide in scope, and so criteria had to be established to determine the species whose ribosomal RNA sequences would be most useful for the objectives outlined above. Dinoflagellates that were given sequencing priority included:

- Taxa presently underrepresented in phylogenetic trees
- Taxa suspected of being early branches in the dinoflagellate lineage
- Dinoflagellate taxa that are either completely non-dinokaryotic or dinokaryotic only during some life stages

- Non-photosynthetic species and species with aberrant plastid types (published phylogenetic trees of dinoflagellates are heavily biased towards photosynthetic species)
- Taxa likely to break up long branches in published trees
- Groups likely to be polyphyletic in their current definition (e.g. Gymnodiniales, Blastodinales, Phytodinales, *Gymnodinium*, *Gyrodinium*, *Amphidinium*).
- Genera of uncertain taxonomic position

Most photosynthetic dinoflagellate species were obtained from unialgal, non-axenic culture collections; exceptions were *Amylax diacantha*, *Ceratium hirundinella*, *Pyrodinium bahamense* and *Thecadinium kofoidii*. *Oxyrrhis marina* (a non-photosynthetic species) was also obtained as a culture, but one that also contained its prey organism, the green alga *Dunaliella* sp. (see Chapter 2). Other species were collected as follows: *Haplozoon axiothellae* was obtained from the gut of its host, the maldanid polychaete *Axiothella rubrocincta*, collected in Argyle Lagoon, San Juan Island, Washington, USA; *Ceratium hirundinella* was isolated by hand from a plankton bloom at Egg Lake, San Juan Island, Washington, USA; *Amylax diacantha*, the three *Protoperidinium* species, *Amphidinium longum* and *Gymnodinium* sp. were provided by either Susanne Menden-Deuer (University of Washington) or Suzanne Strom (University of Western Washington) from cultures isolated in Puget Sound, Washington, USA; *Pyrodinium bahamense* was provided

by Tony Wagey from cultures isolated by Rhodora Azanza in Manila Bay, the Philippines; and *Thecadinium kofoidii*, *Amphidinium semilunatum*, *Roscoffia capitata* and *Thecadinium dragescoi* were isolated by hand from intertidal sand flats at List Harbour, Sylt, North German Wadden Sea by Mona Hoppenrath. All species that originated from culture collections were harvested through centrifugation, their DNA was then extracted using the DNeasy Plant DNA Purification Kit (Qiagen). In all other cases, between 40 and 250 cells (or ca. 50 multicellular specimens of *Haplozoon*) were micropipetted from their environment and washed repeatedly. Isolated cells were centrifuged and stored at room temperature in the lysis buffer of the purification kit indicated above until their DNA could be extracted.

Whenever possible, the 18S (nuclear SSU) rRNA gene was amplified as a single fragment using a polymerase chain reaction with two eukaryotic universal SSU rRNA primers (5'-CGAATTCAACCTGGTTGATCCTGCCAGT-3' and 5'-CCGGATCCTGATCCTTCTGCAGGTTACCTAC-3', 96°C, 3min + 35x(96°C, 1min+ 58°C, 40s+ 72°C, 1min) + 72°C, 1min + 4°C, ∞). However, in many cases two overlapping fragments had to be produced using internal primers designed to match existing eukaryotic SSU rRNA sequences (4F: 5'-CGGAATTCCAGTC-3' and 11R: 5'-GGATCACAGCTG-3'). PCR products were either sequenced directly (material originating from unialgal cultures only, overlapping segments identical) or cloned into pCR-2.1 vector using the TOPO TA cloning kit (Invitrogen). Sequencing reactions were completed with both of 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.

Table 1.1

Source organisms of the SSU rRNA sequences obtained in this study.

Taxon	Ordinal classification in Fensome et al. 1993	Strain Number	GenBank Accession Number
<i>Adenoides eludens</i> (Herdman) Balech	uncertain	CCCM 683	AF274249
<i>Amphidinium asymmetricum</i> Kofoid and Swezy	Gymnodiniales	CCCM 067	AF274250
<i>Amphidinium britannicum</i> (Herdman) Lebour (as <i>Amphidinium</i> <i>asymmetricum</i> var. <i>compactum</i>)	Gymnodiniales	CCCM 081	AY443010
<i>Amphidinium carterae</i> Hulburt	Gymnodiniales	CCMP 1314	AF274251
<i>Amphidinium corpulentum</i> Kofoid and Swezy	Gymnodiniales	UTEX LB 1562	AF274252
<i>Amphidinium herdmannii</i> Kofoid and Swezy	Gymnodiniales	CCCM 532	AF274253
<i>Amphidinium longum</i> Lohmann ²	Gymnodiniales	none	AF274254
<i>Amphidinium massartii</i> Biecheler	Gymnodiniales	CCCM 439	AF274255

<i>"Amphidinium operculatum"</i> Claparede & Lachmann ⁴	Gymnodiniales	CCMP 1342	AY443011
<i>"Amphidinium rhynchocephalum"</i> Anissimowa ⁴	Gymnodiniales	UTEX LB 1688	AY443012
<i>Amphidinium semilunatum</i> Herdman ²	Gymnodiniales	none	AF274256
<i>Amylax diacantha</i> Meunier	Gonyaulacales	none	AY443013
<i>Ceratium hirundinella</i> (O. F. Müller) Dujardin	Gonyaulacales	none	AY443014
<i>Glenodiniopsis steinii</i> (Lemmermann) Woloszynska ³ (as <i>Glenodiniopsis uliginosa</i>)	Peridinales	NIES 463	AF274257
<i>Gonyaulax cochlea</i> Meunier	Gonyaulacales	CCMP 1592	AF274258
<i>Gymnodinium</i> sp. ²	Gymnodiniales	none	AF274260
<i>Gyrodinium dorsum</i> Kofoid and Swezy	Gymnodiniales	UTEX LB 2334	AF274261
<i>Gyrodinium instriatum</i> Freudenthal & Lee	Gymnodiniales	CCMP 431	AY443015
<i>Gyrodinium uncatenum</i> Hulburt	Gymnodiniales	CCCM 533	AF274263
<i>Haplozoon axiothellae</i> Siebert ²	Blastodinales	none	AF274264
<i>Hemidinium nasutum</i> Stein ¹	Phytodinales	NIES 471	AY443016
<i>Heterocapsa niei</i> (Loeblich) Morrill & Loeblich III ¹	Peridinales	CCMP 447	AF274265
<i>Heterocapsa pygmaea</i> Loeblich III,	Peridinales	CCCM 681	AF274266

Schmidt and Sherley			
<i>Heterocapsa rotundata</i> (Lohmann) Hansen	Peridinales	CCCM 680	AF274267
<i>Karenia brevis</i> (Davis) Hansen & Moestrup	Gymnodinales	CCMP 718	AF274259
<i>Karlodinium micrum</i> (Leadbeater & Dodge) J. Larsen ¹	Gymnodinales	CCCM 555	AF274262
<i>Kryptoperidinium foliaceum</i> (Stein) Lindemann ¹	Peridinales	UTEX LB 1688	AF274268
<i>Lingulodinium polyedrum</i> (Stein) Dodge	Gonyaulacales	CCCM 202	AF274269
<i>Oxyrrhis marina</i> Dujardin ²	In the dinoflagellate lineage but not a member of the group	CCCM 534	AF482425
<i>Pentapharsodinium</i> sp. Indelicato & Loeblich III (as <i>Scrippsiella faeroense</i>)	Peridinales	CCMP 771	AF274270
<i>Peridinium polonicum</i> Woloszynska	Peridinales	NIES 500	AY443017
<i>Peridinium wierzejskii</i> Woloszynska	Peridinales	NIES 502	AY443018
<i>Peridinium umbonatum</i> Stein ³ (as <i>Peridinium inconspicuum</i>)	Peridinales	UTEX LB 2255	AF274271
<i>Peridinium willei</i> Huitfeld-Kaas ³	Peridinales	NIES 304	AF274272

<i>Peridinium willei</i> Huitfeld-Kaas ³ (as <i>Peridinium volzii</i>)	Peridinales	NIES 365	AF274280
<i>Prorocentrum gracile</i> Schütt	Prorocentrales	CCCM 765	AY443019
<i>Protoceratium reticulatum</i> (Claparède & Lachmann) Bütschli	Gonyaulacales	CCCM 535	AF274273
<i>Protoperidinium conicum</i> (Gran) Balech ²	Peridinales	none	AY443020
<i>Protoperidinium excentricum</i> (Paulsen) Balech ²	Peridinales	none	AY443021
<i>Protoperidinium pellucidum</i> Bergh ²	Peridinales	none	AY443022
<i>Pyrocystis lunula</i> (Schütt) Schütt	Gonyaulacales	CCCM 517	AF274274
<i>Pyrodinium bahamense</i> Plate	Gonyaulacales	none	AF274275
<i>Pyrophacus steinii</i> (Schiller) Wall & Dale	Gonyaulacales	NIES 321	AY443024
<i>Roscoffia capitata</i> Balech ²	uncertain	none	AF521101
<i>Scrippsiella sweeneyae</i> Balech ex Loeblich III	Peridinales	CCCM 280	AF274276
<i>Scrippsiella trochoidea</i> (Stein) Loeblich III	Peridinales	CCCM 602	AF274277

<i>Symbiodinium</i> sp. in <i>Aiptasia pallida</i> (= " <i>Symbiodinium bermudense</i> ")	Suessiales	none	AY443023
<i>Symbiodinium</i> sp. (free-living, not described)	Suessiales	CCMP 421	AF274279
<i>Thecadinium dragescoi</i> Balech ²	Gonyaulacales	none	AY238479
<i>Thecadinium kofoidii</i> (Herdman) Larsen	Gonyaulacales	none	AY238478
<i>Thecadinium mucosum</i> Hoppenrath & Taylor	Gonyaulacales	CCCM 682	AY238477
<i>Thoracosphaera heimii</i> (Lohmann) Kamptner ¹	Thoracosphaerales	CCCM 670	AF274278
<i>Woloszynskia leopoliensis</i> (Woloszynska) Thompson	Gymnodiniales	NIES 619	AY443025

1: Partial small subunit sequences existed before the present work.

2: Non-photosynthetic species.

3: Fresh water species for which the nomenclature of Popovsky and Pfiester 1990 was used.

4: Species defined on equivocal characters (N. Daugbjerg, personal communication).

1.2.3. Phylogenetic Analysis

The new sequences, as well as many others available in public databases (including sequences from environmental samples, López-García et al. 2001, Moon-van der Staay et al. 2001) were added to the alignment of van de Peer et al. (1998). The final alignment contained 98 dinoflagellate species, *Perkinsus*, *Parvilucifera* and several ciliate and sporozoan sequences that were used as outgroups. Sequences for *Oxyrrhis marina* (including a sequence from a second isolate of the species recently released to GenBank, NIES 494) were initially included in the analyses. However, early on it became clear that the *Oxyrrhis* sequences were likely to distort the phylogenetic trees obtained (see Chapter 2), and so they were excluded from subsequent analyses. Phylogenetic trees including *Oxyrrhis marina* are more relevant to Chapter 2, they will be further discussed there. Only unambiguously-aligned sections of the molecule (1479 characters) were used in the phylogenetic analyses that included all outgroups. A separate set of analyses of SSU rRNA data was performed excluding all ciliate and apicomplexan taxa (*Perkinsus* was used as the outgroup); by doing so we were able to align confidently a larger portion of the SSU rRNA molecule, 1649 sites.

Small-subunit sequences were also concatenated with published sequences for sections of the LSU. Concatenated alignments that included SSU rRNA and domains D1-D3 of the LSU rRNA included 25 alveolate species (22 of them dinoflagellates) and 2418 nucleotides, while alignments with SSU rRNA and

domains D1-D2 of the LSU rRNA included 34 species (31 of them dinoflagellates) and 2100 nucleotides. Phylogenetic trees based on LSU only were also calculated for comparison, and in those the choice of sites used was extremely conservative, only 447 sites for alignments that used domains D1-D2, 718 sites for those that used domains D1-D3.

Distances were calculated with PUZZLE 5.0. (Strimmer and von Haeseler 1996) using the HKY substitution frequency matrix, a standard model frequently used for nuclear genes. Nucleotide frequencies and transition/transversion ratios were estimated from the data, and site-to-site variation was modeled on 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 were inferred as described for 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 rRNA sequences (40 species, 35 of them dinoflagellates). They were inferred under an HKY model incorporating a discrete gamma distribution to correct for rate heterogeneity (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 one hundred bootstrap data sets in the case of the concatenated data.

1.3. RESULTS

1.3.1. 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 difficult to establish whether the dinoflagellate clade was monophyletic in our trees or not. The environmental sequences, however, always grouped in two clades. One of them (group II in López-García et al. 2001) generally included all known sequences of Syndiniales (*Hematodinium* and three species of *Amoebophrya*; in the Fitch-Margoliash tree, Figure 1.2, *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 (Figure 1.1) and Weighbor trees (not shown) branched basal to all other dinoflagellates but not to the *Perkinsus/Parvilucifera* grouping (in the Fitch-Margoliash tree, Figure 1.2, this clade branched after the Syndinians and *Noctiluca*). Assuming that all these environmental sequences come from true dinoflagellates, then the dinoflagellate clade is supported in all trees by bootstrap values of 60-65%.

The placement of *Noctiluca* in all trees was very unstable. In BioNJ and Weighbor trees (e.g. Figure 1.1), 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 rRNA trees including only dinoflagellates and *Perkinsus* that utilized more sites (Figure 1.4) invariably placed *Noctiluca scintillans* in a clade with all non-syndanian dinoflagellates that also included two putative members of the Blastodinales, *Amyloodinium* sp. and *Haplozoon axiothellae*.

A large part of that clade is composed of very short-branched members of the orders Gymnodiniales, Peridinales, Prorocentrales and Dinophysiales, the so-called GPP complex (Saunders et al. 1997), along with *Thoracosphaera* (Thoracosphaerales), *Hemidinium* (Phytodinales), *Amyloodinium* and *Haplozoon* (Blastodinales) and *Pfiesteria*. The order Dinophysiales, represented by the genus *Dinophysis*, is the only one that groups strongly as a distinct clade within the GPP complex (Edwardsen et al. 2003). The Prorocentrales resolves as at least 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, no bootstrap support). 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 the 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* (concern exist that the sequence included in all SSU trees might be from a misidentified *Gymnodinium chlorophorum*, G. Saunders, personal communication); the second, members of *Karenia* and *Karlodinium* but also *Amphidinium herdmannii*; and the third, three putative members of *Gyrodinium* (*G. instriatum* and *G. dorsum* have identical SSU rRNA sequences that differ from that of *G. uncatenum* by only 3 nucleotides out of 1755, in SSU rRNA phylogenies these three species do not group with the type species of *Gyrodinium*, *G. spirale*, B. Leander and B. Olson, unpublished data). The sequences for *Amphidinium cf. operculatum*, *Amphidinium massartii* and *Amphidinium rhynchocephalum* are also identical, they differ by 8 nucleotides (from a total of 1752) from that of *A. carterae* (the taxonomic identity of many species of *Amphidinium* is being reassessed, N. Daugbjerg, pers. comm., the nomenclature of the strains investigated here is sure to change in the future)

In some trees (e.g. the Fitch tree, Figure 1.2), 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* and three species of *Peridinium*: *P. polonicum*, *P. umbonatum* and *P. wierzejskii* (in Weighbor and BioNJ trees this clade is interrupted by gymnodinialean and/or prorocentralean groups). Nevertheless, several peridinialean taxa never group with the bulk of the order. These include a well supported clade of the three *Protooperidinium* species and a

well supported grouping of three *Peridinium* species (*Peridinium* sp., *P. willei* and *P. bipes*) that sometimes includes *Glenodiniopsis steinii* (e.g. Figures 1.1, 1.2.). The diatom-bearing genera *Kryptoperidinium* and *Durinskia* form a weakly-supported clade in BioNJ trees (Figure 1.1), 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 *Amphidinium 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, Figures 1.1, 1.2), although it is never well supported. The phytodinialean genus *Halostylodinium* is the only non-gonyaulacalean taxon that consistently branches within the clade. Within the Gonyaulacales (Table 1.2), several groupings appear consistently, for example one containing *Alexandrium*, *Fragilidium*, *Ostreopsis*, *Pyrocystis*, *Pyrodinium* and *Pyrophacus* (suborder Goniodominae, 50-60% bootstrap support) and another containing all *Ceratium* species (Ceratiineae, 75-90% bootstrap support). Members of the Gonyaulacineae (*Amylax*, *Ceratocorys*, *Gonyaulax*, *Lingulodinium* and *Protoceratium*) consistently branch at the base of the Gonyaulacales, always as a paraphyletic group that gives rise to the Ceratiineae and Goniodominae and that also contains *Crypthecodinium* and *Halostylodinium*.

Figure 1.2. Phylogenetic tree constructed with the Fitch-Margoliash algorithm 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. Bootstrap values are given when higher than 60%.

Figure 1.3. Maximum-likelihood phylogenetic tree constructed from 40 alveolate SSU rRNA sequences, 35 of them dinoflagellates, and corrected for rate heterogeneity.

Figure 1.4. 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 given when higher than 60%

Table 1.2

Classification of the order Gonyaulacales according to Fensome et al. 1993,
including only genera for which SSU rRNA sequence data are available.

SUBORDER	FAMILY	SUBFAMILY	GENUS
Gonyaulacinae	Gonyaulacaceae	Cribroperidinioideae	<i>Protoceratium</i>
			<i>Lingulodinium</i>
		Gonyaulacoideae	<i>Gonyaulax</i>
			<i>Amylax</i>
	Ceratocoryaceae		<i>Ceratocorys</i>
Ceratiineae	Ceratiaceae		<i>Ceratium</i>
Goniodomineae	Goniodomaceae	Gambierdiscoideae	<i>Ostreopsis</i>
			<i>Coolia</i>
		Helgolandinioideae	<i>Alexandrium</i>
			<i>Fragilidium</i>
			<i>Pyrophacus</i>
		Pyrodinioideae	<i>Pyrodinium</i>
	Pyrocystaceae		<i>Pyrocystis</i>
Uncertain	Crypthecodiniaceae		<i>Crypthecodinium</i>
	Uncertain		<i>Thecadinium</i>
Order Phytodinales in Horiguchi et al. 2000			<i>Halostylodinium</i>

1.3.2. LSU rRNA Phylogeny

Phylogenetic trees based on LSU data were generally similar to those based on SSU rRNA. As LSU rRNA sequences for *Perkinsus*, Syndiniales, Noctilucales or Blastodinales are unavailable (and in the case of *Oxyrrhis* the amount of data that exists is insufficient to include the species in the analysis, Lenaers et al. 1991), the trees obtained consisted of a large, poorly resolved group of very short-branched taxa (the GPP complex, Gymnodinales, Peridinales, Prorocentrales and Dinophysiales) and a monophyletic grouping of longer-branched members of the order Gonyaulacales (Figure 1.5). Within the GPP complex, groupings well supported in SSU rRNA trees are also well supported here, e.g. the *Gymnodinium fuscum* group (henceforth *Gymnodinium* sensu stricto, Daugbjerg et al. 2000), the *Karenia/Karlodinium* group, the expanded Suessiales (including several *Gymnodinium* species), and Dinophysiales. There are, however, several differences from SSU rRNA trees. In at least some LSU trees, all Prorocentrales do group together (e.g. in the Weighor tree, Figure 1.5, the dataset does include species from both the 'planktonic' and the 'benthic' groups), and whereas the *Amphidinium carterae* group still holds together with good support and a relatively long branch, it is not at the base of the Gonyaulacales (in Weighor and Fitch trees it interrupts a badly supported clade of Peridinales, e.g. Figure 1.5). 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

Figure 1.5. Phylogenetic tree constructed with weighted neighbour-joining from a gamma-corrected distance matrix of domains D1 and D2 of the LSU rRNA gene (447 nucleotides) from 71 alveolates, 69 of them dinoflagellates. Bootstrap values are given when higher than 60%.

alignments contain different species of the genus: *W. pseudopalustris* in LSU, *W. leopoliensis* in SSU).

The Gonyaulacales also hold together in most LSU trees, albeit with modest bootstrap support (in the ML tree, the genus *Ceratium* branches with the Apicomplexan outgroup). The majority of the gonyaulacalean species for which LSU data are known are members of the Goniodominae (*Alexandrium*, *Coolia*, *Fragilidium* and *Ostreopsis*), and they do form a clade to the exclusion of all other taxa, although with low bootstrap support (the sequence for *Ceratium furca* interrupts a strongly supported clade of many *Alexandrium* species in all trees; it is likely that this is a sequencing/laboratory 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, but this is not the case in the Fitch trees, where Goniodominae appear to give rise to Gonyaulacinae and *Ceratium*. *Protoceratium* and *Gonyaulax*, the only Gonyaulacinae in the trees, were never sisters.

1.3.3. Combined rRNA Phylogeny

Phylogenetic trees based on combined datasets generally show the basic structure discussed above (Figure 1.6): a poorly supported backbone of short-branched taxa (the GPP complex) that includes some well-supported subgroups, and the Gonyaulacales, longer-branched taxa that invariably form a clade, here 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 rRNA trees. Within the Gonyaulacales, the Gonyaulacinae (*Gonyaulax* and *Protoceratium*) generally branch as sisters to a group that contains *Ceratium* and the Goniodominae (Figure 1.6, in the Fitch and Weighbor trees based on SSU/D1/D2/D3 concatenations the *Gonyaulax/Protoceratium* clade is not retained, data not shown). One major difference between the concatenated and single gene trees is that in all concatenated trees the two *Heterocapsa* species (*H. rotundata* and *H. triquetra*) branch before the bulk of the GPP complex with bootstrap support between 43 and 71%; in the Weighbor trees and in the Fitch and ML trees based on the SSU/D1/D2/D3 concatenation the two *Heterocapsa* species are sisters, in the other trees they are not. Many nodes have better bootstrap support than in the single gene based trees. It is unclear whether this is a consequence of the smaller numbers of taxa or the additional sequence data.

Figure 1.6. 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. Bootstrap support values are given when higher than 60% and on the branch that separates *Heterocapsa* from the rest of the dinoflagellates.

CHAPTER 2: MOLECULAR DATA AND THE PHYLOGENETIC POSITION OF *OXYRRHIS MARINA* AND *PERKINSUS MARINUS*

2.1. INTRODUCTION

As mentioned before, the alveolates form a large and diverse assemblage of protists that include three major lineages: ciliates, dinoflagellates, and apicomplexans. However, in addition to these well-defined and relatively well-studied groups, there are also a number of species that display alveolate features like cortical alveoli but lack characteristics that would specifically ally them with any one of the three subgroups. These organisms, sometimes referred to as protalveolates (Cavalier-Smith 1991, 1998), are often regarded as intermediates between the major alveolate groups, and are therefore potentially instrumental in reconstructing the origin and evolutionary history of the characteristics that define ciliates, dinoflagellates, and apicomplexans.

Oxyrrhis marina is a heterotrophic flagellate commonly found in marine and brackish nearshore waters, including rock pools, estuaries, and marshes. The species has often been regarded as a dinoflagellate (e.g. Kofoed and Swezy 1921, Dodge 1984, Sournia 1986), but has also been explicitly excluded from the group in other classification schemes (Fensome et al. 1993). It has a number of characters very different from those of true dinoflagellates. In *Oxyrrhis* the mitotic spindle is intranuclear and originates from numerous plaques on the nuclear envelope (Triemer 1982, Gao and Li 1986); in dinoflagellates the spindle is

extranuclear and its microtubules are located within cytoplasmic channels that traverse the nucleus (Kubai and Ris 1969, Ris and Kubai 1974, Dodge 1987). The nuclear organization in *Oxyrrhis* is very atypical: it contains a large number of long, thin chromosomes, separated by numerous electron-dense bodies that could be small chromosome fragments (Dodge and Crawford 1971). This organization is different from the thick, continuously condensed, fibrillar chromosomes in the dinokaryon of typical dinoflagellates. Other differences between *Oxyrrhis* and true dinoflagellates are the lack of a girdle, a sulcus or pusules in *Oxyrrhis* (some dinoflagellates have secondarily lost the girdle and/or sulcus, Fensome et al. 1993). The phylogenetic position of *Oxyrrhis* has not been substantially investigated using molecular data. Only one report includes sequence data from *Oxyrrhis* (Lenaers et al. 1991), with only 235 nucleotides from two domains of the large subunit ribosomal RNA gene were used (D1 and D8). Phylogenetic trees inferred from this sequence and that of 12 dinoflagellates and one ciliate placed *Oxyrrhis* basal to the dinoflagellates (apicomplexans were not included). An SSU sequence from a Japanese strain of *Oxyrrhis* became available as the present study was being made. It is included in the analysis presented here.

Perkinsus marinus, another protalveolate, is the causative agent of "dermo", an important disease of oysters and many other species of bivalves (Perkins 1976). The taxonomic placement of *Perkinsus* has always been problematic; over the years the genus has been considered to be a member of the fungi, labyrinthulids and haplosporidians. Eventually, ultrastructural data led to

the conclusion that *Perkinsus* represents an early lineage of the apicomplexans (Levine 1978, Perkins 1996). This was based largely on the fact that its flagellated stage contains an apical organelle with similarities to the apicomplexan conoid, an apical structure composed of microtubular units arranged in a helical coil forming a truncated cone. In *Perkinsus*, however, this "conoid" is open along one side, a feature that led to a reinterpretation of the significance of the structure for the taxonomy of the genus (Siddall et al. 1997). The motile life stage of *Perkinsus marinus* has, like dinoflagellates, two dissimilar flagella that insert ventrally, one of them with mastigonemes along one side (Perkins 1996). Cell division in *Perkinsus* also appears to be dinoflagellate-like: the nuclear envelope remains intact during mitosis and deep channels are formed, continuous with the cytoplasm and lined by the nuclear membrane. The mitotic spindle runs through these channels and attaches to kinetochore-like structures on the nuclear envelope (Perkins 1996). However, the interphase nuclear ultrastructure of this species is unlike that of typical dinoflagellates: chromatin appears as electron-dense aggregates of varying density, not as the fibrillar structures of typical dinokaryons (Perkins 1996). Most recently, *Perkinsus* was placed in its own alveolate phylum, the Perkinsozoa, together with a newly described parasite of dinoflagellates, *Parvilucifera infectans* (Norén et al. 1999).

Two independent gene phylogenies (SSU rRNA and actin) have provided fairly convincing evidence that *Perkinsus* is more closely related to dinoflagellates than to any other alveolates (Goggin and Barker 1993, Reece et al. 1997). Nevertheless, the support for this position is sometimes equivocal in SSU rRNA

trees (e.g. Siddall et al. 1997), and other analyses of SSU rRNA have also shown *Perkinsus* and *Parvilucifera* branching at the base of the apicomplexans (Norén et al. 1999). In actin phylogenies the very divergent sequences of ciliates fall far from either dinoflagellates or apicomplexans (e.g. Keeling 2001), making it difficult to draw any firm conclusions on the position of *Perkinsus* based solely on this gene.

To investigate further the origins of *Oxyrrhis* and *Perkinsus*, genes encoding SSU rRNA, actin, alpha-tubulin, and beta-tubulin from *Oxyrrhis marina* and actin, alpha-tubulin, and beta-tubulin from a variety of dinoflagellates were sequenced. Phylogenies of these genes individually and in combination were inferred to determine the relationships between *Oxyrrhis*, *Perkinsus*, and other alveolates, and to begin to reconstruct the nature of the ancestors of dinoflagellates.

2.2. MATERIALS AND METHODS

The SSU rRNA gene from *Oxyrrhis marina* was amplified and sequenced as described in the previous chapter. Protein genes from the organisms listed in Table 2.1. were amplified using the following primers:

GAGAAGATGACNCARATHATGTTYGA and

GGCCTGGAARCAYTTNCGRTGNAC for actin,

TCCGAATTCARGTNGGNAAYGCNGGYTGGA and

CGCGCCATNCCYTCNCCNACRTACCA for alpha-tubulin, and
GCCTGCAGGNCARTGYGGNAAYCA and
TCCTCGAGTRAAYTCCATYTCRTCCAT for beta-tubulin, all in PCR reactions
using genomic DNA.

PCR products were cloned into pCR-2.1 vector using the TOPO TA cloning kit (Invitrogen), and several clones of each gene were sequenced on both strands. Protein-coding gene sequences were translated and added to existing alignments of eukaryotic sequences (Keeling 2001, Fast et al. 2002). Only unambiguously aligned characters were used in the phylogenetic analyses, resulting in data sets of 244, 384 and 395 characters for actin, alpha-tubulin, and beta-tubulin respectively. Phylogenetic trees were inferred both using comprehensive alignments containing a large number of taxonomically diverse eukaryotes to confirm the alveolate nature of *Oxyrrhis* and *Perkinsus*, and also with smaller subsets of these alignments that contained only alveolate taxa, so that more sophisticated analyses could be performed. In the larger, global analyses *Oxyrrhis* and *Perkinsus* sequences were always most closely related to apicomplexans and dinoflagellates, so for most of the smaller data sets ciliates were used as the outgroup. This was not the case in the actin data set: ciliate actin sequences are so divergent that they do not form a group with other alveolates (e.g. Keeling 2001). In this case heterokonts were used as outgroups, as these seem to be the nearest relatives to alveolates in actin phylogenies (e.g. Baldauf et al. 2000, Keeling 2001). In addition to the single-gene data sets, an alignment composed of concatenated sequences of actin, alpha-tubulin and beta-

tubulin was also produced (1023 amino acids). It contained only alveolate taxa for which the complete sequence of all three genes are known: three ciliates, three apicomplexans, *Oxyrrhis*, *Perkinsus*, and the only dinoflagellate for which all three genes are known, *Heterocapsa triquetra*.

Phylogenies from the single-gene and the concatenated data sets were inferred using distance and maximum likelihood methods of tree reconstruction. Distance matrices were calculated with TREE-PUZZLE 5.0. (Strimmer and von Haeseler 1996) using the WAG substitution matrix. Amino acid frequencies were estimated from the data. The among site rate variation was modeled on a gamma distribution with invariable sites plus eight variable rate categories, and the alpha shape parameter estimated from the data. Distance trees were constructed using weighted neighbor joining using WEIGHBOR (Bruno et al. 2000) and Fitch-Margoliash using FITCH (Felsenstein 1993). One hundred bootstrap data sets were constructed using SEQBOOT, and distances calculated using PUZZLEBOOT (by M. Holder and A. Roger: www.tree-puzzle.de) with the alpha shape parameter, amino acid frequencies, and transition/transversion ratio from the initial tree enforced on the 100 replicates. Protein maximum likelihood trees were inferred using ProML (Felsenstein 1993) with the JTT substitution frequency matrix, global rearrangements, and 10 input order jumbles. Site-to-site rate variation was modeled using the r option with the frequencies and rates calculated by TREE-PUZZLE. Protein maximum likelihood bootstrapping was performed as above, with the rates and rate categories from the original data set enforced on each replicate.

Table 2.1

Accession numbers for the new protein-gene sequences. Michelle McEwan, an undergraduate student under my supervision, obtained the sequences marked with an asterisk. Sources of organisms as described in Table 1.1.

TAXON	Actin	Alpha-tubulin	Beta-tubulin
<i>Amphidinium corpulentum</i>	--	--	AF482405*
<i>Amphidinium herdmanii</i>	--	AF482406* AF482407*	--
<i>Gyrodinium instriatum</i>	--	--	AF482408*
<i>Heterocapsa rotundata</i>	AF482409*	AF482410*	--
<i>Karenia brevis</i>	AF482415 AF482416 AF482417 AF482418	AF482419	--
<i>Oxyrrhis marina</i>	AF482402	AF482403*	AF482404
<i>Peridinium willei</i>	AF482420	--	AF482421*
<i>Woloszynskia leopoliensis</i>	--	--	AF482422*
<i>Symbiodinium</i> sp. (free-living, not described, CCMP 421)	AF482423	--	AF482424*

2.3. RESULTS AND DISCUSSION

2.3.1. SSU rRNA phylogeny of *Oxyrrhis* and *Perkinsus*

The SSU rRNA sequence of *Oxyrrhis marina* proved to be much more divergent than those of any dinoflagellates (Figures 2.1 and 2.2). In all phylogenetic analyses, the two *Oxyrrhis* isolates branched together with high support. In analyses encompassing many of the recognized eukaryotic lineages (Figure 2.1), and in a more local analysis restricted to alveolates (Figure 2.2), *Oxyrrhis* generally branched with the dinoflagellate order Gonyaulacales, and this relationship was relatively well-supported (e.g. 81% and 76% bootstrap support uniting *Oxyrrhis* and the gonyaulacalean *Gonyaulax spinifera* in Figure 2.1). The only analysis where this relationship did not appear was the maximum likelihood tree of alveolates-only, where *Oxyrrhis* branches from within the dinoflagellates, but not specifically with Gonyaulacales (not shown). However, the divergent nature of the *Oxyrrhis* sequences makes it difficult to draw any firm conclusions about the phylogenetic placement of the species. The *Oxyrrhis* branch lengths in the weighted neighbor joining distance tree of Figure 2.1 are for example almost eight times as long as that of *Gonyaulax spinifera*. Accordingly, the SSU rRNA phylogeny must be considered cautiously, especially as *Oxyrrhis* tends to branch with the Gonyaulacales, an otherwise morphologically coherent group with relatively divergent SSU rRNA sequences compared with other dinoflagellates (Saunders et al. 1997). Both the eukaryote-wide (Figure 2.1) and the alveolate-only data sets (Figure 2.2) contained sequences from an unidentified group of

marine alveolates (Moon-van der Staay et al. 2001); in all analyses these taxa failed to ally with *Oxyrrhis*. Although the divergent nature of the *Oxyrrhis* sequence makes firm conclusions difficult, these analyses obviously do not support the notion that these unidentified organisms are closely related to *Oxyrrhis* (Moon-van der Staay et al. 2001). Nevertheless, as shown below, the position of *Oxyrrhis* in SSU rRNA phylogenies is likely incorrect; its true position is probably at the base of the dinoflagellates. Accordingly, a possible relationship between *Oxyrrhis* and these picoplanktonic alveolates cannot be excluded.

The position of *Perkinsus* in the SSU rRNA analyses (basal to dinoflagellates) is consistent with most previously published results (Goggin and Barker 1993, Siddall et al. 1997). However, this position is not well supported by bootstrap analyses in the current study (e.g. 58% and less than 50% in Figure 2.1), and other analyses of SSU rRNA have reported conflicting positions for *Perkinsus* and the related *Parvilucifera* (Norén et al. 1999). The combination of poor bootstrap support and conflicting results indicates that SSU rRNA data are insufficient to resolve the position of perkinsids, and other data must be sought. It is also noteworthy that the SSU rRNA sequence from *Colpodella*, another protalveolate unrelated to *Perkinsus* or *Oxyrrhis*, branches at the base of the apicomplexans, as seen previously in other analyses (Siddall et al. 2001, Kuvardina et al. 2002, Leander et al. 2003).

Figure 2.1. Phylogenetic tree constructed with weighted neighbor-joining from a gamma-weighted distance matrix of SSU rRNA sequences (1488 nucleotides) from 78 phylogenetically diverse eukaryotic species. Bootstrap values, based on Weighbor (top) and Fitch-Margoliash (bottom) phylogenetic trees (gamma-corrected distances), are shown above selected internodes. Alveolate groups are marked. Accession numbers in GenBank are given for sequences from environmental samples with undetermined taxonomic identity and for the Japanese isolate of *Oxyrrhis marina*.

Figure 2.2. Phylogenetic tree constructed with weighted neighbour-joining from a gamma-corrected distance matrix of SSU rRNA sequences (1488 nucleotides) from 97 alveolate species, including 52 dinoflagellates and 27 undescribed species from environmental samples that are identified by their GenBank accession number. Bootstrap support values are given when higher than 60%.

2.3.2. Phylogenetic position of *Oxyrrhis* and *Perkinsus* based on actin, alpha-tubulin and beta-tubulin sequences

Given the difficulties imposed by the divergent *Oxyrrhis* SSU rRNA sequences and the general lack of support for the topologies of the various SSU rRNA trees for dinoflagellates, the relationships between *Oxyrrhis*, *Perkinsus*, dinoflagellates and apicomplexans were examined using three protein-coding genes: actin, alpha-tubulin and beta-tubulin; genes coding for all three proteins were determined from *Oxyrrhis*, and both alpha-tubulin and beta-tubulin sequences were acquired from *Perkinsus*. As only one alpha-tubulin, four actin- and two beta-tubulin sequences have been previously characterized from dinoflagellates, these three genes were amplified from several members of the group (actin from four species, alpha-tubulin from three, and beta-tubulin from six; Table 2.1.). In many species of dinoflagellates more than one copy of a particular gene was found. For example, at least two different copies of alpha-tubulin exist in *Amphidinium herdmannii*, two beta-tubulin genes in both *Heterocapsa triquetra* and *Perkinsus marinus*, and four distinct actin genes in *Karenia brevis*.

In contrast to the SSU rRNA gene, none of the three protein-coding genes sampled from *Oxyrrhis* were found to be particularly divergent. In all of the eukaryote-wide phylogenetic trees based on actin (Figure 2.3), alpha-tubulin (Figure 2.4), and beta-tubulin (Figure 2.5) both *Oxyrrhis* and *Perkinsus* join the alveolate clade, confirming their general taxonomic position within this group. However, unlike the SSU rRNA trees, the protein trees almost never show

Oxyrrhis branching within the dinoflagellates (in the weighted neighbor joining tree of actin, Figure 2.3, the dinoflagellate *Cryptothecodinium cohnii* branches below an *Oxyrrhis/Perkinsus* clade without bootstrap support); both maximum likelihood and distance trees based on alveolate-only sequences from all three proteins (Figures 2.7, 2.8 and 2.9) place *Oxyrrhis* and *Perkinsus* as sister to all dinoflagellates. In general, actin phylogenies (Figures 2.4 and 2.7) produce consistent and strong support for both *Oxyrrhis* and *Perkinsus* branching with the dinoflagellates, but little or no support for the node uniting dinoflagellates to the exclusion of *Oxyrrhis* and *Perkinsus*. Alpha-tubulin trees (Figures 2.5 and 2.8) are probably the most robust of the three protein-coding genes, and consistently show high support for both *Oxyrrhis* and *Perkinsus* branching as sisters to the dinoflagellates. In beta-tubulin phylogenies of alveolates, it has previously been shown that ciliates are paraphyletic (Fast et al. 2001), and the same is found here (Figures 2.6 and 2.9). Nevertheless, *Oxyrrhis* branches with the dinoflagellates with variable levels of support in different analyses, and *Perkinsus* also branches at the base of the dinoflagellates in analyses restricted to alveolates (in the large weighted neighbor joining tree, Figure 2.5, it also branches at the base of dinoflagellates, but as sister to a ciliate, *Stylonychia*). Moreover, in all beta-tubulin trees, the dinoflagellates form a strongly supported clade to the exclusion of both *Perkinsus* and *Oxyrrhis* (97% to 100% bootstrap support). Lastly, in trees based on concatenated actin, alpha-tubulin, and beta-tubulin sequences (Figure 2.9), there is strong support for a clade containing *Perkinsus*, *Oxyrrhis*, and the dinoflagellates (100% bootstrap support), but this data set could not address

whether either taxon branched within the dinoflagellates since only one dinoflagellate was represented.

The phylogenies described above consistently support the conclusion that both *Perkinsus* and *Oxyrrhis* diverged from common ancestors with the dinoflagellates, but the order in which *Perkinsus*, *Oxyrrhis*, and the true dinoflagellates evolved remains equivocal. Among the trees, examples can be found in which *Oxyrrhis* branches earlier than *Perkinsus* and the dinoflagellates (e.g. Figure 2.4), in which *Perkinsus* branches earlier than *Oxyrrhis* and the dinoflagellates (e. g. Figures 2.6 and 2.8), or even where *Perkinsus* and *Oxyrrhis* are sisters (e.g. Figures 2.3 and 2.7). In cases where *Perkinsus* and *Oxyrrhis* are sisters, there is little support for the node uniting them. Similarly, there is typically little support for the node separating them in other analyses, although trees placing *Perkinsus* deeper tend to have slightly higher support. The concatenated data set proved to be useful for addressing this question, since the relative branching order of *Perkinsus*, *Oxyrrhis*, and dinoflagellates could still be discerned from these trees even though only one dinoflagellate was represented. In this case (Figure 2.9) there is consistent and relatively high bootstrap support for *Perkinsus* branching first, with *Oxyrrhis* and the dinoflagellate *Heterocapsa* resolved as sister lineages.

The results obtained from the SSU rRNA phylogenetic trees are not congruent with those obtained from any of the protein-gene trees: whereas in SSU rRNA-based trees *Oxyrrhis marina* appears to have evolved from within the

Gonyaulacales, in all of the protein-based trees it branches as a sister taxon to the dinoflagellates. The highly divergent nature of the *Oxyrrhis marina* SSU rRNA sequences is likely causing them to branch artificially with the Gonyaulacales, because they too have divergent SSU rRNA genes compared with other dinoflagellates (e.g. Saunders et al. 1997). In contrast, the *Oxyrrhis* protein-coding gene sequences are generally no more or less divergent than the dinoflagellate homologues, and produce congruent phylogenetic trees that strongly support *Oxyrrhis* branching at the base of the dinoflagellates.

Figure 2.3. Phylogenetic tree constructed with weighted neighbor-joining from a gamma-corrected distance matrix of actin sequences (244 amino acids) from 85 phylogenetically diverse eukaryotic species. Bootstrap values based on weighted neighbor-joining (top) and Fitch-Margoliash (bottom) are shown above selected internodes. Alveolate groups and heterokonts are marked.

Figure 2.4. Phylogenetic tree constructed with weighted neighbor-joining from a gamma-corrected distance matrix of alpha tubulin sequences (384 amino acids) from 50 phylogenetically diverse eukaryotic species. Bootstrap values based on weighted neighbor-joining (top) and Fitch-Margoliash (bottom) are shown above selected internodes. Alveolate groups are marked.

Figure 2.5. Phylogenetic tree constructed with weighted neighbor-joining from a gamma-corrected matrix of beta-tubulin sequences (395 amino acids) from 56 phylogenetically diverse eukaryotic species. Bootstrap values based on weighted neighbor-joining (top) and Fitch-Margoliash (bottom) are shown above selected internodes. Alveolate groups are marked.

Figure 2.6. Gamma-corrected protein maximum-likelihood phylogenetic tree based on actin sequences (245 amino acids) from dinoflagellates, apicomplexans, *Perkinsus* and *Oxyrrhis*. Heterokonts are used as the outgroup. Bootstrap values based on protein maximum-likelihood (top), weighted neighbor-joining (centre) and Fitch-Margoliash (bottom) are shown above selected internodes.

Figure 2.7. Gamma-corrected protein maximum-likelihood phylogenetic tree based on alpha-tubulin sequences (405 amino acids) from alveolates. Bootstrap values based on protein maximum-likelihood (top), weighted neighbor-joining (centre) and Fitch-Margoliash (bottom) are shown above selected internodes.

Figure 2.8. Gamma-corrected protein maximum-likelihood phylogenetic tree based on beta-tubulin sequences (395 amino acids) from alveolates. Bootstrap values based on protein maximum likelihood (top), weighted neighbor-joining (centre) and Fitch-Margoliash (bottom) are shown at selected internodes.

Figure 2.9. Gamma-corrected protein maximum-likelihood tree based on concatenated actin, alpha-tubulin and beta-tubulin sequences (1023 amino acids) from alveolates. Bootstrap values based on protein maximum-likelihood (top), weighted neighbor-joining (centre) and Fitch-Margoliash (bottom) are shown above selected internodes.

CHAPTER 3: PROPOSING A PHYLOGENETIC FRAMEWORK FOR UNDERSTANDING THE EVOLUTIONARY HISTORY OF DINOFLAGELLATES

Figure 3.1 outlines a hypothesis on the evolutionary history of dinoflagellates and their close relatives. It is based on the features of molecular trees that are largely well supported and/or congruent with one-another, and on morphological and paleontological information.

3.1. *Perkinsus*, *Oxyrrhis* and the Syndiniales

The relative positions of *Perkinsus*, *Parvilucifera*, the dinokaryotic dinoflagellates and the apicomplexans are well supported by data from many different genes coding for both ribosomal RNAs and proteins (e.g. Reece et al. 1997, Norén et al. 1999, Fast et al. 2001). The relationship between *Colpodella* and the apicomplexans also seems to be relatively stable: even if the only type of molecular data in this case are limited to sequences from the SSU rRNA gene, the topologies that are recovered correlate well with morphological data (e.g. Kuvardina et al. 2002, Leander et al. 2003, Leander and Keeling 2003).

The phylogenetic position of *Oxyrrhis* has been more problematic. Phylogenies based on the SSU rRNA gene place it among the dinokaryotic dinoflagellates with 76-81% bootstrap support for an association with *Gonyaulax spinifera* (see Chapter 2). Protein-gene data give a very different

Figure 3.1. A 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 paleontological information.

result: all protein-gene phylogenies presented to date (actin, alpha- and beta tubulin as described above, but also HSP90, B. Leander, personal communication) place *Oxyrrhis* at the base of the dinoflagellates. Considering the highly divergent nature of the *Oxyrrhis* SSU rRNA sequence and the congruence in the protein-gene phylogenies (and also a short LSU fragment, Lenaers et al. 1991), it is more likely that *Oxyrrhis* is a sister taxon to the dinokaryotic dinoflagellates.

But where does the taxon branch with respect to *Perkinsus* and the syndinians? In many molecular phylogenies (e.g. Figures 2.6, 2.8, 2.9 and phylogenetic trees based on HSP90, B. Leander, personal communication), *Perkinsus* branches with good support at the base of an *Oxyrrhis*/Dinokaryota clade. One of the implications of this topology is that *Oxyrrhis* would then have had to experience a change (reversal?) in the position of its mitotic spindle: whereas apicomplexans, *Colpodella*, *Perkinsus*, and the dinokaryotic dinoflagellates all have external spindles (and open, semi-open or closed mitoses), *Oxyrrhis*, like the ciliates, has an internal one. Nevertheless, alternative positions of the species would imply assumptions of morphological changes that are much more improbable. For example, placing *Oxyrrhis* as a sister taxon to a *Perkinsus*/dinoflagellate clade, as a sister to the apicomplexans or as a sister to a dinoflagellate/apicomplexan clade would require a parallel loss of nucleosomal histones (*Oxyrrhis*, like dinokaryotic dinoflagellates, appears to lack nucleosomal histones, Li 1984), something clearly more unlikely. One important caveat to this, however, is that it is not known whether *Perkinsus* has nucleosomal histones or

not, even if ultrastructural features of their nuclei are consistent with them being present (see Chapter 4 for an in-depth discussion of this issue). All in all, because of the strength of the molecular data and the improbability that histones were lost more than once, it is likely that *Oxyrrhis* branches between *Perkinsus* and the dinokaryotic dinoflagellates.

Syndinians are a group that has been suspected in the past of being polyphyletic (e.g. Hollande 1974). The only molecular data that have been obtained for described members of the group are SSU rRNA sequences from two genera: *Amoebophrya* and *Hematodinium*, taxa classified by Fensome et al. (1993) in different families. No data are yet available for the morphologically most aberrant group of Syndiniales, the Duboscquellaceae. *Amoebophrya* and *Hematodinium* generally form a clade in phylogenetic trees (albeit always with weak support), but in a few analyses they can fall separately. Interestingly, the diversity of the order might be underestimated: many SSU rRNA sequences obtained from picoplanktonic environmental samples cluster with 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 couldn't be stated categorically that they were from syndinians. The addition of *Hematodinium* to the data set greatly strengthens this assumption, as this syndinian branches at the base of the clade that contains several strains of *Amoebophrya* and the picoplanktonic taxa. Nevertheless, the type species of the taxon, *Syndinium*, has yet to be included in phylogenetic analyses, so caution in this question is still warranted (syndinians may be polyphyletic). It is 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 that clade, it is impossible to say whether these sequences are from syndinians (or indeed dinoflagellates) or not. They always branch after *Perkinsus* and so they are members of the dinoflagellate lineage, but it is not possible to include them in any phylogenetic framework until their morphology is known.

The relative positions of the syndinians and *Oxyrrhis* can't be determined on the base of the available molecular data alone: only SSU rRNA sequences are known for syndinians, and the *Oxyrrhis* sequence for that gene is misleading (see above). Considering that *Perkinsus* branches at the base of the lineage, there are only three possible topologies of phylogenetic trees including dinokaryotes, syndinians and *Oxyrrhis*. 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, I (weakly) favour a topology where syndinians are sisters to a clade comprising *Oxyrrhis* and the dinokaryotic dinoflagellates. Nevertheless, much more data are needed to confirm this.

3.2. Noctilucales and Blastodiniales

In the most recent general classification of dinoflagellates (Fensome et al. 1993) Noctilucales and Blastodiniales are contained in 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 that stain brightly with alkali fast green, a chemical reagent that colors basic proteins (histones of typical eukaryotic nuclei are easily stained by it, dinokaryons are not). Nevertheless, these species have life stages with real dinokaryons: 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 1971, 1972). The dinokaryotic cells that are produced in this manner have the typical appearance of dinoflagellates; in *Noctiluca* they have been shown to function as gametes (Saito et al. 2002).

Molecular sequences (in this case only SSU rRNA) 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, although never with good bootstrap support (e.g. Figures 1.1, 1.2 and 1.3). However, in analyses with few outgroups and more aligned sites it always joins the GPP complex (Figure 1.4, no support).

The position of the taxon is thus highly unstable in SSU rRNA based phylogenetic trees. *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 banding pattern consistent with that of completely dinokaryotic dinoflagellates, not with eukaryotic, histone-containing nuclei (Li 1984). In other words, *Noctiluca* may lack typical core histones throughout its life cycle, suggesting that the alkali fast green stain in the trophont's nucleus is revealing other basic, non-core-histone proteins. As a consequence of these two observations, the basal position of *Noctiluca* (and by extension the rest of the Noctilucales) within the dinokaryotic dinoflagellates should be reexamined: the two main arguments for proposing such a basal position have been shown to be either equivocal (SSU rRNA-based phylogenetic analyses), or probably wrong (the ostensible presence of histones in the nuclei of feeding stages). Three morphological features of *Noctiluca* and other Noctilucales argue for a relationship of the order to at least some groups of gymnodinial dinoflagellates. First, young trophonts and/or dinospores of several of the less morphologically derived noctilucalean taxa (e.g. *Kofoedinium* and *Spatulodinium*) are practically indistinguishable from a number of athecate dinoflagellate genera, especially *Amphidinium* (Cachon and Cachon 1968). More importantly, *Noctiluca* shares two rare morphological features with members of the genus *Gymnodinium* sensu stricto (Daugbjerg et al. 2000). One is that *Gymnodinium* and the gametes of *Noctiluca* are the only dinoflagellates that have been shown to lack a transverse striated flagellar root (Hansen et al. 2000). Furthermore, the nuclear envelope of both *Gymnodinium* and the trophont of *Noctiluca* have peculiar chambers

(ampullae) in which the nuclear pores are situated (Afzelius 1963, Dodge and Crawford 1969, Soyer 1969). 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 they would provide an important morphological connection between the two groups. It is unknown whether other Noctilucales have ampullae around the nucleus, and also whether the “double wall” that exists around the nucleus of *Gyrodinium spirale* and some of its close relatives (e.g. Kofoed and Swezy 1921) is a structure related to those ampullae.

There are no morphological reasons to suspect that the order Noctilucales is polyphyletic, but the Blastodiniales almost certainly are (e.g. Chatton 1920, Fensome et al. 1993). *Amyloodinium* and *Haplozoon* never branch together in our trees, although both are always members of the GPP complex (in some trees *Amyloodinium* may branch at the base of the dinokaryotic dinoflagellates as a whole, e.g. Figure 1.1, but see also Figures 1.2, 1.3 and 1.4). Furthermore, although several members of the order have, like Noctilucales, non-dinokaryotic nuclei in some life stages (e.g. *Amyloodinium*, *Blastodinium*, *Caryotoma*, *Crepidodinium* and *Oodinium*, 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 *Haplozoon* and *Piscinoodinium* (trophonts in these last two genera are dinokaryotic, Siebert and West 1974, Lom and Schubert 1983, and dinospores have never been shown to have anything other than a dinokaryon in dinokaryotic dinoflagellates). Other genera are understudied, e.g.

Apodinium, *Cachonella* and *Sphaeripara*; the true phylogenetic affinities of these taxa are unclear.

The derived position of *Amyloodinium* in SSU rRNA 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 the order Peridiniales (Landsberg et al. 1994, Steidinger et al. 1996, Fensome et al. 1999). Could other Blastodinales also belong in the Peridiniales? This could be the case for taxa like *Oodinium* (like *Amyloodinium*, the trophont of *O. fritillariae* has thecal plates, but unlike it also ampullae around the nucleus, Cachon and Cachon 1977) and especially *Protoodinium*, where even the trophont has peridinialean tabulation (Cachon and Cachon 1971). Other Blastodinales seem to share more similarities with athecate dinoflagellates, e.g. *Crepidoodinium* and *Haplozoon*, both with many polygonal alveoli in surface view, and probably also *Piscinoodinium* (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 members of the Blastodinales (e.g. the small, polygonal alveoli) have been observed in their trophonts, an often heavily modified life stage. The morphology of their dinospores should be much more helpful in determining their true phylogenetic affinities, as shown by the example of *Amyloodinium ocellatum*. In summary, Blastodinales remain vastly understudied, and much more data are needed to establish their phylogenetic affinities. Nevertheless, the probable absence of histones in the nucleus of the *Noctiluca* trophont (and also in the non-dinokaryotic genus *Oxyrrhis*) could also extend to the blastodinialean stages without obvious

dinokaryotic chromosomes. If this is the case, proposals placing either order outside the dinokaryotic dinoflagellates are not warranted.

3.3. 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 different taxa at those basal positions, and bootstrap support is never strong. Nevertheless, there are tendencies that warrant comparison with the available morphological and paleontological data.

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 the plastids of haptophytes and peridinin- and 19-hexanoyloxyfucoxanthin-containing dinoflagellates (photosynthetic dinoflagellates contain different types of chloroplasts of which the peridinin type is by far the most common, see Chapter 4). They found, as expected, a strong phylogenetic relationship between haptophyte plastids and those of *Karenia* and *Karlodinium*, the two genera with 19-hexanoyloxyfucoxanthin-containing plastids (Tengs et al. 2000, Ishida and Green 2002). Surprisingly, they also found that in *psaA* and

psbA-based trees, peridinin-containing dinoflagellates group strongly either as a sister-taxon to *Karenia* and *Karlodinium* (combined psaA and psbA dataset), or embedded within a clade with 19-hexanoyloxyfucoxanthin-containing ancestors (psbA dataset). Based on these data they proposed an early tertiary endosymbiosis event for the dinoflagellate lineage, and a later transformation of that same plastid into the peridinin-containing type of the majority of the extant photosynthetic dinoflagellates.

The *Karenia/Karlodinium* clade is one of the groupings that does sometimes branch at the base of the dinokaryotic dinoflagellates in SSU rRNA-based phylogenetic trees (e.g. Figure 1.4). 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 Yoon et al.'s (2002) model for the origins of peridinin-containing plastids and the phylogenetic position of the 19-hexanoyloxyfucoxanthin-containing dinoflagellates. First, as is correctly noted in their paper, the divergence rate of all the dinoflagellate genes examined is noticeably greater than that of the rest of the taxa included in the analyses. As a consequence, a concern exists that the dinoflagellate sequences may be attracted to one-another not because of a real phylogenetic signal, but because they share long branches. The authors attempted to correct for this attraction, but nevertheless the concern remains. Furthermore, 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 comparable to those of their outgroups, unlike the plastid-encoded genes, which are unprecedentedly divergent and relatively phylogenetically uninformative in dinokaryotes (Zhang et al. 2000).

The genus *Heterocapsa* occupies a basal position within the Dinokaryota surprisingly often in phylogenetic trees, especially those based on LSU (maximum likelihood) and alpha-tubulin; in combined SSU/LSU trees *Heterocapsa* also consistently occupied such a position, albeit with 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 reasonable that this genus may have diverged before the split between those two orders.

The phylogenetic history of gymnodinial 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 possess tabulations that make them obvious members of other orders (e.g. Biecheler 1938 for *Gymnodinium*/*Cryptocodinium cohnii*, Hansen 1995 for *Katodinium*

rotundatum/Heterocapsa rotundata and Montresor et al. 1999 for *Polarella glacialis*, see also the Appendix for *Gymnodinium elongatum/Lessardia elongata*). These tabulations are difficult to discover using light microscopy alone, and so it is a virtual certainty that several (perhaps many) of the taxa that are currently classified in the 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 the phylogenetic history of the group. Furthermore, small, nonserially-arranged amphiesmal vesicles do not necessarily imply the absence of a theca, 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 only liquid (e.g. *Karlodinium micrum*, Leadbeater and Dodge 1966; *Amphidinium 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 the Gymnodiniales is phylogenetically informative remains unknown.

Molecular data always show a number of separate gymnodinialean clades originating from within the GPP complex, generally separated from thecate forms by weak bootstrap supports and not necessarily sisters to them. A more interesting question, however, is whether the Gymnodiniales *senso stricto* (i.e.

dinoflagellates with numerous small alveoli arranged non-serially) is polyphyletic or not, and whether the reason for the polyphyly of the Gymnodiniales *sensu lato* is only the fact that the group contains species with unrecognized non-gymnodinial tabulations. Molecular data seem to suggest that even Gymnodiniales *sensu stricto* is polyphyletic: well-studied taxa with small alveoli (e.g. *Amphidinium carterae*, *Karenia brevis*, *Gymnodinium fuscum*) never ally in phylogenetic trees.

The fact that in virtually all molecular trees gymnodinial species arise from within the GPP complex, separated from thecate taxa by 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 or only liquid would therefore represent intermediate stages of thecal loss. The alternative would be that the Gymnodiniales (or at least some of their subgroups) is the sister group to the other dinokaryotic dinoflagellates. This view is supported by the fact that the small alveoli of the 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, they cannot determine whether some Gymnodiniales are ancestral and others derived. Paleontology is not very helpful in this regard either: gymnodinial cysts are often 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, 1999); the earliest unequivocal gymnodinial fossils, skeletal

elements from Actiniscaceae and Dicroerismataceae, are from relatively recent Tertiary formations.

Paleontological data suggest an early origin for another order of dinokaryotic dinoflagellates, the Suessiales. They comprise organisms with alveoli arranged in seven to ten 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 (there are earlier fossils from the Silurian and Devonian that have been proposed as thecate dinoflagellates, but their identification as such is still controversial; the gonyaulacalean Shublikodiniaceae, like the earliest suessialean fossils, are from the mid-Triassic, Fensome et al. 1999). Molecular trees presently do not support any particular position for the Suessiales: although the group appears rarely 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 small-subunit 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. *Gymnodinium beii*, *Gymnodinium simplex*, *Gymnodinium corii* and *Woloszynskia*

pseudopalustris). Preliminary data show the presence of a thin theca in *Gymnodinium simplex* (FJR Taylor, unpublished data); whether this theca is arranged in a suessialean pattern, and whether this arrangement extends to the other species mentioned above remains to be determined.

In summary, the problem of the earliest-branching dinokaryotic dinoflagellates is far from being resolved: morphological and paleontological data could be interpreted as pointing towards gymnodinialean and suessialean taxa for these positions, whereas molecular data tend to indicate *Heterocapsa*. What is quite clear is that Gymnodiniales as currently circumscribed is a polyphyletic group; many gymnodinialean taxa appear to be more closely related to thecate forms than to other gymnodinialeans and thecal loss in dinoflagellates could have been a common event. 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 during that explosion.

3.4. 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 should be assigned 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 Peridinales). Small-subunit rRNA sequences exist for three dinoflagellate species formally classified in the Phytodinales: *Gloeodinium viscum*, *Halostylodinium arenarium* and *Hemidinium nasutum*. *H. nasutum* and the type species of *Gloeodinium*, *G. montanum*, have very similar coccoid stages, the two species have even been proposed to be conspecific (Popovsky 1971). A fourth species in our trees, *Glenodiniopsis steinii*, is currently classified in the Peridinales, but has a coccoid life stage strongly reminiscent of the *Gloeodinium*-like stage of *Hemidinium nasutum* (Popovsky and Pfister 1990). Given that the thecal patterns of the motile stages of these species are not known, it is premature to make the genera *Hemidinium* and *Gloeodinium* synonymous (similarities in the morphology of coccoid stages are common among different species), for that reason the name *Gloeodinium viscum* (and not "*Hemidinium viscum*") will be used in this work.

Halostylodinium arenarium groups with gonyaulacalean taxa in all phylogenetic trees examined, a placement that is congruent with most tabulational features of the species as interpreted by Horiguchi et al. (2000). *Hemidinium*, *Glenodiniopsis* 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 including *Gloeodinium viscum* and *Glenodiniopsis* sometimes occur, e.g. Figure 1.4, but a clade including

Hemidinium nasutum and *Gloeodinium viscum* was never recovered). This placement is congruent with the peridinialean tabulation of the motile stage of *Glenodiniopsis*, and suggests that once the tabulations of *Hemidinium nasutum* and *Gloeodinium viscum* are fully determined (only a partial tabulation is known for *Hemidinium nasutum*, no tabulational data exist 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.

3.5. Thecate dinoflagellates: Peridiniales, Gonyaulacales, Dinophysiales and Prorocentrales

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 (Fensome et al. 1993). Furthermore, paleontological and morphological evidence points to a close relationship between the Peridiniales, Dinophysiales and Prorocentrales. Paleontological data yielded very strong evidence linking the 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 and hyposomal features, but its epitheca has distinct peridinialean traits, very different from those of other Dinophysiales (Piel and Evitt 1980, Fensome et al. 1993). Within the Peridiniales, the groups with the most similarity to *Nannoceratopsis* are the fossil Comparodiniaceae and the extant Oxytoxaceae (Fensome et al. 1993).

Molecular data are lacking for *Oxytoxum* or its relatives, but a close relationship between Peridiniales and Dinophysiales is weakly apparent in molecular phylogenetic trees: in our trees Dinophysiales is 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 rRNA trees the order splits into at least 2 groups (Grzebyk et al. 1998). Despite this split, this morphologically very cohesive order is likely to be monophyletic, tabulation patterns within it are both homogenous and radically derived. Interestingly, at least some LSU trees (notably Weighbor) resolve the Prorocentrales as monophyletic. The phylogenetic origins of the group are more difficult to discern. The fact that Prorocentrales is a member 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, as well as the arrangement of the small platelets around the flagellar pores are common features of Prorocentrales and Dinophysiales; they may be closely related to each other (Taylor 1980, 1987). Nevertheless, no intermediate fossil forms exist to shed light on this hypothesis.

Thus, a relationship between the Peridiniales and the Dinophysiales/Prorocentrales on the one hand, and the Gonyaulacales on the other, is supported by available data. But were the first thecate dinoflagellates Peridiniales, Gonyaulacales or neither? The earliest dinoflagellate fossils are, except for controversial Silurian and Devonian forms, members of either the Suessiales or the thecate family Shublikodiniaceae (Fensome et al. 1999).

Fensome et al. (1993) point 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. In other words, the lines between the two orders blur at this level. Molecular data tend to yield trees in which a paraphyletic order Peridinales is ancestral to the monophyletic 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 Peridinales, genera like *Acanthodinium*, *Amphidoma*, *Cladopyxis* or *Palaeophalacroma* (Gonyaulacales), or *Glenodinium* (Peridinales). The genus *Heterocapsa* (Peridinales) 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 ribosomal RNA genes. The implication of this position would be that Peridinales is indeed ancestral to Gonyaulacales, a hypothesis 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 based on mediocre support at the relevant nodes, it is dangerous to assume that a lack of peridinialean fossils from the Triassic implies that the group was completely absent then.

One more dinoflagellate "order" appears to be closely related to Peridinales: the Thoracosphaerales. The principal life-stage of *Thoracosphaera*, the only genus in this monotypic 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 Peridinales 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 the species (Tangen et al. 1982). Molecular data tend to support a relationship between *Thoracosphaera* and several genera of Peridinales, including *Scrippsiella*. This position in molecular trees (as well as the calcareous cyst wall) would indicate 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 Peridinales and of the Calciodinelloideae.

If the scenario presented above is correct, the Peridinales would take an important placement in regard to the phylogeny of the dinoflagellates as a whole, it would be a paraphyletic order that gave rise not only to the other thecate taxa (Dinophysiales, Prorocentrales and possibly Gonyaulacales), but to many athecate and putatively athecate forms as well (many lineages of Gymnodiniales, Thoracosphaerales, as well as possibly Noctilucales and Blastodinales).

Whereas branching orders within Peridinales are not resolved in any of our trees, within Gonyaulacales the rate of evolution of both the large- and the small-

subunit ribosomal RNA genes is faster, and as a consequence branches of the resulting phylogenetic trees are longer and with better resolved lineages.

Felicitously, the Gonyaulacales is also a group with a good fossil record, and the tabulation patterns of extant and fossil members are well known. For these reasons, the group provides a good model to contrast taxonomic schemes based on morphology (i.e. tabulation) with those based on molecular data.

Two of the three gonyaulacalean suborders for which there are SSU rRNA sequences are normally recovered in phylogenetic trees: Goniodomineae (50-60% bootstrap support) 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 *Crypthecodinium* and *Thecadinium* (and the formally phytodinialean genus *Halostylodinium*). LSU gene trees (e.g. Figure 1.5) also generally support the monophyly of Goniodominae, although with weak bootstrap support and one important caveat: it always includes the sequence from *Ceratium furca* (SSU rRNA and especially morphological data very strongly suggest that this species is a Ceratiineae, it is likely that the LSU sequence data for this species represents either a misidentification or a laboratory error). The other Ceratiineae (i.e. the rest of the genus *Ceratium*) group strongly with each other, and *Gonyaulax* and *Protoceratium*, 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 *Amylax*, *Gonyaulax*

and *Lingulodinium* in the family Gonyaulacaceae, but rather with *Ceratocorys* (Ceratocoryaceae, 100 % bootstrap support).

One genus within the Gonyaulacales was studied in considerable detail, *Thecadinium*, a taxon that has had a very complicated taxonomic history (the results of that study, a collaboration with a German group, have been submitted for publication in Hoppenrath et al. 2004). Three of the seven members of the genus (one of them previously undescribed) were included in SSU rRNA phylogenetic trees, and that information was combined with detailed analyses of thecal plate arrangements in all members of the genus. We concluded that one species, *Thecadinium dragescoi*, is particularly divergent from all others and should be excluded from the genus. The other six species, all Gonyaulacales, form an extremely heterogeneous grouping, but we were not able to demonstrate a polyphyly that we suspect. For the sake of nomenclatural stability we decided to maintain the genus until more data are gathered. *Thecadinium dragescoi* was shown to be a close relative of the genus *Amphidiniopsis* (Peridinales), but was not formally transferred to that genus because a revision of *Amphidiniopsis* is imminent and we wanted to avoid two consecutive name changes for *T. dragescoi*.

3.6. Rates of evolution, the structure of dinoflagellate phylogenetic trees and the Mesozoic radiation

There is a striking asymmetry of evolutionary rates in the ribosomal genes of dinoflagellates, more pronounced in the small-subunit genes but also present in the domains of the large-subunit considered here. As a consequence, both SSU- and LSU-based phylogenetic trees for the group present a characteristic structure: a large group of very short-branched GPP species, and a clade with medium- to long-branched species. As far as these two groupings are concerned, the differences in evolutionary rate are certainly correlated with the phylogenetic history of the group: the Gonyaulacales contain only medium to long-branched species, and there are usually no Gonyaulacales elsewhere in the trees. Nevertheless, other medium- to long branched species that are not Gonyaulacales are present in the ribosomal gene phylogenies: *Amoebophrya*, *Haplozoon*, *Oxyrrhis* and *Protoperidinium* in SSU rRNA trees, and the *Amphidinium carterae* clade in both SSU- and LSU-based phylogenies. The fact that, with the exception of *Oxyrrhis* and in a few trees the *A. carterae* clade these long-branched taxa do not generally intrude in the Gonyaulacales is a sign that the grouping may be formed based on a real phylogenetic signal, not only because of long-branch attraction. It is interesting to note that none of the protein gene sequences for *Crypthecodinium cohnii* (one of very few gonyaulacalean genera for which protein genes are known) is 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; there appear to be few substitutions in the data set separating one well-supported clade from another. This is consistent with a rapid, early dinoflagellate radiation into the major forms we see today. The fossil record gives a similar picture (Fensome et al. 1999): although trace fossils with possible dinoflagellate affinities and dinoflagellate-like acritarchs exist from the Paleozoic, 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 dinoflagellates were already present. Early experimentation, later stabilization, and the early presence of "missing links" are all features of this Mesozoic radiation event (Fensome et al. 1999). Nevertheless, the dinoflagellate fossil record is heavily biased towards groups that produce fossilizable cysts (ca. 15% of extant species of dinoflagellates, Head 1996), other groups are poorly represented, and as a consequence it was unclear whether the rapid increase in gonyaulacalean and peridinialean morphological types in the early Jurassic was caused by a true radiation of the whole group. The congruence of the patterns suggested by the fossil record and the rRNA trees, which include non-cyst-formers, implies a general early radiation that included athecate forms.

This has important consequences for the interpretation of Palaeozoic fossils that have been postulated as having dinoflagellate origins, i.e. several acritarchs and a number of biogeochemical traces containing dinosteranes (Moldowan and Talyzina 1998). Among extant organisms, dinosterols (membrane

compounds that fossilize as dinosteranes) occur almost exclusively in dinoflagellates (Withers 1987). However, dinosteranes in the fossil record appear in important quantities during the late Proterozoic and the Paleozoic (Cambrian, Ordovician and Silurian), as well as during the Phanerozoic, with a significant lull between the Devonian and the Permian (Moldowan et al. 1996). The abundance profile of acritarch species in the Palaeozoic follows a similar profile, and so it has been proposed that palaeozoic dinosteranes were produced by dinoflagellate-like organisms, some of which fossilized as acritarchs (Moldowan and Talyzina 1998). The molecular data examined here suggest (but do not prove) that the dinosterane-producing organisms from the Palaeozoic were not dinokaryotic dinoflagellates, all the (extant) lineages of that group, including athecate forms, are apparently part of the same radiation event that gave rise to the Peridiniales and Gonyaulacales. Ancestors of dinokaryotic dinoflagellates, however, must have existed during the Palaeozoic, they could have been the source of those dinosteranes.

CHAPTER 4: ORIGIN AND EVOLUTION OF DINOFLAGELLATE FEATURES

Having proposed a putative framework for the phylogenetic history of dinoflagellates, the evolutionary history of some of the morphological features of the group is now considered.

4.1. The Nucleus

Dinokaryotic dinoflagellates have chromosomes that are always condensed, even in interphase; when examined with TEM they present a characteristic fibrillar ultrastructure (e.g. Dodge 1987). Their nuclei are also biochemically different from those of other eukaryotes: dinokaryotic nuclei 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, Sigeo 1986) and up to 70% of the thymine in their DNA is replaced by 5-hydroxymethyluracil (Rae 1976). They divide through a type of mitosis characteristic for the group: chromosomes are always 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.

Biochemical features of the nuclei of protalveolates have not been well studied, and so it is not yet possible to determine when in the evolutionary history

of dinoflagellates the thymine in DNA started to be replaced by 5-hydroxymethyluracil. On the other hand, the question of the presence or absence of histones in the dinoflagellate lineage has interested many researches over the years. The paradigm on the absence of typical histones in dinoflagellates 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, Wang et al. 2003), and to date there are three sequences available, from *Alexandrium fundyense*, *Cryptothecodinium cohnii* and *Lingulodinium polyedrum* (all Gonyaulacales). Homologies of these histone-like proteins of dinoflagellates (HLP's) to other proteins are not obvious, but Kasinsky et al. (2001) reported a 31% similarity in amino acid composition between the complete HCc2 of *Cryptothecodinium 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 an important feature in the study of the

phylogenetic questions of interest here, 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: dinokaryons do not stain with alkali fast green, whereas the nuclei of most eukaryotes, including syndinians, *Oxyrrhis* and the trophonts of taxa like *Noctiluca*, *Blastodinium* and *Oodinium* do (references in Table 4.1). The ultrastructure of those nuclei is also quite different to that of dinokaryons, 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 histone-like proteins, 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 *Solenodinium* and *Syndinium* the chromosomes are stained, whereas in *Amoebophrya* and *Duboscquella* only the nucleoli are. Unfortunately, of these four genera only *Amoebophrya* is represented in molecular based phylogenetic trees, so it is uncertain whether the order is really monophyletic (there is no real reason to suspect polyphyly). Regardless, the staining pattern in *Amoebophrya* and *Duboscquella* is more consistent with the

presence of histone-like proteins in these organisms rather than real histones. Ciliates and apicomplexans clearly have histones (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*, but their nuclei look more eukaryote-like than dinoflagellate-like in ultrastructural studies. This is a gap that needs to be filled by future research.

Table 4.1.

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)
<i>Colpodella</i>	No (Brugerolle 2002a)	?	Semiopen (Brugerolle 2002a)
<i>Acrocoelus</i>	No (Fernández et al. 1999)	?	?
<i>Perkinsus</i>	No (Perkins 1996)	?	Closed, with channels and external spindle (Perkins 1996)
<i>Parvilucifera</i>	No. Has an outer layer of fibrils around the chromatin in the zoospore nucleus (Norén et al. 1999)	?	?
<i>Rastrimonas</i>	No (Brugerolle 2002b)	?	Closed, external spindle and no channels (in anaphase nuclear envelope disappears in median zone, Brugerolle 2002b)

<i>Colponema</i>	No (Mignot and Brugerolle 1975)	?	?
<i>Syndinium</i>	No.Chromatin masses that do not correspond in number to chromosomes (Ris and Kubai 1974, Soyer 1974)	Staining: Yes (Also in <i>Solenodinium</i> . In <i>Amoebophrya</i> and <i>Duboscquella</i> only the nucleoli stain, Hollande 1974) Histones: ?	Closed, one channel (Ris and Kubai 1974)
<i>Oxyrrhis</i>	Yes, but not banded as in typical dinokaryons	Staining: Yes Histones: Probably not (Li 1984, Kato et al. 1997)	Closed, intranuclear spindle (Triemer 1982)
<i>Noctiluca</i>	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)
<i>Blastodinium</i>	TROPHONT: No ZOOSPORE: Yes (Hardly any interphase during sporulation) (Soyer 1971)	TROPHONT: Staining: Yes Histones: ? ZOOSPORE: No (Soyer 1971)	Closed, channels (Soyer 1971)
<i>Oodinium</i>	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 (Dodge 1987)

4.2. Mitosis

The ancestral type of mitosis for alveolates is difficult to determine. Cryptomonads, haptophytes and most heterokonts, including the early-branching labyrinthulids, have an open (sometimes semi-open) mitosis, but oomycetes, another early branch of the heterokonts (e.g. Cavalier-Smith and Chao 1996), have a closed one (Raikov 1994). 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, Raikov 1994). The dinoflagellate lineage, however, is very consistent in this respect (Table 4.1): *Perkinsus*, the syndinians, *Oxyrrhis* and the dinokaryotic dinoflagellates all have a closed mitosis, mostly with an external spindle (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* has not been described). So, although a closed mitosis could have originated early in the evolutionary history of alveolates, prior to the divergence of the ciliates (Figure 3.1), the mitotic channels probably originated at the base of the dinoflagellate lineage. The external spindle probably originated prior to the divergence of the apicomplexans. The only way to explain the state of these characters in *Oxyrrhis* while taking into account the molecular data on the phylogenetic position of the genus 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 MTOC's (microtubule organizing centers). 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 MTOC's 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.

4.3. Plastids and Photosynthesis

4.3.1. The photosynthetic ancestry of dinoflagellates

On the face of it, the presence of photosynthetic organelles in roughly half of the species of dinoflagellates (Taylor 1987) is quite an aberration: none of the dinoflagellates' close relatives are photosynthetic. Furthermore, the type of plastids that exist in the different lineages of photosynthetic dinoflagellates can be extremely different from one-another (e.g. Schnepf and Elbrächter 1992, 1999): 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.

Photosynthetic dinoflagellates are usually mixotrophic (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). The first clues to this arose when a plastid remnant was found in apicomplexans, the closest relatives to dinoflagellates (Wilson et al. 1991). Since then it has been shown that both the apicomplexan and the dinoflagellate peridinin plastid are derived from a red algal endosymbiont (McFadden and Waller 1997, Zhang et al. 2000). Phylogenetic trees based on plastid genes from both dinoflagellates and apicomplexans tend to cluster the plastid genes from the two groups. However, these plastid genomes are extremely derived, and long branches cannot be excluded as an explanation for their association in molecular trees (Takishita and Uchida 1999, Zhang et al. 2000). Fast et al. (2001) used nuclear encoded, plastid-targeted genes with more conclusive results: they argued that there has 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-targeted 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.

4.3.2. Plastid loss and replacement in dinoflagellates

With the exception of *Amoebophrya*, *Hematodinium* and in many trees also *Noctiluca*, all non-photosynthetic dinoflagellates in the trees (*Amphidinium semilunatum*, *A. longum*, *Amyloodinium*, *Crypthecodinium*, *Haplozoon*, *Lessardia*, *Pfiesteria*, *Protoperidinium*, *Roscoffia*, and *Thecadinium dragescoi*) were generally scattered among the photosynthetic lineages (exceptions are *Haplozoon axiothellae* in a few uncorrected ML trees, *Amphidinium semilunatum* in many ML trees, and the *Pfiesteria*/*Amyloodinium* clade in some BioNJ and Weighbor trees, e.g. Figure 1.1). In Kishino-Hasegawa tests, alternative trees where each individual non-photosynthetic species (or well-supported group of exclusively non-photosynthetic species) was placed between the syndiniales and the rest of the dinoflagellates were generally not rejected at the 5% confidence level (the exception being *A. longum*). However, Kishino-Hasegawa tests did resoundingly reject alternative trees where all non-photosynthetic dinoflagellates are grouped together (with or without *Amoebophrya* and *Noctiluca*), irrespective of their position in the trees. Because a close relationship between all non-photosynthetic dinoflagellates is rejected by the phylogenies and the Kishino-Hasegawa tests, at least some non-photosynthetic dinoflagellates must have originated after the latest possible common ancestor of all peridinin-containing dinoflagellates, making plastid losses within the group a virtual certainty.

While SSU rRNA phylogeny does support plastid loss in *Amphidinium semilunatum*, *A. longum*, *Amyloodinium*, *Crypthecodinium*, *Haplozoon*, *Lessardia*, *Pfiesteria*, *Protoberidinium*, *Roscoffia* and *Thecadinium dragescoi*, (and in some trees also in *Noctiluca*), it is not sufficiently firmly resolved to be compelling in the absence of additional data. In the case of *Noctiluca*, the instability of the taxon in phylogenetic trees and its lack of obvious close relatives except for other Noctilucales makes it difficult to make strong statements about whether it experienced plastid loss or not. Fortunately, for many other taxa there are clear morphological signs of their evolutionary origin. *Lessardia*, *Protoberidinium*, *Roscoffia* and *Thecadinium dragescoi* are, for example, clearly Peridinales (Fensome et al. 1993, Hoppenrath et al. 2003, see also appendix), a group that includes many photosynthetic forms. Therefore at least one (possibly more) instance of plastid loss is likely to have occurred within the group. *Crypthecodinium cohnii* has a gonyaulacoid tabulation, although somewhat atypical (Fensome et al. 1993). In some molecular studies, this species branched conspicuously early (e.g. Litaker et al. 1999), but in the majority of our trees, *Crypthecodinium* appears to be related to the Gonyaulacales, a placement consistent with its tabulation. It is thus likely that this species is secondarily heterotrophic and that its anomalous position in previously published trees was an artifact of its long branch coupled with sparse taxon sampling.

Amphidinium semilunatum is likely to be an athecate dinoflagellate (*A. longum* is probably not, personal observations on SEM). In spite of the fact that in SSU rRNA phylogenetic trees the Gymnodinales never form a monophyletic

group, all members of the order do branch after the syndiniales, usually scattered among thecate, photosynthetic forms. This scattering suggests that the non-photosynthetic members of the order probably had photosynthetic ancestors. The position of *Amphidinium semilunatum* within the photosynthetic dinoflagellates is not very stable (see for example Figure 1.3), but there are no morphological reasons to consider it to be particularly early-diverging. The case for plastid loss in *A. longum* is much stronger, since alternative trees with this species diverging before the latest possible common ancestor of peridinin-containing dinoflagellates were rejected by the Kishino-Hasegawa test.

Haplozoon axiothellae is a very unusual, non-photosynthetic, multicellular, parasitic dinoflagellate (Shumway 1924, Siebert and West 1974, Leander et al. 2002), and its phylogenetic position has never been clear. No position of *Haplozoon* is strongly supported by SSU rRNA phylogeny, this organism can be placed essentially anywhere within dinokaryotes without causing the resulting tree to be rejected by the Kishino-Hasegawa test. Nevertheless, altogether it seems most likely that *Haplozoon* is probably descended from photosynthetic ancestors. The position of the branch that includes *Amyloodinium* and *Pfiesteria* is also uncertain, but since those two genera have motile stages with unquestionably peridinialean tabulation (Landsberg et al. 1994; Steidinger et al. 1996; Fensome et al. 1999) it too is most likely that they are secondarily heterotrophic, as most of the trees weakly suggest.

Several groups of dinoflagellates contain plastids that differ in pigmentation from the typical peridinin plastids. Small-subunit-based trees contain four dinoflagellate taxa with true aberrant plastids: *Lepidodinium viride*, the *Kryptoperidinium foliaceum*/*Durinskia baltica* clade, the 19'-hexanoyloxyfucoxanthin group (*Karenia* and *Karlodinium*) and the genus *Dinophysis*. Trees based on large-subunit data also contain *Gymnodinium chlorophorum*. All of these typically branch after the latest possible common ancestor of peridinin-containing dinoflagellates (exceptions are many ML trees where either the 19-hexanoyloxyfucoxanthin group or *Kryptoperidinium foliaceum* fall between *Amoebophrya/Noctiluca* and the rest of the dinoflagellates, e.g. Figure 1.3, and one Fitch tree where *Durinskia* occupied that position). Alternative trees with all aberrantly-pigmented dinoflagellates, or *Dinophysis* or *Lepidodinium* alone placed in basal positions were rejected by Kishino-Hasegawa tests at the 5% confidence levels; trees with *Kryptoperidinium/Durinskia* or the 19'-hexanoyloxyfucoxanthin group in those positions were not. Nevertheless, morphological features in the aberrantly-pigmented dinoflagellates make it unlikely that they arose prior to the peridinin-containing plastid: *Lepidodinium*, like *Gymnodinium chlorophorum*, is similar to several peridinin-containing members of the genus *Gymnodinium*, and *Kryptoperidinium* and *Durinskia* have peridinialean tabulations, albeit somewhat atypical. The case for the 19-hexanoyloxyfucoxanthin group is weaker, since there are no obvious morphological features linking them to another dinoflagellate taxon. However, the (weakly supported) group that contains them also includes a peridinin-containing species (*Amphidinium herdmanii*). It is thus likely that all dinoflagellates with

aberrant plastids had peridinin-containing ancestors, and that they all replaced one type of plastid for another.

The degree to which new plastids are integrated varies greatly. The replacement process can be thought to be "in progress" in *Kryptoperidinium foliaceum* and in *Durinskia baltica*, both organisms with a raphid pennate diatom endosymbiont (Chesnick et al. 1997). In both cases, as well as in *Peridinium quinquecorne* (Horiguchi and Pienaar 1991) the endosymbiont appears to be relatively complete, having a nucleus, mitochondria and other organelles but lacking a cell wall or obvious mitotic spindle (Dodge 1983). They also carry a probable remnant of the old peridinin-containing plastid in the form of an eyespot surrounded by three membranes (Jeffrey and Vesk 1976, Horiguchi and Pienaar 1991, Schnepf and Elbrächter 1999). In the other three replacement instances discussed here, the plastids themselves are all that remains of the endosymbiont: *Lepidodinium viride* and *Gymnodinium chlorophorum* contain green plastids of probable prasinophyte origin with chlorophyll a and b (Schnepf and Elbrächter 1999, it is unclear whether the two species represent one or two endosymbiosis events), the 19'-hexanoyloxyfucoxanthin-containing species carry plastids derived from haptophytes (Tengs et al. 2000), and *Dinophysis* has phycobilin-containing plastids derived from cryptomonads (Hackett et al. 2003).

Saunders et al. (1997) found a non-photosynthetic species (*Polykrykos schwartzii*) as a sister to *Karenia mikimotoi* (100% bootstrap support, unpublished SSU rRNA sequence). If this position is correct, then haptophyte-containing

dinoflagellates may have had non-photosynthetic ancestors. This would imply a replacement of peridinin-containing plastids by haptophyte-derived plastids through non-photosynthetic intermediate stages, a situation possibly very different from the replacement process in *Kryptoperidinium* and *Durinskia* if their eyespot is indeed a remnant of the old plastid.

In summary, photosynthetic forms only appear relatively late in the evolutionary history of dinoflagellates, early-branching taxa of the lineage (i.e. *Perkinsus*, *Parvilucifera*, the syndinians and *Oxyrrhis*) are all non-photosynthetic. That all peridinin-containing dinoflagellates must have a common ancestor is beyond doubt, peridinin probably originated only once (Saunders et al. 1997). 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 plastid loss. Using this 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.

Plastid replacement differs fundamentally from secondary symbiogenesis in that it probably occurs by the recruitment of preexisting 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 dinoflagellates 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.

4.4. Flagella and the definition of dinoflagellates

The definition for dinoflagellates used by Fensome et al. (1993) is based on flagellar characters. The transverse flagellum of dinoflagellates is very distinctive 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 has a wavy appearance. In addition to these ultrastructural features, the fact that both flagella insert laterally is a 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* may be an exception, Appleton and Vickermann 1997). The same is true for at least some species of *Colpodella*, a sister taxon to the apicomplexans (B. Leander, personal communication, 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 ancestral 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.

A paraxial rod (striated strand) 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 species, *Amoebophrya*, it has not been seen in the apicomplexan lineage, *Perkinsus* or *Parvilucifera* (references in Table 4, a vestigial paraxonemal structure does exist at the base of the transversal flagellum of at least some species of *Colpodella*, Brugerolle 2002a). However, the ultrastructures of the paraxial rod/striated strand of *Oxyrrhis* and the dinokaryotic dinoflagellates are different, 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. This is found in several members of the dinoflagellate lineage, e.g. in *Parvilucifera* as well as in some dinokaryotic dinoflagellates, especially Gonyaulacales (Leadbeater and Dodge 1967, Maruyama 1982, Norén et al. 1999). Additional features of the flagellar apparatus 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), but data are still scarce and comprehensive analyses of their evolutionary history seem premature.

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 Blastodinales) but in *Oxyrrhis* and possibly syndinians as well. If the phylogenetic framework presented here turns out to be correct, this feature could be added to the flagellar definition of the dinoflagellate taxon.

Table 4.2.

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. 2000

<i>Colpodella</i>	Subapical	<i>C. vorax</i> : Paraxonemal structure in the proximal portion. No mastigonemes reported (Brugerolle 2002a). <i>C. edax</i> : Mastigonemes present (Leander et al. 2003)	Unremarkable. No mastigonemes.	Brugerolle 2002a, Leander et al. 2003
<i>Acrocoelus</i>	Ventral	Both flagella are posteriorly directed. No mastigonemes.	Unremarkable. No mastigonemes.	Fernández et al. 1999
<i>Perkinsus</i>	Subapical	Only in zoospore. Filamentous mastigonemes present along one side forming tufts, spur-like unit at the base of each tuft. No mention of a paraxial rod.	Only in zoospore. Unremarkable. No mastigonemes.	Perkins 1996
<i>Parvilucifera</i>	Subapical	Only in zoospore. Short mastigonemes on one side, long, thin hairs on the other. No paraxial rod.	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
<i>Rastrimonas</i>	Subapical	Anterior flagellum shorter. Mastigonemes "have not been satisfactorily demonstrated"	Longer. Terminates in a thin filament.	Brugerolle 2002b
<i>Colponema</i>	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
Syndinians	Ventral	No mention of mastigonemes in <i>Hematodinium</i> , mastigonemes present in <i>Amoebophrya</i> .	"Rudder-like" in <i>Hematodinium</i> , no mastigonemes in either <i>Hematodinium</i> or <i>Amoebophrya</i> .	Appleton and Vickerman 1998, W. Coats, pers. comm.
<i>Oxyrrhis</i>	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

Dinokaryotes	Ventral	Single row of fine hairs, contains a striated strand	Usually smooth. A paraxial rod (striated strand) and/or diffuse paraflagellar material can be present.	Leadbeater and Dodge 1967, Maruyama 1982
--------------	---------	--	--	--

CONCLUDING REMARKS

The molecular data that are currently available for dinoflagellates have not fully clarified the internal relationships of the taxa that compose the taxon; even some of the most fundamental questions on the branching orders within this group have remained unresolved. Nevertheless, important insights have been obtained through the present work. They are:

1. Molecular and palaeontological data sets suggest that dinoflagellates experienced an explosive radiation of forms. Palaeontological data provide a timing for this event, the early Mesozoic, but the molecular data examined here show that it probably involved all groups of dinokaryotic dinoflagellates, not only the ones that produce fossilizable cysts.
2. Dinoflagellates have experienced repeated instances of plastid loss and at least four instances of plastid replacement. No other group of eukaryotes shows this level of trophic adaptability.
3. *Oxyrrhis marina* is unlikely to have had dinokaryotic ancestors. Its lineage probably arose prior to that of all dinokaryotic dinoflagellates, almost certainly after the *Perkinsus* lineage and possibly also after the syndinians.
4. Histones were probably lost in the dinoflagellate lineage earlier than previously assumed, after the divergence of the *Perkinsus* lineage but

before the appearance of the syndinians. It is, however, very important that the histone profiles from *Oxyrrhis* and *Noctiluca* be confirmed, and that those for *Perkinsus* and the syndinians be determined.

5. The order Peridiniales is likely paraphyletic, occupying a central position in the evolution of dinokaryotic dinoflagellates. It probably gave rise to many other dinoflagellate orders, including the Dinophysiales, Prorocentrales, many lineages of Gymnodiniales and Blastodiniales, and possibly also the Gonyaulacales.
6. The order Gonyaulacales is monophyletic and distinct from the Peridiniales. Morphological and molecular data agree on the composition of a great majority of its constituent subgroups.

APPENDIX: *LESSARDIA ELONGATA* AND THE TAXONOMIC POSITION OF THE GENUS *ROSCOFFIA*

A.1. INTRODUCTION

As discussed above, genera of athecate dinoflagellates have been suspected for many years to be polyphyletic, and the boundaries between them are widely understood to be arbitrary. In spite of this, genera like *Gymnodinium*, *Gyrodinium*, *Amphidinium* and *Katodinium* continue to be used, mainly because insufficient data are available for meaningful revisions. There is now a concerted effort to use ultrastructural and molecular data to clarify the phylogenetic relationships between these organisms and to classify them accordingly (e.g. Daugbjerg et al. 2000). As first steps toward that end, the type species of some of the larger genera are being investigated thoroughly (e.g. *Gymnodinium fuscum*, Hansen et al. 2000) and the phylogenetic relationship of some of the other members of those genera to the type species is being reassessed (Daugbjerg et al. 2000, see also the preceeding chapters). As a consequence, several new genera of naked dinoflagellates have been recently established, e.g. *Akashiwo*, *Karenia* and *Karlodinium* (Daugbjerg et al. 2000). Nevertheless, large genera like *Gymnodinium* still remain polyphyletic assemblages that contain many poorly studied, ostensibly naked species (e.g. Saunders et al. 1997, Chapter 3).

In addition to the naked forms, gymnodinoid taxa have also historically contained cryptically thecate forms that had not been recognized as such. This was shown to be the case, for example, in *Katodinium rotundatum*, a thecate species recently reclassified to the peridinialean genus *Heterocapsa* (Hansen 1995), and in the genus *Pfiesteria*, a taxon that appears athecate under the light microscope but that has been shown to contain a clear thecal plate pattern (Landsberg et al. 1994, Fensome et al. 1999). Here, an organism is investigated that closely resembles "*Gymnodinium elongatum*" as depicted by Hope (1954). It contains thin thecal plates in a pattern consistent with the order Peridiniales.

Birkenes (1941) and Braarud (1945) noted an elongated, non-photosynthetic dinoflagellate during surveys of the phytoplankton of the Oslo Fjord, Norway, and recorded it as either "*Gymnodinium 1*" or "*Gymnodinium elongatum*" (Braarud 1945, Table 17, page 73). Brigit Hope (1954) named this same species (references were given to Birkenes' and Braarud's work) more formally as *Gymnodinium elongatum*, but provided neither a description nor a diagnosis for it, only two small drawings with little detail and no scale bar or other indication of size. This does not satisfy the requirements valid at the time for publication of a new name under either the ICBN or the ICZN (see discussion). A dinoflagellate species very similar to the one shown in Hope (1954) has been recorded since then from the Danish coasts of the Skagerrak and Kattegat (Hansen and Larsen 1992), from several locations in the NW Atlantic (Georges Bank, Baffin Bay; E. Lessard and C. Lovejoy, personal communications; Gulf of Maine, Shapiro et al. 1989) and from the NE Pacific (Oregon Coast, Sherr and

Sherr 2002; Bering Sea, E. Lessard, personal communication; Gulf of Alaska, Shapiro et al. 1989). It has usually been designated as *Gymnodinium elongatum* Hope (e.g. Hansen and Larsen 1992). The species has also been shown to fluoresce green (wavelength ca. 535 nm) after excitation with blue light (ca. 460 nm, Shapiro et al. 1989), and has been used as a model for carbon to volume relationships in heterotrophic dinoflagellates (as *Bernardinium* sp. in Menden-Deuer and Lessard 2000).

The organism investigated here is probably conspecific with that "*Gymnodinium*". Scanning electron microscopy and calcofluor white staining were used to observe and elucidate a delicate thecal pattern that is very similar to that of the peridinialean family Podolampaceae. A similar thecal plate pattern is also present in the genus *Roscoffia*, a taxon of uncertain taxonomic position that also has a thecal plate pattern reminiscent of the Podolampaceae (Horiguchi and Kubo 1997, Hoppenrath and Elbraechter 1998). In order to test a putative relationship between this "*Gymnodinium elongatum*" (or *Lessardia elongata*, as the species is now called) and the genus *Roscoffia* as represented by the sand-dwelling, marine *Roscoffia capitata*, the small-subunit (SSU) ribosomal RNA gene of both organisms was sequenced and phylogenetic trees inferred.

A.2. MATERIALS AND METHODS

A.2.1. Organisms and Culture Conditions

Lessardia elongata was collected in August 1991 in Georges Bank (NW Atlantic, off the coast of Massachusetts, USA) by Dr. Evelyn Lessard (University of Washington, Seattle, USA) using a flow cytometer sorting on green fluorescence; it has been kept in culture at her laboratory since then. The cultures are grown at 16-18°C in 30 psu saltwater medium, enriched with f/2 vitamins and f/200 trace metals. They are fed once a week with the cryptomonad *Rhodomonas lens* at a concentration of ca. 4000 *Rhodomonas* cells•mL⁻¹ of *Lessardia* culture. A culture of *Lessardia elongata* derived from Dr. Lessard's collection now also exists at the Canadian Centre for the Culture of Microorganisms (CCCM 865) at the University of British Columbia, Vancouver. *Roscoffia capitata* Balech was isolated by Mona Hoppenrath (Wattenmeerstation Sylt) from the intertidal sand flats of the island of Sylt, Germany. Approximately 50 cells were micropipetted from their environment and washed repeatedly in filtered seawater.

A.2.2. Light Microscopy

Cells were observed under a cover slip fixed in place with "VALAP" (equal parts of vaseline, lanolin, and paraffin wax, Kuznetsov et al. 1992). Light micrographs were produced with a Zeiss Axioplan 2 Imaging microscope

connected to a Q-Imaging, Microimager II, black and white digital camera. For plate pattern identification, cells were stained with calcofluor white (Fritz and Triemer 1985) and observed with ultraviolet light.

A.2.3. Scanning Electron Microscopy

A small volume (10 ml) of cells in seawater medium was transferred into a small Petri dish that contained a piece of filter paper, saturated with 4% OsO₄, mounted on the inner surface of the lid. The lid was placed over the chamber and the cells were fixed by OsO₄ vapors for 30 min. Six drops of both 8% glutaraldehyde and 4% OsO₄ were added directly to the seawater and the cells were fixed for an additional 30 min. Cells were transferred onto an 8 µm polycarbonate membrane filter (Corning Separations Div., Acton, MA), dehydrated with a graded series of ethyl alcohol, and critical point dried with CO₂. Filters were mounted on stubs, sputter coated with gold, and viewed under a Hitachi S4700 Scanning Electron Microscope. Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

A.2.4. Transmission electron microscopy

Cells were concentrated into Eppendorf tubes and fixed in 2% glutaraldehyde, 0.1 M cacodylate buffer (pH = 7.2), and 250 mM sucrose at 4 °C

for 1 h. Pelleted cells were washed twice in the buffer (with added sucrose) for 15 minutes and post-fixed with 1% OsO₄ at 4 °C for 1 h. Pellets were washed with distilled water, dehydrated with a graded series of ethyl alcohol, bathed twice with acetone, infiltrated with acetone-resin mixtures, and embedded with pure Epon resin. Blocks were polymerized at 60°C and sectioned on a Leica UltracutT Ultramicrotome. Ultrathin sections were post-stained with uranyl acetate and lead citrate and viewed under a Hitachi H7600 Transmission Electron Microscope. All SEM and TEM work described in this chapter was done in close collaboration with Dr. Brian Leander.

A.2.5. Molecular Phylogenetic Analysis

The SSU genes of *Lessardia elongata* and *Roscoffia capitata* were sequenced and analysed as described in section 1.2.2.

A.3. TAXON DESCRIPTIONS

A.3.1. Description of *Lessardia* Saldarriaga et Taylor

Aphotosynthetica thecata dinoflagellata cum cingulo planissimo. Sulcus planus. Dexter antapicalis discus cum spina.

Non-photosynthetic, thecate dinoflagellate with a weakly impressed cingulum. Sulcus not impressed. The right antapical plate carries a spine.

Eymology: The genus is named after the provider of the culture, Dr. Evelyn Lessard, who has made important contributions to the understanding of the ecology of heterotrophic dinoflagellates.

Type Species: *Lessardia elongata* Saldarriaga et Taylor

A.3.2. Description of *Lessardia elongata* Saldarriaga et Taylor

Biconica dinoflagellata, epitheca exigue maior quam hypotheca a qua separata est cingulo que quod locatum posterius aequatore cellae. Cingulus non tortum, sulcus planus. Thecati disci levi plerumque sed transiti paucis trichocystis apertionibus. Formula disci Po Pi CP 3' 1-2A 5'' 3C 6S 4''' 3'''''. Dexter antapicalis discus (3''''') cum spina. Apicalis pori structura habens conicale caput cum 6 depressis in disco pori et cum canale alto ad latum ventralem quod tangit longum angustum primum apicalem discum.

Biconical dinoflagellate, epitheca slightly larger than the hypotheca, separated from it by a cingulum that is located posteriorly from the cell equator. No cingular displacement, sulcus flat. Thecal plates generally smooth but traversed by a few trichocyst openings. Plate formula Po Pi CP 3' 1-2A 5'' 3C 6S 4''' 3'''''. Right

antapical plate (3'') with a spine. Apical pore complex in the form of a conical cap with 6 indentations on the pore plate and a deep groove towards the ventral side that contacts the long, narrow first apical plate.

Holotype: The block for transmission electron microscopy Le-1 is hereby designated as the *typus* for *Lessardia elongata* Saldarriaga et Taylor. It is deposited at the Herbarium of the University of British Columbia (UBC) in Vancouver, Canada.

Iconotype: Figure 5.4: a-f

Type Locality: Georges Bank, NW Atlantic Ocean.

Habitat: Marine.

Distribution: The organism has been reported as a planktonic species in the Northern Atlantic and Pacific Oceans: the Norwegian coast, Skagerrak, Kattegat (Denmark/Scandinavia), Baffin Bay (Canada/Greenland), Georges Bank (off Massachusetts, USA), the Oregon Coast, the Gulf of Alaska and the Bering Sea.

Etymology for the specific epithet: Refers to the elongated shape of the cell

A.4. RESULTS

A.4.1. Morphological examination

Live *Lessardia elongata* are 20-32 μm long (mean: 27.9 ± 2.19 , $n=100$) and 7-14 μm wide at the cingulum (mean: 10.1 ± 1.46 , $n=100$), but they shrink by up to 30% in fixatives like lugol or glutaraldehyde (E. Lessard, personal communication). Cells are transparent and lack chloroplasts (Figures A.1a-c, A.1g), recently ingested prey can often be seen in the antapical half of the cell within very conspicuous vacuoles (e.g. Figure A.1b,g, A.2i). The nucleus is situated in the apical half of the cell (Figure A.1c, A.1g), and contains typically dinokaryotic chromosomes. The cell fluoresces green when excited with blue light of ca. 460 nm wavelength (not shown) and shows distinct thecal plates when stained with calcofluor white (Figures A.1d, A.1e). Cells divide through desmoschisis (not shown).

Examination with SEM revealed two flagella with characteristics typical of dinoflagellates (Gaines and Taylor 1985, Figures A.1f, A.2a) and a structure at the insertion point of the flagella that could be a peduncle (Figure A.2a). Under the cell membrane lie smooth, undecorated thecal plates (Figure A.2j) arranged in a pattern described by the formula $\text{Po Pi CP } 3' \text{ } 1\text{-}2\text{A } 5'' \text{ } 3\text{C } 6\text{S } 4''' \text{ } 3''''$ (Figures A.1d-e, A.3a-d, A.4a-f) and containing relatively few trichocyst openings (Figures A.2b-c, A.2f, A.3a-d). The apical pore complex appears as a small horseshoe-shaped cap with six indentations on the pore plate (Pi), a conical cover plate that was

seen to fall off in a few occasions (Po), and a deep mid-ventral groove subtended by a canal plate (CP, Figures A.3e, A.3f). The first apical plate and the anterior sulcal plates are both extremely long and narrow, they connect the apical pore complex to the sulcal region (Figures A.3a, A.4a, A.4e); the other two apical plates are much broader (Figures A.3a-d, A.4a-e). At least one small anterior intercalary plate is always present (dorsal-right side, Figures A.3c, A.4c, A.4e); on one occasion a second, much larger one was also seen (see dotted lines in Figures A.4c-e; in specimens with just one anterior intercalary plate, this region is covered by a lobe of plate 2"). There are five precingular plates, generally similar in size. The cingulum is ca. 3 μ m wide, very weakly impressed and shows no displacement; it is composed of three rectangular plates that are continuous with a very large right sulcal plate that reaches into the hyposome. Six plates make up the sulcal region (Figures A.2b, A.2c). The large right sulcal (Sr) and the narrow anterior sulcal (Sa) plates were mentioned above, neither of these lies entirely within the sulcus. The same is true for the posterior sulcal plate (Sp), located further antapically and next to two of the antapical plates. Other sulcal plates include a small plate bordered by the right, anterior and posterior sulcal plates and by the flagellar pore (Srp), the median sulcal plate (Sm), surrounding the flagellar pore on three sides and carrying a conspicuous bulge (Figures A.2b, A.2c); and a relatively large left sulcal plate (Ss), separating the median sulcal from both the cingulum and the postcingular series on the left side. The four postcingular plates are roughly similar in shape and size (Figures A.3a-d, A.4a-d, A.4f). Three plates form the antapical end of the cell, the right one (3''') carrying a spine (Figures A.3g, A.4f).

The interior of the cells contains large numbers of vacuoles (e.g. Figures A.1g, A.2i), including a very large one in the antapical half of the cell that often contains partially digested prey. At least two types of trichocysts are present in *Lessardia*, the smaller type of which tends to be scattered along the sides of the cells (e.g. Figures A.2b-c, A.2f). The larger trichocysts are square in transversal section (Figure A.2h) and are arranged in batteries perpendicular to the cell membrane (Figure A.2g). They tend to be concentrated at either end of the cells (Figure A.2d), and large trichocyst openings tend to be a feature of the thecal plates in these regions (e.g. Figures A.3b, A.3d, A.3g). On the apical end, plate 2' carries very conspicuous openings for these large trichocysts, but interestingly, the opening of the trichocysts were always on the left side of the cell, none were seen on the right side, i.e. on plate 3'. Large trichocyst openings are present in all three antapical plates (Figures A.3a-e, A.3g).

Figure A.1. General morphology of *Lessardia elongata*. a: Differential interference contrast (DIC) light micrograph showing the transverse flagellum (arrow). Bar: 12.5 μ m. b: DIC light micrograph showing the digestive vacuole with ingested prey (arrow). Bar: 12.5 μ m. c: DIC light micrograph showing the dinokaryotic nucleus (arrow), the digestive vacuole, and one flagellum. Bar: 12.5 μ m. d: Ventral view of *L. elongata* stained with calcofluor white and illuminated with UV light. Note the sulcal region. Bar: 6 μ m. e: Dorsal view of *L. elongata* stained with calcofluor white and illuminated with UV light. Bar: 6 μ m. f: SEM micrograph of *L. elongata* with the plasmalemma and the two flagella present. Bar: 5 μ m. g: TEM micrograph of *L. elongata*, longitudinal section. Note the nucleus with dinokaryotic chromosomes (N), the digestive vacuole (DV), and mitochondria with tubular cristae close to the apical and antapical ends (M). Bar: 2 μ m.

Figure A.2. Details in the morphology of *Lessardia elongata*. a: SEM micrographs of the sulcal region of the cell; plasmalemma and flagella are still present. The structure at the base of the flagella (arrow) is interpreted to be the peduncle. Bar: 3 μ m. b and c: Thecal plate pattern of the sulcal region. Arrows indicate small trichocyst openings. Bars: b: 2 μ m. c: 1.5 μ m. d: DIC light micrograph of a living cell with expanded large trichocysts. Bar: 25 μ m. e: TEM micrograph of an expanded trichocyst. Bar: 0.1 μ m. f: TEM micrograph of a small trichocyst, longitudinal section. Bar: 0.5 μ m. g: TEM micrograph of large trichocyst batteries close to the apical end of the cell. Bar: 0.5 μ m. h: Square transversal sections of large trichocysts. Bar: 0.5 μ m. i: Transversal section in the antapical half of the cell showing a digestive vacuole (DV) with prey. Cr: cryptomonad prey. Bar: 2 μ m. j: Amphiesma of the cell showing the plasmalemma, two alveolar boundaries, and several thecal plates. Bar: 0.5 μ m.

Figure A.3. Scanning electron micrographs of the thecal plate pattern of *Lessardia elongata*. Thecal plate margins have been marked with white lines in b, c, and d. a: Ventral view. b: Left side view. c: Dorsal view. d: Right side view. Bar: 5 µm. e: Apical complex, dorsal/right side view. Bar: 0.5 µm. f: Apical complex, ventral/left side view. Arrow shows the trichocyst opening on plate 2'. Bar: 0.5 µm. g: Antapical end of the cell. Note the large trichocyst openings (arrow) and the spine. Bar: 1 µm. Pi: Inner pore plate, Po: Outer pore plate. CP: Canal plate. 3': Third apical plate.

Figure A.4. Line drawings of the thecal plate patterns of *Lessardia elongata*. a: Ventral view, b: Left side view. c: Dorsal view. d: Right side view. e: Apical view. f: Antapical view.

A.4.2. Molecular Phylogenetic Analysis

Small-subunit ribosomal RNA gene sequences were obtained from both *Lessardia elongata* and *Roscoffia capitata*. Phylogenetic analyses showed both species branching within the GPP-complex in almost all maximum likelihood and distance trees, sometimes forming a clade to the exclusion of all other taxa, albeit with weak bootstrap support. Very often, however, the putative blastodinialean *Haplozoon axiothellae* branched between *Lessardia* and *Roscoffia* (e.g. Figures 1.1, 1.2, 1.4) with a very long branch (other positions of *Haplozoon* in phylogenetic trees are also common, no single position is supported by bootstrap numbers). Unfortunately, SSU rRNA sequences for established podolampaceans are not yet available, and so the relationship between *Lessardia*, *Roscoffia* and the Podolampaceae could not be tested with molecular phylogenies.

A.5. DISCUSSION

Lessardia elongata could very well be the same species as the organism named "*Gymnodinium elongatum*" by Hope (1954), in very general terms, the morphology of *Lessardia* is consistent with the drawings shown in that work. However, given the paucity of morphological data provided, it is difficult to be absolutely sure (photographs are provided in Shapiro et al. 1989 and Hansen and Larsen 1992, much better evidence as to the identity of the species treated there). It is certain, however, that the name "*Gymnodinium elongatum*" should be treated

as a nomen nudum: Hope's discussion of the species provides neither a description nor a diagnosis, only the two drawings, and this does not satisfy the requirements for valid publication of either the ICBN (Articles 32.1.c and 42.3) or the ICZN (Article 13) valid at the time.

The genus *Lessardia* as defined here is monotypic. However, it is very likely that *Pronoctiluca rostrata* Taylor 1976, a planktonic organism from the Northern Indian Ocean, may actually be a second species in the genus. It shares many of the characteristics of *L. elongata*, including the biconical shape (here more elongated than in *Lessardia*), a delicate theca and a spine at the antapical end (the figure in Taylor 1976b is inverted). A cingulum was not seen in *Pronoctiluca rostrata*, but this is not different from the situation in *Lessardia*, where it is very difficult to distinguish a girdle with light microscopy. *Pronoctiluca rostrata* is 115-128 μm long, almost 4 times as long as *L. elongata*.

The genus *Gymnodinium* was recently re-defined to include athecate dinoflagellates with a horseshoe-shaped apical groove running in an anticlockwise direction, a nuclear envelope with vesicular chambers, a displaced cingulum and a nuclear fibrous connective (Daugbjerg et al. 2000). *Lessardia elongata* lacks most of those features (the presence of a nuclear fibrous connective in the species cannot be ruled out but is unlikely) and has a well-defined theca; it is certainly not closely related to *Gymnodinium*. Its thecal plate arrangement is instead consistent with that of the dinoflagellate order Peridiniales (Fensome et al. 1993). The first apical plate, although morphologically quite derived (extremely

long and thin), is essentially symmetrical, reflecting the fact that the cingulum is not displaced. The apical pore complex is also reminiscent of the features of peridinial genera: it is not triangular or teardrop shaped, but conical and with a deep groove pointing mid-ventrally.

Within the Peridiniales, the thecal plate arrangement of *Lessardia* most closely resembles that of the family Podolampaceae (Fensome et al. 1993, Carbonell-Moore 1994). In fact, the thecal arrangements in *Lessardia* and the Podolampaceae (Figure A.5) are identical except for one feature: Podolampaceae have one antapical plate, while *Lessardia* has three. *Lessardia* has also only 4 postcingular plates, while the majority of the Podolampaceae have 5, but a number of species in the podolampacean genus *Blepharocysta* do have 4 postcingular plates (Carbonell-Moore 1994). *Lessardia* also shares with the Podolampaceae the relatively rare feature of a broad, flat cingulum located posteriorly from the cell equator; in the Podolampaceae the cingulum is completely flattened out and has not always been recognized as such (Podolampaceae have traditionally been considered to lack a cingulum altogether, but plate homology studies show that the cingular plates are actually present and fused with at least some of the postcingular ones, Fensome et al. 1993).

The only other dinoflagellate genus with extensive similarities in thecal plate patterns to *Lessardia* is *Roscoffia*, a genus that has also been suggested to be related to the Podolampaceae (Horiguchi and Kubo 1997, Hoppenrath and Elbraechter 1998). The epithecae of the two genera have essentially identical

plate patterns (although an anterior intercalary plate has only been observed in *Roscoffia minor*, Figure A.5, it may or may not exist in *Roscoffia capitata*, Horiguchi and Kubo 1997, Hoppenrath and Elbraechter 1998). Nevertheless, *Lessardia* is also different from *Roscoffia* in its possession of three antapical plates, *Roscoffia*, like the established Podolampaceae, has only one.

Lessardia can easily be accommodated in the Podolampaceae, the broad, flat cingulum of the genus is a feature characteristic of this family. The fact that *Lessardia* has three antapical plates rather than one is not problematic: the closest peridinialean family to the Podolampaceae, the Protoperidiniaceae (formerly Congruentidiaceae, see Fensome et al. 1998 for a nomenclatural discussion), has members with both one and two antapical plates, this is a feature that appears to vary easily. The Protoperidiniaceae is the only other taxon that could reasonably house *Lessardia*. However, members of the Protoperidiniaceae consistently have 6 or even 7 precingular plates, never 5, and, more importantly, they always have a strongly impressed cingulum. They also tend to divide through eleutheroschisis, while *Lessardia*, like at least one member of the Podolampaceae (*Podolampas bipes*, Hoppenrath and Elbraechter 1998), does so through desmoschisis. We have inferred phylogenetic trees that included unpublished sequences from three species of the genus *Protoperidinium* (not shown). Neither *Lessardia* nor *Roscoffia* ever formed a clade with any members of *Protoperidinium*.

Roscoffia is much more difficult to place confidently in the Podolampaceae. The main reason for this is the fact that, although perhaps somewhat broader than usual, the cingulum in this genus is just as distinctly imprinted as in most dinoflagellates. In addition, many aspects of the biology of this genus are poorly understood: it is not known for example whether *Roscoffia* divides through desmoschisis (like the Podolampaceae) or eleutheroschisis. However, the thecal plate pattern of *Roscoffia* is virtually identical to that of the Podolampaceae, a feature that strongly argues for the inclusion of this genus in the family. Our molecular results also support this view: if *Roscoffia* and *Lessardia* are closely related (as suggested with weak support by the majority of our phylogenetic trees) and *Lessardia* is in the Podolampaceae, it is very likely that *Roscoffia* is closely related to the family as well. The genus *Roscoffia* will not be formally included in the Podolampaceae for two reasons. First, it lacks the most characteristic feature of the family, the flat cingulum. Secondly, and perhaps more importantly, many features of the biology of *Roscoffia* are poorly known, including its mode of division (desmoschisis or eleutheroschisis?).

When compared to the established Podolampaceae (genera like *Podolampas*, *Blepharocysta* and *Lissodinium* among others), both *Lessardia* and *Roscoffia* appear to possess plesiomorphic states for the cingulum. In *Roscoffia*, the presence of a deeply imprinted cingulum is a feature that allies it to dinoflagellates outside of the family. In *Lessardia*, this feature appears to be at an intermediate stage between that of the Podolampaceae and the rest of the dinoflagellates: the cingulum in this genus is only weakly imprinted, but not

completely flat as is the case in the other Podolampaceae. Molecular data from other genera in the Podolampaceae and the Protoperidiniaceae should be helpful in resolving the phylogenetic position of these two genera. It would be for example interesting to determine whether *Lessardia* and especially *Roscoffia* diverge early with respect to the other Podolampaceae, as the morphological data suggest.

Haplozoon axiothellae, a species that very often branches together with both *Lessardia* and *Roscoffia* in phylogenetic trees, is morphologically extremely different from either of those two species. It is a parasite whose main life stage is a syncytial trophont with a multicellular appearance, made up of three fundamental units (terminology according to Shumway 1924): an anterior "trophocyte" that attaches the organism to its host through a characteristic stylett, a row of "gonocytes" in the midregion, and posterior "gonocytes" that can detach and become gymnodinoid spores. The different "cells" of the organism are however not completely independent, a common plasmalemma that does not extend to the separations between the "cells" (alveolae only) covers the whole organism. The surface of the whole organism is covered with large numbers of small, roughly polygonal alveolae, each one containing a very thin thecal plate (Leander et al. 2002). The morphology of this organism has nothing in common with that of *Lessardia*, *Roscoffia* or the Podolampaceae, a phylogenetic relationship between these taxa seems thus extremely unlikely, in spite of the (unsupported) phylogenetic data.

Figure A.5. Line drawings of the epithecae and hypothecae of (a and b) *Roscoffia capitata*, modified after Horiguchi and Kubo 1997; (c and d) *Lessardia elongata*; and (e and f) *Blepharocysta* sp., a member of the Podolampaceae, modified after Carbonell-Moore 1994.

A.6. NOTE ON THE AUTHORSHIP OF THE MATERIAL PRESENTED IN THE APPENDIX

The material presented in the appendix was published in the Journal of Phycology as a multiauthored paper: Saldarriaga JF, Leander BS, Taylor FJR and Keeling PJ 2003: *Lessardia elongata* gen. et sp. nov. (Dinoflagellata, Peridiniales, Podolampaceae) and the taxonomic position of the genus *Roscoffia*. J. Phycol. 39: 368-378. Patrick Keeling obtained the SSU rRNA sequence for *Lessardia elongata*, and Brian Leander produced the TEM photographs of the work; he also showed me how to fix and dehydrate the material for SEM and helped me organize the figure plates. I obtained the SSU sequence for *Roscoffia capitata*, did all the phylogenetic analyses, produced the SEM photographs and, with the aid of F.J.R. Taylor, worked out the tabulation of *Lessardia*. I also wrote the paper and produced the line drawings. All four authors contributed greatly to the content of the paper.

LITERATURE

Afzelius BA 1963: The nucleus of *Noctiluca scintillans*; aspects of nucleocytoplasmic exchanges and the formation of nuclear membranes. J. Cell Biol. 19: 229-238.

Appleton PL and Vickerman K 1998: In vitro cultivation and developmental cycle in culture of a parasitic dinoflagellate (*Hematodinium sp.*) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters. Parasitology 116: 115-130.

Archibald JM, Rogers MB, Toop M, Ishida KI and Keeling PJ 2003: Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigeloviella natans*. Proc. Natl. Acad. Sci. USA. In press.

Ausseil J., Soyer-Gobillard M.-O., Géraud M.-L., Bhaud Y., Perret E., Barbier M., Albert M., Plaisance L. and Moreau H. (2000): Dinoflagellate centrosome: associated proteins old and new. Europ. J. Protistol. 36, 1-19.

Baldauf SL, Roger AJ, Wenk-Siefert I and Doolittle WF 2000: A kingdom-level phylogeny of eukaryotes based on combined protein data. Science 290: 972-977.

Ben Ali A, De Baere R, Van der Auwera G, De Wachter R and Van de Peer Y 2001: Phylogenetic relationships among algae based on complete large-subunit rRNA sequences. Int. J. Syst. Evol. Microbiol. 51: 737-749.

Bernhard D and Schlegel M 1998: Evolution of histone H4 and H3 genes in different ciliate lineages. J. Mol. Evol. 46: 344-354.

Birkenes E 1941: Fitoplanktonundersøkelser (og hydrografiske observasjoner) i Oslofjorden sommeren 1939. Thesis, University of Oslo, Norway.

Braarud T 1945: A phytoplankton survey of the polluted waters of inner Oslo fjord. Hvalrådets Skrifter 28: 1-142.

Biecheler B 1938: Sur un péridinien cuirassé incolore nouveau *Crypthecodinium* n.g. *setense* n. sp. et la famille nouvelle des Crypthecodiniacées. Société zoologique de France, Bulletin 63 (1): 9-13.

Brugerolle G 2002a: *Colpodella vorax*: ultrstructure, predation, life cycle, mitosis and phylogenetic relationships. Europ. J. Protistol. 38: 113-125.

Brugerolle G 2002b: *Cryptophagus subtilis*: a new parasite of cryptophytes affiliated with the Perkinsozoa lineage. Europ. J. Protistol. 37: 379-390.

Brugerolle G 2003: Apicomplexan parasite *Cryptophagus* renamed *Rastrimonas* gen. nov. Europ. J. Protistol. 39: 101.

Bruno WJ, Socci ND and Halpern AL 2000: Weighted neighbor joining: a likelihood-based approach to distance-based phylogeny reconstruction. Mol Biol Evol 17: 189-197.

Cachon J and Cachon M 1968: Contribution a l'étude des Noctilucidae Saville-Kent. I: Les Kofoidininae Cachon J. et M. Évolution morphologique et systématique. Protistologica 3: 427-443.

Cachon J and Cachon M 1971: *Protoodinium chattoni* Hovasse: manifestations ultrastructurales des rapports entre le Péridinien et la méduse hôte: fixation, phagocytose. Arch. Protistenk 113: 293-305.

Cachon J and Cachon M 1977: Observations on the mitosis and on the chromosome evolution during the life cycle of *Oodinium*, a parasitic dinoflagellate. *Chromosoma* (Berl.) 60: 237-251.

Cachon J and Cachon M 1987: Parasitic dinoflagellates. In: Taylor FJR (ed.): The biology of dinoflagellates. Botanical Monographs Volume 21, Blackwell Scientific Publications, Oxford.

Carbonell-Moore MC 1994: On the taxonomy of the family Podolampadaceae Lindemann (Dinophyceae), with descriptions of three new genera. Review of Palaeobotany and Palynology 84: 73-99.

Cavalier-Smith T 1991: Cell diversification in heterotrophic flagellates. In: Patterson DJ and Larsen J (eds.): The biology of free-living heterotrophic flagellates. Syst. Assoc. Special Vol. 45, Clarendon Press, Oxford.

Cavalier-Smith T 1993: Kingdom Protozoa and its 18 phyla. Microbiological Reviews 57 (4): 953-994.

Cavalier-Smith T 1998: A revised six-kingdom system of life. *Biol Rev Camb Philos Soc* 73, 203-66.

Cavalier-Smith T 1999: Principles of protein and lipid targeting in secondary symbiogenesis: euglenoid, dinoflagellate, and sporozoan plastid origins and the eukaryote family tree. *J Eukaryot Microbiol* 46: 347-366

Cavalier-Smith T 2000: Membrane heredity and early plastid evolution. *Trends Plant Sci* 5: 174-182.

Cavalier-Smith T 2003: Genomic reduction and evolution of novel genetic membranes and protein-targeting machinery in eukaryote-eukaryote chimaeras (meta-algae). *Philos. T. Roy. Soc. B* 358 (1429): 109-133.

Cavalier-Smith T and Chao EE 1996: 18S rRNA sequence of *Heterosigma carterae* (Raphidophyceae), and the phylogeny of heterokont algae (Ochrophyta). *Phycologia* 35: 500-510

Chatton E 1920: Les péridiniens parasites: morphologie, reproduction, ethologie. *Archives de zoologie expérimentale et générale*, Tome 59, pp 1-475. Reprint 1975 Otto Koeltz Science Publishers, Koenigstein, Germany.

Chesnick JM, Kooistra WHCF, Wellbrock U and Medlin LK 1997: Ribosomal RNA analysis indicates a benthic pennate diatom ancestry for the endosymbionts of the dinoflagellates *Peridinium foliaceum* and *Peridinium balticum* (Pyrrophyta). *J Euk Microbiol* 44: 314-320.

Chudnovsky Y, Li JF, Rizzo PJ, Woodland-Hastings J and Fagan TF 2002: Cloning, expression and characterization of a histone-like protein from the marine dinoflagellate *Lingulodinium polyedrum* (Dinophyceae). *J. Phycol.* 38: 543-550.

Corliss JO 1975: Nuclear characteristics and phylogeny in the protistan phylum Ciliophora. *Biosystems* 7: 338-349.

Crawford RM and Dodge JD 1971: The dinoflagellate genus *Woloszynskia*. II. The fine structure of *W. coronata*. *Nova Hedwigia* 22: 699-719.

Crawford RM, Dodge JD and Happey CM 1970: The dinoflagellate genus *Woloszynskia*. I. Fine structure and ecology of *W. tenuissima* from Abbot's Pond, Somerset. *Nova Hedwigia* 19: 825-840.

Creedon KA, Kaslow DC, Rathod PK and Wellems TE 1992: Identification of a *Plasmodium falciparum* histone 2A gene. Mol. Biochem. Parasitol. 54 (1), 113-115.

Daugbjerg N, Hansen G, Larsen J and Moestrup Ø 2000: Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39: 302-317.

Dodge JD 1965: Chromosome structure in the dinoflagellates and the problem of the mesokaryotic cell. Excerpta Med. Int. Congr. Ser. 91: 339-341.

Dodge JD 1966: The Dinophyceae. In: Godward MBE (ed.): The chromosomes of the algae, pp. 96-115. Arnold, London.

Dodge JD 1983: A re-examination of the relationship between unicellular host and eukaryotic endosymbiont with special reference to *Glenodinium foliaceum* Dinophyceae. In: Schwemmler W and Schenk HEA (eds): Endocytobiology II. de Gruyter, Berlin.

Dodge JD 1984: Dinoflagellate taxonomy. In: Spector DL (ed.) Dinoflagellates. Academic Press, Orlando.

Dodge JD 1987: Dinoflagellate ultrastructure. In: Taylor FJR (ed.): The biology of dinoflagellates. Botanical Monographs Volume 21, Blackwell Scientific Publications, Oxford.

Dodge JD and Crawford RM 1968: Fine structure of the dinoflagellate *Amphidinium carteri* Hulbert. Protistologica 4: 231-242.

- Dodge JD and Crawford RM 1969: The fine structure of *Gymnodinium fuscum* (Dinophyceae). *New Phytol.* 68: 613-618.
- Dodge JD and Crawford RM 1971: Fine structure of the dinoflagellate *Oxyrrhis marina*. II: The flagellar system. *Protistologica* 7: 399-409.
- Drebes G 1981: Possible resting spores of *Dissodinium pseudolunula* (Dinophyta) and their relation to other taxa. *Br. Phycol. J.* 16: 207-215.
- Edwardsen B, Shalchian-Tabrizi K, Jakobsen KS, Medlin LK, Dahl E, Brubak S and Paasche E 2003: Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analyses of rDNA. *J. Phycol.* 39: 395-408.
- Fast NM, Kissinger JC, Roos DS and Keeling PJ 2001: Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol Biol Evol* 18: 418-426.
- Fast NM, Xue L, Bingham S and Keeling PJ 2002: Re-examining alveolate evolution using multiple protein molecular phylogenies. *J. Eukaryot. Microbiol.* 49: 30-37.
- Felsenstein J. Phylip (Phylogeny Inference Package) 3.57c. Distributed by the author, Seattle, Washington, USA.
- Fensome RA, Taylor FJR, Norris G, Sarjeant WAS, Wharton DI and Williams GL 1993: A classification of living and fossil dinoflagellates. *Micropaleontology Special Publication 7*, Sheridan Press, Hanover, Pennsylvania, USA.
- Fensome RA, Bujak J, Dale B, Davies EH, Dodge JD, Edwards LD, Harland R, Head MJ, Lentin JK, Lewis J, Matsuoka K, Norris G, Sarjeant WAS, Taylor FJR

and Williams GL 1998: Proposal to conserve the name Protoperidiniaceae against Congruentidiaceae, Diplopsalaceae and Kolkwitzellaceae (Dinophyceae). Taxon 47: 727-730

Fensome RA, Saldarriaga JF, Taylor FJR 1999: Dinoflagellate phylogeny revisited: reconciling morphological and molecular based phylogenies. Grana 38: 66-80.

Fernández I, Pardos F, Benito J and Arroyo NL 1999: *Acrocoelus glossobalani* gen. nov. et sp. nov., a protistan flagellate from the gut of the enteropneust *Glossobalanus minutus*. Europ. J. Protistol. 35: 55-65.

Fritz L and Triemer RE 1985: A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. J. Phycol. 21: 662-664.

Gaines G and Taylor FJR 1985: Form and function of the dinoflagellate transverse flagellum. J. Protozool. 32: 290-296.

Gajadhar AA, Marquardt WC, Hall R, Gunderson J, Ariztia-Carmona EV and Sogin ML 1991: Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Cryptosporidium parvum* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. Mol. Biochem. Parasitol. 45, 147-154.

Gao XP and Li JY 1986: Nuclear division in the marine dinoflagellate *Oxyrrhis marina*. J Cell Sci 85: 161-176.

Gascuel O 1997: BioNJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol 14: 685-695.

Gibbs SP 1981: The chloroplasts of some algal groups may have evolved from endosymbiotic eukaryotic algae. *Ann. N. Y. Acad. Sci.* 361, 193-208.

Goggin CL and Barker SC 1993: Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. *Mol Biochem Parasitol* 60, 65-70.

Grzebyk D, Sako Y and Berland B 1998: Phylogenetic analysis of nine species of *Prorocentrum* (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological comparisons and description of *Prorocentrum panamensis* sp. nov. *J. Phycol.* 34: 1055-1068.

Gunderson JH, Goss SH and Coats DW 1999: The phylogenetic position of *Amoebophrya* sp. infecting *Gymnodinium sanguineum*. *J Euk Microbiol* 46: 194-197.

Hackett JD, Maranda L, Yoon HS and Bhattacharya D 2003: Phylogenetic evidence for the cryptophyte origin of the plastid of *Dinophysis* (Dinophysiales, Dinophyceae). *J. Phycol.* 39: 440-448.

Hallegraeff GM and Lucas IAN 1988: The marine dinoflagellate genus *Dinophysis* (Dinophyceae): photosynthetic, neritic and non-photosynthetic, oceanic species. *Phycologia* 27: 25-42.

Hansen G 1995: Analysis of the thecal plate pattern in the dinoflagellate *Heterocapsa rotundata* (Lohmann) comb. nov. (= *Katodinium rotundatum* (Lohmann) Loeblich). *Phycologia* 34 (2): 166-170.

Hansen G and Larsen J 1992: Dinoflagellater i danske farvande. In: Thomsen, H.A. (ed.): Plankton i de indre danske farvande. Havforskning fra Miljøstyrelsen, Copenhagen, p. 45-155.

Hansen G, Moestrup Ø and Roberts KR 2000: Light and electron-microscopical observations on the type species of *Gymnodinium*, *G. fuscum* (Dinophyceae). *Phycologia* 39(5): 365-376.

Head MJ 1996: Modern dinoflagellate cysts and their biological affinities. In: Jansonius J and McGregor DC (eds.): *Palinology: principles and applications*, Volume 3, pp. 1197-1248. American Association of Stratigraphic Palynologists Foundation, Dallas.

Herzog M and Maroteaux L 1986: Dinoflagellate 17S rRNA sequence inferred from the gene sequence: evolutionary implications. *Proc. Nat. Acad. Sci. USA* 83: 8644-8648.

Hillis DM and Dixon MT 1991: Ribosomal DNA: molecular evolution and phylogenetic inference. *Quart. Rev. Biol.* 66: 411-453.

Hollande A 1974: Étude comparée de la mitose syndinienne, de celle des péridiniens libres et des hypermastigines; infrastructure et cycle évolutif des syndinides parasites de radiolaires. *Protistologica* 10 (3): 413-451.

Hollande A and Corbel JC 1982: Ultrastructure, cycle évolutif et position systématique de *Caryotoma bernardi* Holl. et Enj. (Dinoflagellés, Oodinides), parasite endocapsulaire des Thalassicolles (Radiolaires). *Protistologica* 18 (1): 123-133.

Hope B 1954: Floristic and taxonomic observations on marine phytoplankton from Nordåsvatn, near Bergen. *Nytt Mag. Bot.* 2: 149-153

Hoppenrath M and Elbraechter M 1998: *Roscoffia capitata* (Dinophyceae) refound: notes on morphology and biology. *Phycologia* 37(6): 450-57.

Hoppenrath M, Saldarriaga JF, Schweikert M, Elbrächter M and Taylor FJR 2004: Revision of the genus *Thecadinium* (Dinoflagellata) and description of *Thecadinium mucosum* sp. nov., a new sand-dwelling, marine dinoflagellate. J. Phycol. Submitted.

Horiguchi T and Pienaar RN 1991: Ultrastructure of a marine dinoflagellate, *Peridinium quinquecorne* Abé (Peridinales) from South Africa with special reference to its chrysophyte endosymbiont. Botanica Marina 34: 123-131.

Horiguchi T and Kubo F 1997: *Roscoffia minor* sp. nov. (Peridinales, Dinophyceae), a new sand-dwelling, armoured dinoflagellate from Hokkaido, Japan. Phycological Research 45(2): 65-9.

Horiguchi T, Yoshizawa-Ebata J and Nakayama T 2000: *Halostylodinium arenarium* gen. et sp. nov. (Dinophyceae), a coccoid, sand-dwelling dinoflagellate from subtropical Japan. J. Phycol. 36: 960-971.

Ishida K and Green B 2002: Second- and third-hand chloroplasts in dinoflagellates: phylogeny of oxygen-evolving enhancer 1 (psbO) protein reveals replacement of a nuclear-encoded plastid gene by that of a haptophyte tertiary endosymbiont. PNAS 99 (14): 9294-9299.

Jeffrey SW and Vesik M 1976: Further evidence for a membrane-bound endosymbiont within the dinoflagellate *Peridinium foliaceum*. J Phycol 12: 450-455.

John U, Fensome RA and Medlin LK 2003: A new ribotype in the *Alexandrium tamarense* species complex (Dinophyceae) and the application of a molecular clock to explain the biogeographic distribution of the genus. J. Phycol., in press.

Kasinsky HE, Lewis JD, Dacks JB and Ausió J 2001: Origin of H1 linker histones. The FASEB Journal 15: 34-42.

Kato KH, Moriyama A, Huitorel P, Cosson J, Cachon M and Sato H 1997: Isolation of the major basic nuclear protein and its localization on chromosomes of the dinoflagellate *Oxyrrhis marina*. Biology of the Cell 89: 43-52.

Keeling, PJ 2001: Foraminifera and cercozoa are related in actin phylogeny: two orphans find a home? Mol. Biol. Evol. 18: 1551-1557.

Kofoed CA and Swezy O 1921: The free-living unarmored dinoflagellata. University of California Memoirs, vol. 5. University of California Press, Berkeley, California.

Kubai DF and Ris H 1969: Division in the dinoflagellate *Gyrodinium cohnii*. J Cell Biol 40, 508.

Kuvarina ON, Leander BS, Aleshin VV, Mylnikov AP, Keeling PJ and Simdyanov TG 2002: The phylogeny of colpodellids (Alveolata) using small-subunit rRNA gene sequences suggests they are the free-living sister group to apicomplexans. J. Eukaryot. Microbiol. 49: 498-504.

Kuznetsov SA, Langford GM and Weiss DG 1992: Actin-dependent organelle movement in squid axoplasm. Nature 356: 722-725.

Landsberg JH, Steidinger KA, Blakesley BA and Zondervan RL 1994: Scanning electron microscope study of dinospores of *Amyloodinium* cf. *ocellatum*, a pathogenic dinoflagellate parasite of marine fish, and comments on its relationship to the peridiniales. Dis Aquat Org 20: 23-32.

Leadbeater B and Dodge JD 1966: The fine structure of *Woloszynskia micra* sp. nov., a new marine dinoflagellate. Br. Phycol. Bull. 3: 1-17.

Leadbeater B and Dodge JD 1967: An electron microscope study of dinoflagellate flagella. J. Gen. Microbiol. 46: 305-314.

Leander BS, Saldarriaga JF and Keeling PJ 2002: Surface morphology of the marine parasite *Haplozoon axiothellae* Siebert (Dinoflagellata). Europ. J. Protistol. 38: 287-297.

Leander BS, Kuvardina ON, Aleshin VV, Mylnikov AP and Keeling PJ 2003: Molecular phylogeny and surface morphology of *Colpodella edax* (Alveolata): insights into the phagotrophic ancestry of apicomplexans. J. Euk. Micro. 50: 334-340.

Leander BS and Keeling PJ 2003: Morphostasis in alveolate evolution. Trends Ecol. Evol. 18: 395-402.

Lenaers G, Scholin C, Bhaud Y, Saint-Hilaire D and Herzog M 1991: A molecular phylogeny of dinoflagellate protists (Pyrrhophyta) inferred from the sequence of 24S rRNA divergent domains D1 and D8. J. Mol. Evol. 32: 53-63.

Levine ND 1978: *Perkinsus* gen. n. and other new taxa in the protozoan phylum Apicomplexa. J Parasitol 64, 549.

Li JY 1984: Studies on dinoflagellate chromosomal basic protein. BioSystems 16: 217-225.

Litaker RW, Tester PA, Colorni A, Levy MG and Noga EJ 1999: The phylogenetic position of *Pfiesteria piscicida*, Cryptoperidiniopsis sp. , *Amyloodinium ocellatum*

and a *Pfiesteria*-like dinoflagellate to other dinoflagellates and apicomplexans. J Phycol 35: 1379-1389.

Lom J 1981: Fish-invading dinoflagellates: a synopsis of existing and newly proposed genera. Folia Parasitologica (Praha) 28: 3-11.

Lom J and Lawler AR 1973: An ultrastructural study on the mode of attachment in dinoflagellates invading gills of cyprinodontidae. Protistologica 9: 293-309.

Lom J and Schubert G 1983: Ultrastructural study of *Piscinoodinium pillulare* (Schäperclaus, 1954) Lom, 1981 with special emphasis on its attachment to the fish host. J. Fish Dis. 6: 411-418.

Lom J, Rohde K and Dyková I 1993: *Crepidoodinium australe* n. sp., an ectocommensal dinoflagellate from the gills of *Sillago ciliata*, an estuarine fish from the New South Wales coast of Australia. Dis Aquat Org 15: 63-72.

López-García P, Rodríguez-Valera F, Pedrós-Alió C and Moreira D 2001: Unexpected diversity of small eukaryotes in deep-sea Antarctic phytoplankton. Nature 409: 603-607.

Maroteaux L, Herzog M and Soyer-Gobillard MO 1985: Molecular organization of dinoflagellate ribosomal DNA: molecular implications of the deduced 5.8S rRNA secondary structure. BioSystems 18: 307-319.

Maruyama T 1982: Fine structure of the longitudinal flagellum in *Ceratium tripos*. J. Cell Science 58: 109-123.

McFadden GI and Waller RF 1997 Plastids in parasites of humans. BioEssays 19: 1033-1040.

Menden-Deuer S and Lessard EJ 2000: Carbon to volume relationships for dinoflagellates, diatoms and other protist plankton. *Limnol. Oceanogr.* 45 (3): 569-79.

Mignot JP and Brugerolle G 1975: Étude ultrastructurale du flagellé phagotrophe *Colponema loxodes* Stein. *Protistologica* 11: 429-444.

Moldowan JM and Talyzina NM 1998: Biogeochemical evidence for dinoflagellate ancestors in the early Cambrian. *Science* 281: 1168-1170.

Moldowan JM, Dahl J, Jacobson SR, Huizinga BJ, Fago FJ, Shetty R, Watt DS and Peters KE 1996: Chemostratigraphic reconstruction of biofacies: molecular evidence linking cyst-forming dinoflagellates with pre-Triassic ancestors. *Geology* 24: 159-162.

Montresor M, Procaccini G and Stoecker DK 1999: *Polarella glacialis* gen. nov., sp. nov. (Dinophyceae): Suessiaceae are still alive! *J. Phycol.* 35: 186-197.

Moon-van der Staay SY, De Wachter R and Vaultot D 2001: Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* 409: 607- 610.

Netzel H and Dürr G 1984: Dinoflagellate cell cortex. In: Spector DL (ed.): *Dinoflagellates*. Academic Press, Orlando.

Norén F, Moestrup Ø and Rehnstam-Holm AS 1999: *Parvilucifera infectans* Norén and Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. *Europ. J. Protistol.* 35: 233-254.

Perkins FO 1976: Zoospores of the oyster pathogen, *Dermocystidium marinum*. I. Fine structure of the conoid and other aporozoan-like organelles. J Parasitol 20: 959-974.

Perkins FO 1996: The structure of *Perkinsus marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. Journal of Shellfish Research 15 (1): 67-87

Perkins FO, Barta JR, Clopton RE, Peirce MA and Upton SJ 2000: Apicomplexa. In: Lee JJ, Leedale GF and Bradbury P (eds.): An illustrated guide to the protozoa, second edition. Society of Protozoologists. Allen Press Inc., Lawrence, Kansas, USA

Perret E., Davoust J., Albert M., Besseau L. and Soyer-Gobillard M.-O. 1993: Microtubule organization during the cell cycle of the primitive eukaryote dinoflagellate *Cryptothecodinium cohnii*. J. Cell Sci. 104, 639-651.

Piel KM and Evitt WR 1980: Paratabulation in the Jurassic dinoflagellate genus *Nannoceratopsis* and a comparison with modern taxa. Palynology 4: 79-104.

Popovsky J 1971: Some remarks to the life cycle of *Gloeodinium montanum* Klebs and *Hemidinium nasutum* Stein (Dinophyceae). Arch. Protistenk. 113: 131-136.

Popovsky J and Pfiester LA 1990: Dinophyceae (Dinoflagellida). Süßwasserflora von Mitteleuropa, Band 6. Gustav Fischer Verlag, Jena, Stuttgart.

Rae PMM 1976: Hydroxymethyluracil in eukaryote DNA: a natural feature of the pyrrrophyta (dinoflagellates). Science 194: 1062-1064.

Raikov IB 1994: The diversity of forms of mitosis in protozoa: a comparative review. *Europ. J. Protistol.* 30: 253-269.

Reece KS, Siddall ME, Bureson EM and Graves JE 1997: Phylogenetic analysis of *Perkinsus* based on actin gene sequences. *J. Parasitol.* 83: 417-423.

Rees AJJ and Hallegraeff GM 1991: Ultrastructure of the toxic, chain-forming dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *Phycologia* 30: 90-105.

Ris H and Kubai DF 1974: An unusual mitotic mechanism in the parasitic protozoan *Syndinium* sp. *J. Cell Biol.* 60: 702-720.

Rizzo PJ 1981: Comparative aspects of basic chromatin proteins in dinoflagellates. *BioSystems* 14: 433-443.

Rizzo PJ 1991: The enigma of the dinoflagellate chromosome. *J. Protozool.* 38: 246-252.

Rizzo PJ and Noodén LD 1973: Isolation and chemical composition of dinoflagellate nuclei. *J. Protozool.* 20: 666-673.

Roberts KR 1991: The flagellar apparatus and cytoskeleton of dinoflagellates: organization and use in systematics. In: Patterson DJ and Larsen J (eds.): The biology of free-living heterotrophic flagellates. Systematics Association Special Volume 45, Clarendon Press, Oxford.

Saito H, Lirdwitayaprasit T, Fukuyo Y and Furoya K 2002: Sexual reproduction of *Noctiluca scintillans* with an endosymbiont green flagellate, *Pedinomonas noctilucae*. 10th International Conference on Harmful Algae, Book of Abstracts, St. Pete Beach, Florida, pg. 247.

Sala-Rovira M, Geraud ML, Caput D, Jacques F, Soyer-Gobillard MO, Vernet G and Herzog M 1991: Molecular cloning and immunolocalization of two variants of the major basic nuclear protein (HCc) from the histone-less eukaryote *Crypthecodinium cohnii* (Pyrrhophyta). *Chromosoma* 100: 510-518.

Saunders GW, Hill DRA, Sexton JP, Andersen RA (1997) Small-subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. *Pl Syst Evol [Suppl]* 11:237-259.

Schnepf E and Elbrächter M 1988: Cryptophycean-like double membrane-bound plastid in the dinoflagellate *Dinophysis* Ehrenb.: evolutionary, phylogenetic and toxicological implications. *Botanica Acta* 101:196-203.

Schnepf E and Elbrächter M 1992: Nutritional strategies in dinoflagellates: a review with emphasis on cell biological aspects. *Eur J Protistol* 28:3-24.

Schnepf E and Elbrächter M 1999: Dinophyte plastids and phylogeny: a review. *Grana* 38: 81-97.

Shapiro LP, Haugen EM and Carpenter EJ 1989: Occurrence and abundance of green-fluorescing dinoflagellates in surface waters of the Northwest Atlantic and Northeast Pacific oceans. *J. Phycol.* 25: 189-91.

Sherr BF and Sherr EB 2002: Microzooplankton distribution in relation to phytoplankton community succession in the upwelling ecosystems off Oregon and northern California. *EOS* 83(4), Ocean Sciences Meet. Suppl., Abstract # OS31D-56.

Shumway W 1924: The genus *Haplozoon*, Dogiel. Observations on the life history and systematic position. *J. Parasitol.* 11, 59-77.

Siddall ME, Reece KS, Graves JE and Bureson EM 1997: Total evidence refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. *Parasitology* 115 (2): 165-176.

Siebert AE and West JA 1974: The fine structure of the parasitic dinoflagellate *Haplozoon axiothellae*. *Protoplasma* 81:17-35.

Sigee DC 1986: The dinoflagellate chromosome. *Advances in Botanical Research* 12: 205-264.

Sournia A 1986: Atlas du Phytoplankton Marin Volume I: Introduction, Cyanophycees, Dictyochophycees, Dinophycees et Raphidophycees. Éditions du Centre National de la Recherche Scientifique, Paris.

Soyer MO 1969: L'enveloppe nucléaire chez *Noctiluca miliaris* Suriray (Dinoflagellata). I: Quelques données sur son ultrastructure et son évolution au cours de la sporogénèse. *J. Microscopie* 8: 569-580.

Soyer MO 1971: Structure du noyau des *Blastodinium* (dinoflagellés parasites): division et condensation chromatique. *Chromosoma (Berl.)* 33: 70-114.

Soyer MO 1972: Les ultrastructures nucléaires de la noctiluque (dinoflagellé libre) au cours de la sporogénèse. *Chromosoma (Berl.)* 39: 419-441.

Soyer MO 1974: Étude ultrastructurale de *Syndinium* sp. Chatton, parasite coelomique de copépodes pélagiques. *Vie Milieu* 24: 191-212.

Steidinger K 1993: Some taxonomic and biologic aspects of toxic dinoflagellates. In: Falconer IR (ed.): *Algal toxins in seafood and drinking water*. Academic Press

Steidinger KA, Burkholder JM, Glasgow Jr. HB, Hobbs CW, Garrett JK, Truby EW, Noga EJ and Smith SA 1996: *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. J Phycol 32:157-164

Stoecker DK 1999: Mixotrophy among dinoflagellates. J. Eukaryot Microbiol 46:397-401.

Strimmer K and von Haeseler A 1996: Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. Mol Biol Evol 13: 964-969.

Swofford DL 1999: Phylogenetic analysis using parsimony (and other methods) PAUP* 4.0 (test version). Sinauer, Sunderland, Massachusetts.

Takishita K and Uchida A 1999: Molecular cloning and nucleotide sequence analysis of psbA from the dinoflagellates: origin of the dinoflagellate plastid. Phycological Research 47: 207-216.

Tangen K, Brand LE, Blackwelder PL and Guillard RRL 1982: *Thoracosphaera heimii* (Lohmann) Kamptner is a dinophyte: observations on its morphology and life cycle. Marine Micropaleontology 7: 193-212.

Taroncher-Oldenburg G and Anderson DM 2000: Identification and characterization of three differentially expressed genes, encoding S-adenosylhomocysteine hydrolase, methionine aminopeptidase and a histone-like protein in the toxic dinoflagellate *Alexandrium fundyense*. Applied and Environmental Microbiology 66: 2105-2112.

Taylor FJR 1976a: Flagellate phylogeny: a study of conflicts. J. Protozool. 23: 28-40.

Taylor FJR 1976b: Dinoflagellates from the International Indian Ocean Expedition. *Bibliotheca Botanica*, 132: 1-234, pl. 1-46.

Taylor FJR 1980: On dinoflagellate evolution. *BioSystems* 13: 65-108.

Taylor FJR 1987: General group characteristics, special features, short history of dinoflagellate study. In: Taylor FJR (ed.): *The biology of dinoflagellates*. Botanical Monographs Volume 21, Blackwell Scientific Publications, Oxford.

Tengs T, Dahlberg OJ, Shalchian-Tabrizi K, Klaveness D, Rudi K, Delwiche CF and Jakobsen KS 2000: Phylogenetic analyses indicate that the 19' hexanoyloxyfucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol Biol Evol* 17: 718-729.

Triemer RE 1982: A unique mitotic variation in the marine dinoflagellate *Oxyrrhis marina* (Pyrrhophyta). *J. Phycol.* 18: 399-411.

Triemer RE and Fritz L 1984: Cell Cycle and Mitosis. In: Spector DL (ed.): *Dinoflagellates*. Orlando, Academic Press, pp. 149-179.

Van de Peer Y, van der Auwera G and de Wachter R 1996: The evolution of stramenopiles and alveolates as derived by substitution rate calibration of small ribosomal subunit RNA. *J. Mol. Evol.* 42: 201-210.

Van de Peer Y, Caers A, de Rijk P and de Wachter R 1998: Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research* 26: 179-182.

Watanabe MM and Sasa T 1991: Major carotenoid composition of an endosymbiont in a green dinoflagellate, *Lepidodinium viride*. *J. Phycol* 27 (Suppl.): 75.

Whatley JM, John P and Whatley FR 1979: From extracellular to intracellular: the establishment of mitochondria and chloroplasts. *Proc. Roy. Soc. Lond. B* 204:165-187.

Wilcox LW, Wedemayer GJ and Graham LE 1982: *Amphidinium cryophilum* sp. nov. (Dinophyceae), a new freshwater dinoflagellate. II. Ultrastructure. *J. Phycol.* 18: 18-30.

Wilson RJM, Gardner MJ, Feagin JE and Williamson DH 1991: Have malaria parasites three genomes? *Parasitol. Today* 7: 134-136.

Withers NW 1987: Dinoflagellate sterols. In: Taylor FJR (ed.): *The biology of dinoflagellates*. Botanical Monographs Volume 21, Blackwell Scientific Publications, Oxford.

Wolters J 1991: The troublesome parasites – molecular and morphological evidence that Apicomplexa belong to the dinoflagellate-ciliate clade. *BioSystems* 25: 75-83.

Wong JTY, New DC, Wong JCW and Hung VKL 2003: Histone-like proteins of the dinoflagellate *Cryptothecodinium cohnii* have homologies to bacterial DNA-binding proteins. *Eukaryotic Cell* 2: 646-650

Yoon HS, Hackett JD and Bhattacharya D 2002: A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. *PNAS* 99 (18): 11724-11729.

Zardoya R, Costas E, López-Rodas V, Garrido-Pertierra A and Bautista JM 1995: Revised dinoflagellate phylogeny inferred from molecular analysis of large-subunit ribosomal RNA gene sequences. *J. Mol. Evol.* 41: 637-645.

Zhang Z, Green BR and Cavalier-Smith T 1999: Single gene circles in dinoflagellate plastid genomes. *Nature* 400:155-159.

Zhang Z, Green BR and Cavalier-Smith T 2000: Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: a possible common origin for sporozoan and dinoflagellate plastids. *J. Mol. Evol.* 51: 26-40.