Susumu Ohtsuka · Toshinobu Suzaki
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Fabrice Not *Editors*

Marine Protists

Diversity and Dynamics

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Editors
Susumu Ohtsuka
Takehara Marine Science Station
Setouchi Field Science Center
Graduate School of Biosphere Science
Hiroshima University
Takehara, Hiroshima, Japan

Takeo Horiguchi
Department of Biological Sciences
Faculty of Science
Hokkaido University
Sapporo, Hokkaido, Japan

Fabrice Not
Centre National de la Recherche
Scientifique – CNRS
University Pierre and Marie Curie – UPMC
Station Biologique de Roscoff
Roscoff, France

Toshinobu Suzaki
Graduate School of Science
Kobe University
Kobe, Hyogo, Japan

Noritoshi Suzuki
Institute of Geology and Paleontology
Graduate School of Science
Tohoku University
Sendai, Miyagi, Japan

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Preface

Among the eight major superphyla of eukaryotes, only a handful of organisms have developed multicellularity. Indeed, most of eukaryotic life on Earth is composed of protists, a large portion of which are marine. Thanks to the recent advent of molecular and cellular techniques, we have partially unveiled their potential for both applied and fundamental sciences. Protistology is entering an exciting time, a time of major discoveries, opening up avenues in a variety of scientific fields such as evolution of life, global ecology and biodiversity, and cell biology, among others.

One major drawback to fully understanding the implications of protists for global ecology and fundamental biology, however, is the scattered knowledge inherent in human sectionalism. Bridges between scientific domains are often difficult to build. Fossilized protistan taxa such as foraminifers and radiolarians have traditionally been investigated by paleontologists, while non-fossilized ones are usually studied by biologists and ecologists. Parasitic marine protists such as apicomplexans and myxozoans are separately addressed by fish pathologists. Scientists are well aware of the great complexity of the functioning of ecosystems and the importance of biotic interactions. Holistic approaches to integrate knowledge from various taxonomic groups but also environmental, contextual data are clearly required to address current global issues.

The Great East Japan Earthquake disaster has made us gravely aware that our modern industrialized societies are a tinderbox. We have realized that now is a critical turning point for innovation of new social systems that accelerate wise use of nature. Our ancestors had locally experienced many "collapses" without recognition of these essential laws. Moreover it reminds us of the war movie entitled *Valkyrie* in which we realize we must make compensation for our own actions. The development of protist biology and ecology has a tremendous potential to contribute to resolving current and forthcoming major issues faced by humans in terms of shortages of natural resources for food and energy.

Following a series of joint meetings of the Plankton Society of Japan and the Japanese Association of Benthology, we recognized the importance of putting together our knowledge to further contribute to the development of protistology. We then drew up a list of eminent experts who kindly accepted our request for

contributions, making this unique book on the diversity and dynamics of marine protists possible. We would like to express our sincere thanks to Springer, particularly to Ms. Mei Hann Lee, who offered us this valuable opportunity. We could not have accomplished our tasks without their constant encouragement and support. This book aims at providing students and researchers a multidisciplinary overview of the science of protists in order to establish the basis of modern, integrated protistology.

Takehara, Japan — Susumu Ohtsuka
Kobe, Japan — Toshinobu Suzaki
Sapporo, Japan — Takeo Horiguchi
Sendai, Japan — Noritoshi Suzuki
Roscoff, France — Fabrice Not

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Part I
Diversity, Biology, and Ecology

Chapter 1
Protistan Diversity in Environmental Molecular Surveys

Ramon Massana

Abstract Marine protists include a heterogeneous collection of phototrophic and heterotrophic unicells covering a wide cell size range and belonging to virtually all eukaryotic lineages. They have been identified by microscopy, which allows a reasonable level of resolution for the larger specimens but is clearly insufficient for the smallest ones. Moreover, as occurs with their prokaryotic counterparts, a large majority of marine protists are uncultivable. Molecular tools have revolutionized field studies of protists' diversity, allowing exhaustive species inventories especially when combined with high-throughput sequencing technologies. These surveys have shown that natural assemblages are very diverse, including novel phylogenetic lineages that had remained uncharacterized despite their evident ecological significance. The extent of diversity and novelty is largest within the assemblage of the smallest protists, the picoeukaryotes. The information gathered by sequencing phylogenetic marker genes has been combined with an array of complementary molecular methods such as fingerprinting tools to study diversity changes along spatial and temporal gradients, fluorescence in situ hybridization (FISH) to put a face on the novel lineages and perform specific cell counts, and metagenomics to explore ecological adaptations on the basis of the genetic potential. This chapter presents an overview of the molecular approaches currently applied to gain knowledge on the diversity and function of protists in the environment.

Keywords Environmental molecular surveys • Fingerprinting • FISH • High throughput sequencing • Metabarcoding • Metagenomics • rDNA genes • Single cell genomics • Uncultured microorganisms

1.1 Introduction

A major scientific achievement in the past century has been the realization of the prevalence of the invisible microbial world (Maloy and Schaechter 2006), which

R. Massana (✉)
Department of Marine Biology and Oceanography, Institut de Ciències del Mar (CSIC), Passeig Marítim de la Barceloneta 37-49, E-08003 Barcelona, Catalonia, Spain
e-mail: ramonm@icm.csic.es

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has emerged as a critical component in disciplines as diverse as ecology, evolution, biotechnology and medicine. Microorganisms embrace a huge amount of biological diversity and comprise prokaryotic and eukaryotic unicells. Broadly speaking, prokaryotes have based their evolutionary and ecological success on metabolic versatility, whereas in eukaryotes the success has been based on structural and behavioral innovations, resulting in larger and more intricate unicells and multicellular organisms (Massana and Logares 2013). Eukaryotic microbes are largely protists, a category that includes all eukaryotes except plants, animals and fungi (O'Malley et al. 2013). Being defined by exclusion, it is not surprising that protists do not form a coherent phylogenetic assemblage. Instead, they account for the vast majority of eukaryotic diversity, while plants, animals and fungi only represent three separate branches in a leafy tree (Baldauf 2003). This new eukaryotic tree of life (Fig. 1.1), mostly obtained by multigene phylogeny, is the necessary backbone for environmental sequencing studies (Pawlowski et al. 2012). Indeed, molecular

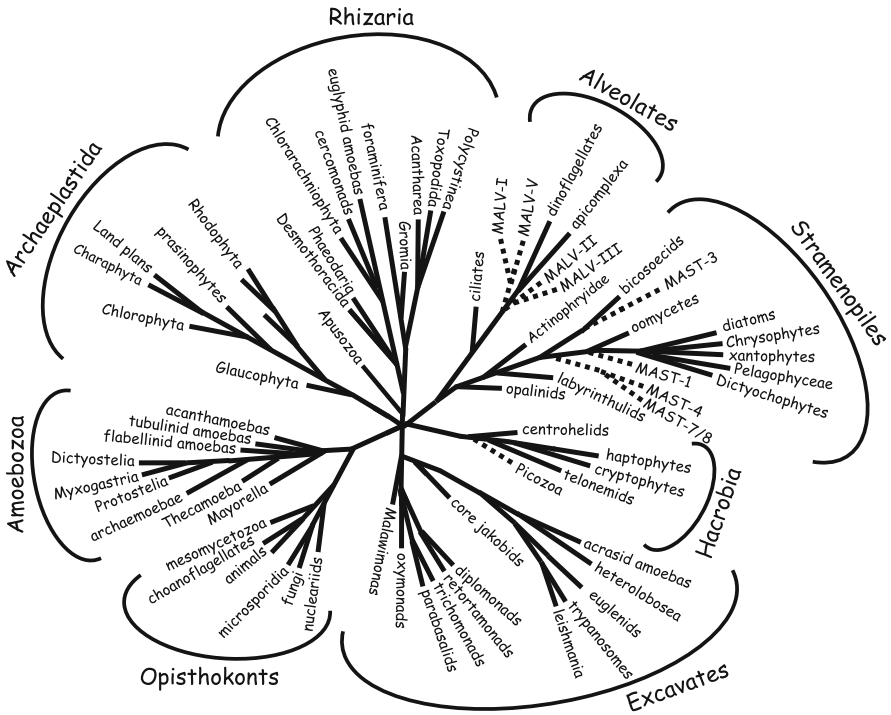

Fig. 1.1 Schematic representation of eukaryotic tree of life displaying the main phylogenetic lineages comprised within each supergroup (Modified from Baldauf 2003). The supergroup Hacrobia has been proposed recently but is not completely consolidated (Burki et al. 2012). *Dotted lines* indicate uncultured lineages detected in environmental surveys

approaches have revolutionized microbial ecology in the last two decades, revealing that most microbes, including microeukaryotes, had escaped culturing and thus formal characterization. Today, molecular tools are providing unique opportunities for exploring the phylogenetic and functional diversity of marine protists. A few recent reviews in this active research discipline present the main taxonomic groups from a phylogenetic and ecological perspective (Vaulot et al. 2008; Massana 2011; Caron et al. 2012). In this chapter I take a different view, focusing on some of the most popular molecular tools, describing their potential to unveil microbial diversity, and highlighting the main results when applied to marine planktonic protists.

1.2 Protists in Marine Environments

Oceans cover about 70 % of the Earth's surface and are critical in processes that have global ecological and socioeconomic importance (Bigg et al. 2003). Life originated in the oceans, which have been the main sites of evolution, and today harbor organisms from a large range of phylogenetic affiliations, body sizes, and trophic modes (Sala and Knowlton 2006). Marine organisms span eight orders of magnitude in cell size, from the smallest bacteria (0.2 μm) to the largest whales (20 m), and protists cover at least half of this size spectrum (Caron et al. 2012), including an amazing and beautiful diversity of cell forms and behavioral traits. Moreover, protists occupy all sorts of marine habitats: water columns from the photic surface to the dark ocean, sediments in the whole bathymetric range, particular ecosystems like light-independent hydrothermal vents, and other marine organisms as symbionts or parasites. Truly, protists are integral members of marine ecosystems (Sherr et al. 2007).

Perhaps it is the plankton where the importance of microbial life is more apparent. In fact, tiny photosynthetic cells suspended in the upper skin of the oceans are responsible for roughly half of the Earth's primary production (Field et al. 1998). Planktonic microbes are categorized into logarithmic size classes: picoplankton (0.2–2 μm), nanoplankton (2–20 μm) and microplankton (20–200 μm). The two larger size classes are composed essentially of eukaryotes, whereas the picoplankton, first thought to be composed of prokaryotes only, was soon recognized to contain also eukaryotic unicells (Johnson and Sieburth 1982). Protist primary producers are present in the three size classes, and the actual size spectrum in a given region has strong implications for the structure of microbial food webs and for carbon export to the deep ocean. In some cases, primary producers are directly consumed by metazoan grazers, such as large diatoms or dinoflagellates being eaten by copepods. In many other cases, however, primary producers are too small and can only be grazed by other protists (Calbet and Landry 2004). Moreover, heterotrophic bacterial production is an important process in plankton, and bacteria can only be eaten by the smallest protists. Food webs established from marine primary and secondary

production depend largely on phagotrophic protists, which abound in the three microbial size classes (Sherr and Sherr 2002). Therefore, protists play fundamental roles as primary producers, consumers, decomposers and trophic linkers in marine food webs.

1.3 Identification of Marine Planktonic Protists

The primary approach for identifying marine protists is morphological inspection, generally done by inverted light microscopy. A few examples within the three size classes are shown in Fig. 1.2. Many of the larger protists have outer conspicuous ornaments and can be readily identified by morphology, this phenotypic appearance often being sufficient for formal species descriptions (Mann 1999). This is the case for many diatoms, dinoflagellates, ciliates, acantharians and foraminifera that dominate the microplankton. In some cases, even for these large cells, standard light microscopy is not enough to recognize closely related species and more elaborated protocols are required, such as infraciliature silver staining for ciliates, or scanning electron microscopy for the silica shell designs in diatoms (Amato et al. 2007). But the true limitation of morphological identifications appears when dealing with the smallest protists. Within the nanoplankton, many cells can be at best assigned to a broad taxonomic group, whereas the problem is more severe within picoeukaryotes,

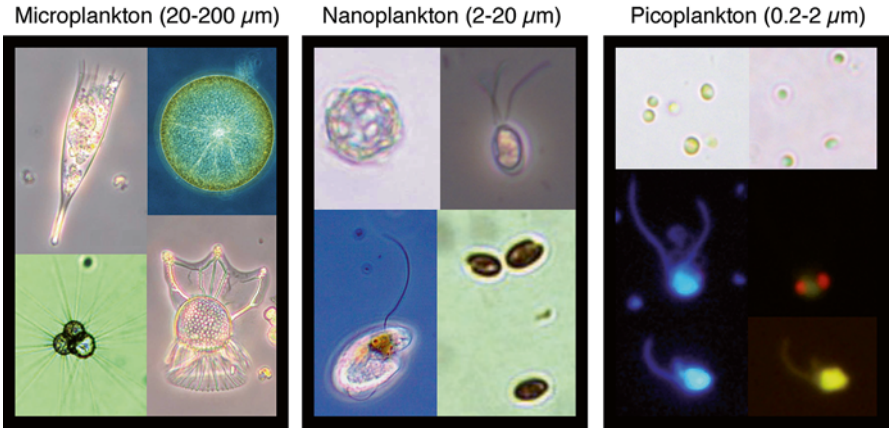

Fig. 1.2 Light microscopy pictures of marine protists. Names listed clockwise starting *upper left*. Microplankton: *Dadayiella ganymedes* (ciliate), *Coscinodiscus* sp. (diatom), *Ornithocercus magnificus* (dinoflagellate), *Globigerinella* sp. (foraminifera). Nanoplankton: unidentified coccolithophorid, unidentified flagellate, *Rhodomonas salina* (cryptophyte; culture), *Oxyrrhis marina* (dinoflagellate; culture). Picoplankton: *Pelagomonas calceolata* (pelagophyte; culture), *Micromonas pusilla* (mamiellophyte; culture), unidentified phototrophic flagellate (*middle*) and heterotrophic flagellate (*lower*), observed by epifluorescence under UV radiation (*left*) or blue light (*right*) (Images courtesy of L Arin, A Calbet, AM Cabello, and I Forn)

which are hardly seen by inverted microscopy. A proper quantification of picoeukaryotes requires epifluorescence microscopy, an approach that provides less morphological characters for inspection, just cell size, general shape, and the presence of plastids and flagella (Fig. 1.2). As a consequence, and with a few exceptions, picoeukaryotes cannot even be classified into a high-rank taxonomic group.

Classical microbiology has addressed the identification problem of bacteria, archaea and small protists by isolating these organisms in clonal cultures, which allows an inspection of their metabolism and ecophysiology, macromolecular composition, gene marker sequences and, in eukaryotes, the internal ultrastructure by transmission electron microscopy. Thus, many eukaryotic taxa have been defined by analyzing cultured strains, and sometimes cells that are apparently the same on superficial morphological inspection turn out to belong to very different lineages (Potter et al. 1997). A critical caveat with this approach, however, is that natural microbes are often reluctant to be isolated in clonal cultures, as revealed by seminal molecular approaches, constituting the uncultured microbial majority (Rappé and Giovannoni 2003). This implies that culturing-driven studies are ignoring the true diversity of natural assemblages.

New insights into the microbial world arrived with molecular tools a few decades ago (Giovannoni et al. 1990). They are independent of morphology and cultivability, and instead use the information of DNA sequences to characterize organisms and, eventually, to reconstruct the evolutionary paths of life. Using a conserved gene like the one coding the RNA of the small subunit of the ribosome (18S rDNA in eukaryotes), it is possible to compare all extant living beings. This gene is widely used in ecological and evolutionary studies, since it is universal, functionally conserved, highly expressed in ribosomes, and includes conserved and variable regions ideal for probe design (Amann et al. 1995). Molecular tools have diversified, and there is a tool best suited for different questions (Fig. 1.3). This ranges

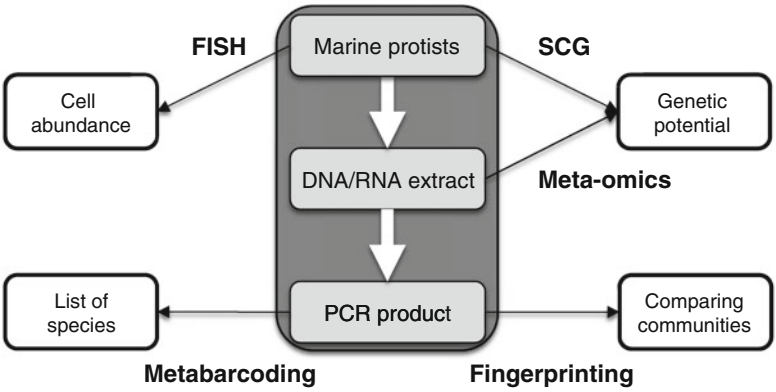

Fig. 1.3 Simplified representation of molecular approaches for microbial ecology studies, including the material assayed (*central boxes*) and the techniques used (*names in bold*) to address specific questions (*outer boxes*). *FISH* fluorescence in situ hybridization, *SCG* single-cell genomics, *Meta-omics* metagenomics and metatranscriptomics

from identifying the species list in a given sample (metabarcoding), comparing communities (fingerprinting), counting the cell abundance of given taxa (FISH), or analyzing the genetic potential of cells (SCG) and communities (meta-omics). This multifaceted approach is now used to investigate the abundance, diversity, and function of marine protist assemblages.

1.4 Environmental Sequencing of Phylogenetic Marker Genes

Seminal molecular studies of marine protists' diversity were based on sequencing 18S rDNA genes directly from the environment (Díez et al. 2001; López-García et al. 2001; Moon-van der Staay et al. 2001). This is done by extracting DNA from the microbial assemblage, amplifying mixed 18S rRNA genes by PCR (polymerase chain reaction) using eukaryotic primers, cloning and sequencing the PCR products, and comparing the sequences with public databases. The first reports targeted the picoeukaryotic fraction and revealed an astonishingly large diversity, a view confirmed in later studies targeting other genes, such as the plastidic 16S rDNA, rbcL, and psbA (Massana 2011). Thus, morphologically similar picoeukaryotes under the microscope belong to groups scattered throughout the eukaryotic tree of life. Furthermore, while some of these sequences match well-known species, others form phylogenetic clades (sets of related sequences) that are distant from any characterized lineage and so represent novel and unexplored diversity. These striking results have opened avenues for new ecological and evolutionary explorations.

Many sequences detected in picoeukaryote surveys form novel clades that are sisters to dinoflagellates collectively named Syndiniales or marine alveolates. They comprise five major groups (MALV-I to -V), which are in turn highly subdivided (Guillou et al. 2008). Often they explain the majority of clones in each sample, although they are clearly overrepresented in DNA-based surveys (see later). Also common in picoeukaryote surveys is a collection of novel lineages within basal heterotrophic stramenopiles, named marine stramenopiles or MAST. Recent reports indicate the existence of 18 independent MAST clades (Fig. 1.4), and aspects of their ecological attributes are inferred by analyzing the origin of sequences (Massana et al. 2014). Thus, some groups are aerobic picoeukaryotes (black dots in Fig. 1.4), while others have been found only in anoxic waters and sediments (white dots). The remaining groups are more complex, and this can be explained by their internal diversity. Thus, two lineages within MAST-2 and -12 have made the marine–freshwater transition, and several MAST-1 and -3 clades include larger cells adapted to anoxic systems. MAST lineages remain uncultured, with the exception of the recently described *Incisomonas marina* within MAST-3 (Cavalier-Smith and Scoble 2013). The picobiliphyta (Not et al. 2007), now known as Picozoa (Seenivasan et al. 2013), is another interesting group, not because of its clonal abundance but because

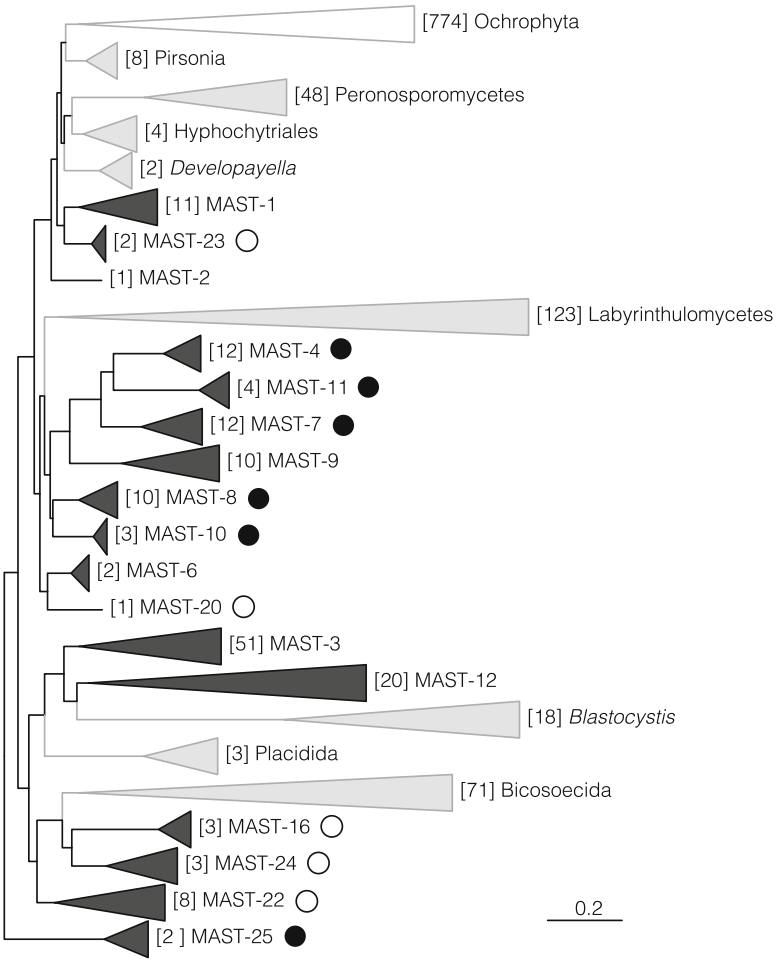

Fig. 1.4 High-rank phylogenetic tree of stramenopiles using the 18S rDNA gene (Redrawn from Massana et al. 2014). Formal classes are shown in collapsed *grey triangles* and MAST ribogroups in *black triangles* (ochrophyta is in *white* because it contains many formal classes). Triangle width is proportional to the phylogenetic diversity of the group. The number of sequences within groups (clustered at 98 % similarity) is indicated in *brackets* before the names. Ribogroups that are clearly surface marine picoeukaryotes are marked with *black dots*, whereas those that are anaerobic protists are noted with *white dots*

it represents a high-rank novel taxon, with an unclear position in the eukaryotic tree of life. Finally, molecular surveys have also identified novelty within known groups, such as novel diversity within prasinophytes (Viprey et al. 2008), choanoflagellates and chrysophytes (del Campo and Massana 2011). Indeed, the novel eukaryotic diversity detected in marine protist assemblages occurs at all phylogenetic scales.

The cloning and sequencing approach is critical for a fine characterization of the phylogenetic diversity of marine protists. Complete 18S rDNA sequences of the known and novel taxa are collected and compiled into reference datasets, which are crucial backbones for later studies (Guillou et al. 2013). Indeed, a comprehensive phylogeny of all taxa, and specially of the novel ones, requires complete and well-curated sequences. However, clone libraries have a limited sequence output and are insufficient for a full description of microbial diversity. This is now possible by high-throughput sequencing (HTS) approaches (Margulies et al. 2005), which are revolutionizing all biological sciences disciplines. These do not require the cloning step, and massive sequencing is done in parallel (Metzker 2010). They started with pyrosequencing (454 Life Sciences/Roche) and today the Illumina technology (Illumina/Solexa), with its capacity to provide millions of sequences up to 400 bp for a reasonable price, is more popular. Given the huge sequence output, bioinformatics (computers, software, and human power) is emerging as a new necessity for many microbial ecology labs. In diversity studies, HTS targets a short gene fragment from the mix of community genomes and the whole process is known as tag sequencing or metabarcoding. Basic routines in metabarcoding analyses include a strict check of sequence quality, clustering sequences in OTUs (Operational Taxonomic Units) based on similarity, and phylogenetic classification of OTUs using reference databases. For the first time, HTS provides exhaustive inventories of protists' diversity and allows exploration of the rare biosphere (Pedrós-Alió 2012). Rare taxa always numerically dominate the OTU list, although it is not always easy to differentiate between real diversity and sequencing errors (Kunin et al. 2010).

Here I use data from the BioMarKs project (www.biomarks.eu) for an overview of the phylogenetic diversity of coastal marine protists. The dataset derives from six European sites sampled for environmental DNA in three size fractions (Logares et al. 2012). About 3.5×10^5 pyrosequences (pyrotags from now) have been obtained, and here I show the relative abundance of pyrotags affiliating to phylogenetic groups, at a class level for the known diversity (Adl et al. 2012), averaging the data from all samples (Fig. 1.5). The microplankton is dominated by only five groups: dinoflagellates, radiolaria, diatoms, ciliates and MALV (in this order), which together account for 98 % of the pyrotag signal. Within the nanoplankton, three taxa are particularly important: dinoflagellates, diatoms and MALV, explaining 77 % of the signal, still giving a place for other groups at moderate relative abundance. The picoplankton include many groups, with MALV and dinoflagellates accounting for 56 % of the signal and some groups in relative abundances above 4 %: mamiellophytes, ciliates, radiolaria, MAST and cryptophytes. The remaining groups generally contribute more in the picoplankton than in the other two size fractions. Clearly picoeukaryotes are the most diverse in terms of the presence and relative abundance of phylogenetic lineages. This overview provides a first glimpse of the taxonomic composition of coastal protists, still considering that sequence abundance is not directly linked to cell abundance (see later). Moreover, similar data for the open ocean are urgently needed.

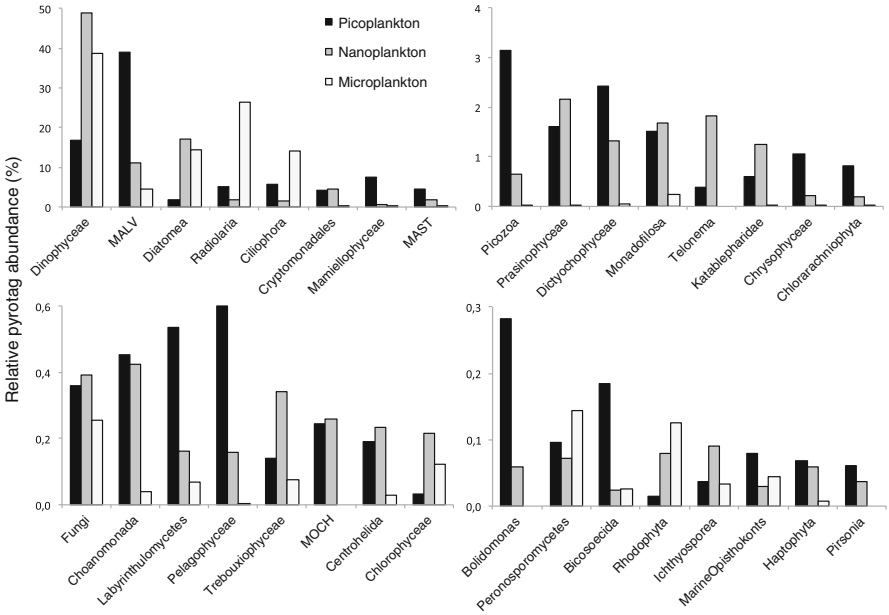

Fig. 1.5 Analysis of the phylogenetic diversity, mostly at class level, of marine protists as reported by relative pyrotag abundance (average of 13 samples) within three size fractions: picoplankton (*black bars*), nanoplankton (*grey bars*) and microplankton (*white bars*). Data derive from pyrosequencing environmental DNA collected at six European coastal sites during the BioMarKs project (www.biomarks.eu) (Note the scale change between panels)

1.5 Fingerprinting Analysis for Community Comparison

Phylogenetic diversity studies by sequencing marker genes were soon accompanied by fingerprinting tools, which provide a characteristic banding pattern per sample (bands representing taxa) for fast community comparisons. The most common techniques are denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), and automated ribosomal interspacer analysis (ARISA). All start with a PCR amplification of marker genes from community DNA. Individual sequences in the PCR mixture are then separated by electrophoresis based on their melting domains (DGGE), the location of their first restriction site (T-RFLP), or the size of their ITS region (ARISA). Based on simultaneous sample fingerprints, a matrix is constructed with the presence (and relative intensity) of each band in all samples, which is then used to compare communities based on their similarity by clustering or ordination methods (Fromin et al. 2002). Moreover, fingerprinting tools allow a rough phylogenetic classification of the bands observed, both by direct sequencing (DGGE) and by comparing band sizes

with reference datasets (T-RFLP and ARISA). Although not very accurate, the phylogenetic identification of bands is very useful for a preliminary study of the dynamics of specific taxa.

Fingerprinting tools, generally targeting rDNA genes, have been used to assess changes in protist diversity along spatial and temporal gradients in the sea. DGGE analyses indicate that the most important factor determining the composition of picoeukaryotes is depth in the water column, while the composition at the same water layer may be relatively homogeneous over broad geographic distances (Díez et al. 2004; Not et al. 2008). In the first study, two main diversity assemblages appear along the vertical profile in one Antarctic station, samples up to 100 m depth and deeper samples, as shown in dendrograms grouping samples (Fig. 1.6a). At the surface, community composition is rather stable along the large geographic range

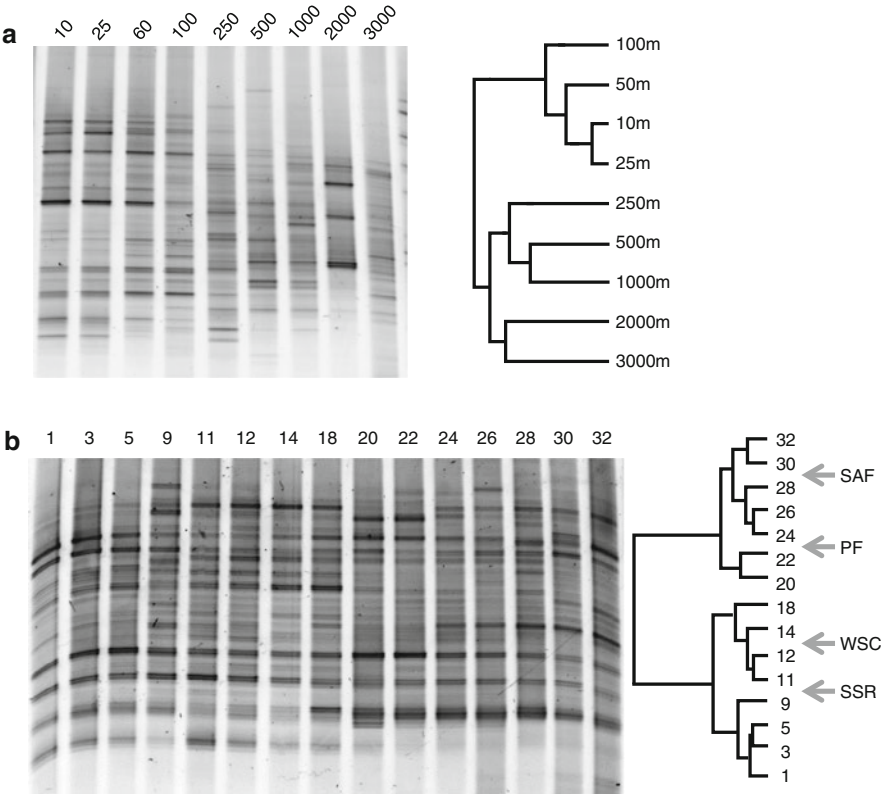

Fig. 1.6 Diversity changes of marine picoeukaryotes in the Southern Ocean analyzed by DGGE gels and dendrograms (From Díez et al. 2004). (**a**) Vertical profile (from 10 to 3000 m depth) at station 18 in the Antarctic zone. (**b**) Horizontal profile at surface from the Weddell Sea close to the ice edge (station 1, latitude 63°S) to the sub-Antarctic zone (station 32, 55°S), crossing the South Shetlands ridge (*SSR*) and several oceanographic fronts: Weddell–Scotia confluence (*WSC*), polar front (*PF*) and sub-Antarctic front (*SAF*)

studied (over 1000 km from the Weddell Sea to the sub-Antarctic zone), but punctuated when crossing oceanographic fronts or bathymetric features (Fig. 1.6b). Temporal changes during several years have been studied recently by T-RFLP in a coastal station (Kim et al. 2014). The study highlights modest month-to-month changes in protist composition, and a large interannual variability, pointing to a significant non-periodic temporal component. However, for specific depths and particular temporal windows, there is also the evidence of seasonality, indicating an annual resetting of protist taxa.

The modest spatial and temporal variation observed in natural protist communities contrasts with the drastic changes observed in a few days in bottle incubations or mesocosms, driven by the modification of grazing pressure or simply by bottle effects (Countway et al. 2005). In particular, the fact that picoeukaryotes remain stable over large oceanic areas and short time scales corroborate their participation in the ocean's veil (Smetacek 2002). On a global scale, and combined with identical sequences retrieved in distant sites, it can be suggested that few geographic barriers limit the dispersal of marine picoeukaryotes, so the same species can be found when conditions are similar. Above this ocean's veil, larger cells may display more stochastic dynamics, and truly biogeographic barriers may limit gene flow or species dispersal within larger protists like microplanktonic diatoms (Casteleyn et al. 2010).

Nowadays, HTS has the potential to eclipse traditional fingerprinting tools thanks to the development of cheaper and faster HTS routines, including the optimization of user-friendly bioinformatic pipelines. For that, a separate PCR is done per sample with a primer having a short identifying sequence (a barcode), PCR products are mixed and sequenced together, and during OTU clustering the number of tags per OTU is computed per sample. The generated OTU table is similar to the matrices derived from DGGE or T-RFLP profiles, and can be used for multivariate analyses. And, interestingly, each OTU is now not simply a band but a high-quality sequence with an unambiguous phylogenetic placement. Nevertheless, it is unlikely that traditional fingerprinting methods will be completely replaced, since they will always be faster, simpler and cheaper for initial sample comparisons. Studies using the comparative and phylogenetic potential of HTS are still scarce (Stoeck et al. 2009). Recently, Logares et al. (2014) have used Illumina to assess protist diversity in BioMarKs samples. Protists' assemblages group mostly by size and the rare biosphere is a prevalent feature, apparently following quite stable assembling rules, in these assemblages.

1.6 Beyond Sequences and Back to the Cells

Sequence datasets provide fundamental views of in situ diversity, but may be far from yielding quantitative estimates of the detected taxa. Some factors modifying the conversion of sequence-to-cell relative abundance are methodological, such as inefficient DNA extraction of particular lineages, or biases occurring during the PCR, by which some sequence types are over- or under-amplified resulting in PCR

pools not reflecting initial ratios (Wintzingerode et al. 1997). Other factors derive from intrinsic features of the samples, such as the retention of dissolved or detrital DNA in the filters, or large variations in the rDNA operon copy number among taxa, up to several orders of magnitude among similarly sized cells (Zhu et al. 2005; Gong et al. 2013). Environmental sequencing using RNA extracts (via reverse transcription to cDNA) avoids some problems, such as the detrital bias (RNA is more labile than DNA), or the varying rDNA copy number (the number of ribosomes is a better proxy of cell biomass). Thus, protist surveys of the same sample via DNA or RNA yield different results (Stoeck et al. 2007). In picoeukaryotes, differences are mostly driven by large overestimations in DNA surveys of MALV, likely having very high rDNA copy numbers, and radiolaria (Not et al. 2009). Within the BioMarKs dataset, MALV-I and MALV-II account for 15.4 and 19.7 % of pyrotags based on DNA but only 1.8 and 2.0 % based on RNA. Radiolaria (excluding Acantharea) are less represented in these coastal sites but still its relative abundance decreases 15 times (from 0.46 to 0.03 %).

Surveys based on RNA extracts are probably closer to in situ diversity, but to obtain true cell abundances a quantitative method such as FISH (fluorescence in situ hybridization) is required. FISH effectively closes the rRNA approach that starts with sequencing surveys, taking the reverse path, from sequences back to cells (Amann et al. 1995). During FISH, fixed microbial cells immobilized on a filter are hybridized with a taxon-specific 18S rDNA probe labeled with a fluorochrome. Probes attach to the ribosomes of target cells, which are then "illuminated" by epifluorescence microscopy. Crucial steps are probe design, the selection of a short sequence (~20 bp) specific for a given group (a species, class, or domain), and probe specificity tests, done with cultured cells as negative and positive controls. When a culture is not available (as in novel diversity), a natural sample where these sequences have been found is used as a positive control, and results should be considered critically. Once optimized, FISH allows quantification of specific taxa within natural samples, but it is time-consuming since it targets one taxon at a time. When combined with experiments and direct observations, FISH gives access to the function and ecological role of specific taxa.

The novel eukaryotic clades detected in molecular surveys were obvious targets for seminal FISH studies, primarily to put a face (rough morphology) to these unknown cells and then to give data on their abundance and distribution. This technique has revealed that cells from three MAST ribogroups have a size of 2–5 μm (Fig. 1.7a), lack chloroplasts, often have one flagellum, and are widely distributed in the sea at abundances of tens to hundreds of cells per milliliter (Massana et al. 2006). Applying FISH after short-term ingestion experiments using fluorescently labeled food proves that MAST cells are able to ingest bacteria (Fig. 1.7b) and unveils differences in grazing rates and prey spectra (Massana et al. 2009). The typical cell size, distributional patterns and grazing activity indicate functional diversity of each particular ribogroup, which likely occupies a different ecological niche. MAST cells appear to represent a significant share in heterotrophic flagellates assemblages (HF), and a single group, MAST-4, accounts for 9 % of HF cells on average.

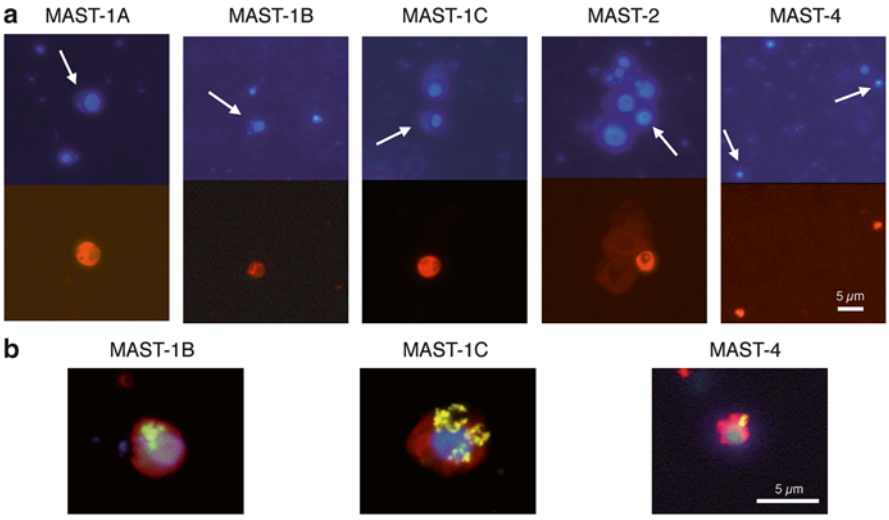

Fig. 1.7 (**a**) Epifluorescence micrographs of MAST cells belonging to five ribogroups. *Upper panels* show DAPI-stained microeukaryotes and *bottom panels* show the same field with FISH-stained cells. Only a few cells stained by DAPI (*white arrows*) are labeled with the specific MAST probe. (**b**) Bacterivory of MAST cells. Picture overlays of the same cell observed under UV radiation (blue nucleus after DAPI staining), green light (red cytoplasm after FISH) and blue light excitation (yellow fluorescently labeled bacteria) (Note that each MAST cell has ingested a different number of labeled bacteria)

With respect to MALV ribogroups, which include among others the parasite *Amoebophrya* sp. (Coats and Park 2002), the FISH technique has allowed co-localizing of MALV cells within putative hosts. Thus, the life cycle of MALV-II cells has been reconstructed, initiated by a free-living dinospore that infects a dinoflagellate, proliferates within the host cell, and forms a trophont that leaves the host to disaggregate into new dinospores (Chambouvet et al. 2008). Combined with single-cell analysis of host cells, it turns out that MALV-II cells parasitize mostly dinoflagellates, whereas MALV-I cells have a wider host spectrum, including ciliates, radiolarians, and fish eggs (Harada et al. 2007). Furthermore, FISH confirms the large MALV overestimation in DNA surveys, as counts of MALV-II dinospores are generally low (0.4–3.1 % of picoeukaryotes) in open sea samples (Siano et al. 2010). Still, these moderate counts, together with high prevalence (2–10 % of dinoflagellate cells appear infected) and a wide range of host species, suggests that MALV may be important parasites in the marine realm. Thus, parasitism emerges as an interaction that can severely influence microbial food webs, community structure and population dynamics.

The third novel group analyzed by FISH was the high-rank novel lineage Picozoa. Initially they were described as picoeukaryotes with a phycobilin-containing chloroplast (Not et al. 2007). However, further studies showed that these cells are generally larger (Cuvelier et al. 2008) and lack chloroplasts (Yoon et al. 2011), facts

confirmed with the first representative culture of the phylum (Seenivasan et al. 2013). Finally, FISH has also been applied to assess the abundance and distribution of well-known groups in the environment, such as mamelliophytes (Not et al. 2004), prymnesiophytes, pelagophytes, and chrysophytes (Jardillier et al. 2010). These four groups seem to dominate photosynthetic picoeukaryotes. Presently, the FISH approach is limited by the probes available, so major efforts in probe design and optimization are needed.

1.7 The Genomic Era

All previous sections in the chapter are based on the study of a single gene, most notably the 18S rDNA, and it is obvious that assessing complete genomes would provide more detailed insights into marine protists' diversity and dynamics. From an evolutionary perspective, this allows performance of multigene phylogeny for a better reconstruction of the ancient events in eukaryotic evolution (Burki et al. 2012). Moreover, the nuclear genome is a mosaic of genes with divergent evolutionary histories, including prokaryotic genes imported during primary endosymbiotic events that originated plastids and mitochondria, and eukaryotic genes taken during secondary or tertiary endosymbiosis (Timmis et al. 2004). In addition, horizontal gene transfer among eukaryotes can also occur, like the acquisition of fungi-like features in oomycetes (Richards et al. 2011). Genome analysis is thus fundamental for understanding the complex histories of eukaryotic evolution. From an ecological perspective, genomes can provide glimpses of the potential of eukaryotic cells. General issues include finding genes involved in sexuality and thus population structuring forces, in processing organic matter as food (lysozymes, chitinases), in uptaking small molecules (transporters), or in signal reception and transduction (kinases). New metabolic repertoires are not expected in eukaryotes, which are rather uniform as compared with the huge flexibility of prokaryotes. Genomes are analyzed from different perspectives, and may lead to hypotheses to be tested experimentally. Together, genomic and ecophysiological studies contribute to identify the traits that make an organism successful in the environment (Litchman and Klausmeier 2008).

Currently there are >300 eukaryote genomes completed (http://www.genomesonline.org/), and most are from multicellular organisms (82.5 %) or microeukaryotes with applied interest like parasites or pathogens (9.5 %). The remaining 8 % are free-living protists and include only cultured strains, which are grown in the lab to obtain enough DNA for genome sequencing. They belong to widely separated eukaryotic groups such as chlorophytes (7 genome projects), amoebozoans (4), ciliates (3), cryptophytes (3), diatoms (2), cercozoans (2), and haptophytes, pelagophytes, apusozoans and choanoflagellates (1 each). Only a few of these free-living species with the genome completed (and some additional ones in the list of ongoing projects) can be seen as ecologically relevant marine protists. Given this shortage, and the fact that eukaryotic genomes can be large and structurally complex, the

Marine Microbial Eukaryote Transcriptome Sequencing Project initiative (MMETSP, http://marinemicroeukaryotes.org/) was launched recently to provide the transcriptome (sequencing pool of total mRNA) of marine microeukaryotes. The program aims to increase the genomic knowledge by creating catalogs of genes that specify how these organisms thrive in diverse habitats and influence marine ecosystems. Currently 535 projects have been sequenced and are ready for bioinformatic analysis. Most (82 %) are from algal species—diatoms, dinoflagellates, prymnesiophytes, and prasinophytes being the most represented groups.

Both genome and transcriptome projects of microbial eukaryotes are based on cultured strains. The culturing requirement explains the low representation of heterotrophic protists within MMETSP projects, which appear to be much more prone to culturing bias than algal species (del Campo et al. 2013). Thus, important bacterivorous heterotrophic flagellates, such as the diverse, widespread and abundant MAST cells, have mostly escaped culturing and therefore genome characterization. The same occurs within larger heterotrophic flagellates, which are much less abundant but sometimes important biomass contributors (Arndt et al. 2000), and even within some algal species difficult to culture. Today, single-cell genomics (SCG) provides the opportunity to access microbial genomes avoiding the culturing step (Stepanauskas 2012). It combines the sorting capacity of modern flow cytometers, which deposit single cells in wells in a high-throughput manner, and new routines for whole-genome amplification, able to generate micrograms of DNA from minute (femtograms) quantities of single cells. Sequencing of single-cell genomes has been successfully applied to marine prokaryotes (Woyke et al. 2009) and only recently to heterotrophic protists (Yoon et al. 2011). Currently it has some limitations, such as amplifying contaminant DNA, uneven genome coverage during amplification, or the production of chimeric genome arrangements. Solutions to these limitations are being explored (Rodrigue et al. 2009), and it is expected that SCG will give access to the genomes of the microbial uncultured majority.

An important target of the genomic era is the gene content of microbial communities (DeLong and Karl 2005). Metagenomics, direct sequencing of environmental DNA, reveals community gene repertoires and metabolic potential, while metatranscriptomics, sequencing of community RNA, provides insights into realized functions. Seminal studies cloned and sequenced large DNA fragments, allowing to study complete operons and to link phylogenetic and functional markers (Béjà et al. 2000). In a second phase, small DNA fragments were cloned by routine methods and sequenced at random. A large database of marine metagenomes has been obtained by shotgun sequencing during the Global Ocean Sampling expedition (Rusch et al. 2007). This has fueled the microbial research agenda for years, but has given little access to protists, often removed by filtration before the analysis. The recent -omics efforts are taking full advantage of HTS methods. Analyzing metagenomes using Illumina sequencing involves several steps, starting with a strict control to keep only high-quality reads, which are then assembled in contigs. This is perhaps the largest challenge given the shortness of the reads (just a few hundred base pairs) and the large diversity of natural assemblages. Contigs are separated by taxonomic origin to retain putative eukaryotic contigs, gene prediction is done by

standard genomic tools and genes are annotated into functional categories. This process highlights again two of the bottlenecks of the field: the necessity of bioinformatic tools and experience, and the paucity of reference datasets (here genomes of ecologically relevant marine protists) to identify and annotate metagenomic information. Metagenomic surveys of marine protists have not been yet published, but several are in progress.

1.8 Closing Remarks

Protists are of paramount importance for the eukaryotic tree of life, since they comprise the majority of extant eukaryotic lineages. The marine plankton harbors a large variety of protists, which play fundamental roles as primary producers, grazers, organic matter decomposers, and parasites. Many marine protists cannot be identified morphologically or by culturing, so their study essentially requires molecular tools, which provide detailed views of protist diversity within size classes. The picoeukaryotes emerge as the most diverse and the most unknown. Molecular techniques have opened new possibilities in protist ecology, allowing identification of the cells that inhabit an environment, to study temporal and spatial community dynamics, and to infer their genomic content and functional adaptations.

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Chapter 2
Unusual Features of Dinokaryon, the Enigmatic Nucleus of Dinoflagellates

Yasuhiro Fukuda and Toshinobu Suzaki

Abstract The dinoflagellate is a member of the eukaryotes that belong to a diverged protist group, Alveolata. Because the nuclei show several unusual features not observed in other eukaryotes, the nucleus in the "core" or typical dinoflagellate is especially called a "dinokaryon". Chromosomes in the dinokaryon are condensed throughout the whole cell cycle and show a cholesteric liquid crystal organization. Its nucleosome lacks the "beads on a string" structure which is commonly observed in eukaryotic nuclei. Despite the existence of canonical histone protein-coding genes, those proteins are not found in the dinokaryon, while certain proteins showing amino acid sequences similar to bacterial or viral proteins are abundantly contained in the dinokaryon. The dinoflagellate genome carries some rare bases such as 5-hydroxymethyluracil, and TTTT repeats exist as a possible transcription initiator element instead of the TATA box. A comprehensive transcriptome analysis of the EST database using a variety of dinoflagellates including ancestral to divergent species showed that SL trans-splicing is required for transcript maturation.

Keywords Dinoflagellate • Dinokaryon • Cholesteric liquid crystal • Chromosome • *Oxyrrhis* • *Noctiluca* • *Hematodinium* • NP23 • DVNP • HCc

2.1 Introduction

Dinoflagellates are essentially biflagellated protists and inhabit most marine and freshwater aquatic environments, from tropical to polar waters. Dinoflagellates are a member of the Alveolata (Cavalier-Smith 1991, 1993; Adl et al. 2005, 2012). More than 4,500 species, about half of which are fossils, have been recorded.

Y. Fukuda (✉)
Department of Biodiversity Science, Division of Biological Resource Science, Graduate School of Agricultural Science, Tohoku University, 232-3, Yomogida Naruko-onsen, 989-6711, Osaki, Miyagi, Japan
e-mail: yasufuku@bios.tohoku.ac.jp

T. Suzaki
Graduate School of Science, Kobe University, Kobe, Hyogo, Japan
e-mail: suzaki@kobe-u.ac.jp

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Half of the species possess photosynthetic capability, while the other species are non-photosynthetic or parasitic (Taylor et al. 2008). The photosynthetic species play important roles in the marine ecosystem, because they are second only to diatoms as primary producers (Field et al. 1998). Some photosynthetic species known as "zooxanthellae" are beneficial intracellular endosymbionts of corals, sea anemones, and jellyfish, and the symbionts provide their photosynthetic products to the host (Chap. 17). Their transient massive propagation gives rise to the red tide that sometimes kills fish or shellfish (Chap. 24). Moreover, some dinoflagellates are known to cause fish and shellfish to be lethally toxic to humans (Steidinger 1983; Taylor 1989). The diversity of dinoflagellates was well reviewed by Taylor et al. (2008).

Dinoflagellates possess several characteristics in the cell. Dinoflagellates are essentially biflagellated, and the two flagella are differentiated morphologically and functionally (Fig. 2.1a). A single layer of flattened vesicles (alveoli or amphiesmal vesicles) lies beneath the cell membrane (Fig. 2.1b). Plastids of typical dinoflagellates are originated from the red algae via secondary endosymbiosis (Fig. 2.1c) (Falkowski et al. 2004; Keeling 2004; Zhang et al. 1999), whereas in several dinoflagellate lineages, their plastids have been superseded by other plastids from various algal species including dinoflagellates via tertiary endosymbiosis (Gabrielsen et al. 2011; Ishida and Green 2002). Thus, the dinoflagellates have been regarded as a laboratory of endosymbiosis and establishment of organelles from endosymbionts

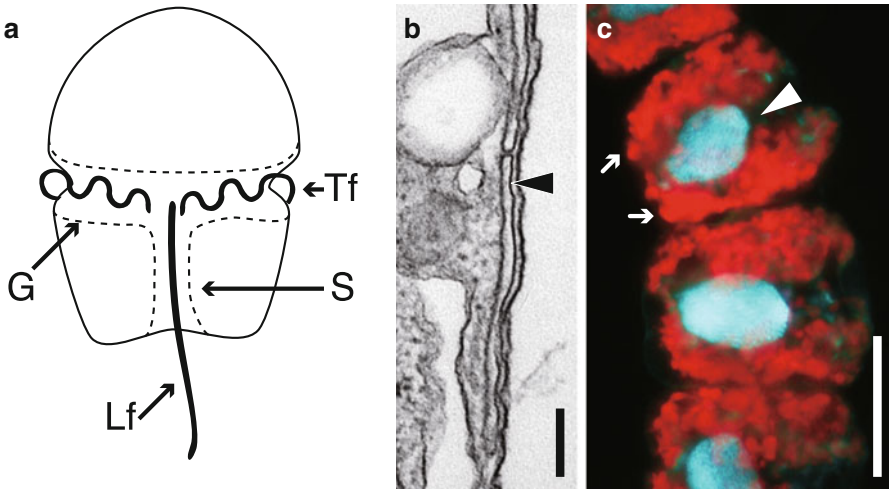

Fig. 2.1 Morphological characters of dinoflagellate cells. (**a**) A schematic illustration of a typical dinoflagellate cell as viewed from the ventral side. *Tf* transverse flagellum, *Lf* longitudinal flagellum, *G* girdle (cingulum), *S* sulcus. (**b**) An electron micrograph showing amphiesmal vesicles (*black triangle*) in *Oxyrrhis marina* (bar = 100 nm). (**c**) Fluorescence micrograph of a chain of *Gymnodinium catenatum* cells. Granular structures in *red color* (*arrows*) represent autofluorescence of plastid. A nucleus is indicated with a *white triangle*, which is stained with DAPI (bar = 50 μm)

(Morden and Sherwood 2002). Like Apicomplexans, being a sister group to dinoflagellates, the mitochondrial genome carries only three protein-coding genes for the electron transport chain (cytochrome oxidase subunit 1, cytochrome oxidase subunit 3, and cytochrome b) and fragmented large-subunit and small-subunit rRNA genes. Extensive substitutional RNA editing and RNA trans-splicing are required to make proper transcripts (Jackson et al. 2007; Nash et al. 2008; Waller and Jackson 2009).

In particular, the nucleus of the dinoflagellates shows several unusual features that are extremely different from those of most other eukaryotes. For instance, typical histone proteins are not detectable in the nucleus, while small basic proteins resembling bacterial HUs exist abundantly. Chromosomes are continuously condensed throughout the whole cell cycle, and stacked arcs of filaments appear in the chromosome (see Fig. 2.4c). Nuclear envelopes do not disappear during mitosis, while cytoplasmic tunnels crossing through the nucleus are formed, and mitotic spindle microtubules pass within the tunnels. Chromosomes are connected to and divided with the spindle, being mediated by the nuclear envelope. Nucleofilaments are not the type of well-known "beads on a string" structure, but are smooth and thin filaments (e.g., Rizzo and Nordan 1974; Hamkalo and Rattner 1977; Li 1984; Vernet et al. 1990). Because of such unique characteristics, the nucleus is specially called the dinokaryon (Spector 1984). Since these nuclear features, especially the lack of histone proteins and the difference in nucleosome organization, resemble those of prokaryotes, dinoflagellates were previously ascribed to "Mesokaryotes" which was considered as an intermediate group of organisms between prokaryotes and eukaryotes (Dodge 1965). However, development of molecular phylogenetic analysis and detailed ultrastructural studies finally concluded that the mesokaryote concept was incorrect (Raikov 1995). Dinoflagellates unmistakably belong to the eukaryotes, and their chromosomes showing unusual features are considered to have evolved from the typical eukaryotic and histone-containing chromosomes.

Here, we will summarize the accumulated knowledge on the nuclear features, especially in the chromosome architecture and nuclear proteins.

2.2 Phylogeny of Dinoflagellates

First, we have to introduce the phylogenetic relationship between dinoflagellates and their neighbors, because the unusual unclear features as described below are in good correlation to their phylogenetic positions. The dinoflagellates belong to a monophyletic group, the Alveolata, which consists of three major groups (apicomplexans, ciliates, and dinoflagellates) and minor groups such as perkinsids, colpodellids, and *Chromera*. Figure 2.2 shows a simplified schematic relationship and representative species of these organisms (Cavalier-Smith 1991, 1993; Adl et al. 2005, 2012).

Apicomplexans, being obligate parasitic protists, are characterized by their apical complex structure. The name of the phylum Apicomplexa is derived from this unique cell structure. Representative species of the apicomplexans are *Plasmodium*

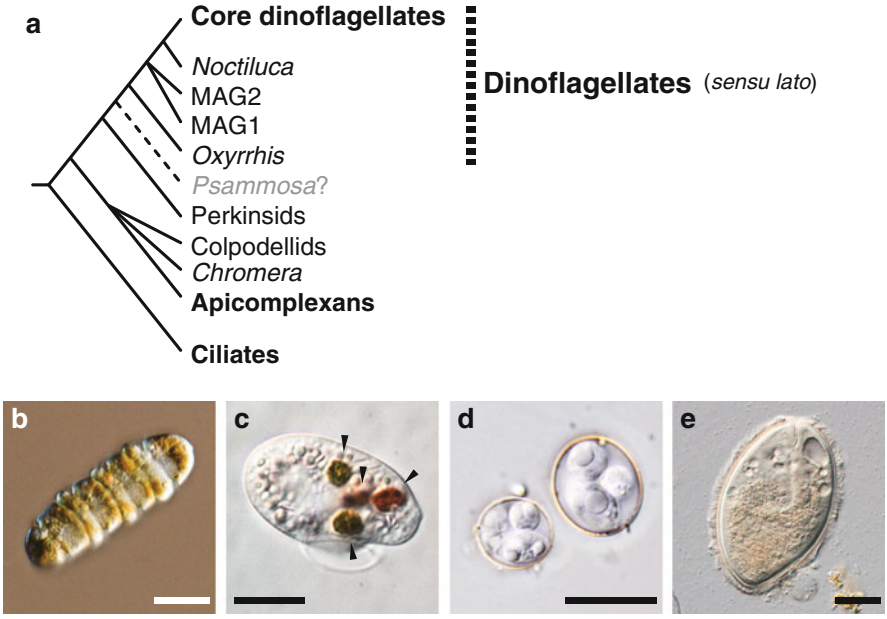

Fig. 2.2 (**a**) Schematic phylogenetic relationship among representative Alveolata lineages. (**b**) A four-cell chain of the core dinoflagellate *Gymnodinium catenatum* (bar = 50 μm). (**c**) The most ancestral dinoflagellate, *Oxyrrhis marina*. A swimming cell contains four food green algae (*triangles*) (bar = 10 μm). (**d**) Oocysts of *Eimeria* sp., a representative apicomplexan parasite that causes the intestinal disease, coccidiosis (bar = 10 μm). (**e**) A rumen ciliate *Dasytricha* sp. isolated from cattle. The symbiotic ciliates contribute to digestion of cellulose in the ruminant (bar = 50 μm)

falciparum, causing the serious and fatal disease malaria, and *Eimeria tenella*, an intestinal parasite for chicken coccidiosis. Ciliates are protists bearing numerous cilia on their cell surface and carrying two functionally and genetically differentiated nuclei within a single cell. *Tetrahymena thermophila* and *Paramecium tetraurelia* are well-known ciliates used as model organisms. Among the alveolates, the dinoflagellates are more closely related to the apicomplexans than to the ciliates. Flattened vesicles (alveoli) lying beneath the plasma membrane are a notable common characteristic of the alveolates, and they share a unique cortical protein called alveolin, which appears on the alveolus membrane (Gould et al. 2008). Flagellar or ciliary pits and tubular cristae in mitochondria are also conserved among the alveolates (Adl et al. 2012).

Nowadays, it is proposed that the branches near the base of the alveolate lineages and the groups such as colpodellids, *Chromera*, perkinsids, syndiniales, and *Oxyrrhis* are grouped together as the protoalveolata (Adl et al. 2012). However, the protoalveolata is not a monophyletic group. Perkinsids, syndiniales, and *Oxyrrhis* are lineages closely related to dinoflagellates, and the last two groups (syndiniales and *Oxyrrhis*) are sometimes regarded as primitive members of the dinoflagellates

(Saldarriaga et al. 2001, 2003; Leander and keeling 2004; Bachvaroff et al. 2014). In contrast, colpodellids and *Chromera* are phylogenetically more closely related to apicomplexans than dinoflagellates and are placed as early branches in the apicomplexan lineage (Leander et al. 2003; Moore et al. 2008). Therefore the concept of the protoalveolata is inconclusive and requires more discussion.

A large number of molecular phylogenetic surveys on dinoflagellate diversity have been performed, and their relationship has been moderately resolved (e.g., Lenaers et al. 1991; Saunders et al. 1997; Saldarriaga et al. 2001, 2003; Leander and Keeling 2004; Zhang et al. 2005; Hoppenrath and Leander 2010; Bachvaroff et al. 2014). The *Oxyrrhis* branch is recognized as the most ancestral dinoflagellate or pre-dinoflagellate lineage. Of note, Okamoto et al. (2012) identified two novel species, *Psammosa pacifica* and *P. atlantica. Psammosa* possess apical complex structures and possibly branched out before *Oxyrrhis* diverged. However, their phylogenetic relationships have not been elucidated sufficiently.

Following *Oxyrrhis* branching, MAG1 (Marine alveolate group 1) and MAG2 (Marine alveolate group 2) diverge. Previously, those two groups were only known either from environmental DNA sequence analysis or as uncultured picoplankton, and their details had been unclear (López-García et al. 2001; Moon-van der Staay et al. 2001; Groisillier et al. 2006). However, it was revealed recently that some species previously known as parasitic and endosymbiotic dinoflagellates in fact belong to MAG1 and MAG2, respectively. The syndiniales are also included in this groups (Harada et al. 2007; Bråte et al. 2012) (for details, see Chap. 17). Followed by noctilucid dinoflagellates in which *Noctiluca scintillans* is known as the representative species (Fukuda and Endoh 2008; Ki 2010; Gómez et al. 2010), the core dinoflagellates carrying typical dinokaryons show great diversity.

In *Oxyrrhis marina*, chromosomes are condensed permanently as the typical dinoflagellates, while the stacked arcs, which are well organized in the dinokaryon, do not appear in this species (Fig. 2.3a) (Dodge and Crawford 1971). During mitosis, a large number of nuclear plaques are formed on the nuclear envelope, and intra-nuclear spindle microtubules are organized from the plaques. Then, chromosomes divide with the aid of these microtubules (Gao and Li 1986). *Psammosa* and some other species belonging to MAGs possess syndinian-like nuclei which are characterized by a centrally located nucleolus and peripherally lying condensed chromatins (Fig. 2.3b [from Okamoto et al. 2012]). Resembling *Oxyrrhis*, their mitosis is carried out with intra-nuclear spindle microtubules (Cachon and Cachon 1987; Okamoto et al. 2012). *Noctiluca scintillans* shows complicated nuclear features. Chromosomes of the noctilucid trophont, which is a vegetative diploid state, are not condensed during the interphase (Figs. 2.3d and 2.3e, compare with Fig. 2.4c), and their condensation occurs when mitosis begins. This phenomenon resembles typical eukaryotic mitosis (Ishikawa 1898; Calkins 1899; Pfiester 1984). There are many nucleolus-like structures (Afzelius 1963). Just beneath the nuclear envelops, a large number of vesicles exist, and some of the vesicles are integrated with the nuclear envelopes (Afzelius 1963; Soyer 1969). Alkali fast green staining was performed to detect basic proteins in the nucleus of the trophont and the result

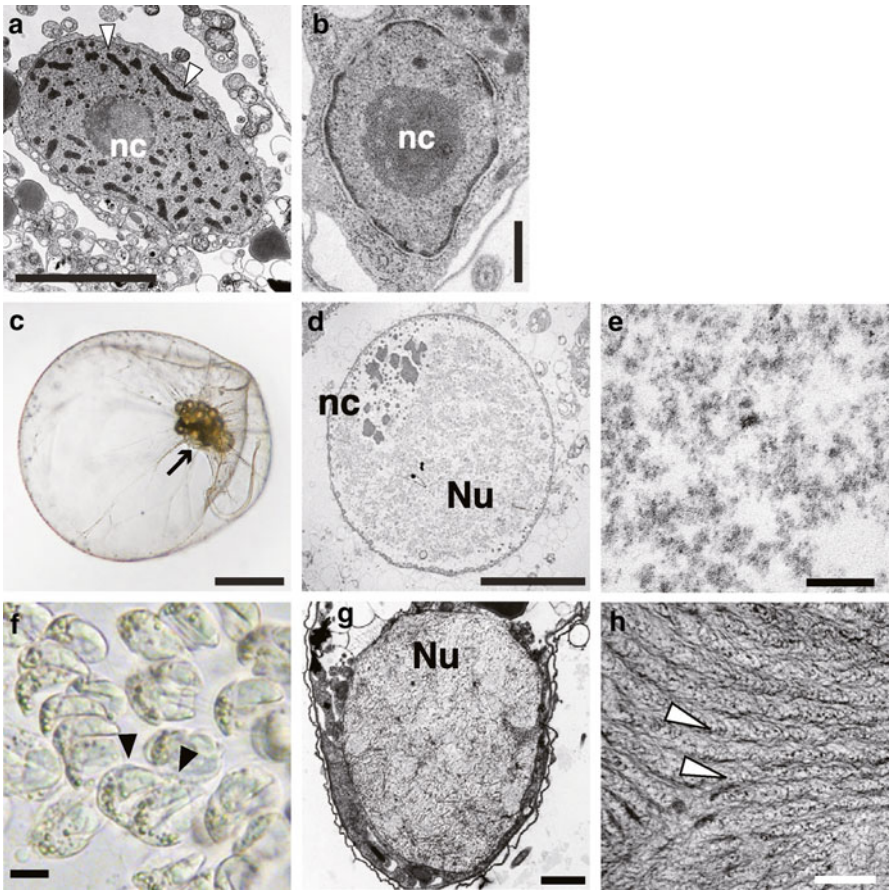

Fig. 2.3 Comparison of nuclear morphology among dinoflagellates. (**a**) A section of a nucleus in *Oxyrrhis marina*. A large nucleolus indicated with "*nc*" is located centrally in the nucleus. *Triangles* indicate condensed ribbon-like chromosomes (bar = 5 μm). (**b**) A representative morphology for syndinian-like nuclei, showing a nucleus of *Psammosa pacifica*. Similar to *Oxyrrhis*, a large nucleolus (nc) is present at the center, and condensed chromatin exists at the periphery of the nucleus (Okamoto et al. 2012) (bar = 500 nm). (**c**) A side view of a mature trophont cell of *Noctiluca scintillans*. The trophont has an eggplant-like shape. The nucleus (*arrow*) is located at the center of the cell, behind the brown oil drops (bar = 100 μm). (**d**) A whole image of the *Noctiluca* nucleus in the trophont. Condensed chromosomes are not present, while chromatin bodies are visible as black dots (Nu). Nucleolus bodies are shown with "nc" (bar = 10 μm). (**e**) A magnified image of the chromatin body. Black dots correspond to the chromatin bodies. The stacked arc appearance of the typical dinokaryon is not present (bar = 100 nm). (**f**) Matured gametes on a gametogenic cell of *N. scintillans*. From 256 to 1024 gametes (*triangles*) are developed from one trophont via gametogenesis (bar = 10 μm). (**g**) A transverse section through a nucleus in the gamete. Chromatin fibers expand into the whole nucleus (Nu) (bar = 1 μm). (**h**) A magnified image of the chromatin fibers in the noctilucid gamete. Stacked arcs (*triangles*) exist in the gamete nucleus (bar = 100 nm)

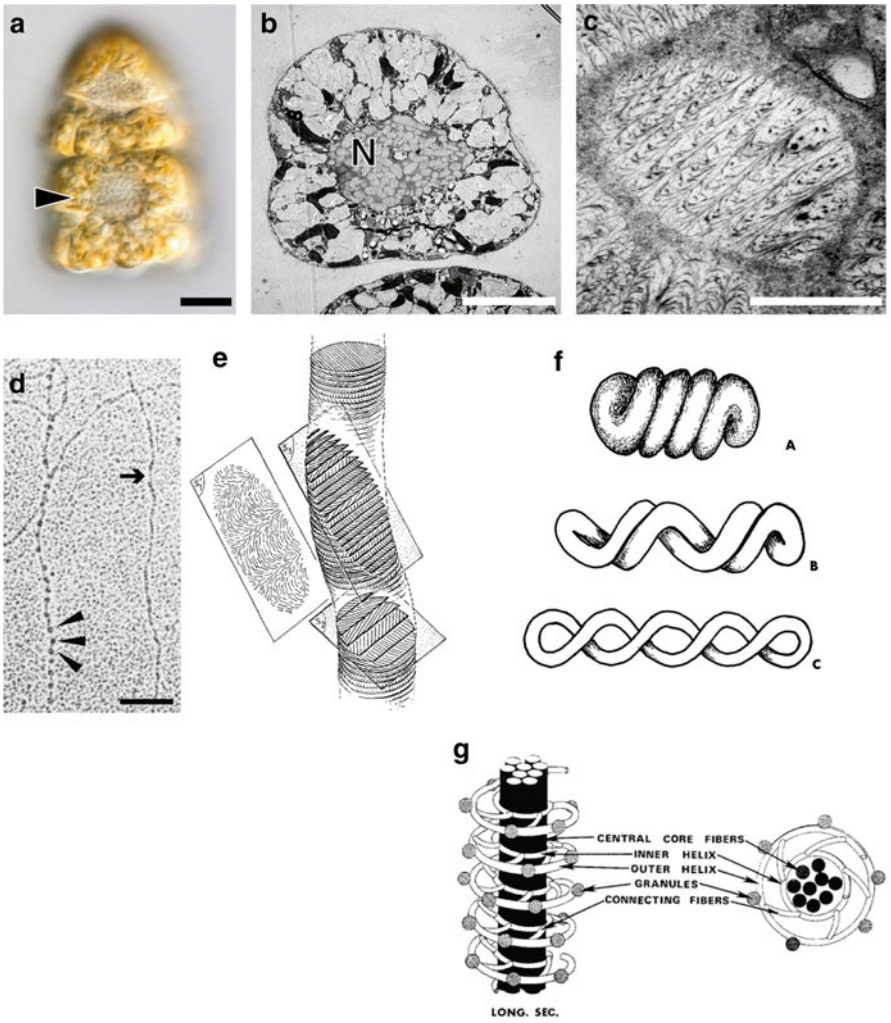

Fig. 2.4 (**a**) A ventral view of *Gymnodinium catenatum* forming a two-cell chain. Tiny rods (*triangle*) are liquid crystal chromosomes (bar = 10 μm). (**b**) A longitudinal section through the chain of *Gymnodinium catenatum* cells. A dinokaryon (N) is located at the center of the cell. The dinokaryon is filled with many oval-shaped chromosomes (bar = 10 μm). (**c**) An enlarged electron micrograph of a chromosome in *Gymnodinium catenatum*. Stacked arcs occupy the chromosome (bar = 500 nm). (**d**) Nucleofilaments of the dinoflagellate *Peridinium balticum* and those of its diatom endosymbiont. *Triangles* represent nucleosomes with the "beads on a DNA string" structure, which is originated from the endosymbiotic diatom that carries a typical eukaryotic nucleus. *Arrow* indicates the nucleofilament of the host's dinokaryon. The smooth 6.5-nm filament does not bear nucleosomes (bar = 100 nm). After Rizzo and Burghardt (1980). (**e**). A diagram representing a proposed model for cholesteric liquid crystal organization in the dinoflagellate chromosome. The chromosome consists of a stack of disks which are formed by chromosome fibers arranged in parallel, and the angle of the chromosome fibers changes by a constant small angle from one disc to the following one. For this reason, an oblique section would show stacked arcs as the projected image, as schematically demonstrated on the *left*. After Bouligand et al. (1968). (**f**) Diagrams representing the model organization for the oval-shaped chromosome by a thick toroidal bundle, chromonema. *A*: A tightly coiled chromonema represents one whole chromosome in living cells. *B*: Partially uncoiled chromonema. *C*: Loosened (relaxed) chromonema. After Oakley and Dodge (1979). (**g**) A diagram representing the detailed structure of the chromonema. After Spector et al. (1981)

was positive, while staining of the gamete nucleus showed a negative result, indicating possession of histone-like proteins in the nucleus of the noctilucid trophont (Li 1984). When gametogenesis starts (Fig. 2.3f), chromosome condensation occurs, and the condensing chromosomes are segregated with the extra-nuclear spindle microtubules running through the cytoplasmic tunnels. Then, the nucleus of the mature gamete is ubiquitously filled with a stacked arc as chromosomes of the typical dinokaryon, while obvious chromosome bodies are not present (Fig. 2.3g, h) (Zingmark 1970; Soyer 1972; Fukuda and Endoh 2006).

2.3 The Dinokaryon

2.3.1 Genome, Chromosome, and DNA

Although there are some exceptions, dinoflagellates are haploid during the vegetative phase, containing a large amount of DNA. The content ranges from 3 to 250 pg/cell, corresponding to approximately $3–250 \times 10^6$ bp (Spector 1984). Thus, the size of the dinoflagellate genome is 1–80 fold that of the human haploid genome which is approximately 3×10^6 bp. According to the draft genome sequencing in *Alexandrium ostenfeldii*, tandem repeat sequences are massively present, occupying 58 % of the determined genome (Jaeckisch et al. 2011). By contrast, in the symbiotic alga *Symbiodinium minutum*, only less than 10 % of the genome is estimated to be composed of tandem repeat sequences (Shoguchi et al. 2013). Complete whole genome data from several other dinoflagellates would explain the reason behind the immense size of the dinoflagellate genome.

Dinoflagellate chromosomes remain condensed throughout the cell cycle, and are obviously visible even at the interphase (Fig. 2.4a, b). The chromosomes vary in numbers depending on the species, from 4 to 200 (Spector 1984). Based on electron microscopic observations, stacked arcs of filaments appear in the chromosomes (Fig. 2.4c), which presumably correspond to the nuclear filaments.

Unusual substitution occurs in the dinoflagellate genomic DNA. In addition to 5-methylcytosine and N^6-methyladenine, 12–68 % of thymine bases are replaced by 5-hydroxymethyluracil (HOMeU) (Rae 1973 and 1976; Steele and Rae 1980; Blank et al. 1988). An incorporation experiment of radioactive nucleotides has shown that HOMeU does not appear as post-replication modifications. HOMeU is synthesized in the dinoflagellate cell and incorporated into its genomic DNA by DNA polymerization during genome replication (Galleron 1984). HOMeU is a rare base and some bacterial phages such as *Bacillus subtilis* bacteriophage SP8 carry HOMeU in their genomes, whereas the base is hardly detectable from most of the eukaryotic genome (Kallen et al. 1962). In these phages, HOMeU is regarded to function as a protector of the phage genome from restriction enzymatic activities of the host. Biological roles of the base, however, have not been explained for dinoflagellates.

2.3.2 *Transcription and Spliced Leader* Trans-*Splicing*

In general, condensed chromosomes do not express any genes. Experiments using radioactive isotopes showed that mRNA transcription occurs only on the DNA loops protruding from chromosomes (Sigee 1984). Z-DNA, which is a left-handed double helix of DNA, is localized at the chromosomal peripheries. The locally formed Z-DNA seems to disentangle tightly packed DNA strings in the chromosome and protrude coding regions from the condensed chromosomes (Soyer et al. 1990). Z-DNA is also localized on chromosome segregation forks in the M phase of the cell cycle. This may disentangle the replicated DNA filaments, possibly mediating the chromosome segregation. Intriguingly, formation of the protruding DNA loops occurs prominently at the G1 phase, suggesting that mRNAs are actively transcribed at the G1 phase (Bhaud et al. 2000).

To make matured mRNAs in dinoflagellates, transcribed mRNA precursors are required to be transplanted with a small RNA sequence called spliced leader (SL) RNA at the 5′ ends of the pre-mRNAs (Zhang et al. 2007). The SL trans-splicing occurs in some other eukaryotes such as kinetoplastids, cnidarians, and flatworms, but the SL trans-splicing in dinoflagellates is notable for the following reasons. First, the dinoflagellate SL sequence, DCCGUAGCCAUUUUGGCUCAAG (D = U, A, or G), is strictly conserved in all dinoflagellate lineages examined, including the most ancestral species *Oxyrrhis marina*. Second, the SL donor RNA genes are coded as tandem arrays, as reported for kinetoplastids, but transcribed SL RNA is unusually short (50–60 bp). Third, the conserved SL-binding motif which interacts with the recipient's premature transcripts is not present in the intron of the SL donor gene. Instead, a putative spliceosomal binding motif (AUUUUGG) exists within the exon. The SL trans-splicing possibly works as generating mRNAs from polycistronic mRNA precursors, sanitizing the mRNAs, and regulating gene translation (Hastings 2005; Zhang and Lin 2009; Lin et al. 2010).

2.3.3 *Mitosis*

Dinoflagellates divide their nuclei through a unique process of mitosis. The nuclear envelope persists completely throughout mitosis, while chromosomes are separated by the aid of extra-nuclear spindle microtubules. After mitosis starts, spindle-shaped chromosomes split progressively, and transform into Y- or V-shapes (Bhaud et al. 2000). In the metaphase, the nucleus becomes a disc-like configuration, and cytoplasmic tunnels are formed that penetrate through the nucleus from the anterior to the posterior ends. Then, extra-nuclear spindle microtubules are formed and pass through the cytoplasmic tunnels. Chromosomes are attached to the inner surface of the nuclear envelopes, and the chromosome-attaching site becomes connected to the spindle microtubules running through the tunnels. Thus, chromosomes are linked to the spindle through the mediation of the nuclear envelope. From electron

microscopic observations, electron-dense materials located on the envelope connect condensed chromosomes to the extra-nuclear spindles, whereas distinct kinetochore structures are not observed on the chromosome (Spector 1984). At the late anaphase, V- or Y-shape chromosomes are separated into two spindle-shape chromatids, and two disc-like nuclei are produced. After the completion of the nuclear division, cytoplasmic tunnels and extra-nuclear spindles disappear, and the nucleus regains its original globular shape (Bhaud et al. 2000).

2.3.4 Nucleofilament

In eukaryotes, with the exception of dinoflagellates, the nucleosome is the elementary unit of the nucleofilament, and is formed by a repeating structure in which DNA (2 nm) wraps around the core histone octamer (10 nm in diameter). Consequently, nucleosomes without linker histone show an appearance resembling beads on a string of DNA (Olins and Olins 1974). In contrast, the nucleofilament isolated from the dinoflagellate chromosome appears as a smooth thread of approximately 6.5 nm in diameter, and repeating subunits are absent in the filament (Fig. 2.4d [from Rizzo and Burghardt 1980]). The smooth filament (6.5 nm) is thicker than the naked DNA filament (2 nm), but thinner than the size of the core histone octamer (10 nm). When dinoflagellate nuclei were treated with micrococcal endonuclease, digested DNA did not form a ladder of discrete bands corresponding to the nucleosome repeat lengths, but a smear pattern appeared. Micrococcal endonuclease digested only about 10 % of the nuclear genomic DNA, and the remainder seemed to be intact. These results indicated that the dinoflagellate DNA is uniformly and tightly bound by proteins smaller than the core histone octamer (Hamkalo and Rattner 1977; Bodansky et al. 1979; Rizzo and Burghardt 1980). For this reason, the nucleofilament of dinoflagellates is regarded to be organized as a smooth 6.5-nm filament, in which the DNA is well protected from the nuclease digestion (Herzog and Soyer 1981).

2.3.5 Proposed Models for Chromosome Architecture

The dinoflagellate chromosomes are composed of stacked rows of parallel arcs, which is one of the prominent features of the dinokaryon (Fig. 2.4c). Regarding the chromosome conformation, several models have been proposed (see Raikov 1978; Spector 1984), and representative models are summarized below.

A cholesteric liquid crystal organization model which was proposed by Bouligand et al. (1968) is the most well-known conformation mode for the dinoflagellate chromosome (Fig. 2.4e [from Bouligand et al. 1968]). According to this model, the dinoflagellate chromosome is formed with disk layers in which nucleofilaments consisting of DNA and DNA binding proteins are closely aligned. Each disk is

composed of nucleofilaments aligned at slightly different angles, and the disks stack in a left-handed manner to yield the stacked arc morphology (e.g., Bouligand et al. 1968; Livolant and Bouligand 1978).

Oakley and Dodge (1979) proposed a different model for the higher-order chromosome architecture (Fig. 2.4f [from Oakley and Dodge 1979]). According to their model, one dinoflagellate chromosome consists of one thick toroidal bundle called a chromonema, and an oval-shaped chromosome is formed with tightly and right-hand coiled chromonema. The chromonema represents a fiber of 127 nm in diameter, and is a complex made of several different thin filaments and granules. A bundle of 9-nm filaments forms the central core of the chromonema, and twin helices made of 2.5-nm filaments and 9-nm granules wind the core (Spector and Triemer 1981; Spector et al. 1981) (Fig. 2.3g [from Spector et al. 1981]).

Herzog and Soyer (1983) experimentally loosened the dinoflagellate chromosome by using cation chelators (EDTA and EGTA), and validated the chromosome conformation. They revealed that the dinoflagellate chromosome was formed by helical compaction of nucleofilaments in a hierarchy of six organization levels, and that Ca^{2+} and Mg^{2+} were required for their stabilization. From their observations, the lowest (Level 1) is the elemental 6.5-nm smooth filament corresponding to the nucleofilament. Level 2 is the 10-nm fiber which is formed of two twisted 6.5-nm nucleofilaments. The 10-nm fiber tightly twists and forms a single helical 18-nm fiber of the third level. Level 4 is the 25- to 31-nm double helices, formed of two level-3 fibers twisted in a left-hand manner. To be level 5, the level-4 fiber coils left-handedly and tightly, becoming 43–56 nm in diameter. Finally, two level-5 fibers form a right-hand double-helical bundle, corresponding to the whole chromosome (level 6).

The cholesteric liquid crystal organization model well explains both ultrastructural morphologies and geometric properties (Rill et al. 1989). Therefore, the model has been accepted and widely authorized in spite of a few unsolved problems, such as the fact that no interpretation has been made to account for the nature of the hierarchic six organizations of this model. Further studies are necessary to reveal the chromosome conformation in dinoflagellates.

2.3.6 Nuclear Proteins

Several unusual respects have been reported for dinokaryon proteins. The DNA/basic protein ratio is approximately 1:1 in most eukaryotic nucleus, whereas the ratio usually reaches 10:1 or more in dinoflagellates (Rizzo et al. 1982; Spector 1984; Vernet et al. 1990). Furthermore, as already mentioned above, the dinokaryon does not contain nucleosomes due to the absence of typical core histone proteins, and this feature is the only exception among eukaryotes. These amazing features have attracted a number of investigators, and some unique proteins are identified and characterized for dinokaryons as summarized in Table 2.1.

Table 2.1 Representative nuclear proteins in dinoflagellates

Protein name	Species	Molecular weight (kDa)	Nuclear localization	Predicted function	Reference
HCc1 and HCc2	*Crypthecodinium cohnii*	14	Periphery of chromosomes	Regulation of transcription	Sala-Rovira et al. (1991)
(In the former, these proteins were described as HCcα, β and γ in Vernet et al. 1990)			Nucleolar organizing region		
Dinap1	*C. cohnii*	44.8	Nucleoplasm	Regulation of transcription	Bhaud et al. (1999)
Dinap2	*C. cohnii*	40.5	–	–	Bhaud et al. (1999)
Dip1	*C. cohnii*	45	–	Potential ligand of Dinap1	Guillebault et al. (2001)
(In addition to Dip1, four proteins [Dip2–5] have been found for potential ligand for Dinap1)				Regulation of transcription	
DapC	*C. cohnii*	43	–	Potential ligand of Dip1	Guillebault et al. (2001)
(In addition to DapC, three proteins [DipB, C and G] have been found for potential ligand for Dip1)				Regulation of transcription	
Lamins	*Amphidinium carterae*	62 and 58	Nucleolus, lamina, interchromosomal nucleoplasm	Maintenance of nuclear structure	Mínguez et al. (1994)
Lamin-related protein	*A. carterae*	50	Nucleolus, lamina, interchromosomal nucleoplasm	Maintenance of nuclear structure	Mínguez et al. (1994)
DVNP	*Hematodinium* sp	13.4–20.2	Chromosome	DNA binding	Gornik et al. (2012)
NP23	*Oxyrrhis marina*	23	Chromosome	DNA binding	Kato et al. (1997)
Type II topoisomerase	*Crypthecodinium cohnii*	–	–	Regulation of transcription	Mak et al. (2005)
TBP-like protein	*C. cohnii*	24.6[a]	–	–	Guillebault et al. (2002)
RCC1	*Symbiodinium minutum*	–	–	–	Shoguchi et al. (2013)
p16, p16.5, and p17	*Crypthecodinium cohnii*	16, 16.5 and 17 (respectively)	–	–	Vernet et al. (1990)

The core histone proteins and histone-related proteins are not listed here

[a]Molecular weight deduced from translated amino acid sequences in this review

2.3.7 HCc Family

The HCcs (Histone-like proteins from *Crypthecodinium cohnii*) are the most well analyzed nuclear proteins of dinoflagellates. HCcs were initially identified from a well-studied dinoflagellate, *Crypthecodinium cohnii* (Rizzo and Nordan 1974). The earlier studies revealed that the molecular weights of HCcs were lower (approximately 16 kDa) and their basic amino acid compositions were different as compared with typical eukaryotic histone proteins. Rizzo and Morris (1984) reported that HCcs were lysin rich and arginine rich, and only these amino acids could account for 80 % of the acid-extracted nuclear proteins. Vernet et al. (1990) showed that HCcs consisted of three different molecular species, and these proteins were named HCcα, HCcβ, and HCcγ. By conventional SDS-PAGE, HCcα and HCcβ were migrated into a similar position, and HCcγ was located at a slightly larger position (Vernet et al. 1990). Later, two different genes encoding HCcs were found, and their sequences were also identified (Sala-Rovira et al. 1991). From *HCc1*, it was deduced that the gene encoded a 133-amino-acid and lysin-rich (17.7 % of amino acid residues) basic protein. The deduced amino acid sequence showed that its approximate molecular weight was 12.5 kDa, being well concordant with the previous results reported by Rizzo and Morris (1984). Immunohistochemical labeling by using HCc antibodies revealed that HCcs are localized in the nucleoplasm surrounding the chromosomes. By electron microscopy, HCcs were found to be present at the periphery of chromosomes and aligned along the DNA loops, which are protruding filaments from the chromosomes (Sala-Rovira et al. 1991). In HCc3, a member of additionally identified HCcs by Wong et al. (2003), DNA binding activity is confined to the C-terminal domain, and its N-terminal domain is responsible for dimer formation. HCc3 dimer induces DNA aggregation under a high concentration (Chan and Wong 2007). Consequently, it was concluded that HCcs functions to maintain DNA loops from condensed chromosomes. Furthermore, HCcs are abundantly present in the nucleolus, especially around the nucleolus-organizer regions, suggesting a possible involvement in the transcription of ribosomal RNA genes (Sala-Rovira et al. 1991).

Although HCc genes carry introns (Yoshikawa et al. 1996), their deduced amino acid sequences show a significant homology to the bacterial histone-like HU proteins, a major component of the bacterial nucleoid. The molecular phylogenetic relationship between dinoflagellate HCcs and bacterial HUs indicates that dinoflagellate HCc homologs and proteobacterial long HU form a well-supported monophyletic group (Wong et al. 2003; Hackett et al. 2005; Chan et al. 2006). These results suggest that the ancestor of dinoflagellates might have acquired the proteobacterial HU via lateral gene transfer, and that they might have retained and adapted the gene to their eukaryotic lineage.

2.3.8 Dinaps and Their Associating Proteins

In addition to HCcs, two other major proteins of 48 kDa and 46 kDa have been detected in the nuclear extract of *Crypthecodinium cohnii* and named as Dinap1 (Dinoflagellate nuclear associated protein 1) and Dinap2 (Bhaud et al. 1999),

respectively. Dinap1 consists of 400 amino acids with a suggested molecular weight of 44.8 kDa, and does not show homology to any known proteins. According to its deduced molecular structure, Dinap1 is divided into two domains; the N-terminal region containing approximately 250 amino acids and the C-terminal region of about 150 amino acids. The N-terminal region contains two putative zinc finger repeats, and the C-terminal region carries two WW domains. Dinap1 is synthesized in a cell cycle-dependent manner, and is only detectable during the G1 and S phases of the cell cycle. Guillebault et al. (2001) found five potential ligands for Dinap1 (Dips: Dinap1 interacting proteins) and also four potential ligands for Dip1 (Daps: Dip1 associated proteins) in *C. cohnii*. Dip1 and DapC have been extensively examined and well ascertained. The WW domain recognizes proline-rich sequences as PPXY, and Dip1 harbors the motif (Guillebault et al. 2001). In addition, the protein carries a putative one zinc finger motif and two WW domains. Deduced amino acid sequences and conserved motif organization suggests a similarity between Dip1 and the mammalian pre-mRNA splicing factor FBP21 (Formin-binding protein 21) (Guillebault et al. 2001). DapC, which possesses PPXPXGX repeats in its N-terminal region and one putative leucine zipper in the C-terminal region, does not resemble any known proteins (Guillebault et al. 2001). Dip1 and DapC are co-immunoprecipitated with Dinap1, and these interactions are confirmed by reverse immunoprecipitation. Though Dinap1, Dip1 and DapC exist in the nucleus at G1 phase, these three proteins do not bind directly to DNA. Moreover, recombinant Dinap1 protein activated up to 40 % of the basic transcription activity of *C. cohnii* in an *in vitro* condition. For these reasons, it has been suggested that these three proteins (Dinap1, DapC, and Dip1) are subunits of a large nuclear complex which functions in the regulation of transcription (Guillebault et al. 2001).

Dinap2, by contrast, is a 40.5 kDa protein and possesses neither particular repeats nor structural domains. Dinap2 shows low similarity to SCP-X (sterol carrier protein) in rat and mouse (Bhaud et al. 1999), but no further information is available.

2.3.9 Lamin and Lamin-Like Proteins

Lamins, which belong to intermediate filaments, are components of the nuclear lamina. The nuclear lamina not only exists as a structural framework for the nucleus, but also plays a pivotal role for chromatin organization (Dechat et al. 2008). Nuclear matrix extracts from *Amphidinium carterae* showed three bands corresponding to 62, 58 and 50 kDa by Western blotting with anti-lamin and anti-intermediate filament antibodies (Mínguez et al. 1994). The 62- and 58-kDa proteins were abundant in the nuclear matrix and reacted with both anti-lamin and anti-intermediate filament antibodies. Dinoflagellates possess a well-developed nuclear lamina, and these two proteins seem to be its major constituents. The 50-kDa protein also reacted with anti-lamin antibodies, suggesting that this protein also corresponds to a lamin or a related intermediate filament protein.

2.3.10 NP23 and DVNP

NP23/DVNP is the only identified protein that most likely constructs the chromosome organization. In *Oxyrrhis marina,* a nuclear protein representing approximately 23 kDa by conventional SDS-PAGE massively exists in the nucleus, and the protein was named NP23 (Li 1984; Kato et al. 1997). NP23 contains many basic amino acid residues (22 % of the total amino acids), being especially rich in lysine (17.9 % of the total amino acids). From immunoelectron microscopic observation using an antibody against NP23, the protein was found to be present in the chromosome and the nucleolus, being colocalized with DNA in the chromosome (Kato et al. 1997). Therefore, it has been suggested NP23 is a protein that binds with DNA and the DNA-NP23 complex consists of the chromosome organization.

Gornik et al. (2012) found a protein presumably homologous to NP23 in the parasitic dinoflagellate *Hematodinium* sp. and identified its coding genes. The protein was named DVNP (Dinoflagellate viral nucleoproteins), because its deduced amino acid sequence surprisingly showed a strong homology to viral proteins. DVNPs, including several molecular variants, consist of 124–186 amino acids and are rich in basic amino acids. Their molecular weights are estimated as 13.4–20.2 kDa. A nuclear translocation signal is present in their N-terminal region, and anti-DVNP antibody was found to label the chromosomes in *Hematodinium* sp. From the homologous gene survey, its homologs were found in a lot of dinoflagellates examined. Conversely, DVNP orthologous genes were detected in neither other eukaryotes nor prokaryotes. Judging from its small size and strong binding capability to DNA, it is suggested that the smooth 6.5-nm filament found in the dinoflagellate chromosome is formed by binding of DVNPs to DNA. A number of phosphorylation sites are predicted in DVNP, and phosphorylated DVNP is reported to exist in *Hematodinium* sp., suggesting that phosphorylation of DVNP might regulate the chromosome conformation and gene expression in dinoflagellates.

It is noteworthy that a part of DVNP sequences without a nuclear translocation signal at the N-terminal shows a strong homology to some hypothetical proteins of phycodnaviruses such as *Ferdmania* sp. virus and *Ectocarpus siliculosus* virus. Amino acid alignment of DVNPs and the viral hypothetical proteins revealed that all the proteins share many amino acid residues. Therefore, Gornik et al. (2012) proposed the following possible evolutionary scenario. A common dinoflagellate ancestor became infected by a phycodnavirus carrying the origin of DVNP, followed by acquisition of the viral gene via lateral gene transfer. It turn, this event somehow triggered the deletion of the typical histone proteins and transformation of the chromosome architecture during the history of dinoflagellate evolution.

2.3.11 Histones and Histone-Related Proteins

The core histone octamer and its DNA wrapping consist of the nucleosome structure which is an elementary unit of eukaryotic chromosomes. Histone-related proteins such as histone modification enzymes or histone chaperones play important roles in epigenetic regulation of gene expression as well as other functions involving DNA repair and replication. Conventional electrophoresis, biochemical approaches, and electron microscopies had raised a presumption that dinoflagellates lack typical histone proteins (e.g., Rizzo and Noodén 1974; Li 1984). However, Okamoto and Hastings (2003) discovered a histone H3-coding gene in the transcriptomes of *Pyrocystis lunula*, and a number of transcriptomes, ESTs, and genome analyses revealed that all dinoflagellates examined so far carry a full suite of genes for core histone proteins (Hackett et al. 2005; Lin et al. 2010; Roy and Morse 2012; Bayer et al. 2012). Some dinoflagellates carry several histone variants in their genome (Roy and Morse 2012; Bayer et al. 2012). Comparison of deduced amino acid sequences of the histone proteins indicates that dinoflagellates and well-studied eukaryotes share functional motifs and amino acid residues. For instance, the histone H2A.X contains its signature SQ motif (SQ, D/E, M/Y/F) in the C-terminal region, and the motif functions for the repair of DNA breakage and chromosome recombination. Phosphorylation at a serine residue in the SQ motif leads nonhomologous end-joining repair. The SQ motif is well conserved in the presumable histone H2A.X sequences of dinoflagellates. On the histone H2B, H3, and H4 proteins, a number of posttranslational modification sites for gene expression and chromatin remodeling exist and are conserved among dinoflagellates (Lin et al. 2010; Roy and Morse 2012; Bayer et al. 2012). Furthermore, genes coding for various histone modification enzymes such as histone acetyltransferases, deacetylases, methyltransferase, methylases, and demethylases, and those coding for histone chaperones are identified in dinoflagellate transcriptomes (Roy and Morse 2012; Bayer et al. 2012). Taken together, the dinoflagellates indeed possess a full set of genes for the typical eukaryotic chromosome organization.

Roy and Morse (2012) applied 20 μg of the acid-extracted protein from *Lingulodinium polyedrum* for immunoblotting against histone H2B and H3 proteins. The protein amount used in the assay was 300 times greater than that for the control experiment using yeast extract, but no band corresponding to the size of histone proteins was detected. The authors used LC-MS/MS for more specific identification, but none of the core histone proteins were found at all, in spite of the detection of several histone-like proteins. Thus, Roy and Morse (2012) speculated that the translation of the histone proteins is strongly repressed by post-transcriptional regulation in the dinoflagellates. The authors further analyzed whether the cell cycle-dependent mRNA accumulation of the histone genes occur. In typical eukaryotes, some of the histone genes are regulated by the cell cycle, and the transcripts tend to accumulate at S phase. However, in the dinoflagellate *Lingulodinium,* the levels of these mRNAs remained unchanged regardless of the stage of the cell cycle.

In spite of the fact that the genes coding for core histone proteins are present in the genome, the amount of histone proteins, if translation of mRNAs in fact occur, is likely to be very low and below the level of detection. The possible roles of such a small quantity of histone proteins, if present, might include a contribution for the DNA repair pathway and transcriptional regulation of the gene (Hackett et al. 2005; Roy and Morse 2012).

2.3.12 Other Nuclear Proteins

In dinokaryon, type II topoisomerase, PCNA (Proliferating cell nuclear antigen), and a TBP (TATA box binding protein)-like protein have been found.

Type II topoisomerases manage DNA supercoiling and separate interlocked chromosomes. Firstly, the enzyme cuts the backbones of both strands of the target DNA, allowing one of the strands to be transported through the break. Finally, it relegates the cut of DNA. These sequential enzymatic activities results in decreasing or increasing the DNA coiling. In eukaryotic nuclei, one of the major functions of type II topoisomerase is disentangling of supercoiled nucleofilaments after the S phase. This activity is required for resolution and segregation of the replicated genome into two sister chromatids during mitosis (Watrin and Legagneux 2003). In dinoflagellates, specific inhibitors for type II topoisomerase caused G1 phase delay as well as G2/M phase arrest. Furthermore, stacked-arcs within the chromosomes disappeared after the drug treatment (Mak et al. 2005). As described above, dinoflagellates actively undergo transcription during the G1 phase on DNA loops protruded from the condensed chromosomes. Taken together, the type II topoisomerase is suggested to participate in maintenance of the chromosome organization and formation of the DNA loop (Mínguez et al. 1994; Mak et al. 2005).

The PCNA, which functions in DNA replication and repair (Essers et al. 2005), has also been studied in dinoflagellates. PCNA of the dinoflagellate *Pfiesteria piscicida* is not very different from the typical eukaryote PCNA (Zhang et al. 2006). In *Karenia brevis*, PCNA is located in the nucleus throughout the whole cell cycle, whereas it is representing molecular weight and intra-nuclear localization of the protein change in accordance with the phases of the cell cycle. At the G1 and G2/M phases, PCNA shows 28 kDa, and its location dominates in the nuclear periphery. At the S phase, the size of PCNA increases to 37 kDa, and the protein translocates to the chromosomes concomitantly with the change in size. PCNA is usually regulated by posttranslational protein modifications with SUMO (small ubiquitin-related modifier) or monoubiquitination in vertebrates (Hoege et al. 2002). The shift in the band size of the dinoflagellate PCNA is approximately 9 kDa, roughly corresponding to the size of SUMO (12 kDa) and ubiquitin (8.5 kDa). From these results, PCNA is suggested to be involved in DNA replication, and its activity seems to be controlled by SUMO or ubiquitin modifications like other eukaryotes (Brunelle and Dolah 2011).

Neither canonical TATA box nor any other known consensus promoter elements have been found in the dinoflagellate genome (Li and Hastings 1998). Nonetheless, dinoflagellates carry a TBP-like protein (Guillebault 2002). The dinoflagellate TBP-like protein is molecular phylogenetically suggested as representing an intermediate state between typical eukaryotic TBPs and TLFs (TBP-like factor). Interestingly, strictly conserved four phenylalanine residues, which participate in binding to the canonical TATA box, are mutated in the dinoflagellate TBP-like protein. By contrast, dinoflagellate TBP-like protein carries conserved four glycine residues, which are located in the N- and C-terminal repeats and are required for maintaining the three-dimensional structure of TBP. Due to four phenylalanine mutations, dinoflagellate TBP-like protein can no longer bind to the canonical TATA, but it moderately binds to thymidine repeats (potential TTTT box) in a high-salt condition. Further analyses will be required to clarify the binding site of the dinoflagellate TBP-like protein and its functions.

Whole-genome sequencing has been performed on *Symbiodinium minutum*, a symbiont of tropical sea anemones. The result showed the presence of a number of copies of genes coding for RCC1 (Regulator of chromosome condensation)-like proteins in the genome (Shoguchi et al. 2013). Human RCC1 is known to bind to the nucleosome and plays an important role for regulating chromosome condensation during mitosis (Makde et al. 2010). A phylogenetic tree for eukaryotic and bacterial RCCs indicates that *S. minutum* carries both eukaryotic and bacterial RCC1 orthologs, and *S. minutum* RCC1s are highly diverged. The dinoflagellate RCC1 proteins might provide us with the key to understand the molecular machinery for maintaining permanently condensed chromosomes in dinoflagellates.

2.4 Concluding Remarks

Let us consider nuclear proteins existing in the dinokaryon again. Dinoflagellates possess eukaryotic nuclear lamins, whereas DVNP, possibly originated from phycodnavirus, is an elemental part of their chromosome. Dinoflagellate TBP-like protein carries mutated sites which are located at the conserved amino acid residues, and it seems to be a highly modified promoter protein for the dinoflagellate genome that generally lacks canonical TATA boxes. Dinoflagellates have acquired HCc from eubacteria via lateral gene transfer, which is a unique DNA-associating protein required for the DNA looping from condensed chromosomes. PCNA and type II topoisomerase shows expression patterns which are different from other eukaryotes. Furthermore, dinoflagellates, like other eukaryotes, possess a complete set of genes encoding for core histone proteins and a number of histone-related proteins, which might be involved in transcriptional regulation and DNA repairing machinery. Taken together, the dinokaryon can be regarded as a hybrid nucleus which consists of the following three components; (1) a conserved and typical eukaryotic part; (2) acquired molecules from other organisms or viruses via lateral gene transfer; and (3) unique molecules individually evolved in dinoflagellates.

Regarding the plastid, its situation seems to be similar to that of the dinokaryon. Some dinoflagellates possess plastids retained from a common ancestor of Alveolata, and others are completely heterotrophic due to the loss of plastids. Tertiary endosymbiotic events also occur, which result in the formation of new plastids originated from various algae. Some species carry transient plastids by kleptoplasty.

In conclusion, it may be reasonable to assume that dinoflagellates favor acquiring cellular systems from other organisms; they have aggressively acquired, modified, and integrated systems of other organisms into their own use. Further studies will be needed to fully understand this unique ability of the dinoflagellate.

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Chapter 3
Diversity of Microbial Eukaryotes in Deep Sea Chemosynthetic Ecosystems Illuminated by Molecular Techniques

Kiyotaka Takishita

Abstract Chemosynthetic ecosystems found in deep sea hydrothermal vents and cold seeps rely on the biological conversion of carbon dioxide or methane into organic matter using the oxidation of hydrogen sulfide or methane as a source of energy, rather than sunlight as in photosynthesis. It is known that communities of chemosynthetic ecosystems include endemic animals, such as mussels, clams, and tubeworms, along with bacterial and archaeal primary producers oxidizing reduced chemical species that support the survival of these animals. On the other hand, microbial eukaryotes (i.e., protists and fungi) in chemosynthetic ecosystems have not been investigated as thoroughly as the animals and prokaryotes. Nevertheless, mainly based on molecular techniques such as culture-independent PCR and fluorescence in situ hybridization, it has become more clearly understood that a phylogenetically broad range of microbial eukaryotes occurs in chemosynthetic ecosystems and that these microbes play a significant role as grazers, decomposers, or parasites. Furthermore, the existence of novel microbial eukaryotes exclusively or mainly inhabiting chemosynthetic ecosystems has also been suggested, stimulating future studies on their evolution and physiology.

Keywords Anaerobic • Chemosynthetic • Deep sea • Parasites • Seeps • Vents

3.1 Introduction

Photosynthesis is the process of converting light energy into chemical energy and producing organic matter from carbon dioxide. This process occurs in plants and algae, and these photosynthetic organisms play a significant role in primary production. Chemosynthesis is another means of biological conversion of molecules

K. Takishita (✉)
Japan Agency for Marine-Earth Science and Technology (JAMSTEC),
Yokosuka, Kanagawa 237-0061, Japan
e-mail: takishitak@jamstec.go.jp

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with one carbon (usually carbon dioxide) into carbohydrates, such as sugars, using energy and the reducing power produced by the oxidation of reduced forms of inorganic compounds. The reduced inorganic compounds used in chemosynthesis are hydrogen sulfide, molecular sulfur, ammonia, nitrous acid, molecular hydrogen, and iron, and organisms capable of synthesizing organic matter with chemical energy generated from such inorganic molecules are called chemoautotrophs. Chemoautotrophs include sulfur-oxidizing bacteria, nitrifying bacteria, hydrogen-oxidizing bacteria, and iron-oxidizing bacteria. For example, the overall chemical reaction involved in chemosynthesis by sulfur-oxidizing bacteria is $6CO_2 + 6H_2S + 6H_2O + 6O_2 \rightarrow C_6H_{12}O_6 + 6H_2SO_4$, while the chemical reaction of photosynthesis is $6CO_2 + 6H_2O$ (+ light energy) $\rightarrow C_6H_{12}O_6 + 6O_2$. In addition, methane-oxidizing bacteria (methanotrophs) can grow on methane as their sole source of carbon and energy. Methane is a single-carbon organic compound rather than an inorganic molecule. Therefore, methanotrophs are classified as chemoheterotrophs rather than as chemoautotrophs. However, methanotrophs generally utilize methane produced by abiotic chemical processes through volcanic activity as well as by a biotic process of methanogens, and therefore these bacteria sometimes play a role as primary producers in certain ecosystems, as in the case of chemoautotrophs.

Ecosystems where chemoautotrophs and/or methanotrophs, rather than plants and algae, are involved in primary production are defined as chemosynthetic ecosystems, and biological communities formed in such ecosystems are called chemosynthetic communities. Chemosynthetic ecosystems/communities are mainly divided into two types, hydrothermal vent ecosystems/communities and cold-seep ecosystems/communities, both of which are normally found on the deep sea floor (Fig. 3.1). The former depend on mineral-rich seawater heated to high temperatures by the magma under the earth's crust and are found at volcanically active sites where tectonic plates are moving apart such as mid-oceanic ridges. The latter depend on unheated seeping seawater rich in hydrocarbons such as methane and are found at convergent plate boundaries such as trenches and troughs, methane hydrate-bearing sediments, mud volcanoes, and submarine oil fields. In addition to these two types of ecosystem, large organic matter that falls to the deep sea floor, such as whale carcasses and wood logs, are known to release a large amount of reduced chemical species such as hydrogen sulfide during their anaerobic decomposition, and to form chemosynthetic ecosystems.

In chemosynthetic ecosystems, there is a wide variety of symbiotic relationships between chemosynthetic bacteria (i.e., chemoautotrophs and methanotrophs) and endemic animals. Animals representative of chemosynthetic ecosystems, such as the clams *Calyptogena* spp., the mussels *Bathymodiolus* spp., the sea snails *Alviniconcha* spp., and siboglinid worms (including tubeworms), harbor chemosynthetic intracellular or extracellular bacterial symbionts and depend on the nutrients supplied by their symbionts. These animals closely associated with the primary

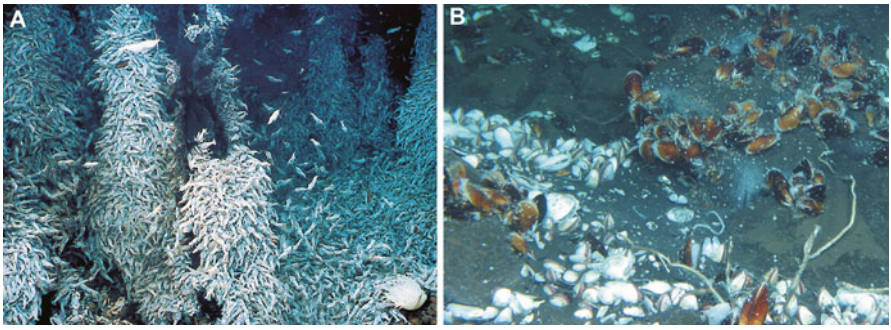

Fig. 3.1 (**a**) A dense population of the shrimp *Rimicaris kairei* at the Kairei hydrothermal vent field of the Central Indian Ridge. (**b**) Colonies of the bivalves *Calyptogena* spp. (*white clams*) and *Bathymodiolus* spp. (*brown mussels*) at the cold-seep site in Sagami Bay

production of chemosynthetic bacteria densely colonize the sites of hydrothermal vents and cold seeps, contrasting sharply with the scarcely populated "normal" deep sea environment. For example, the vent shrimp *Rimicaris exoculata* has been observed in very high densities (up to 2500 individuals/m^2) on active chimney walls at several sites of the Mid-Atlantic Ridge (Komai and Segonzac 2006). The population density of *Calyptogena* spp. at the seep site off Hatsushima in Sagami Bay, Japan, has also been estimated to be high (128 individuals/m^2) (Fujikura et al. 2002). Furthermore, it has also recently been revealed that anaerobic oxidation of methane by the syntrophic association between two different prokaryotes, "anaerobic" methane-oxidizing archaea (ANME) and sulfate-reducing bacteria, is an ecologically important biological process in anoxic and methane-rich seep sediments (e.g., Orphan et al. 2001).

Unlike in the case of animals and prokaryotes (archaea and bacteria), there is still limited information on the diversity and ecology of unicellular eukaryotes composed of protists and fungi inhabiting deep sea hydrothermal vents and cold seeps. Given that protists and fungi are known to play a significant role as primary producers, grazers, decomposers, or parasites in ecosystems depending on photosynthesis, such microbial eukaryotes should also have key ecological functions in chemosynthetic ecosystems. In the last 10 years, molecular techniques such as culture-independent PCR of eukaryotic rRNA genes with environmental DNA have been applied to elucidate the diversity of microbial eukaryotes inhabiting chemosynthetic ecosystems (summarized in Table 3.1). Based on the results of such surveys, I review recent progress in the understanding of microbial eukaryotes in these environments, to which relatively little attention has been paid, and give perspectives on this research area.

Table 3.1 Deep sea chemosynthetic ecosystems surveyed with molecular techniques to elucidate the diversity of microbial eukaryotes

Location	Depth (m)	Source(s)	Reference(s)
Hydrothermal vents			
Guaymas Basin	2,000	Sediments	Edgcomb et al. (2002), Coyne et al. (2013)
Mid-Atlantic Ridge	750–2,200	Sediments, seawater, bivalves, rocks	López-García et al. (2003, 2007), Le Calvez et al. (2009), Sauvadet et al. (2010)
Southern East Pacific Rise	2,500–2,700	Seawater, bivalves	Sauvadet et al. (2010)
Cold seeps			
Kuroshima Knoll (Japan)	650	Sediments	Takishita et al. (2006)
Sagami Bay (Japan)	900–1,200	Sediments, bivalves	Takishita et al. (2007, 2010), Nagahama et al. (2011), Noguchi et al. (2013)
Gulf of Mexico	1,000	Bivalves	Sauvadet et al. (2010)

3.2 Microbial Eukaryotes in the Ecosystems of Hydrothermal Vents

Regarding microbial eukaryotes inhabiting deep sea hydrothermal vents, culturing and/or microscopic studies remain very sparse. Atkins et al. (2000) isolated and cultured several flagellated protists from four vent sites, Juan de Fuca Ridge, Guaymas Basin, and 21°N and 9°N on the East Pacific Rise. Based on microscopy and small-subunit (SSU) rRNA gene sequences, those protists were identified as an ancyromonad (*Ancyromonas sigmoides*), bicosoecids (*Cafeteria* sp. and *Caecitellus parvulus*), a cercozoan (*Massisteria marina*), a choanoflagellate (*Monosiga* sp.), a chrysomonad (*Paraphysomonas* sp.), and kinetoplastids (*Rhynchomonas nasuta* and *Bodo saliens*), most of which are ubiquitous members of marine, freshwater, and terrestrial environments worldwide. On the other hand, in the vent fields of Juan de Fuca Ridge, Kouris et al. (2007) found morphologically unique folliculinid ciliates (*Folliculinopsis* sp.) that form dense bright-blue mats and are likely associated with chemosynthetic bacteria, suggesting their endemism and physiological adaptations to this peculiar environment. These culturing and microscopic studies are indispensable to determine the morphological, physiological, and ecological characteristics of each microbial eukaryote. However, such studies often fail to reveal the diversity of microbial eukaryotes in an environment, because many of them are difficult to culture and remain uncultured, and morphologically nondescript organisms such as ovoid- or ellipsoid-shaped yeasts are occasionally difficult to identify precisely based on microscopy alone.

In recent years, there has been increasing interest in the diversity of microbial eukaryotes from a wide range of environments based on culture-independent PCR surveys using eukaryotic SSU rRNA gene as a marker. Studies using this approach have unveiled an unexpectedly high diversity of microbial eukaryotes in sediments and/or seawater around the deep sea hydrothermal vents of the Guaymas Basin (Edgcomb et al. 2002), Mid-Atlantic Ridge (López-García et al. 2003, 2007), and Southern East Pacific Rise (Sauvadet et al. 2010). For example, "phylotypes" (biological types classified by phylogenetic analyses of retrieved gene sequences) belonging to fungi, green algae, stramenopiles, alveolates, and radiolarians have been found in anoxic sediments of the Guaymas vent field (Edgcomb et al. 2002), while fungi, stramenopiles, alveolates, euglenozoans, cercozoans, radiolarians, and Heterolobosea have been shown to inhabit sediments, carbonates, and fluid–seawater interfaces of Mid-Atlantic Ridge hydrothermal sites (López-García et al. 2003, 2007). In particular, the latter environments have been suggested to harbor a large diversity of divergent lineages within the alveolate radiation. In addition to organisms belonging to these eukaryotic groups, the SSU rRNA gene sequences from amoebozoans, centrohelids, apusomonads, and ancyromonads have also been obtained from seawater surrounding vent chimneys of the Southern East Pacific Rise (Sauvadet et al. 2010).

Intriguingly, many phylotypes very closely related to aerobic eukaryotes globally distributed in marine environments, along with phylotypes of anoxic specialists such as the ciliates *Metopus contortus* and *Trimyema compressum*, were unexpectedly recovered from anoxic Guaymas Basin sediments, regardless of the fact that bacteria detected in the same sediments were composed of species typically found in anoxic and hydrocarbon-rich environments. These findings suggest that the corresponding microbial eukaryotes are potentially tolerant to anaerobic conditions. Alternatively, these organisms (at least part of them) possibly originated from dead sunken or specific life stage (cyst) cells. The latter case may be especially true for photosynthetic microbial eukaryotes including green algae and diatoms of which phylotypes have been encountered in the samples of Edgcomb et al. (2002). Phylotypes closely related to such microalgae have been more frequently recovered from anoxic sediment near active fumaroles on the submarine caldera floor of Kagoshima Bay, Japan, where the chemical characteristics are similar to those of the Guaymas vent field but the depth is much shallower (about 200 m) (Takishita et al. 2005), supporting the hypothesis that not all phylotypes obtained are necessarily indigenous to the ecosystems of hydrothermal vents. Nevertheless, based on the phylotypes of a wide variety of heterotrophic protists and fungi identified through those molecular studies, microbial eukaryotes are highly likely to act as grazers and decomposers in sediments and seawater around deep sea hydrothermal vents, and play key roles in these ecosystems.

It is also remarkable that phylotypes closely related to parasitic protists such as perkinsids, apicomplexans (including gregarines), and kinetoplastids, along with "marine Alveolata groups" (including Syndiniales) have frequently been detected in sediments and/or seawater of the Guaymas and Mid-Atlantic Ridge vents (Edgcomb et al. 2002; López-García et al. 2003). Based on these findings, Moreira and

López-García (2003) pointed out that such diverse parasitic protists could infect the dense animal communities thriving around hydrothermal vents and that some of them may act as causative agents of the enigmatic sudden mortality of these animals. In particular, it is known that perkinsids infect a variety of bivalves and sometimes kill their hosts. One phylotype of perkinsid has been recovered from an artificial substrate for experimental colonization near a bed of the mussel *Bathymodiolus azoricus* in the vent field of the Mid-Atlantic Ridge, suggesting that the perkinsid parasitizes these hydrothermal bivalves. Sauvadet et al. (2010) conducted a culture-independent PCR survey on the liquid of the pallial cavity of *Bathymodiolus thermophilus* and *Calyptogena magnifica* from the Southern East Pacific Rise and of *B. azoricus* from the Mid-Atlantic Ridge to detect microbial eukaryotes associated with these bivalves. In that study, PCR using a non-metazoan universal PCR primer (Bower et al. 2004) along with a eukaryotic universal primer (hereafter referred to as "non-metazoan PCR") was applied to prevent the amplification of the bivalve SSU rRNA gene. The phylotypes of ciliates along with those of cercozoans and/or (probably heterotrophic) dinoflagellates have been constantly and predominantly retrieved from two bivalve species from the Southern East Pacific Rise. Among these ciliate phylotypes, close relatives of *Parauronema virginianum*, *Uronema marina*, and *Entodiscus borealis* could be parasites or commensals. Interestingly, a novel eukaryote having no affiliation with known major eukaryotic lineages predominantly occurs in the pallial cavity of some individuals of *B. azoricus* from the Mid-Atlantic Ridge, but it remains unclear whether this organism is a parasite, a commensal, a mutualistic symbiont, or merely a contaminant from seawater. Although those molecular surveys did not indicate the presence of potentially pathogenic fungi in hydrothermal environments, Van Dover et al. (2007) found that a *Capronia*-like black yeast belonging to the ascomycete order Chaetothyriales specifically infects *Bathymodiolus brevior* at the Mussel Hill hydrothermal vent in the Fiji Basin. This fungus has been shown to elicit an intense hemocytic immune response from *B. brevior* and to be apparently associated with severe tissue degradation of the host in histological studies and using the fluorescent in situ hybridization technique, raising the possibility of massive mortality of this mussel at Mussel Hill. Collectively considering all these observations, it is reasonable to assume that parasitic protists and fungi actually flourish in the ecosystems of deep sea hydrothermal vents and potentially affect the community structure and population dynamics of animals and other organisms inhabiting those sites.

Many phylotypes recovered from these habitats were identical or closely related to those from other environments such as surface seawater and were likely opportunistic or cosmopolitan. Nevertheless, whether microbial eukaryotes endemic to hydrothermal vent ecosystems exist is an attractive and controversial topic. Based on phylogenetic analyses, López-García et al. (2007) proposed a potential hydrothermal and/or anaerobic lineage belonging to bacidiomycetes, but this lineage actually includes sequences from soil and human skin (Moon-van der Staay et al. 2006; Findley et al. 2013), excluding the possibility of such endemism. Le Calvez et al. (2009) found a high diversity of fungi at several vent sites with culture-independent PCR using "fungal-specific primers" and suggested a deeply branching

novel lineage specifically thriving in deep sea hydrothermal vent fields within the chytridiomycete clade. Likewise, Coyne et al. (2013) surveyed the ciliate communities in anoxic sediments of the Guaymas Basin hydrothermal vents through culture-independent reverse transcription PCR (RT-PCR), in which environmental RNA rather than environmental DNA is used as a template, with primers specific to this protist group, and also reported a novel lineage composed of sequences from hydrothermal vent fields along with cold seeps (this lineage is also discussed in the next section). However, this issue cannot be resolved until more thorough PCR surveys in environments other than hydrothermal vent areas and cell isolation/cultivation followed by physiological studies of the microbial eukaryotes in question have been conducted.

3.3 Microbial Eukaryotes in the Ecosystems of Cold Seeps

In the context of "comprehensive" surveys of microbial eukaryotes in cold-seep ecosystems, pioneering microscopic work was conducted at sites in Monterey Bay, California, USA. In bacterial mats on the sediments of these studied sites, foraminifers, euglenozoans, and ciliates were found to dominate microbial eukaryotes. Bernhard et al. (2001) identified 38 species of living benthic foraminifers there, all of which also occur in areas other than seep environments. Buck and Barry (1998) found many species of seep euglenozoans and ciliates with either intracellular or extracellular symbiotic bacteria at the light microscopic level, and Buck et al. (2000) reported that at least three discrete morphotypes among the euglenozoans observed harbored extracellular symbiotic bacteria completely covering the surface of the euglenid cell, based on scanning electron microscope images. The bacteria associated with euglenozoans contained membranes surrounding sulfur inclusions and are likely to be sulfur oxidizers, although phylogenetic and physiological data are lacking. These bacteria have been suggested to support the host euglenozoans by providing themselves as food and/or by detoxifying the immediate surrounding environment through the oxidation of hydrogen sulfide. The chemoautotroph-bearing euglenozoans found in seep habitats in Monterey Bay may belong to "Symbiontida", a recently established subclade of Euglenozoa (Yubuki et al. 2009). All symbiontid species described (i.e., *Calkinsia aureus*, *Bihospites bacati*, and *Postgaardi mariagerensis*) are similarly covered with extracellular symbiotic bacteria and exist in oxygen-depleted and sulfidic marine environments. In addition to the studies conducted in the Monterey Bay seep area more than 25 years ago, it should also be noted that Buck et al. (2014) have recently found novel microbial communities including amoebae, euglenozonas, ciliates, and unidentified protists in polysaccharide sheaths of the sulfur-oxidizing bacteria *Thioploca* spp. flourishing in the same habitat by microscopic observation.

Culture-independent PCR surveys using the eukaryotic SSU rRNA gene as a marker were conducted in oxygen-depleted sediments of methane seep sites located on the Kuroshima Knoll and in Sagami Bay, Japan (Takishita et al. 2006, 2007,

2010). Unexpectedly, the sequence from the basidiomycetous fungus *Cryptococcus curvatus* was predominantly recovered at both sites, in contrast to the microscopic studies of the Monterey Bay cold seeps. These findings suggest that *C. curvatus* dominates microbial eukaryotic communities in these environments. This fungus is frequently isolated from terrestrial habitats, and thus we first suspected contamination when its sequence was obtained. However, that possibility seemed unlikely after we checked the experimental devices, DNA extraction kit, and liquid nitrogen tank in which the sediment samples were stored. Indeed, fungus-like cells have been observed in sediment samples. On the other hand, Nagahama et al. (2011) suggested that the phylotypes of "basal fungi" (the early diverging fungal assemblage other than ascomycetes and basidiomycetes) occupy a major portion of the fungal community in the seep sediments of Sagami Bay and did not detect the sequence of *C. curvatus*. These basal fungi were composed of the cryptomycote clade (also referred as the environmental clade LKM11) (Jones et al. 2011) and a novel clade newly found in the study mentioned. It should be noted that Nagahama et al. (2011) performed culture-independent PCR with fungal-specific primers, while eukaryotic universal primers were used in previous studies. Therefore, the PCR bias due to different primer selection may have at least partially affected the incongruence between the results reported by Takishita et al. (2007) and Nagahama et al. (2011), in spite of the fact that the same seep area was surveyed. Otherwise, the fungal community may not be uniformly distributed in this seep area, since the sampling points were slightly different between those two studies. In any case, the fungi detected are suggested to be ecologically important in cold-seep ecosystems as decomposers or consumers. In addition, an unknown relationship between *C. curvatus* and methane may have to be considered, because the sediments we analyzed included methane abundantly seeping from the seafloor.

Although the above findings were based on culture-independent PCR surveys using environmental DNA, we conducted further culture-independent RT-PCR surveys using environmental RNA in the cold-seep sediments in Sagami Bay (Takishita et al. 2010). In sharp contrast to the results at the DNA level mentioned above, the phylotypes mostly encountered at the RNA level were from ciliates. Given that RNA is much more transient than DNA and that environmental RNA surveys would be expected to detect viable indigenous organisms in the environment more sensitively, these ciliates are metabolically active in the sediments. Since most ciliates are known to be bacterivorous, it has been suggested that the ciliates of which sequences were recovered play a role in the food web of this ecosystem as grazers of prokaryotes abundantly present in the sediments such as ANME and sulfate-reducing bacteria. The ciliate sequences detected were phylogenetically diverse, representing eight known classes as well as undesignated lineages. Notably, phylotypes belonging to one of the undesignated lineages have been frequently encountered from deep sea chemosynthetic ecosystems (i.e., seep sediments in Sagami Bay, sheaths of *Thioploca* colonizing the Monterey Bay seep sediments, and anoxic sediments of the Guaymas Basin hydrothermal vents), as shown in Fig. 3.2, although this type has also been found in non-chemosynthetic environments (e.g., the Antarctic deep sea).

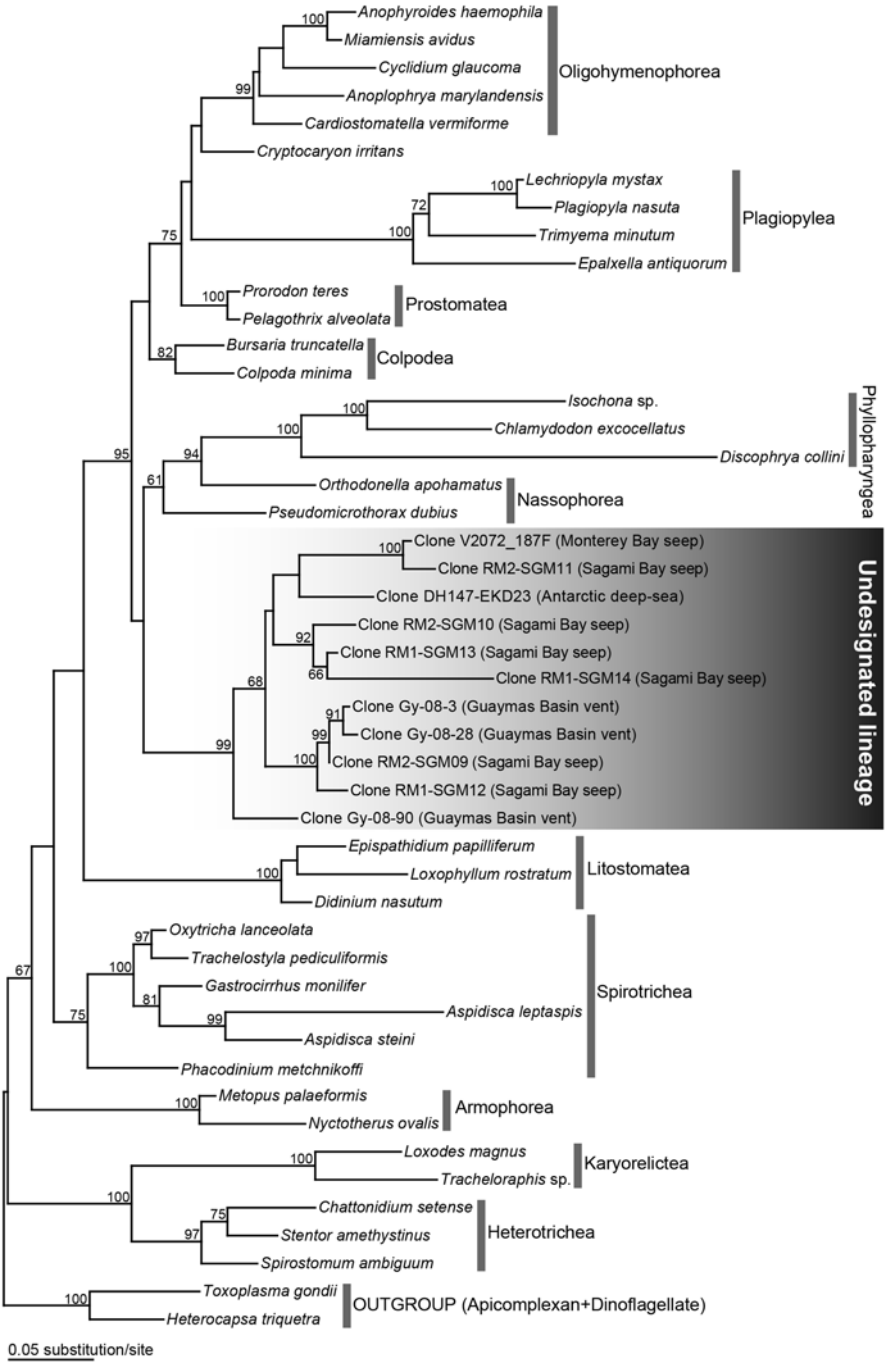

Fig. 3.2 Maximum-likelihood tree of SSU rRNA gene sequences (1,318 positions of 46 ciliates, one apicomplexan, and one dinoflagellate) reconstructed using RAxML 7.2.8 (Stamatakis 2006) with a GTRGAMMAI model selected as the best-fit model with Modeltest 3.7 (Posada and Crandall 1998). An undesignated ciliate lineage mainly composed of environmental sequences from chemosynthetic ecosystems is shaded. Bootstrap probabilities are shown for nodes with support greater than 60 %

In addition to *C. curvatus* and ciliates, the phylotypes of diverse heterotrophic cercozoans and stramenopiles have also been recovered from the seep sediments in Sagami Bay at both DNA and RNA levels, suggesting that the corresponding organisms feed on prokaryotes in seep ecosystems (Takishita et al. 2007, 2010). We have attempted culturing and microscopic observations of the Sagami Bay samples, and a flagellated organism was successfully enriched under anaerobic culture conditions (Takishita et al. 2007). The SSU rRNA gene sequence from this flagellate was almost identical to the environmental sequence (C1_E027) from the Guaymas Basin hydrothermal vent and was positioned within the radiation of Fornicata based on phylogenetic analyses. Fornicata is one of the Excavata lineages, composed exclusively of anaerobic species such as diplomonads and retortamonads (Simpson 2003). Subsequently, this flagellate has been repeatedly isolated from a wide range of oxygen-depleted environments other than the seep sediments in Sagami Bay and has been designated as *Kipferlia bialata* (Kolisko et al. 2010). *K. bialata* may not be very abundant in the Sagami Bay seep sediments, because its environmental sequence has not been detected with culture-independent PCR methods. Nevertheless, it seems likely that this type of anaerobic flagellate also contributes to the consumption of prokaryotes, along with the ciliates and heterotrophic cercozoans/stramenopiles mentioned above.

As in the molecular studies of hydrothermal vent ecosystems, the phylotypes of potentially parasitic protists such as Syndiniales, Apicomplexa, and Ichthyosporea were retrieved from the seep sediments in Sagami Bay (Takishita et al. 2007). These findings suggested that dense animal populations inhabiting this ecosystem could also host such parasitic protists. Using molecular techniques, Noguchi et al. (2013) investigated whether such microbial eukaryotes actually occur inside the bodies of the bivalves *Calyptogena* spp. and *Bathymodiolus* spp. from Sagami Bay. Through a non-metazoan PCR survey, the SSU rRNA gene sequence from a specific protist was obtained from the gill tissues of a significant fraction of these bivalves. Unexpectedly, this sequence was not among the phylotypes detected in sediments from the same habitat. Based on the phylogeny, the corresponding organism was shown to belong to the Alveolata, but its precise affiliation remains unclear as the detected sequence branched deeply within the Syndiniales, for which the monophyly was consistently recovered but not robustly supported. Furthermore, whole-mount fluorescence in situ hybridization of the *Bathymodiolus* gill tissue demonstrated that this novel alveolate occurs inside epithelial bacteriocytes occupied by methanotrophic bacterial endosymbionts (Fig. 3.3). Based on such localization, it is possible that the protist in question consumes the bacterial endosymbionts as food or depends on nutrition produced by the endosymbionts. It was found that the gill tissue harboring this alveolate was unlikely to have decayed or been damaged. Thus, this newly found protist is possibly an endo-commensal rather than a virulent endoparasite of these bivalves, even if it has some degree of physiological and ecological impact on the bivalves. Using the non-metazoan PCR method, Sauvadet et al. (2010) examined protist communities in the pallial cavity fluids (not inside the bodies) of *Bathymodiolus brooksi* and *B. childressi* collected at seep sites in the Gulf of Mexico, together with those of bivalves inhabiting hydrothermal vent

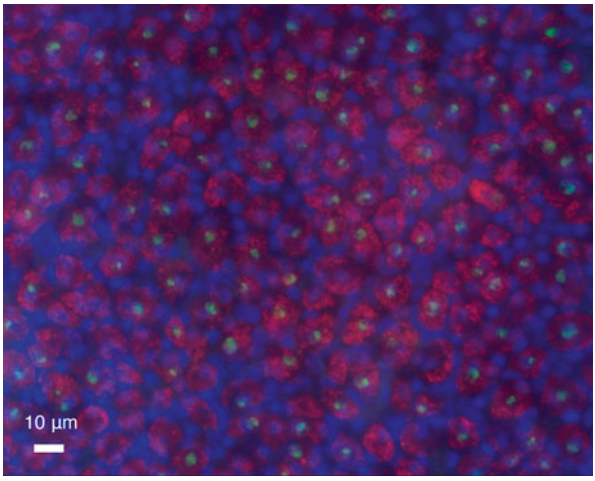

Fig. 3.3 *Bathymodiolus platifrons*. Fluorescence micrograph of whole-mount fluorescence in situ hybridization on the gill filaments. Image of red and green fluorescence signals with an Alexa555-labeled probe (specifically targeting methanotrophic endosymbiotic bacteria) and an Alexa488-labeled probe (specifically targeting the novel protist belonging to Alveolata) of the gill filament, respectively, with DAPI staining. The protist exists inside bacteriocytes occupied by methanotrophic bacteria

areas as mentioned in the previous section. However, the sequence from the alveolate found by Noguchi et al. (2013) or from its closely related organism was not detected. The host specificity and potential dispersal (i.e., cosmopolitan distribution or endemism) of this alveolate remain enigmatic. In addition, it is also still uncertain which organisms are potential hosts of phylotypes closely related to the parasitic protists frequently encountered in culture-independent PCR surveys of the seep sediments in Sagami Bay. Although the precise nature of the relationship remains to be resolved, these findings stress the under-considered ecological significance of eukaryotic parasites in cold-seep ecosystems, as is the case of hydrothermal vent ecosystems.

3.4 Perspectives

General problems and perspectives of environmental surveys at the molecular level including culture-independent PCR are not mentioned here. Instead, I present views on the expected future studies of microbial eukaryotes in deep sea chemosynthetic ecosystems.

Culture-independent PCR surveys of hydrothermal vents and cold seeps have uncovered the huge diversity of protists and fungi inhabiting these ecosystems. However, it is often difficult to infer the morphological, metabolic, and ecological characteristics of such organisms based only on the retrieved marker gene sequences.

Thus, to obtain information on these biological characteristics, the techniques of microscopy in combination with in situ hybridization based on the retrieved sequences and cell isolation/cultivation followed by physiological and biochemical experiments are indispensable. To apply these techniques, samples in which the organisms investigated are alive or unbroken must be collected. When the seep sediment samples of Sagami Bay—in which rRNA sequences from phylogenetically diverse ciliates were predominantly encountered—were observed with microscopes, no ciliated cells could be found (Takishita et al. 2010). Since these sediment samples from the depth of 900–1,200 m were recovered without maintaining hydraulic pressure, the cells of ciliates were possibly broken due to the drastic pressure change. This problem may be true for other microbial eukaryotes lacking a tough cell wall. To prevent the cells from bursting and/or dying before microscopic observation, a sampling device capable of maintaining high hydraulic pressure (or of gradually decreasing the pressure) may have to be utilized. A device for "in situ fixation" of the cells in environments using fixatives could be a powerful tool to protect the cell morphology of microbial eukaryotes and to precisely identify the organisms under a microscope. Samples obtained with such a fixation device could also be subjected to in situ hybridization experiments. Furthermore, a cultivation system under high-pressure conditions may have to be developed to keep the targeted protists and fungi alive. Such sampling devices and cultivation systems have already been applied to deep sea "piezophilic" prokaryotes (reviewed in Kato 2011), but similar attempts have not yet been made for microbial eukaryotes inhabiting chemosynthetic ecosystems and other deep sea environments. To infer the metabolic and ecological aspects of microbial eukaryote communities in these ecosystems, metatranscriptome sequencing that is targeted at eukaryotic microbes through poly(A)+RNA selection may also be effective. On the other hand, eukaryotic sequences could not be efficiently recovered with metagenomics, as prokaryotic sequences are supposed to be overwhelmingly dominant in the metagenomic data.

A significant fraction of phylotypes of protists and fungi retrieved from chemosynthetic ecosystems and their corresponding organisms are also found in non-chemosynthetic environments, suggesting that these microbial eukaryotes are cosmopolitan. Nevertheless, there are also novel eukaryotic lineages exclusively or mainly composed of environmental sequences from areas of hydrothermal vents and/or cold seeps, as mentioned above. Thus, one of the most intriguing subjects in this research area may be discovery of microbial eukaryotes exclusively or mainly inhabiting chemosynthetic ecosystems. One possible physiological characteristic common to these organisms is energy production via chemosynthesis. Such microbial eukaryotes could metabolize reduced compounds such as hydrogen sulfide and methane by themselves. Otherwise, chemosynthetic symbionts could be harbored, as in the case of various animals endemic to hydrothermal vents and cold seeps such as *Calyptogena* spp., *Bathymodiolus* spp., and tubeworms. If the former case is true, it is possible that genes associated with the oxidization of reduced molecules were laterally transferred from a chemosynthetic prokaryote to a microbial eukaryote. If the latter is the case, that makes these "chemosynthetic microbial eukaryotes" more attractive in the context of evolution. In multicellular eukaryotes including animals,

the germ line is separated from the soma. This reproductive system probably reduces the likelihood of fixation of foreign DNA (e.g., a laterally or endosymbiotically transferred gene) in a population. There is no evidence that the genomes of animals with chemosynthetic bacteria include genes derived from their symbionts. In contrast to multicellular eukaryotes, such reproduction is not very common among microbial (unicellular) eukaryotes, and any transferred gene is necessarily inherited by its gene recipient's offspring. Therefore, once the chemosynthetic endosymbionts are found in microbial eukaryotes, the possibility that they are "chemosynthetic organelles" with a genome reduced due to endosymbiotic gene transfer to a host (i.e., microbial eukaryote) genome would be considered. Methods to isolate and cultivate chemosynthetic prokaryotes could serve as references to establish cultures of microbial eukaryotes with the metabolic capacity for chemosynthesis.

Finally, it is highly likely that ecologically, evolutionarily, and metabolically interesting protists and fungi in the chemosynthetic ecosystems are still hidden. For example, considering their reduced environments, novel anaerobic microbial eukaryotes with mitochondria degenerated to various degrees would exist. We identified a novel alveolate endobiont in the gill tissue of the *Bathymodiolus* bivalve as mentioned above (Noguchi et al. 2013). However, this may be the tip of the iceberg of symbionts, commensals, and parasites in these ecosystems, and various types of such organisms will possibly be discovered in the future. Identifying the cells individually based on morphology and metabolic capacity is laborious, making it difficult to determine the total organismal diversity in an environment. However, such studies will clarify the ecology of microbial eukaryotes in chemosynthetic ecosystems, which is becoming within our grasp using molecular techniques.

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Chapter 4
Biology, Diversity and Ecology of Free-Living Heterotrophic Flagellates

Takeshi Nakayama

Abstract The free-living heterotrophic flagellates are phylogenetically very diverse, and their phylogenetic diversity is nearly equal to that of all eukaryotes. They constitute the microbial loop together with bacteria and other protists, and play an indispensable and important role in aquatic ecosystems. However, their diversity has not been fully understood. In addition, recent environmental DNA studies suggest that there are many unidentified heterotrophic flagellate lineages in nature. This chapter describes the current knowledge on the diversity, biology and ecology of various groups of free-living heterotrophic flagellates.

Keywords Diversity • Ecology • Heterotrophic flagellates • Phylogeny • Protists

4.1 Introduction

All heterotrophic flagellates (HF) were classified in the Zoomastigophorea and regarded as ancestral representatives of "animals". However, this traditional taxonomic and evolutional view on HF was changed drastically by ultrastructural and molecular studies. Recent molecular phylogenetic studies indicate that there are several supergroups within the eukaryotes (e.g., Walker et al. 2011; Adl et al. 2012). HF are situated in all these supergroups and some HF represent independent lineages that cannot be included in any supergroups (Table 4.1). Phylogenetically, the diversity of HF is nearly equal to that of all eukaryotes. The basic architecture of HF is considered to be the ancestral organization of eukaryotes (e.g., Cavalier-Smith 2002). Therefore, recognition of the diversity and phylogeny of HF is necessary to understand those of eukaryotes.

Most free-living HF are phagotrophic; they engulf and digest particulate foods. Non-living organic matter, bacteria (including cyanobacteria), eukaryotic algae, protozoans, and sometimes parts of land plants or metazoans are consumed by various flagellates (e.g., Sanders 1991). Some flagellates feed on a wide range of food,

T. Nakayama (✉)
Faculty of Life and Environmental Sciences, University of Tsukuba,
1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
e-mail: algae@biol.tsukuba.ac.jp

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Table 4.1 A classification system of eukaryotes especially for heterotrophic flagellates

Opisthokonta
Phylum Amoebidiozoa (“Mesomycetozoa”, ichthyosporids)
Phylum **Choanozoa** *s.s.* (choanoflagellates) … e.g., ***Acanthoeca, Codosiga, Desmarella, Diploeca, Monosiga, Protospongia, Salpingoeca, Stephanoeca***
Metazoa
Fungi (including Microsporidia)
Incertae sedis
Class Corallochytrea (*Corallochytrium*)
Class Filasterea (*Capsaspora, Ministeria*)
Class Cristidiscoidea (e.g., *Fonticula, Nuclearia*)
Amoebobiota
Phylum **Amoebozoa**
Class Tubulinea (e.g., *Amoeba, Arcella*)
Class Flabellinea (Discosea)* (e.g., *Paramoeba, Vannella*)
Class **Archamoebea** (Pelobiontea) … e.g., *Entamoeba,* ***Mastigamoeba***, ***Mastigella***, ***Mastigina***, ***Pelomyxa***, ***Phreatamoeba***
Class **Protostelea***
Class Dictyostelea
Class **Myxogastrea** (Myxomycetes)
Incertae sedis
Order **Holomastigida** … ***Multicilia***
Order **Phalansteriida** … ***Phalanstelium***
etc.
Excavata
Phylum **Metamonada**
Fornicata … e.g., ***Carpediemonas***, ***Chilomastix***, ***Dysnectes***, *Enteromonas,* ***Ergobibamus***, *Giardia,* ***Hexamita***, ***Hicanonectes***, ***Kipferlia***, ***Trepomonas***
Parabasalia … e.g., ***Ditrichomonas***, ***Honigbergiella***, ***Lacusteria***, ***Pseudotrichomonas***, ***Tetratrichomonas***, *Trichomonas, Trichonympha*
Preaxostyla (Anaeromonadea) … e.g., *Oxymonas*, ***Trimastix***
Phylum **Loukozoa** (jakobids) … e.g., ***Histiona***, ***Jakoba***, ***Reclinomonas***
Phylum **Percolozoa** (heteroloboseans *s.l.*)
Class **Pharyngomonadea** … ***Pharyngomonas***
Class **Heterolobosea** *s.s.* … e.g., *Acrasis, Naegleria,* ***Psalteriomonas***, ***Percolomonas***, ***Stephanopogon***
Phylum **Euglenozoa**
Class **Euglenophyceae** … e.g., ***Astasia***, ***Entosiphon***, ***Euglena***, *Eutreptiella,* ***Peranema***, ***Petalomonas***, ***Phacus,*** *Rapaza*
Class **Postgaardea** (Symbiontida) … ***Calkinsia***, ***Bihospites***, ***Postgaardi***
Class **Diplonemea** … ***Diplonema***, ***Rhynchopus***
Class **Kinetoplastea** … e.g., ***Bodo***, *Icthyobodo,* ***Neobodo***, ***Parabodo***, *Trypanosoma*
Incertae sedis
Class Tsukubea … *Tsukubamonas*
Class Malawimonadea … *Malawimonas*

(continued)

Table 4.1 (continued)

Plantae (Archaeplastida)
Phylum Glaucocystophyta (Glaucophyta)
Phylum Rhodophyta (red algae)
Phylum **Chlorophyta**
Class Trebouxiophyceae
Class Ulvophyceae
Class **Chlorophyceaes** … e.g., *Chlamydomonas*, *Oedogonium,* ***Polytoma***, ***Polytomella***, *Scenedesmus*
etc.
Phylum Streptophyta (land plants and relatives)
Heterokonta (stramenopiles)
Phylum Labyrinthulomycota (e.g., *Diplophrys, Thraustochytrium*)
Phylum Opalozoa (e.g., *Blastocystis, Opalina, Proteromonas*)
Phylum **Pseudofungi***
Class **Bigyromonadea** … ***Developayella***
Class Hyphochytridiomycetes
Class Oomycetes
Incertae sedis
Order **Pirsoniales** … ***Pirsonia***
Phylum **Ochrophyta** (Heterokontophyta)
Class Bacillariophyceae (diatoms)
Class **Dictyochophyceae** … e.g., ***Ciliophrys***, ***Actinomonas***, Pedinella, ***Pteridomonas***
Class Eustigmatophyceae
Class **Picophagea** … ***Picophagus***
Class **Chrysophyceae** … e.g., ***Anthophysa***, Dinobryon, Ochromonas, ***Paraphysomonas***, ***Spumella***
Class Raphidophyceae
Class Xanthophyceae
Class Phaeophyceae (brown algae)
etc.
Incertae sidis
Class **Bicosoecea** (Bikosea) … e.g., ***Adriamonas, Boroka, Bicosoeca, Caecitellus*, *Cafeteria*, *Filos, Halocafeteria*, *Labromonas, Nerada, Paramonas, Pseudodendromonas, Symbiomoans***
Class **Placididea** … ***Placidia, Wobblia***
Class **Nanomonadea** (MAST-3) … ***Incisomonas***, ***Solenicola***
Class Nucleohelea (actinophryids)
Incertae sidis
Order **Rictida** … ***Rictus***
Alveolata
Phylum Ciliophora (ciliates)
Phylum **Colponemidia** … ***Colponema***
Phylum **Acavomonidia** … ***Acavomonas***

(continued)

Table 4.1 (continued)

Phylum **Apicomplexa**
Colpodellids* … e.g., ***Alphamonas, Chilovora, Colpodella, Voromonas***
Class Coccidea
etc.
Phylum **Dinozoa** (Dinoflagellata)
Class Perkinsea
Class **Oxyrrhea** … ***Oxyrrhis***
Class Ellobiopsea
Class Syndinea (MALV II)
Class **Noctiluciphyceae** (Noctilucea) … e.g., ***Kofoidinium, Noctiluca, Spatulodinium***
Class **Dinophyceae** … e.g., *Ceratium*, ***Gyrodinium, Phalacroma, Protoperidinium***, *Symbiodinium*
Incertae sedis
Eduboscquellidae (MALV I)
Rhizaria
Phylum Radiozoa (*Sticholonche* and radiolarians excl. phaeodarians)
Phylum Foraminifera
Phylum **Cercozoa***
Class **Skiomonadea** … ***Tremula***
Class **Chlorarachniophyceae** (Chlorarachnea) … e.g., *Bigelowiella*, ***Minorisa***
Class **Metromonadea*** … ***Metopion, Metromonas, Micrometopion***
Class Granofilosea* … e.g., *Clathrulina, Limnofila, Massisteria, Nanofila*
Class **Sarcomonadea*** … e.g., ***Brevimastigomonas, Cavernomonas, Cercomoans, Eocercomonas, Metabolomonas, Nucleocercomonas, Paracercomonas*****,** ***Agitata*****,** ***Aurigamonas*****,** ***Allantion*****,** ***Allapsa*****,** ***Bodomorpha*****,** ***Cholamonas*****,** ***Dujardina*****,** ***Flectomonas*****,** ***Helkesimastix*****,** ***Mollimonas*****,** ***Neohetromita*****,** ***Proleptomonas*****,** ***Sainouron*****,** ***Sandona*****,** ***Teretomonas***
Class **Thecofilosea*** … e.g., ***Botuliforma, Cryothecomonas*****,** ***Ebria,*** *Lecythium***,** ***Mataza, Protaspis*****,** *Pseudodifflugia, Rhogostoma*, ***Ventrifissura, Verrucomonas***
Class Phaeodarea
Class **Imbricatea*** … ***Allas, Auranticordis, Clautriavia, Discomonas, Esquamula,*** *Euglypha****, Gyromitus, Nudifila,*** *Paulinella****,*** *Peregrinia, Pseudopirsonia,* ***Reckertia, Spongomonas, Thaumatomastix, Thaumatomonas***
Class Phytomyxea (plasmodiophorids)
Class Gromiidea
Class Ascetosporea
Class Proteomyxidea*
Eukarya incertae sedis
Phylum **Cryptophyta**
Class **Goniomonadea** … ***Goniomonas***
Class **Cryptophyceae** … e.g., *Chroomonas*, ***Cryptomonas*** (***Chilomonas***), *Guillardia*
Phylum **Katablepharida** … ***Hatena, Katablepharis*** (***Kathablepharis***)***, Leucocryptos, Platychilomonas, Roombia***

(continued)

Table 4.1 (continued)

Phylum Haptophyta (Pavlovophyceae and Coccolithophyceae)
Class Pavlovophyceae
Class Coccolithophyceae (Prymnesiophyceae) … e.g., *Chrysochromulina*, *Emiliania*, *Haptolina*, *Pleurochrysis, Prymnesium*
Phylum Heliozoa (centrohelids)
Phylum **Picozoa** … ***Picomonas***
Phylum **Telonemia** … ***Telonema***
Phylum **Apusozoa***
Class **Thecomonadea** (apusomonads) … ***Amastigomonas, Apusomonas, Manchomonas, Mantamonas*, *Multimonas, Podomonas, Thecamonas***
Class **Hilomonadea** (ancyromonads)… ***Ancyromonas, Fabomonasa, Nutomonas, Planomonas***
Incertae sedis
Class **Palpitea** … ***Palpitomonas***
Class **Breviatea** … ***Breviata, Subulatomonas***
Class **Diphyllatea** … ***Collodictyon, Diphylleia, Sulcomonas***
etc.

Taxa in *bold face* include heterotrophic flagellates, *underlined* taxa include mixotrophic flagellates (only phagotrophic taxa). *Asterisks* mean the taxa may be not monophyletic

but most flagellate feed more selectively. They feed on small particles suspended in water, on those loosely attached to substrata, or on larger prey by various strategies (e.g., Sleigh 2000). Some photosynthetic flagellates also can engulf particulate food (mixotrophy *s.s.*). Although most of these phagotrophic flagellates probably absorb organic small molecules (osmotrophy), some flagellates are obligate osmotrophs (e.g., *Polytoma*, "*Chilomonas*"). In aquatic ecosystems, these flagellates (especially phagotrophs) play important roles as consumers of bacteria and producers. They are especially fundamental components for transfer of the bacterial production based on dissolved organic matter to higher trophic levels (a microbial loop) (e.g., Azam et al. 1983). Therefore, understanding of the ecological role of HF is necessary to understand aquatic ecosystems.

The diversity of HF in natural environments has been studied using culturing and microscopy. However, recent environmental DNA studies indicate that there are many new and unexpected protistan lineages in aquatic environments (Massana, Chap. 1; Takishita, Chap. 3). Furthermore, these studies suggest that culturing bias sometimes hides the natural diversity of HF (e.g., del Campo and Massana 2011; del Campo et al. 2013). Therefore, further environmental DNA studies, new culturing efforts, and combined techniques such as FISH should be applied to reveal the real diversity and ecological role of HF.

In this chapter, I will give an overview of our current knowledge about the diversity, biology and ecology of various groups of HF.

4.2 Opisthokonta

Two large and diversified multicellular groups of eukaryotes, Metazoa and Fungi, form a supergroup of eukaryotes called the Opisthokonta. In addition, some protistans such as choanoflagellates, filastereans, ichthyosporids and nucleariids also belong to this supergroup. The flagellate cells of opisthokonts possess a single posteriorly directed flagellum inserted in the posterior end of the cell (*opisthios* = posterior, *kontos* = flagellum). In contrast, other eukaryotes basically possess two flagella inserted anteriorly to laterally (ventrally). Mitochondrial cristae are flat in most opisthokonts. Opisthokonts are exclusively heterotrophic and no taxa have chloroplasts, but some species harbor symbiotic or kleptoplastidic algae (e.g., lichens, reef-building corals, sacoglossan sea slugs). Molecular data sometimes suggest a close relationship between Opisthokonta and Amoebozoa and this clade is termed "unikonts" (Richards and Cavalier-Smith 2005). However, this relationship is not confirmed because the root of the eukaryote tree is not resolved at present.

In the Opisthokonta, the choanoflagellates is the only group of flagellates. They are unicellular or colonial, planktonic or benthic, and free-living in marine and freshwater habitats. The single flagellum of choanoflagellates is surrounded by a collar composed of many fine "tentacles" (microvilli) supported by actin microfilaments. The flagellum produces a water current from base to tip and food particles (usually bacteria, but see Vørs et al. 1995) adhere to and are ingested by microvilli (e.g., Leadbeater 1991). This cell architecture is very similar to that of sponge choanocytes, and suggests a close relationship to Metazoa. Multigene phylogenetic analyses support the sister relationship between choanoflagellates and Metazoa (e.g., Shalchian-Tabrizi et al. 2008; Torruella et al. 2012). Because of this phylogenetic position, choanoflagellates have received attention to understand the origin of Matezoa and the nuclear genome has been sequenced for *Monosiga brevicollis* (King et al. 2008). The genome contains cell adhesion and signaling protein domains that are otherwise restricted to metazoans. Choanoflagellates have been classified based on the composition and structure of cell covering. Members of the Codosigidae appear to be naked when examined by light microscopy, but have a fine organic coat (glycocalyx) visible only by electron microscopy (e.g., Leadbeater 2008). The cell of the Salpingoecidae is encased in a firm flask-, cup-, or tube-shaped organic theca that is visible by light microscopy. However, molecular phylogenetic analyses indicate that the Codosigidae and Salpingoecidae are indistinctly mixed phylogenetically (Carr et al. 2008). In contrast, molecular data support the monophyly of another traditional choanoflagellate taxon based on the periplast feature, the Acanthoecidae, which contains species with an organic protoplast sheath and a basket-like lorica composed of siliceous ribs (costae). Acanthoecid choanoflagellates exclusively inhabit marine environments and are considered to be important components in the microbial food web (e.g., Leakey et al. 2002). In addition, nonloricate choanoflagellates (and other flagellates such as *Bicosoeca*) attached to large phytoplankton such as diatoms may constitute important pelagic bacteriovores in aquatic ecosystems (Simek et al. 2004).

Other opisthokont protistan groups contain no flagellate species (but the peduncle of a filasterean, *Ministeria vibrans*, may be a modified flagellum; see Cavalier-Smith and Chao 2003). However, free-living amoeboid opisthokonts (filastereans, nucleariids) probably play a similar role (bacteriovores or eukaryovores) to HF in aquatic ecosystems. In addition, unicellular parasitic opisthokonts (e.g., ichthyosporids, basal fungi such as Cryptomycota and chytrids) usually produce flagellate cells (zoospores) as a dispersal stage and they are frequently detected in environmental DNA studies (e.g., Karpov et al. 2014). These parasitic opisthokonts are probably important components in aquatic ecosystems (Schweikert, Chap. 21; Yokoyama et al. Chap. 22).

4.3 Amoebozoa (Amoebobionta)

The Amoebozoa is a supergroup of eukaryotes including canonical amoebae, mycetozoans (dictyostelids, myxogastrids and polyphyletic protostelids) and some flagellates. Amoebozoans have no filopodia but usually possess lobopodia or its derivatives. The mitochondria usually have branching tubular cristae (thus sometimes referred to as ramicristae), but are reduced into hydrogenosomes or mitosomes in some taxa. Amoebozoans are exclusively heterotrophic and common in aquatic (both marine and freshwater) and soil environments. They are usually components of benthic ecosystems but are sometimes found in plankton samples. Trophic cells of amoebozoans usually lack flagella, but some taxa are flagellates. Some amoebozoan flagellates have a single anterior flagellum directed anteriorly and a simple flagellar apparatus composed of a single basal body giving rise to a cone and ribbon of microtubules (e.g., pelobionts) (Walker et al. 2001). However, the flagellate stage of mycetozoans usually has two flagella (anteriorly directed immature and posteriorly directed mature flagellum; Gely and Wright 1986) and a complex flagellar apparatus that is probably homologous to that of most other eukaryote groups excluding opisthokonts ("bikonts") (Spiegel 1991; Walker et al. 2003; Yubuki and Leander 2013).

Pelobionts (e.g., *Mastigamoeba*, *Mastigella*) are microaerophilic or anaerophilic amoeboflagellates forming lobopodia in an eruptive manner. Cells usually possess a single anterior flagellum without outer dynein arms that beats languidly. They have no typical Golgi body and mitochondria but organelles bounded by double membranes that are probably reduced mitochondria (Walker et al. 2001; Hampl and Simpson 2008). Most pelobionts are free-living and inhabit microoxic to anoxic environments such as marine and freshwater sediments (e.g., Bernard et al. 2000). Molecular data suggest that pelobionts form a clade (Archaeamoebae) with entamoebae, which are mostly parasitic anaerophilic amoebae without flagella (e.g., *Enatamoeba histolyca*). *Phalansterium* is a bacteriovorous, free-living, freshwater or soil flagellate producing extracellular soft globular matrices that sometimes form colonial organization (Hibberd 1983; Ekelund 2002). The cell has a single anterior flagellum surrounded by a continuous cytoplasmic color. The cytoplasm includes a

Golgi body and mitochondria with tubular cristae. *Phalansterium* is superficially similar to choanoflagellates (Opisthokonta) and *Spongomonas* (Rhizaria), but molecular data suggest it is related to schizoplasmodiids "protostelids" (Shadwick et al. 2009). *Multicilia* is a eukaryovorous marine flagellate that usually possesses 20–30 (sometimes two or up to 200) flagella emerging randomly over the cell surface (Mikrjukov and Mylnikov 1998). The flagella beat weakly without coordination but the basal body is connected to the neighboring one by a microtubular root. The cells have no polarity and many Golgi bodies. Mitochondrial cristae are tubular. The phylogenetic position of *Multicilia* in the Amoebozoa is uncertain (Nikolaev et al. 2006; Lahr et al. 2011).

4.4 Excavata

Excavates are predominantly unicellular HF that typically have a ventral feeding groove supported by the complex cytoskeleton derived from microtubular flagellar roots. They usually possess an anterior (immature) and a posterior (mature) flagellum with a complex flagellar apparatus inserted in the anterior end of the ventral groove (e.g., Simpson 2003; Yubuki et al. 2013a). Three microtubular roots (R1, R2 and singlet root), in which R2 is divided into outer and inner components, extend from the posterior basal body and support the ventral groove. R3 extending clockwise from the anterior basal body is associated with an array of cytoskeletal microtubules usually supporting the dorsal side of the cell (dorsal fan). Some fibrous components (e.g., A, B, C, I fiber) are associated with these microtubular roots. Although some excavates lack this typical cell architecture (e.g., Parabasalia, Euglenozoa), their flagellar apparatus can be assumed to be derived forms from this basic structure. Interestingly, almost all eukaryote groups possess some of these characteristics in flagellar apparatus. These similarities suggest that the basic architecture of the excavate flagellar apparatus is a prototype of most eukaryote flagellar apparatus (Yubuki and Leander 2013). In excavates, mitochondria possess discoid, tubular or rarely flat cristae, but are sometimes reduced to lacking cristae and a mitochondrial genome. Excavates are important components in aquatic ecosystems as free-living bacteriovores and eukaryovores but many excavates are symbiotic (including parasitic). Excavates includes two major groups, Metamonada and Discoba, and an isolated minor lineage represented by *Malawimonas*.

The Metamonada is composed of three anaerobic/microaerophilic flagellate groups, Fornicata (diplomonads and relatives), parabasalids and Preaxostyla (anaeromonads). All metamonads have no typical mitochondria but probable relics of mitochondria (hydrogenosomes or mitosomes) are detected in some taxa (e.g., Dolezal et al. 2005; Schneider et al. 2011; Takishita et al. 2012). Most known species of metamonads are symbiotic (sometimes parasitic) flagellates in metazoans, including humans, but some free-living species inhabiting low-oxygen marine and freshwater environments are known. Fornicates other than diplomonads are characterized by the presence of an arched B fiber connecting R1 and R2 (e.g., Yubuki

et al. 2013a). In addition, most fornicates lack typical Golgi bodies. In the Fornicata, some free-living flagellates inhabiting marine sediments such as *Carpediemonas*, *Dysnectes*, *Ergobibamus*, *Hicanonectes* and *Kipferlia* represent a paraphyletic basal grade (e.g., Takishita et al. 2012). These flagellates called *Carpediemonas*-like organisms (CLOs) possess two flagella in which the paraxial vanes are on the posterior flagellum. The phylogenetic position of CLOs indicates that these flagellates are important to understand the evolutional degeneration of mitochondria and origin of diplomonads. Most diplomonads are symbiotic (parasitic) flagellates such as *Giardia intestinalis* (*G. lamblia*) causing giardiasis in humans, but some species of *Trepomonas* and *Hexamita* are free-living and relatively common bacteriovorous flagellates in low-oxygen environments such as marine and freshwater sediments (e.g., Mylnikov 1991; Bernard et al. 2000). Diplomonads usually possess two nuclei each of which is associated with a flagellar apparatus including four basal bodies (karyomastigont). However, some species (enteromonads) have only a single karyomastigont system (e.g., Brugerolle and Müller 2000; Brugerolle and Regnault 2001). Retortamonads is another amitochondriate fornicate group possessing four (*Chilomastix*) or two (*Retortamonas*) flagella in which the posterior one has vanes. Although almost all retortamonads are symbiotic, a free-living species, *C. cuspidate*, has been reported from marine and freshwater low-oxygen environments (Bernard et al. 1997). Recent molecular data suggest that retortamonads are paraphyletic in the Fornicata (Takishita et al. 2012).

Parabasalids are amitochondriate but have hydrogenosomes to function in respiration and metalloprotein biosynthesis (e.g., Schneider et al. 2011). This flagellate group is characterized by the presence of a parabasal body that is a striated root extending posteriorly from the basal bodies and associated with developed Golgi cisternae (e.g., Brugerolle and Müller 2000). Parabasalids are sometimes aflagellate (e.g., *Dientamoeba*) or multiflagellate (hypermastigids), but basically have four flagella, in which the posterior flagellum may be attached to the cell body by an undulating membrane. The feeding groove is reduced in parabasalids. In the flagellar apparatus, an anterior pelta composed of a sheet of cross-linked microtubules (probably homologous to the dorsal fan of typical excavates) continue to an axostyle extending posteriorly (e.g., Brugerolle 1991a; Brugerolle and Müller 2000). No other microtubular roots are detected but some fibrous roots are developed. Nuclear division is performed by closed mitosis with an extranuclear spindle (pleuromitosis). Although most parabasalids are symbionts (sometimes parasites) of metazoans, especially termites and vertebrates, some bacteriovorous free-living species are reported from marine and freshwater low-oxygen environments (e.g., Brugerolle 1991b; Yubuki et al. 2010a).

The Preaxostyla (Anaeromonadea), including oxymonads and *Trimastix*, are characterized by the I fiber with a preaxostylar substructure (a latticework paracrystalline layer with a single outer layer) (Simpson 2003). Oxymonads are exclusively symbionts of metazoans, especially termites and their relatives. *Trimastix* is a bacteriovorous free-living quadriflagellate that has the typical excavate architecture such as a ventral feeding groove, flagellar vanes and complex flagellar apparatus (e.g., Simpson et al. 2000). *Trimastix* inhabits low-oxygen marine and freshwater environments and lacks typical mitochondria but has hydrogenosome-like organelles (e.g., Zubáčová et al. 2013).

Discoba is an informal name for the clade composed of the Euglenozoa, Percolozoa (heterolobosean), Loukozoa (jakobids) and *Tsukubamonas*. They are mostly unicellular HF with mitochondria. No phenotypic apomorphies are detected for this clade but molecular phylogenetic studies strongly support the monophyly of Discoba (e.g., Yabuki et al. 2011).

The Euglenozoa are composed of four flagellate groups: euglenids (Euglenophyceae *s.l.*), kinetoplastids, diplonemids and symbiontids (probably equivalent to Postgaardea; Yubuki et al. 2013b). They typically have two heterodynamic flagella inserted into the anterior flagellar pocket. The flagella usually contain proteinaceous paraxial (paraxonemal) rods along axonemes and possess fine nontubular hairs along the outside. Cytoskeletal microtubules arising from the R3 support the flagellar pocket and cell surface. Euglenozoans lack a feeding groove typical for excavates, but have the feeding apparatus supported by the R2 beside the flagellar pocket. The nucleus with a permanent nucleolus is divided by closed mitosis. At least kinetoplastids and diplonemids utilize spliced leader transcripts in nuclear gene expression (e.g., Sturm et al. 2001). Mitochondrial cristae are usually discoid and are unique in eukaryotes. Kinetoplastids possess a unique multichromosomal mitochondrial genome, and this feature may be universal in the Euglenozoa (Roy et al. 2007).

The Euglenids are a large group characterized by the presence of proteinaceous pellicular strips arranged longitudinally or spirally just beneath the cell membrane. Taxa with many pellicular strips usually show characteristic squirming movements (metaboly, euglenoid movement). Euglenids include well-known photosynthetic algae such as *Euglena* but this group is basically heterotrophic (e.g., Leander et al. 2007). Photosynthetic euglenids have plastids derived from the secondary endosymbiosis with a green alga (possibly Pyramimonadales, Chlorophyta; e.g., Hrdá et al. 2012) and represent a subclade nested within heterotrophic euglenids. Recently, a mixotrophic euglenid, *Rapaza viridis*, has been reported and it probably represents an earliest photosynthetic euglenid (Yamaguchi et al. 2012). Early divergent euglenids are bacteriovorous, and they have rigid cells with a few (<12) pellicular strips (e.g., *Petalomonas, Entosiphon, Ploeotia*). Eukaryovorous euglenids usually possess many pellicular strips (20–60) arranged spirally and are capable of metaboly (e.g., *Peranema*, "*Dinema*" = *Dinematomonas*). Phototrophic and primary osmotrophic euglenids derived from eukaryovorous ancestors independently from each other are basically metabolic (e.g., *Eutreptiella*, *Astasia*) but include secondarily rigid taxa (e.g., *Phacus*, *Menoidium*). In addition some photosynthetic euglenids secondarily lost photosynthetic ability and are osmotrophic (e.g., "*Astasia*" *longa* = *Euglena longa*). Phagotrophic euglenids possess a feeding apparatus sometimes supported by robust rods and vanes, but that of phototrophic and osmotrophic euglenids is highly reduced. Heterotrophic euglenids are very common in both marine and freshwater environments and important components in the microbial food web. Most heterotrophic euglenids are gliding on substrata but (both primary and secondary) osmotrophic euglenids are usually not gliding.

Kinetoplastids include infamous parasites (e.g., *Trypanosoma*) but are basically free-living bacteriovorous flagellates such as *Bodo*, *Neobodo*, *Parabodo* and

Rhynchomonas. These flagellates possess the kinetoplast, an aggregated mass of mitochondrial genome including maxicircles and minicircles. Glycolysis is performed in specialized peroxysomes, glycosomes (e.g., Opperdoes 2010). Molecular phylogenetic analyses recognize five lineages in kinetoplastids (e.g., Deschamps et al. 2011) in which two (prokinetoplastids and trypanosomatids) are symbiotic (sometimes parasitic) and three are composed of mostly free-living flagellates that are very common in marine, freshwater and soil environments. *Bodo saltans*, *Neobodo designis*, *N. saliens* and *Rhynchomonas nasuta* are taxa of the twenty most commonly reported HF (Patterson and Lee 2000). However, it should be noted that only a few (morpho-)species have been described but molecular data show the huge genetic diversity in these species (von der Heyden and Cavalier-Smith 2005).

Diplonemids are eukaryovorous flagellates found in marine and freshwater. They are metabolic biflagellates with a euglenozoan cell architecture and a complex feeding apparatus that is similar to some phagotrophic euglenids, but have some unusual features for the Euglenozoa such as flagella without paraxial rods and mitochondria with flat cristae. They are recorded as predators, penetrating and digesting the cells of planktonic diatoms and deteriorating leaves of aquatic plants, or parasites of crustaceans (Triemer and Ott 1990; Schnepf 1994; von der Heyden et al. 2004). Although only two genera (*Diplonema*, *Rhynchopus*) with a few species are known in the Diplonemea, environmental DNA studies suggest that this group has a hidden large diversity especially in the deep ocean (e.g., Lara et al. 2009). Symbiontids (Postgaardea) are unique biflagellates possessing numerous episymbiotic bacteria on the cell surface (Yubuki et al. 2009, 2013b; Breglia et al. 2010). They have no typical mitochondria but probably homologous organelles without cristae are situated just beneath the cell surface. Symbiontids are found in low-oxygen marine environments and are probably eukaryovorous.

Percolozoans (heteroloboseans *s.l.*) typically have amoeboid and flagellate trophic stages, but either stage is reduced in many taxa (e.g., Harding et al. 2013). Amoeboid cells move rapidly with eruptive lobopodia. Flagellate cells usually possess two or four flagella inserted in the anterior end of the feeding groove that is largely modified in some taxa. Flagellar apparatus shows a characteristic "double bikont" system with two R2 and a rhizoplast. Most percolozoans (Heterolobosea *s.s.*, Tetramitia) have parallel basal bodies and no R1 but *Pharyngomonas* (Pharyngomonadea) possesses orthogonal basal bodies and developed two R1 (Park and Simpson 2011). Mitochondria with discoid cristae are frequently associated with rough endoplasmic reticulum. They lack a typical Golgi apparatus. Percolozoans inhabit various habitats including marine, freshwater, soil and some extreme environments (e.g., hypersaline waters) (e.g., Park and Simpson 2011). *Percolomonas* is a relatively common bacterivorous quadriflagellate in marine environments. A novel multiflagellated eukaryovore, *Stephanopogon,* which is superficially very similar to ciliates but nested in the Heterolobosea (or *Percolomonas*; Yubuki and Leander 2008), is found in marine interstitial environments.

Jakobids (Loukozoa) are heterotrophic bacteriovorous flagellates including a few genera and species (*Andalucia, Histiona, Jakoba* and *Reclinomonas*) (e.g., O'Kelly 1993, 1997; Simpson and Patterson 2001; Lara et al. 2006). The cell has the typical

excavate architecture and two flagella in which the posterior one possesses a dorsal vane. Mitochondrial cristae are mostly tubular but flat in *Jakoba*. It is noteworthy that the mitochondrial genome of a jakobid (*R. americana*) retains several primitive (bacterial) features such as retaining more protein-coding genes than other mitochondria and the presence of some subunits of a bacterial-type RNA polymerase (Lang et al. 1997; Gray et al. 2004). Jakobids are plankton or benthos in marine and freshwater habitats. *Tsukubamonas* is a freshwater flagellate representing the forth lineage of Discoba (Yabuki et al. 2011). This bacteriovorous flagellate possess a vacuolated cell with many excavate features such as a ventral feeding groove, splitting R2 and singlet root. The precise phylogenetic position of *Tsukubamonas* in Discoba is unresolved.

Malawimonas is another typical excavate (O'Kelly and Nerad 1999). This freshwater biflagellate possesses a laterally flattened cell, a single ventral vene on the posterior flagellum, and discoidal mitochondrial cristae. The phylogenetic position of *Malawimonas* is not resolved in the molecular phylogenetic analyses; this flagellate may be related to the Metamonada, Discoba or external to the excavates (e.g., Zhao et al. 2012).

4.5 Archaeplastida (Plantae)

Archaeplastids are organisms with plastids originated by primary endosymbiosis with a cyanobacterium. This supergroup of eukaryotes is composed of glaucophytes, red algae and green plants (green algae and land plants; Viridiplantae). The organization level of archaeplastids is very diversified, from simple unicellular to complex multicellular. Most members of the archaeplastids are photosynthetic, but some parasitic taxa are known in red algae and green plants (e.g., *Benzaitenia*, *Helicosporidium*, *Rafflesia*). In archaeplastids, only some species of the Volvocales (Chlorophyta) are HF (e.g., *Polytoma, Polytomella*). These osmotrophic organisms possessing two or four isokont flagella inhabit freshwater and soil environments. Heterotrophic archaeplastids are exclusively osmotrophic and the ability of phagocytosis is usually considered to be lost in archaeplastids. However, some members of the Pyramimoandales (Chlorophyta) may have the ability to ingest particles such as bacteria (e.g., Bell and Laybourn-Parry 2003).

4.6 Stramenopiles

Stramenopiles (Heterokonta) include a photosynthetic group (Ochrophyta, Heterokontophyta or stramenochromes) and many heterotrophic lineages such as fungi-like organisms (Oomycetes, Hyphochytridiomycetes, Labyrinthulea), heliozoan-like protists (actinophryds), intestine parasites (Opalozoa) and free-living HF. Flagellate cells of stramenopiles typically possess two heterodynamic

heterokont flagella inserted anteriorly to ventrally; a posteriorly directed mature flagellum is smooth but an anteriorly directed immature flagellum is ornamented by characteristic tripartite (base, shaft and terminal hairs) tubular hairs (*stramen* = straw, *pilus* = hair). Recent studies have identified a protein of the flagellar hairs (Honda et al. 2007; Yamagishi et al. 2007). Because the tubular flagellar hairs reverse the water current produced by normal flagellar beats, preys are drawn to the cells from the anterior area in phagotrophic stramenopiles. The flagellar transitional region sometimes includes the characteristic transitional helix. Stramenopiles typically possess four microtubular roots (R1–R4) and in phagotrophic species preys are usually ingested by the cytostome formed between the splitting R2 (e.g., Moestrup and Andersen 1991). In the flagellate cells, the basal bodies are closely associated with the nucleus, and a Golgi body is situated at the dorsal side of the nucleus. Mitochondria possess tubular cristae. Molecular data indicate that stramenopiles form a clade (SAR clade, subkingdom Harosa) with Alveolata and Rhizaria in eukaryotes.

Bicosoecids are one of the most common heterotrophic flagellate groups in aquatic environments, at least under microscopy (e.g., *Caecitellus, Cafeteria*) (e.g., Patterson and Lee 2000; but see del Campo and Massana 2011). They ingest small preys and are mostly bacteriovorous (e.g., Vørs et al. 1995). Bicosoecids are usually unicellular biflagellates and the posterior flagellum is directed to the left-posterior side of the cell. At the base of R2, microtubules are arranged in an L-shape (basically 8 + 3 + x-fiber) (e.g., O'Kelly and Patterson 1996). Most bicosoecids possess no transitional helix in the flagellar transitional region. Many bicosoecids usually attach to the substrata by the tip of the posterior flagellum (e.g., *Cafeteria*) or lorica (*Bicosoeca*). Some bicosoecids (e.g., *Paramonas*, *Siluania*, *Symbiomonas*) are planktonic and the posterior flagellum is reduced in these genera. In the bicosoecids, *Caecitellus* is unusual in that the cell is gliding and the anterior flagellum is smooth (O'Kelly and Nerad 1998). In addition, some freshwater bicosoecids (*Adriamonas*, *Cyathobodo*, *Pseudodendromoans*) possess two nearly equal flagella, both of which lack hairs (e.g., Verhagen et al. 1994). The unequal flagella of *Rictus lutensis* found in low-oxygen environments also lack hairs (Yubuki et al. 2010b). Although molecular data weakly support the basal position of *R. lutensis* in the bicosoecids, the ultimate phylogenetic position in the stramenopiles remains unknown.

Placidids are also unicellular heterotrophic biflagellates and very similar to some bicosoecids such as *Cafeteria*. However, placidids are different from bicosoecids in the presence of a posterior flagellum directed to the right side in the attaching cell, a double helix in the flagellar transitional region, and a U-shaped arrangement of microtubules (7 + 3) in R2 (Moriya et al. 2000, 2002). They are attaching, gliding or free swimming flagellates found in marine environments. Although only two genera and species (*Placidia cafeteriopsis* and *Wobblia lunata*) have been described so far, recent studies suggest that there is a hidden diversity in placidids (Park and Simpson 2010). Molecular data suggest that the Placididea is an early divergent lineage of stramenopiles as bicosoecids, labyrinthulids and opalozoans (e.g., Yubuki et al. 2010b). However, the interrelationships among

these protists are not resolved. *Developayella* is another bacteriovorous flagellate possessing the double transitional helix and is relatively common in marine environments. Cells possess a ventral depression in which two flagella directed posteriorly are situated in stationary cells (Tong 1995). Molecular data suggest that *Developayella* is related to oomycetes and hyphochtridiomycetes, but the accurate phylogenetic position of this flagellate is not settled. Labylinthulids (labyrinthulomycetes), another heterotrophic stramenopile group, have no flagella in the trophic stage. However, they are probably important decomposers in marine ecosystems (Raghukumar and Damare 2011; Nakai and Naganuma, Chap. 13).

The ochrophytes, including diatoms, brown algae, golden algae and yellow-green algae, are usually photosynthetic and possess chloroplasts originated by the secondary endosymbiosis with a red alga. However, some ochrophytes, especially in the Chrysophyceae and Dictyochophyceae, are heterotrophic. In addition, some members of these classes (e.g., *Dinobryon, Ochromonas, Pedinella*) are mixotrophic; they can perform photosynthesis using chloroplasts but also can use organic material. Mixotrophic ochrophytes may play important roles in aquatic ecosystems (e.g., Frias-Lopez et al. 2009; Unrein et al. 2014). In the ochrophytes, the Chrysophyceae are characterized by the presence of lateral filaments on the flagellar hairs and the producing of siliceous walled stomatocysts (statospores) in the life cycle. The Chrysophyceae include many planktonic or benthic heterotrophic genera such as *Anthophysa*, *Paraphysomonas* and *Spumella*. Photosynthetic chrysophyceans are ubiquitous in freshwater environments, but heterotrophic chrysophyceans are common in both marine and freshwater. The Dictyochophyceae are characterized by the presence of a shallow depression of the nucleus in which the basal bodies are situated and microtubules extend from the nuclear surface. Heterotrophic dictyochophyceans such as *Ciliophrys* and *Pteridomonas* are also common in marine environments (Patterson and Lee 2000).

Environmental DNA studies indicate that unidentified lineages of stramenopiles, MAST (*ma*rine *st*ramenopiles), are important components in marine ecosystems (e.g., Massana et al. 2004). Now about 18 MAST groups are recognized and at least some of them are considered to be HF (Massana et al. 2006, 2014). Only the organisms in the MAST-3 (Nanomonadea) have been cultured and revealed to be HF (Cavalier-Smith and Scoble 2013). The abundance of MAST in marine environments indicates that their biological information is necessary to understand marine ecosystems. In addition, these data also indicate that we know about only a part of the diversity of stramenopiles so far. Taxonomic and phylogenetic studies on MAST are indispensable to clarify the diversity and evolution of stramenopiles.

4.7 Alveolata

The Alveolata are a supergroup of eukaryotes composed of three large protistan lineages, the Ciliophora (ciliates), Apicomplexa and Dinozoa (dinoflagellates). They possess flat vesicles called alveoli (inner membrane complex, amphiesmal vesicles) immediately beneath the plasma membrane. The alveoli are not part of

other endomembrane systems, and contain an unique family of structural proteins, alveolins (Gould et al. 2008). In addition, the alveoli sometimes include organic or calcified plates. The alveolate cell is usually covered by many alveoli arranged in a taxa-specific manner, and small depressions performing pinocytosis (micropore) and ejectile organelles (trichocysts) are sometimes situated between these alveoli. Mitochondrial cristae are basically tubular. Interestingly, most alveolates reproduce by oblique to transversal (not longitudinal) binary fission.

Ciliates are "specialized flagellates" that thrive in almost all aquatic environments. The ciliate cells have one of the most complex cell architectures in life. They usually have many cilia (flagella) arranged in species-specific rows (kineties) and the cilia sometimes form bundles for walking (cilli) or sheets creating water currents (membranelle). Ciliate nuclei exhibit a unique dimorphism; somatic polyploid macronuclei are active transcriptionally to control cell functions and physiology, and germline diploid micronuclei perform genetic recombination during conjugation. Ciliates are basically heterotrophic and important bacteriovores and eularyovores in aquatic ecosystems with flagellates and amoebae. In addition, some ciliates harbor symbiotic or kleptoplastidic algae and may be important producers in some aquatic ecosystems (e.g., Stoecker et al. 1987).

In alveolates, the Apicomplexa and Dinozoa form a robust clade called Miozoa or Myzozoa. Recently, some eukaryovorous biflagellates (*Acavomonas*, *Colponema*) have been shown to be early divergences of the Miozoa (Tikhonenkov et al. 2014). This finding suggests that the common ancestor of Miozoa (and probably Alveolata) would be a eukaryovorous flagellate. Apicomplexans are mostly parasitic (symbiotic) organisms including *Cryptosporidium*, *Toxoplasma* and *Plasmodium*. They possess a unique organelle, an apical complex, composed of a microtubular cone (conoid or pseudoconoid), a ring-shaped MTOC (polar ring) and secretive vesicles (rhoptries and micronemes). The apical complex is used to attach and invade the host cell. Many apicomplexans have non-photosynthetic plastids (apicoplasts) surrounded by four membranes. The apicoplasts probably have a common secondary endosymbiotic origin with dinoflagellate chloroplasts (peridinin-type). Furthermore, some early divergences of the Apicomplexa are photosynthetic and possess typical chloroplasts (chromerids, e.g., Oborník and Lukeš 2013). Therefore, the common ancestor of the Apicomplexa would be photosynthetic. Colpodellids *s. l.* (e.g., *Chilovora, Colpodella, Voromonas*), another early divergence of the Apicomplexa, exhibit an intermediate lifestyle between carnivores and parasites (e.g., Brugerolle 2002; Cavalier-Smith and Chao 2004; Mylnikov 2009). The flagellate cells of colpodellids attach to the prey cell and suck its contents by myzocytosis. Colpodellids are probably important components in both marine and freshwater ecosystems as predators of eukaryotes.

Dinozoans exhibit various trophic modes, phototrophy, mixotrophy and heterotrophy. Flagellate cells of dinozoans usually possess an anterior (transverse) wrapping flagellum and a posteriorly directed longitudinal flagellum inserted ventrally. Some dinozoans (perkinsids, *Psammosa*) possess the apical complex that supports the sister relationship between Apicomplexa and Dinozoa (e.g., Okamoto et al. 2012). In most dinozoans, the nuclear genome is uniquely condensed with DVNP (dinoflagellate/viral nucleoprotein) (excl. perkinsids) (Gornik et al. 2012).

Interestingly, most early divergent lineages of the Dinozoa (e.g., perkinsids, ellobiopsids, syndineans) are endo- or ectoparasites of various organisms such as copepods, mollusks, appendicularians and protists including dinoflagellates. In these parasitic dinozoans, syndineans (*m*arine *alv*eolates II = MALV II) and eudubosc-quellids (MALV I) are dominant components in environmental DNA studies (e.g., Guillou et al. 2008). However, they are probably not free-living because all known species of these groups are parasitic. Because parasitic organisms generally produce numerous dispersal units, these environmental sequences are probably derived from zoospores of these organisms. In either case, the abundant occurrence of MALV in DNA studies indicates their ecological importance and that biological information on these parasitic dinozoans is necessary to understand marine ecosystems (Horiguchi, Chap. 16). Other early divergent dinozoans (e.g., Noctilucales, *Oxyrrhis, Psammosa*) are free-living phagotrophic flagellates (Fukuda and Suzuki, Chap. 2). *Noctiluca* feeds on prey particles using mucoid filtration and is an important component of the coastal ecosystem (Omori and Hamner 1982; Uhlig and Sahling 1990). Almost all photosynthetic dinozoans are classified in the Dinophyceae and they typically possess chloroplasts surrounded by three membranes and containing a unique carotenoid, peridinin. Although this type of chloroplast is considered to be derived from secondary endosymbiosis with a red alga, some dinophyceans have chloroplast originated from green alga, haptophyte or diatoms. The photosynthetic dinophyceans are common and important producers in aquatic (especially marine) environments, but many photosynthetic dinophyceans are also capable of uptaking foods (mixotrophs). In addition, about half of the known species of the Dinophyceae are obligate heterotrophic (Gaines and Elbrächter 1987). They are important eukary-ovores in marine ecosystems (e.g., Sherr and Sherr 2007) and exhibit three types of feeding mechanism (e.g., Hansen and Calado 1999). Many dinophyceans feed by typical phagocytosis (direct engulfment) in which a whole prey is engulfed usually through the sulcus. The second type of feeding mechanism, myzocytosis (tube feeding), is also common in heterotrophic dinophyceans. They suck the contents of the prey through a peduncle (a protoplasmic strand protruding from the sulcus) or phagopod (a non-cytoplasmic tube formed in the antapical part of the cell). Some thecate dinophyceans (e.g., *Protoperidinium*) feed on prey (sometimes large filamentous diatoms) by the third type of feeding mechanism, pallium feeding. In these dino-phyceans, a specialized pseudopodium called a pallium (feeding veil) extends from the sulcus and envelops prey. The prey is digested in the pallium outside the cell body and only liquefied cell contents are absorbed.

4.8 Rhizaria

The Rhizaria are a supergroup of eukaryotes including various protists such as radiolarians, foraminifers, plasmodiophorids, chlorarachnids, euglyphids and many HF. The monophyly of the Rhizaria is robustly supported by molecular data and many rhizarians possess filo-, reticulo- or axopodia and mitochondria with tubular cristae.

However, no phenotypic apomorphy for this supergroup has been detected so far. The Rhizaria are composed of three large groups, Radiozoa, Foraminifera and Cercozoa. Radiozoans have axopodia and siliceous or strontium sulfate skeletons typically with a central capsule dividing the cell into endoplasm and ectoplasm (Suzuki and Not, Chap. 8). Foraminifers possess reticulopodia and calcareous tests (Kimoto, Chap. 7). The trophic stage of radiozoans and foraminifers has no flagella, but they are important heterotrophic plankton and benthos in marine ecosystems as HF. In addition, many species harbor symbiotic or kleptoplastidic algae (e.g., *Zooxanthella*) and represent producers in the ocean.

The Cercozoa are a large protistan group, recognizable only by molecular characters, and divided into two subphyla, the Endomyxa and Filosa, but the monophyletic relationship between them may be questioned (e.g., Sierra et al. 2013). The Endomyxa includes fungi-like parasites (phytomyxids), sporozoan parasites (haplosporids) and various "amoebae" (e.g., *Gromia*, *Filoreta*, vampyrellids) but no flagellate members are known so far. In contrast, the Filosa include many HF together with some "amoebae" (e.g., euglyphids, desmothoracids, phaeodareans) and algae (chlorarachnids). Currently these cercozoan flagellates are classified into several classes such as the Metromonadea, Sarcomonadea, Thecofilosea, and Imbricatea. However, the molecular support is weak and the phenotypic apomorphy is not recognized for each class. These flagellates are mostly biflagellate and may be planktonic (e.g., *Eblia*) or gliding (e.g., *Metopion*), amoeboid (e.g., *Cercomoans*) or stiff (e.g., *Clautriavia*), naked (e.g., *Allapsa*) or covered by scales (e.g., *Thaumatomastix*) or theca (e.g., *Protaspis*), bacteriovorous (e.g., *Neoheteromita*) or eukaryovorous (e.g., *Metromonas*). In addition, environmental DNA studies indicate that there are many unidentified cercozoan lineages (e.g., Bass and Cavalier-Smith 2004). Cercozoan flagellates are very common in marine, freshwater and soil environments, and certainly important components in microbial food web. However, the biological information on these flagellates is very limited so far, and further studies on cercozoan flagellates are necessary to understand microbial ecosystems.

4.9 Other Flagellates

The Cryptista are a group of free-living biflagellates possessing a unique cell covering (periplast) and novel ejectile organelles called ejectisomes that are probably originated from endosymbiotic bacteria (Yamagishi et al. 2012). The Cryptista are composed of two groups, cryptomonads and kathablepharids. In cryptomonads (Cryptophyta), the periplast includes a proteinaceous single sheet or multiple plates just beneath the plasma membrane, the ejectisome composed of two coils, and flat mitochondrial cristae. Cryptomonads include Goniomonadea, Cryptophyceae, and some unidentified lineages recognized only by the environmental DNA (CRY1 and 3; e.g., Kim and Archibald 2013). *Goniomonas*, the only known genus of the Goniomonadea, is a bacteriovorous flagellate and is common in both marine and freshwater environments. On the other hand, most cryptophyceans are

photosynthetic with chloroplasts originated by secondary endosymbiosis with a red alga. They are common and important primary producers in both marine and freshwater environments, and are sometimes dominant especially in cold, deep or dark environments (e.g., Spaulding et al. 1994; Tirok and Gaedke 2007). Some species of *Cryptomonas* (formerly *Chilomonas*) secondarily lost photosynthetic ability and are osmotrophic. In addition, some photosynthetic cryptopyceans engulf bacteria and therefore may be mixotrophic (Kugrens and Lee 1990; Urabe et al. 2000). Kathablepharids (katablepharids) possess the periplast with a bilayered sheath on a plasma membrane, the ejectisome composed of a single coil, and the tubular mitochondrial cristae. Kathablepharids are important eukaryovores in both marine and freshwater environments, and they engulf prey through a complex apical feeding apparatus composed of cytopharyngeal rings and longitudinal arrays of microtubules. A species of kathablepharids, *Hatena arenicola*, is known to harbor kleptoplastidic alga (*Nephroselmis*) (Okamoto and Inouye 2006). Recent molecular data suggest that a free-living heterotrophic marine biflagellate, *Palpitomonas* (Palpitea), is closely related to the Cryptista (Yabuki et al. 2014).

Haptophytes possess yellowish chloroplasts derived from secondary endosymbiosis with a red alga. They have a characteristic flagellum-like appendage, haptonema, which may function in attachment, avoidance, or feeding responses and is usually covered by organic and/or calcareous scales (e.g., Inouye and Kawachi 1994). Haptophytes with calcareous scales (coccoliths) are called coccolithophores and are considered to be important primary producers in marine environments (Hagino and Young, Chap. 12). Some coccolithophores may be heterotrophic but further studies need to confirm it (Marchant and Thomsen 1994). Recent studies suggest that non-coccolithophore haptophytes, such as *Chrysochromulina*, are more abundant in marine environments. Their ability of mixotrophy may be an important factor for their abundance in open oceans (e.g., Frias-Lopez et al. 2009; Liu et al. 2009).

The Picozoa were a group recognized by environmental DNA and considered to possess chloroplasts containing phycobilins (thus called picobiliohytes) (Not et al. 2007). However, single-cell genomics later showed these organisms to be not photosynthetic but heterotrophic (Yoon et al. 2011). Finally, an actual organism (*Picomonas*) of "picobiliphytes" was isolated (Seenivasan et al. 2013). *Picomonas* is a small (approximately 3 μm), planktonic, marine heterotrophic biflagellate, but its feeding behavior and food source are still unclear (feeding colloids?). Furthermore, picozoans may exhibit various trophic strategies because environmental DNA studies indicate a huge diversity in the Picozoa. *Telonema*, the only known genus of the Telonemia, is a planktonic flagellate with a complex subcortical lamina composed of microtubules and fibers (e.g., Yabuki et al. 2013). Two equal flagella with tripartite tubular hairs emerge from beside the antapical rostrum. *Telonema* is a common bacterio- or eukaryovorous flagellate in marine environments. In addition, environmental DNA studies indicate that there are many undescribed telonemids in marine and freshwater habitats (Shalchian-Tabrizi et al. 2007). Molecular data sometimes suggest that the Picozoa and Telonemia are related to the Cryptista and Haptophyta with Heliozoa (centrihelids)

(e.g., Okamoto et al. 2009). However, the precise phylogenetic positions of these flagellates have not been resolved.

Breviates (*Breviata*, *Subulatomonas*) are microaerophilic gliding amoeboflagellates with a single anterior flagellum (Walker et al. 2006; Katz et al. 2011). They are superficially similar to pelobionts (Amoebozoa), but different in the shape of pseudopodia, the presence of outer dynein arms and the structure of the flagellar apparatus. Breviates inhabit marine and freshwater low-oxygen environments and their mitochondria lack cristae. Collodictyonids are freshwater planktonic flagellates with a deep ventral feeding groove and two (*Diphylleia*) or four (*Collodictyon*) flagella inserted apically (e.g., Brugerolle et al. 2002). Mitochondrial cristae are tubular. They engulf large preys such as other protists and cyanobacteria, and play an important role in reduction of cyanobacterial blooms (Kobayashi et al. 2013). The precise phylogenetic positions of breviates and collodictyonids are not resolved, but molecular data suggest that they are one of the earliest divergences in eukaryotes (Katz et al. 2011; Zhao et al. 2012).

Apusozoans are gliding biflagellates with an organic cell covering. They are basically bacteriovores common in marine, freshwater and soil environments. Apusomonads (Thecomonadea) and ancyromonads (Hilomonadea) are usually classified in the Apusozoa, but the monophyletic relationship between them is uncertain (e.g., Paps et al. 2013). Mitochondrial cristae are tubular (apusomonads) or flat (ancyromonads). Apusozoans have receive attention for understanding of the early evolution of eukaryotes because they have some "bikont" features (biflagellate organization, dihydrofolate reductase-thymidylate synthetase gene fusion) but molecular phylogenetic studies suggest a close relationship to the Opisthokonta (e.g., Paps et al. 2013).

In addition, many described HF remain to be clarified with evident affinities with other eukaryotes (e.g., Hemimastigophora). Furthermore, environmental DNA studies indicate that many protistan lineages remain to be identified.

HF are indispensable pieces for understanding of the phylogeny of eukaryotes and the microbial loop in ecosystems. HF are phylogenetically very diverse, and exhibit a huge diversity in various biological aspects such as their ultrastructure, genetics, physiology and ecology. Because their phylogenetic diversity is nearly equal to that of all eukaryotes, much unexpected biological knowledge would be obtained from studies on HF.

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Chapter 5
Basal Lineages of Green Algae – Their Diversity and Phylogeny

Stuart D. Sym

Abstract The basal lineages of the green algae (Chlorophyta) were historically merged in a single class of usually scaly green algal members, the Prasinophyceae. While ultrastructural data were more or less supportive of this idea, modern molecular phylogenetics is showing that a single class is untenable and members are currently accommodated in up to five new classes, (the Chlorodendrophyceae, Mamiellophyceae, Mesostigmatophyceae, Nephroselmidophyceae and Pedinophyceae), with more still to come. This chapter considers the characteristics of, and phylogenetic relationships between, these new classes and other related groups, as well as their representatives and, briefly, their ecology.

Keywords Distribution • Ecology • Mixotrophy • Novel green classes • Phylogeny • "Prasinophytes" • Sexuality • Symbiosis • Systematics

5.1 Introduction

The class Prasinophyceae Moestrup and Throndsen (1988) accommodated the deepest rooted, predominantly chlorophyte members of the Viridiplantae. A keystone phylogenetic analysis (Steinkötter et al. 1994; 18S rDNA) undermined its validity as a monophyletic group, even though the taxa used were restricted. The Pedinophyceae Moestrup 1991 were the first to be formally removed into a separate class, a move corroborated by molecular phylogenetic analysis, placing it together with the Chlorodendrophyceae and the "core chlorophytes," the Trebouxiophyceae, Ulvophyceae and Chlorophyceae (the TUC clade; Marin 2012). *Mesostigma* Lauterborn 1894 was then moved to its own class, the Mesostigmatophyceae Marin and Melkonian 1999, in the Streptophyta.

Nakayama et al. (1998) sampled a greater range of taxa among the prasinophytes in their analysis and initiated a clade-numbering system based on the order of divergence, the deepest with the lowest number. Four monophyletic clades were

S.D. Sym (✉)
School of Animal Plant and Environmental Sciences, University of the Witwatersrand, Private Bag 3, WITS 2050, Johannesburg, South Africa
e-mail: Stuart.sym@wits.ac.za

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recovered, the Pyramimonadales Chadefaud 1950, Mamiellales Moestrup 1984, Chlorodendrales *sensu* Melkonian (1990) and Pseudoscourfieldiales *sensu* Melkonian (1990), although the last clade was later shown to be the Nephroselmidales Nakayama, Suda, Kawachi et Inouye 2007 (Fawley et al. 1999). Their numbering system was supplemented in studies that followed and accommodated new members either as subtaxa of the now-defunct Prasinophyceae, or as taxa at levels appropriate to their status from modern phylogenetic inference. In addition, it took on material recovered from environmental samples, which revealed a greater diversity at levels ranging from intrageneric up to class (e.g., Viprey et al. 2008). Supplementary numbering of the large clades followed the order in which they were encountered, and so a phylogenetic basis to their order has been lost.

The prasinophytes therefore are paraphyletic with ten separate lineages (12 including the Mesostigmatophyceae and Pedinophyceae). While each lineage is generally robust, there is often little support for their branching order relative to one another and variation is considerable (e.g., Nakayama et al. 1998; Guillou et al. 2004; Viprey et al. 2008), often as a function of the choice of the outgroup (Marin and Melkonian 2010). The latter authors recognize three main divergences in the "prasinophytes": (1) a basal Mamiellophyceae/Pyramimonadales/and possibly Prasinococcales group; (2) an intermediate Nephroselmidophyceae/Pycnococcaceae/*Picocystis* and allies group; and (3) a late diverging Chlorodendrophyceae lineage rooted in the core chlorophytes (Fig. 5.1).

This account considers the diversity of green algae traditionally considered as "prasinophytes". It adopts and builds on the nine lineages presented by Viprey et al. (2008).

5.2 Classification of Primitive Green Algae

The current nomenclature employed for "prasinophytes" is a hybrid of old and new systems. Essentially, all of the first order numbered taxa, with the possible exception of the Palmophyllales, should be treated equally, at class level.

5.2.1 Streptophyta

5.2.1.1 Class Mesostigmatophyceae Marin et Melkonian 1999

This group originally included the freshwater genera *Mesostigma* Lauterborn 1894 (Fig. 5.2a) and *Chaetosphaeridium* Klebahn 1892 based on SSU rDNA sequences and the exclusive presence of maple-leaf-like scales on their parallel flagella (Marin and Melkonian 1999). However, there is now more support for a sister relationship with *Chlorokybus* (e.g., Rodríguez-Ezpeleta et al. 2007) as an early branch of the Streptophyta, rather than, as once thought (Lemieux et al. 2000), a divergent lineage

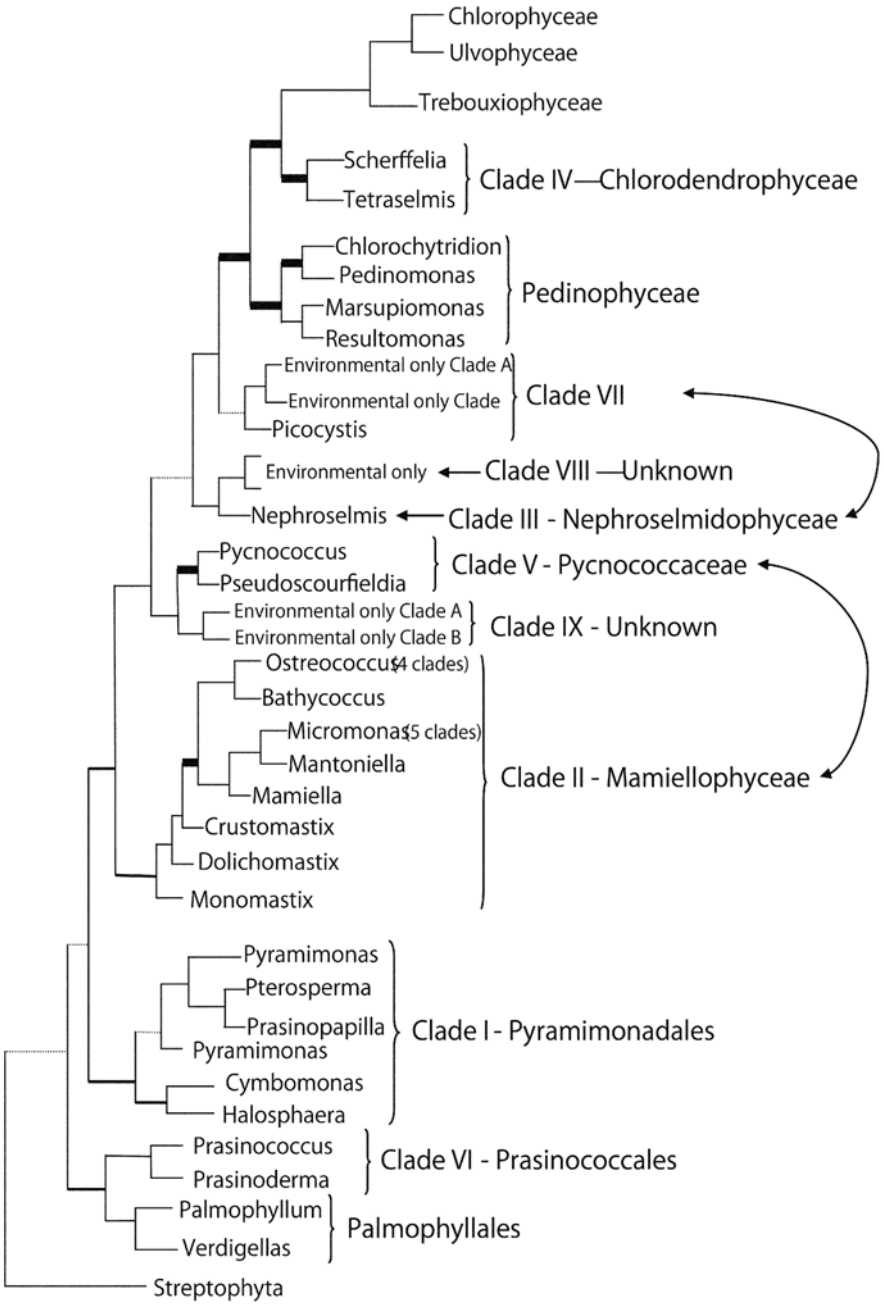

Fig. 5.1 A hybrid of phylogenetic trees of the "prasinophytes" based on SSU rDNA sequences (after Viprey et al. 2008; Marin and Melkonian 2010; Zechman et al. 2010). *Thickness of branches* shows relative level of support (moderate is thinnest) and *broken lines* indicate no support. Branching pattern mostly follows that of Marin and Melkonian (2010), but *loops* indicate which clades are reversed in the scheme of Viprey et al. (2008). Branch length has no relevance and many of the deeper branches would probably collapse as polytomies

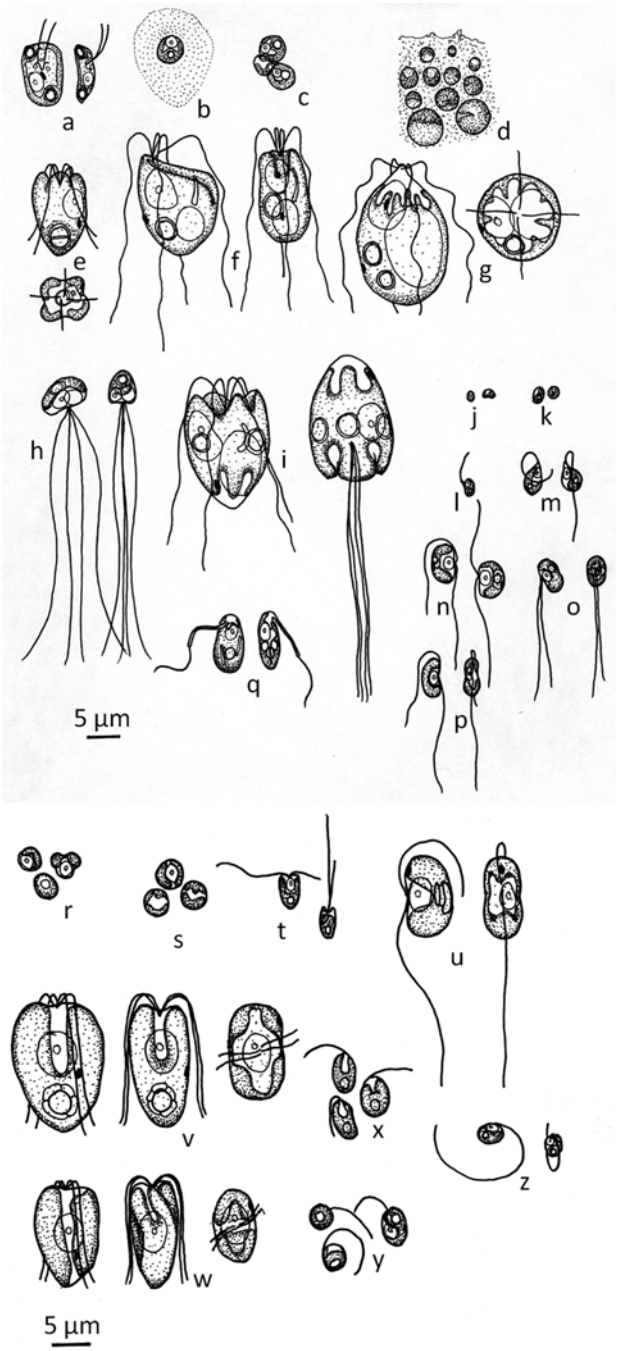

Fig. 5.2 Various representatives of the "prasinophytes" as seen at the light microscope level (drawn to scale): (**a**) *Mesostigma*, (**b**) *Prasinococcus*, (**c**) *Prasinoderma*, (**d**) Section of *Palmophyllum* thallus, (**e**) *Pyramimonas*, (**f**) *Cymbomonas*, (**g**) *Prasinopapilla*, (**h**) *Pterosperma*, (**i**) *Halosphaera*, (**j**) *Ostreococcus*, (**k**) *Bathycoccus*, (**l**) *Micromonas*, (**m**) *Mantoniella*, (**n**) *Mamiella*, (**o**) *Dolichomastix*, (**p**) *Crustomastix*, (**q**) *Monomastix,* (**r**) *Picocystis*, (**s**) *Pycnococcus*, (**t**) *Pseudoscourfieldia*, (**u**) *Nephroselmis*, (**v**) *Tetraselmis*, (**w**) *Scherffelia*, (**x**) *Pedinomonas*, (**y**) *Marsupiomonas*, (**z**) *Resultomonas*

prior to the split between the Chlorophyta and the Streptophyta. *Mesostigma* (Fig. 5.2a) is the sole genus in the order Mesostigmatales Cavalier-Smith 1998 emend. Marin et Melkonian 1999 and the family Mesostigmataceae Marin et Melkonian (not Mesostigmaceae *sensu* Fott 1974, which included *Monomastix*).

5.2.2 Chlorophyta

5.2.2.1 Order Prasinococcales (Clade VI)

The genera *Prasinococcus* Miyashita et Chihara 1993 and *Prasinoderma* Hasegawa et Chihara 1996 (Fig. 5.2b, c), have small (2–4 μm), yellow-green, thick- walled (multilayered) and aflagellate cells, without basal bodies or scales. A branch of the mitochondrion invades the pyrenoid and the pigment complement includes micromonol (Miyashita et al. 1993; Jouenne et al. 2011). Division is unequal and by budding, with only the smaller of the daughter cells escaping the parent cell wall before forming its own (Miyashita et al. 1993; Hasegawa et al. 1996). *Prasinococcus* is monospecific (*P. capsulatus* Miyashita et Chihara 1993; Miyashita et al. 1993) and differs from *Prasinoderma* by having a gelatinous envelope secreted though holes in a collared rim of the cell wall (Miyashita et al. 1993). *Prasinoderma* has two species, *P. coloniale* Hasegawa et Chihara 1996 and *P. singularis* Jouenne 2011 (Hasegawa et al. 1996). Originally considered a sister to the Mamiellophyceae (Miyashita et al. 1993), this group is now viewed as an early chlorophyte branch (Turmel et al. 2009; Jouenne et al. 2011; Fig. 5.1) or even as a clade basal to the dichotomy between the Chlorophyta and the Streptophyta (Viprey et al. 2008), although support at this level is poor. Numerous novel environmental sequences affiliated with these different species have been recovered from numerous habitats (Jouenne et al. 2011).

5.2.2.2 Order Palmophyllales Zechman, Verbruggen, Leliaert, Ashworth, MA Buchheim, Fawley, H Spalding, Pueschel, JA Buchheim, Verghese et Hanisak 2010

This group is considered at this juncture because of its possible link with the Prasinococcales (Zechman et al. 2010). Its marine members are macroscopic and benthic, at great depth (≥100 m). Their deep-green thalli are encrusting or stalked and comprise many subspherical unicells (6–10 μm) embedded mostly at the edges of a firm, gelatinous matrix (Fig. 5.2d). The cells lack pyrenoids, motile stages or even basal bodies. It has a single family, the Palmophyllaceae Zechman, Verbruggen, Leliaert, Ashworth, MA Buchheim, Fawley, H Spalding, Pueschel, JA Buchheim, Verghese et Hanisak 2010, with two confirmed members, *Verdigellas* Ballantine et JN Norris 1994 and *Palmophyllum* Kützing 1849, but probably also includes *Palmoclathrus* Womersley 1984 (Zechman et al. 2010). This is a well-supported clade, but its position varies according to the molecular data used; either, with moderate support, alone as the first branch in the Chlorophyta or, together with the

Prasinococcales (Sect. 5.2.2.1), as an unresolved branch relative to the streptophytes and the chlorophytes (Zechman et al. 2010). Morphological links with the Prasinococcales (and Pseudoscourfieldiales) are obvious (Zechman et al. 2010). This clade has no representation in environmental sequences but this is, no doubt, a consequence of their benthic nature.

5.2.2.3 Pyramimonadales sensu Melkonian 1990 (Clade I)

This includes *Cymbomonas* Schiller 1913 (Fig. 5.2f), *Halosphaera* Schmitz 1878 (Fig. 5.2i), *Prasinopapilla* nomen nudum (Fig. 5.2g), *Pterosperma* Pouchet 1893 (Fig. 5.2h) and *Pyramimonas* Schmarda 1849 (Fig. 5.2e). They, or their stages, have four, or multiples of four, flagella. Complex and different layerings of scales cover the body and flagella, each usually with different scale types (reviewed in Sym and Pienaar 1993), but they also have up to three types of flagellar hairs (T-hairs, P_l-hairs and P_t-hairs; Marin and Melkonian 1994). The group has a variably invaded pyrenoid, 2–4 dictyosomes and a blind duct. Subplasmalemmal extrusomes (e.g., Moestrup et al. 2003), puncta or trichocysts (Sym and Pienaar 1993) are common. They have an asymmetric flagellar apparatus with cruciate microtubular roots, the 1d of which associates with a fiber encircling the opening of the duct or with an MLS (reviewed by Sym and Pienaar 1993).

With the exception of *Pyramimonas*, all genera only have one species with an ultrastructural characterization: *Halosphaera minor* Ostenfeld 1898, *Prasinopapilla vacuolata* nomen nudum, *Pterosperma cristatum* Schiller 1925 and *Cymbomonas tetramitiformis*. *Pyramimonas tetrarhynchus* Schmarda 1849 is the only corroborated freshwater species despite numerous other descriptions existing. There are about 41 confirmed marine species. This clade is robustly supported in most 18S rDNA analyses (e.g., Guillou et al. 2004, Vipreye et al. 2008). *Pyramimonas*/*Pterosperma* and *Cymbomonas*/*Halosphaera* clades are common (e.g., Nakayama et al. 1998) with a variable rooting for *Prasinopapilla*. Numerous environmental sequences associated with this clade have been recovered, especially ones for unknown species of *Halosphaera* and *Pyramimonas* (Viprey et al. 2008).

5.2.2.4 Mamiellophyceae Marin et Melkonian 2010 (*Clade II*)

Molecular support for this class is strong, but no universal set of morphological characters exists for this class, except that scaled members lack the small underlayer scales found elsewhere (Marin and Melkonian 2010).

5.2.2.4.1 Mamiellales Moestrup 1984 emend. Marin et Melkonian 2010

Representatives here are exclusively marine, ≤7 μm, uni-, bi- or aflagellate, with roots only on bb1 and, if scaly, with cobwebbed scales. They have an unusually large variation in the size of two chromosomes (Subirana et al. 2013). Like the

Prasinococcales and Pycnococcaceae, they have prasinoxanthin and micromonal (Marin and Melkonian 2010; Jouenne et al. 2011).

Mamiellaceae Moestrup 1984 emend. Marin et Melkonian 2010

Members have two basal bodies, at least one of which subtends a flagellum with T-hairs with double-rowed shafts (Marin and Melkonian 1994, 2010). Eyespots, if present, are monolayered (Marin and Melkonian 2010). *Mamiella* and *Mantoniella* both possess a fiber associated with the 1d or R1 root (Nakayama et al. 2000). Confirmed species of this family include *Mamiella gilva* (Parke et Rayns) Moestrup 1984 (Fig. 5.2n), *Mantoniella squamata* (Manton et Parke) Desikachary 1972 (Fig. 5.2m), *Mantoniella antarctica* Marchant 1989 and *Micromonas pusilla* (Butcher) Manton et Parke 1960 (Fig. 5.2l), but the diversity of *Micromonas* is considerable; up to five clades have been recovered, possibly representing the earliest example of cryptic speciation (Šlapeta et al. 2006).

Bathycoccaceae Marin et Melkonian 2010

Cells here lack basal bodies or eyespots. Only the scaly *Bathycoccus prasinos* Eikrem et Throndsen 1990 (Fig. 5.2k) and the naked *Ostreococcus tauri* Courties et Chrétiennot-Dinet 1995 (Fig. 5.2j) have ultrastructural descriptions, but there are two cryptic species, *O. lucimarinus* nomen nudum (Palenik et al. 2007) and *O. mediterraneus* Marin et Grimsley 2013 (Subirana et al. 2013). *Ostreococcus* is the smallest known eukaryote (<1 μm) with a highly reduced genome (10.2 Mb; Courties et al. 1998) and a pigment signature including Mg-3,8-D but lacking prasinoxanthin. Most of the cell is occupied by a single, pyrenoid-less chloroplast (Chrétiennot-Dinet et al. 1995). Four clades of *Ostreococcus* have been recovered from environmental sequences (Guillou et al. 2004), but to date *Bathycoccus* has been poorly represented (e.g., Guillou et al. 2004; Viprey et al. 2008).

5.2.2.4.2 Dolichomastigales Marin et Melkonian 2010

Members are small (≤6 μm), kidney-shaped and biflagellate, with subequal, long (10–30 μm), slightly divergent flagella. Microtubular roots are only present on bb2. They have short lateral and terminal flagellar T-hairs with single-rowed shafts, and a few P_I-hairs restricted to the base of one flagellum. Micromonal is absent (Marin and Melkonian 2010).

Dolichomastigaceae Marin et Melkonian 2010

This family only contains the marine, prasinoxanthin-containing *Dolichomastix* (Fig. 5.2o) (Marin and Melkonian 2010), with three of its four species only known by their scales. *Dolichomastix tenuilepis* Throndsen et Zingone 1997 has trailing

flagella during swimming, or separates them out during lateral gliding with the short (F1) flagellum trailing, or wraps them around the cell at rest. The olive-green chloroplast has a pyrenoid and a monolayered eyespot. Both the body and flagella are covered by plate-like scales and the latter also have short tubular T- and tip hairs and a few P_l hairs at the base of F2 (Throndsen and Zingone 1997). Environmental sequences reveal other representatives in two clades, one including *D. tenuilepis* (Viprey et al. 2008; Marin and Melkonian 2010).

Crustomastigaceae Marin et Melkonian 2010

The only genus here is *Crustomastix* Nakayama, Kawachi et Inouye 2000 (Fig. 5.2p), with two characterized species; the type, *C. didyma* Nakayama, Kawachi et Inouye 2000 (Nakayama et al. 2000) and *C. stigmatica* Zingone 2002 (Zingone et al. 2002). The cell is yellow-green and kidney-shaped. It lacks a pyrenoid and a yellow eyespot is only found in *C. stigmatica*. The hair-pointed flagella exhibit flagellar beating, the long (F1) flagellum trailing and F2 recurving around the cell anterior. No scales other than hair scales (P_l- hairs on the proximal end of F2, T-hairs and tip-hairs) are present. The cell is covered by a thin crust, which is secreted via a duct in *C. didyma*, and only this species has a 1d root (R1) with a duct fiber and an MLS (Nakayama et al. 2000; Zingone et al. 2002). Shared characters (MLS and duct system) with the Pyramimonadales led Nakayama et al. (2000) to consider this genus the most primitive of the Mamiellales. Environmental data indicate that numerous other species exist in three member-rich clades (A–C). Clade A incorporates *C. didyma* and *C. stigmatica*, numerous other marine representatives and also three sequences from freshwater habitats (Marin and Melkonian 2010).

5.2.2.4.3 Monomastigales Marin et Melkonian 2010

These freshwater, unicellular flagellates, 3–21 μm long, have one emergent, scaleless flagellum (F2) with a hair point and one barren basal body (bb1). They are ovate, laterally flattened, with an anterior contractile vacuole, usually two pyrenoids, a dorsal eyespot, and obvious, linear, mucilagenous trichocysts. The cell is covered by adherent, cross-hatched, oval scales. The F2 is inserted in a groove and extends posterolaterally during swimming, and bb1 is angled or opposite in orientation. There is a 4-2-4-2 cruciate root system (Marin and Melkonian 2010). The single family, the Monomastigaceae Marin et Melkonian 2010, has three species, *Monomastix opisthostigma* Scherffel 1912 (the type), *M. minuta* Skuja (Fig. 5.2q) and an isolate, confirmed by molecular work (Marin and Melkonian 2010). Environmental data indicate the presence of many more monomastigaleans in two clades (Marin and Melkonian 2010).

5.2.2.5 Clade VII sensu Guillou et al. 2004

This is a weakly supported sister clade of the core chlorophytes (Guillou et al. 2004) with three subclades, two of which (A and B) are only known mostly from marine environmental sequences (e.g., Guillou et al. 2004; Viprey et al. 2008). Cultures of two members of clade A exist and they are coccoid, scaleless and have usual green carotenoids (Guillou et al. 2004).

5.2.2.5.1 The *Picocystis* Clade

The remaining subclade (C) comprises the minute (1.5–5 μm), aflagellate, walled (trilaminate) *Picocystis* (Fig. 5.2r). The bi-lobed, pyrenoid-less chloroplast and nucleus dominate the cell, making it trilobed at times when stressed (Krienitz et al. 2012). Its pigments are typical for greens and it lacks Mg-3,8-D. Division is by autosporulation, usually resulting in two daughter cells. Only one species has been described (*Picocystis salinarum* RA Lewin 2000), but there is some variation in SSU rDNA sequences, and material from Mono Lake (California) in particular has been resolved as discrete from other clones (Krientiz et al. 2012).

5.2.2.6 Pycnococcaceae Guillard emend. Fawley 1999 (Clade V)

This "family," erected to accommodate the first coccoid, walled prasinophyte, *Pycnococcus provasolii* Guillard 1991 (Fig. 5.2s), also briefly contained the Prasinococcales (Miyashita et al. 1993; Sects. 5.2.2.1). An original link between *Pycnococcus provasolii* and *Pseudoscourfieldia marina* (Throndsen) Manton 1965 (Fawley 1992) was undermined by the latter's affiliation with *Nephroselmis* in many early phylogenetic analyses (Steinkötter et al. 1994; Melkonian and Surek 1995; Nakayama et al. 1998) because a culture of *Nephroselmis pyriformis* was mislabeled as *Pseudoscourfieldia* (Fawley et al. 1999; Nakayama et al. 2007). The close affiliation of *Pycnococcus* with *Pseudoscourfieldia* was later confirmed and they could even transpire to be life cycle stages of one organism (Fawley et al. 1999).

The minute (4 μm), biflagellate cell of *Pseudoscourfieldia* (Fig. 5.2t) is flattened and conical, with a shallow flagellar pit (Moestrup and Throndsen 1988). The anisokont cell, originally considered a rapid swimmer (Moestrup and Throndsen 1988), has also been demonstrated to display slow gliding, where the long flagellum (F1) drives and trails and the shorter is extended anteriorly (Nakayama et al. 2007; Fig. 5.2t, cell on left). The cell body and flagella are scale-covered (Moestrup and Throndsen 1988), the latter including unusual T-hairs and a few P_1-hairs at the tip of F2 (Nakayama et al. 2007). The yellow-green cell lacks an eyespot and has a pyrenoid invaded by the mitochondrion (Moestrup and Throndsen 1988).

Pycnococcus is coccoid, aflagellate, scaleless and with a monolayered wall with an operculum-like structure (Guillard et al. 1991). It has similar pigmentation, dimensions and pyrenoid invasion to those of *Pseudoscourfieldia*. Cell division is achieved as in the Prasinococcales (Hasegawa et al. 1996). A rare, short-lived motile phase (5 μm) was observed with a single recurved flagellum of similar dimension and dynamics to the F1 of *Pseudoscourfieldia* (Guillard et al. 1991).

This "family" is one of the most closely affiliated clades with the "core chlorophytes" but the branching order is not clearly resolved (e.g., Nakayama et al. 2007; Fig. 5.1). Environmental sequences of this group are recovered in one of three subclades (Viprey et al. 2008), showing a greater diversity than currently described.

5.2.2.7 Nephroselmidophyceae Cavalier-Smith 1993 (Clade III)

This class, with one order, the Nephroselmidales Nakayama, Suda, Kawachi et Inouye 2007 and family, the Nephroselmidaceae Skuja ex P C Silva 1980, contains only *Nephroselmis* Stein 1878, with ten formally described species (Nakayama et al. 2007). Cells here are biflagellate and laterally compressed, with a trailing, longer (F1) flagellum and the F2 directed anteriorly during swimming (Fig. 5.2u). The cell body and flagella are covered by complex layers of different scales, the most distinctive of which are stellate (e.g., Nakayama et al. 2007). The flagella additionally have T-hairs (on both), P_I-hairs (on one side of F2 only) and complex tip hairs (on F1 only in *N. pyriformis*) (Marin and Melkonian 1994; Nakayama et al. 2007). The 2s root is lacking and an MLS-like structure is present at the base of the 2d root. The pyrenoid of all but one species (*P. spinosa* Suda 2003) is penetrated by thylakoids, and an eyespot may be present (Nakayama et al. 2007).

The genus has four distinct clades (SSU rDNA data; Faria et al. 2012), the earliest diverging of which is *N. pyriformis* (N Carter) Moestrup 1983 and therefore treated as the subgenus *Microsquamata* Yamaguchi, Nakayama et Inouye 2013. The other three clades are unresolved, so they have been retained in a single subgenus, *Nephroselmis*, including the type, *N. olivacea* Stein 1878 (Yamaguchi et al. 2013). The class forms a sister to the *Picocystis* clade (clade VII) (Nakayama et al. 2007). Environmental sequences have not brought to light the presence of any further material that belongs to this class.

5.2.2.8 Chlorodendrophyceae Massjuk 2006 (Clade IV)

Only two genera are found here, *Tetraselmis* Stein 1878 (Fig. 5.2v) and *Scherffelia* Pascher 1911 (Fig. 5.2w) in a single family, the Chlorodendraceae Oltmanns 1904, with a single order, the Chlorodendrales Fritsch 1917, both in a more restricted sense (Melkonian 1990) than as described by Moestrup and Throndsen (1988). The thecate cells have four isokont flagella covered by two layers of scales including T-hairs (Marin et al. 1993) and held in pairs on opposite broad sides of the cell. The two genera are differentiated by the chloroplast, which is single and with a pyrenoid in *Tetraselmis*. They have extremely short X-2-X-2 roots. The morphologically based

subgenera of the numerous species of *Tetraselmis*, including the type, *Tetraselmis cordiformis* (HJ Carter) Stein 1878, are not robust and the genus is in need of revision (Marin et al. 1993). However, there is molecular support for at least eight species (Arora et al. 2013). *Scherffelia* has six species, but only the type, *S. dubia* (Perty) Pascher 1911 emend. Melkonian et Preisig 1986, has been included in molecular phylogenies, where it is either a basal branch (Arora et al. 2013) or embedded within the *Tetraselmis* lineage (Guillou et al. 2004; Viprey et al. 2008). The class is commonly recovered as an immediate sister of the core chlorophytes (e.g., Marin 2012; Fig. 5.1). Some additional clades in this class have been recovered from environmental sequences (two in Guillou et al. 2004; four in Viprey et al. 2008).

5.2.2.9 Pedinophyceae Moestrup emend. Marin 2012

Cells of members of this class are oval, compressed, unscaled and uniflagellate. The flagellum (F1), covered with fine hairs, is subapical in insertion, recurved around the cell during rest but with flagellar beat when active. A nascent basal body (bb2) is present and the two bbs are antiparallel and counterclockwise in orientation with cruciate microtubular roots and a rhizoplast. They have a single chloroplast with a pyrenoid and a monolayered eyespot. There are two orders.

5.2.2.9.1 Pedinomonadales Moestrup emend. Marin 2012

This is found in freshwater, including soils. Cells are non-thecate and the flagellum is up to twice the length of the cell. The bbs are displaced almost by the width of one bb. Mucilage-covered palmelloid stages are possible. There is one family, Pedimonadaceae Korshikov 1938, with only two genera, the type *Pedinomonas* Korshikov 1923 (Fig. 5.2x) and *Chlorochytridion* Vischer 1945.

5.2.2.9.2 Marsupiomonadales Marin 2011

This brackish or marine order has two families, the Marsupiomonadaceae Marin 2011 and the Resultomonadaceae Marin 2011, only distinguishable by molecular characteristics. Its members are small (≤3 μm) and may have a dorsal theca. The flagellum is more than twice cell length and the bbs are barely displaced. The type of the Marsupiomonadaceae is *Marsupiomonas pelliculata* Jones, Leadbeater et Green 1994 (Fig. 5.2y) and that of the Resultomonadaceae Marin 2011 is *Resultomonas moestrupii* Marin 2011 (= *Resultor micron* (Throndsen) Moestrup 1991; Fig. 5.2z).

Once placed as an integral part of the core chlorophytes (Turmel et al. 2009), this class has now convincingly been shown to rather be a sister of the TUC-Chlorodendrophyceae clade (Marin 2012). Environmental samples indicate many novel groups within each of the orders and families, but they are not always robustly supported (Marin 2012).

5.2.2.10 Clades VIII and IX

The morphology of organisms here is unknown because they are based entirely on environmental sequences (Viprey et al. 2008). However, clade VIII is weakly linked with the Nephroselmidophyceae (clade III). Clade IX is well represented by environmental sequences and quite diverse, with representatives belonging to one of two well-supported subclades. It is weakly linked with the Pycnococcaceae (clade V) (Viprey et al. 2008).

5.3 Ecology of Primitive Green Algae

The great majority of "prasinophytes" are encountered in marine environs or waters of elevated salinity. Individual taxa, like *Ostreococcus*, may be euryhaline (Chrétiennot-Dinet et al. 1995), particularly ice algae (e.g., Daugbjerg 2000), which need to accommodate the extreme variations in salinity associated with the thawing of ice floes and freezing of seawater, while *Picocystis* is restricted to hypersaline water (Krienitz et al. 2012).

A definite polar flora of "prasinophytes" exists, incapable of surviving temperatures higher than approximately 10 °C (e.g., Daugbjerg 2000; Balzano et al. 2012), while *Picocystis* is adapted to water temperatures between 30 and 40 °C (Krienitz et al. 2012). Some, like *Ostreococcus,* survive a broad range (4–25 °C; Courties et al. 1998) between these extremes. Temperature tolerances obviously impact on seasonality. Many genera of the Mamiellophyceae occur throughout the year in temperate zones (e.g., Romari and Vaulot 2004) and, surprisingly, a *Micromonas*-like member of the picoplankton was reported to persist through the long, dark winter of Arctic Canada (Lovejoy et al. 2007). For the most part, though, an elevated presence is recorded in summer (e.g., Romari and Vaulot 2004).

Many marine "prasinophytes" contribute to inshore plankton, where nutrients are more readily available (e.g., *Prasinoderma singularis*, Jouenne et al. 2011; *Ostreococcus, Bathycoccus* and *Micromonas*, Romari and Vaulot 2004; Worden 2006; Lovejoy et al. 2007). More protected stretches, such as lagoons, favor some groups (e.g., *Ostreococcus* Chrétiennot-Dinet et al. 1995) although they face the associated pressures of salinity change. Less-productive oceanic waters, long considered diatom dominated or dinoflagellate dominated, can have the Prasinococcales as a major contributor (e.g., Miyashita et al. 1993; Jouenne et al. 2011). A highly reduced, coccoid growth form dominates planktonic green algae, and is thought, by its independent expression in separate lineages (Prasinococcales, Pseudoscourfieldiales, Mamiellophyceae), to be a distinct advantage (Potter et al. 1997). The resultant increase in the surface area to volume ratio enhances transport, metabolism and cell division, in turn leading to greater competitiveness in an environment that can be trophically challenged. A reduced size also presents more of a challenge to predators and promotes buoyancy (Potter et al. 1997).

Many "prasinophytes" are found in the top 40–50 m of the water column (e.g., Romari and Vaulot 2004) and seem to prefer high levels of light (e.g., Jouenne et al. 2011) yet tolerate levels at least two orders of magnitude lower (Chrétiennot-Dinet et al.1995). *Pycnococcus* (Guillard et al. 1991) and some *Ostreococcus* (Subirana et al. 2013) and *Micromonas* strains (e.g., Lovejoy et al. 2007) are considered low intensity (*c*. 10 μmol photons.m^{-2}.s^{-}1) and/or short wavelength adapted. The ability to harvest light at low levels is often attributed to high levels of siphonein or siphonoxanthin (McFadden et al. 1986), violaxanthin (Chrétiennot-Dinet et al. 1995), Mg-3,8-D (Fawley 1992), or chlorophyll b (Leliaert et al. 2011). This ability enables the development of the deep chlorophyll maximum often observed in open seas (e.g., Estrada et al. 1993) and accounts for records of prasinococcaleans, mamiellaleans and palmophyllaleans beyond 50 m (e.g., Jouenne et al. 2011; Zechman et al. 2010); indeed, *Pycnococcus* was named after the pycnocline in which it was originally discovered (Guillard et al. 1991). In addition to the Palmophyllales, *Pyramimonas* (e.g., Sym and Pienaar 1991), *Nephroselmis* (Faria et al. 2012), *Tetraselmis* (because of its division strategy) and probably many more "prasinophytes" can be benthic, a habitat largely untapped in environmental sequencing (e.g., Guillou et al. 2004).

The Prasinococcales are widespread, generally in oligotrophic waters (Hasegawa et al. 1996; Miyashita et al. 1993; Juoenne et al. 2011). The Mamiellales and Dolichomastigales (Throndsen and Zingone 1997; Romari and Vaulot 2004; Balzano et al. 2012; Subirana et al. 2013) are also global, but more in coastal water. The Pycnococcaceae (Guillard et al. 1991; Moestrup and Throndsen 1988; Nakayama et al. 2007), all genera of the Pyramimonadales, except *Prasinopapilla* (e.g., Daugbjerg 2000; Moestrup et al. 2003; Viprey et al. 2008), *Nephroselmis pyriformis* (Nakayama et al. 2007) and *Tetraselmis*, are cosmopolitan in the marine environment, while the monomastigaleans (Marin and Melkonian 2010) and pedinomonadaleans (Marin 2012) are cosmopolitan in freshwater. The genus *Picocystis*, while restricted to hypersaline inland lakes, is widespread (Krienitz et al. 2012). Despite their global presence, "prasinophytes" are rarely implicated in bloom events, and reports are restricted to some members of the Prasinococcales (in oceanic waters), *Ostreococcus*, *Picocystis*, *Pyramimonas* and *Tetraselmis* (e.g., Jouenne et al. 2011; Chrétiennot-Dinet et al. 1995; Krienitz et al. 2012).

Surprisingly, *Cymbomonas* (Maruyama and Kim 2013) and *Pyramimonas gelidicola* (Bell and Laybourn-Parry 2003) have limited mixotrophic capacity as bacterivores when light is limited. "Prasinophytes" are also partners in symbiotic associations. *Tetraselmis* is a facultative symbiont of *Symsagittifera roscoffensis* (= *Convoluta*; Serôdio et al. 2011) and is also a competent symbiont of the radiolarian *Spongodrymus* (Viprey et al. 2008; Arora et al. 2013) as is *Nephroselmis* in the katablepharid *Hatena* (Okamoto and Inouye 2006). An environmental clade of the Resultomonadaceae (Pedinophyceae) was recovered from sponge tissue (Marin 2012) and two organisms relegated to *Pedinomonas* are symbiotic in *Noctiluca* (Sweeney 1976) or the radiolarian *Thalassolampe* (Cachon and Caram 1979). The affiliation of the latter two needs re-investigation because all currently sequenced members of the Pedinomonadales are freshwater forms. There is strong evidence

that the secondary chloroplasts of euglenids are derived from the Pyramimonadales (Turmel et al. 2009) and that those of *Lepidodinium* are not of "prasinophyte", but rather core chlorophyte, origin (Matsumo et al. 2011).

Finally, circumstantial evidence for sexuality in "prasinophytes" is increasing. Uniflagellate stages, potentially gametes, have been found in *Pyramimonas tychotreta* (Daugbjerg et al. 2000), *P. olivacea* (Sym et al. 2005) and *Cymbomonas tetramitiformis* (Moestrup et al. 2003). Furthermore, the genome of *Prasinoderma coloniale* exhibits two peaks suggestive of a haplo-diplontic life cycle (Jouenne et al. 2011), and *Ostreococcus* and *Micromonas* possess sex-related and meiosis-specific genes in their genomes (e.g., Derelle et al. 2006). Marker loci on two chromosomes of several strains of *Ostreococcus tauri* revealed that they were all haploid and that differences between strains could only be accounted for by sexual exchanges, although the data indicate that such events would be rare (Grimsley et al. 2010). Such rarity is expected in the sea, where "prasinophytes" are presumed to have arisen, because it is well buffered against environmental change. There is thus far less pressure on an organism to resort to sexual reproduction here than in fresh water.

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Chapter 6
Typical Features of Genomes in the Mamiellophyceae

Nigel Grimsley, Sheree Yau, Gwenaël Piganeau, and Hervé Moreau

Abstract In surveys of marine phytoplankton diversity, prasinophyte algae are commonly found and are often a major component, especially in coastal areas. They form a paraphyletic assemblage of at least nine clades, with the Mamiellophyceae (prasinophyte clade II) being a major group. In the order Mamiellales, the three genera *Bathycoccus*, *Micromonas* and *Ostreococcus* are the dominant picoeukaryotic organisms in many different oceanic areas, as witnessed by their abundance in analyses of environmental DNA samples. Each of the six complete genomes analyzed from cultured representatives of this group has revealed densely packed coding sequences, with strong evolutionary divergence from its nearest phylogenetically defined neighbors. These species lie at the base of the green lineage, but various metabolic processes reflect their marine lifestyles and distinguish them from land plants, including a high proportion of selenoprotein enzymes and C4 photosynthesis. They all possess two unusual chromosomes with lower GC content and atypical gene content, whose function so far remains enigmatic.

Keywords Mamiellophyceae • Green algae • Algal genomics • Picoeukaryotes • Prasinovirus

6.1 Introduction to Mamiellophyceae

Marine phytoplankton represent about half of the planet's biomass production and is ensured by unicellular organisms (Field et al. 1998). Among phytoplankton, picoorganisms, with a size below 3 μm, are one of the major groups by abundance (Buitenhuis et al. 2012, 2013).

Picophytoplankton include cyanobacteria (essentially the marine genera *Prochorococcus* and *Synechococcus*) and picoeukaryotes, which are very diverse, including species of all known lineages of the crown eukaryotes, except unikonts (Vaulot et al. 2008). Among these picoeukaryotes, the class Mamiellophyceae is

N. Grimsley • S. Yau • G. Piganeau • H. Moreau (✉)
CNRS-UPMC UMR 7232, Oceanological Observatory of Banyuls (OOB),
1 avenue Fontaule, 66650 Banyuls sur mer, France
e-mail: h.moreau@obs-banyuls.fr

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found in all metagenomic studies focused on the pico- size and appears more and more of quantitative importance. It is a monophyletic class emerging early in the green lineage, inside the polyphyletic group of prasinophytes. This clade has been recently revisited and is finally divided into three orders, the Dolichomastigales, Mamiellales and Monomastigales (Fig. 6.1) (Marin and Melkonian 2010). This class globally includes small unicellular algae presenting loosely defined morphological characteristics such as scaly or non-scaly cells, and flagellated or non-flagellated cells. In fact, they are astonishingly morphologically diverse for such small cells.

These species are grouped together mainly on molecular criteria using concatenated gene sequence comparisons of nuclear- and plastid-encoded rRNA operons. The orders Dolichomastigales and Monomastigales include both marine and freshwater species, and searches in environmental databases show an unexpected diversity of Monomastix freshwater species (Marin and Melkonian 2010).

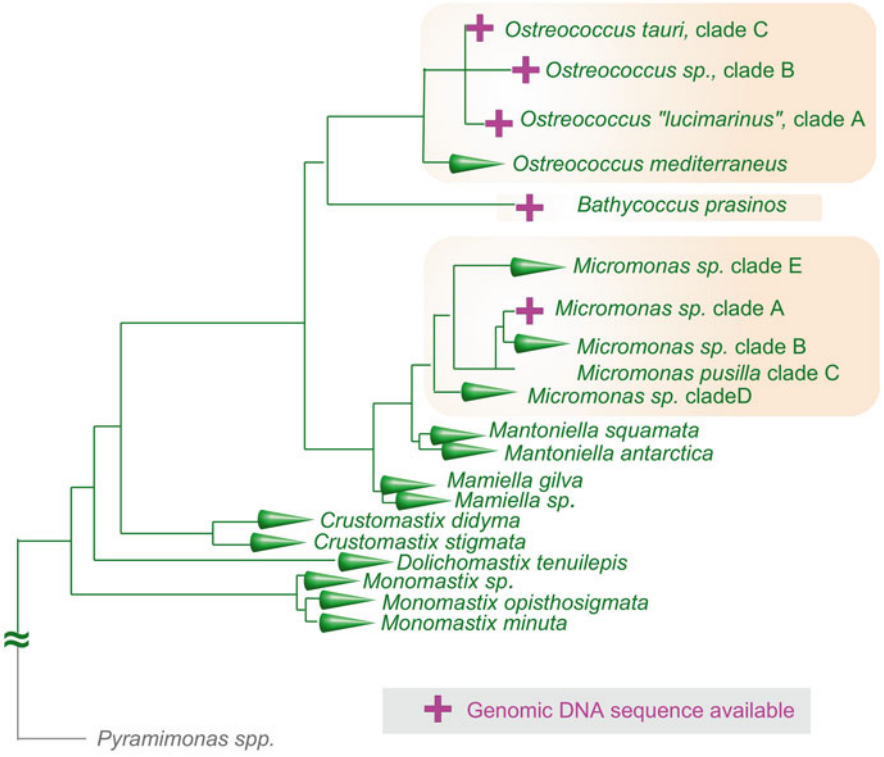

Fig. 6.1 Phylogenetic analyses based on 18S rDNA within the class of Mamiellophyceae. Complete genomes are known for six species (in *shaded backgrounds*) (From Marin and Melkonian (2010). Modified and from Piganeau et al. (2011))

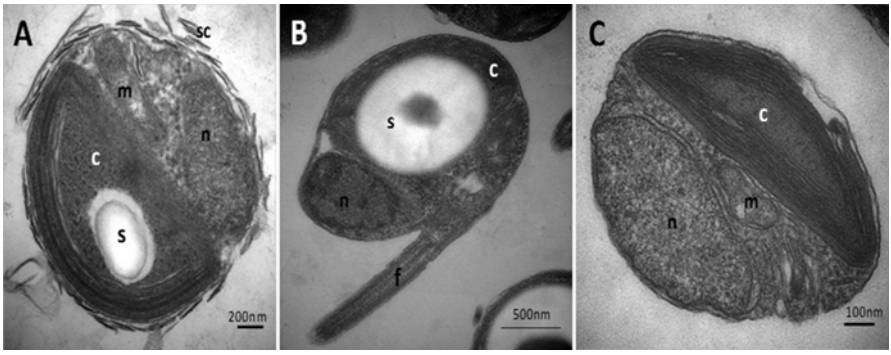

Fig. 6.2 EM pictures of the three most environmentally relevant genera of Mamiellales: (**a**) *Bathycoccus*; (**b**) *Micromonas*; (**c**) *Ostreococcus. c: chloroplast; f: flagellum; m: mitochondria; n: nucleus; sc: scales.* Pictures from ML Escande, BIOM, Oceanological Observatory of Banyuls, France

The order of Mamiellales is exclusively marine and includes the three genera *Bathycoccus*, *Micromonas* and *Ostreococcus*, which are quantitatively important in most of the environmental genomic studies on marine waters (Fig. 6.2). *Bathycoccus* is a ubiquitous genus whose abundance has probably been underestimated (Johnson and Sieburth 1982), *Micromonas* dominates coastal and polar areas (Foulon et al. 2008; Kilias et al. 2014). *Ostreococcus* appears more sporadically, and is able to develop blooms in coastal areas (O'Kelly et al. 2003). These three genera are characterized by a small size (1–2 μm) and a minimal cellular organization (one mitochondrion and one chloroplast) and small genomes (from 13 to 23 Mb). *Micromonas* (Butcher 1952) is a naked cell with one flagellum whereas the two other genera are nonmotile. *Ostreococcus* (Courties et al. 1994; Chrétiennot-Dinet et al. 1995) is also naked whereas *Bathycoccus* (Eikrem and Throndsen 1990) is covered with scales around the plasmic membrane. The complete genome of at least one representative of each of these three genera is known (see below).

6.2 Genome Characteristics of Mamiellales

6.2.1 The Genomes of the Mamiellales Are Small

Six complete genomes of the Mamiellales (in the class Mamiellophyceae) are available (Derelle et al. 2006; Palenik et al. 2007; Worden et al. 2009; Moreau et al. 2012), ranging in size from 13 Mb (*O. tauri*) to 22 Mb (*M. pusilla*) (Fig. 6.3). The *O. tauri* genome is packed with about 8,000 predicted genes that must encode all of genetic information necessary for a free-living photosynthetic unicellular

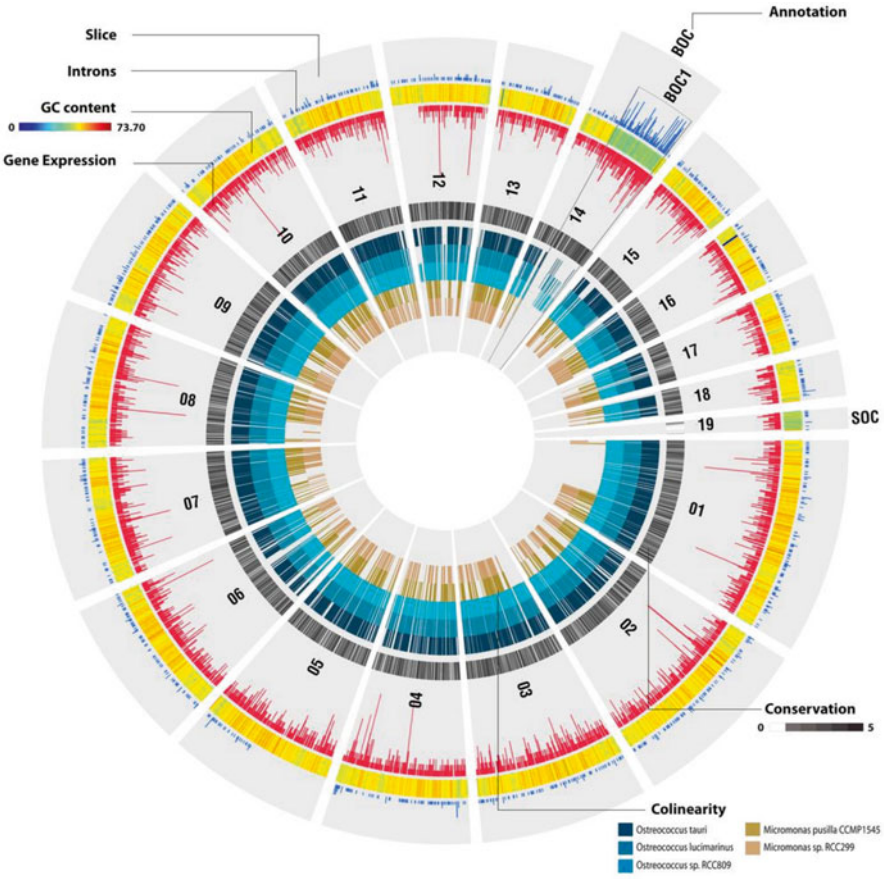

Fig. 6.3 Comparison of Mamiellophyceae genomes (From Moreau et al. 2012)

eukaryote. On average, less than 200 bp of non-coding DNA sequences separate genes, compared with nearly 1 kb in *Schizosaccharomyces pombe* (Wood et al 2002); about three quarters of these are supported by similarities to known genes and about half of the genes are homologous to plant genes. Baker's yeast (*Saccharomyces cerevisiae*) has the same genome size as *O. tauri*, despite yeast's lack of the panoply of genes necessary for photosynthesis, but yeast cells are about 50 times as voluminous as those of *O. tauri. O. tauri's* cells do not even have enough space for a classical spindle apparatus in mitotic division (Henderson et al. 2007).

Phylogenetically, the Mamiellales branch very early from the green lineage and usually the number of gene duplications is reduced to a minimum, compared with higher plants. They are thus attractive models for evolutionary comparisons, which can be made easily using the pico-PLAZA online database (Vandepoele et al. 2013, http://bioinformatics.psb.ugent.be/versions/pico-plaza/). As an example, using "dynamin" (a component involved in cellular trafficking) (Fujimoto and Ueda 2012)

as a text query shows that there is only one copy of the dynamin central domain in *O. tauri*, but higher numbers in many other species, ranging up to through 10 in the model plant *Arabidopsis thaliana* to 19 in the genome of the moss *Physcomitrella patens* (Rensing et al. 2008), where several copies are involved in plastid division (Sakaguchi et al. 2011) (Table 6.1). Since the Mamiellales have such streamlined genomes, often retaining only one or two copies of certain genes, they can be useful models for functional studies (Deveaux et al. 2008; Moulager et al. 2010).

Analyses of several other genomes in the order Mamiellales revealed genus *Ostreococcus* to have the smallest known genome in this group (12–14 Mb (Palenik

Table 6.1 Copy numbers of dynamin-containing proteins in the green lineage

Description	Dynamin central region IPR000375	Dynamin GTPase domain IPR001401	Dynamin GTPase effector IPR003130	Dynamin GTPase conserved site IPR019762
Total number of gene concerned	84	178	66	24
Number of species carrying the gene	19	19	19	8
Ostreococcus lucimarinus	2	5	51	0
Ostreococcus RCC809	1	4	1	0
Ostreococcus tauri	1	3	1	0
Bathycoccus prasinos	1	5	1	0
Micromonas pusilla CCMP1545	2	6	2	1
Micromonas sp. RCC299	2	6	2	1
Coccomyxa sp. C169	3	7	3	0
Chlorella sp. NC64A	3	10	2	0
Volvox carteri	4	8	4	0
Chlamydomonas reinhardtii	6	15	5	1
Arabidopsis thaliana	10	15	10	5
Oryza sativa	13	16	9	2
Physcomitrella patens	19	25	14	12
Cyanidioschyzon merolae	1	3	1	0
Paeodactylum tricornutum	2	6	1	1
Fragilariopsis cylindrus	1	8	1	0
Thalassiosira pseudonana	1	6	1	1
Ectocarpus siliculosus	5	11	0	0
Aureococcus anophagefferens	7	19	3	0

et al. 2007) and unpublished data), the more recently sequenced *Bathycoccus* sp. genome being slightly larger (15 Mb) (Moreau et al. 2012) and the two *Micromonas* spp. genomes being about twice as large as that of *O. tauri* (Worden et al. 2009). Phylogenetic analyses suggest that *Micromonas* spp. are the most deeply branching members of this group, while the genus *Bathycoccus* arrived later, *Ostreococcus* then arising within the family Bathycoccaceae (Marin and Melkonian 2010). The most parsimonious explanation for evolution of the different species is thus that their cells and genomes are under selective pressure to reduce their sizes to a minimum, with gene family numbers becoming more and more reduced, sometimes disappearing completely if they are not absolutely necessary for survival. Examples of such losses are that *Ostreococcus* lacks several genes involved in flagellum biosynthesis and is missing cell wall proteins that are found in many other green algae. Transcription factors, which may show expanded copy numbers in higher plants as they have been recruited to control different cellular control processes, are also often found in reduced numbers or even absent in *Ostreococcus* spp. We might speculate that the evolutionary force driving size reduction could be selection to increase the surface to volume ratio, in order to maximize the efficiency of nutrient uptake (see Okie 2013 and references therein), or to escape grazing by filter feeders such as oysters, which prefer larger prey (Dupuy et al. 2000). *Ostreococcus* spp. are abundant in lagoons where oysters are cultured.

One likely consequence of selection for minimalism is that these algae might also lose some distinguishing morphological characters, so we might expect that morphological convergence occurred in biologically different species that may be adapted to different environments, such as nutrient conditions or differing temperatures as observed (Rodriguez et al. 2005; Slapeta et al. 2006; Subirana et al. 2013; and see below). However, we cannot exclude the alternative hypothesis that these species evolved only through niche adaptation from identical ancestors.

6.2.2 Similar Cellular Morphologies Cloak Phylogenomic Divergence

In the genus *Ostreococcus*, two species have been described officially (Courties et al. 1994; Chrétiennot-Dinet et al. 1995; Subirana et al. 2013), but current genomic data (Rodriguez et al. 2005; Palenik et al. 2007), and currently the complete genome of *O. mediterraneus* is being analyzed (Keeling et al. 2014; and unpublished data), leave no doubt that there are at least four species of *Ostreococcus* represented in cultures, and other uncultured species may yet remain to be discovered. After the discovery, description and complete genome analysis of *O. tauri*, isolated from a coastal Mediterranean lagoon, the first big surprise came to light after analyzing the complete genome of a second strain of *Ostreococcus* isolated from the North Pacific Ocean, close to the Californian Coast (Palenik et al. 2007). Although the cellular morphologies of the Pacific and Mediterranean strains seen by electron microscopy

were identical, their complete genomes showed only 70 % overall sequence diversities (Jancek et al. 2008), a comparable divergence to that seen between chicken and humans. This observation was unexpected, because their 18S ribosomal RNA sequences, the phylogenetic markers generally used for species' descriptions, are 99.8 % identical. The overall level of divergence between these genomes means that these lineages must represent biologically different populations (see Coyne and Orr 2004). The strain from the Pacific Ocean was then nominated to be *"Ostreococcus lucimarinus*," but in the absence of morphological characters to distinguish it from *O. tauri*, no formal description of this strain as a separate species was reported. Subsequently the two further "ecotypes" of *Ostreococcus* spp. (Rodriguez et al. 2005), which must in fact represent separate species (Subirana et al. 2013), have been described and analyzed at the genome level (unpublished data). The strain RCC809 is a clonal lineage obtained from RCC141 that was collected from 105 m deep in the tropical Atlantic Ocean during the EUMELI 3 cruise in 1991 by culture in K medium (F. Partensky, unpublished data; see Vaulot et al. 2004), and the genome of RCC2590 representing the species *O. mediterraneus* (Subirana et al. 2013) is being sequenced.

In a similar way, the comparison of the complete genomes of two *Micromonas* spp. revealed that they most probably represent different species (Worden et al. 2009). A remarkable new kind of repeated element named called an "introner element" (IE), lacking many of the characteristics of classical transposons but rather behaving like fully functional spliceosomal introns, was discovered in one of the strains of *Micromonas*. IEs with relatively well conserved sequences were found at novel positions in genes, were classified in four sub-families and doubled the number of introns in the CCMP1545 strain. A detailed study (Verhelst et al. 2013) revealed other families of IEs, and these authors hypothesized that each IE class may have originated from a single ancestral IE, likely by a process involving reverse splicing.

Only one species of the genus *Bathycoccus* has so far been sequenced (Moreau et al. 2012), although it seems likely that cryptic species exist (Vaulot et al. 2012; Monier et al. 2013). *Bathycoccus prasinos* cells are covered with scales that resemble spider's webs in electron micrographs, a feature shared with certain other members of the Mamiellophyceae (e.g., Moestrup 1984, 1990) but a morphological trait distinguishing it clearly from *Ostreococcus* spp. Although no transposons were found in the *Bathycoccus* genome, four gene families, including sialyltransferases, sialidases, ankyrin repeats and zinc ion-binding genes, have expanded extensively, and more than 400 predicted genes have probably been acquired by horizontal gene transfers from other eukaryoytes. It is possible that the expansion of genes involved in glycoconjugate pathways (sialyltransferases and sialidases) is associated with the production of cell surface polysaccharides, as these are perhaps components of scales (Melkonian et al. 1991), but this remains to be shown experimentally.

In summary, in the Mamiellales the extent of phylogenomic divergence between different unicellular eukaryotic species is greatly underestimated when ribosomal RNA genes are used as standard genetic markers for species' diversities. Piganeau

et al. (2011) showed this observation to be true in general for numerous pairwise comparisons of phylogenomic divergences in protists, whereas the ribosomal gene divergences between different multicellular organisms more closely represented their genomic divergences, and that this situation might arise because of difference in the effective population sizes. Identical 18S ribosomal RNA gene sequences can thereby conceal biologically different species (e.g., Bendif et al. 2014).

6.2.3 *Two Outlier Chromosomes Have Enigmatic Functionalities*

Analysis of the genome sequence of *O. tauri* revealed that two of the 20 chromosomes were quite different to the others (Derelle et al. 2006). We refer to these henceforth as the "big outlier chromosome" (BOC, chromosome 2 in *O. tauri*) and the "small outlier chromosome" (SOC, chromosome 19 in *O. tauri*). Continued efforts to sequence complete genomes of different species in the Mamiellales over the last 7 years have increased the importance of this initial observation, since this feature has been found in all of the genomes of the Mamiellales so far, despite their phylogenomic divergence, implying that it evolved in a common ancestor. Several articles have addressed this topic in different contexts (reviewed in Piganeau et al. 2011; Moreau et al. 2012; Subirana et al. 2013), so this overview will be brief.

Whereas the overall GC content of *O. tauri's* genome is rather high (59 %) the GC content of these outliers is much lower. All of the SOC sequence has a lower GC content (54 %), and about a half of the BOC has a lower GC content (52 %). Besides this remarkable biochemical difference, the gene content of these OCs is atypical. Taken together, in *O. tauri* these two aberrant chromosomes carry 77 % of the >400 transposable elements (TEs), or relics thereof, which are identified in the genome (about 60% in the BOC and 20 % in the SOC). In the SOC, fewer than 20 % of the genes are related to the green lineage, and a high proportion of them encode proteins involved in biosynthesis of glycoconjugates and surface membrane proteins.

Likewise, the complete genomes of *O. lucimarinus*, the two *Micromonas* spp., and *Bathycoccus* sp. all carry these lower GC-content regions.

Detailed analyses (see Moreau et al. 2012 for an overview) have revealed further peculiarities of these chromosomes. The BOC is usually composed of two quite different regions; about a half of it (region BOC1) is more conserved in its gene content between different members of the Mamiellales compared with other chromosomes, while BOC2 is less conserved and has a lower GC content. BOC1 has a higher proportion of housekeeping functions and is more strongly expressed than other regions of the genome. The SOC is known to vary in size between individuals of the same biological species (Subirana et al. 2013). In an NGS analysis of 13 independent clonal wild-type lines of *O. tauri*, a good realignment coverage of all of the chromosomes except for the SOC was possible (R. Blanc-Mathieu,

unpublished data), suggesting that the sequence content of this chromosome is too variable to assemble to the reference genome using high-throughput Illumina-type sequencing technology. Further work must be pursued, either by using the PacBio-type sequencing approach (Huddleston et al. 2014), or by using more classical approaches.

6.3 Metabolic Processes or Life Styles Inferred from Genomes

Complete genome sequences have provided insights into potential Mamiellales metabolisms and adaptations, although most of them are still awaiting experimental evidence. For example, Mamiellales biochemical studies showed that the same light-harvesting chlorophyll-binding proteins (LHCs) were associated with both PSI and PSII. However, the availability of *Micromonas* and *Ostreococcus* genomes confirmed the existence of an unusual Mamiellales-specific LHC, probably associated with both photosystems (Cardol et al. 2008), but also revealed the presence of specific PSI and PSII subunits, which are expressed at low levels and which were not detected biochemically (Six et al. 2005). Because Mamiellales are considered as early diverging green algae, these findings had important evolutionary implications and supported the hypothesis of an ancient origin of LHCI genes from which LHCII and the major Mamiellales LHC genes probably evolved, consolidating data into a coherent evolutionary scenario. Still on photosynthesis, genes encoding all of the enzymes required for C4-photosynthesis are present in both the *Micromonas* and *Ostreococcus* genomes indicating that, like diatoms, these algae may use this pathway as a CO_2 concentration mechanism (Derelle et al. 2006; Worden et al. 2009). This may, therefore, be a strategy general to planktonic microalgae, raising the possibility that, despite its high energetic cost, C4-photosynthesis might give a critical ecological advantage in CO_2-limiting conditions, such as in phytoplankton blooms.

In silico analysis of nutrient acquisition has also been investigated by comparative analysis of genes involved in nitrogen metabolism. Eight of the genes involved in nitrate uptake and assimilation, as well as four genes for urea assimilation, are found next to each other in two clusters located on two chromosomes in the three sequenced *Ostreococcus* (Derelle et al. 2006; Palenik et al. 2007) (Fig. 6.4). A comparable clustering of nitrate assimilation genes is also observed in the sequenced *Micromonas* strain CCMP2545. In *Bathycoccus* and in the second *Micromonas* sequenced (strain RCC299), the cluster is reshuffled with fewer genes: the five genes of the 3′ extremity are lacking in both species and these genes are found dispersed in the genome, as in the green alga *Chlamydomonas reinhardtii* (Derelle et al. 2006). This organization indicates a possible selective pressure for optimization of nitrate and urea uptake and assimilation in the *Ostreococcus* species and in some strains of *Micromonas*.

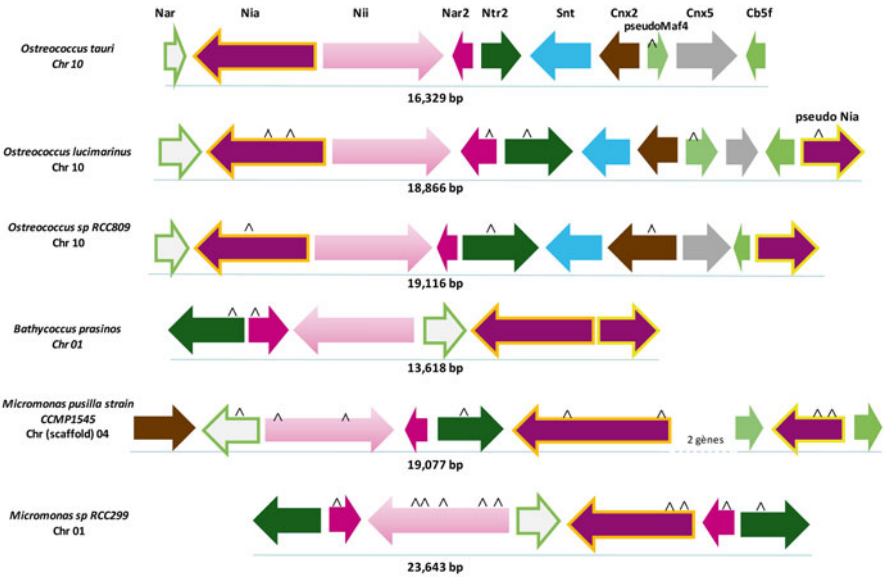

Fig. 6.4 Comparison of the nitrate gene cluster assimilation among Mamiellales. Nar, plastid nitrite transporter; Nia, nitrate reductase apoenzyme; Nii, plastid-targeted nitrite reductase apoenzyme; Nar2, nitrate high-affinity transporter accessory protein; Ntr2, nitrate high-affinity transporter; Snt, putative molybdate transporter; Cnx2, molybdenum cofactor biosynthesis protein; Maf4, Maf4-related hypothetical protein; Cnx5, molybdenum cofactor biosynthesis protein (molybdopterin synthase sulfurylase); Cb5f, cytochrome b5 reductase. ^: introns

Interestingly, four genes of urea assimilation are also clustered in *Ostreococcus* species and in the CCMP 2545 *Micromonas* strain originating from the English channel. These genes are completely absent in the strain RCC299 originating from New Caledonia (Fig. 6.5), and this corresponds experimentally to a loss of growth of this strain on urea as a nitrogen source (unpublished Moreau personal observation). A recent study in *Micromonas* showed a high degree of "mixed lineage gene affiliations" for nitrogen transport and assimilation genes (McDonald et al. 2010), suggesting ancient origin, a complex evolution pattern of gene duplication and gene loss, or horizontal gene transfer for some of these genes.

These *in silico* analysis are still in their infancy and many metabolisms, potential adaptations and/or evolutive signatures still remain to be elucidated. Besides portals dedicated to the annotation of each of the different genomes, web sites focused on comparative analysis between the different organisms belonging to the green lineage, such as PLAZA (http://bioinformatics.psb.ugent.be/plaza/) (Proost et al. 2009) and its derivative pico-PLAZA (http://bioinformatics.psb.ugent.be/pico-plaza/) (Vandepoele et al. 2013) are now available. Such tools for comparative approaches, coupled to the development of new sequencing possibilities for both new genomes and metagenomes from various environments, open the door to a better understanding of the adaptations of the organisms to their environment.

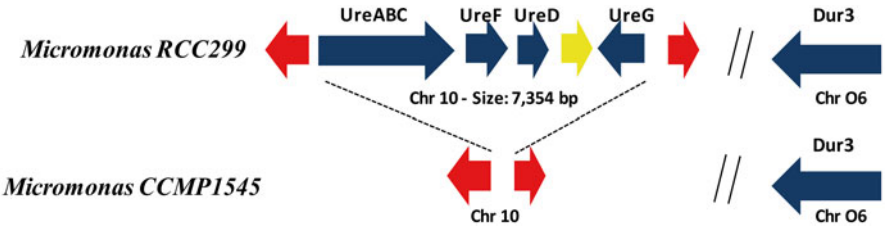

Fig. 6.5 Urea cluster present in one strain of *Micromonas* and absent in the other. *Blue arrows*: genes involved in urea metabolism; *yellow arrow*: gene not involved in urea metabolism; *red genes*: genes not involved in urea metabolism and flanking the urea cluster. Note that the 5′ and 3′ genes flanking the cluster are the same in the two *Micromonas* strains. UreABC, Ni-dependant urease apoenzyme. The A, B and C subunits are encoded by three separate genes in bacteria but form a single gene here, as in higher plants; UreG and ureaD, urease accessory proteins G and D. Note these two genes are fused together in the *Ostreococcus tauri* genome; UreF, urease accessory protein F, which forms a complex with G and D and with apo-urease to allow nickel insertion, resulting in activation of urease. Dur3, urea high-affinity symporter

6.4 Organellar Genomes

Mamiellophyceae organelles, like the rest of the cell, tend toward minimalism. Cells usually contain a single chloroplast (cp) and mitochrondrion (mt) (Marin and Melkonian 2010). The genome copy number is similarly estimated to be low at 4–6 times that of the nuclear genome (unpublished observations). This overall reduction marks the evolutionary history of the cp, which is characterized by gene loss that parallels the simplification of cell structure (Turmel et al. 2009). Given its small size and high coding density, this likely applies to the mt as well.

6.4.1 Common Features of Mamiellophyceae Organellar Genomes

All the Mamiellophyceae organelle genomes sequenced to date are circular, have a common gene repertoire and low GC content and, most notably, are compact. Indeed, *O. tauri* organellar genomes are thus far the smallest of the Mamiellophyceae (cp: 71,666 bp, mt: 44,237 bp), with the cp also being the smallest known within Chlorophyta. Shared coding sequences include the ribosomal genes, the full tRNA complement necessary for translation and select genes involved in photosynthesis and oxidative phosphorylation in the cp and mt, respectively. The low GC content is related to a preference for codons with A or U in the third base position (Robbens et al. 2007). AT enrichment is common in all known organelle genomes (reviewed in Smith 2012), so in this regard the Mamiellophyceae are not an exception;

however, the precise evolutionary forces driving AT enrichment are still elusive. The availability of both nuclear and organelle genomes for *O. tauri* revealed that at least one mt gene and several cp genes are present in the nucleus, thus gene transfer to the nucleus was a driver of organelle genome size reduction (Robbens et al. 2007). It is likely that simple gene loss has played a role as well. The rate of change in the organelles appears to have slowed and stabilized for a long period in the more recent past as divergence between organelle orthologous genes within *Osteococcus* spp. is lower than that of the nuclear genome (Piganeau and Moreau 2007).

6.4.2 Variable Features of Mamiellophyceae Organellar Genomes

The organellar genomes vary most in their organization and the presence of repeated and mobile elements. From the sequences currently available, the Mamiellales share the same overall structure while cp of the most phylogenetically distant of the Mamiellophyceae, *Monomastix*, is the most divergent. *O. tauri* was the first chlorophyte found to possess identical inverted repeats (IR) in both the cp and the mt (Robbens et al. 2007). Subsequent genome sequencing has shown this to be common to the other Mamiellales (Worden et al. 2009; Moreau et al. 2012) but notably absent in *Monomastix* (Turmel et al. 2009). The IRs divide the genome into a quadripartite structure intervening between a large and a small single-copy region that varies in its genetic content. The quadripartite organization is further shared with Streptophyta (reviewed in Green 2011) and is likely ancestral in the green lineage. It is curious that repetition for such a large proportion of the organelle genome is retained, given the apparent selection for small size, implying that the IRs provide some function. In the *O. tauri* mt in particular, 44 % comprises IRs (Robbens et al. 2007). It was suggested this allows the generation of subgenomic circles influencing gene dosage, as has been observed in land plants (Robbens et al. 2007), or that IRs may be necessary if replication is recombination dependent (Bendich 2004).

The cp sequence of *Monomastix* revealed an interesting exception in the trend toward overall genome reduction in two ways. First, although the gene content of the *Monomastix* cp is not much greater than *Ostreococcus*, it has five group I introns that are likely mobile elements (Turmel et al. 2009), which are absent in the organelles of Mamiellales. Second, in contrast to organelles from Mamiellales that have small to absent intergenic sequences, the *Monomastix* cp has large intervening spacers of 524 bp on average. These spacers consist primarily of short dispersed repeats of 19–58 bp and are the principal source of the size expansion in the cp DNA (Turmel et al. 2009).

6.4.3 *Population Genomic Insights into Organelle Inheritance and Evolution*

Although Mamiellophyceae genomes are haploid, genes for meiosis and the evidence of recombination in the nuclear genome suggest sexual reproduction occurs (Grimsley et al. 2010). Population genome data for *O. tauri* revealed evidence of recombination in the cp, but not in the mt (Blanc-Mathieu et al. 2013). This implies that inheritance of the cp is biparental in Mamiellophyceae, unlike the majority of the green lineage where organelles are inherited uniparentally. Since the Mamiellophyceae are at the base of the divergence of Chlorophyta and Streptophyta, this raises the possibility that biparental cp inheritance is an ancestral state. This brings to light factors influencing genome evolution that lead to maintenance of recombination in one organelle, which was estimated to occur at a similar frequency as in the nuclear genome, but not in the other. A possible benefit to maintaining biparental inheritance in the cp is that it is more efficient at removing deleterious mutations, consistent with lower ratio of synonymous to non-synonymous polymorphisms observed in the cp than in the mt. On the other hand, this makes the cp more vulnerable to spread selfish elements, which accords with their presence in the cp and absence in the mt (Blanc-Mathieu et al. 2013).

6.5 Metagenomics of Mamiellales

Metagenomic studies have shown that Mamiellales are ubiquitous in marine waters (Chap. 1). These studies either make use of the amplification or nucleotide hybridization of probes of single taxonomic marker genes, typically the small ribosomal subunit (SSU) gene, or use random "shotgun" sequencing of whole genomic fragments. Cells for shotgun sequencing have been generally captured by filtration or by fluorescence-activated cell sorting. The latter method is more effective at targeting Mamiellophyceae, as the prokaryotic component overwhelms size-fractionated datasets (Marie et al. 2010).

6.5.1 *Linking Marker Gene Diversity, Physiology and Ecology*

The initial sequencing of the 18S SSU gene from coastal and open ocean marine samples gave the first indication of a hidden breadth of genetic diversity within the Mamiellales and their relatively high abundance in picoeukaryotic communities (Guillou et al. 2004). Interestingly, both *Ostreococcus* and *Micromonas* were shown to comprise several heterogeneous clades, while *Bathycoccus* formed a single clade with little SSU sequence divergence (Guillou et al. 2004) despite the fact *Bathycoccus* likely has a wider distribution and greater abundance in the oceans (Johnson and

Sieburth 1982). Subsequent studies have examined the diversity of Mamiellales SSU sequences in various marine systems including the Mediterranean coast (Zhu et al. 2005), the Pacific Ocean (Shi et al. 2009), the Sargasso Sea (Treusch et al. 2012), the Atlantic Ocean (Kirkham et al. 2011) and the Arctic Ocean (Balzano et al. 2012). Overall, their abundance has large spatial and temporal variability with certain strains being apparently restricted in range, such as the *Micromonas* clade E that dominates the Arctic Ocean (Lovejoy et al. 2007).

There is additional evidence that *Ostreococcus* and *Micromonas* clades represent ecotypes, i.e., genotypes whose distribution is determined by environmental factors. For example, molecular surveying of *Micromonas* clades A, B and C indeed found that although all clades are sympatric, their relative abundance varies with environmental gradients (Foulon et al. 2008). Similarly, *Ostreococcus* clades could be divided into high- and low-light-adapted clades in laboratory conditions (Rodriguez et al. 2005). The distribution of *Ostreococcus* clades in the ocean, however, appeared more complex and related to further factors such as temperature, salinity and nutrient concentrations (Demir-Hilton et al. 2011). Molecular surveying has proven to be a very powerful tool for starting to uncover what influences Mamiellales species distributions. Thus far, all studies have limitations in their phylogenetic or spatio-temporal resolution and undoubtedly further investigation linking genetic diversity with physiology and biogeographical patterns is required to build a complete picture.

6.5.2 Whole Genomic Sequence from "Wild" Populations

Just as marker gene surveys uncovered species diversity within morphospecies groups, shotgun metagenomes have afforded glimpses into hitherto unseen genomic richness and enabled insights into genome evolution. *Ostreococcus* genomic fragments retrieved from Sargasso Sea metagenomes shared 85 % nuclear sequence identity in orthologous genes with the available *O. tauri* and *O. lucimarinus* reference genomes and thus likely represented a different species. This Sargasso *Ostreococcus* species consisted of at least two strains, which was not evident from 18S rDNA gene diversity analysis, and allowed determination of the degree of constraint on intronic sites (Piganeau and Moreau 2007). Similarly, *Bathycoccus* metagenomic sequences from a coastal Chilean upwelling site (Vaulot et al. 2012) and from the tropical Atlantic (Monier et al. 2012) revealed the first evidence of genome-wide variation in *Bathycoccus* species. Amino acid sequence identity was much higher between the Chilean samples (~96 % identity) and the Mediterranean *Bathycoccus* reference genome than between the Atlantic metagenome and the reference (84.4 %) (Vaulot et al. 2012). Intriguingly, the Atlantic *Bathycoccus* has an SSU gene sequence identical to the reference, despite having greater divergence in protein-coding regions (Vaulot et al. 2012). Although there appears to be a higher degree of conservation within *Bathycoccus* than between *Ostreococcus* species, these metagenomic sequences suggest concealed species or ecotypes in

Bathyococcus. Thus the availability of even fragments of genomes from the environment is providing some in-roads into answering big questions such as "what level of genetic diversity constitutes a different species in the Mamiellophyceae?" and "how much genetic diversity is there in each species?"

6.6 Viruses

6.6.1 Prasinoviruses Are Diverse and Abundant

Large DNA viruses of the Mamiellales (prasinoviruses) are found very commonly together with their host species and no description of the genomics of this group would be complete without mentioning them. They carry a large amount of genetic information (200–250 genes per virus) and undoubtedly play an important role in the evolution of their host species. The first viruses infecting *Micromonas pusilla* were discovered 35 years ago by Mayer and Taylor (1979) who found that 0.2 μm filtrates of water samples from the Strait of Georgia would readily lyse cultures of this species kept at the University of British Colombia. *Micromonas* virus life cycles have been investigated (Waters and Chan 1982; Cottrell and Suttle 1991; Brussaard et al. 1999) and host cell lysis appears to occur in the daytime (Brown et al. 2007). All prasinoviruses encode a gene for DNA polymerase, which has been used as a marker for studying phylogeny and environmental diversity (Chen and Suttle 1995, 1996; Chen 1996; Short and Short 2008; Bellec et al. 2009, 2010; Short 2012; Manrique et al. 2012) and a recent survey shows that they are the most abundant large viruses of eukaryotic marine plankton (Hingamp et al. 2013).

Over the last 6 years, prasinoviruses infecting *Bathycoccus prasinos* and *Ostreococcus* spp. have also been discovered, and the complete genomes of numerous prasinoviruses have been sequenced (Derelle et al. 2008; Weynberg et al. 2009; Moreau et al. 2010; Weynberg et al. 2011). Remarkably, the complete genomes of *Bathycoccus*, *Micromonas* and *Ostreococcus* viruses show greater overall similarity than that observed between the complete genomes of their host species (Moreau et al. 2010). Prasinoviruses are thus either evolving more slowly than their host species, or they have switched between hosts after the speciation of these genera in the evolution of the class Mamiellophyceae.

Given that the cells of these host species are only about 1–2 μm in diameter, and that the cytoplasm of the cell represents less than 30 % of the cell volume, prasinoviruses are enormous, about 0.11 μm between opposing faces of their icosahedral particles. Nine particles would thus traverse the diameter of one cell. The burst size is accordingly small, averaging around 25 particles in *Ostreococcus* (Derelle et al. 2008). Over a half of prasinovirus genes encode products with no known biological functionalities. Numerous predicted genes are probably involved in viral DNA replication and the metabolism of DNA, including some methylases, but no restriction enzymes have so far been found. About a quarter of the known predicted genes are probably involved in protein metabolism. Several genes, not previously observed in

viruses of eukaryotes, encoding the biosynthesis of certain amino acids (aa), seem to have been acquired by *Ostreococcus* and *Micromonas* viruses, whereas *Bathycoccus* viruses encode two huge proteins (from 3100 to 5700 aa) with unknown functionalities. Numerous prasinovirus genes show the greatest similarities to prokaryotic genes, and relatively few show close similarity to host genes, so these genes may have been recruited from other genomes, but there is no evidence that these kinds of exchange are very recent. Nevertheless such exchanges might be important on an evolutionary time scale, and a few genes were probably recruited from host genomes.

6.6.2 *Is the Evolution of Host and Viral Genomes Driven by an Arms Race?*

In diverse organisms the evolution of new virulent strains of pathogens is countered by the development of resistant host strains (e.g., Flor 1955, 1971; Dawkins and Krebs 1979), forcing evolutionary divergence in both partners. Two kinds of laboratory observations support the notion that evolution in the Mamiellophyceae is also subject to this kind of selection. First, spontaneous resistance of algal cell lines to virulent prasinoviruses occurs frequently in culture and appears to be genetically stable (Thomas et al. 2011), suggesting that the host cell can quickly evolve to escape subsequent infection.

Second, prasinoviruses show rather strict specificity, usually infecting only 10–20 % of wild-type strains (Clerissi et al. 2012, see Fig. 6.6) within the biologi-

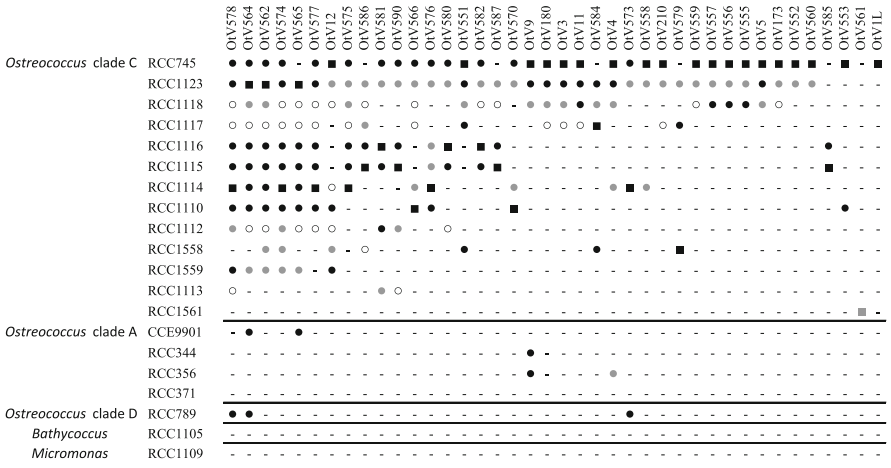

Fig. 6.6 Specificity of *Ostreococcus* viruses (From Clerissi et al. 2012, copyright © 2012, American Society for Microbiology). *Minus sign*—no lysis; *black filled squares*—lysis on the host used for the isolation of the virus; *black filled circles*—high lysis; *gray filled circles*—intermediate lysis; *open circles*—low lysis

cally defined species of *O. tauri* (Grimsley et al. 2010), or within different clades of the genus *Micromonas* (Bellec et al. in press). Given the diversity of specificities encountered among different host–virus combinations tested, a countless diversity of specificities is likely to be present among the astronomical numbers of algal cells that populate our oceans, witnessing continuous evolution of virulence.

6.7 Perspectives

The enigmatic variable regions of genomes in the Mamiellales provide a challenge to advancing knowledge about this group of species, as they are more difficult to assemble using high-throughput technologies. However, they might represent specific adaptations of individual strains to their environments, and thus be particularly important for understanding the biology of these species. Clerissi et al. (2012) presented very preliminary evidence that variations in the size of the SOC might be associated with different viral susceptibility specificities, but in general little is known about how populations of plankton adapt to the changing oceanic environment. The tiny genomes of cosmopolitan Mamiellales provide important tools for future interdisciplinary investigations in this research field.

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Chapter 7
Planktic Foraminifera

Katsunori Kimoto

Abstract Planktic foraminifera are single-celled marine eukaryotes characterized by having calcareous shells. They are holoplankton with 40–50 identified species in the world ocean. Their biology, diversity, and shell chemistry are sensitive to changes in the oceanic environment, and therefore their carbonate shells are useful climatic tracers of temperature, water mass, and other chemical indicators of global change. Production of carbonate shells plays an important role in marine biogeochemical cycles involving carbon and is closely related to the Earth's climate systems. Geochemical studies combined with culture experiments using planktic foraminifera provide much useful information about future biological and climatic responses on Earth. Molecular biology based on DNA has become a major tool for all ecological, biological, and evolutional studies of foraminifera. Molecular phylogenic analyses have revealed that morphologic classification of the planktic foraminifera resulted in underestimation of their biodiversity.

Keywords Planktic foraminifera • Rhizaria • Photosymbiosis • Reproduction • Geochemistry • Molecular phylogeny • Culture experiment

7.1 Introduction

7.1.1 What Are Foraminifera?

The foraminifera are a group of single-celled organisms living in the world ocean and are among the most successful, highly diversified amoeboid protists. The name "Foraminifera" originates from the Latin "foramen" (perforation) and "fero" (bear). Foraminifera are classified broadly on the basis of two types of habitat: planktic and benthic ("planktonic" is the customary term but "planktic" is a correct usage as a derivation from a Latin noun). Planktic foraminifera live at surface to intermediate

K. Kimoto (✉)
Japan Agency for Marine-Earth Science and Technology (JAMSTEC),
2-15 Natsushima-cho, Yokosuka 237-0061, Japan
e-mail: kimopy@jamstec.go.jp

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water depths (to about 1,000 m) throughout the world ocean. In contrast, benthic foraminifera live at all depths, from brackish marshes to the deep-sea floor, on seagrass, and in sediments of the world ocean. A group of organic-walled and agglutinated benthic foraminifera was identified in the Challenger Deep in the Mariana Trench (about 11,000 m) (Akimoto et al. 2001; Todo et al. 2005). The extant foraminifera have been classified morphologically into at least 6,000 species worldwide (Jones 1994). Of these, the planktic foraminifera comprise only 40–50 species (Hemleben et al. 1989; Schiebel and Hemleben 2005).

A typical feature of the foraminifera is the creation of a hard skeleton (shell or test) made of calcium carbonate ($CaCO_3$), organic material, or mineral particles from bottom sediments ("agglutinated"). After foraminifera die, their whole shells and shell fragments accumulate in deep-sea sediments. Of the total amount of biogenic carbonate produced in the world ocean, planktic foraminifera account for a $CaCO_3$ deposition of 0.36–0.88 Gt/y (Schiebel 2002). This is roughly 32–80 % of the $CaCO_3$ deposited in the deep ocean (Erez 2003). Such carbonate depositions consisting of foraminiferal shells are called "carbonate ooze" (or *Globigerina* ooze), and they cover about 50 % of the ocean floor (140×10^6 km^2). These fossil species therefore play an important role in the global oceanic carbon cycle and become useful climatic recorders of past oceanographic conditions.

On the basis of recent molecular phylogenetic studies, the foraminifera can be classified into the supergroup Rhizaria, one of the eight supergroups in the eukaryotes. The Rhizaria include two phyla: Cercozoa and Retaria. The Cercozoa include Filosa and Endomyxa and the Retaria include Foraminifera and Radiolaria (Cavalier-Smith and Chao 2003). Rhizaria are characterized by reticulate rhizopodia (pseudopodia) that protrude outward as a web-like structure (Pawlowski 2009). This molecular phylogenetic category also includes some naked or soft-shelled freshwater and terrestrial amoeboid foraminifera (Pawlowski et al. 1999; Pawlowski and Holzmann 2002).

This chapter presents an overview of the biology and ecology of carbonate-shelled modern planktic marine foraminifera, as well as a review of relevant geochemical and phylogenetic studies. Discussions of their geologic and paleoceanographic applications are kept to a minimum. The general usage of planktic foraminifera for biostratigraphy and paleoceanographic reconstructions is covered in references presented for further reading.

7.1.2 Evolution over Geological Timescales

According to the fossil record, benthic foraminifera had already appeared by the Early Cambrian Period (560 million years ago [Ma]) (Howell and Dunn 1942; Culver 1991). These species were Allogromida, with an agglutinated test. The first fossil calcareous foraminifera appeared in the Middle Devonian but not in the Ordovician, according to recent studies (see Suzuki and Oba 2015). The first fossil planktic foraminifers appeared between the Toarcian (182–174 Ma) and the late

Bajocian (169–168 Ma) (See Suzuki and Oba 2015). Although there is still some debate about the timing of the first appearance of planktic foraminifera, recent geologic evidence suggests that the origin dates to the Late Triassic–Early Jurassic (Boudagher-Fadel 2012). Morphologic similarity suggests that the first planktic foraminifera evolved from related benthic species of Rotaliida (Boudagher-Fadel 2012). These early planktic species secreted shells of aragonite (the second most common polymorph of natural calcium carbonate) and were sometimes agglutinated (Oberhauser 1960). It is widely recognized that the first planktic foraminifer with a carbonate shell was *Globuligerina oxfordiana*, which appeared in the Middle Jurassic (170 Ma) (Tappan and Loeblich 1988). It appears in sediments that reflect shallow and neritic environments around the Tethys Sea (the paleo-Mediterranean Sea between the Gondwana and Laurasia supercontinents in the Mesozoic Era) and never appears in deep-sea sediments (Riegraf 1987). Consequently, the first planktic foraminifera might have been meroplankton—mostly benthic but with a temporary drifting lifestyle.

The actual divergence to worldwide planktic foraminifera started in the Early Cretaceous (around 118 Ma). This period is generally thought of as a "greenhouse" period, with relatively high atmospheric carbon dioxide (CO_2) levels and high global mean temperatures. Average sea surface temperatures (SSTs) during this period, reconstructed by using organic geochemical methods, were around 26–32 °C (Littler et al. 2011). In such a greenhouse world, the planktic foraminifera evolved and adapted to not only surface water but also to deeper conditions by the time of the Early Cretaceous. By the end of the Early Cretaceous (100 Ma), planktic foraminifera were distributed through a wide range of habitat depths. Consequently, the carbonate shells of planktic foraminifera had many variations of form, such as single and double bordering ornamentation around the shell (keel), peripheral elongation, multiserial spiraling, and shell hypertrophy (e.g., Boersma 1978).

At the end of the Cretaceous (65.5 Ma), drastic climate changes occurred in the so-called K-Pg (Cretaceous–Paleogene) boundary event. This event is well documented as the "Dinosaur extinction" resulting from a meteorite impact in Yucatan, Mexico (Hildebrand et al. 1991; Smit 1999), and many other terrestrial and marine species became extinct. About 90 % of the planktic foraminiferal species also became extinct because of this environmental catastrophe (e.g., Smit 1982; D'Hondt et al. 1996; Molina et al. 1998). The extinct species were characterized by large and complex morphology (Keller 1988). In contrast, the surviving species were cosmopolitan generalists with small and simple forms, like the globigerinid and serial foraminifera (Keller 1988; Keller and Abramovich 2009). Such primitive-shaped, smaller foraminifera became the stock species for evolution in the Cenozoic era (65.5 Ma to the present).

Although there were some major and minor extinctions following the abrupt climate changes over the transition from a greenhouse world to an icehouse world in the Cenozoic (e.g., Zachos et al. 2001), planktic foraminifera remained alive over the long geological timescales. The first spinose species (Globigerinidae) adapted to the photic zone appeared in the Paleogene (65.5–23 Ma). Spinose species have photosymbiotic microalgae in their cells (see Sect. 7.4) and photosynthesize effectively

by adapting to surface water. Flat chamber species developed a keel on the periphery (Globorotaliidae) in the Neogene (23–2.6 Ma). Species of this type adapted to subsurface and intermediate water in the ocean. Their general morphological features had appeared by the early Neogene and continue into the modern living planktic foraminiferal lineages.

Hence, the morphological evolutionary history of planktic foraminifera over geological timescales is a history of adaptation to varying environmental conditions. In fact, some of the same morphologic features of planktic foraminifera appeared in the Cretaceous, Paleogene and Neogene, even though mass extinctions meant that there was no genetic continuity (Saito et al. 1981; Banner 1982). This indicates the conservative genetic characteristics of planktic foraminifera (Hemleben et al. 1989).

7.1.3 Research History of Living Planktic Foraminifera: An Overview

Early ecological studies of planktic foraminifera were achieved mainly through the geological record rather than through biology. Murray (1897) first described the latitudinal distribution of planktic foraminifera by using deep-sea surface sediment and plankton tow records. Fossil foraminiferal assemblages were useful for evaluating sediment age and depositional environment; therefore in the early twentieth century the petroleum industry studied the correlation and assignment of sedimentary ages by using foraminifera (biostratigraphy). The biological study of living foraminifera by academia began in the mid-1950s and involved biology, ecology, paleoceanography, and other related climatological research (e.g., Phleger 1945, 1954). It was known that the faunal composition of the dead shells of planktic foraminifera in the surface sediments were consistent with corresponding surface water masses. This means that the geographic distribution of fossil species in the sediments reflects past oceanographic conditions. The most remarkable result from early paleoceanographic studies was the reconstruction of the SST during the last glacial age (about 24,000–18,000 years ago; Last Glacial Maximum [LGM]) by multiple regression analysis based on the worldwide geographic distribution of fossil foraminiferal species (CLIMAP 1976). This analysis provided the first quantitative estimates of past sea surface temperatures and indicated that the global average SST was 2 °C lower than at present. Faunal compositional changes in deep-sea sediments are clear and concrete indicators of climate change over geological timescales and are still essential in research today; e.g., in the Deep-Sea Drilling Program (DSDP, 1963–1983) and later in the International Ocean Discovery Program (IODP, ongoing since 2014).

Biological studies of living planktic foraminifera collected by plankton tow were performed in parallel with the early paleoceanographic studies (e.g., Parker 1962; Bé 1959, 1960; Bradshaw 1959; Ujiié 1968). In particular, Bé and Tolderlund (1971), Bé (1977) and Bé et al. (1977) collected various types of living planktic

foraminifera in the field and described behavior and ecological responses as observed with both light microscopy and scanning electron microscopy (SEM). Their work involved various topics related to living planktic foramininfera, i.e., their growth, feeding activity, calcification processes, shell architecture, reproduction, and symbiosis. Later, Hemleben et al. (1989) presented biological and ecological studies of modern planktic foraminifera and compiled all knowledge available at the time in a "milestone" book of modern planktic foraminifera.

Time-series observations using sediment traps provide an alternative approach to clarifying short-term faunal responses of planktic foraminifera. Sediment traps can be moored to collect the sinking particle flux in the water column at a given location. A common design of sediment trap consists of a collecting funnel, a baffle (to minimize turbulence), a rotating sample collector, and a computerized controller (Honjo and Doherty 1988). The collecting cups in this type of trap are automatically moved into alignment with the collecting funnel and can collect sinking particles over programmed intervals—typically weekly or bi-monthly. Dead shells of planktic foraminifera settle in the water column as aggregated sinking particles (known as "marine snow") and are collected in the receiving cup positioned at the bottom of the sediment trap. The faunal compositions of planktic foraminifera in sediment traps were examined to resolve seasonal ecological responses and evaluate the contribution of foraminifera to the total carbonate flux (e.g., Berger and Soutar 1967; Thunell and Honjo 1981; Curry et al. 1983; Thunell et al. 1983; Thunell and Sautter 1992; Eguchi et al. 1999; Kincaid et al. 2000; King and Howard 2001; Kawahata et al. 2002; Kuroyanagi et al. 2002, 2008; Lin et al. 2004; Xu et al. 2005; Asahi and Takahashi 2007; Yamasaki et al. 2008; Storz et al. 2009). These studies made great progress toward understanding carbon cycles and the ecology and biology of planktic foraminifera in the world ocean.

7.2 Shell Morphology and Structure

7.2.1 Morphology-Based Classification

Planktic foraminiferal shells are tiny (around 1.0 mm) and are composed of multiple hemispherical calcareous chambers; morphological details can therefore be observed by both stereomicroscopy and SEM. The major species of extant planktic foraminifera show a range of morphologies (Figs. 7.1, 7.2, 7.3, and 7.4). On the basis of the morphological features of shell structures, planktic foraminifera are divided into three superfamilies within Globigerinida (Kucera 2007): Globigerinoidea, Globorotaloidea, and Heterohelicoidea (Fig. 7.5). The Globigerinoidea are characterized by a trochospiral coil and are spinose. The Globorotaloidea are trochospiral to streptospiral (with a twisted pivot axis of coiling) and non-spinose. The Heterohelicoidea are non-spinose, with microperforations on the surface, and display biserial and triserial coiling. Family-level taxonomy is still under discussion,

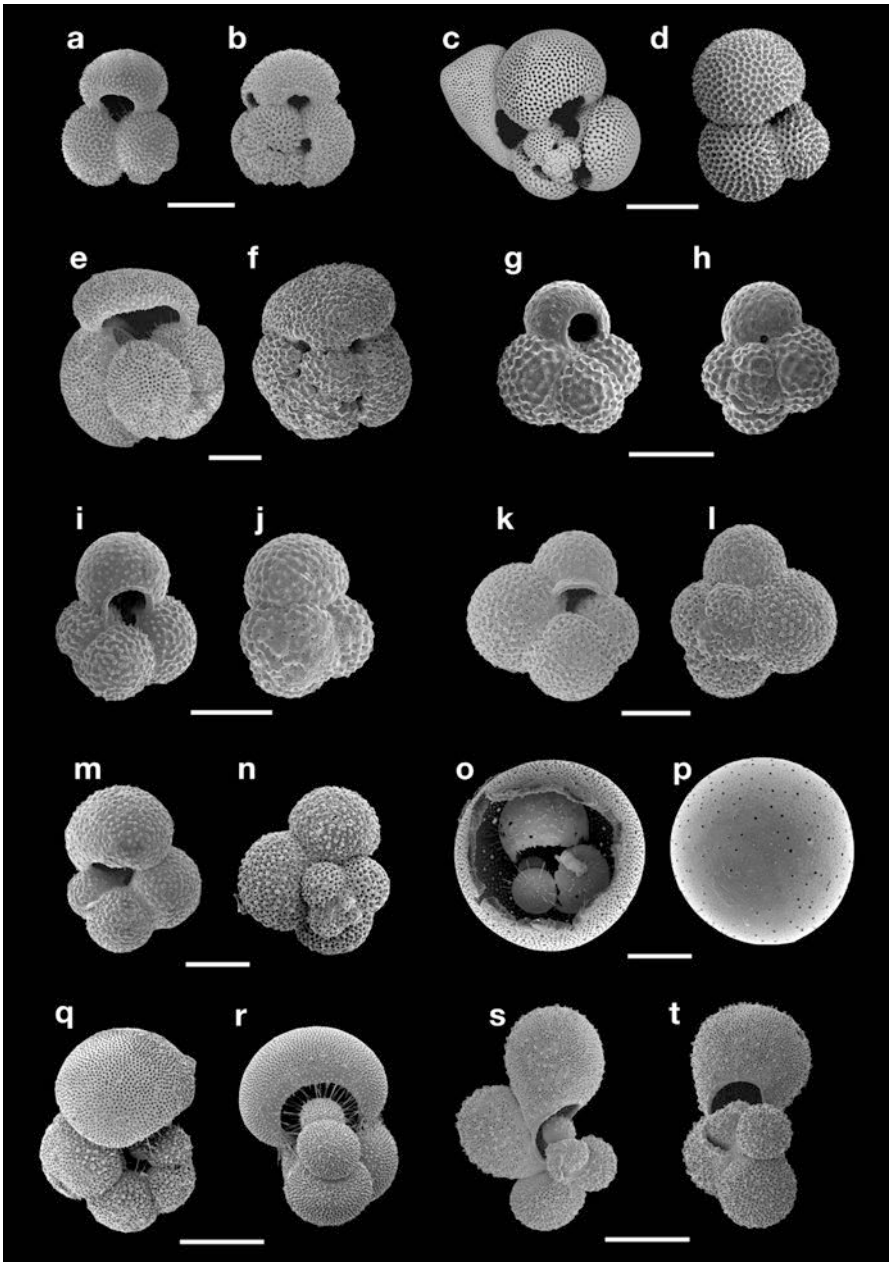

Fig. 7.1 Scanning electron microscopy (*SEM*) images of modern planktic foraminifera: (**a**, **b**) *Globigerinoides ruber*; (**c**, **d**) *Globigerinoides sacculifer*; (**e**, **f**) *Globigerinoides conglobatus*; (**g**, **h**) *Globigerinoides tenellus*; (**i**, **j**) *Globigerina rubescens*; (**k**, **l**) *Globigerina falconensis*; (**m**, **n**) *Globigerina bulloides*; (**o**, **p**) *Orbulina universa;* (**q**, **r**) *Globigerinella siphonifera*; (**s**, **t**) *Globigerinella calida* (scale bars: 100 μm for Fig. 7.1e–n, q; 200 μm for Fig. 7.1a–d, o–t)

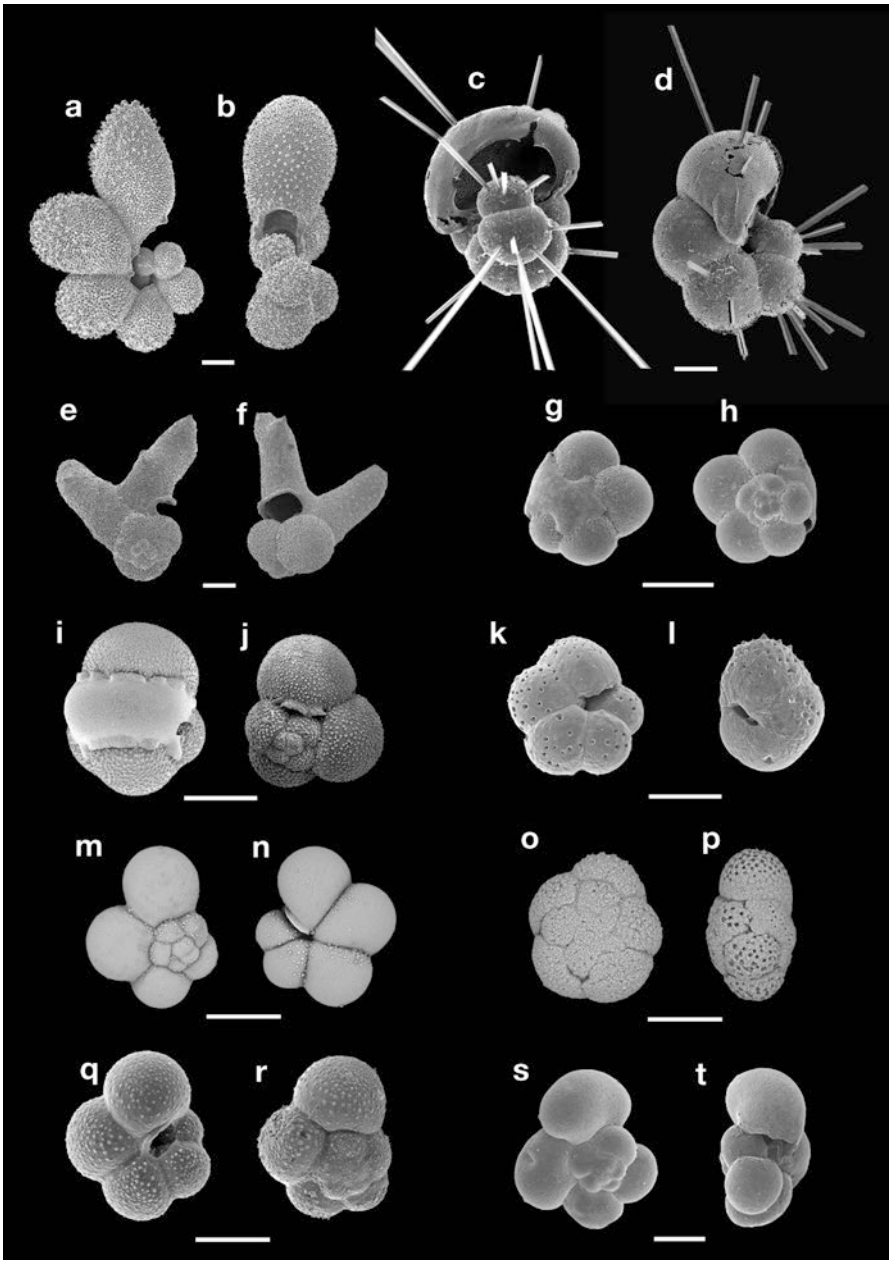

Fig. 7.2 (**a**, **b**) *Hastigerinella adamsi*; (**c**, **d**) *Hastigerina pelagica*; (**e**, **f**) *Hastigerinella digitata*; (**g**, **h**) *Globigerinita iota*; (**i**, **j**) *Globigerinita glutinata*; (**k**, **l**) *Turborotalita clarkei*; (**m**, **n**) *Tenuitella parkerae*; (**o**, **p**) *Turborotalita humilis*; (**q**, **r**) *Turborotalita quinqueloba*; (**s**, **t**) *Globorotalia anfracta* (scale bars: 50 µm for Fig. 7.2q–t; 100 µm for Fig. 7.2a–p)

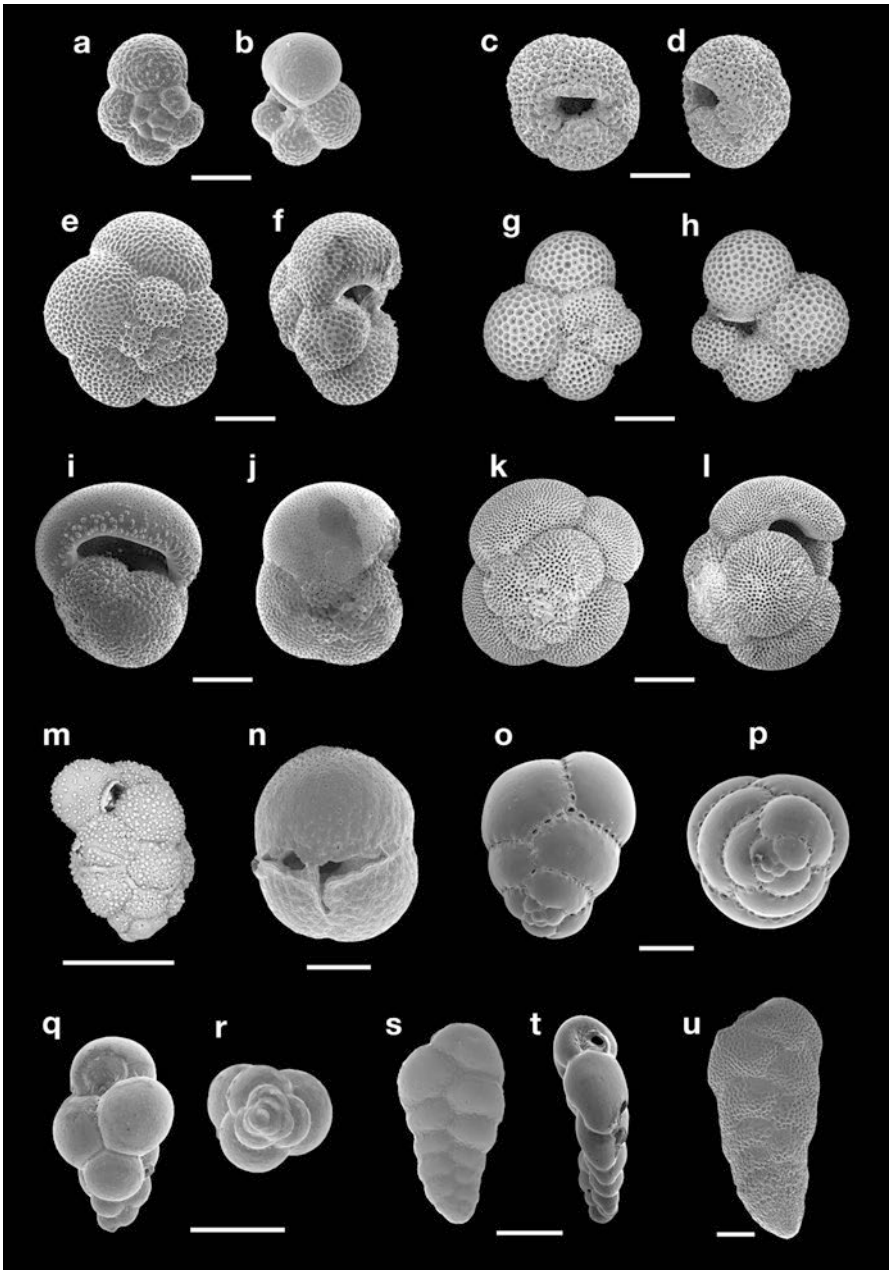

Fig. 7.3 (**a**, **b**) *Neogloboquadrina incompta*; (**c**, **d**) *Neogloboquadrina pachyderma*; (**e**, **f**) *Neogloboquadrina dutertrei*; (**g**, **h**) *Globorotaloides hexagonus*; (**i**, **j**) *Pulleniatina obliquiloculata*; (**k**, **l**) *Globoquadrina conglomerata*; (**m**) *Globigerinella uvula*; (**n**) *Sphaeroidinella dehiscens*; (**o**, **p**) *Candeina nitida*; (**q**, **r**) *Gallitellia vivans*; (**s**, **t**) *Streptochilus globulosus*; (**u**) *Streptochilus globigerus* (scale bars: 50 μm for Fig. 7.3q–u; 100 μm for Fig. 7.3a–j, m, o–p; 200 μm for Fig. 7.3k–l, n)

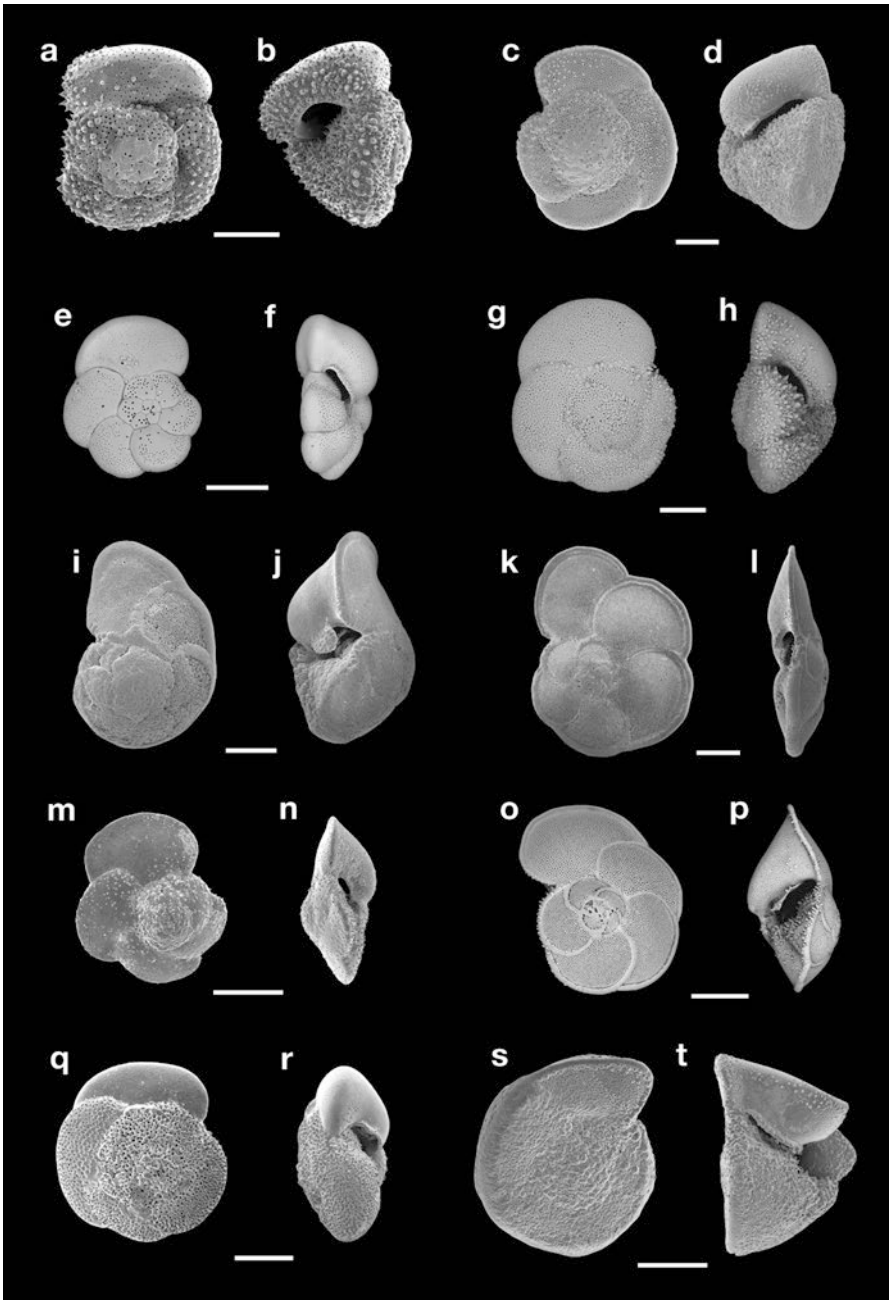

Fig. 7.4 (**a**, **b**) *Globorotalia inflata*; (**c**, **d**) *Globorotalia crassaformis*; (**e**, **f**) *Globorotalia bermudezi*; (**g**, **h**) *Globorotalia crassula*; (**i**, **j**) *Globorotalia tumida*; (**k**, **l**) *Globorotalia menardii*; (**m**, **n**) *Globorotalia hirsuta*; (**o**, **p**) *Globorotalia ungulata*; (**q**, **r**) *Globorotalia scitula*; (**s**, **t**) *Globorotalia truncatulinoides* (scale bars: 100 μm for Fig. 7.4a–h; 200 μm for Fig. 7.4i–t)

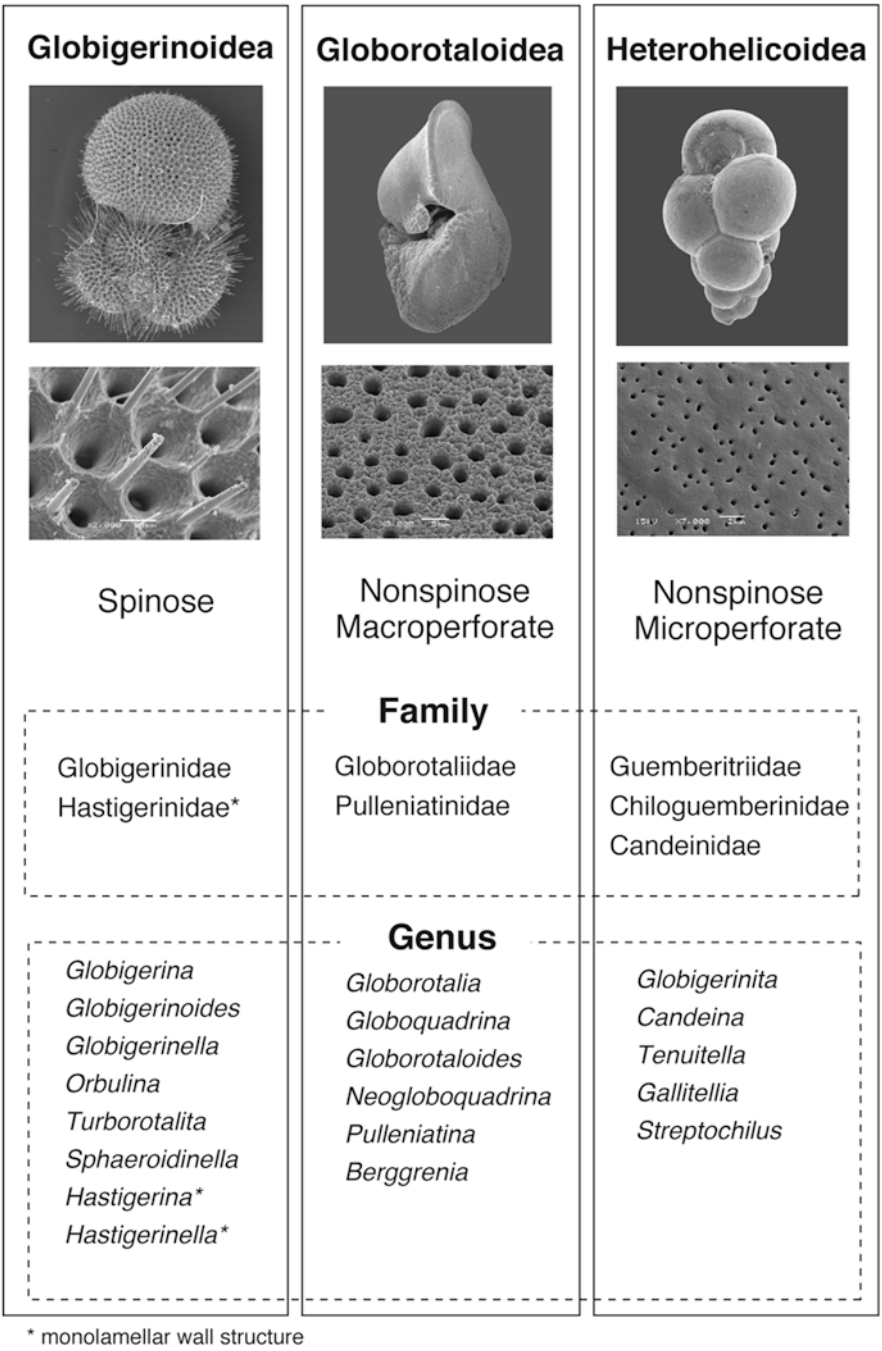

Fig. 7.5 Classification of modern planktic foraminifera, Globigerinida (modified from Kucera 2007). The *upper panels* show representative species of each superfamily and their surface structures. The seven families and 19 genera of modern planktic foraminifera are shown classified into each superfamily

and some families are located in different taxonomic categories on the basis of morphologic features. For example, Schiebel and Hemleben (2005) concluded that the Hastigerinidae, which form a monolamellar shell, could not belong to the existing Globigerinoidea because of their bilamellar wall structure. The establishment of systematics for the planktic foraminifera will require bilateral coordination between morphological taxonomy and molecular phylogeny (see also Schiebel and Hemleben 2005; Aurahs et al. 2009; Pawlowski 2009; Boudagher-Fadel 2012).

Species-level classification is based on more detailed shell features. The major features and terminology of modern planktic foraminiferal shells are shown in Fig. 7.6. The morphology of the outer shell, the surface and inside structure, the chamber arrangement, coiling direction, number, and shape, and the location of apertures are the keys to identifying each species. For more details about the genus- and species-level concepts, the reader is referred to the work of Parker (1962), Saito et al. (1981), Kennett and Srinivasan (1983), Loeblich and Tappan (1988), and Hemleben et al. (1989).

7.2.2 Shell Structure and Ontogeny

Foraminiferal shell growth during the juvenile stage is poorly understood, but chamber formation in mature foraminifera has been well documented for some planktic species under laboratory culture conditions (Hemleben et al. 1977; Bé et al. 1979, 1980; Spero 1988). Chamber formation progresses through several

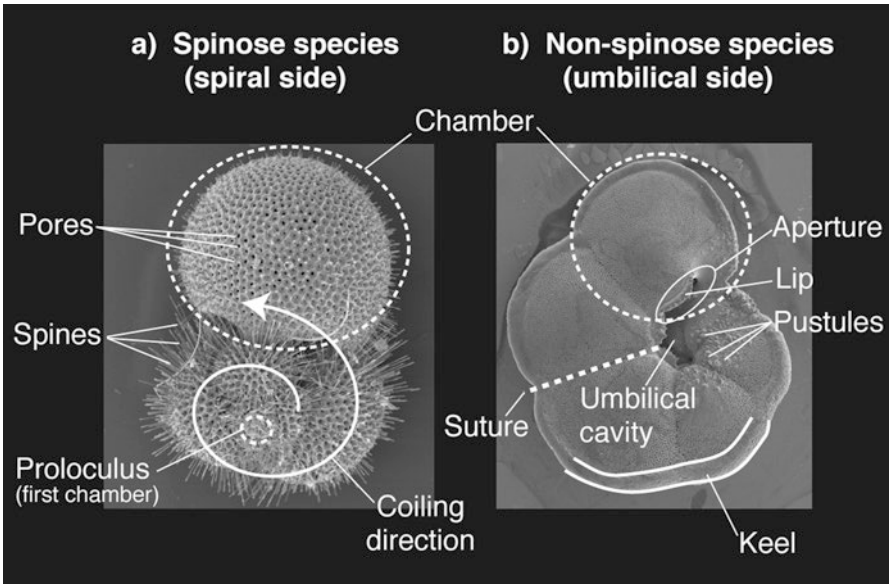

Fig. 7.6 Major morphological features and terminology of spinose and non-spinose planktic foraminifera: (**a**) *Globigerinoides sacculifer*; (**b**) *Globorotalia menardii*

steps (Fig. 7.7a–c): First, a thick rhizopodial tract expands outward from the aperture to make a cytoplasmic bulge. Several hours later, the outline of the bulge becomes a smooth border; this is the so-called "primary organic sheet" (POS: Erez 2003. Fig. 7.7d) and is a template (mold) for a new chamber. Next, calcification occurs on both sides of the POS. The primary calcite wall is built in about a half-day, but the calcification for thickening (the dense bilamellar wall) continues for a few days. Many pores with diameters from 2 to 7 μm can be observed on the shell surface (Fig. 7.7d). Pores are created by resorption of previously deposited calcite at fixed sites on the chamber wall. The pores play a role in metabolic exchange and excretion (Bé et al. 1980) and are used as a gateway for photosymbionts. For instance, when the spinose species *Globigerinoides sacculifer* is in the light, it draws out photosymbionts from inside its shell, passing them through the pores

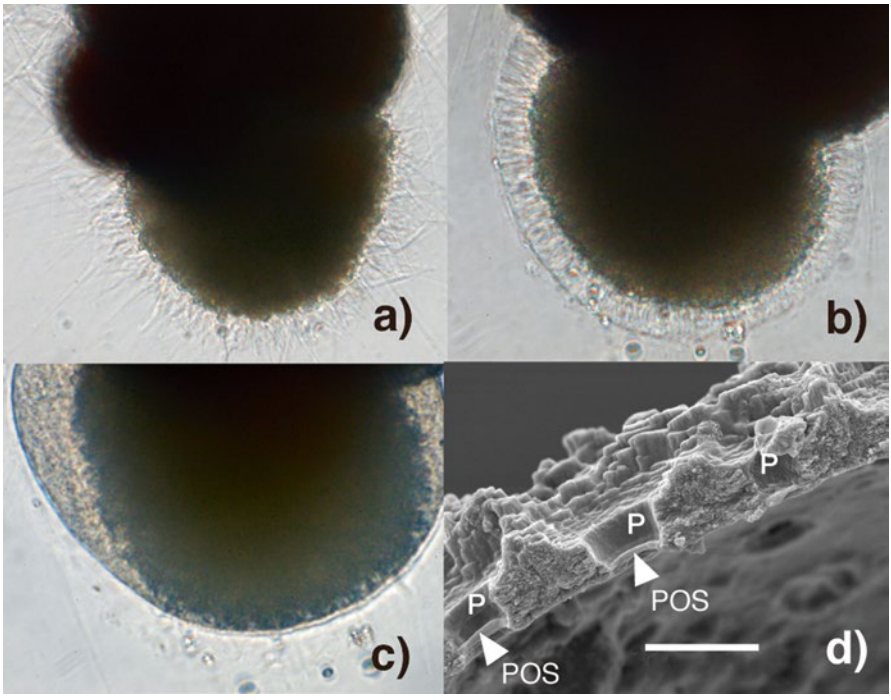

Fig. 7.7 Chamber formation process in non-spinose planktic foraminifera *Neogloboquadrina incompta*. (**a**) Rhizopodia expand outward from the aperture and cytoplasm to form a cytoplasmic bulge (the "anlage"). (**b**) The primary organic sheet (*POS*) has developed and calcification has started. The POS is an organic template for precipitation of calcium carbonate. (**c**) The outline of a newly formed chamber has developed and cytoplasm intrudes into the new chamber. Several hours are still needed to thicken the chamber by calcification. (**d**) Cross-sectional image of early stage of shell wall of *Globigerinoides sacculifer* by SEM. Plate-like crystals are forming at the shell surface. The POS is present at the lower part of the pores (*P*) (scale bar = 5 μm; photos of a–c are courtesy of H. Takagi)

and attaching them to the spines (see Sect. 7.4). Recent studies indicated that pore density and distribution might be the key to distinguishing cryptic species (see details in Sect. 7.6).

Although the function of the shell in planktic foraminifera is not fully understood, it is likely related to protection of the cell and to cell buoyancy. Surface-dwelling planktic foraminifera have spines made of calcium carbonate. Spines develop inside the stacked rhizopodia and precipitate within a few hours. The spines often reach lengths more than ten times the shell diameter. The main function of spines is considered to be buoyancy, increasing hydrodynamic resistance to seawater to maintain a suitable depth, much like a sea anchor. Before reproduction, some spinose species shed all spines (Hemleben et al. 1979) and secrete an additional carbonate crust over the outside of their shells (Caron et al. 1990). This additional carbonate precipitation is called gametogenic calcite. Planktic foraminifera sink to greater water depths for gamete release to avoid competition for survival and predation on gametes in the surface water (Hemleben et al. 1979); the planktic foraminifera make their shells heavier by secreting additional carbonate. In contrast, non-spinose and deep-dwelling species such as those in the genus *Globorotalia* have large, thick and heavy shells. This might help them to maintain their greater habitat depth.

7.3 Horizontal and Vertical Distributions

Planktic foraminifera have adapted to oceanic environments from the polar to the equatorial ocean; they are abundant in the open ocean but rare in coastal waters. Their horizontal distribution is regulated mainly by SST and surface productivity (Bé 1977). Horizontal distributions can be basically separated into five provinces in both the Northern and the Southern Hemisphere: tropical, subtropical, transitional, subpolar, and polar (Fig. 7.8). The highest species diversity of planktic foraminifera is in the tropical and subtropical regions (up to 30 species), gradually decreasing toward the polar regions. In addition, some species distinctly occur in coastal or equatorial upwelling regions. For example, the spinose *Globigerina bulloides* is well known as a nutrient-sensitive species. Although it is dominant in the subpolar regions, it frequently occurs around coastal and equatorial upwellings, and in seasonal upwelling conditions at low latitudes during the monsoon period (e.g., Prell and Curry 1981; Thunell and Sautter 1992; Conan and Brummer 2000).

Foraminiferal habitats include a wide vertical range of depths. Plankton tow studies indicate that most planktic foraminifera live in the euphotic zone during their juvenile stage and then descend to deeper water as adults. Bé (1977) summarized planktic foraminiferal habitat depths and distinguished three depth ranges: shallow water (approximately 0–50 m), intermediate water (50–100 m), and deep water (below 100 m). Because shallow and intermediate species are photosymbiotic bearing or facultative host species, they live in the euphotic zone to maintain their photosymbiotic algae. Almost all deep-water species are without photosymbionts, and some settle to depths of around 2,000 m (Schiebel and Hemleben 2005).

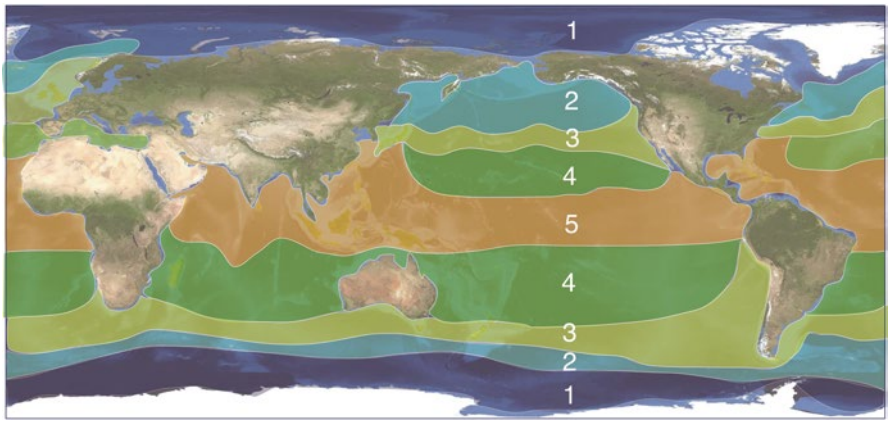

Fig. 7.8 Major faunal provinces of living planktic foraminifera (modified from Bé 1977): 1, Polar provinces; 2, subpolar provinces; 3, transitional provinces; 4, subtropical provinces; 5, tropical provinces. These provinces are horizontally distributed along latitude lines and are symmetrical between both hemispheres

In general, the relative abundance of living planktic foraminifera is high above the thermocline, and that of empty shells (dead specimens) increases exponentially below the thermocline.

Faunal responses and habitat depth are clearly linked to seasonal changes in water masses and food or nutrient availability (e.g., Berger and Soutar 1967; Fairbanks and Wiebe 1980; Thunell and Honjo 1981; Curry et al. 1983; Thunell et al. 1983; Thunell and Sautter 1992; Eguchi et al. 1999; King and Howard 2001; Kuroyanagi and Kawahata 2004). In general, phytoplankton and zooplankton biomass is concentrated in the deep chlorophyll maximum (DCM) located in the lower photic zone, and the DCM depth depends on the seasonal pycnocline (a distinct water density gradient related to temperature and salinity). Plankton tow observations show that planktic foraminiferal abundances follow chlorophyll distributions (Fairbanks and Wiebe 1980; Schiebel et al. 2001).

Little is known about any daily vertical migrations of planktic foraminifera, but the effects of such migrations must be rare or negligible. Instead, small-scale patchiness in the distributions of planktic foraminifera strongly influences their population dynamics. Such patchiness is possibly related to small-scale eddies and associated changes in water-mass structure (Hemleben and Spindler 1983).

7.4 Biology

7.4.1 Cytoplasm

Inside their shells, healthy planktic foraminifera are filled with cytoplasm; under the microscope they therefore appear to have orange to pale yellow shells (Fig. 7.9). These colors are generally characteristic of the cytoplasm but sometimes originate

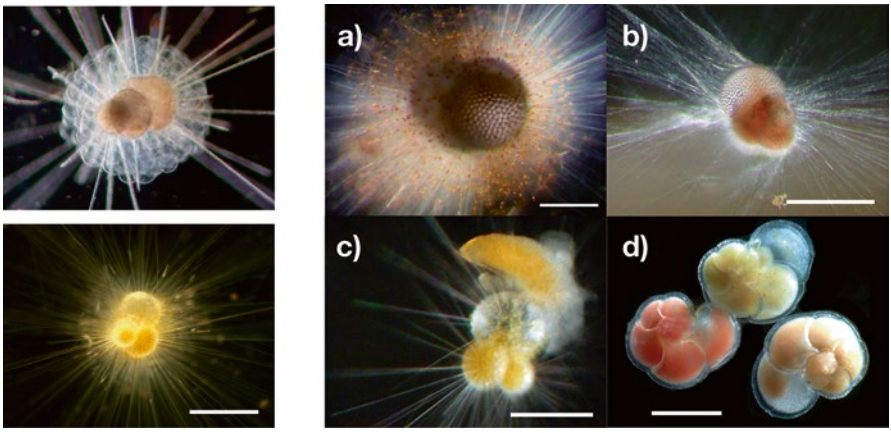

Fig. 7.9 Living planktic foraminifera. (**a**) *Globigerinoides sacculifer* with spines and photosymbiotic algae (dinoflagellate). (**b**) *Neogloboquadrina dutertrei* with rhizopodia. (**c**) *Globigerinoides ruber* (*lower*) with captured food (*Artemia* nauplii, *upper*). Artemia is bent because of the stickiness and pulling force of the rhizopodia. (**d**) Three cytoplasmic color types of *Globorotalia menardii* (scale bars = 500 μm for all)

from the food or the pigments of photosymbiotic algae incorporated within the cytoplasm. Unhealthy or weakened foraminifers have a reduced cytoplasm volume and an increased numbers of empty chambers.

Part of the cytoplasm protrudes outward as a web-like structure. This outward array is called the rhizopodia (pseudopodia: Fig. 7.9b). Rhizopodia move by cytoplasmic streaming. The shell of planktic foraminifera is enveloped by a cytoplasm–rhizopodial network; thus the carbonate shell does not come into direct contact with the surrounding seawater. Rhizopodial activity is closely related to trophic activity and metabolic exchange. Laboratory observations show that the rhizopodia of spinose foraminifera extend all around the shell when suspended in the water. The rhizopodia of healthy individuals are strongly adhesive, therefore planktic foraminifera can easily capture larger and motile zooplankton (e.g., copepods) or phytoplankton (Fig. 7.9c). Thus the rhizopodia act as a web to capture food. The rhizopodia penetrate the bodies of captured organisms and withdraw their tissues, then finally move the captured food into the cytoplasm. Rhizopodia are also used for the elimination of waste. Substances to be excreted are brought from inside the cell to the outside by rhizopodia, where the substances are discharged along with the cytoplasm that surrounds the waste materials.

7.4.2 Predation

The trophic activity of some planktic foraminifera has been studied in culture experiments and by observing food vacuoles with transmission electron microscopy (TEM) (Caron and Bé 1984; Hemleben et al. 1989). These studies show that spinose species are carnivorous and non-spinose species are omnivorous. Hemleben et al. (1989) listed the varieties of prey consumed by different species of planktic

foraminifera in culture. Spinose species prey on copepods, tintinnids, ciliates, tunicates, and polychaete larvae. In contrast, non-spinose species feed mainly on diatoms, dinoflagellates, eukaryotic algae, and thecate algae; unidentified animal tissues are also found (Hemleben et al. 1989). It is likely that non-spinose species prey not only on phytoplankton but also on sinking organic matter (marine snow) in the water column. Planktic foraminifera consume only biological materials that become attached to their rhizopodia; they always try to move this material into their cytoplasm. As a result, planktic foraminifera often cannibalize each other under culture conditions. Cannibalism has been observed in non-spinose species, but not in spinose species (Hemleben et al. 1989).

7.4.3 Symbiosis

It has been known since the late nineteenth century that some planktic foraminifera have photosymbiotic algae within their cells (endosymbionts) (e.g., Murray 1897). The beginning of endosymbiotic relationships between planktic foraminifera and algae is inferred to be the Late Cretaceous from the results of stable isotope records in fossil species (Houston and Huber 1998). The presence or absence of endosymbionts in planktic foraminifera follows no clear rule relative to their ecology or taxonomic category; therefore, morphologic and habitat variations are not reliable indicators of symbiosis. Within the extant species, those living in tropical to subtropical waters from the surface to the mixed layer have photosymbionts, and some thermocline species are facultative hosts. In contrast, deeper-living species and subpolar and polar species do not have photosymbionts (e.g., Hemleben and Spindler 1983; Hemleben et al. 1989; Gastrich 1987).

Physiological interactions between symbiont and host are poorly known, but some reports indicate that photosymbiotic activities are positively associated with planktic foraminiferal growth, calcification, survival time, and reproductive behavior (Caron et al. 1981; Bé et al. 1982). Likewise, symbionts may benefit by receiving a supply of nutrients and protection from the host organism (Caron 2000). Photosymbiotic algae are spread out around the shell by the rhizopodia in the light (the so-called symbiont "halo") but are withdrawn in the dark (Bé 1982). Such a circadian movement of photosymbionts would function to enable effective photosynthesis by the algal cells and transfer of organic substances from the symbionts to the host. For example, the photosynthetic rates of dinoflagellates within the symbiotic halo (about 1.0 mm from the shell) and inside the shell in *Globigerinoides sacculifer* are approximately 2.7 mmol O_2/l/min and 3.8 mmol O_2/l/min, respectively, under light conditions (photon flux of 400 μEinst/m^2/s) (Jorgensen et al. 1985). This equates to an oxygen level double that of ambient seawater, with an increase in pH to around 8.6. In contrast, the oxygen level at the shell surface drops by 50 % under dark conditions (Jorgensen et al. 1985). Similar results have been found in microelectrode experiments with another spinose species harboring photosymbionts, namely *Orbulina universa* (Rink et al. 1998).

The advantages of endosymbiosis were also confirmed experimentally in the laboratory. The lifespan and chamber growth rate were measured for the symbiont-bearing *G. sacculifer* under normal seawater conditions with light, and with added 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosynthesis inhibitor. The results suggested that both the lifespan and shell length were greater under natural conditions than with DCMU (Bé 1982). The beneficial role of photosynthesis by photosymbiotic algae can be explained as helping in the removal of metabolic CO_2 and elevation of the oxygen level, while increasing the local pH around the foraminifera and enhancing calcification, which occurs more easily under more alkaline conditions (e.g., Kohler-Rink and Kühl 2005).

Some algal groups of symbionts are known for extant foraminifera species—mainly dinoflagellates, chrysophytes (Anderson and Bé 1976a; Gastrich 1987; Spero 1987; Faber et al. 1988), chlorophytes and prymnesiophytes (Gast and Caron 2001). The relationship between host and symbiont is normally one-to-one; there are no reports of multiple species of symbionts in a single host species (Gast and Caron 2001). It is common, however, for a symbiont to have a relationship with multiple host species. For instance, the most common endosymbiont in spinose planktic foraminifera is *Pelagodinium béii* (dinoflagellate), and this species is observed in *Globigerinoides conglobatus, G. ruber*, *G. sacculifer*, and *O. universa*.

It is not known when planktic foraminifera capture symbionts from the environment, but it is likely in the early life stages because juvenile planktic foraminifera (2 or 3 chambers) already contain 3–5 symbiont cells (Hemleben et al. 1989). Symbionts increase in number through cell division during their symbiosis in the foraminiferal cell as the host matures. A large adult *O. universa* has approximately 2×10^4 cells inside the shell and is expected to fix carbon at 3.4×10^{-2} μM/h at the light-saturated photosynthetic rate (Spero and Parker 1985). Thus, planktic foraminifers endosymbiotic algae behave as primary producers in surface waters.

Most host foraminifera (e.g., *G. sacculifer*, *O. universa*, and *Globigerinella siphonifera*) have been observed to digest photosymbionts just before gamete formation (e.g., Bé et al. 1983). Planktic foraminifera in the pre-reproductive phase do not ingest any food by using their rhizopodia; therefore, digestion of photosymbionts might be a source of nutrients.

7.4.4 Reproduction and Life Cycles

Circumstantial evidence from plankton tow sampling and culture experiments suggests that some planktic foraminifera have life cycles with a lunar period. Spindler et al. (1979) showed that 85 % of 717 individuals of *Hastigerina pelagica* released gametes within the 5-day period between 3 and 7 days after the full moon. Erez et al. (1991) performed plankton tow observations and found that young individuals of spinose planktic foraminifera (roughly 200 μm along the main axis) appeared in surface water 7–8 days after the full moon and subsequently sank below the photic

zone as they matured. The life cycle of spinose planktic foraminifera is shown schematically in Fig. 7.10. Kawahata et al. (2002) confirmed these periodic cycles in *G. sacculifer* through the year from sediment trap samples collected in the western equatorial Pacific Ocean. These studies indicate that some planktic foraminifera have a lifespan of 1 month; however, the lifespan may depend on species, food availability, and water temperature. Experiments by the authors showed that the cold-water species *Neogloboquadrina pachyderma* lived a maximum of 200 days at water temperatures of 4 °C.

The reproductive process in planktic foraminifera is well documented (Bé and Anderson 1976; Hemleben et al. 1989). In general, planktic foraminifera create the next generation by sexual reproduction (gametogenesis), which is frequently observed under laboratory culture conditions. In the case of *O. universa*, the sexual reproductive process proceeds as follows (Fig. 7.11):

1. Approximately 24 h before reproduction, the host cells shed their spines and stop catching food. Rhizopodia lose their stickiness and become shortened. During this process, all photosymbionts (dinoflagellates) are digested by the host.
2. About 12 h before reproduction, the cytoplasm loses its color and becomes pale yellow to whitish. Production of gametes begins.
3. Six hours later, gametes can be observed wiggling inside the host cell.

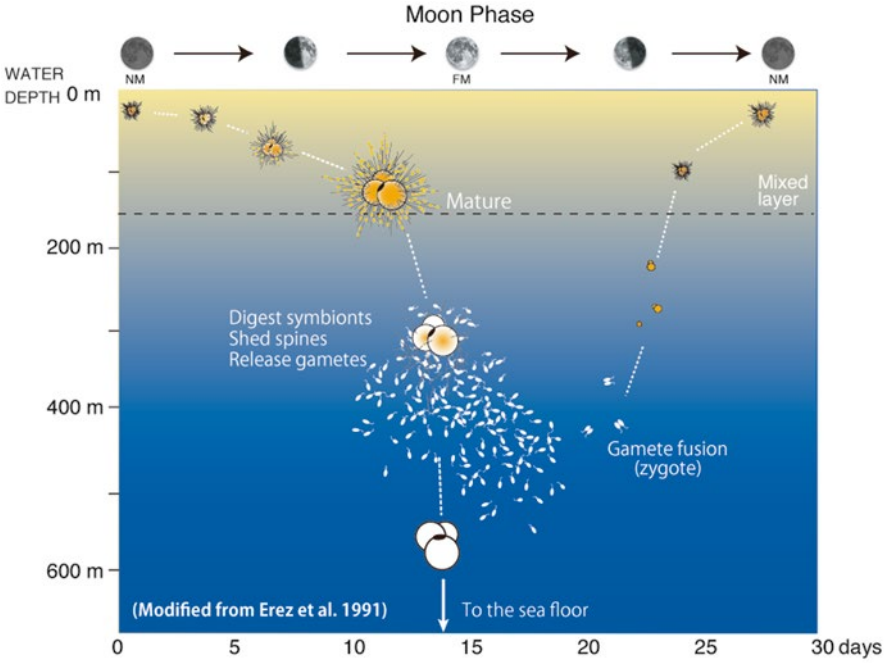

Fig. 7.10 Schematic of the lunar cycle of planktic foraminifera *Globigerinoides sacculifer* (modified from Erez et al. 1991). The lifespan of released gametes is several hours. Gamete fusion has not been observed under natural or laboratory conditions

4. Gamete release begins. Gametes are released gradually, swimming out from the host shell, although they are sometimes released explosively all at once. The released gametes are about 2–5 μm and have two flagella. Gametes swim actively and quickly disperse in the water. Five hours after the release of gametes, the cytoplasm of the host is partially or completely lost, leaving only the carbonate shell.

Some species of both spinose and non-spinose planktic foraminifera follow the reproductive process described above (e.g., Hemleben et al. 1989; Caron et al. 1990). However the reproductive process for many planktic species is still unknown and poorly understood. For example, the fusion of released gametes (syngamy) and consequent growth of planktic foraminifera are unconfirmed under laboratory conditions. There is one report of a gamete fusion-like phenomenon in *H. pelagica* under culture conditions, but the details of the process were not clearly described

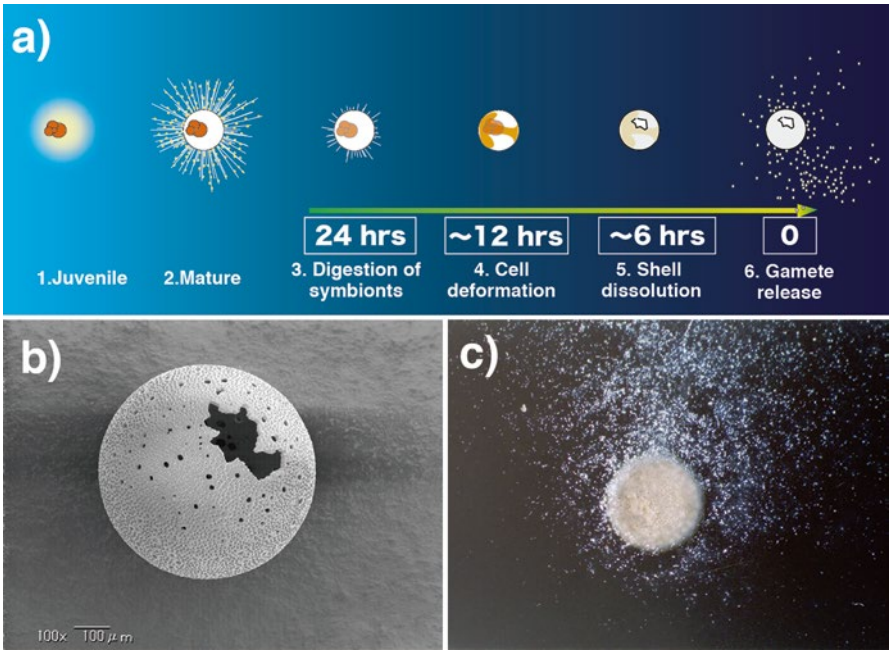

Fig. 7.11 (**a**) Schematic illustration of time-series changes in reproduction phases of *Orbulina universa*. (1) Juvenile specimen. Spherical final chamber is forming. (2) Mature specimen. Spherical chamber is complete and specimen actively preys on food. (3) 24 h before reproduction. Reproduction phase has started. Spines have shrunk and rhizopodia have lost stickiness. Digestion of symbionts has started. (4) 12 h before reproduction. Cell deformation has started; the cell does not maintain its original shape. The cell has expanded inside the spherical chamber and generation of gametes has started. (5) 6 h before gamete release. Gametes have been generating and fill the entire spherical chamber. Part of the spherical shell has dissolved. (6) Gamete release has started. Release of all gametes from the mother shell takes several hours. (**b**) Dissolved spherical chamber just after gamete release. (**c**) Release of gametes (gametogenesis). This image was taken 30 min after the start of gametogenesis. In this case, gametogenesis continued for about 4 h in total

(Ketten and Edmond 1979). Furthermore, it had been believed that planktic foraminifera reproduce only sexually (gamete release), but asexual reproduction also occurs, as in other protists. This has been reported for only one species, the cold-water *Neogloboquadrina pachyderma* (the right-coiled form; now renamed *N. incompta*), which reproduced asexually fairly infrequently under laboratory culture conditions. Kimoto and Tsuchiya (2006) counted 138 individual two-chamber (deuteroconch) gamonts released from the mother shell (Fig. 7.12). Only eight of the offspring matured in culture, and four of these released gametes. They reported that asexual reproduction by this species accounted for less than 1 % of its total reproductive activity.

Although the reproductive cycles of planktic foraminifera are still poorly understood, tentative life cycles for planktic foraminifera have been proposed (Fig. 7.13). Many observations indicate that the primary life cycle of planktic foraminifera includes sexual reproduction, with the gamont creating motile gametes. In very rare cases (probably fewer than 1 %) they pass through an asexually reproducing phase.

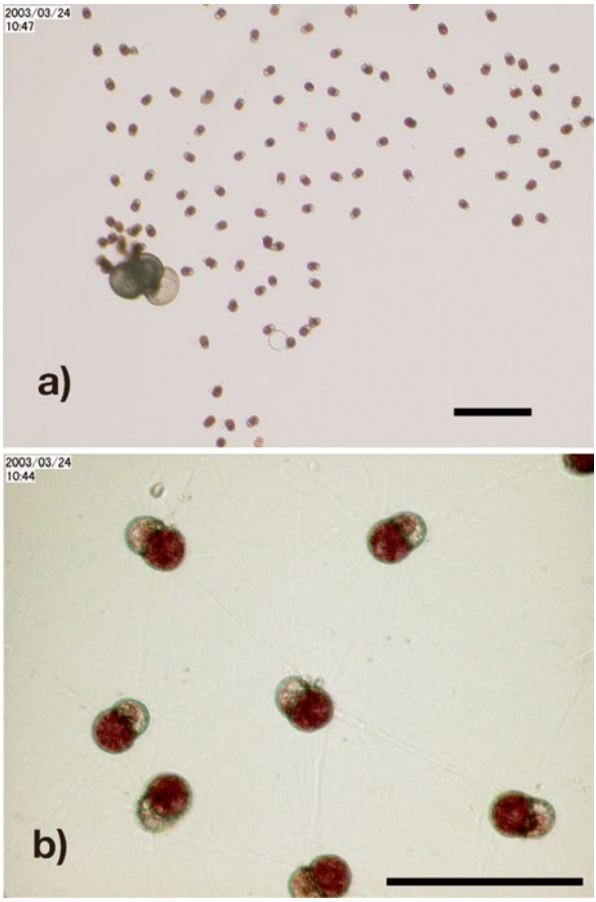

Fig. 7.12 The case of asexual reproduction of *Neogloboquadrina incompta*. (**a**) Image just after reproduction. 138 gamonts were observed under the microscope (scale bar = 200 μm). (**b**) Close view of gamonts (offspring). The rhizopodia stretched from the deuteroconch (2-chamber form) and its length was approximately four times that of the proloculus (scale bar = 100 μm)

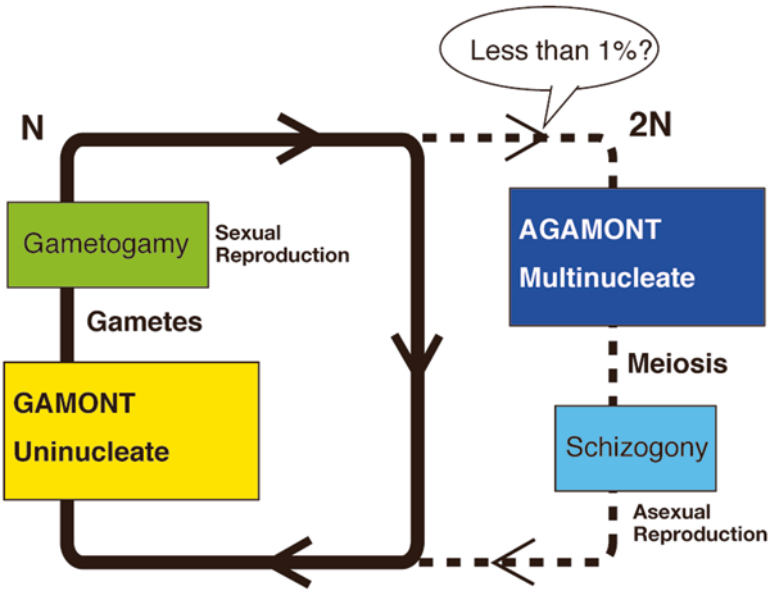

Fig. 7.13 Diagram of the life cycle of planktic foraminifera. The most common life cycle, represented by the black square, is the (gamont) haploid generation. The diploid generation (agamont) phase seems to be very rare: fewer than 1 % of captured specimens are in this phase

In the next generation, the agamont returns to the sexually reproducing phase. Additional research is needed to fully understand the reproductive process in planktic foraminifera.

7.5 Shell Geochemistry

7.5.1 Advances in Geochemistry and Paleoceanography

Over the last three decades there have been dramatic developments in geochemical techniques using the carbonate geochemistry incorporated in foraminiferal shells. In particular, progress in isotope ratio mass spectrometry (IR-MS), inductively coupled plasma mass spectrometry (ICP-MS), and related preprocessing techniques has produced a very large geochemistry dataset and advanced the understanding of the relationships between the carbonate chemistry of foraminiferal shells and environmental changes (e.g., Shackleton et al. 1983; Delaney et al. 1985; Boyle and Keigwin 1985; Nürnberg 1995; Rosenthal et al. 1997). These geochemical studies were performed as part of paleoceanographic reconstructions using fossil foraminifera, and they have resulted in extensive knowledge of the climatic history and environmental systems of the Earth since the Cretaceous.

The basic concepts behind using shell geochemistry for climatic reconstructions are as follows: (1) foraminifera live in their preferred water masses (or water depths); (2) the chemical composition of foraminiferal shells reflects the seawater chemistry at the time of shell formation; (3) using the relationships between seawater chemistry and chemical compositions of shells, (paleo) ocean chemistry can be reconstructed from fossil foraminiferal shell compositions. In other words, the accuracy of chemical proxies for paleoceanographic reconstruction depends on regression formulas for the relationships between the chemical proxies and environmental factors.

The interactions between chemical proxies and biological responses were investigated intensively by H. Spero, University of California Davis, and his colleagues. They integrated biological and geochemical techniques and achieved breakthroughs in foraminiferal biogeochemistry (e.g., Spero and Parker 1985; Spero et al. 1997; Bemis et al. 1998; Vetter et al. 2013). At the same time, there were studies of the biomineralization of planktic foraminifera and the chemical reactions in their cells, using various geochemical tracers, but many of these reactions are still enigmatic. The current understanding of foraminiferal biomineralization (see Erez 2003) suggests that materials for calcification are obtained from ambient seawater and brought to separate vacuoles by endocytosis. Calcium and carbonate are stored in such intracellular pools (the internal calcium pool) and modified to be used for precipitating the first calcium carbonate during the initial stage of calcification (granular calcite). In the calcium pool are concentrations much higher than that of actual Ca depositions. The materials for the second stage of carbonate precipitation (laminated calcite) are induced directly from the ambient seawater.

Recent cytological studies indicate that the pH in the cytoplasm of benthic foraminifera is locally elevated to above 9.0 (de Nooijer et al. 2009). This indicates that foraminifera can precipitate carbonate particles within their cells under controlled pH conditions. Bentov et al. (2009) reported a similar phenomenon in observations of cultured benthic foraminifera. In other words, foraminifera create places within the cytoplasm that are supersaturated with respect to calcium carbonate (alkaline conditions) and with an elevated CO_3^{2-} concentration, and then carbonate reactions occur in the cell. Biologically controlled processes in carbonate precipitation are complex and are not in accordance with thermodynamic reactions. Such chemical disequilibria are known as "vital effects" (Urey et al. 1951), and they strongly affect the geochemical record in shells. Therefore, such vital effects are an obstacle for paleoceanographic reconstruction using geochemical tracers in foraminiferal shells (e.g., stable isotopes and trace metals).

7.5.2 Oxygen and Carbon Isotopes

Stable oxygen and carbon isotope ratios are traditional and useful chemical tracers for clarifying foraminiferal biology, ecology, and the paleochemistry of the ocean. Oxygen and carbon isotopes in carbonate ($\delta^{18}O$ and $\delta^{13}C$) are represented by a relative comparison between a sample and reference material as follows:

$$\delta^{18}O = \left[\left(^{18}O/^{16}O\right)_{sample} - \left(^{18}O/^{16}O\right)_{ref}\right] \Big/ \left(^{18}O/^{16}O\right)_{ref} \times 1{,}000 \quad (7.1)$$

$$\delta^{13}C = \left[\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{ref}\right] \Big/ \left(^{13}C/^{12}C\right)_{ref} \times 1{,}000 \quad (7.2)$$

The relationship between temperature and the oxygen isotope ratio of carbonates precipitated from seawater under thermodynamic equilibrium conditions is represented by the following equation (Kim and O'Neil 1997):

$$T = 16.1 - 4.64(\delta c - \delta w) + 0.09(\delta c - \delta w)^2 \quad (7.3)$$

where T (°C) is the temperature during calcification and δc and δw (‰) are oxygen isotope ratios of calcite (shells) and ambient sea water (Pee Dee Belemnite [PDB] scale), respectively.

If foraminifera construct their shells at thermodynamic equilibrium between seawater and carbonate, the $\delta^{18}O$ of the carbonate depends strongly on the water temperature. However, some biological effects in foraminifera induce isotopic disequilibrium and increased disjunction from the original seawater $\delta^{18}O$. The ranges of $\delta^{18}O$ disequilibrium have been empirically determined by plankton tow observations (compiled by Niebler et al. 1999: Table 7.1) and culture experiments (e.g., Bemis et al. 1998). The disequilibrium of $\delta^{18}O$ values has a large variation resulting from several biological factors, including photosynthesis by symbionts (Spero and Williams 1989; Spero and Lea 1993), shell ontogeny (Spero and Lea 1996), and carbonate chemistry in the seawater (Spero et al. 1997). As Eq. 7.3 indicates, a difference of 1.0‰ in $\delta^{18}O$ equals a difference in estimated temperature of about 4 °C, and therefore foraminiferal isotopic disequilibrium cannot be ignored.

The carbon isotope composition of marine carbonates ($\delta^{13}C$) primarily reflects the carbon isotopes of total dissolved inorganic carbon (DIC) in the seawater (e.g., Urey 1947; Emiliani 1955); it therefore has the potential to yield information for reconstructing global carbon cycles in the ocean. The $\delta^{13}C$ in bulk shells of foraminifera may also reflect the DIC of seawater (Williams et al. 1977; Berger et al. 1978). Global carbon isotopic signals of foraminifera have been continuously reconstructed from the present to the Late Cretaceous (about 70 Ma) (e.g., Zachos et al. 2001). However, the carbon isotopes in individual planktic foraminiferal shells do not reflect thermodynamic equilibrium with ambient water. As with oxygen isotopes, individual and interspecific trends of $\delta^{13}C$ include environmental and physiological biases, mainly for the following reasons: (1) physiological changes due to differences in calcification rates (e.g., Spero and Parker 1985; Faber et al. 1988); (2) cellular controls on the composition of the intracellular fluid at the time of calcite precipitation (Erez 2003); (3) the carbonate chemistry of seawater (Spero et al. 1997); and (4) photosynthetic activity by symbionts (e.g., Spero and Williams 1989; Spero and Lea 1993; Birch et al. 2013). These multiple factors are not independent, which makes it difficult to distinguish $\delta^{13}C$ data from the shells of individual planktic foraminifera.

Table 7.1 Observed oxygen isotope disequilibrium for planktic foraminiferal species collected by plankton tow (Niebler et al. 1999)

Spinose species	Disequilibrium $\delta^{18}O$ (‰)	Size (μm)
Globigerinoides ruber	±0.0 to −1.0	200–400
Globigerinoides sacculifer	±0.0 to −0.6	>200
Globigerinoides conglobatus	<−0.3	>270
Orbulina universa	<−0.4	200–400
Globigerina bulloides	±0.0 to +0.5	200–400
Globigerinella calida	−0.2 to −0.6	>125
Globigerinella siphonifera	±0.0 to −0.4	120–500
Non-spinose species		
Neogloboquadrina dutertrei	±0.0 to −0.53	>350
Neogloboquadrina incompta &	−0.7 to −0.8	>125
Neogloboquadrina pachyderma		
Globorotalia menardii	−0.2	>200
Globorotalia inflata	−0.4 to +0.4	>200
Globorotalia tumida	±0.0	>200
Globorotalia hirsuta	−0.5 to +0.2	>200
Globorotalia scitula	<−0.4	>150
Globorotalia crassaformis	±0.0 to +0.2	250–500
Globorotalia truncatulinoides	−0.3 to +0.2	>250
Pulleniatina obliquiloculata	<−0.4	>250

Neogloboquadrina incompta and *N. pachyderma* are listed as one species by Niebler et al. (1999). At present, these species are distinguished genetically as different species

7.5.3 Trace Metals in Foraminiferal Shells

Trace metals in foraminiferal carbonate shells are important for understanding the biomineralization mechanisms of trace elements and reconstructing the paleoceanographic conditions of seawater. Trace elements dissolved in seawater are incorporated in foraminiferal shells at very low concentrations, ranging from 1×10^{-2} to 1×10^{-9} mol/mol-Ca (Lea 1999: Fig. 7.14a). Generally, trace metal concentrations are represented as a ratio with calcium (metal/Ca, mol/mol).

The theoretical basis for the incorporation of trace metals follows thermodynamics (Lea 1999). Trace metals are incorporated in a reaction wherein divalent metal ions (Me^{2+}) combine with carbonate ions (CO_3^{2-}), and calcium ions (Ca^{2+}) are released:

$$CaCO_3 + Me^{2+} \longleftrightarrow MeCO_3 + Ca^{2+} \tag{7.4}$$

where 'Me' represents a trace metal. The equilibrium constant K for the above reaction is represented by:

$$\left(aMeCO_3 / aCaCO_3\right)_{calcite} = K\left(aMe^{2+} / aCa^{2+}\right)_{seawater} \tag{7.5}$$

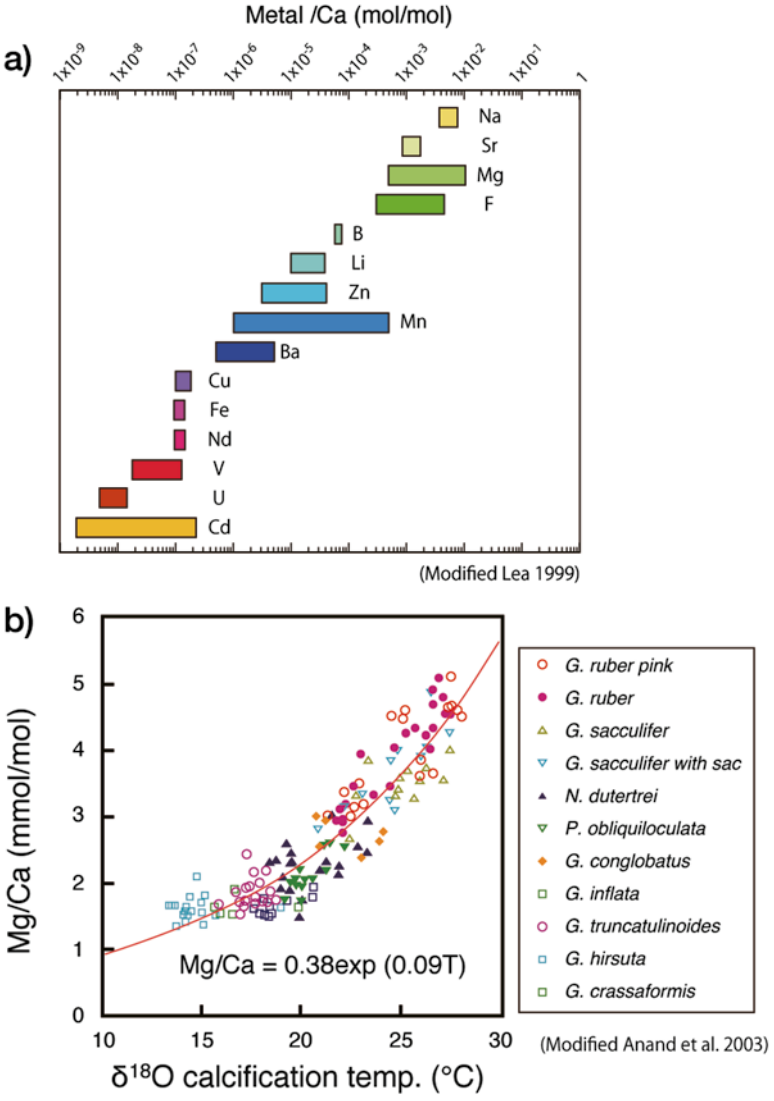

Fig. 7.14 (**a**) Concentrations of trace metals relative to that of calcium in foraminiferal shells (modified from Lea 1999). (**b**) Relationship between calcification temperature, as derived from the oxygen isotope record of shells, and Mg/Ca in planktic foraminifera (modified from Anand et al. 2003). Different symbols represent different species

where "a" indicates the activity of the solid and dissolved phases. This thermodynamic relationship can be simplified by the following empirical equation:

$$\left(\left[\mathrm{Me}\right]/\left[\mathrm{Ca}\right]\right)_{\mathrm{calcite}} = D \times \left(\left[\mathrm{Me}\right]/\left[\mathrm{Ca}\right]\right)_{\mathrm{seawater}} \tag{7.6}$$

where the square brackets indicate the concentration. The constant D is defined as the empirical distribution coefficient, and it reflects the degree to which the trace metals in seawater are concentrated into the foraminiferal calcite shells. The approximate residence time of Ca in seawater is over 1 million years (Broecker and Peng 1982); therefore, Ca concentrations in seawater, at least, should have been constant over the last million years. If the relationship between D and environmental parameters can be clarified, it could serve as a paleo-proxy for estimating the concentrations of trace metals in seawater and related climatic conditions in the past ocean.

Some trace elements in foraminiferal shells show a relationship with a specific oceanic environmental parameter (Table 7.2). For example, magnesium (Mg) in foraminiferal shells has a close relationship with the water temperature at the time and location of shell formation (Savin and Douglas 1973; Cronblad and Malmgren 1981; Nürnberg 1995; Nürnberg et al. 1996). The relationship between water temperature and Mg/Ca has been confirmed not only for living species under natural conditions but also under laboratory conditions (e.g., Delaney et al. 1985; Anand et al. 2003; von Langen et al. 2005). The residence time of Mg in the ocean is about 10 million years and its distribution is heterogeneous (Broecker and Peng 1982). The Mg/Ca ratio in seawater is almost constant at 5.17 (mol/mol) and Mg/Ca in foraminiferal shells ranges from 0.5 to 5 mmol/mol (Lea 1999). In other words, the D values of Mg (D_{Mg}) for foraminiferal shells are roughly 0.0001–0.001 and depend on seawater temperature. Mg/Ca of extant planktic foraminifera increases exponentially with increasing water temperature (T) (Fig. 7.14b). It is represented by the following equation:

$$\mathrm{Mg}/\mathrm{Ca} = b\exp\left(aT\right) \tag{7.7}$$

where a and b are the exponential constant and pre-exponential, respectively. Almost all modern planktic foraminifera show this exponential relationship (Lea et al. 1999; Anand et al. 2003). This universal relationship can be applied to fossil species of planktic foraminifera in deep-sea sediment cores from the world ocean basins, and paleo-SSTs have been precisely reconstructed (e.g., Lea et al. 2000; de Galidel-Thoron et al. 2005). Mg/Ca in foraminiferal shells is little affected by other climatic factors, and so Mg/Ca-based temperature is one of the more reliable chemical tracers of paleotemperature.

7.5.4 Sub-micron-Order Geochemical Analysis

The mechanisms of vital effects in foraminifera are extremely complicated because of multiple related factors. Clarification of such biological processes would greatly improve our understanding of biomineralization and paleoceanography. Beginning in the early twenty-first century, geochemical analysis of foraminiferal shells at the micron to submicron scale became practical with specialized instrumentation and techniques, namely the electron probe micro-analyzer (EPMA) (e.g., Eggins et al.

Table 7.2 Summary of typical stable isotopes and trace metals in shells of planktic and benthic foraminifera used as proxies for chemical and environmental parameters

	Element	Notation	Proxy	References
Stable Isotopes	Carbon	$\delta^{13}C$	Carbon isotope of DIC	Epstein et al. (1953)
				Shackleton et al. (1983)
				Spero and Lea (1993)
	Oxygen	$\delta^{18}O$	Temperature	Epstein et al. (1953)
				Bemis et al. (1998)
				Niebler et al. (1999)
	Boron	$\delta^{11}B$	pH	Hemming and Hanson (1992)
				Sanyal et al. (1995)
				Wolf-Gladrow et al. (1999)
				Henehan et al. (2013)
	Magnesium	$\delta^{26}Mg$	Seawater chemistry	Pogge von Strandmann (2008)
	Calcium	$\delta^{44}Ca$	Seawater temperature	Nagler et al. (2000)
				Heuser et al. (2005)
Trace metals	Lithium	Li/Ca	Seawater chemistry	Delaney et al. (1985)
				Hall and Chan (2004)
				Lear et al. (2010)
	Magnesium	Mg/Ca	Temperature	Delaney et al. (1985)
				Nürnberg (1995)
				Lea et al. (1999)
	Strontium	Sr/Ca	Seawater chemistry	Bender et al. (1975)
				Delaney et al. (1985)
				Rosenthal et al. (1997)
				Lea et al. (1999)
	Boron	B/Ca	Carbonate chemistry (pH dependence)	Yu and Elderfield (2007)
				Allen et al. (2012)
	Zinc	Zn/Ca	Seawater chemistry, water circulation	Marchitto et al. (2002, 2005)
	Cadmium	Cd/Ca	Seawater chemistry, P concentration	Boyle (1981)
				Boyle (1988)
				Mashiotta et al. (1997)
	Barium	Ba/Ca	Nutrients, alkalinity	Lea and Boyle (1991)
				Lea (1993)
				Hönisch et al. (2011)
	Vanadium	V/Ca	Seawater chemistry	Russell et al. (1994)
	Uranium	U/Ca	Seawater chemistry	Hastings et al. (1996)
				Russell et al. (2004)

DIC dissolved organic carbon

2003; Sadekov et al. 2005; Pena et al. 2008; Hathorne et al. 2009; Tachikawa et al. 2013), secondary ion mass spectrometry (SIMS) (e.g., Allison and Austin 2003; Kunioka et al. 2006; Weidel et al. 2007; Kozdon et al. 2009), and laser-ablation inductive coupled plasma mass spectrometry (LA-ICP-MS) (e.g., Eggins et al. 2003; Rathmann et al. 2004; Raitzsch et al. 2011). Recent trends in foraminiferal biogeochemistry have shifted to sub-micron-order geochemical analysis of the insides of shells and cells to determine the interactions between foraminiferal cells and their carbonate shells and the surrounding microenvironments.

Micron-scale chemical analysis indicates that the concentrations of trace elements within the shells have large variations. Mg/Ca, Mn/Ca, Ba/Ca, and Zn/Ca showed heterogeneous distributions in the shell and some elements showed a distinct banding distribution pattern. These studies clearly indicate that trace metals incorporated in foraminiferal shell walls are distributed with a high degree of heterogeneity (Eggins et al. 2003; Sadekov et al. 2005; Kunioka et al. 2006). Such banding of trace metals might be attributed to (1) temperature changes resulting from foraminiferal migration in the water column (e.g., Lohmann 1995); (2) carbonate chemistry modification resulting from photosymbiotic activity (Eggins et al. 2004); or (3) changes in the carbon source within the cell (Erez 2003). Larger intra-test variations in foraminiferal metal/Ca could not be interpreted with respect to natural environmental variations. For example, Hönisch et al. (2011) concluded that foraminiferal Ba/Ca is not affected by surrounding environments or symbiotic activity. Therefore, they are likely related to biological processes rather than ambient environmental changes (e.g., Hathorne et al. 2009).

Recently studies have reported a higher variability of $\delta^{18}O$ in foraminiferal calcite from different locations within the shell. Kozdon et al. (2009) investigated intra-test oxygen isotope variations in the cold-water species *Neogloboquadrina pachyderma* (sinistral coiling) by using SIMS. They found that, at the micron scale, oxygen isotopes showed very large variations (about 1.8‰) between ontogenic calcite (inner calcite layer) and crust (outer calcite layer). Furthermore, the $\delta^{18}O$ of ontogenic calcite and crust of this species showed that they were precipitated out of equilibrium, and they displayed negative and positive disequilibrium (vital effects), respectively. More recently, Vetter et al. (2013) investigated micron-scale shell-wall oxygen isotope and trace metal variations in cultured planktic foraminifera (*Orbulina universa*) by using SIMS with a 3-μm analytical focus and high precision (±0.6‰/single spot, 2 SD). They found immediate chemical responses between a trace metal (Ba/Ca) and $\delta^{18}O$ within 1–2 μm of calcite that had been precipitated during several hours. These chemical variations were synchronized, and the $\delta^{18}O$ values were consistent with those predicted. These studies show that the combination of both stable isotope and trace metal incorporation in foraminiferal shells at the micron scale can provide new insight into calcification over the course of a day; they also suggest the possibility of using foraminifera to resolve daily environmental changes.

The study of the submicron-scale intra-shell geochemistry of foraminifera and its relationship with surrounding environments is still in the early stages, but these new analytical techniques have great potential to unlock the “black box” of biogeochemical responses in carbonate chemistry.

7.6 Molecular Biology

7.6.1 Evolution

The use of molecular biological techniques has led to spectacular progress in the last two decades in the study of foraminifera. The availability of large subunit (LSU) and small subunit (SSU) ribosomal DNA (rDNA) sequences opened the way to understanding the phylogenetic relationships between the foraminifera and other protists. The development of techniques to amplify genomic fragments by polymerase chain reaction (PCR) allowed the detection of very small amounts of foraminiferal DNA. To date, partial sequences of the terminal 3′ end of SSU rDNA and internally transcribed spacer (ITS) rDNA regions from a single cell of foraminifera have been used to recognize distinct genotypes and phylogenetic relationships between benthic and planktic taxa and among different species (e.g., Pawlowski et al. 1994; Darling et al. 1996; de Vargas et al. 1999; Morard et al. 2009; Ujiié et al. 2010; Aurahs et al. 2011). At present, the foraminifera represent the most intensely studied group of non-cultured protozoa (Pawlowski and Holzmann 2002).

7.6.2 Origin of the Modern Planktic Foraminifera

Molecular biological analyses of foraminifera have also made a substantial impact on studies of the evolution of extant species. According to the foraminiferal phylogenetic tree based on molecular studies, all extant planktic foraminiferal species are in the clade of Rotaliida, a class of multilocular foraminifera (e.g., Pawlowski et al. 2003). The other extant foraminifera in Rotaliida are benthic species; therefore, planktic foraminifera seem to have diverged from the lineages of benthic foraminifera. This conclusion is supported by micropaleontological information that was established by using classical shell morphology.

On the other hand, molecular phylogenetic analysis has revealed that planktic foraminifera are not monophyletic in origin but instead evolved via multiple transitions within the clade of Rotaliida. Early studies of molecular phylogeny identified three apparent benthic ancestral lines for the major planktic foraminiferal species (spinose, non-spinose and microperforate) by using the SSU rDNA gene (e.g., Darling et al. 1997; de Vargas et al. 1997). Additionally, recent molecular phylogenetic studies of triserial and biserial planktic foraminifera belonging to Heterohelicoidea have revealed a lineage distinct from the three major lineages. Ujiié et al. (2008) found that the triserial planktic species *Gallitellia vivans* is phylogenetically very close to triserial benthic foraminiferal species. They concluded that the origin of *G. vivans* is completely different from those of known planktic foraminiferal lineages and that this species evolved from the similarly formed benthic foraminifera *Stainforthia* and *Virgulinella*. Furthermore, Darling et al. (2009) analyzed the molecular phylogeny of the biserial planktic foraminifer *Streptochilus globigerus* and concluded that it was the same species as the biserial benthic foraminifer *Bolivina variabilis*.

Geochemical evidence of shells suggests that the abovementioned triserial and biserial species have a planktic lifestyle throughout their lives (Kimoto et al. 2009; Darling et al. 2009). These studies present intriguing results that help in understanding the processes of evolution in foraminifera from benthic to planktic, as well as their morphologic relationships over geological history. Hence, so far, five polyphyletic origins have been identified in total for extant planktic foraminifera.

7.6.3 Genetic Diversity

Recent molecular phylogenetic analysis using ITS regions and partial alignment of SSU rDNA of planktic foraminifera has discovered different species within what were previously single species as identified morphologically (morphotypes). These hidden species are so-called "cryptic species". Data in the rDNA molecular phylogenetic tree indicate that a distinct clade has no gene flow among lineages; therefore, cryptic species within one morphotype are regarded as different species. During the last two decades, many genotypes have been identified within morphologically identical species, and genetically defined cryptic diversification is a hot topic in the molecular phylogeny of planktic foraminifera. A review by Darling and Wade (2008) and recent articles up to 2013 indicate that there are more than 60 cryptic species of extant planktic foraminifera; the total number of planktic foraminiferal species is at least 100 (Table 7.3) and continues to increase. In other words, more genotypes exist in the planktic foraminiferal population than had been expected on the basis of morphological features.

7.6.4 Geographic and Vertical Distribution of Genetic Diversity

Molecular phylogenetic studies provide important insights about concepts such as planktic foraminiferal divergence, evolution, and interspecies gene flow. Plankton tow studies of large-geographical-scale distributions of planktic foraminiferal genotypes suggest that cryptic species are more segregated in the surface ocean than are morphotypes of each species (e.g., de Vargas et al. 1999, 2001, 2002; Darling et al. 2000, 2004, 2007). It was necessary to investigate the relationship between water-mass hydrography and cryptic species distribution patterns in order to clarify the reasons for the segregation of genotypes. The results showed that the distribution and abundance of planktic foraminifera are determined not only by hydrographic factors such as patterns of ocean currents, temperature, and salinity (Darling et al. 2004, 2007) but also by ecological factors such as chlorophyll concentrations (de Vargas et al. 1999; Morard et al. 2009) and competition among sibling species with similar ecological demands (Aurahs et al. 2009). Recently, Seears et al. (2012) suggested that the relationship between sea surface primary productivity and symbiotic associations of planktic foraminifera is the main factor driving segregation.

Table 7.3 Summary of the general ecology of living planktic foraminifera

Spinose species	Habitat depth	Provinces	Photosymbionts	Nutrition	Number of genotypes[a]
Globigerinoides ruber	Mixed layer <50 m	Tropical–subtropical	Dino	Carnivorous	7
Globigerinoides ruber (pink)	Mixed layer < 50 m	Tropical–subtropical	Dino	Carnivorous	1
Globigerinoides sacculifer	Mixed layer <50 m	Tropical–subtropical	Dino	Carnivorous	1
Globigerinoides conglobatus	Mixed layer <50 m	Tropical–subtropical	Dino	Carnivorous	2
Orbulina universa	Mixed layer <100 m	Tropical–subtropical	Dino	Carnivorous	3
Globigerina falconensis	Mixed layer <100 m	Subtropical	Barren	–	1
Globigerina bulloides	Mixed layer <100 m	Subtropical–subpolar	Barren	Carnivorous	11
Globigerina rubescens	Mixed layer <50 m	Tropical–subtropical	Dino?	–	1
Globigerinella siphonifera	Mixed layer <100 m	Tropical–subtropical	Chryso/prym	Carnivorous	13
Turborotalita quinqueloba	Mixed layer <50 m	Transitional–subpolar	Barren	–	6
Hastigerina pelagica	Mixed layer <100 m	Tropical–subtropical	Barren	Carnivorous	3
Hasrigerinella digitata	Deep >100 m	Tropical	–	Carnivorous	1
Sphaeroidinella dehiscens	Deep >100 m	Tropical	–	–	1
Non-spinose species					
Pulleniatina obliquiloculata	Mixed layer <100 m	Tropical–subtropical	Chryso/facultative	Herbivorous	3
Globorotaloides hexagonus	Deep >100 m	Tropical–transition	–	–	–
Neogloboquadrina dutertrei	Mixed layer <100 m	Tropical–subtropical	Chryso/facultative	Omnivorous	3
Neogloboquadrina incompta	Mixed layer <100 m	Transition–subpolar	Barren	Herbivorous	2
Neogloboquadrina pachyderma	Mixed layer <100 m	Subpolar/polar	Barren	Herbivorous	7
Globorotalia truncatulinoides	Deep >100 m	Subtropical	Barren	Omnivorous	5
Globorotalia crassaformis	Deep >100 m	Tropical/subtropical	–	–	1
Globorotalia menardii	Deep >100 m	Tropical/subtropical	Chryso/facultative	Omnivorous	1
Globorotalia inflata	Deep >100 m	Transitional/subpolar	Chryso/facultative	Omnivorous	2

(continued)

Table 7.3 (continued)

Spinose species	Habitat depth	Provinces	Photosymbionts	Nutrition	Number of genotypes[a]
Globorotalia tumida	Deep >100 m	Topical/subtropical	Barren	–	1
Globoritalia hirsuta	Deep >100 m	Transition	Chryso	–	1
Globorotalia scitula	Deep >100 m	Tropical–subpolar	Barren	–	1
Globorotalia ungulata	Mixed layer <100 m	Tropical–subtropical	–	–	1
Microperforation					
Candeina nitida	Mixed layer <100 m	Tropical/subtropical	–	–	1
Globigerinita glutinata	Mixed layer <100 m	Tropical–subpolar	Chryso/facultative	Herbivorous	1
Globigerinita uvula	Mixed layer <100 m	Transition–subpolar	–	–	1
Gallitellia vivans	Mixed layer <100 m	Coastal	Barren	Herbivorous	1
Streptochilus globulosus	Mixed layer <100 m	Tropical/subtropical	Barren	Herbivorous	–
Streptochilus globigerus	Mixed layer <100 m	Tropical/subtropical	–		1

Ecology is based on the following studies: Bé (1977), Hemleben et al. (1989), Schiebel and Hemleben (2005), Kuroyanagi et al. (2008), Darling et al. (2009), Kimoto et al. (2009), Hull et al. (2011), Birch et al. (2013), and unpublished data of author

Designation: *dino* Dinoflagellates, *chryso* Chrysophytes, *prym* Prymnesiophytes

[a]Based on SSU rDNA data from Darling et al. (2006), Darling and Wade (2008), Aurahs et al. (2009), Seears et al. (2012), Weiner et al. (2012, 2014), and André et al. (2014)

On the other hand, there are reports of vertical niche partitioning of genotypes (depth-parapatric speciation) in the world ocean; this partitioning is likely related to water column structure. Ujiié et al. (2012) investigated the biogeographic and phylogenetic distributions of three genotypes of *Pulleniatina obliquiloculata* in the Indo-Pacific Warm Pool (IPWP). They found longitudinal and vertical gradients in the frequencies of each *P. obliquiloculata* genotype inhabiting the IPWP, and they suggested that passive transportation by ocean currents was not sufficient to account for the genotype distributions. Weiner et al. (2012) found three genetic types of *Hastigerina pelagica* without morphological distinction but with discriminable vertical habitats above 700-m water depths in the Atlantic, Mediterranean, and Pacific oceans. They inferred that vertical speciation of *H. pelagica* was the result of adaptation through competition in different environmental conditions. These results strongly suggest that cryptic species in planktic foraminifera have different ecologies or habitats, and that horizontal or vertical niche partitioning could lead to species segregation.

7.6.5 Reassessment of Shell Morphology

Individual foraminiferal specimens can be precisely analyzed for their morphologic shell features and their genetics. Huber et al. (1997) used molecular phylogenetic analysis to show that *Globigerinella siphonifera* Type I and Type II shared a cryptic relationship. They also determined that the cryptic species could be distinguished by the density of pores on their shell surfaces: the density in Type I was 10 % higher than that in Type II. De Vargas et al. (1999) and Morard et al. (2009) suggested that three cryptic species of *Orbulina universa* can also be identified by the size and density of perforations on the shell surface. Aurahs et al. (2011) succeeded in separating right cryptic species of *Globigerinoides ruber* by performing a statistical analysis of shell morphometry (chamber length); they proposed that the traditional subspecies name *Globigerinoides elongatus* should be reinstated for the genetic Type IIa. Quillévéré et al. (2013) proposed using the statistical analysis to detect different genetic types (Types I to V) of *Globorotalia* (*Truncorotalia*) *truncatulinoides*. These studies suggested that it could be possible to distinguish other cryptic species on the basis of morphometric analysis, and this encouraged the application of morphometric analysis to fossil assemblages.

At the same time, however, molecular phylogenetic analysis has revealed cases of taxonomic overinterpretation. *Globigerinoides sacculifer*, a tropical species, has various morphologic types in the world ocean. André et al. (2013) carried out a world survey of two ribosomal DNA regions (SSU and ITS-1) in different morphotypes of *G. sacculifer*, including *G. trilobus*, *G. immaturus*, *G. quadrilobatus*, and typical *G. sacculifer* itself. Each morphospecies is distinguishable by its chamber arrangement, growth ratio of chambers, and shape of the last chamber. Surprisingly, however, *G. sacculifer* has only one genotype, and there is no relationship between the morphologic divergence and genetics (André et al. 2013). From the viewpoint of molecular phylogeny, this suggests a delimitation of classification based on tradi-

tional morphology. At the same time, an understanding of the relationships between molecular phylogeny and morphology is important for understanding morphologic diversification and cryptic diversity in planktic foraminifera.

The different coiling directions (sinistral and dextral) of the planktic foraminiferal shell is the most remarkable and decisive feature of their morphology. Planktic foraminifera, except for the biserial coiling species, have both coiling forms, and some species show repeated switches in coiling direction at geological timescales (Bandy 1960; Bolli 1971; Saito 1976; Brummer and Kroon 1988; Xu et al. 1995). As one example, the change in coiling direction of the extant cold-water species *Neogloboquadrina pachyderma* was widely believed to be controlled by water temperature: the dextral and sinistral coiled morphotypes predominated in warmer and colder water, respectively, with a transition temperature of about 7.2 °C (Ericson 1959). Specifically, it was believed that the coiling direction of *N. pachyderma* would change from dextral to sinistral at around 7.2 °C, and vice versa. This idea dominated the micropaleontological community for a long time and was used to reconstruct paleoceanographic history. Recent molecular analysis, however, indicates that the sinistral and dextral coiled *N. pachyderma* are genetically distinct and each coiling form is a separate species (Bauch et al. 2003; Darling et al. 2004, 2006). Molecular and morphological analyses of *N. pachyderma* indicate that opposite-coiled types occur in nature at a rate of about 3 % and should be referred to as "aberrant coiling" (Darling et al. 2006). Darling et al. (2006) concluded that *N. pachyderma* (sinistral coiled) should be renamed "*N. incompta*," which was originally described as a right-coiling form in 1961 (Cifelli 1961). On the other hand, coiling dimorphism is found in a single genetic type of a planktic foraminifer *G. truncatulinoides* (Ujiié and Asami 2013). They showed that coiling direction does not phenotypically change depending on water temperature. Left–right asymmetry of planktic foraminifera likely has a genetic basis. Analysis of fossil assemblages of planktic foraminifera indicates that coiling direction is a heritable rather than an environmentally controlled trait (Brummer and Kroon 1988; Norris and Nishi 2001). The meaning and mechanism of coiling direction changes are not well understood and still present open questions, but coiling direction is likely controlled by genetics.

In conclusion, molecular biology based on DNA has become a major tool for all ecological, biological, and evolutional studies of foraminifera. Molecular phylogenic analyses have revealed that morphologic classification of the planktic foraminifera resulted in underestimation of their biodiversity. Cryptic speciation of planktic foraminifera appears to be a common phenomenon in nature. However, it is still unclear why speciation occurs within a given water mass. Temperature, salinity, and water-mass boundaries have the potential to provide physical separation for speciation. These parameters may affect faunal components, abundance, and diversity. Geographic isolation at geological timescales also becomes a physical boundary. However, the geographic and vertical distributions of cryptic species are not exactly consistent with water-mass or geographic boundaries. Ecological factors could have a strong effect on the generation of cryptic diversity in water masses.

Recognition of cryptic species and their morphologic relationships is important for understanding the relationship between environmental factors and species diver-

sity and for improving climate proxies and paleoclimate reconstruction. However, these datasets are still incomplete and further investigations are needed.

7.7 Collection and Cultivation

Culture experiments with planktic foraminifera in the laboratory are important for understanding their biology, ecology, and relationships with surrounding environments. However, planktic foraminifera are generally found in the open ocean, so it is necessary to use a boat or ship to collect living specimens.

As described in an early work by Anderson et al. (1979), the use of SCUBA gear may be the best method for collecting planktic foraminifera because it can eliminate physical damage to the live organisms during sampling operations (SCOR/IGBP Working Group 138 2011: Collection by SCUBA divers is documented in a short movie by the Scientific Committee on Oceanic Research/International Geosphere–Biosphere Programme [SCOR/IGBP] Working Group 138 [SCOR/IGBP Working Group 138 2011].) On the other hand, traditional collection by plankton tow is also a good method because it is simple and low cost and enables the collection of many living species safely and reliably. This section describes the essence of collecting operations by plankton tow, with some tips for maintenance of cultures.

7.7.1 Collection of Living Specimens

When a small boat is used to collect planktic foraminifera, the following items are needed: (1) plankton net (45, 63 and 100 μm mesh are generally used) with sample bottles; (2) rope; (3) portable cooler with cool-gels or ice-packs; (4) bucket; (5) water containers to collect culture water (e.g., plastic carboys); (6) temperature sensor or portable conductivity–temperature–depth sensor package (CTD); and (7) global-positioning system (GPS) logger. For the plankton net, a large-volume sample bottle (e.g., 2,000 ml) is recommended for the cod end (i.e., the terminus of the net) to avoid any stress to specimens during sampling operations.

Fresh samples are best collected far from shore—at least several kilometers. A small plankton net (mouth opening diameter, 45 cm) is useful for easy operation on a small boat. The plankton net is gently deployed from the boat and allowed to drift (Fig. 7.15a). Deployment of the plankton net to depths of 5–10 m is sufficient to collect spinose (surface water) species. The rope leading from the net is fixed tightly to the handrail on the boat. Shorter collection times are recommended (5 min maximum) to avoid the collection of excessive amounts of plankton.

If a winch is available, vertical towing from greater depths is possible. In this case, the winch retrieval speed should be less than 1 m/s. Sampling locations should be recorded by the GPS logger. To determine the culture conditions for the onshore laboratory, in situ temperature and salinity (or conductivity) should be measured by

using a CTD equipped a thermistor and conductivity cell. Recently, smaller CTD sensors have become available, which can be attached to the frame of the plankton net. After sampling, data from the CTD are downloaded from its memory.

The sample bottles containing the collected samples are removed from the plankton net, filled with fresh seawater, and immediately placed in the cooler with cool-gel. The cod end of the plankton net should be modified to accept screw-cap-type sample bottles for easy exchange operations on the ship. Sample bottles should be kept in the cooler with cold packs and returned to the laboratory as soon as possible.

7.7.2 Preparation in the Laboratory

The onshore laboratory should be prepared ahead of time for sample separation. All glassware and plastic-ware that will be used in culture experiments should be heat sterilized or steam sterilized to reduce contamination by bacteria. Upon their arrival on shore, samples should be transported to the laboratory immediately and kept cool until the beginning of sample preparation. Just after collection, planktic foraminifera sink and stay on the bottom of the sample bottles. For the effective collection of planktic foraminifera, sample bottles should be allowed to rest for about 10 min to separate foraminifera from other zooplankton. The supernatant seawater toward the top of sample bottle will include copepods and other motile "swimmers;" therefore, the supernatant should be decanted carefully into another large glass container and only the remnant seawater with settled contents used for studying living planktic foraminifera. The key to successful culture experiments is finding and using healthy individuals. Healthy individuals have dark red to orange cytoplasm that fills all chambers. Spinose species sometimes shed their spines during sampling operations. Healthy specimens will regrow their spines within several hours in culture.

Quick separation from other biologic materials is the most important consideration for keeping live planktic foraminifera for a long time in culture. To separate the planktic foraminifera, the remnant seawater and settled material in the sample bottle are poured into a medium-sized plastic bowl (a polypropylene bowl with a diameter of about 20 cm is useful). If the bowl is gently swirled, the planktic foraminifers (with other carbonate particles) will collect at the center of the bowl. Living planktic foraminifera are picked from this aggregation by using a sterilized Pasteur pipette under a light stereomicroscope. Individual specimens are transported one at a time to sufficiently large petri dishes containing seawater that has been filtered through a 0.22-μm membrane filter.

Healthy spinose and non-spinose planktic foraminifera frequently use their rhizopodia to adhere tightly to the surface of the glass dishes and the inside of the pipette, making it difficult to transport specimens to culture vessels. This is difficult to avoid, but quick transfer operations can relieve this problem. For example, an adhering planktic foraminifer can be washed gently by a stream of water from a pipette, and then quickly transferred using a Pasteur pipette when it becomes free in the seawater.

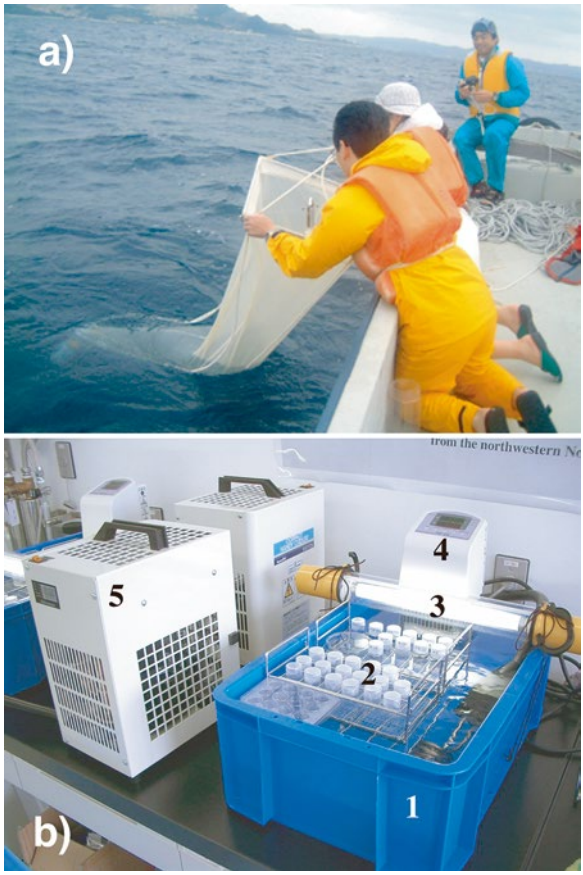

Fig. 7.15 (**a**) Plankton tow sampling using a small boat near shore. (**b**) Smallest version of the culture system for planktic foraminifera. 1, Water bath; 2, glass vials (culture vessels); 3, fluorescent lamp; 4, water circulator with heater; 5, chiller

Some spinose species (e.g., *Hastigerina*, *Globigerinella*, *Globigerina*, and *Globigerinoides*) sometimes retain very long spines (more than ten times the shell length), and the rhizopodia extend around the shell. In these cases, a thick plastic Pasteur pipette with the tip cut off is useful for transport. In all cases, it is important to avoid spending too much time on these procedures.

7.7.3 Maintenance of Living Specimens

For culture experiments, a single, separated planktic foraminifer is transferred to each of the culture vessels by using a Pasteur pipette. Small culture vessels are useful if the only goal is observation of the activity of living specimens. Small glass petri dishes or columnar glass cylinders (e.g., 2.5-cm diameter, 7-cm height, with approximately 30 ml seawater) are useful for culture and observational experiments. A glass petri dish is advantageous for observing cytoplasmic flow and activity of

rhizopodia through an inverted stereomicroscope. The seawater used for culture should be collected from the same location as the specimens. Seawater should be filter sterilized by being passed through a 0.22-μm membrane filter. It is better to keep the culture temperature the same as that at the collecting location. In many cases, cultured planktic foraminifera reach gametogenesis under these conditions (Fig. 7.15b).

7.7.4 Feeding and Maintenance

As earlier studies have determined (e.g., Anderson and Bé 1976b; Spindler et al. 1984; Hemleben et al. 1989), suitable foods for planktic foraminifera in culture are mainly nauplii of the branchiopod *Artemia salina* (brine shrimp) for carnivorous species (e.g., *Globigerina*, *Globigerinoides*, *Orbulina*, *Globigerinella*), and diatoms cultured in the laboratory (*Coscinodiscus*, *Chaetoceros* and *Thalassiosira* are easier for cultivation) for herbivorous species (e.g., *Neogloboquadrina*, *Pulleniatina*). Details of phytoplankton culture methods can be found in any textbook of algal culturing techniques (e.g., Andersen 2005). For feeding foraminifer specimens, *Artemia* nauplii or cultured algae are placed gently onto the cultured foraminifera by using a Pasteur pipette. Healthy planktic foraminifera have long and sticky rhizopodia around the shell, and they can catch food easily. In many cases, however, *Artemia* nauplii move actively just after hatching and planktic foraminifera sometimes cannot catch them. In those cases, *Artemia* can be placed on a glass plate and heated for several seconds with an alcohol lamp. *Artemia* nauplii are quickly killed and can then be given to cultured foraminifera.

Providing excessive nutrition may be detrimental for foraminiferal metabolism. Aberrant shell chambers sometimes occur in conditions with excess food (e.g., Hemleben et al. 1989). Furthermore, leftover food should be removed from the culture vessel as soon as possible to avoid fouling of the culture media (seawater) and contamination with microzooplankton and bacteria. Microzooplankton and bacteria frequently attack and cause damage to cultured foraminifera. Planktic foraminifera are separated and cultured individually (one per culture vessel), because they are carnivorous and in many cases will prey on each other under culture conditions.

7.7.5 Sample Fixation

For purposes of morphologic and geochemical analyses, it is important to properly store carbonate shells to avoid physical and chemical modification after collection. To fix foraminiferal shells, a high concentration of ethyl alcohol (>90 %) is best for long-term preservation. The specimens should be separated from seawater by using 63-μm mesh, otherwise the ethanol will dissolve organic matter in the seawater and

become clouded. For the purpose of molecular phylogenetic analysis, specimens should be cleaned by brush in the filtered seawater to remove attached materials before fixation. Cleaned specimens are preserved in individual tubes with 50 μL of guanidium isothiocyanate (GITC*) DNA extraction buffer and kept at −20 °C. (e.g., Morard et al. 2009)

7.7.6 Advanced Systems for Ecological Study: A Flow-Through Culture Approach

A flow-through culture system is a type of continuous culture in which the limiting nutrient is present in the inflowing medium. Such systems are designed mainly for the study of phytoplankton (e.g., chemostats), but the concept and equipment can also be applied to the study of foraminifera in culture. Flow-through culture systems have long been attempted for culture studies of marine protozoa, including foraminifera (e.g., Zach 1993). However, they have not been used for the culture of planktic foraminifera, because these organisms are positively buoyant and can easily flow out of the culture system through the drain or overflow. Flow-through culture systems, with their monitoring of chemical parameters, have the potential to follow the biological and geochemical influences of dissolved gases (e.g., O_2, CO_2) and related carbonate system parameters (pH, alkalinity, and carbonate species) in seawater.

Figure 7.16 is a conceptual illustration of a flow-through culture system designed for the ecological study of planktic foraminifera, and in particular for high-CO_2-exposure experiments on the effects of ocean acidification. In this system, each planktic foraminifer is encased in a cell strainer (Becton, Dickinson and Company, East Rutherford, NJ, USA) wrapped in nylon mesh (40-μm openings). This strainer is made from polypropylene and floats in seawater; therefore, planktic foraminifera can be exposed to controlled seawater conditions without escaping from the culture vessels (Fig. 7.17). The culture system is completely closed, and recirculating seawater is pumped from a holding tank at a constant flow rate by an electromagnetic metering pump with a PTFE diaphragm (no chemical or organic contaminants). This design has certain benefits, because a large volume of seawater can be used for culture experiments, minimizing any chemical gradients in the microenvironments surrounding the foraminifera. The system has been successfully used to culture some planktic foraminifera, including both spinose (e.g., *G. bulloides*, *G. sacculifer*, *G. siphonifera*, *O. universa*, and *H. pelagica*) and non-spinose species (*N. pachyderma* and *N. incompta*).

As the information presented in this section shows, living specimens of planktic foraminifera for culture experiments can be collected by plankton tow from coastal to open ocean waters. Culturing planktic foraminifera is not unusually difficult with the appropriate preparations and methodology. The most important considerations for long-term cultivation are to (1) reduce any damage during sample collection;

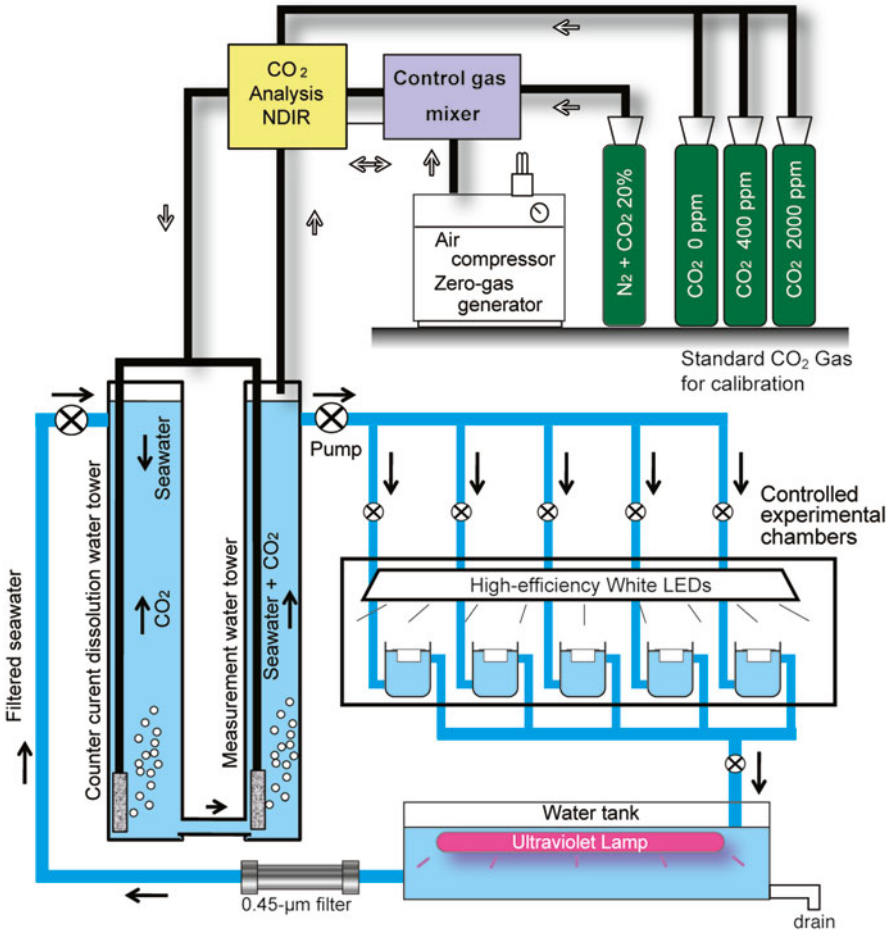

Fig. 7.16 Schematic diagram of the flow-through culture system used for pCO_2 exposure experiments and installed at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). Original concept of this system was made by Kimoto Electric Co., Ltd., Osaka, Japan. Water circulation is controlled by peristaltic and electromagnetic pumps (PTFE diaphragm pump). pCO_2 is adjusted by mixing CO_2-free gas (zero gas) and N_2-based 20 % CO_2. Mixed gas flow is controlled by a mass-flow controller unit and pCO_2 is measured by a non-dispersive infrared (*NDIR*) sensor. The pCO2 is adjustable from approximately 100 to 4,000 ppm in this system

and (2) keep the time as short as possible for transportation and isolation phases. Culture experiments should be designed for ultra-high resolution analysis to contribute to our understanding of biogeochemistry, biomineralization, and paleoceanography.

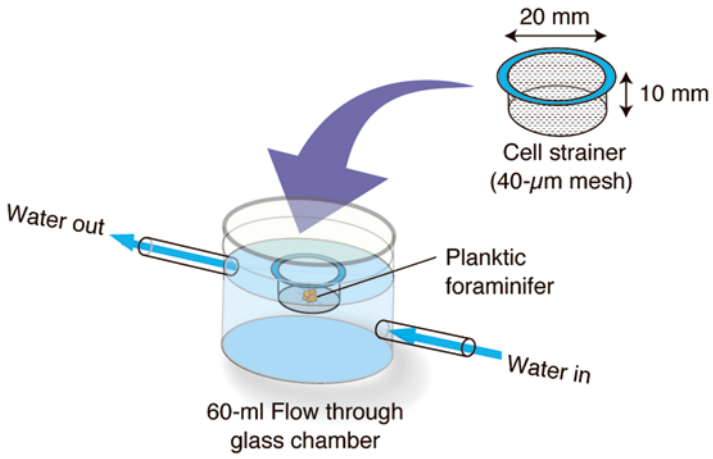

Fig. 7.17 Culture vessel designed for flow-through culture of a single specimen. A living planktic foraminifer is held in a floating cell strainer (40-μm mesh), which is placed into a flow-through glass chamber (60-ml). The top of the glass chamber is closed tightly to prevent seawater evaporation and gas exchange between the seawater and air. Seawater within the system is completely replaced every 10 min

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Chapter 8
Biology and Ecology of Radiolaria

Noritoshi Suzuki and Fabrice Not

Abstract Radiolaria are unicellular holoplanktonic protozoa with siliceous or strontium sulfate skeletons. Mainly studied by micropaleontologists because of their excellent fossil record, they are also key members of planktonic communities and play important roles in various oceanic ecosystems. This chapter presents an overview of the current knowledge on living Radiolaria (orders Acantharia, Collodaria, Nassellaria, Spumellaria and Taxopodia). Besides general considerations on Radiolaria as a whole, it focuses on the taxonomy, biology, and ecology of each radiolarian order. Finally this chapter provides insights on research perspectives to improve our knowledge of living radiolarians and their ecological role in marine ecosystems.

Keywords Acantharia • Collodaria • Nassellaria • Plankton • Radiolaria • Rhizaria • Spumellaria • Taxopodia • Biology • Ecology

8.1 Overview of Radiolarian Features

The advent of molecular phylogeny and phylogenomics is changing our vision of the taxonomy and evolution of eukaryotic lineages. The eukaryotic super-phylum Retaria (within the Rhizaria lineage) includes the Foraminifera and the Radiolaria, the latter being composed of five extant and well established orders: the Acantharia, Taxopodia, Spumellaria, Nassellaria, and Collodaria (De Wever et al. 2001; Suzuki and Aita 2011; Sierra et al. 2013) (Fig. 8.1). Phaeodaria, which were initially

N. Suzuki (✉)
Institute of Geology and Paleontology, Graduate School of Science,
Tohoku University, Sendai 980-8578, Miyagi, Japan
e-mail: norinori@m.tohoku.ac.jp

F. Not
Centre National de la Recherche Scientifique – CNRS,
University Pierre and Marie Curie – UPMC, Station Biologique de Roscoff,
Place Georges Teissier, 29680 Roscoff, France
e-mail: not@sb-roscoff.fr

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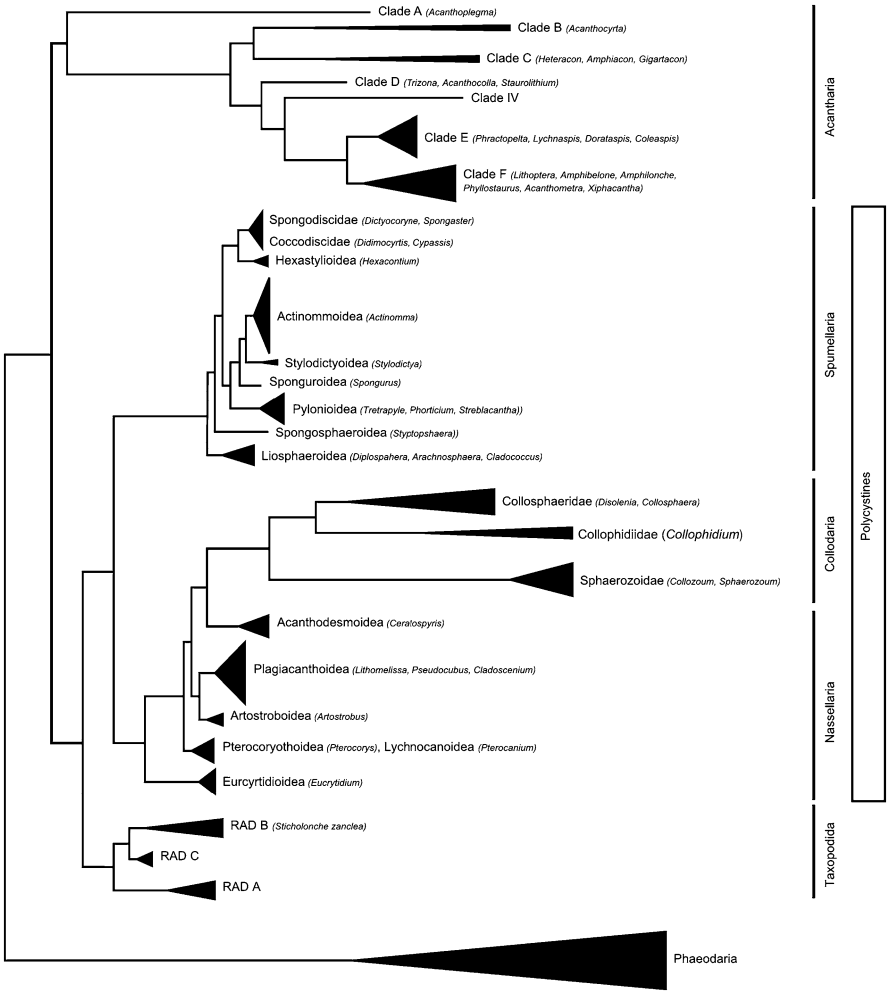


Fig. 8.1 Schematic tree based on an assemblage of genetic and morphological information representing the current understanding of evolutionary relationships between major radiolarian groups. The *vertical length* of the triangles represents a relative number of extant species, while the *horizontal length* of the triangles is our estimation of evolutionary time between each lineage but does not represent a true phylogenetic distance. Phaeodaria belong to Cercozoa and are represented as an outgroup. Extant radiolarians encompass orders Acantharia, Collodaria, Nassellaria, Spumellaria and Taxopodia

regarded as a part of the Radiolaria are now considered to belong to the Cercozoa, still within the Rhizaria (Nakamura and Suzuki 2015). On the other hand, Taxopodia, which has long been thought to be closely related to Heliozoa, now belongs to Radiolaria (Sierra et al. 2013). Because of these latest developments, one has to be careful when using the term "Radiolaria" in scientific contributions as it can encompass quite different meanings. Radiolaria presenting a siliceous test (i.e., Spumellaria,

Nassellaria and Collodaria) are traditionally grouped under the informal taxonomic unit called "polycystine", essentially based on paleontological studies (De Wever et al. 2001) (Fig. 8.1).

Radiolaria are holoplanktonic amoeboid protists with skeletons made of opaline silica (SiO_2 nH_2O) or strontium sulfate ($SrSO_4$) (Fig. 8.2). Their size ranges from tens to several hundreds of micrometers. Radiolaria possess a common cytological structure of double-layered cytoplasm (endoplasm and ectoplasm) and pseudopodia (Figs. 8.2 and 8.3, Table 8.1). The endoplasm is the inner cytoplasmic part comprising the nucleus (or nuclei), Golgi, ribosomes, mitochondria and other organelles and is bounded by a capsular chitinous membrane called the capsular wall (for polycystines) or thin mesh-like membrane (for Acantharia). The ectoplasm surrounds the endoplasm and consists of food vacuoles, algal symbiotic vacuoles and other cytoplasmic parts. There are several types of pseudopodia. Axopodia are straight pseudopodia structured with an axoneme and microtubuline-associated cytoskeleton. They originate from the endoplasm and extend all the way outside of the ectoplasm. Pseudopodia without axoneme are considered as part of the ectoplasm and are called the reticulopodia network or filopodia (Suzuki and Aita 2011). Radiolarians can

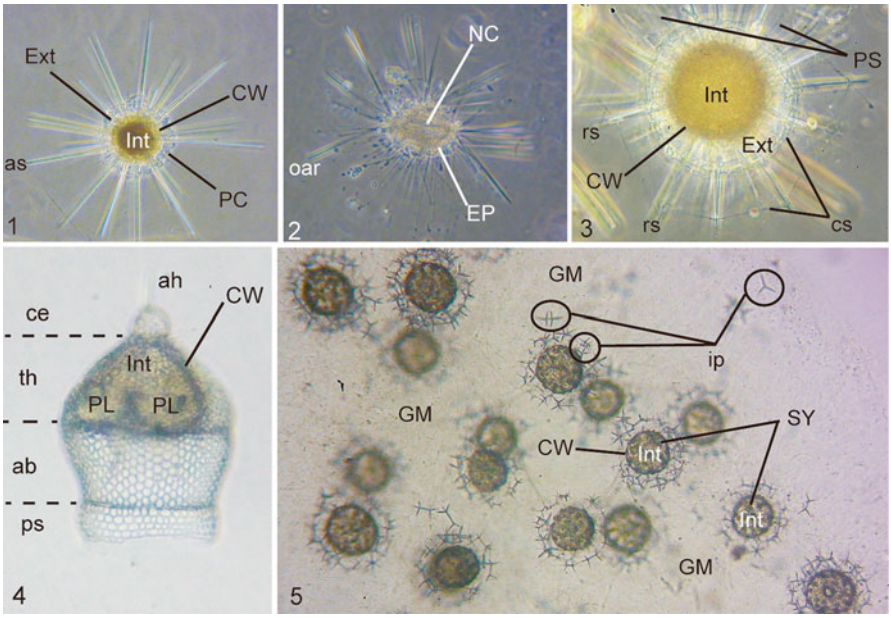


Fig. 8.2 Images of living radiolarians obtained by optical microscopy: (*1*) Acantharia; (*2*) Taxopodia; (*3*) Spumellaria; (*4*) Nassellaria; and (*5*) Collodaria. The scale for each image is variable. Their classification is essentially based on examination of internal structures and is therefore very challenging on living specimens. Briefly, Acantharia have a strontium sulfate skeleton, Taxopodia have characteristic pseudopodia, Spumellaria have a concentric internal structure of their skeleton, Nassellaria are characterized by a heteropolar skeleton, and Collodaria form colonies with gelatinous matrix. Detailed morphological features reported on this figure are listed in Table 8.1

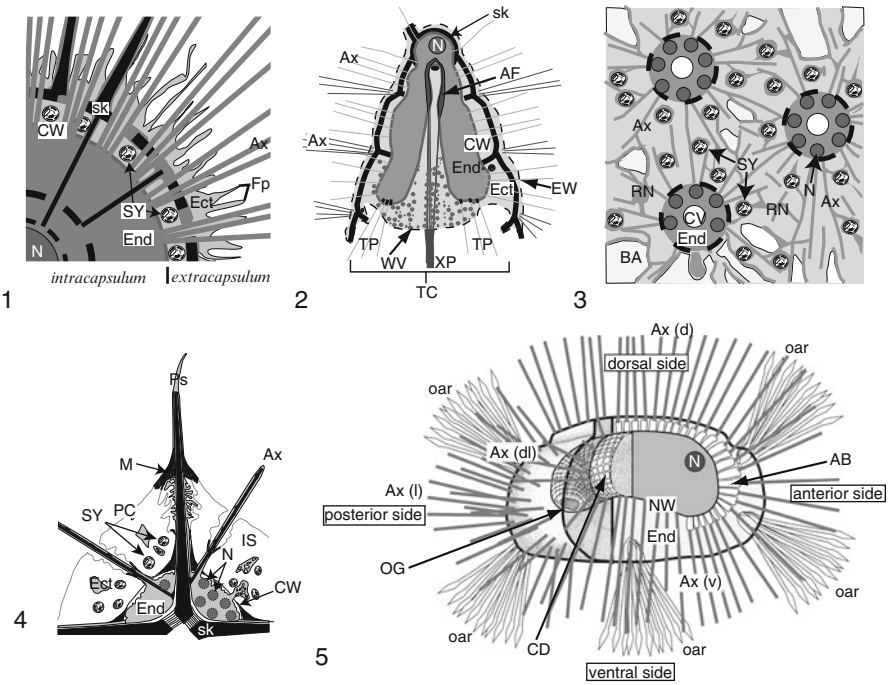


Fig. 8.3 Overall cytoplasmic structure of radiolarian orders (from Anderson 1983; Cachon et al. 1989; Suzuki and Aita 2011): (*1*) spherical Spumellaria; (*2*) multi-segmented Nassellaria; (*3*) colonial Collodaria; (*4*) Acantharia with multi-nuclei; (*5*) Taxopodia. Detailed morphological features reported on this figure are listed in Table 8.1

control their buoyancy by contraction and elongation of pseudopodia. The position of the endoplasm and ectoplasm is sometimes so complex that any cytoplasmic positioned outside the capsular membrane is often named extracapsulum, whereas when it is positioned inside the capsular membrane it is called intracapsulum (Figs. 8.2 and 8.3 and Table 8.1).

Radiolarians are difficult to keep alive in culture and knowledge on their life cycle is therefore fragmentary. The longevity of solitary radiolarians is assumed to be a few months based on the seasonal variation in vertical flux of selected species. Lunar cycles like the one observed in planktonic foraminifers have not been detected in any radiolarian groups so far. Reproduction by binary fission is not assumed to be common but cannot be excluded since it has been documented for the Collodaria (Anderson and Gupta 1998). Instead, release of small bi-flagellated cells (2–5 μm) of unknown ploidy has been more frequently observed for several spumellarian, collodarian and acantharian species after having turned whitish (Anderson 1983; Kimoto et al. 2011; Decelle et al. 2012a). The fate of these swarmers is unknown, and whether they are gametes or not is unclear. The small size of these swarmers together with the lack of chlorophyll fluorescence demonstrates that symbiotic

Table 8.1 List of abbreviations

Taxonomic specification	Abbreviations	Terms
All Radiolaria		
	ax	Axopodia
	CW	Capsular wall
	Ect	Ectoplasm
	End	Endoplasm
	EP	External cytoplasm
	Ext	Extracapsulum
	FP	Filopodia
	Int	Intracapsulum
	N	Nucleus
	RN	Reticulopoida network
	PS	Pseudopoida
	sk	Mineralized skeleton
	SY	Photosymbiont
	WV	Waste vacuole
Nassellaria		
	ab	Abdomen
	ah	Apical horn
	ce	Cephalis
	PL	Protoplasmic lobe
	ps	Post-abdominal segment
	TC	Terminal cone
	TP	Terminal projection
	th	Thorax
	XP	Axial projection
Acantharia		
	as	Acantharian spicule
	EW	Extracapsular membrane
	IS	Internal empty
	M	Myoneme
Spumellaria		
	AF	Axoflagellum
	cs	Cortical shall
	rs	Radial spine
Collodaria		
	BA	Bubble-like alveoli
	CV	Central vacuole
	GM	Gelatinous matrix
	ip	Isolated spicule

(continued)

Table 8.1 (continued)

Taxonomic specification	Abbreviations	Terms
Taxopodia		
	AB	Axopod base
	Ax(d)	Dorsal axopodia
	Ax(dl)	Dorsolateral axopodia
	Ax(l)	Lateral axopodia
	Ax(v)	Ventral axopodia
	CD	Cap-like depression
	NC	Nuclear capsule
	NW	Nuclear capsular wall
	oar	Oar-like axopodia
	PC	Periplasmatic cortex

radiolarians very likely transmit their symbionts horizontally (e.g., de novo acquisition at each host generation, Decelle et al. 2015).

Ontogenetic growth of radiolarians is poorly understood and usually indirectly reconstructed after pieces of information collected from fossils and observations in living conditions (Fig. 8.4). The siliceous matter of polycystines is amorphous silica, so-called opal (SiO_2 nH_2O), and the siliceous skeleton forms within a cytoplasmic sheath called the silica deposition vacuole (SDV) as endoskeleton (not exoskeleton) (Anderson 1994). Silica deposition can be studied in living polycystines using the fluorescent compound PDMPO, an acronym for 2-(4-pyridyl)-5-[(4-(2-dimethyl-aminoethylaminocarbamoyl)methoxy)-phenyl] oxazole (Ogane et al. 2009). The model of siliceous skeleton formation consists of primary growth (PG), secondary growth (SG) and skeletal-thickening growth (STG) (Ogane et al. 2010) (Fig. 8.4). PG forms the principal framework of skeletons and SG forms a new siliceous part on the already existing skeleton (Anderson and Bennett 1985). STG thickens the already existing skeletons but never forms a new skeleton part like PG and SG (Ogane et al. 2009) and endoplasm increases in size with growth. Two silicification processes have been identified, an intermittent silicification process and a continuous silicification process (Ogane et al. 2010). The intermittent silicification process is recognized to occur in a variety of Spumellaria and Nassellaria, while the continuous silicification process is only known for pylonioid Spumellaria and at the proximal portion (cephalis, thorax and the upper portion of abdomen) of Nassellaria (Ogane et al. 2010; Suzuki et al. 2013).

Radiolarians are ubiquitous and frequently encountered in the oceans but paradoxically little is known about their feeding behavior. Radiolarians are mainly heterotrophic protists and tend to be carnivorous, ciliates tintinnids, being a prey of choice, in the photic zone (Anderson et al. 1984; Swanberg et al. 1986; Swanberg and Eide 1992). Food preferences of mesopelagic and bathypelagic radiolarians are currently unknown. Except for colonial Collodaria, radiolarians consume preys by external assimilation of digested material in food vacuoles located in

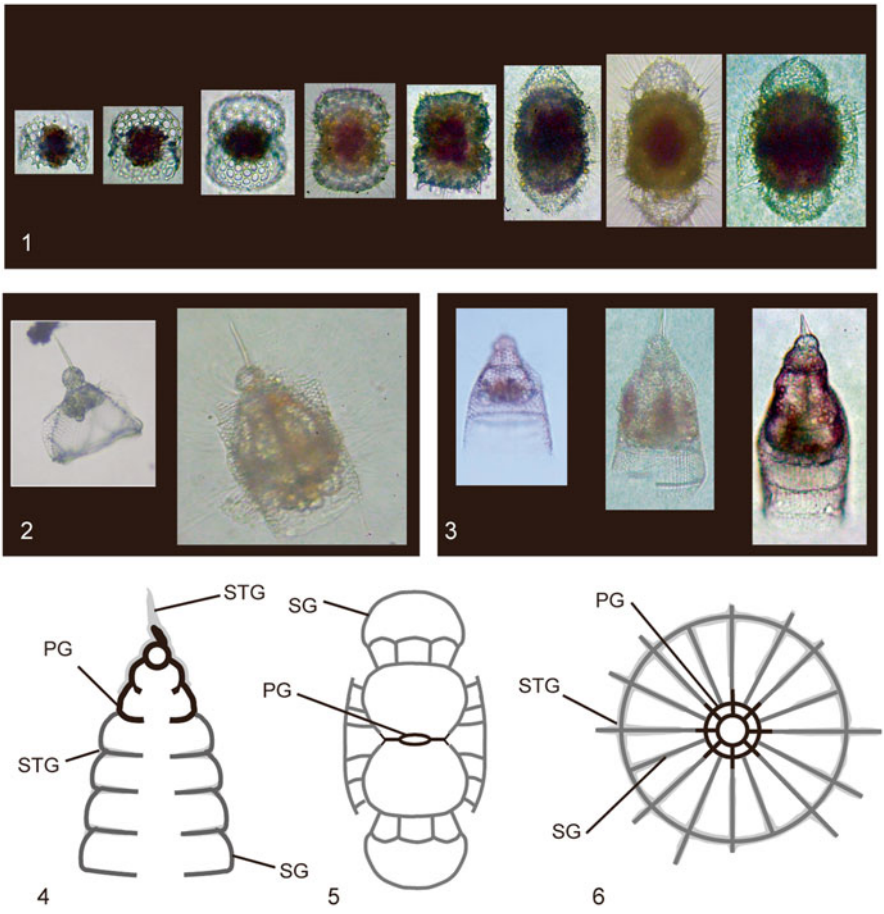


Fig. 8.4 Ontogenetic growth of Spumellaria (*1*) and Nassellaria (*2, 3*). The skeletogenesis patterns of Nassellaria (*4*) and Spumellaria (*5, 6*) are also illustrated. The spumellarian *Didymocyrtis tetrathalamus* (Fig. 8.4 (1, 5)) develops a new skeleton when cytoplasm fills the already existing skeleton. Nassellaria develop a new skeleton, but an empty space with cytoplasm always remains in the proximal portion of the test. Skeletogenesis is divided into the primary growth (PG), secondary growth (SG) and skeletal-thickening growth (STG) stages

the ectoplasm. There are only a few reports of Radiolaria serving as food particles for other planktonic organisms such as large copepods or Euphausiacea (Eskinazi-Sant'anna 2006).

Besides heterotrophic feeding, a significant number of radiolarian species inhabiting the photic zones of the oceans do bear symbiotic microalgae. A variety of associated photosynthetic organisms, including cyanobacteria, prasinophytes, haptophytes, and dinoflagellates have been reported to live in symbiosis with Radiolaria (Anderson 1993; Anderson et al. 1998; Decelle et al. 2015). The photosymbiont most commonly observed is the dinoflagellate genus *Brandtodinium*

nutriculum (Probert et al. 2014), originally described as "*Zooxanthella nutricula* Brandt" in the collodarian host *Collozoum inerme* (Müller) (Brandt 1883). Parasites are often reported from various groups of Radiolaria (Koeppen 1894; Borgert 1898; Hollande and Enjumet 1955; Cachon 1964; Cachon and Cachon 1987; Suzuki et al. 2009a; Bachvaroff et al. 2012; Coats et al. 2012). Parasites can be found in both the ectoplasm and/or endoplasm. The parasites most commonly observed, or reported, based on molecular sequences, belong to marine alveolates such as the genera *Amoebophrya*, *Merodinium*, or *Syndinium* (Hollande and Enjumet 1955; Dolven et al. 2007).

Out of the 600–800 extant radiolarian species, about 100–150 species can be found coexisting at a single location, except for the Arctic Ocean where local species richness is lower. Radiolaria live exclusively in marine environments with one exception so far, the brackish water species *Lophophaena rioplatensis* Boltovskoy et al. (Boltovskoy et al. 2003). They can be found from the surface down to 8,000 m depth (Reshetnyak 1955). The average abundances of single cells in seawater is 100 to 200 cells m^{-3} for Spumellaria and Nassellaria, 10s to 100s of colonies m^{-3} for Collodaria, 100s to 1000s of cells m^{-3} for Acantharia, and 10,000 cells m^{-3} for Taxopodia in specific conditions (Tan et al. 1978; Kling 1979; Swanberg 1983; Michaels et al. 1995).

8.2 Acantharia

8.2.1 Cell Structure and Taxonomy

Acantharia are the only group of organisms known to have a strontium sulfate ($SrSO_4$) skeleton during their entire lives. Acantharians actively accumulate strontium from depleted seawater and make celestite precipitate in cytoplasmic envelope. Each spicule consists of a single crystal (Wilcock et al. 1988). The precipitation process of acantharian celestite is unknown. All acantharian species, with a few exceptions, appear to have twenty radial spicules or ten radial spicules intersecting at the center of the cell and consistently arranged following a symmetry rule called Müller's laws (Haeckel 1862) after the first description by Müller (1859) (Fig. 8.5). The ontogenetic growth of Acantharia occurs through strontium sulfate crystal elongation and development of accessory lateral spicules eventually leading to cortical shells, resulting in an increased endoplasm volume (Schewiakoff 1926). Cyst-forming acantharians (Fig. 8.6) undergo a profound re-organization of their skeleton when entering cyst formation. The cell moves its spicules in one direction and then forms either a rounded or oblong-shaped cyst entirely made of strontium sulfate. The cell size changes from a few tens to a few hundreds of μm in diameter. Acantharian cells are typically divided into endoplasm and ectoplasm parts and have a periplasmatic cortex being a characteristic feature of Acantharia (Fig. 8.3). The major body of the endoplasm is situated in the intracapsulum but small parts of

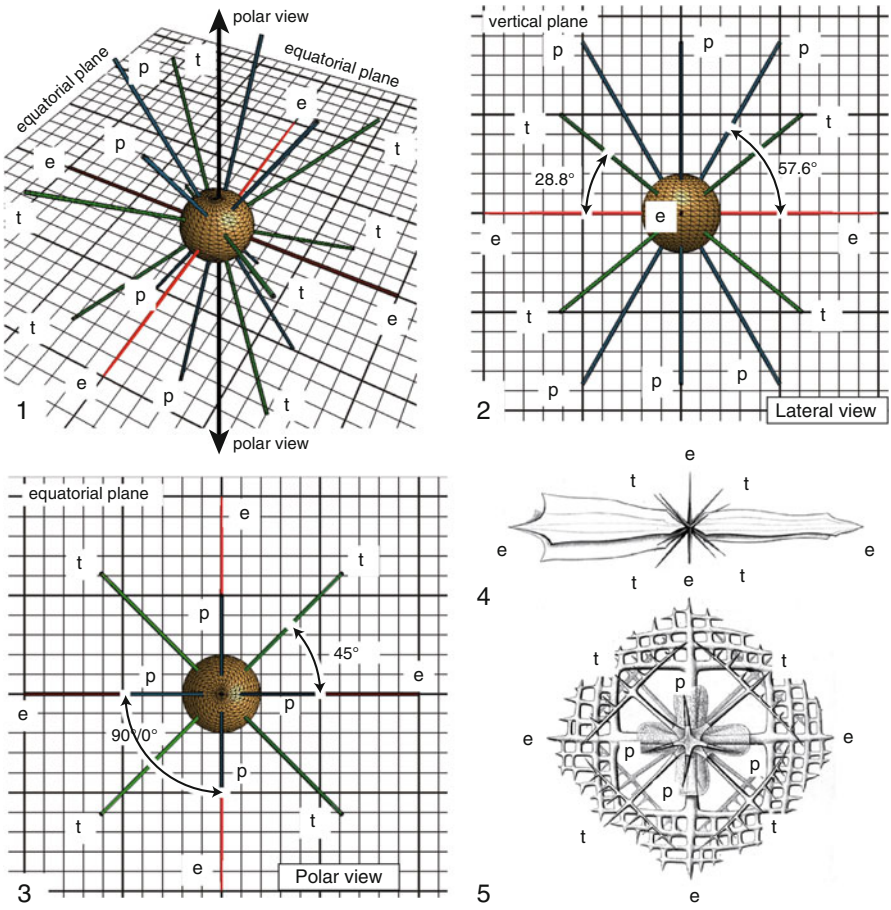


Fig. 8.5 Müller's law for the arrangement patterns of acantharian spicules. Acantharia have a total of twenty spicules. The central part is the C_4 symmetry axis; four equatorial spicules "e" are placed in a virtual equatorial plane, eight tangential spicules "t" are inclined at 28.8° and eight polar spicules "p" are inclined at 57.6° to both sides of the equatorial spicules. (*1*) Tangential view; (*2*) lateral view; (*3*) polar view; (*4*) example of spicules types for the genus *Amphibelone* (figure from Haeckel 1887); (*5*) example of spicule types for the genus *Lithoptera* (figure from Haeckel 1887)

the endoplasm are protruded as extracapsulum out of the endoplasm. Ectoplasm is made of algal and food vacuoles which are located between the perispicular membrane and the periplasmatic cortex. Acantharian cells can display a variety of cytoplasm colors, but this feature is not used as a taxonomic character as it varies depending on cell conditions. Acantharians are also characterized by the presence of tens of nuclei in a single cell (Suzuki et al 2009b).

Classical taxonomy is essentially based on the work by Schewiakoff (1926) which distinguished four orders, 16 families, 49 genera and 145 species and subspe-

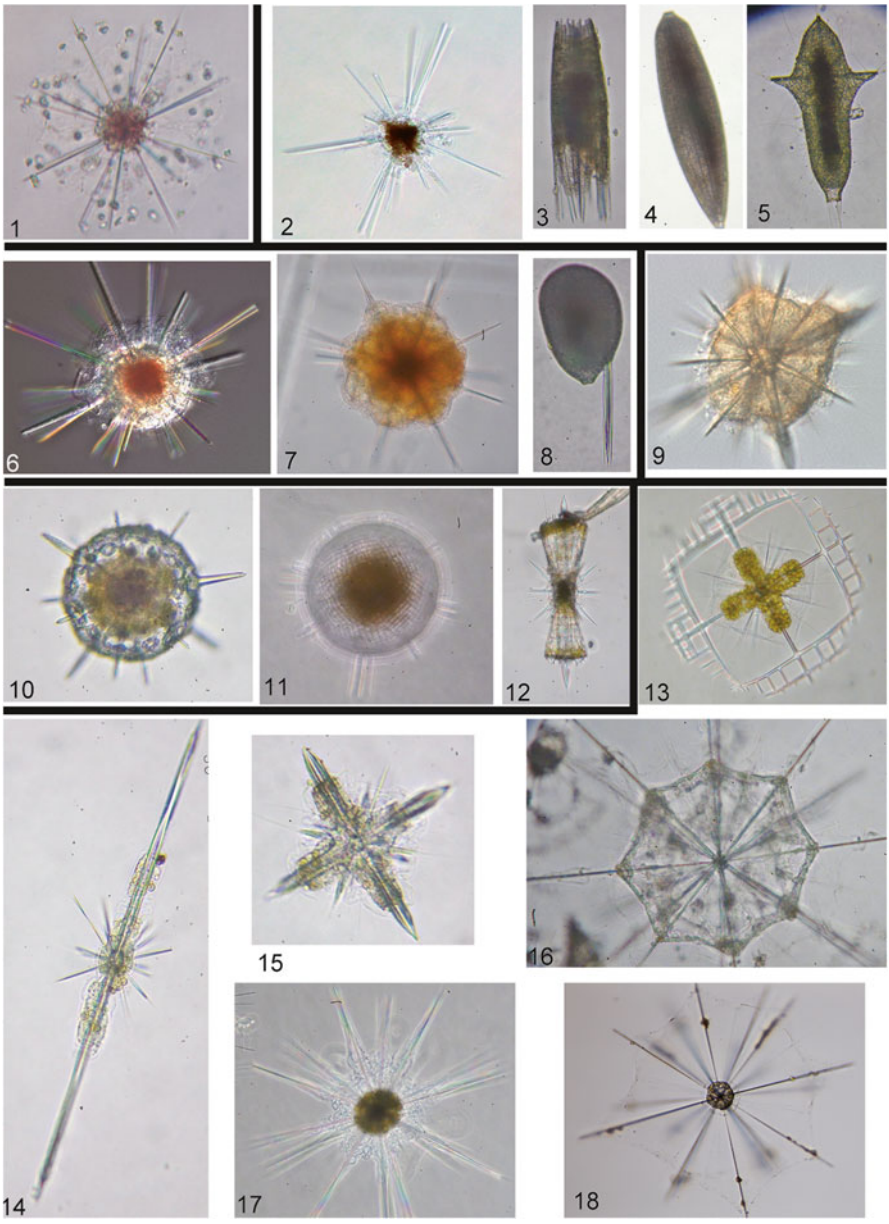


Fig. 8.6 Major acantharian groups and cysts classified according to the clades indicated by Decelle et al. (2012a, c). (*1*) Clade A (Holacanthida *partim* in the classical taxonomy: *Acanthoplegma* sp.); (*2–5*) clade B (Holacanthida *partim* in the classical taxonomy (*2*: *Acanthochiasma fusiforme* (Haeckel), *3*, *4*: clade B1 cysts; *5*: clade B2 cyst)); (*6–8*) clade C (Chaunacanthida: 6: *Litholopus muelleri* (Haeckel), *7*: *Acanthocyrta haeckeli* Schewiakoff, *8*: Clade C4 cyst); (*9*) clade D (Holacanthida *partim* in the classical taxonomy: *Staurolithium* sp. A); (*10–12*) clade E (Symphiacanthida and Arthracanthida in the classical taxonomy: *10*: *Dorataspis*

cies by examination of living acantharians in the Naples bay (Fig. 8.6). As Schewiakoff (1926) did not designate type species for most genera, Campbell (1954) formalized acantharian taxonomic names under the International Code of Zoological Nomenclature (ICZN, 1999). Campbell (1954) listed all the known acantharians above the genus level to arrange the Suborder "Acantharina" into two divisions, five superfamilies, 15 families and 71 genera. The taxonomic systematics proposed by Schewiakoff (1926) has been only slightly amended with the establishment of a new order, Plegmacantha (Reshetnyak 1981; Bernstein et al 1999). In the classic taxonomic scheme, the orders Hollacanthida and Chaunacanthida present intersected radial spicules or twenty easily disconnected radial spicules, lack algal symbionts (Fig. 8.6), and correspond to the cyst-forming taxa (Decelle et al. 2013). In contrast, the orders Symphiacanthida and Arthracanthida have tightly connected spicules and always harbor algal symbionts (Fig. 8.6). Morphological classification at the genus and species levels matches only partially with the molecular phylogeny (Decelle et al. 2012c). Based on molecular clock and co-phylogeny of host and symbionts, it has been shown that the onset of the symbiotic relationship took place at a time of extreme oligotrophy in the oceans ca. 175 Ma (the Toarcian Event) and corresponds to skeleton complexification of the Acantharia (Decelle et al 2012a, b).

8.2.2 Reproduction and Motility

Two types of reproductive behaviors are observed in Acantharia (Febvre 1989; Decelle et al. 2013). The first consists of the release of small bi-flagellated cells called swarmers by symbiont-bearing Acantharia. The acantharian endoplasm turns whitish before release of swarmers. Although the actual ploidy of these cells is not formally demonstrated, they are released after successive nuclear divisions and are usually considered as gametes (Decelle et al. 2013). The second reproductive type is carried out by the cyst-forming acantharian groups, which always lack algal symbionts and have disconnected radial spicules (Decelle et al. 2013). Once formed, the cyst sinks to the ocean depth where swarmer cells are released. Juvenile acantharians show the highest abundance between 500 and 900 m water depths in the Atlantic Ocean (Massera-Bottazzi and Andreoli 1982), which is consistent with the

Fig. 8.6 (continued) *loricata* Haeckel [the *reddish part* is the endoplasm], *11*: *Phatnaspis tabulatus* (Müller), *12*: *Diploconus tridentatus* Popofsky [Although photosymbionts colored *lemon-yellow* in this figure are concentrated at the end of tubular reversed-cone equatorial "spicules", photosymbionts can be also distributed on groove of the tubular "spicule"]); (13–18) clade F (*13*) clade F1 (Symphiacanthida and Arthracanthida in the classical taxonomy: *Lithoptera fenestra* Müller [the *square membrane* outside the mass of *yellowish green* granular photosymbionts is the periplasmatic cortex]); (*14*) clade F2 (the same with Fig. 8.6 (13): *Amphilonche elongatum* (Müller); (*15–18*) clade F3 (the same with Fig. 8.6 (13): *15*: *Quadristaurus nordgaardi* (Jørgensen), *16*: *Acanthometron pellucidum* (Müller), *17*: *Phyllostaurus siculus* (Haeckel), *18*: *Acanthonia cuspidata* (Haeckel))

hypothesis suggesting that cyst-forming acantharians reproduce at depth (Decelle et al. 2013). The cell size within a single species can vary from a few tens to a few hundreds of μm in diameter.

Acantharia possess a unique structure, the periplasmatic cortex (Fig. 8.3), a cytoplasmic membrane allowing extremely rapid contraction and expansion of its cellular volume. The periplasmatic cortex is connected along each acantharian spicule with two to twelve myonemes (Fig. 8.3) that are composed of a dense bundle of protein filaments like animals muscle (Febvre 1971, 1974). Periplasmatic cortex stretches with the upward movement of myoneme along the spicule (Febvre 1972). The myoneme relaxes very slowly (0.02 of its length s^{-1}) and contracts as quickly as 7–8 of its length s^{-1}, and the contraction–relaxation cycles of these mobile myonemes are controlled by concentration of calcium (relaxation occurs at 10^{-7} M and contraction at over 5×10^{-6} M of calcium) (Febvre 1981). The function of inflation and deflation of periplasmatic cortex is presumed to modify the buoyancy of the cell and/or to eject waste material from the cell.

8.2.3 Trophic Characteristics and Biotic Interactions

Very little is known about food preferences of Acantharia. They have been observed to consume ciliate tintinnids rather than copepods and their larvae (Schewiakoff 1926; Swanberg and Caron 1991). Also, the fate of acantharian cells in the food web is currently unclear.

The main symbionts in Acantharia are identified as belonging to the haptophyte genus *Phaeocystis* (Decelle et al. 2012a, 2015). Using genetic markers, no species-specific relationship could be revealed and any symbiotic acantharian species can take any *Phaeocystis* genera as a symbiont. Using a set of five genetic markers, *Phaeocystis* cells found in symbiosis are identical to free-living ones. The number of symbionts within one acantharian cell varies usually between 12 and 30, but can exceed 150–300 symbionts in some species (Khmeleva 1967). The actual nature of the relationship between *Phaeocystis* and its host is unclear but it does not seem to be a simple mutualism. The microalgae *Phaeocystis* is known to be a major producer of DMSP (dimethylsulfoniopropionate), which is a precursor of the climatically active gas DMS. Measured concentration of particulate DMSP in acantharian-*Phaeocystis* holobionts is 1000 times higher than for solitary *Phaeocystis* cells. If *Phaeocystis* is the dominant symbiont for most symbiotic Acantharia, the acantharian clade C has a mix of different kinds within one cell (Decelle et al. 2013). Up to now, Acantharia have been documented as being infected by marine alveolates of the Syndiniophyceae (*Dogelonidium ovoides* (Cachon) Loebl. et A. R. Loebl.) or Amoebophryaceae (*Amoebophrya acanthometridae* Borgert) (Théodoridès 1982).

8.2.4 *Environmental Distribution*

Acantharia are extremely abundant in nearly every open ocean water sample observed live. This contrasts drastically with the observation of similar samples preserved in regular fixatives (e.g., formalin) in which Acantharia rapidly dissolve after death because of under-saturation of strontium in seawater. Essentially because of the fixative procedure Acantharia are highly elusive and have been overlooked in ecological studies. Acantharian cells preserve well in 100 % alcohol or in a solution of formalin buffered with strontium chloride at a final concentration of 80 mg L^{-1} (Beers and Stewart 1970; Febvre 1989).

Yet a few studies using culture independent molecular survey or studies focused on Radiolaria highlighted global trends. Acantharia are commonly distributed in tropical and subtropical oceans (Not et al. 2007; Lovejoy and Potvin 2011; Decelle et al. 2012a). Plankton samples from the Arctic Ocean also contain acantharians belonging to *Acanthometron* and *Litholophus* (Hülsemann 1963). Acantharia with algal symbionts tend to be abundant at the surface in oligotrophic tropical–subtropical pelagic oceans. For instance, 90 % of acantharian cells were found from the surface to 20 m depths off Bermuda (Michaels 1988). Carbon fixation in acantharians was <4 % of the total primary productivity in oligotrophic oceans and 6–35 % of the primary production by plankton larger than 100 μm (Michaels 1991). Symbiotic acantharians can show diel vertical migration patterns being deeper at night (Massera-Bottazzi and Andreoli 1982). Acantharians also show a seasonal pattern, being twice as abundant in summer and autumn compared with winter in Bermuda (Michaels 1991) (Fig. 8.7). In the East China Sea, standing stocks of acantharians increased rapidly from nearly a few individuals m^{-3} to 5.9×10^4 individuals m^{-3} during mid-June when temperature and salinity both reach 22 and 32 °C, respectively, and then slowly decreased in abundance down to 100 individuals m^{-3} from July to October (Tchang and Tan 1964). The distribution of Acantharia without algal symbionts is poorly known. Sediment-trap records of acantharian cyst sinking events have been reported to occur at high latitudes after a phytoplankton bloom. These events contribute significantly to carbon export and are important for the strontium biogeochemical cycle (Anita et al. 1993; Martin et al. 2010; Decelle et al 2013).

8.3 Taxopodia

8.3.1 *Cell Structure and Taxonomy*

Taxopodia cells present a thick inner cytoplasmic membrane dividing the cell in two parts, an endoplasm and an ectoplasm. The Taxopodia endoplasm, which has a peanut shape, is believed to hold the nucleus and is consequently named the nuclear capsule (Cachon et al. 1977). Taxopodia possess two types of pseudopodia: normal

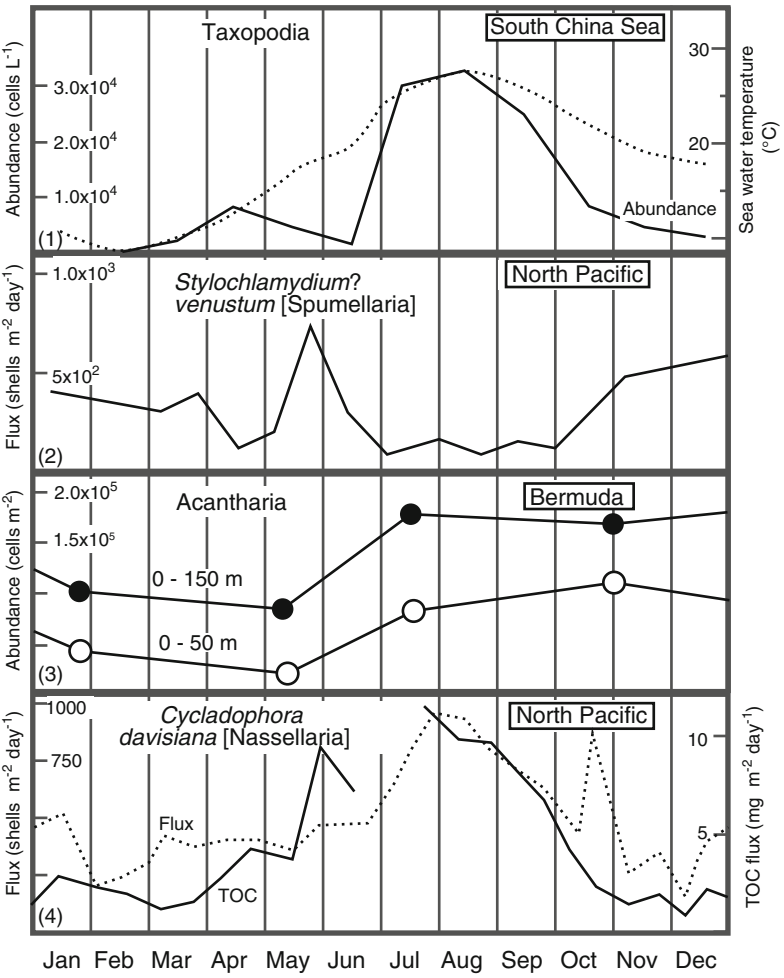


Fig. 8.7 Seasonality of selected radiolarian taxa. Acantharia and Taxopodia are known to become abundant abruptly from several observations. Seasonal changes in the abundance of spumellarian and nassellarian polycystines are rarely reported. No significant seasonality in abundance has been clearly detected in almost any of the studied polycystines so far. (*1*) Taxopodia (Tan et al. 1978); (*2*) *Stylochlamydium*? *venustum* [epipelagic Spumellaria from the subarctic North Pacific] (Itaki and Takahashi 1994); (*3*) Acantharia (Michael 1991); (*4*) *Cycladophora davisiana* [mesopelagic Nassellaria from the subarctic North Pacific] (Okazaki et al. 2003)

axopodia with thick bundle of axoneme, and oar-like axopodia (Figs. 8.2 and 8.3). Opaline silica is found in the oar-like axopodia (Takahashi and Ling 1980). A total of 320 oar-like axopodia are arranged into six rows and arise from the surface of the nuclear capsular wall (Cachon et al. 1977). The checkerboard appearance of the nucleus membrane comes from cuplike depressions in the stem of each axopodium (Fig. 8.3). Larger oar-like axopodia located at on the anterior end of the cell, whereas

no axopodia are found on the postero-ventral portion of the cell. Only one species (*Sticholonche zanclea*) has been described so far, but many environmental sequences displaying a high diversity above genus level are found in molecular phylogenetic analyses (Not et al. 2007; unpublished data).

8.3.2 Reproduction and Motility

Taxopodia swim actively by rowing their oar-like axopodia, which are moved through calcium-controlled, contractile, non-actin-containing microfilaments located at the base of each axopodium (Cachon et al. 1977). The oar-like axopodia can move along an arc of 120 degrees in less than 0.1 s. The Taxopodia swim technique entails three stroke cycles: translational motion, transitory pause, and axopodial return. The organism moves forward with one active stroke (translational motion), followed by a short pause (transitory pause), and then their axopodia return to their start position with a contraction of the 1/3–1/4 part of the nuclear capsule (axopodial return). Although very little is known about Taxopodia reproduction, binary division has been observed. The cell divides into two portions along the plane across the dorsal and ventral lines (Hollande and Enjumet 1954). Yet, ontogenetic growth of Taxopodia has not been reported because they die rapidly after collection.

Taxopodia can lose and reform their normal axopodia (Cachon et al. 1977; Cachon and Cachon 1980). Experimental observation showed that the reconstruction of microtubules starts from the periphery, at the base of the axopodium; it is then followed by the elongation of all microtubules in the axopodium, and the elongation ends after the microtubules located in the central part of the axopodium become the longest (Cachon and Cachon 1980).

8.3.3 Trophic Characteristics and Biotic Interactions

No photosymbionts have been described in Taxopodia so far and food preferences are unknown. Copepods (e.g., *Cetropages velicatus* and *Paracalanus parvus*) and Euphausiacea (*Euphausia* sp.) can feed on *Sticholonche zanclea* (Taxopodia) under limiting food concentrations (Eskinazi-Sant'anna 2006). Taxopodia are commonly infected by *Amoebophrya sticholonchae*, a Marine Alveolata Group II (Fol 1883; Borgert 1898; Dolven et al. 2007). Release of parasite gametes is well documented. *A. sticholonchae* becomes larger at the sporogenesis stage, as a single conical mass whose sharp tip is always oriented toward the opposite side of the central capsule of Taxopodia, and this conical mass abruptly changes its form into a worm to burst through the cytoplasmic membrane in 1 or 2 min (Fol 1883; Borgert 1898).

8.3.4 Environmental Distribution

Taxopodia are abundant worldwide in epipelagic and mesopelagic zones but can also be found in neritic environments where they tend to be abundant seasonally, as in the frontal zone between cold and warm water currents in the Pacific Ocean (Tan et al. 1978; Takahashi and Ling 1980; Henjes et al. 2007). Taxopodia abundance is tightly associated with the chlorophyll-*a* maximum zone in the equatorial Pacific (Takahashi and Ling 1980) and at shallower depths in the East China Sea (Tan et al. 1978). In the Polar Frontal Zone of the Southern Ocean, Taxopodia are less abundant during the austral winter and increase in abundance from the early spring to autumn (Klaas 2001), and are concentrated below 80 m depths, close to the pycnocline (Henjes et al. 2007). In the East China Sea, Taxopodia show a significant seasonality with abundances reaching up to 3.0×10^4 cells m^{-1} from June to September (Tan et al. 1978) (Fig. 8.7).

8.4 Spumellaria

8.4.1 Cell Structure and Taxonomy

Spumellaria is a diversified group of Radiolaria whose skeleton is characterized by its concentric structure (Figs. 8.2, 8.3 and 8.8). The overall appearance of the skeleton is spherical, elliptical, flat, or cruciate, and is made of double medullary shells (microsphere and macrosphere) and cortical shells, with radial beams connecting shells. Radial spines can arise from the internal radial beams, and very short spicular by-spines can also be present. The endoplasm is generally in the innermost part of the skeleton (also called the test or shell) while the position of the ectoplasm is variable. Straight pseudopodia, probably axopodia, emerge from the endoplasm and radiate throughout the test (Fig. 8.3). One long, thick pseudopodium, called axoflagellum, extends in some spumellarian species (e.g., flat-shaped spongodiscid spumellarians) (Fig. 8.8). Algal symbionts, if present, are located inside the cortical shell or attached along pseudopodia. Some Spumellaria are presumed to belong to

Fig. 8.8 (continued) *Didymocyrtis tetrathalamus tetrathalamus* (Haeckel) [*5*: live image, *6*: skeleton. An axoflagellum extends from one polar part of the skeleton]; (*7*) *Calcaromma morum* (Müller); (*8*) *Hexacontium* cf. *asteracanthion* (Haeckel); (*9*) *Tetrapetalon echinaster* (Haeckel) [the *golden yellow dots* on the distal part of axopodia are photosymbionts]; (*10*) *Saturnalis circularis* Haeckel [skeleton]; (*11*) *Actinomma* aff. *langii* (Dreyer) [skeleton]; (*12*) *Haliommetta quadrisphaera* (Dogiel); (*13*) *Flustrella dujardinii* (Haeckel); (*14*) *Stylochlamydium asteriscus* Haeckel [skeleton looks similar to (*13*) but they can be distinguished by their internal structure]; (*15*, *16*) *Spongurus cylindricus* Haeckel [*15*: live image, *16*: skeleton]; (*17*) *Cladococcus* cf. *irregularis* Popofsky; (*18*, *19*) *Diplosphaera hexagonalis* (Haeckel) [*18*: skeleton, *19*: live image. A spherical cortical shell is encrypted inside the opaque intracapsulum]; (20) *Lithelius* spp.; (21, 22) *Larcopyle buetschlii* Dreyer [*21*: live image, *22*: skeleton]; (*23*) *Plegmosphaerella* cf. *entodictyon* (Haeckel); (*24*) *Plegmosphaera* sp.; (*25*, *26*) *Tetrapyle circularis* Haeckel [*25*: live image, *26*: skeleton]; (*27*, *28*) *Phorticium pylonium* Haeckel group [*27*: live image, *28*: skeleton]

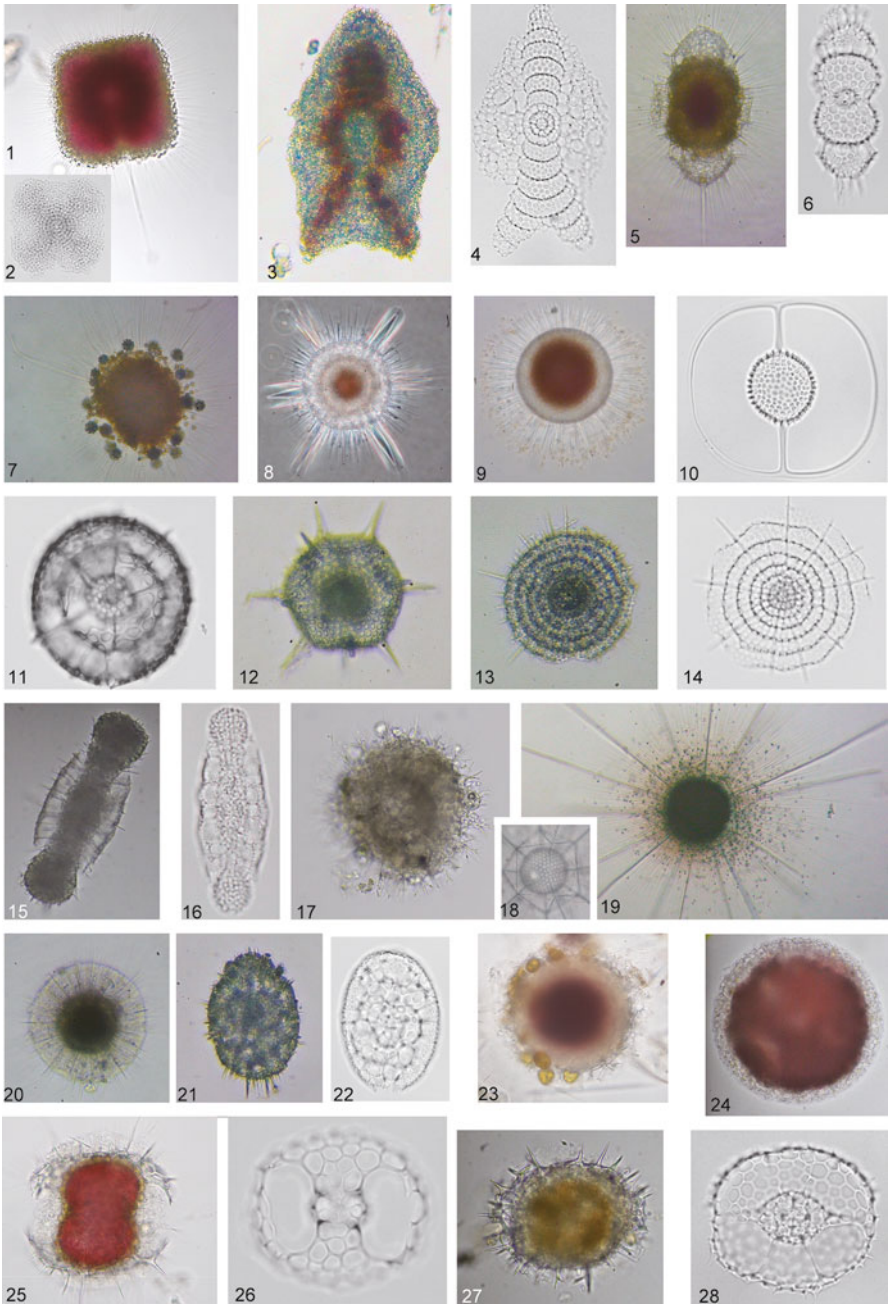


Fig. 8.8 Major spumellarian taxa: (*1–7*, *13*) Spongodiscoidea; (*8–10*) Hexastyloidea; (*11*, *12*) Actinommoidea; (*14*) Stylodictyoidea; (*15*, *16*) Sponguroidea; (*17–19*) Liosphaeroidea; (*20*) Lithelioidea; (*21*, *22*, *25–28*) Pylonioidea; (*23*, *24*) Spongosphaeroidea. Coccodiscidae (Fig. 8.8 (5, 6)) were placed in the superfamily Spongodiscoidea, according to the molecular phylogenetic data. (*1*, *2*) *Spongaster tetras tetras* Ehrenberg [*1*: live image, *2*: skeleton. Axoflagellum elongates from the skeleton]; (*3*, *4*) *Dictyocoryne perforatum* (Popofsky) [*3*: live image, *4*: skeleton]; (*5*, *6*)

the order Entactinaria, which is thought to represent a relict group from the Cambrian (De Wever et al. 2001). Because of strong morphological convergence among spumellarians, the taxonomic classification essentially relies on the innermost structure of the test.

Extant Spumellaria are divided into nine superfamilies, Actinommoidea, Liosphaeroidea, Hexastyloidea, Spongodiscoidea, Stylodictyoidea, Sponguroidea, Pylonioidea, Spongosphaeroidea, and Lithelioidea (Fig. 8.8), based on the taxonomic systematic unification between the morphological and molecular knowledge (Matsuzaki et al. 2015). Although they can be reliably identified only with the examination of the innermost structure, Spongodiscoidea and Stylodictyoidea tend to possess a flat-shaped skeleton, Pylonioidea have the intersected girdle-like structure regardless of the superficial appearance, and Lithelioidea convolve their test. These extant spumellarians are further classified into ~30 families, ~110 genera and ~380 species (Suzuki, unpublished data).

8.4.2 Reproduction and Motility

Release of swarmers has been repeatedly observed in genera *Dictyocoryne* (Spongodiscoidea), *Cypassis* (Coccodiscidae) and other spumellarians. No binary fission has been observed, but "Siamese twinned" spumellarians with exactly the same species have been found since the Middle Triassic (Itaki and Bjørklund 2008; Dumitrica 2013), which suggests asexual reproduction by binary fission.

Young spumellarian cells have double medullary shells and main radial spines, suggesting simultaneous skeletal formation at the PG stage (Fig. 8.4). These radial spines extend with SG and lateral appendages as a part of the outer cortical shell develops on each radial spine. Formation of a new cortical shell completes without intermediate morphotypes from the previous ontogenetic growth stage, suggesting simultaneous shell deposition at the SG stage (Suzuki 2006). SG and STG occur in the whole skeleton outside of the endoplasm or at specific sites on already existing skeleton, depending on species. Endoplasm increases in size with growth.

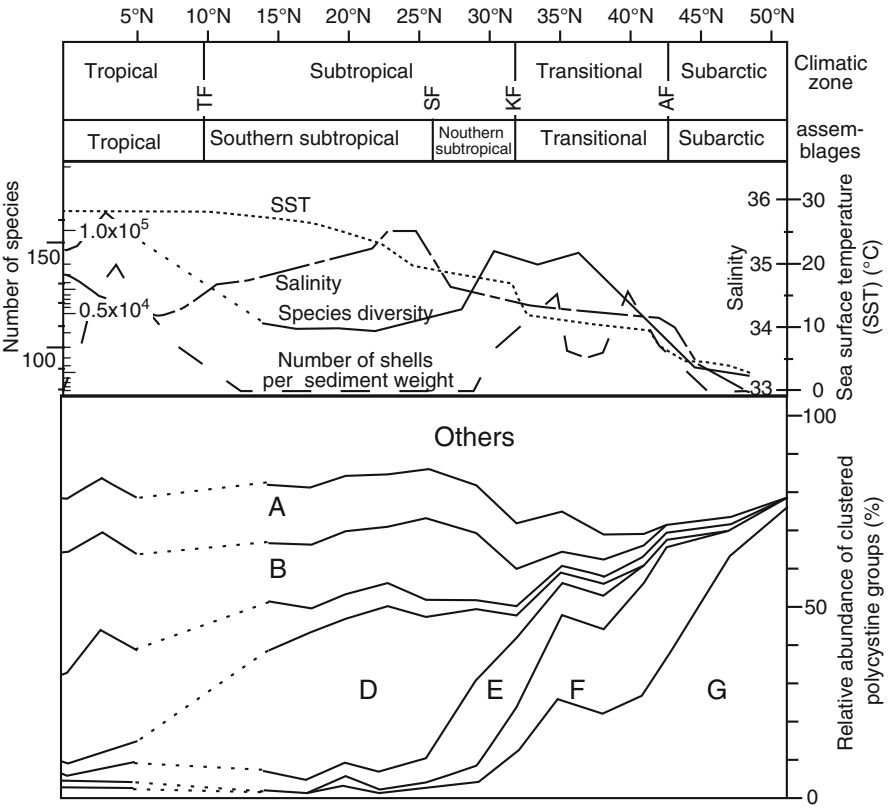
The mobile cytoplasm of Spumellaria is composed of pseudopodia. Pseudopodia are considered to play a role in buoyancy control, escape against attack, enhancement of cytoplasmic streaming inside the endoplasm, prey capture, adhesion, and disposal of unnecessary material (Suzuki 2005; Suzuki et al. 2009a). Pseudopodial response to stimuli is specific depending on taxa. In laboratory experiments, *Dictyocoryne* and *Spongaster* (Spongodiscoidea, Euchitoniidae) quickly withdraw their pseudopodia upon stimuli in order to sink, while *Diplosphaera hexagonalis* (Astrosphaeridae, Liosphaeroidea) has never responded to any strong stimuli (e.g., Suzuki and Sugiyama 2001). *Spongosphaera streptacantha* (Spongosphaeridae, superfamily unknown), *Arachnosphaera hexasphaera*, and *Rhizosphaera trigonacantha* connect their pseudopodia and loosely aggregate one to each other according to species and exchange stained granules of unknown function among them (Suzuki et al. 2012).

8.4.3 Trophic Characteristics and Biotic Interactions

One of the most important functions of pseudopodia in Spumellaria is adhesion. Spumellaria tightly capture swimming preys through adhesion on their pseudopodia (Sugiyama and Anderson 1997a; Suzuki and Sugiyama 2001). The captured prey can be significantly larger than the size of the radiolarian cell itself. Spumellaria mainly capture copepod nauplii larvae and occasionally tintinnids. Many tropical and subtropical spumellarian species have photosynthetic dinoflagellate symbionts (Fig. 8.8) (Takahashi et al. 2003; Ogane et al. 2010). Most known symbionts are present in the ectoplasm, although endocytoplasmic microalgae and bacteroids are found in the endoplasm of the flat-shaped *Dictyocoryne truncatum* (Anderson and Matsuoka 1992). Also, some flat-shaped Spumellaria such as *Dictyocoryne profunda*, *Myelastrum* and *Spongotrochus* possess numerous spherical bodies of 0.5–1.0 μm in diameter next to the capsular membrane. These bodies have thylakoid-like concentric structures and have been identified as cyanobacteria of the *Synechococcus* clade II based on small subunit ribosomal DNA (Yuasa et al. 2012). The *Synechococcus* clade II is known to be abundant in oligotrophic marine environments. Spumellaria have been shown to be infected by marine alveolates of the Syndiniophyceae class such as genus *Keppenodinium* or *Eudubosquella*.

8.4.4 Environmental Distributions

Spumellarian skeletons are well preserved in marine sediments worldwide and the species distribution is mainly inferred from sediment core studies (Lombari and Boden 1985; Kamikuri et al. 2008; Boltovskoy et al. 2010). The vertical and latitudinal distributions of Spumellaria have been well studied in the North Pacific (Matsuzaki et al. 2014) (Fig. 8.9). *Tetrapyle circularis*, *Phorticium pylonium*, *Dictyocoryne* spp., *Spongosphaera streptacantha* and *Didymocyrtis tetrathalamus* dominate in the subtropical photic zone. These species are characterized by the presence of photosynthetic algal symbionts. *Spongaster tetras irregularis* is found in the temperate photic zone only. *Rhizosphaera mediana* occurs mainly in the photic zone of subarctic–transitional latitudes. In the subarctic Pacific region, the photic zone is rarely associated with radiolarian polycystines. Instead, the subphotic zone (75–200 m water depths) is generally characterized by a cold water spumellarian fauna composed of the common *Cladococcus dentata*, *Larcopyle buetschlii*, *Rhizoplegma boreale*, *Spongurus cylindricus*, *Spongodiscus resurgens* and *Stylochlamydium*? *venustum* (Fig. 8.10). In the mesopelagic zone (200–1000 m), *Actinomma boreale* and *Spongopyle osculosa* are found from high to low latitudes (Fig. 8.9). Such distribution is driven by the presence of photosynthetic symbionts for most of the subtropical photic taxa, while it is mostly linked to temperature/depth requirement for non-photosymbiotic species. For instance, *Actinomma boreale* is commonly distributed in very shallow waters (<50 m water depths) in the



Cluster A: *Tetrapyle circularis, Phorticium pylonium, Zygocircus* cf. *piscicaudatus*

Cluster B: *Didymocyrtis tetrathalamus, Acanthodesmia vinculata, Tholospyris* sp., *Lithocircus reticulatus, Dictyocoryne* spp. *Stylochlamydium asteriscus*

Cluster C: *Spongaster tetras tetras, Dictyocoryne elegans, Stylodictya multispina, Heliodiscus* spp.

Cluster D: *Collosphaera huxleyi, Disolenia quadrata, Polysolenia spinosa, Siphonosphaera abyssi, Ellipsoxiphus atractus, Axoprunum stauraxonium*

Cluster E: *Pterocanium praetextum, Pterocorys zancleus, Lithopera bacca, Eucyrtidium acuminatum, Theocorythium trachelium, Thecosphaera inermis, Spongaster tetras irregularis*

Cluster F: *Anomalacantha dentata, Cycladophora bicornis, Rhizosphaera mediana, Lithelius haeckelispiralis, Spongopyle osculosa, Styptosphaera spumacea*

Cluster G: *Ceratospyris borealis, Lithomelissa setosa, Stylodictya validispina, Pseudodictyophimus gracilipes, Spongodiscus resurgens, Stylochlamydium? venustum, Cycladophora davisiana, Larcopyle buetschlii* *Actinomma boreale* group, *Botryostrobus auritus*, *Larcopyle borealis, Siphocampe arachnea*

Fig. 8.9 Species diversity of polycystines based on sea-floor sediment samples along 165°E, in the Pacific Ocean. Color of taxon names: *red* = taxa with photosynthetic symbionts, *blue* = mesopelagic or deeper taxa, *green* = photic taxa without photosynthetic symbionts, *black* = taxa whose water depth preference and existence of photosynthetic symbionts have never been examined. Highest species diversity, exceeding 150 taxa, is recorded in the tropical zone and regularly decreases when going northward. Abbreviations: *TF* (Tropical Front), *SF* (Subtropical Front), *KF* (Kuroshio Front), *AF* (Subarctic Front)

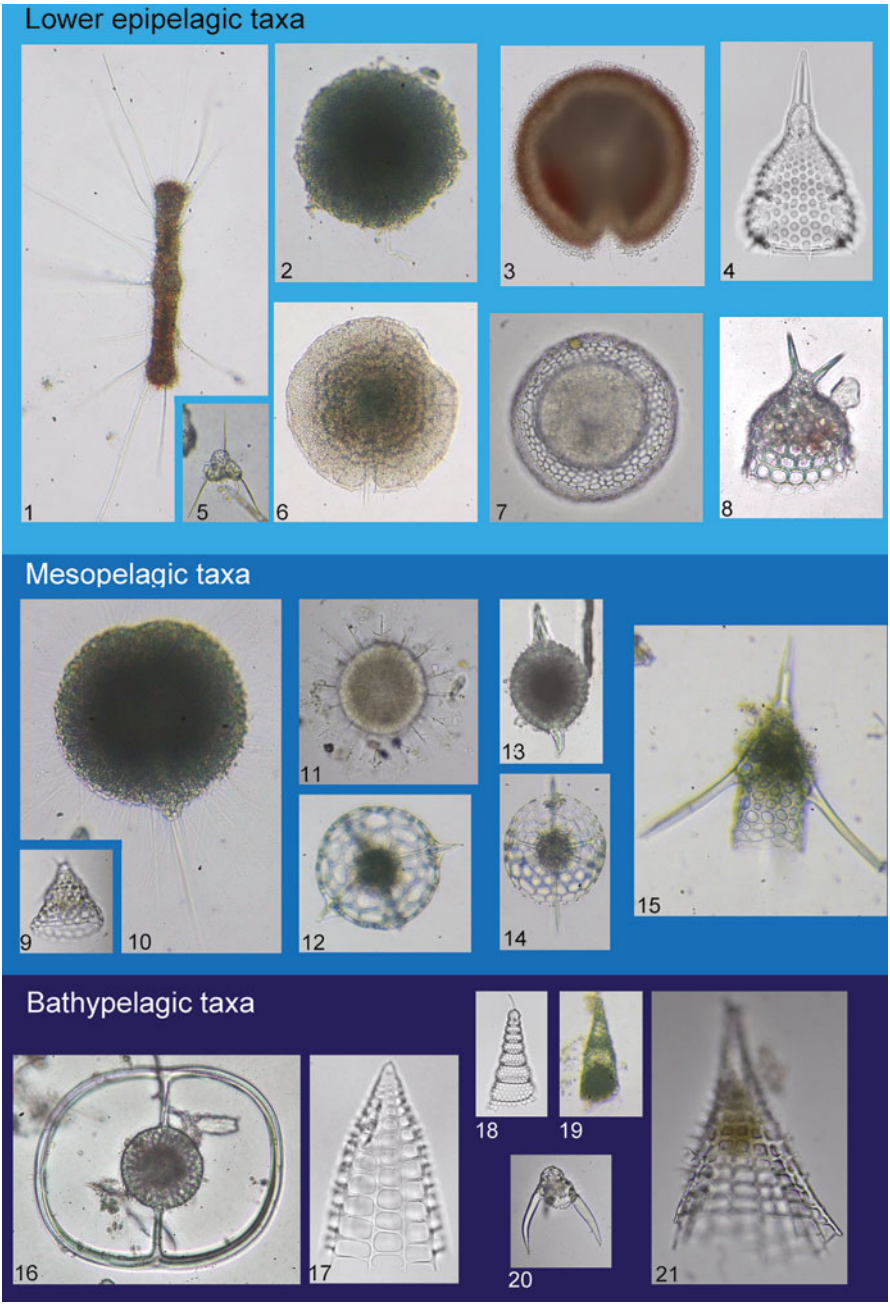


Fig. 8.10 Lower epipelagic (*1–8*), mesopelagic (*9–15*) and bathypelagic (*16–21*) spumellarians and nassellarians in the North Pacific. (*1*) *Spongurus cylindricus* Haeckel group [Sponguroidea]; (*2*) *Spongodiscus resurgens* Ehrenberg [Spongodiscoidea]; (*3*) *Schizodiscus disymmetricus* Dogiel [Stylodictyoidea]; (*4*) *Lamprocyclas maritalis* Haeckel [Pterocorythoidea]; (*5*) *Pseudodictyophimus gracilipes* (Bailey) [Plagiacanthoidea]; (*6*) *Stylochlamydium? venustum* (Bailey) [Spongodiscoidea]; (*7*) *Cyrtidosphaera reticulata* (Haeckel) [Liosphaeroidea]; (*8*) *Cycladophora bicornis* (Popofsky)

Norwegian fjords, but also dominates in deep waters (below 3000 m) in the Sea of Japan. Deep water spumellarian species living in bathypelagic and abyssopelagic zones are regularly found (Reshetnyak 1955). *Saturnalis circularis* is rare but always found in deep waters below 1500 m in the North Pacific (Fig. 8.10). *Plegmosphaera maxima*, *Stylotrochus geddesi*, *Schizodiscus stylotrochoides* and *Styptosphaera stupacea* (not *Stylospahera spumacea*) have been reported from 4000 to 8000 m water depths in the Kuril Trench (Reshetnyak 1955).

Radiolarian distributions at species level have been also studied in the Norwegian Sea and the Southern Ocean in order to examine their anti-tropical (or bipolar) distributions (Stepanjants et al. 2006). Based on morphological identification, anti-tropical distributions have been observed. The cold water–adapted *Actinomma boreale* is common in the Norwegian Sea, the Arctic, the deep water of the North Pacific but also in the Southern Ocean. *Stylochlamydium*? *venustum* live only in the subphotic zone (75–200 m water depths) of the subarctic North Pacific and this species is also common in the Southern Ocean.

Spumellarian species with algal symbionts (*Dictyocoryne truncatum*, *Didymocyrtis tetrathalamus*, *Spongaster tetras tetras*) were tested for their tolerance of salinity, temperature, light intensity and dissolved silica (Anderson et al. 1989, 1990; Matsuoka and Anderson 1992; Sugiyama and Anderson 1997a). These experiments demonstrated a broad tolerance to salinity changes, with some of those species surviving salinities as low as 30.0. They acclimated to water temperature above 31 °C and down to 10 °C, depending on the species investigated. The light intensity had no significant effect and low dissolved silica concentration did not affect the longevity and growth of the specimens.

8.5 Nassellaria

8.5.1 Cell Structure and Taxonomy

The skeleton of Nassellaria is heteropolar with one or more segments aligned along an axis, not a center (Fig. 8.11). From the narrower end, these segments are named cephalis, thorax, abdomen and post-abdominal segments (Fig. 8.2, Table 8.1) (De Wever et al. 2001). The most important skeletal structure for taxonomy at family or higher level is the cephalic internal spicular system consisting of a median bar

Fig. 8.10 (continued) [“theoperids”]; (*9*) *Cycladophora davisiana* Ehrenberg [“theoperids”]; (*10*) *Spongopyle osculosa* Dreyer [Stylodictyoidea]; (*11*) Astrosphaeridae gen. et sp. indet [Liosphaeroidea]; (*12*) *Hexaloche*? *aristarchi* Haeckel [Hexastyloidea]; (*13*) *Amphisphaera tanziyuani* Matsuzaki et Suzuki [Actinommoidea]; (*14*) *Axoprunum strauraxonium* Haeckel [Hexastyloidea]; (*15*) *Lamprotripus hirundo* (Haeckel) [Plagiacanthoidea?]; (*16*) *Saturnalis circularis* Haeckel [Hexastyloidea]; (*17*) *Cinclopyramis circumtextus* (Haeckel) [Acropyramoidea]; (*18*) *Cyrtolagena pectinata* (Haeckel) [superfamily unknown]; (*19*) *Cornutella profunda* Ehrenberg [Acropyramoidea]; (*20*) *Archipilium quasimacropum* Wang et Yang [Plagiacanthoidea?]; (*21*) *Polypleuris gigantheus* (Haecker) [Acropyramoidea]

(***MB***), an apical ray (***A***), a dorsal ray (***D***), a ventral ray (***V***), two lateral rays to the dorsal side (*Ll* and *Lr*), and two lateral rays to the ventral side (*ll* and *lr*) (Petrushevskaya 1971) (Fig. 8.12, Table 8.2: Suzuki et al. 2012). Endoplasm is just hooked on ***MB***, and three or four cytoplasmic lobes of endoplasm hang down from the internal spicular system. Ectoplasm is generally placed inside the test and cytoplasmic membrane surrounds the whole skeleton (Figs. 8.2, 8.3 and 8.11). Pseudopodia extend from the aperture at the distal end of the test and occasionally form a reversed-cone-shaped arrangement (Matsuoka 2007). A very thick single pseudopodium appears from the center of the aperture at the distal end of the test (Figs. 8.3 and 8.11). There are many pores, but only a few pseudopodia pass through these pores (Ogane et al. 2010; Suzuki and Aita 2011).

The taxonomy of Nassellaria at family level is based on the arrangement of the cephalic internal spicular system (Petrushevskaya 1971; De Wever et al 2001), although this is still debated. The extant Nassellaria can be divided into seven superfamilies: Acanthodesmoidea, Acropyramioidea, Artostrobiodea, Cannobtryoidea, Eucyrtidioidea, Lychnocanoidea, Plagiacanthoidea, Pterocorythoidea, and some undefined superfamilies (Fig. 8.11) (Matsuzaki et al. 2015). Overall the current nassellarian taxonomy consists of a total of ~25 families, ~140 genera and ~430 recognized species (Suzuki, unpublished data).

8.5.2 Reproduction and Motility

Little is known about the motility of Nassellaria, compared to other radiolarians. Distinguishable pseudopodia extend from some rods of the cephalic internal spicular system (Sugiyama and Anderson 1997b). When a pseudopodium lengthens from ***A***-ray, pseudopodia from other rays of the cephalic internal spicular systems tend to shrink. Nassellarian pseudopodia are marked by projections from a suture between adjacent segments and the middle part of a barrel-shaped segment (Suzuki and Aita 2011), but the function of these projections is unknown. Pseudopodia emerging from the distal end of the test move very actively. An axial projection extends up to several millimeters like a fishing line and draws back into the test in a repetitive manner. Strong adhesive function is recognized for the distal end of the pseudopodium from ***A***-ray (Fig. 8.12) and of the axial projection. Such function has never been observed in any other pseudopodia so far, but buoyancy control and escape against attack are possible behaviors related with other pseudopodia.

Reproduction of Nassellaria has never been directly observed. Multi-segmented Nassellaria appear to essentially form their segments simultaneously. Initial skeletogenesis, also called "pre-cephalic development", forms the cephalis initial spicular system at the PG stage (Swanberg and Bjørklund 1987), while the formation of the cephalic part is completed at the SG stage. The thorax, or its portion most adjacent to the cephalis, develops at SG (Fig. 8.4). A few segments or some appendage below the final segment occasionally develop in some species (Fig. 8.4). STG stage

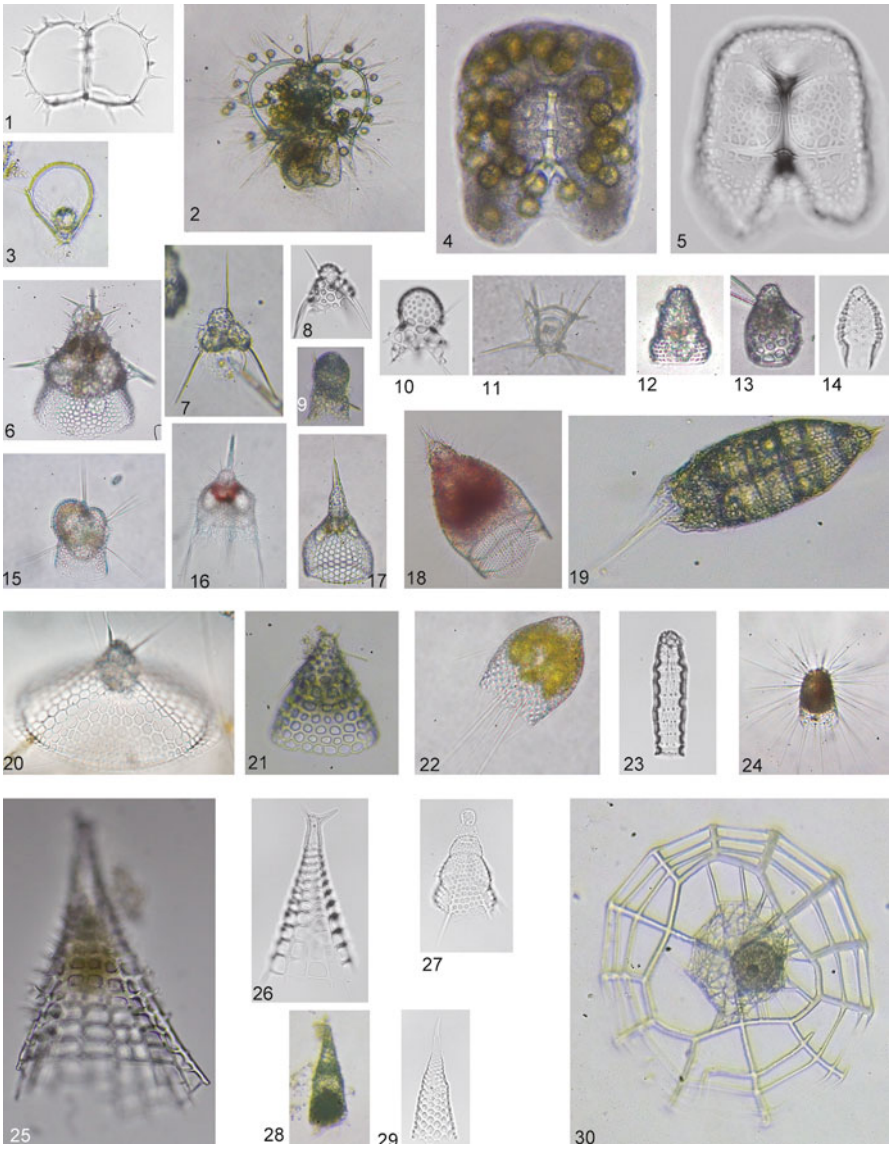


Fig. 8.11 Major taxonomic groups of Nassellaria. (*1–5*) Acanthodesmoidea; (*6–11*, *24*) Plagiacanthoidea; (*12–14*, *23*) Artostrobioidea; (*15*) Cannobotryoidea; (*16*) Lychnocanoidea; (*17*) Pterocorythoidea; (*18, 19*) Eucyrtidioidea; (*20, 21, 30*) "theoperids" (superfamily unknown); (*22*) "carpocaniids" (superfamily unknown); (*25, 26, 28, 29*) Acropyramoidea; (*27*) Superfamily unknown. (*1, 2*) *Acanthodesmia vinculata* (Müller) [*1*: skeleton, *2*: live image]; (*3*) *Zygocircus archicircus* Popofsky; (*4, 5*) *Lithocircus reticulatus* (Ehrenberg) [*4*: live image, *5*: skeleton]; (*6*) *Lipmanella prismatica* (Tan et Tchang); (*7*, *8*) *Pseudodictyophimus gracilipes* (Bailey) [*7*: live cell, *8*: skeleton]; (*9, 10*) *Dimelissa monoceras* (Popofsky) [*9*: live image, *10*: skeleton]; (*11*) *Pseudocubus obeliscus* Haeckel; (*12*) *Botryostrobus auritus* (Ehrenberg); (*13*, *14*) *Tricolocapsa papillosa* (Ehrenberg) [*13*: live image, *14*: skeleton]; (*15*) *Bisphaerocephalina armata*

often occurs on the cephalo-thorax-abdomen segment and tends to occur on the inner side of pores and on the whole final segment (Fig. 8.4) (Ogane et al. 2010).

8.5.3 Trophic Characteristics and Biotic Interactions

Nassellaria have been observed to capture large preys from the distal coiled end of the axial projection (Sugiyama et al. 2008). In these nassellarians, the axial projection lengthens up to several millimeters for a long time. Once the prey, as large as mollusk larvae, is attached on the distal coiled end of the axial projection, the nassellarian quickly withdraws it inside the aperture at the distal end of the conical test. Here, cone-like arranged pseudopodia around the aperture close to form a "fence" that prevents the prey from escaping. Several other marine organisms as large as mollusk larvae are often attached on the axial projection, but these organisms have never been trapped with the axial projection (XP in Fig. 8.3–(2)). Differently from spumellarians, nassellarians have never been documented to capture any nauplii. Some subtropical and tropical trophic taxa such as *Acanthodesmia vinculata*, *Lithocircus reticulatus* and *Dictyocryphalus hispidus* harbor a large amount of photosynthetic algal symbionts that are presumably providing nutrients.

Photosynthetic symbionts of Nassellaria have been identified as dinoflagellates only (e.g., Matsuoka 2007; Ogane et al. 2010). They are always located outside the endoplasm, but their position in the nassellarian test can vary: *Pterocorys zancleus* (Pterocorythoidea), *Lithopera bacca* (Eucyrtidioidea), and *Pterocanium praetextum* have them inside the thorax, *Acanthodesmia vinculata* and *Lithocircus reticulatus* are covered with numerous (50 or more) algal symbionts. No photosymbionts have been found in nassellarians belonging to Acropyramioidea (genera *Cinclopyramis*, *Cornutella*, *Polypleuris*, *Siphocampe*, *Spirocyrtis*, and *Cycladophora*). Probable parasites are Marine Alveolata Group I in *Lamprocyclas gamphonyxa* (Pterocorythoidea) (Dolven et al. 2007) and the dinoflagellate *Syndinium* sp. in *Eucyrtidium* sp. (Hertwig 1879).

8.5.4 Environmental Distributions

Nassellaria preserve well in sediments and are heavily studied by micropaleontologists (De Wever et al. 2001). Their distribution is somewhat similar to that of Spumellaria (Figs. 8.9 and 8.10) (e.g., Matsuzaki et al 2014). The common warm

Fig. 8.11 (continued) Petrushevskaya; (*16*) *Pterocanium praetextum eucolpum* Müller; (*17*) *Anthocyrtidium ophirense* (Ehrenberg); (*18*) *Eucyrtidium tropezianum* (Müller); (*19*) *Eucyrtidium octomeres* (Haecker); (*20*) *Lampromitra coscinodiscus* Ehrenberg; (*21*) *Cycladophora davisiana* Ehrenberg; (*22*) *Carpocanium obliquum* (Haeckel); (*23*) *Siphocampe hyperboreum* (Bailey) [skeleton]; (*24*) *Dictyocryphalus hispidus* (Ehrenberg); (*25, 26*) *Polypleuris gigantheus* (Haecker) [*25*: live image, *26*: skeleton]; (*27*) *Cyrtolagena pectinata* (Haeckel) [skeleton]; (*28, 29*) *Cornutella profunda* Ehrenberg [*28*: live image, *29*: skeleton]; (*30*) *Enneaphormis enneastrus* (Haeckel)

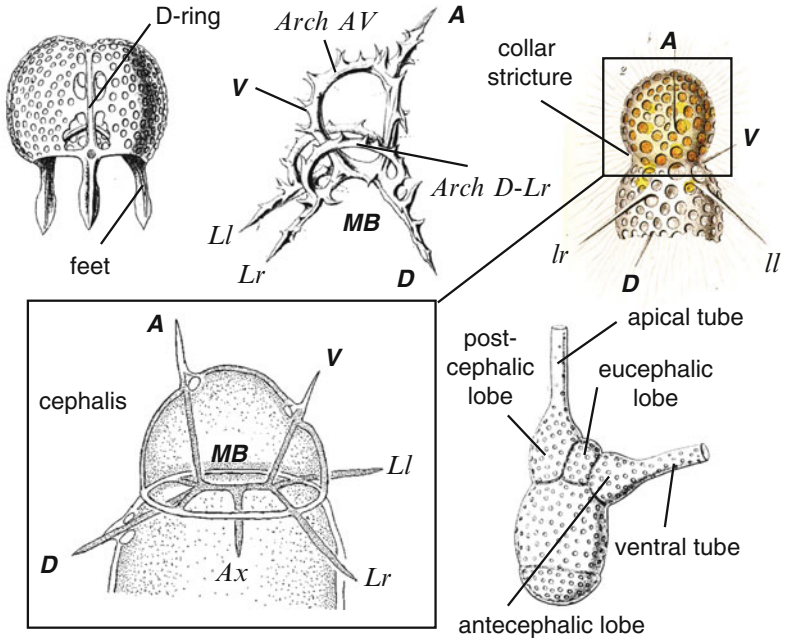


Fig. 8.12 Terminology of internal spicular system (or cephalic structure) for Nassellaria (Suzuki et al. 2012). See Table 8.2 for abbreviations

Table 8.2 Abbreviations of cephalic internal spicular system for Nassellaria

Taxonomic specifidation	Abbreviations	Terms
Nassellaria		
	A	Apical ray
	Arch AV	Arch connecting ***A*** and ***V***
	Arch D-Lr	Arch connecting ***D*** and *Lr*
	Ax	Axobate
	D	Dorsal ray
	D-ring	Dorsal ring
	Ll	Left lateral ray to the ventral side
	Lr	Right lateral ray to the ventral side
	ll	Left lateral ray to the dorsal (apical) side
	lr	Right lateral ray to the dorsal (apical) side
	MB	Median bar
	V	Ventral ray

See also Fig. 8.12

photic nassellarian species is *Acanthodesmia vinculata* but *Lithocircus reticulatus* is also abundant in oligotrophic warm photic waters. In the temperate photic zone, species *Eucyrtidium acuminatum*, *Lithopera bacca*, *Pterocanium praetextum*, *Pterocorys zancleus* and *Theocorythium trachelium* are commonly found. *Pseudodictyophimus gracilipes* lives in the subphotic zone (75–200 m water depths) in the North Pacific, and is often regarded as a subarctic nassellarian species. Representatives of deep water nassellarians are *Cycladophora davisiana*, *Botryostrobus aquilonaris*, *Lamprotripus hirundo* (mesopelagic zone, 200–1000 m), *Cornutella profunda* and *Cinclopyramis circumtexta* (bathypelagic zone, 1000–2000 m), and *Cyrtolagena cuspidata* (below 1500–3000 m). No nassellarian data are available for the deep sea below 3000 m.

Bipolar distributions are known for *Cycladophora davisiana* and *Pseudodictyophimus gracilipes* (Stepanjants et al. 2006), while a heteropolar distribution has been reported for the genus *Antarctissa* (Plagiacanthoidea).

8.6 Collodaria

8.6.1 Cell Structure and Taxonomy

Collodaria is the only radiolarian taxon with colonial representatives. It is currently composed of the three families Collosphaeridae, Collophidiidae, and Sphaerozoidae (Fig. 8.13) (Biard et al. 2015). Collosphaeridae are characterized by a single spherical shell with pit-like pores, and colonies are composed of tens to thousand cells. Sphaerozoidae also form colonies, but each cell is either surrounded by numerous isolated silica spicules or naked. Recent molecular phylogenies demonstrate that the former family Thalassicollidae, composed only of solitary specimens (single cell with or without silica spicules), is not valid as its representatives are scattered throughout other collodarian families (Biard et al. 2015). To date, a total of ~20 genera and ~80 species are considered as taxonomically valid taxa under ICZN. The size of the colonies can range from millimeters to tens of centimeters, its maximum reported length reaching as long as 3 m (Swanberg and Harbison 1980; Swanberg and Anderson 1981). The solitary forms of collodarian have a single, large nucleus, whereas colonial Sphaerozoidae and Collosphaeridae are marked by multi-nuclei in each cell (Suzuki et al 2009b).

In colonial Collodaria, the cells forming the colony exhibit synchronous or cooperating behavior. For instance, intracellular algal symbionts of Collodaria are scattered throughout the gelatinous matrix in daylight to improve photosynthesis efficiency, while they assemble around each radiolarian central capsule at night (Anderson 1983). Albeit that the colony itself is immobile, spontaneous contraction and relaxation of a colony occur upon stimuli. Deep blue bioluminescence (peak emissions between 443 and 456 nm) has been reported in both colonial and solitary forms of Collodaria (Latz et al. 1987; Campbell et al. 1981).

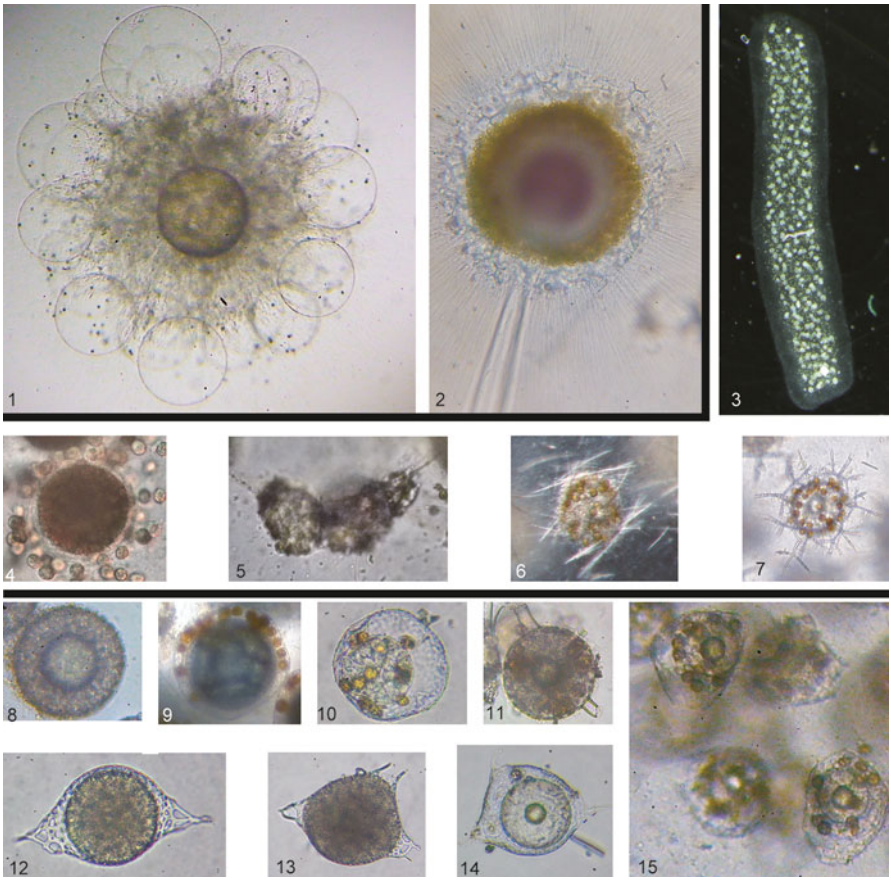


Fig. 8.13 Live collodarian specimens. Figure 9.15(1–3) show typical forms in collodarians: (*1*, *2*) Solitary collodarian form, (*3*) colonial collodarian form. Figure 8.13(4–15) are close-up images of the central capsule. Taxonomic positions at the family level: (*1, 2*) Thalassicollidae; (*3, 4, 6–8*) Sphaerozoidae; (*5, 9–15*) Collosphaeridae. Species level taxa: (*1*) *Thalassicolla nucleata* Huxley; (*2*) *Thalassoxanthium cervicorne* Haeckel; (*3*) *Collozoum* cf. *longiforme* Swanberg et Harbison; (*4*) *Collozoum inerme* (Müller); (*5*) *Collozoum pelagicum* (Haeckel); (*6*) *Rhaphidozoum acuferum* (Müller); (*7*) *Sphaerozoum verticillatum* Haeckel; (*8*) *Collosphaera huxleyi* Müller; (*9*) *Collosphaera tuberosa* Haeckel; (*10*) *Buccinosphaera invaginata* Haeckel; (*11*) *Siphonosphaera socialis* Haeckel; (*12*) *Otosphaera* aff. *polymorpha* Haeckel; (*13*) *Disolenia micractis* (Ehrenberg); (*14*) *Disolenia tenuissima* (Hilmers); (*15*) *Disolenia zanguebarica* (Ehrenberg)

Flashes of 1–2 s with 1×10^9 photons flash^{-1} and reaching up to 1.2×10^{11} photons colony^{-1} were recorded and are not derived from algal symbionts (Campbell et al. 1981). The Ca^{2+}-activated photoprotein (average emission: 440 nm) of the solitary Collodaria *Thalassicolla* is named “thalassicollin” (Campbell et al. 1981).

8.6.2 Reproduction and Motility

Colonial Collodaria with a cortical shell (e.g., *Acrosphaera cyrtodon* Haeckel) have been documented to multiply by binary fission (Anderson and Gupta 1998). Binary fission of *A. cyrtodon* starts before deposition of the siliceous skeleton when a very thin cytoplasmic envelope (cytokalymma) encloses the dividing central capsules. Subsequently to the divisions of the central capsules within the gelatinous matrix, the siliceous skeleton is deposited on the cytokalymma surrounding each central capsule. Once the skeleton is formed, both skeleton and central capsule maintain their size. The exact composition and production process of the gelatinous matrix is unknown, but the overall matrix volume increases in association with the increasing number of central capsules. Collodaria can also produce typical radiolarians swarmers that have a strontium sulfate crystal (2.66×7.04 μm) inside a vacuole of the central capsule (Hughes et al. 1989). The reproductive swarmer of *Sphaerozoum punctatum* is approximately 8–10 μm in length with a pear-like shape and a conical end with two flagella. The conical end contains a nucleus, mitochondria, Golgi, lipid droplet and a vacuole bounded celestite crystal (Yuasa and Takahashi 2014). After repeated binary divisions of the central capsules within a gelatinous matter, the siliceous skeleton deposits simultaneously within the cytokalymma surrounding each central capsule. Occasionally simultaneous shell deposition occurred during binary division and it results in the formation of twined shell (e.g., Sugiyama 1992). Once the skeleton develops by simultaneous shell deposition after binary division, both skeleton and central capsule keep their size.

8.6.3 Trophic Characteristics and Biotic Interactions

Colonial Collodaria thrive essentially in the oligotrophic ocean where they can represent a fair amount of organic matter due to their gelatinous matrix and numerous algal symbionts. Despite being presumably a valuable food source for larger predators, very little is known about the fate of these organisms as no predator has been described to feed on such colonies (Swanberg and Harbison 1980). In contrast, Collodaria have been reported as feeding on tintinnids and mollusk larvae (Swanberg and Caron 1991).

Many algal symbionts are present within each collodarian cell. Symbionts are usually located in the ectoplasm network of the colonies, across the gelatinous matrix, but in Collosphaeridae, they are located in the endoplasm, inside the cortical shell. The symbiont translocation of photosynthates to the host was estimated to be a minimum of 9–16 % of the total primary production by using the ^{14}C-labeling method (Anderson et al. 1983). *Collosphaera huxleyi* is characterized by the presence of a large oil droplet, presumably derived from algal symbionts, in the center of the endoplasm (Anderson 1983). Primary production by symbionts of solitary

and colonial Collodaria has been estimated to be equivalent to the primary production of several liters of seawater without Collodaria (i.e., 36 μg C colony^{-1} h^{-1} at > 10 % of full sun intensity = 12–67 l of seawater in the oligotrophic Sargasso Sea) (Swanberg and Harbison 1980; Caron et al. 1995). The content of chlorophyll-*a* in algal symbionts reaches as much as three times that of the free-living phytoplankton in the Gulf of Aden (Khmeleva 1967), and thus the relative contribution of radiolarians algal symbionts to total primary productivity is likely not negligible although most often not precisely quantified.

Collodaria can be infected by protists such as *Caryotoma bernardi* Hollande and Enjumet (Amoebophryaceae) (Hollande and Corbel 1982) but because of the large size of the colonies it is also the only protistan taxa harboring metazoan parasites. Colonies of *Collozoum longiforme* can bear hyperiid amphipods (*Hyperietta* spp.) and the harpacticoid copepod *Miracia efferata* as obligate parasites in their gelatinous matrix (Swanberg and Harbison 1980). Copepod parasites were observed not to feed on the radiolarian central capsule but to instead eat the matrix, algal symbionts, and peripheral pseudopodia.

8.6.4 Environmental Distributions

Colonies are rather fragile and their quantitative estimates are tedious because they break upon collection and do not preserve well in regular fixatives. Yet, Collodaria are commonly found in oligotrophic tropical to subtropical pelagic oceans such as the central part of oceanic gyres. This distribution is largely explained by the presence of numerous algal symbionts in the matrix of the colonies, thus providing the nutrients required to thrive in an otherwise hostile environment.

With gases inside the matrix, colonial Collodaria present a buoyancy that make them accumulate in the top few meters of the water column on calm days (Anderson 1983; Caron et al. 1995; Dennett et al. 2002). These near-surface abundance maxima are quickly removed by increased winds (Michaels et al. 1995). Large collodarian colonies density can occasionally reach 10 colonies m^{-3}. Both the seasonality and the longer-term fluctuation of standing stock in Collodaria are virtually unknown.

Based on data from shell-bearing, fossilizable collodarians and the fact that all the known species harbor algal symbionts, *Polysolenia spinosa*, *Siphonosphaera abyssi*, *Disolenia zanguebarica* (Fig. 8.13) are considered to be major warm, photic collodarians species (Lombari and Boden 1985; Matsuzaki et al. 2014).

8.7 General Ecology of Radiolaria

Knowledge on the ecology of Radiolaria essentially comes from Spumellaria and Nassellaria. This is because (1) radiolarian studies have been mainly driven by micropaleontologists, and these two groups are the only Radiolaria to be readily preserved in sediments; (2) collodarian colonies are easily broken off upon classical

plankton collection methods; and (3) acantharians, collodarians and taxopod cells are poorly preserved in regular fixation of plankton samples. Furthermore, most micropaleontological studies aiming at understanding radiolarian ecology grouped together spumellarians, nassellarians, and also often included Phaeodaria, making detailed ecological studies difficult. These technical and historical issues are the main reasons for the current radiolarian ecology being largely based on fossilized specimens from sea-floor sediments (e.g., Boltovskoy et al. 2010).

The global "biomass" of fossilized radiolarians is usually estimated by the number of tests in the sediments. The highest abundances were found in the central equatorial Pacific with more than 5.0×10^5 tests per gram of dry, carbonate-free sediment (Fig. 8.9) (Kruglikova 1993). Abundances west of Japan reach up to 1.0–2.0×10^5 tests per gram and abundances at mid-latitudes (i.e., 20–40°N zone) are lowest ($<1.0 \times 10^3$ tests per gram) in the North Pacific.

8.8 Seasonality of Polycystines in Different Regions

The seasonality of polycystine abundances varies between oceans (Fig. 8.14). In the equatorial Pacific, the standing stocks are high during both El Nino (740 shells m^{-3}) and La Nina conditions (670 shells m^{-3}). No significant change in fluxes is detected through the year in the Warm Water Pool of the equatorial Pacific (Fig. 8.14). In the west equatorial Pacific region, the flux of sinking radiolarian skeletons is mainly composed of the species *Tetrapyle circularis*, the *Tholospira cervicornis* group and *Didymocyrtis tetrathalamus tetrathalamus* (Takahashi and Yamashita 2004). In the mid- and high-latitude North Pacific, under marked seasonal cycles for primary productivity and nitrogen dynamics, no clear seasonal patterns are evident in nassellarian and spumellarian fluxes (Gowing 1993). The fluxes of nassellarians and spumellarians were $4.0–8.0 \times 10^3$ shells m^{-2} d^{-1} and $2.0–8.0 \times 10^3$ shells m^{-2} d^{-1}, respectively, in the 0–100 m water depth intervals throughout the year of 1987. Only *Lithopera bacca*, a nassellarian species with algal symbionts, displays a marked peak in the euphotic zone in summer. At high latitude, the inter-annual variations of nassellarians and spumellarians based on 15-year time series are rather closely related to large-scale climate indexes such as the Arctic Oscillations (Fig. 8.14) (Ikenoue et al. 2012). *Stylochlamydium*? *venustum*, a subphotic spumellarian species in the higher latitudinal North Pacific, is the only species to show seasonality in fluxes related to the primary productivity (Itaki and Takahashi 1994) (Fig. 8.7).

Polar oceans show strong seasonality in their physico-chemical status (e.g., sea ice coverage) and primary production. In the Southern Ocean, south of the Polar Frontal Zone, short-term radiolarian shell flux pulses appear during austral summer, reaching 4×10^3 shells m^{-1} d^{-1} which accounts for 90 % of the total annual radiolarian fluxes (Fig. 8.14) (Abelmann and Gersonde 1991; Abelmann 1992). These patterns are linked to the development states of sea ice coverage, timing of phytoplankton productivity, and the impact by zooplankton grazers like krill. In the Arctic, spumellarians and nassellarians in the Chukchi and Beaufort seas of the western Arctic are

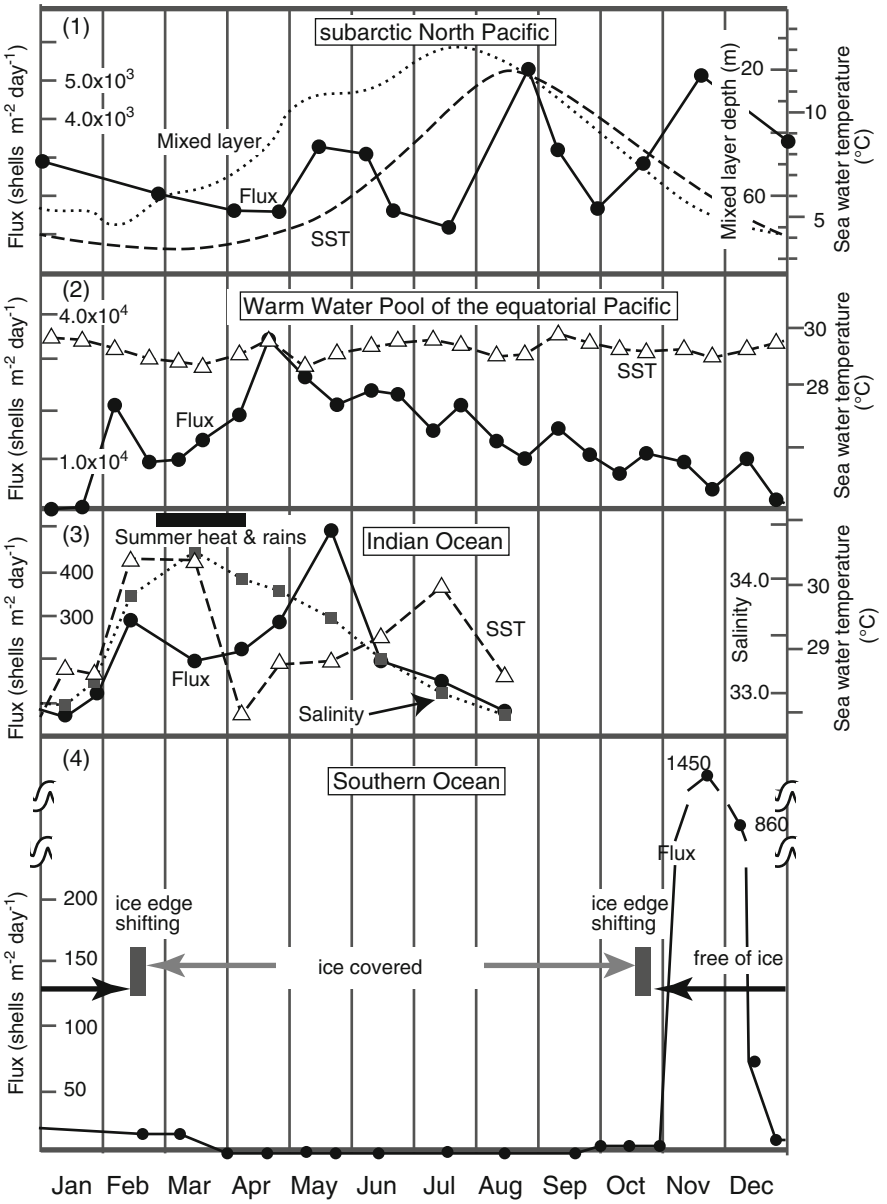


Fig. 8.14 Annual change of polycystine fluxes. (*1*) Fluxes in the subarctic North Pacific tend to drop in the late winter season, when sea surface water temperature is low and the mixed layer is deeper. Seasonal peaks in abundance are recognized in the early summer and the early autumn. (*2*) In the Warm Water Pool of the equatorial Pacific, an abundance peak is recognizable in the spring, but abundances are stable more or less year-round; (*3*) Polycystine abundance data in the Indian Ocean, eastern Bengal Bay. The polycystine abundance in the Bengal Bay increases after the high sea surface temperature and rainy season. (*4*) Reduction of sea ice cover in the early

similar to fluxes that were observed in the Greenland Sea near the sea ice edge and do not contain typical Pacific radiolarians in spite of the inflow of Pacific waters to this region (Itaki et al. 2003). As a consequence of a half year polar night in Arctic and the inflow of low salinity waters from sea ice melting, nassellarian and spumellarian abundances are low in the surface mixed layers (i.e. due to low salinity), and all the Arctic polycystines lack algal symbionts (e.g., *Amphimelissa setosa*, *Actinomma leptodermum* and *Pseudodictyophimus gracilipes*).

In the Atlantic, about 145 nassellarian and spumellarian species are reported, a number roughly equivalent to what is found in the equatorial Pacific (Boltovskoy et al. 1996). The annual vertical fluxes reach up to 2.8×10^4 shells m^{-2} d^{-1}, which is similar to other sinking organisms (e.g., silicoflagellates, dinoflagellates, planktic foraminifers, phaeodarians, tintinnids, ciliate cysts and pteropods) (Boltovskoy et al. 1993, 1996). In the case of the South Atlantic, the flux maximum in July–September coincides with the strongest trade winds, highest South Equatorial Current velocities, minimum sea surface temperatures and a deeper thermocline, while the spike in March–April could reflect dust fallout derived from the Sahara desert as a source of dust-associated nutrients (Boltovskoy et al. 1996).

At a smaller scale, in marginal seas such as the Gulf of California, Sea of Japan, Sea of Okhotsk, and Bering Sea, the ecological patterns exhibited by nassellarians and spumellarians are characteristics. For instance, in the Sea of Okhotsk, the vertical fluxes of nassellarians and spumellarians exhibit significant summer to autumn peaks with lower values during the winter when sea ice is present (Okazaki et al. 2003; Abelmann and Nimmergut 2005). The faunal diversity increases when radiolarian fluxes increase, which is also well correlated with total organic carbon (TOC) fluxes. Several significant seasonalities are detected in the fluxes of *Cycladophora davisiana*, a typical intermediate water dweller in the Sea of Okhotsk and Bering Sea (Fig. 8.7 (4)). This seasonality is linked to surface water inflow of melted water from sea ice and river runoff (Itaki and Takahashi 1994) and species increases are associated with the supply of terrigenous organic material and minerals (Okazaki et al. 2003). The productivity maxima of shallow-living species coincide with spring and autumn phytoplankton blooms in the Sea of Okhotsk (Hays and Morley 2004). In the South China Sea, abundance and species compositions of nassellarians and spumellarians significantly change in relation to drastic change in oceanographic physical properties between summer and winter, in response to the Asian monsoon (Zhang et al. 2009). In particular, *Peromelissa phalacra*, *Botryocyrtis scutum* and *Pterocorys hertwigi* vary in abundance seasonally, despite no seasonal change in the adjacent Pacific Ocean region.

Fig. 8.14 (continued) ice-free season. These graphs are composites for different years, and compiled from published data (Ikenoue et al. 2012, for the subarctic North Pacific; Okazaki et al. 2008, for the Warm Water Pool of the equatorial Pacific; Gupta 2002, for the Indian Ocean; Abelmann 1992, for the Southern Ocean). These data include not only Spumellaria and Nassellaria, but also shell-bearing Collodaria. Okazaki et al. (2008) also include Phaeodaria, but this may not seriously compromise the trends observed in their data

8.8.1 Upwelling Regions

Upwelling regions are characterized by the upward supply of cold, nutrient-rich waters to shallow areas, inducing phytoplankton blooms and ultimately leading to high primary productivity. Upwelling can be classified into three types: coastal upwelling, equatorial upwelling and Southern Ocean upwelling. The total abundance of radiolarians tends to significantly increase in upwelling areas (Thiede 1981), and the species composition seems also to be related to the strength of upwelling in the different oceans (Nigrini and Caulet 1992). The species composition of upwelling polycystine assemblages is divided into three categories: (1) endemic upwelling species; (2) displaced temperate species which are abundant in middle latitudes, but which can be found in tropical areas influenced by upwelling; and (3) enhanced tropical species which are found in tropical regions, but which are more abundant and/or have more robust tests in areas of upwelling (Nigrini 1991).

Endemic Upwelling Species *Dictyophimus infabricatus* and *Inverumbella macroceras* are found mainly in the Benguela, Peru and Somali upwelling regions. The following species are relatively common in the Peru upwelling area: *Pterocanium grandiporus*, *Lamprocyclas maritalis ventricosa*, *Eucyrtidium erythromystax*, *Phormostichoartus schneideri*, and *Pseudocubus warreni. Lamprocyclas hadras* and *Plectacantha cremastoplegma* are found not only in the Peru, but also in the Benguela upwelling region. *Eucyrtidium aderces* and *Anthocyrtidium rectidentatum* are detected only in the Oman upwelling area (Nigrini and Caulet 1992; Jacot Des Combes and Abelmann 2007).

Displaced Temperate Species *Acrosphaera murrayana*, *Pentapylonium implicatum* and *Pterocanium auritum* are recognized as displaced temperate species in the Peru, Oman and Somali upwelling regions (Molina-Cruz 1984; Nigrini and Caulet 1992). In addition, *Acrosphaera murrayana* also becomes abundant in the Gulf of Tehuantepec (Molina-Cruz and Martinez-López 1994). *Arachnocorallium calvata* represents up to 57 % of all the polycystines off West Africa (Boltovskoy et al. 1996) and in the southern California Current regions (Boltovskoy and Riedel 1987), suggesting this to be a displaced temperate species. In the Gulf of California, *Pseudocubus obeliscus* (*Plagiacantha panarium* in the original paper) is dominant (Molina-Cruz et al. 1999). In the region of the Benguela upwelling, the displaced temperate species *Lithomelissa setosa* contributed from 50 to 80 % of the total polycystine assemblages under the subsurface, with temperatures of 10 °C and high nutrient conditions (Matul' 1998). *Lithomelissa setosa* and *Pseudocubus obeliscus* are abundant in the equatorial Pacific upwelling regions (Takahashi and Yamashita 2004). *Collosphaera huxleyi* is recognized as an upwelling species in the Somalia upwelling region (Vénec-Peyré and Caulet 2000).

Enhanced Tropical Species *Pterocorys minythorax*, *Lamprocyrtis nigriniae*, and *Lithostrobus* cf. *L. hexagonalis* belong to this category (Nigrini and Caulet 1992). The upwelling region off the Gulf of Tehuantepec, Mexico, is dominated by *Tetrapyle octacantha* group and *Didymocyrtis tetrathalamus* (Molina-Cruz and Martinez-López 1994).

8.8.2 Major Factors Controlling Polycystine Distributions

No diel vertical migrations have been observed in Nassellaria and Spumellaria (Zhang et al. 2009), but their highest standing stocks are usually observed in close proximity to the deep chlorophyll maximum (DCM). It has been suggested that this pattern would be due to adaptation of symbiont photosynthesis to lower light levels or in response to higher densities of prey at the chlorophyll maximum (Dworetzky and Morley 1987). Yet, it is currently assumed that the spatial distributions of nassellarians and spumellarians, and fluctuations in their standing stocks and fluxes are largely controlled by physical processes in the water masses rather than biotic controls (Welling and Pisias 1998) and large-scale climate changes such as ENSO (El Niño-Southern Oscillation) and AO (Arctic Oscillation) (Ikenoue et al. 2012). Because of the strong link between paleoclimate studies and radiolarian microfossils, sea surface temperature has been one of the main physical parameters investigated to explain polycystine assemblages (Fig. 8.9). Sea surface temperature has been shown to drive relative abundance of polycystine assemblage in the Southern Ocean and in the North Pacific (Cortese and Abelmann 2002; Kamikuri et al. 2008). Based on a data set of relative abundance in surface sediments, a transfer function equation is established and applied to the fossil records. The accuracy of the estimated water temperature in the past mainly depends on the precision of taxonomic classification. If the data and taxonomic classification are accurate, the estimation error of the temperature is within the range of ±0.78 °C ($r^2 = 0.988$) (Matsuzaki et al. 2014). Radiolarians' response to salinity is ambiguous, as only one example was reported where the relative abundance of pylonioid spumellarians (2–18 %) showed a positive correlation to salinity (34.1–34.8) ($r^2 = 0.8245$) (Gupta 2002).

It is generally expected that high dissolved silica content enhances silicification of polycystines, but this expectation is not supported by laboratory experiments which clearly show no responses to enriched dissolved silica (Sugiyama and Anderson 1997a; Suzuki et al. 2013). If dissolved silica becomes higher, pylonioid Spumellaria start to dispel formed siliceous fragments (Suzuki et al. 2013), and if the dissolved silica reaches unusual concentration (up to 150 mM), flat-shaped radiolarians die (Sugiyama and Anderson 1997a). There is thus no significant evidence about the positive relationship between dissolved silica and radiolarian abundances and degree of silicification so far.

8.8.3 Contributions to Inorganic Biochemical Cycle

Because of their role in oceanic food webs, the fact that they sink rapidly after death, and the primary production by their photosynthetic symbionts, radiolarians have a clear impact on the carbon biogeochemical cycle. Radiolarians also play important roles in inorganic chemical cycles in different ways. Acantharia fix strontium and barium in their mineralized skeleton, but these chemicals easily return to seawater after death. Yet, the abundance of Acantharia is such that it impacts the total concentration of strontium and barium in seawater (Bernstein et al. 1992). Acantharians, and in particular the cyst-forming ones, can also contribute significantly to particulate organic carbon flux as deep as 3000 m depth at high latitude and at certain times of the year when surface primary production is important (Martin et al. 2010; Decelle et al. 2013). Silica fixation by Spumellaria and Nassellaria mainly contributes to transport silica to the sea bottom as siliceous sediments worldwide. The contribution of silica fixation by polycystines was very important between the late Carboniferous and the Early Cretaceous as "chert" rock (or radiolarites) was deposited worldwide (see details in Suzuki and Oba 2015).

Heavy metal and rare elements are detected in polycystine skeletons (Chen and Tan 1996). Some of these elements (Al, Fe, Mn, Cu, Cr and Ba) are densely concentrated in the skeleton in comparison with Rare Earth Element (REE) in seawaters and the average of the Earth clay. Polycystine skeletons tend to adsorb cerium (Ce), a process linked to silicification in live specimens, or to selective adsorption from surrounding sediments on remaining shell of dead specimens. If these elements are absorbed by biological process, nassellarians and spumellarians may play an important role for the chemical cycle of depleted elements in seawaters such as Cu, Cr and Ba. Acantharian skeletons contain large quantities of major elements such as Sr and Ba (964.89 ng), Ca (19.60 ng), Zn (4.32 ng), Fe (3.84 ng), Rb (3.40 ng), K (2.87 ng), Pb (2.69 ng) (Brass 1979).

In the North Pacific, the largest fluxes of acantharian skeletons and cysts were detected at 400 m, and minimum to non-detectable fluxes are recorded below 900 m, where the majority of acantharians had dissolved (Bernstein et al. 1987). Yet sinking cysts can reach much deeper depths at specific times of the year (Martin et al 2010, Decelle et al 2013). High barite concentration in marine sediment matches areas of high surface productivity. Formation of marine barite ($BaSO_4$) is closely related with dissolution of acantharian celestite (Bernstein and Byrne 2004). The $(Ba)_t/(Sr)_t$ molar ratio of acantharians is 3.8×10^{-3}, which is approximately 10 times larger than the dissolved $[Ba^{2+}]_T/[Sr^{2+}]_T$ molar ratios of surface ocean waters (Bernstein et al. 1992). This means that acantharian skeletons likely play a larger role in oceanic Ba cycling than in the Sr cycle. Acantharia are the unique biological source of Sr in seawater, so Sr/Ca ratios may be affected by acantharians in the upper 400 m of the oceans (De Deckker 2004).

8.9 Research Perspectives on Living Radiolarians

Since the first description of these beautifully shaped planktonic creatures, study of Radiolaria has been essentially performed by micropaleontologists. The knowledge on living radiolarians is strongly fragmentary and largely comes from collodarians, studied by a handful of dedicated scientists who performed tremendous work in the 1970s and 1980s (Anderson 1983). For most Radiolaria, even very basic information, such as exact taxonomy, life cycle, trophic behavior, and biogeography, is crucially missing, and this hampers the understanding of their biology and ecology, even though many efforts have been made in this direction (Petrushevskaya 1971; De Wever et al. 2001; Suzuki and Aita 2011). With the advent of new technologies and taking advantage of recent progress in various fields of science, we believe radiolarian research is facing an exciting future.

Integrative taxonomy, combining morphology and molecular taxonomic markers, is a powerful approach to address complex taxonomic issues (e.g., the existence and/or position of Entactinaria, which are currently disputed) and simultaneously providing a strong reference framework for environmental studies. At least the morphological classification of Nassellaria and Spumellaria at superfamily level (Matsuzaki et al. 2015) seems to be concordant with the knowledge derived from molecular phylogeny. Radiolarian molecular sequences are among the most represented in culture independent environmental diversity survey of eukaryotes (Not et al 2009; Massana 2015). Being able to assign more accurately these sequences based on a strong morpho-molecular reference framework is determinant for the understanding of large biogeographical patterns, but can also give insight into specific biological processes.

Not being able to go through the radiolarian life cycle ex situ (i.e., to grow them in culture) is currently a major drawback to be worked out. Still, mid-term maintenance is possible and allows experimental work to investigate radiolarian biology. Coupled to experimental set up, advances in single-cell technologies are particularly promising to circumvent the limited amount of biological material available (Decelle et al. 2014). Ecological relevance of Radiolaria for contemporary ecosystem functioning still has to be clearly demonstrated. Appropriate sampling and preservation procedures are required to accurately quantify radiolarians in the environment. Alternatively for large but fragile organisms such as Collodaria, *in situ* imaging technologies are very promising (Dennett et al. 2002). Together with a holistic oceanography approach considering all biotic and abiotic parameters available, these tools can radically change our perception of the role radiolarians play in pelagic ecosystems.

Finally, radiolarians appeared in the late Neoproterozoic, and some of them start to silicify at the Cambrian Explosion (Suzuki and Oba 2015). As many as 2700 genera and 16,000 species of radiolarian fossils are preserved continuously over geological periods in rocks and sediments. Careful combination of molecular phylogenetic and micropaleontological analyses has a great potential for understanding

radiolarian evolution based on the molecular clock (Decelle et al. 2012a). Also, integrative multidisciplinary studies of living Radiolaria based on morphological taxonomy, sediment traps, and environmental sequences are very promising to better estimate ecological preferences of specific taxa, ultimately leading to more accurate equation of transfer functions for paleoceanographic and paleoclimatologic studies.

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Chapter 9
Phaeodaria: Diverse Marine Cercozoans of World-Wide Distribution

Yasuhide Nakamura and Noritoshi Suzuki

Abstract Phaeodarians are unicellular marine protists characterized by the "central capsule" containing the nucleus, the "phaeodium", or mass of brown particles, and a siliceous skeleton called the "scleracoma". Phaeodaria have long been classified as a member of the Radiolaria; however, this protist group now belongs to the phylum Cercozoa. The ancestor of phaeodarians is thought to have appeared in the upper Triassic Epoch according to the fossil record. They reproduce by cell division and swarmer production. These plankton are heterotrophic, and they presumably feed on organic materials suspended in the water column or capture other plankton. Although this group is widely distributed in the world ocean from the surface to deep waters, they have attracted little attention from marine researchers partly because their abundance has long been underestimated. Recent study, however, revealed that phaeodarians are more numerous than expected, and their high abundance is sometimes reported. Considering their occasional high biomass and the fact that their scleracoma is made chiefly from silica, this plankton group plays an important role in local ecosystems and has a large impact in the silica cycle of the ocean. Knowledge of phaeodarian is indispensable for future oceanography; therefore the hitherto-known information on this marine protist is comprehensively reviewed in this chapter.

Keywords Phaeodaria • Tripylea • Cercozoa • Unicellular • Zooplankton • Protist • Deep sea • Matter cycle • Silica

Y. Nakamura (✉)
Plankton Laboratory, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
e-mail: y.nakamura@fish.hokudai.ac.jp

N. Suzuki
Institute of Geology and Paleontology, Graduate School of Science, Tohoku University, Sendai 980-8578, Miyagi, Japan
e-mail: norinori@m.tohoku.ac.jp

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9.1 Introduction

9.1.1 What Are "Phaeodarians"?

Phaeodaria are a group of holoplanktonic marine protists. This cercozoan group adapts to marine environments and plays an important role in local ecosystems. Phaeodaria are heterotrophic plankton which chiefly live in pelagic open oceans from the surface to the deep sea. No phaeodarians have been reported from brackish and high-salinity environments up to the present. Their cell size ranges from several hundred micrometers to a few millimeters and depends on the families to a certain extent. The size of the family Tuscaroridae exceeds 3 mm. By contrast, the family Challengeriidae is generally smaller, reaching a few hundred micrometers at the largest (Fig. 9.1). Some phaeodarians bearing spherical skeletons are similar to polycystines and acantharians, but the group in question is essentially different by possessing more porous and fragile skeletons, a peculiar central capsule and a mass of brown particles, phaeodium. Some phaeodarians of the family Challengeriidae resemble marine dinoflagellates (e.g., *Ceratium gravidum*); therefore these two are occasionally mistaken for each other. Such dinoflagellates, however, can be correctly distinguished by the presence of grooves and the absence of phaeodium.

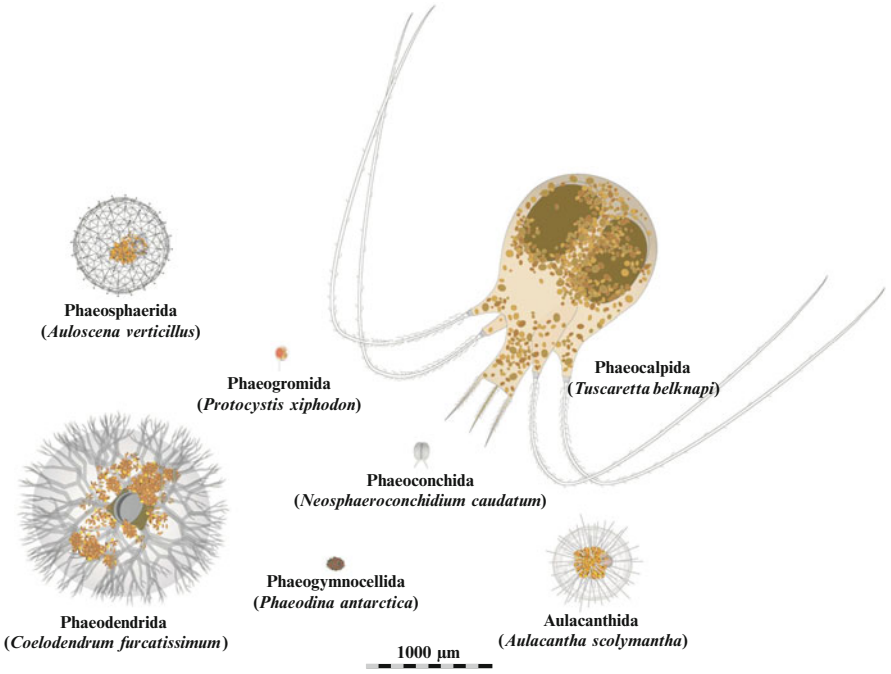

Fig. 9.1 Comparison of cell size between phaeodarian orders

Phaeodarians are often broken by normal sampling methods and are not very abundant in comparison with copepods and ciliates in the euphotic zone, so this protist group has hitherto attracted little attention from plankton researchers as a subject of research. Since the majority of the tremendous taxonomic and ecological studies by Germans ended after World War I, the principal information concerning phaeodarians has been little updated until today. However, our understanding of their taxonomic position and skeletal chemical composition has progressed little by little. They have long been regarded as Radiolaria because of the presence of "ray-like pseudopodia" and a "central capsule" (Calkins 1909), but Phaeodaria are now classified as a subclass of the phylum Cercozoa (Polet et al. 2004; Nikolaev et al. 2004; Yuasa et al. 2005; Howe et al. 2011; Adl et al. 2012; Sierra et al. 2013). This taxonomic revision is important to elucidate the ecological differences between Phaeodaria and "true" Radiolaria (e.g., Suzuki and Aita 2011, Suzuki and Oba, Chap. 15, this volume) and lets us know that we have been unable to estimate the true abundance of phaeodarians with some previous studies, because in such studies, phaeodarians were treated together with the "true" Radiolaria.

Another important point is about the chemical composition of the skeleton (or scleracoma). The phaeodarian skeleton used to be considered as "admixtures of organic matter and silica" or "organic siliceous matter" (e.g., Haeckel 1887). However, it is now confirmed that more than 90 % of their skeleton is actually made up with silica, and organic matter is absent in the structure (Takahashi et al. 1983; Bernstein et al. 1990; Takahashi and Hurd 2007).

9.1.2 History

The first phaeodarian species was described in 1856. Dead specimens of Lirellidae, *Lirella baileyi* Ehrenberg (the original taxonomic name *Cadium marinum* Bailey was replaced by the Ehrenberg name because the former was a junior secondary homonym according to the International Code of Zoological Nomenclature), were collected from surface sediments in the Bering Sea (Bailey 1856), and this species was thought to be a member of Rhizopoda. Living phaeodarians were first illustrated from plankton samples in the Mediterranean Sea (Haeckel 1862). Haeckel (1862) regarded phaeodarians as a member of Radiolaria. Hertwig (1879) clarified their protoplasmic character and named them "Tripylea" after the characteristic three pores on the surface of the central capsule ("Tripyleen" in German is derived from *tri-* Greek three and *pýlē* Greek gate). However, as these structures are not seen in all species, Haeckel (1879) created the taxon name "Phaeodaria" from the presence of "phaeodium."

The high morphological diversity of phaeodarians was recognized thanks to the HMS Challenger Expedition (1872–1876). Preliminary reports of the expedition were published as early as 1876, and these documents described the family Challengeriidae as highly diverse deep-sea marine protists. The discovery of this family is one of the distinguishing fruits of this expedition (Murray 1876). Plankton

sampling from deep layers revealed that the phaeodarians have high species diversity in the deep seas of the Pacific and the South Atlantic Ocean, and they were assumed to be "deep-sea plankton" (Murray 1876; Haeckel 1887). Their global distribution was further investigated by several expeditions from the late nineteenth to the early twentieth century (e.g., the Valdivia Expedition 1898–1899, the Plankton Expedition in 1889, and the Gauss Expedition 1901–1903). The Valdivia Expedition, or "die Valdivia-Tiefsee-Expedition", carried out scientific programs from the African western coast via the East Antarctic coast to the Indian Ocean between July 1898 and April 1899, and various samples were collected down to 6000 m water depths, particularly in the Southern Ocean. Most of the phaeodarians from these samples were studied by Haecker (1904, 1905, 1906, 1907a, b, 1908a, b). The Plankton Expedition targeted plankton in the North Atlantic Ocean reaching to 60°N from July to November 1899, and collected samples down to 3500 m water depths at 33 stations. These samples made remarkable contributions to the biology of almost all the families (Borgert 1905a, b, 1906, 1907, 1909a, b, c, 1910, 1911, 1913, 1922). The Gauss Expedition, or "die Deutsche Südpolarexpedition", investigated around Antarctica, and its samples were also used for the study of phaeodarians (Schröder 1906, 1913). These investigations clarified the taxonomy and ecology of the greater part of this group. The phaeodarians in the South Atlantic were studied with the samples of the German Meteor expedition, or "die Deutsche Atlantische Expedition", (1925–1927), and the vertical distributions of this protist group at family level were documented as deep as 1500 m water depths (Meyer 1933). Deeper vertical distribution was researched down to 8000 m water depths of the Kuril-Kamchatka Trench by the expedition of R/V *Vityaz* between 1949 and 1979 (Reshetnyak 1955). This is the deepest record of phaeodarian sampling (Reshetnyak 1966).

From the 1950s to the 1970s, the cell structure and cell division process were examined by French and German cytologists (e.g., Cachon-Enjumet 1961; Cachon and Cachon 1973; Grell 1953, 1973), focusing on some phaeodarians possessing a large protoplasm with very significantly observable chromosomes. The abundance of radiolarians including phaeodarians has been investigated as a component of marine plankton since the late 1960s (e.g., Beers and Stewart 1969). According to some dozens of "radiolarian" papers which divide "radiolarians" into polycystines, acantharians and phaeodarians, the vertical flux of phaeodarians was estimated in the western North Pacific (e.g., Bernstein et al. 1990), the eastern North Pacific (e.g., Takahashi 1987; Gowing and Coale 1989), the Sea of Okhotsk (e.g., Okazaki et al. 2003), the Bering Sea (Ikenoue et al. 2012), the tropical Atlantic (e.g., Boltovskoy et al. 1993a, b) and the Southern Ocean (e.g., Abelmann 1992; González 1992).

The classification system of phaeodarians has been modified by some authors (Campbell 1954; Cachon and Cachon 1985; Kling and Boltovskoy 1999; Takahashi and Anderson 2000) since Haeckel (1887) established a framework of the phaeodarian taxonomy. These systems were, however, constructed based only on the morphological criteria. Cachon and Cachon (1973) have insisted that Phaeodaria must be clearly separated from Acantharia and Polycystina because of the peculiar

microtubular systems and cytological characters of Phaeodaria. However, researchers have not followed after their opinion (Anderson 1983; De Wever et al. 2001). Molecular studies focusing on protists including phaeodarians started in the first decade of this century (e.g., Polet et al. 2004; Nikolaev et al. 2004), with the result that the taxonomic position of Phaeodaria changed from a member of Retaria to one of Cercozoa, together with Foraminifera and Radiolaria (Polet et al. 2004; Yuasa et al. 2005).

9.2 Classification

9.2.1 Cercozoa

Phaeodaria are now classified as a member of Cercozoa, which are a group of protist including ebridians and chlorarachniophytes, for example. Cercozoa are first recognized as a single supergroup containing some eukaryotes whose taxonomic positions were unknown. The unity of this group was confirmed mainly by molecular analysis of 18S rDNA (Cavalier-Smith 1998). In the current classification system, the phylum Cercozoa taxonomically belongs to the Infrakingdom Rhizaria (Nikolaev et al. 2004; Sierra et al. 2013), and this phylum is divided into 14 groups (Table 9.1). This protist group includes organisms of diverse morphology: filose testate amoebae, zooflagellates, colonial flagellates, protists with axopodia, soft-bodied amoeboflagellates, amoebae with chloroplasts developed from an ingested green alga, testate reticulose amoebae, etc. (Howe et al. 2011).

Table 9.1 Classification system of the phylum Cercozoa (Adl et al. 2012)

Group	Example
Cercomonadidae	*Cercomonas*, *Filomonas*, *Brevimastigomonas, Neocercomonas*
Pansomonadida	*Agitata*, *Aurigamonas*
Glissomonadida	*Allantion*, *Bodomorpha*, *Neoheteromita*
Tremula	*T. longifila*
Metromonadea	*Metromomas*, *Metopion*, *Micrometopion*
Granofilosea	*Limnofila*, *Massisteria*, *Mesofila*, *Nanofila*
Thecofilosea	Cryomonadida, Ebriacea, Ventricleftida, *Phaeodaria*
Imbricatea	Spongomonadida, *Nudifila*, Marimonadidia, Silicofilosea
Chlorarachniophyta	*Chlorarachnion*, *Bigelowiella*, *Cryptochlora*, *Lotharella*
Vampyrellida	*Vampyrella*, *Arachnula*, *Gobiella*, *Hyalodiscus*
Phytomyxea	Plasmodiophorida, Phagomyxida
Filoreta	*F. japonica*, *F. marina*, *F. tenera*
Gromia	*G. sphaerica*, *G. oviformis*
Ascetosporea	Haplosporida, Paramyxida, *Claustrosporidium*, Paradiniidae

Cercozoa are attracting a lot of attention because of their ubiquitous distribution, ecological importance and high morphological variation. These protists have a wide range of habitats: in oceans, in freshwater, in soil and even within the roots of terrestrial plants (Bass and Cavalier-Smith 2004; Urich et al. 2008). Environmental DNA research revealed that cercozoan 18S rDNA is abundant in the sea floor sediments of the Arctic and the Southern Ocean (Pawlowski et al. 2011). Considering its high abundance, this group can be a significant player in the matter cycles and the food webs (Howe et al. 2011). Although their genetic unity is strongly supported, their distinctive morphological characters have not been found out yet (Adl et al. 2012).

9.2.2 Outline of Taxonomy

Phaeodaria are a cercozoan group defined by the presence of (1) a "central capsule" perforated by one astropyle and two parapylae; (2) a "phaeodium" in the extracapsular zone; and (3) a hollow siliceous skeleton called a "scleracoma", in most phaeodarians (Howe et al. 2011).

The latest classification system of Phaeodaria was formulated by examining the morphology of specimens, and the group was divided into seven orders and 18 families (Takahashi and Anderson 2000, Table 9.2). The phaeodarians belonging to the orders Aulacanthida (Figs. 9.2a and 9.3-1) and Phaeosphaerida (Figs. 9.4b and 9.3-3) are characterized by the spherical scleracoma of the radial arrangement of numerous hollow needles, spines and tubes. The Phaeogymnocellida phaeodarians are naked or surrounded with shells of other organisms such as diatoms, polycystines and silicoflagellates instead of their own scleracoma (Cachon-Enjumet 1961; Gowing and Coale 1989) (Figs. 9.4a and 9.3-2). The phaeodarians of Phaeocalpida are large in general, and this order contains five families—Tuscaroridae: flask-like form (Figs. 9.5a and 9.3-4a); Castanellidae: castanet-like sphere with round pores (Fig. 9.3-4b); Circoporidae: spherical or polyhedral with radial spines; Porospathidae: spherical with tubular spines and prolonged peristome (Fig. 9.3-4c); and Polypyramidae: polyhedral covered by pyramidal structure with spines. The Phaeogromida species are relatively small, and this order consists of three families—Medusettidae: campanulate (Fig. 9.3-5c–d); Challengeriidae: flask-like (Figs. 9.5b and 9.3-5a–b); and Lirellidae: elliptical with longitudinal grooves. The order Phaeoconchida contains only the family Conchariidae, characterized by clamshell-like scleracoma composed of two valves (Figs. 9.6a and 9.3-6). Phaeodendrida also include only one family, Coelodendridae, whose central capsule is covered with the "inner shell" extending arboroid structures (Figs. 9.6b and 9.3-7).

Phaeodaria consist of valid ~120 genera and ~500 species (e.g., Campbell 1954; Takahashi and Anderson 2000, Suzuki unpublished synonym database). When we exclude *nomina dubia* with indiscriminable illustrations or without illustrations and the taxa which have never been reported since the first description, a total of ~200

Table 9.2 Classification system of the subclass Phaeodaria (Takahashi and Anderson 2000)

Order	Family	Representative species	Figs.
Aulacanthida	Aulacanthidae	*Aulacantha scolymantha*	9.2a and 9.3-1
	Astracanthidae		
Phaeogymnocellida	Phaeodinidae	*Phaeodina antarctica*	9.4a and 9.3-2
	Phaeosphaeridae		
	Atlanticellidae		
Phaeosphaerida	Aulosphaeridae	*Auloscena verticillus*	9.4b and 9.3-3
	Cannosphaeridae		
	Sagosphaeridae		
Phaeocalpida	Tuscaroridae	*Tuscaretta belknapi*	9.5a and 9.3-4
	Castanellidae		
	Circoporidae		
	Porospathidae		
	Polypyramidae		
Phaeogromida	Challengeriidae	*Protocystis xiphodon*	9.5b and 9.3-5
	Medusettidae		
	Lirellidae		
Phaeoconchida	Conchariidae	*Neosphaeroconchidium caudatum*	9.6a and 9.3-6
Phaeodendrida	Coelodendridae	*Coelodendrum furcatissimum*	9.6b and 9.3-7

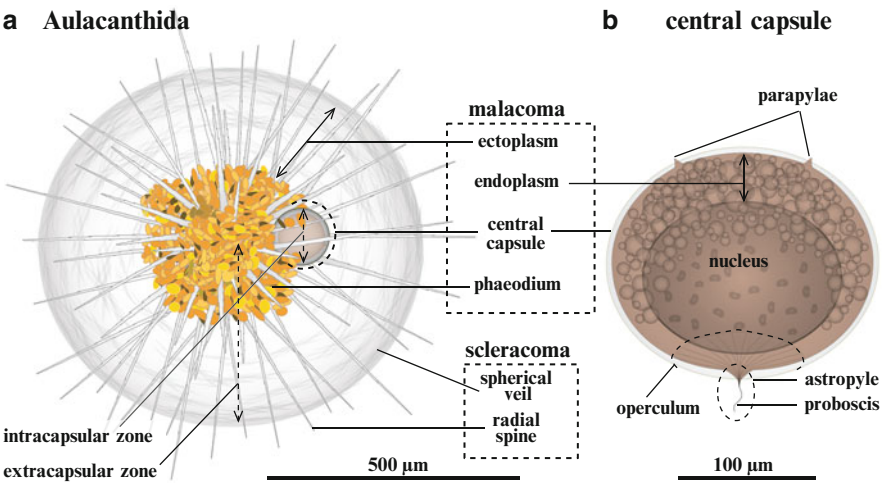

Fig. 9.2 Schematic illustrations of *Aulacantha scolymantha* Haeckel (Aulacanthidae, Aulacanthida): (**a**) overall structure; (**b**) central capsule

species belonging to ~80 genera practically exist (Fig. 9.7). Although molecular analysis allowed development of cercozoan studies, the DNA sequences of phaeodarians have never been analyzed in order to discuss their intra-group phylogenetic relationship.

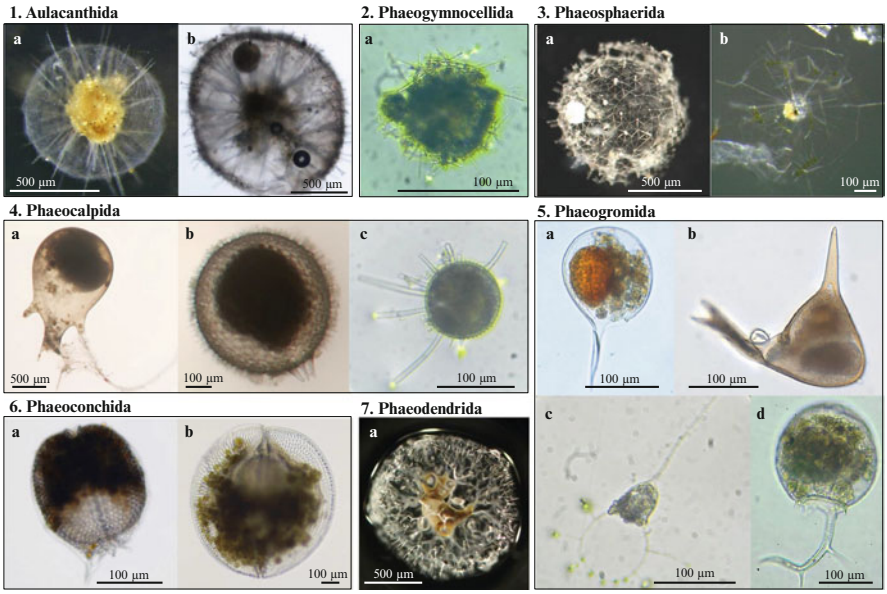

Fig. 9.3 1-a: *Aulacantha scolymantha* Haeckel (Aulacanthidae). 1-b: *Aulographis japonica* Nakamura Tuji and Suzuki (Aulacanthidae). 2-a: *Phaeodina* sp. (Phaeodinidae). 3-a: *Auloscena verticillus* Haeckel (Aulosphaeridae). 3-b: *Coelacantha ornata* Borgert (Cannosphaeridae). 4-a: *Tuscaretta belknapi* (Murray) (Tuscaroridae). 4-b: *Castanella sloggetti* Haeckel (Castanellidae). 4-c: *Porospathis holostoma* (Cleve) (Porospathidae). 5-a: *Protocystis xiphodon* (Haeckel) (Challengeriidae). 5-b: *Protocystis vicina* Reshetnyak (Challengeriidae). 5-c: *Medusetta partheno-paea* Borgert (Medusettidae). 5-d: *Euphysetta lucani* Borgert (Medusettidae). 6-a: *Neosphaeroconchidium caudatum* (Haeckel) (Conchariidae). 6-b: *Conchopsis compressa* Haeckel (Conchariidae). 7-a: *Coelodendrum furcatissimum* Haeckel (Coelodendridae)

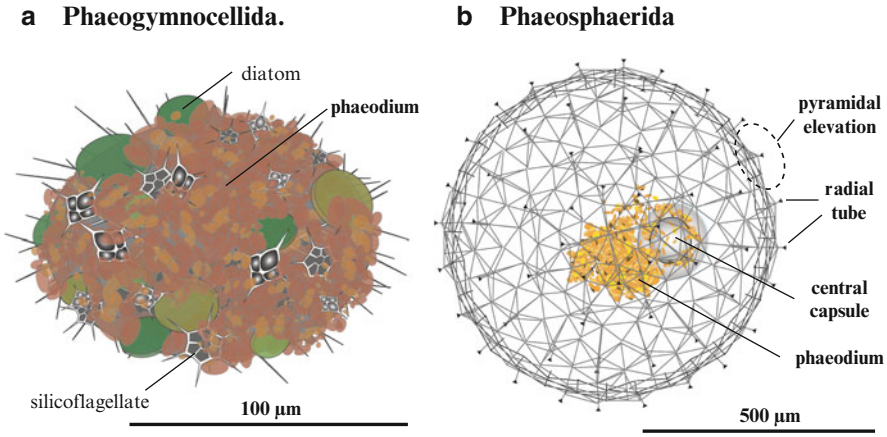

Fig. 9.4 Schematic illustrations of (**a**) *Phaeodina antarctica* (Schroeder) (Phaeodinidae, Phaeogymnocellida) and (**b**) *Auloscena verticillus* Haeckel (Aulosphaeridae, Phaeosphaerida)

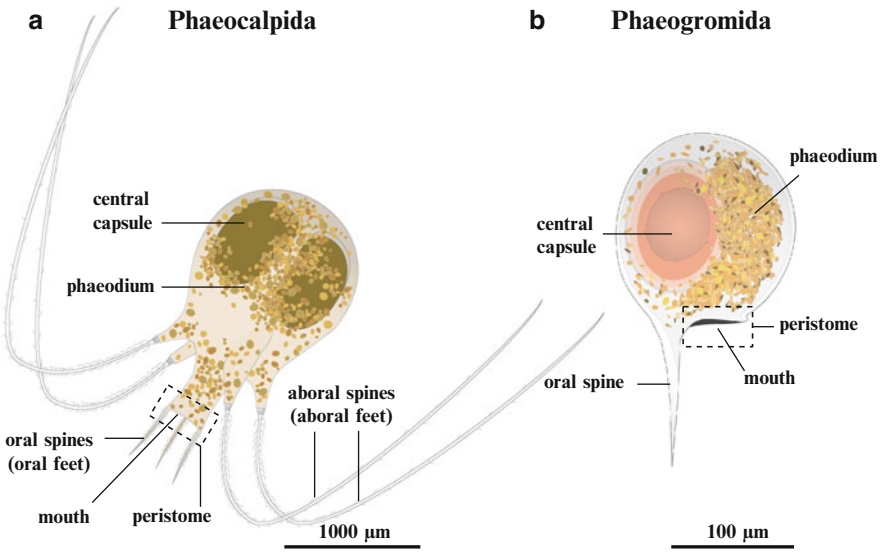

Fig. 9.5 Schematic illustrations of (**a**) *Tuscaretta belknapi* (Murray) (Tuscaroridae, Phaeocalpida) and (**b**) *Protocystis xiphodon* (Haeckel) (Challengeriidae, Phaeogromida)

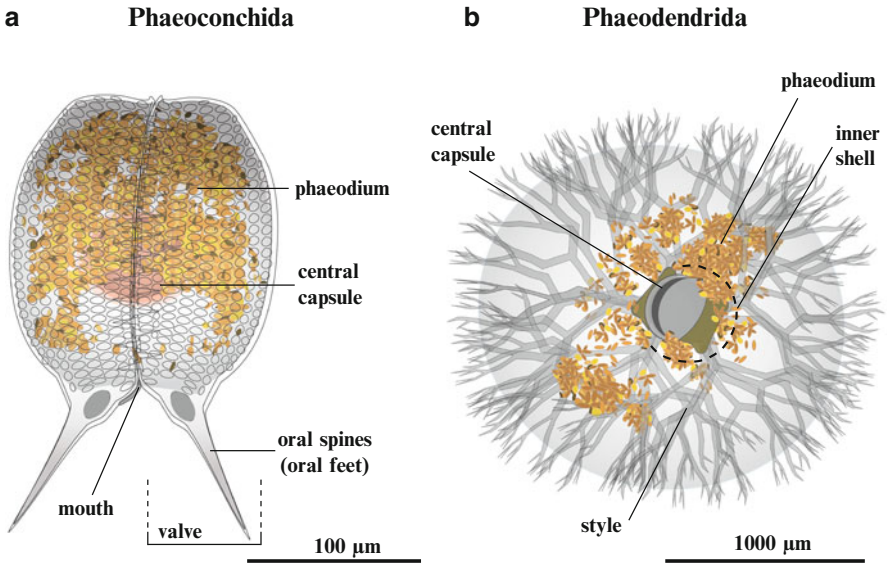

Fig. 9.6 Schematic illustrations of (**a**) *Neosphaeroconchidium caudatum* (Haeckel) (Conchariidae, Phaeoconchida) and (**b**) *Coelodendrum furcatissimum* Haeckel (Coelodendridae, Phaeodendrida)

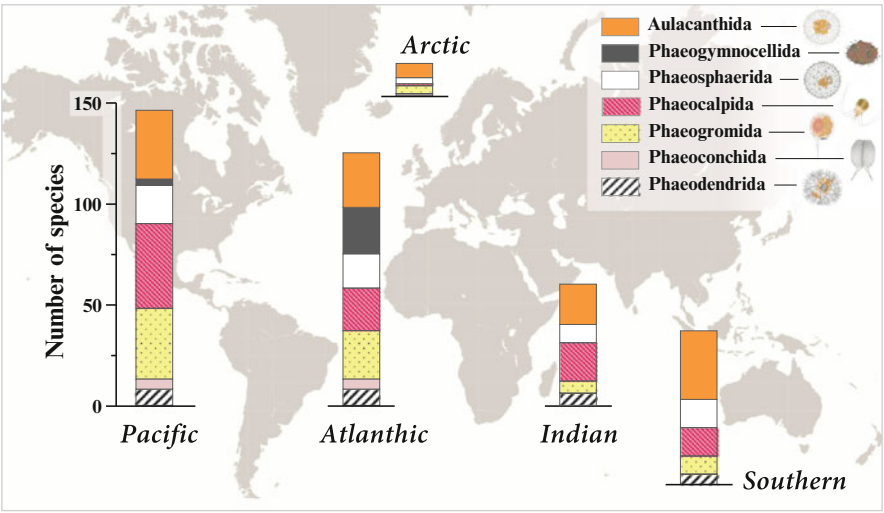

Fig. 9.7 Numbers of phaeodarian species reported from each oceanic region based on the documents published between 1966 and 2014. The composition of the orders is also indicated. Note that the "Pacific" region includes the data from the Bering Sea, the Sea of Okhotsk, the Gulf of California and the Sea of Japan; the "Atlantic" region also contains the data from the Mediterranean Sea

9.3 Cell Structure

Morphological terms concerning the structure of phaeodarians are indicated in Figs. 9.2 and 9.4–6 in accordance with major reviews of this group (Campbell 1954; Cachon-Enjumet 1961; Tibbs 1976; Kling and Boltovskoy 1999; Takahashi and Anderson 2000). Their body is mainly composed of two parts: the scleracoma (hard skeletal parts) and the malacoma (protoplasmic soft part) (Fig. 9.2).

9.3.1 Malacoma

The malacoma is a collective name for soft parts that contain the phaeodium and the protoplasm such as the central capsule and the ectoplasm. The central capsule is a spherical protoplasmic body which has the endoplasm and one or two large spherical nuclei containing a significant number of chromosomes. The endoplasm contains mitochondria, digestive vacuoles, endoplasmic reticulum and several Golgi bodies (Swanberg et al. 1986) (Fig. 9.2). The central capsule is divided from the extracapsular zone by the membranous double capsular wall. The outer wall is relatively thicker than that of radiolarians, whereas the inner wall is thinner, and these two walls are intimately connected with each other (Cachon-Enjumet 1961). This double-walled structure is one of key characteristics in Phaeodaria, which is thought to be derived from "theca", a structure characterizing the class Theofiliosea (Howe et al. 2011). The

central capsule also has three characteristic pore-like structures on its surface, namely, one astropyle (or astropylum) and two parapylae (Cachon and Cachon 1973). These structures are thought to exist for communication between the ecto- and endoplasm. The astropyle is supposed to be a cytopharynx that forms a cone-like structure having cytopharynic screens of microtubules and determines the oral pole of phaeodarians (Cachon-Enjumet 1961). This pore-like structure is surrounded by a conical area called an "operculum" and usually has a tubular extension, a "proboscis". The parapylae are orifices penetrated by the endoplasmic axopodia, which is characterized by large bundles of microtubules with a club-shaped root.

The ectoplasm is sticky and usually covered by the scleracoma. No straight stiff pseudopodia relevant to axopodia of Radiolaria have been observed in any Phaeodaria (Cachon and Cachon 1973). Some species belonging to the family Challengeriidae have two endoplasmic pseudopodia.

Another important structure characterizing the group Phaeodaria is the phaeodium, a mass of brown aggregated particles which does not exist in radiolarians (Gowing 1986, 1989; Gowing and Bentham 1994). The phaeodium are usually suspended in the extracapsular zone near to the astropyle and sometimes in the intracapsular zone (Figs. 9.2 and 9.4-6). These particles are thought to be an assemblage of food and waste vacuoles (Haecker 1908b; Cachon-Enjumet 1961; Gowing 1986, 1989, 1993; Gowing and Silver 1985; Gowing and Bentham 1994). This structure seems to be necessary for the life of phaeodarians because the phaeodium is inherited by daughter cells after the cell division (Haecker 1908b).

The terms "intracapsular zone" and "extracapsular zone" are different from "endoplasm" and "ectoplasm". The endoplasm and the ectoplasm signify the cytoplasm positioned inside or outside of the central capsular wall, respectively. On the other hand, the intracapsular and extracapsular zones indicate the space within and without the wall. For instance, the phaeodium exists in the extracapsular zone, but does not belong to the ectoplasm.

9.3.2 Scleracoma

The scleracoma is a collective name for hard siliceous skeletal parts surrounding and supporting the malacoma, such as the test, spherical veil, spines and tubes (Figs. 9.2 and 9.4-6). This structure is generally porous and substantially different from the radiolarian skeleton. In terms of the chemical components, the scleracoma consists chiefly of silica, but it also contains various metal elements: Al and Si (>1.0 % of the wet weight of the scleracoma), Mg (1.0–0.1 %), Ca, Fe, Mg, Ti and Ag (0.01–0.1 %), V and Ba (0.001–0.01 %) (Reshetnyak 1966).

The appearance of scleracoma is characteristically different among the orders and the families. The scleracoma of the orders Aulacanthida and Phaeosphaerida is generally spherical and composed of the radial arrangement of numerous hollow needles, spines and tubes. Aulacanthida (Figs. 9.2a and 9.3-1) have spheres of meshwork called a "spherical veil", whereas Phaeosphaerida (Figs. 9.4b and 9.3-3) form more geometric spheres made of numerous "tubes". Phaeocalpida, Phaeogromida

and Phaeoconchida have scleracoma forming a relatively firm test. The form of their tests can be flask-like (e.g., Tuscaroridae, Figs. 9.5a and 9.3-4a; Challengeriidae, Figs. 9.5b and 9.3-5a–b), chestnut-like (Castanellidae, Fig. 9.3-4b), campanulate (e.g., Medusettidae, Fig. 9.3-5c–d), clamshell-like (Conchariidae, Figs. 9.6a and 9.3-6) and polyhedral (e.g., Circoporidae). The scleracoma of the order Phaeodendrida is very characteristic. Their central capsule is covered with the "inner shell" extending arboroid, hollow "styles" branching into numerous spines (Figs. 9.6b and 9.3-7). Most of the species belonging to the order Phaeogymnocellida lack the scleracoma, and they are encircled by shells of other organisms such as diatoms, polycystines and silicoflagellates (Cachon-Enjumet 1961; Gowing and Coale 1989) (Figs. 9.4a and 9.3-2). This order seems to select the materials encircling its malacoma: *Miracella ovulum* Borgert (Atlanthicellidae) aggregates silicoflagellates *Dictyocha* and polycystines, while *Phaeodina valdiviae* Haecker (Phaeodinidae) is surrounded with centric diatoms only (Gowing and Coale 1989).

The internal structure of the test-type scleracoma differs among the families. Challengeriidae have a double-walled platy test with an hourglass-like cross-section surface, whereas the tests of Medusettidae have a porous cross-section surface. The tests of Lirellidae and Castanellidae consist of very fine spongy structures. Conchariidae usually have numerous pores on its surface (Takahashi and Hurd 2007).

9.4 Mode of Life

Phaeodarians seem to lack a swimming organ, and this protist is incapable of swimming against the current. They float in the water column presumably by maintaining the neutral buoyancy (Swanberg et al. 1986).

Almost all the phaeodarians are considered to be solitary plankton, but some species belonging to the families Coelodendridae and Tuscaroridae form colonies (Kling and Boltovskoy 1999). The genus *Coelographis* (Coelodendridae) is observed to be sometimes connected to each other by their styles (Swanberg et al. 1986). *Tuscaretta globosa* Borgert and *Tuscaridium cygneum* (Tuscaroridae) are attached on the surface of an intricate "latticed sphere" composed of siliceous rods (Haecker 1908a, Fig. 9.8). The colony of *Tuscaridium cygneum* (Murray) consists of 8–16 individuals, and they hook on the surface of the latticed sphere of 1.2–2.0 cm in diameter (Takahashi 1987, Ling and Haddock 1997).

Little is known about the ecology of phaeodarians, but some interesting features have been reported. Like other deep water marine organisms, *Tuscaridium cygneum* produces a bioluminescent glow probably for distraction to visual predators (Ling and Haddock 1997). Several individuals of *Aulacantha scolymantha* (Aulacanthidae), sampled from the shallows of the northern Mediterranean, showed molt-like behavior. They reconstructed new spherical veils and threw off the original ones which were damaged through the sampling process after the 24 h incubation (Fig. 9.9). This behavior was also seen for a scleracoma-less species of the family Phaeodinidae.

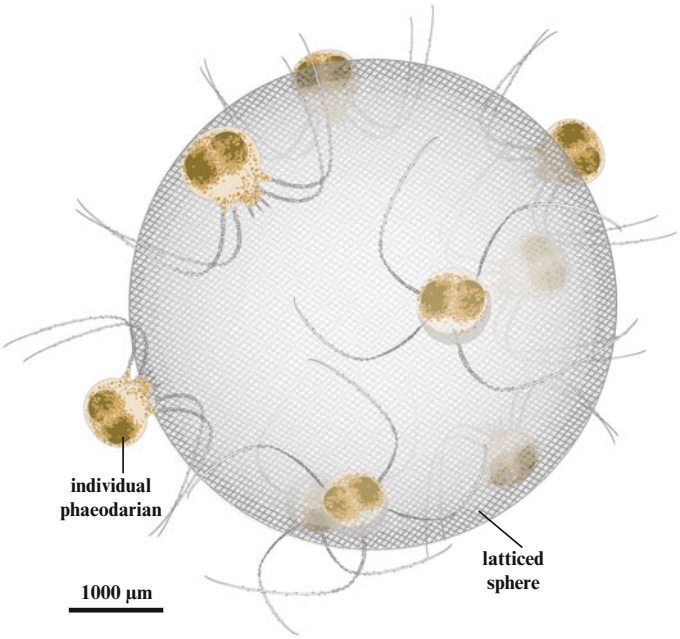

Fig. 9.8 Colony of *Tuscaretta globosa* Borgert (Tuscaroridae), referred from Haecker (1908a)

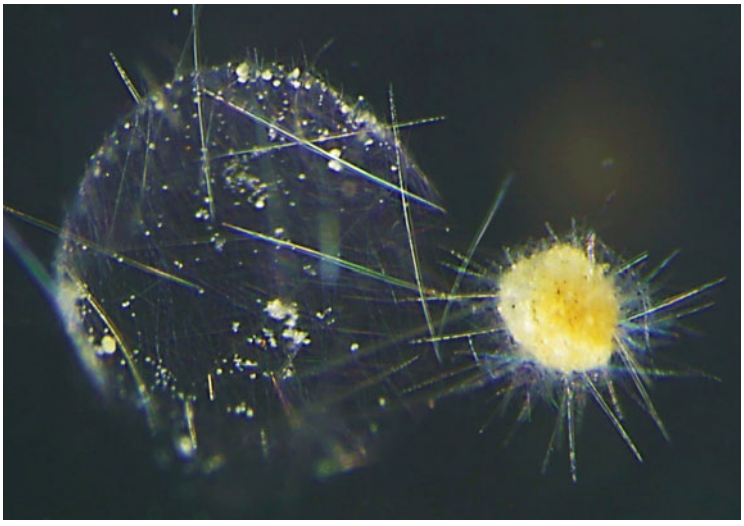

Fig. 9.9 Molt-like behavior of *Aulacantha scolymantha* (Aulacanthidae) sampled in the northern Mediterranean, off the coast of Villefranche-sur-Mer. This individual reconstructed a new spherical veil and got out of the original one damaged through the sampling process after 24 h incubation

9.5 Reproduction

Phaeodarians are thought to reproduce by cell division and by swarmer production (Grell 1973; Hollande 1981; Cachon and Cachon 1985), but the complete life cycle has never been replicated in the laboratory.

9.5.1 Cell Division

Binary division of three genera, *Aulacantha* (Aulacanthidae), *Aulosphaera* (Aulosphaeridae) and *Coelodendrum* (Coelodendridae), was well documented (Cachon-Enjumet 1961). It is confirmed that the division is accompanied by morphological change of chromosomes. Its interpretation has, however, been the subject of controversy whether the division is mitotic (Borgert 1901, 1909d; Bělař 1926; Cachon-Enjumet 1961) or a simple genomic segregation (Grell 1953; Grell and Ruthmann 1964). The most likely process of the nuclear division of *Aulacantha scolymantha* is summarized herein according to Borgert (1909d), Cachon-Enjumet (1961) and Grell (1973).

Metaphase The chromosomes do not align at the equator plane of a spindle unlike the normal binary division. They form a contorted "mother plate" within which they are oriented parallel to the direction of movement and not perpendicular to it. At the end of this process, the nuclear membrane vanishes.

Anaphase The mother plate divides into two daughter plates. The daughter plates are at first still contorted, and then they form plane parallel discs.

Telophase The daughter plates reach a certain distance from each other. The nuclear membrane is reconstructed. The daughter nucleus has a radial structure because of the chromosomes directed toward the outside. The endoplasm splits into two parts. The capsular walls envelop the two masses of the endoplasm covering the daughter nuclei.

Cytokinesis The ectoplasm, phaeodium and scleracoma are completely separated. The original cell becomes two daughter cells.

9.5.2 Swarmer Production

The swarmer production (or sporogenesis) of two species has been reported previously: *Coelodendrum ramosissimum* (Coelodendridae) and *Aulacantha scolymantha* (Aulacanthidae). The capsular membrane and the phaeodium vanish first, and several “plasmatic spheres” of different size appear within the scleracoma. Then, these plasmatic spheres, containing numerous interphasic secondary nuclei, fall apart into small cells. The cells form two long flagella and become swarmers. They

swim out from the scleracoma in the end. Each swarmer possesses crystalline inclusions, therefore they are called "crystal swarmers" (Grell 1973; Cachon and Cachon 1985). Two types of swarmers have been observed: isospores and anisospores. Some researchers suggest that the "anisospores" of *A. scolymantha* are actually parasitic dinoflagellates (Chatton 1934; Hollande and Enjumet 1953). Although the sexuality of phaeodarians is still unknown, the swarmers are thought to be gametes (Borgert 1909d).

9.6 Trophic Interactions

9.6.1 Feeding

Phaeodarians are supposed to be heterotrophic feeders and omnivorous generalists (Gowing 1986, 1989; Swanberg et al. 1986; Gowing and Bentham 1994; Nakamura et al. 2013). As this group cannot swim against the current, most of them seem to float in the water column and to obtain the food coming close to them. They feed generally on particles and cells associated with fecal pellets and aggregates (Gowing 1989; Nöthig and Gowing 1991). *Coelographis* sp. (Coelodendridae) presumably prey on living organisms over a wide range of size. This phaeodarian catches large metazoans such as copepods and salps by the styles, and captures microflagellates by the spathillae (Swanberg et al. 1986).

The captured food is taken into the extracapsular zone, then accumulated within the food vacuoles forming the phaeodium. The food vacuoles of some families (Aulacanthidae, Phaeodinidae, Atlanticellidae, Cannosphaeridae, Lirellidae, Porospathidae, Challengeriidae, Medusettidae and Conchariidae) contain various organic materials: algal cells (diatoms, dinoflagellates, chrysophytes, prasinophytes, *Chlorella*-like cells and algal cysts), microheterotrophs, bacteria, loricae and nematocysts of metazoans, coccoliths, trichocysts, cuticles, amorphous material and siliceous skeletal fragments (Gowing 1986, 1989, 1993; Gowing and Bentham 1994). Vacuoles are present not only in the extracapsular zone but also in the intracapsular zone. The vacuoles filled with partially digested organic matter are observed within the central capsule, suggesting that a part of the food is carried into the intracapsular zone through the astropyle, presumed cytopharynx (Cachon and Cachon 1973; Swanberg et al. 1986).

Phaeodarians inhabit variable depths, so it is presumed that the compositions of the food vacuoles change depending on the depth. The composition of the vacuoles in each depth is similar to that of sinking organic aggregates in the oligotrophic North Pacific, suggesting that phaeodarians selectively feed on organic aggregates rather than bacteria, which is more abundant in the area (Gowing and Bentham 1994).

Scleracoma of some phaeodarians occasionally contains partially ingested diatom frustules, therefore they may directly feed on phytoplankton, depending on the environment (González 1992).

9.6.2 Predators and Symbiosis

Some zooplankton are known to consume phaeodarians but details are not well clarified yet (Raymont 1983; Hopkins 1985; Hopkins and Torres 1989). Only one species has so far been reported as a predator of phaeodarians: a non-selective particle feeding salp, *Salpa thompsoni*, in the western Weddell Sea and near the Antarctic Peninsula (Gowing 1989).

Phaeodarians are often infected with dinoflagellates: *Syndinium borgerti* (host: *Aulacantha scolymantha*, Aulacanthidae), *Syndinium* sp. (host: *Coelodendrum* sp., Coelodendridae), *Atlanticellodinium tregouboffi* (host: *Planktonetta atlantica*, Medusettidae) (Hollande and Enjumet 1955; Cachon and Cachon 1987; Hollande 1974; Théodoridès 1989). These parasitic dinoflagellates are considered to multiply in the central capsule of the host phaeodarian, resulting in the death of the host. Some of these dinoflagellates are apparently similar to the phaeodarian swarmers (Grell 1973). It is also reported that numerous bacteria (50–100 x 10^3 cells g^{-1} of wet phaeodarians) are attached to *Castanidium longispium* (Castanellidae) (Jones 1958). Although many radiolarians have symbiotic microalgae, phaeodarians have been thought to lack symbionts.

9.7 Distribution

9.7.1 Global Distribution

Phaeodarians are largely distributed in the water mass below 150 m in the tropical and subtropical regions, but they also occur in the surface layer of high-latitude regions, such as the Sea of Okhotsk and the Southern Ocean (Nöthig and Gowing 1991; González 1992). Bipolar distributions are recognized for nine species (e.g., *Aulastrum spinosum*, *Sagenoscena irmigeriana* and *Aulacantha laevissima*) (Reshetnyak 1966). Their species diversity is the highest in the Pacific region (approximately 150 species) and the lowest in the Arctic region (approximately 15 species) (Fig. 9.7). These numbers may, however, not reflect the true species diversity, because the species compositions of phaeodarians were thoroughly examined in limited sampling sites.

The composition of phaeodarians at order level is different between the oceanic regions. The species number of the order Phaeodendrida is almost the same in every oceanic region except the Arctic, even though the numbers of other orders differ greatly. The order Phaeogymnocellida also shows a marked difference, they have been found mainly in the Atlantic region. The large part of this order was described in the Mediterranean Sea, with the result that their proportion is relatively high in the Atlantic region (Fig. 9.7).

The zoogeographic zonation of phaeodarians can be divided into three regions, namely (1) the Pacific and North Indian deep water region; (2) the Atlantic deep water region; and (3) the Antarctic deep water region, according to Reshetnyak (1966) (Table 9.3). The Pacific and North Indian deep water region is further subdivided into three sub-regions: the boreal North Pacific, the tropical Pacific and the North Indian. The Atlantic deep water region is also subdivided into three sub-regions: the Arctic, the boreal North Atlantic and the tropical Atlantic. The Antarctic deep water region contains three sectors: the Atlantic, the Indian and the Pacific. This zonation is made based on the degrees of endemism of the phaeodarian assemblages. Since the information concerning their distribution was limited in the time of Reshetnyak (1966), it is necessity to verify these zoogeographic data by examining the reports published after this paper.

9.7.2 Vertical Distribution

Phaeodarians have long been regarded as "deep-sea plankton" since Haeckel (1887). This is partly true because a large part of phaeodarian species live in 1000–4000 m water depth (Reshetnyak 1955), but it is not the case for every species. *Aulacantha scolymantha* (Aulacanthidae) and *Phaeodina* spp. (Phaeodinidae) are abundant in

Table 9.3 Global zoogeographic zonation of phaeodarians (Reshetnyak 1966)

Region	Number of endemic genus	Sub-region	Number of endemic species and subspecies
Pacific and North Indian deep water	10 (e.g., *Auloplegma, Sagoplegma*)	Boreal North Pacific	13 (e.g., *Aulacantha pacifica, Castanissa megastoma*)
		Tropical Pacific	143 (e.g., *Cannobelos calymnata, Aulactinium actinastrum*)
	2 (e.g., *Aulaphacus, Cirorrhegma*)	North Indian	43
Atlantic deep water	0	Arctic	9 (e.g., *Aulosphaera multifurca, Haeckeliana labradoriana*)
	6 (e.g., *Porcupinia, Coelodasea*)	Boreal North Atlantic	9 (e.g., *Aulocoryne zetesios, Cannosphaera antarctica*)
		Tropical Atlantic	160 (e.g., *Cannobelos thalassoplancta, Astracantha heteracanthoides*)
Antarctic deep water	3 (e.g., *Aulodictyum, Conchasma*)	Atlantic sector	4 (e.g., *Sagenoarium anthophorum, Protocystis micropelectus*)
		Indian sector	23 (e.g., *Phaeodina antarctica, Sagoscena pellorium*)
		Pacific sector	7 (e.g., *Aulactinium actinosphaerium, Castanidium antarcticum*)

the warm surface layer of the Mediterranean Sea (e.g., Cachon-Enjumet 1961). The species diversity of phaeodarians increases with the depth, reaching the highest (approximately 50 species) in the deepest layer (6000–8000 m) in the Kuril-Kamchatka Trench (Reshetnyak 1955, 1966). A similar tendency is also reported in the Gulf of California (Zasko and Rusanov 2005). The cell size of deep-living phaeodarians tends to be larger than that of the same or related species dwelling in shallower zones (Haecker 1908a; Reshetnyak 1966; Zasko and Rusanov 2005).

Their vertical distribution is probably species specific. Several species belonging to the family Castanellidae show clearly different vertical distributions (Kling 1976). Challengeriid species were also collected from variable depth intervals in the North Pacific (Okazaki et al. 2004). The vertical distribution of phaeodarians at species level can be categorized into two types: eurybathic (widely distributed from the epipelagic to the hadal zone) and stenobathic (living in limited depth layers). The eurybathic species have wider horizontal distribution than the stenobathic phaeodarians (Zasko 2003; Zasko and Rusanov 2005). The stenobathic species in the Kuril-Kamchatka Trench are subdivided into the following five categories: surface (0–50 m), subsurface (50–200 m), middle bathyal (200–1000 m), bathypelagic (1000–2000 m) and superabyssal (4000–8000 m) (Reshetnyak 1955, 1966).

9.8 Interactions with the Environment

9.8.1 Restricting Factors of Phaeodarian Distribution

The distribution of phaeodarians is partly influenced by the water temperature. Five challengeriid species are sampled from cold waters of less than 5 °C, whereas two different species are collected in the water masses of higher temperature (Meyer 1933) (Table 9.4). Their temperature-dependent distribution is seen in the Sea of Japan too. *Aulographis japonica* (Aulacanthidae) is found only in the water mass of low temperature (approximately 1 °C) (Nakamura et al. 2013). Their vertical distribution pattern is also influenced by the amount of food. As phaeodarians are generalist feeders and presumably feed upon organic aggregates in general, they prefer to be suspended in the zones where plenty of plankton or POM sinking from

Table 9.4 The range of water temperature at the sampling stations of the species belonging to the family Challengariidae (Meyer 1933)

	Water temperature (°C)	
Species	Lowest	Highest
Protocystis micropelecus	2.5	3
Protocystis gravida	3	4
Protocystis swirei	−1	5
Protocystis triangularis	0.5	5
Protocystis thomsoni	2	5
Challengeron willemoesii	5	12
Protocytis varians	5	18

the upper layers. Phaeodarians are abundant in the surface layer of the Weddell Sea, where krill fecal pellets, on which they feed, are more plentiful (González 1992). Another possible restricting factor of their distribution is silica. Certain species change their living depths probably in relation to the concentration of dissolved silica in order to easily build their scleracoma (Bjørklund 1974; Okazaki et al. 2004). These restricting factors could account for the observed patchy distribution of this protist group (Bernstein et al. 1990).

9.8.2 Biomass

The abundance of phaeodarians does not increase with the depth, unlike the species diversity which grows with the depth. Their abundance reaches the maximum in the lower epipelagic or mesopelagic zone of the Antarctic (Morley and Stepien 1984; Abelmann 1992), the North Pacific (Steinberg et al. 2008) and the Sea of Japan (Nakamura et al. 2013). However, the reported abundance of phaeodarians is presumably underestimated because their scleracoma is fragile, and it could easily be broken during net sampling. Most of the coelodendrid phaeodarians have never been caught by normal plankton-net sampling. Aulosphaeridae and Cannosphaeridae are commonly captured in the subtropical shallow waters, but they are often crumpled up. The species of the Order Phaeogymnocellida have been erroneously regarded as "marine snow" or "sinking aggregates" due to the absence of scleracoma or to their body surrounded by siliceous dead remains of diatoms, polycystines and silicoflagellates. These phaeodarians can be identified by dyeing their nuclei using DAPI (Gowing 1989; Gowing and Coale 1989).

Careful examination of phaeodarian abundances revealed that these protists are more numerous than expected, and their high abundance is occasionally reported. Their vertical flux is much higher than that of polycystines in the Panama Basin (Takahashi and Honjo 1983) and on the California coast (Gowing and Coale 1989). The biomass of the families Aulosphaeridae, Sagosphaeridae, Aulacanthidae and Coelodentridae occupies 2.7–13.7 % of the total metazoan biomass in the 150–1000 m layer of the western North Pacific (Steinberg et al. 2008). The dominance of the family Aulacanthidae is also reported in the Sea of Japan. The proportion of *Aulographis japonica* with respect to the total zooplankton biomass is 22.3 % in the 250–3000 m layer, which is the second largest, following that of copepods (Nakamura et al. 2013). Considering their high biomass, especially in deep waters, this protist group would play an important role in the local food webs.

9.8.3 Seasonality

The seasonality of phaeodarian vertical flux seems to depend on the oceanic region. The total phaeodarian flux shows a regular seasonal cycle and peaks in spring and fall–winter at 3198 m depth of the Bering Sea (Ikenoue et al. 2012). The peaks of their flux were seen in summer and spring at 853 m depth in the equatorial eastern

Atlantic (Boltovskoy et al. 1993b). These data are, however, the sum total of their flux, and the flux seasonality of each species possibly differs. Two congeneric species of the family Medusettidae (*Euphysetta staurocodon* and *E. elegans*) show the flux peak in different seasons in the eastern North Pacific (Takahashi 1987).

9.8.4 Importance in the Matter Cycle

The high biomass of phaeodarians suggests that they have a significant influence on the matter cycles of the world ocean. The cell size of some phaeodarians (e.g., Aulacanthidae) is generally larger than that of other heterotrophic siliceous protists such as polycystines (Takahashi and Honjo 1983). Therefore, even though phaeodarians are less abundant than other plankton in number, their contribution to the silica flux could become larger. The large part of silica flux usually comes from diatoms in the western North Pacific, but heterotrophic siliceous protists show higher contributions in several stations because of large phaeodarians (Bernstein et al. 1990). In addition, phaeodarians accumulate ingested organic matter within the phaeodium, and the volume of silica gathered in this part becomes as much as that of their scleracoma (Gowing and Coale 1989). The vertical flux of scleracoma-less phaeodarians is estimated to be almost equal to that of scleracoma-bearing phaeodarians in the eastern North Pacific (Gowing and Coale 1989).

The mini pellets are mass of digested organic matter ejected by plankton, and this substance is thought to play an important role in matter cycle (Gowing and Silver 1985). Phaeodarians could occasionally be a major producer of mini pellets (Gowing and Coale 1989; Nöthig and Bodungen 1989; Buck et al. 1990; González 1992). They transform organic particles into mini pellets sinking into deeper layers in the North Pacific (Gowing and Bentham 1994). The number of mini pellets (4.89×10^5 pieces m^{-3}) is reported to be extraordinarily larger than that of krill feces (8.7 pieces m^{-3}) in the Weddell Sea, and these pellets are important as a nutrition source for marine organisms (González 1992).

9.9 Fossil Records

Phaeodarian scleracoma is so fragile that their fossils are rarely found. Even in the area where they are abundant in the water column, the sediments on the sea floor seldom contain their remains except in the Norwegian Sea (Stadum and Ling 1969). In spite of the large number of "radiolarian" studies (e.g., Suzuki and Aita 2011), phaeodarian fossils are limitedly reported: the Rhaetian (uppermost Triassic) of Japan (Hori et al. 2009); the Upper Cretaceous of Sakhalin (Bragina 2003), Japan (Takahashi 2004), and the Southwest Pacific subsurface sediments (Dumitrica and Hollis 2004); the upper Paleocene of Denmark (Dumitrica and Van Eetvelde 2009);

the upper Eocene (Petrushevskaya and Reshetnyak 1980; Vitukhin 1993); the Miocene of Romania (Dumitrica 1964, 1965) and Kamchatka (Runeva 1974); and the Miocene to the Quaternary of the sea floor sediments in the South Atlantic (Ling 1991) (Fig. 9.10). The family Conchariidae was once reported from the lower Cambrian in China (Hao and Shu 1987; Shu and Chen 1989), but these fossils were later confirmed as parts of extinct metazoans, lobopodians (Chen et al. 1995, Zhang and Aldridge 2007).

The large part of the fossil phaeodarians is occupied by members of the families Challengeriidae and Medusettidae. Only a few reports concern other families. The oldest fossil phaeodarian specimens were sampled in Rhaetian strata (208.5–201.3 million years ago): *Medusetta japonica* and *Triassiphaeodina niyodoensis* (Medusettidae) (Hori et al. 2009, Fig. 9.10). It is noteworthy that the genus *Medusetta* is an extant genus. This Rhaetian sample comes from a deep water sedimentary rock in association with pelagic polycystines, suggesting that these phaeodarians were members of the deep-sea pelagic fauna. The second oldest fossil records jumps to the Cenomanian (the lowest Upper Cretaceous), 100 million years later from the Rhaetian, from the Bykov Formation in southern Sakhalin (Bragina 2003, Fig. 9.10). This report is of particular importance because this is the oldest record of the Challengeriidae. Other genera of this family have been discovered as fossils from the upper Campanian–lower Maastrichtian (Takahashi 2004; Dumitrica

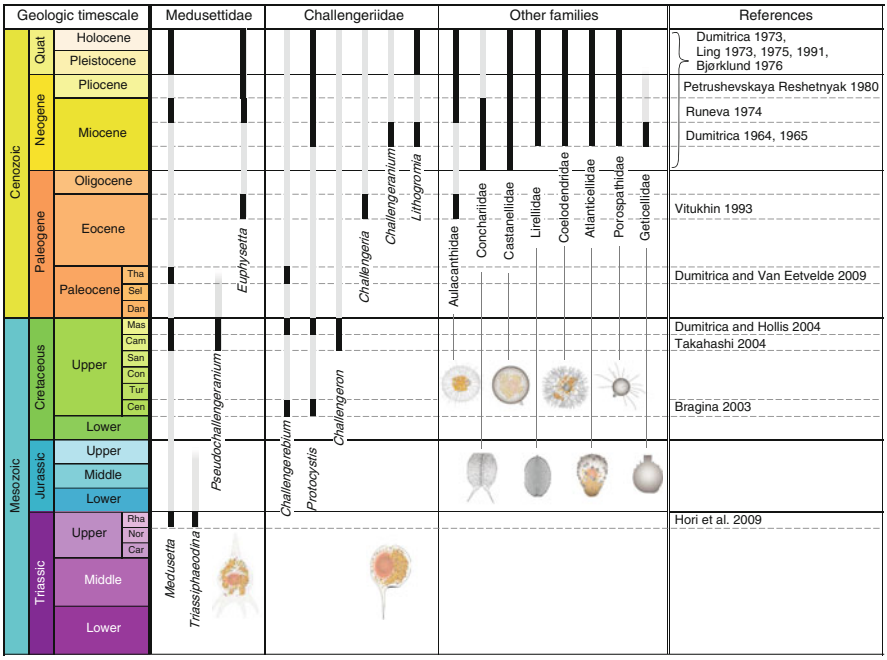

Fig. 9.10 Geologic range of phaeodarians. *Black vertical bar* indicates the age with fossil evidence, whereas the *gray one* shows the age without fossil evidence. Note that all the fossil reports published until 2014 are included in this figure

and Hollis 2004) to the Holocene (Dumitrica 1973), probably because of the intermittent records. Only a few records are reliable concerning other families (Fig. 9.10). The remains of the radial spine probably belonging to the family Aulacanthidae (or morphologically similar families) are found from the upper Eocene (Vitukhin 1993). The oldest records of the families Conchariidae and Castanellidae are noted from the lower Miocene (Dumitrica 1973). From the middle Miocene, the oldest fossils of the following families are sampled: Lirellidae, Coelodendridae, Atlanticellidae and Porospathidae (Dumitrica 1973, Ling 1991). Dumitrica (1965) described a fossil genus *Geticella*, which was decided to belong to the extinct family Geticellidae, from the middle Miocene in Romania. A total of ten genera of the families Challengeriidae and Medusettidae have so far been reported as fossils since the Rhaetian (Fig. 9.10), but extinct genera are only two: *Triassiphaeodina* from the Rhaetian (Hori et al. 2009) and *Pseudochallengeranium* from the upper Campanian–lower Maastrichtian (Dumitrica and Van Eetvelde 2009). The fact that most phaeodarian genera have remained until today suggests that their evolutionary rate is relatively slow for the genus level. The extant genera survived through many global oceanic crises, such as oceanic anoxic events at the Toarcian (Lower Jurassic) and the Cretaceous as well as the Cretaceous–Paleogene mass extinction.

Delicate and fragile fossils are generally found from the Lagerstätte, the strata with extremely well-preserved fossils such as Burgess Shale and Ediacara Hills (see Suzuki and Oba, this volume). The phaeodarians of the Rhaetian, the Maastrichtian and the upper Paleocene are found from nodules or soft sediments, a kind of Lagerstätte (Dumitrica and Hollis 2004; Dumitrica and Van Eetvelde 2009; Hori et al. 2009). These phaeodarians may be preserved under the following conditions (Dumitrica and Van Eetvelde 2009): (1) the mineralogical replacement of the original phaeodarian silica to celestobarite ($BaSrSO_4$), a complete solid solution series between barite ($BaSO_4$) and celestite ($SrSO_4$); (2) exclusive of clays in nodules and concretions; and (3) protection of the siliceous scleracoma from the effects of compaction and destructive pore-fluids percolating through the host sediments. However, other factor could be attributed to the fossilization of phaeodarians because the upper Campanian–lower Maastrichtian phaeodarians are obtained from a "normal" siliceous mudstone sample after chemically sever treatment with hydrofluoric acid (Takahashi 2004).

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Chapter 10
Ecology and Evolution of Marine Diatoms and Parmales

Akira Kuwata and David H. Jewson

Abstract Diatoms and Parmales are microscopic, photosynthetic algal groups that both have siliceous cell walls. It is probable that they shared a common ancestor, but each has evolved along a quite distinct path. Diatoms have undergone extensive adaptive radiation and colonized a wide range of freshwater, terrestrial and marine habitats. They are particularly important in the ecology of the oceans, where it has been estimated that they are responsible for 40 % of global marine production. In contrast, Parmalean algae have remained restricted to more specific niches, mainly in marine coastal areas at high latitudes. They are small (from 2 to 5 μm) and single celled. Until recently, their ecology was largely unknown, but new research has begun to reveal their biology for the first time. In this review, we concentrate on the roles and adaptations of both groups in the marine plankton, highlighting areas for future research.

Keywords Diatom • Parmales • Phytoplankton • Ecology • Evolution • Survival strategies • Resting stages • Adaptive strategies

10.1 Introduction

Diatoms and Parmales are microscopic algae that are eukaryotic and photosynthetic. There are a large number of other algal groups that could be included in such a description, but diatoms and Parmales share one important feature in common; they both have siliceous cell walls (Kröger and Poulsen 2008) (Fig. 10.1). From recent genetic research, we now know that they are close relatives and probably had a common ancestor (Ichinomiya et al. 2011), but each has evolved along a quite distinct path. Diatoms have undergone extensive adaptive radiation, which has resulted in a

A. Kuwata (✉)
Tohoku National Fisheries Research Institute, FRA,
3-27-5 Shinhama-cho, Shiogama, Miyagi 985-0001, Japan
e-mail: akuwata@affrc.go.jp

D.H. Jewson
Freshwater Laboratory, University of Ulster,
Cromore Road, Coleraine BT52 ISA, Northern Ireland, UK
e-mail: d.jewson@btinternet.com

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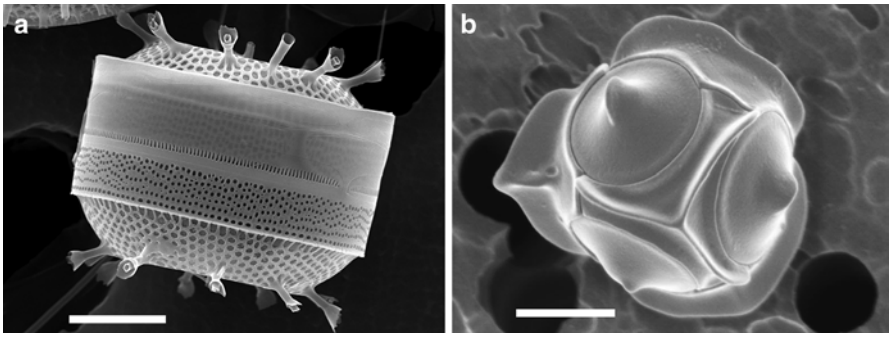

Fig. 10.1 Scanning electron microscope images of a diatom: (**a**) *Thalassiosira nordenskioeldii* (scale bar = 5 μm); and (**b**) a Parmales, *Triparma laevis* (scale bar = 1 μm) (photos taken by A. Kuwata)

wealth of different shapes, sizes and specialized physiological adaptations (e.g., Werner 1977; Round et al. 1990; Smetacek 1999; Sims et al. 2006; Kooistra et al. 2008; Ambrust 2009), enabling them to colonize a wide range of freshwater, terrestrial and marine habitats. In the marine plankton, diatoms may live as single cells or in colonies, particularly as filaments or chains. They range in size from 2 μm up to 2 mm, but are usually between 10 and 200 μm. It has been estimated that they are responsible for 40 % of global marine production (Nelson et al. 1995; Falkowski et al. 1998; Mann 1999). They are the main prey for mesozooplankton and the energy that they fix in photosynthesis is transferred (via grazing) to higher trophic levels, so they are important in maintaining fish production (Ryther 1969). From an evolutionary point of view, the emergence of diatoms with their high productivity may have driven the evolution of crustaceans, pelagic fish and whales, and shaped the modern marine pelagic ecosystem (Parsons 1979). On the other hand, Parmalean algae have remained small (from 2 to 5 μm) and single celled, with a distribution that is mostly restricted to marine coastal areas at high latitudes (Konno and Jordan 2012). Their ecology is still largely unknown, but recent research has revealed aspects of their biology for the first time (Ichinomiya et al. 2011, 2013; Yamada et al. 2014). In this chapter, we concentrate on the roles and adaptations of both groups in the marine plankton, to highlight their different evolutionary paths and also to suggest areas for future research.

10.2 Diatom

10.2.1 History of Study

The study of diatoms has a long tradition, which began in the eighteenth century and mirrored the development of microscopes (Round et al. 1990). The intricate structures of their siliceous cell walls made them attractive to look at and they were relatively easy to preserve and make up into permanent slides, which could be then kept in archived collections and circulated to other diatomists around the world for

taxonomic verification. By the beginning of the twentieth century, ecological studies had started to determine the spatial and seasonal distributions of diatom species in marine habitats and to record biogeographic data (see summaries in Hasle 1976; Guillard and Kilham 1977). More recently, a global diatom database of abundance, biovolume and biomass in the world's oceans has been established and aims to build a dataset of the main plankton functional types to help validate biogeochemical ocean models estimating carbon biomass derived from abundance data (Leblanc et al. 2012).

During the second half of the twentieth century, diatom studies began to increase, although by the time Werner published *The Biology of Diatoms* in 1977, it was still possible to write a review that summarized and reflected the known knowledge of the time. Since then, there has been an enormous upsurge in interest. One stimulus for this came from improvements in dating of sediments during the 1970s, which increased the use of diatoms in paleoecology for reconstructing and quantifying past environmental changes, particularly in terms of acidification and eutrophication of lakes (see Smol and Stoermer 2010). Other developments included the greater use of scanning electron microscopes (SEM), which helped improve descriptions of structural variations in the cell wall. It also contributed to a rapid increase in the number of new species being described and it is now estimated that there are at least 30,000 species, but that possibly over 100,000 may exist (Mann and Droop 1996; Mann and Vannormelingen 2013). One downside to this rapid upsurge in new descriptions is that identification of species has often been based mainly on small variations in cell wall structure, which may not always be the crucial factor in speciation and does not taken into account that cell wall features may vary under different environmental conditions. This is reflected in studies investigating cryptic species, highlighted by the classic work of Mann and colleagues on the benthic freshwater genus *Sellaphora* (Mann et al. 2008; Evans et al. 2008; Poulíčková et al. 2008). Cryptic species are also common in marine habitats such as in the benthic estuarine species *Navicula phyllepta* (Vanelslander et al. 2009) and the cosmopolitan planktonic diatoms *Asterionellopsis glacialis* (Kaczmarska et al. 2014) and *Skeletonema costatum* (Sarno et al. 2005; Kooistra et al. 2008). In living diatoms, it may be possible to resolve some of the underlying problems of cryptic species by including more characters for taxonomy (e.g., genetics and sexual reproduction), but this will not be possible for fossil forms of extinct species found in sediments, where only the remains of the cell wall are found.

10.2.2 Marine Diatom Phytoplankton Ecology

Success and survival of individual species is not down to a single factor but is the result of a combination of factors, often acting at different times during the life cycle (Jewson and Granin 2015). However, investigating this in marine habitats is not easy, because water masses move and intermix with time. Cermeño and Falkowski (2009) pointed out that the extent to which spatial distribution of marine

phytoplankton is controlled by either local environmental selection or dispersal is poorly understood. We need more information on the types of phytoplankton niches and their associated physical and chemical conditions over time. We do know that latitude has an important effect on both the vertical (Fig. 10.2) and horizontal physical environment of seas and oceans. This has created a wide variety of habitats, from the microscale up to large gyres over thousands of kilometers (e.g., d'Ovidio et al. 2010), and diatoms have adapted to colonize the opportunities presented. Most of the primary production in the sea takes place in the upper 100 m, and we know that this is largely sustained by the recycling of nutrients. For example, it has been estimated that every atom of silicon entering the ocean is incorporated into a diatom cell wall approximately 40 times before finally sinking to the seafloor (Tréguer et al. 1995). Another key element is nitrogen, and the depth distribution of its various forms (e.g., ammonia, nitrite and nitrate, Dugdale and Goering 1967) in stratified and mixed water columns, shown in Fig. 10.2, can create a range of different niches. This complexity of habitats with depth in the sea is often overlooked. Other potentially important limiting nutrients include carbon, phosphate and iron (see Sarthou et al. 2005 and further discussion below).

Various attempts have been made to understand how nutrient limitation can influence community composition. One of the earliest researchers to create a more

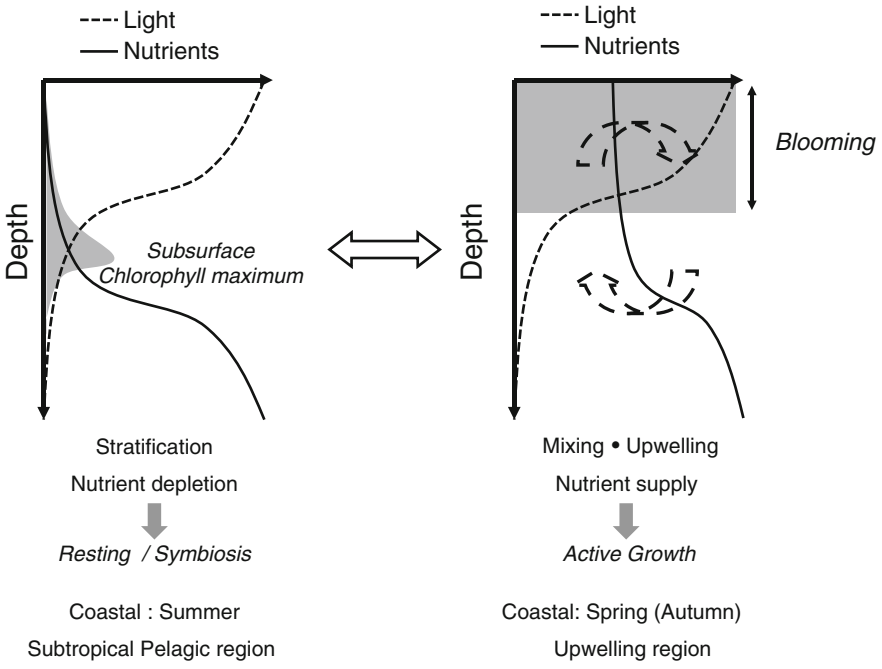

Fig. 10.2 Summary of typical depth distributions of phytoplankton abundances in relation to light and nutrients in different seasonal or geographic locations during either water column mixing or stratification

generalized model was Margalef (1978), who tried to explain how succession might be affected through the supply of nutrients and intensity of turbulence (see also Reynolds 1987; Estrada and Berdalet 1997; Wyatt 2013). Margalef envisaged that major taxonomic groups of phytoplankton occupy different "spaces" or niches within a "mandala" (Fig. 10.3a). In his model, although diatoms are non-motile, their fast potential growth rate and ability to store nutrients in a vacuole enabled them to replace cells at a higher rate than the rate at which they were lost from the population. He noted that they thrive in relatively turbulent, nutrient-rich waters, where any lack of motility is compensated for by resuspension of cells. On the other hand, potential competitors that are motile, such as dinoflagellates, can regulate their position in the water column, which favors survival in stratified waters, particularly where migratory behavior may contribute to the acquisition of nutrients from deeper layers. In some circumstances, some diatom species have compensated for this lack of motility through the use of density changes, which is discussed further below. In Margalef's mandala (1978), a typical phytoplankton succession would follow along a trend from high turbulence-high nutrients to low turbulence-low nutrients. Although Margalef's model provided a useful stimulus and starting point for modeling phytoplankton communities, various researchers (e.g., Smayda and Reynolds 2001) pointed out some of the limitations, including that turbulence and nutrients are independent variables. This helps explain why red tides tend to appear in the rather anomalous situation of high nutrients with low turbulence. A mandala is a useful format to summarize the characteristic dominant diatoms found in different marine planktonic habitats and their respective survival strategies (Fig. 10.3b). In practice, although attempts to formulate a general concept in terms of K and r strategies can be a helpful guide, caution is needed not to oversimplify, because many species can show plastic responses, especially at times of stress.

10.2.3 Resting Stages

One of the adaptations used by diatoms to cope with stressful environmental conditions is the formation of resting stages (Hargraves and French 1983; McQuoid and Hobson 1996; Kuwata and Takahashi 1990; Kuwata et al. 1993; Jewson et al. 2008, 2010). Typically, many coastal marine-centric species, such as *Thalassiosira* and *Chaetoceros* species, which live in turbulent temperate waters, form morphologically distinct resting spores that settle to the bottom to avoid low nutrient concentrations during summer stratification. They rely on being resuspended from the sediments by physical processes (see McQuiod and Hobson 1995; Montresor et al. 2013). Similar resting spores are also important in coastal, ice-related Antarctic habitats, e.g., *Thalassiosira antarctica* and *Porosira glacialis* (Pike et al. 2009). Both of the latter species form resting spores in autumn, when freezing conditions are associated with decreasing irradiance and temperatures but increasing salinity. As well as spores, diatoms have evolved another resting strategy that is based on internal cellular changes, which are called resting cells. Resting cells are more often found in freshwater and

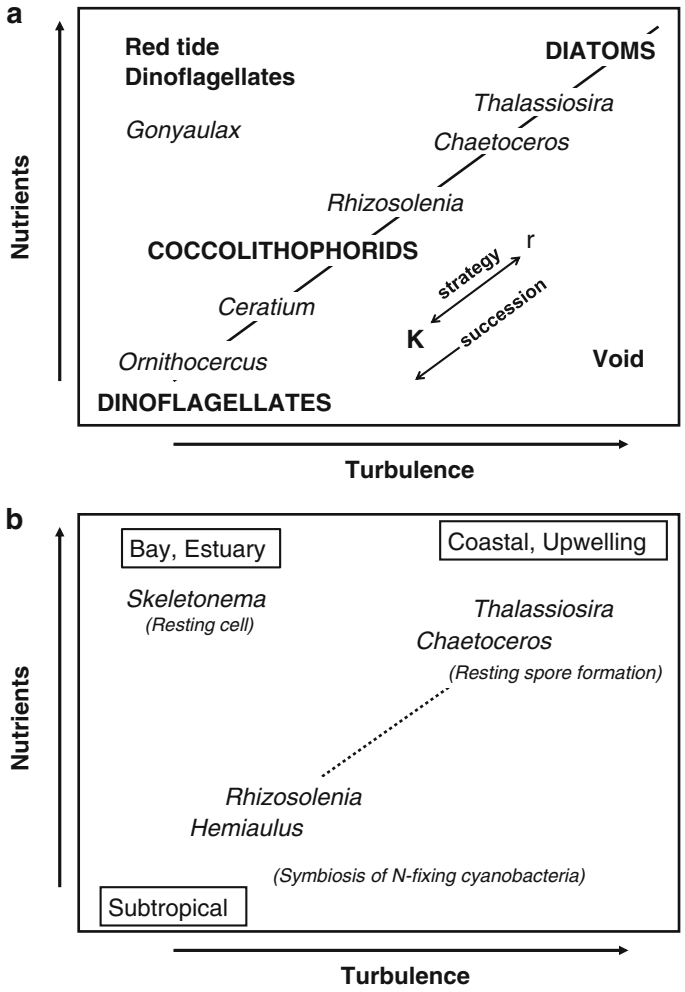

Fig. 10.3 Diagrammatic representation based on (**a**) Margalef's phytoplankton mandala (from Margalef 1978; Estrada and Berdalet 1997), showing a scheme that summarizes the range of ecological niches of marine phytoplankton in relation to variations in nutrient concentration and turbulence; and (**b**) a mandala summarizing characteristic dominant diatoms found in different marine planktonic habitats and their respective survival strategies

pennate diatoms (McQuoid and Hobson 1996). However, some marine species can form both resting spores and resting cells, depending on conditions (Fig. 10.4). For example, *C. pseudocurvisetus* forms resting spores under nitrogen depletion, but this requires large amounts of silica to form the thick spore walls (Kuwata and Takahashi 1990). This thickening may help survival if eaten by copepods (Kuwata and Tsuda 2005). In conditions where silica becomes exhausted, only a part of the population forms resting spores, with the remainder forming resting cells. Each form of resting stage in *C. pseudocurvisetus* shows different degrees of depressed metabolic activity

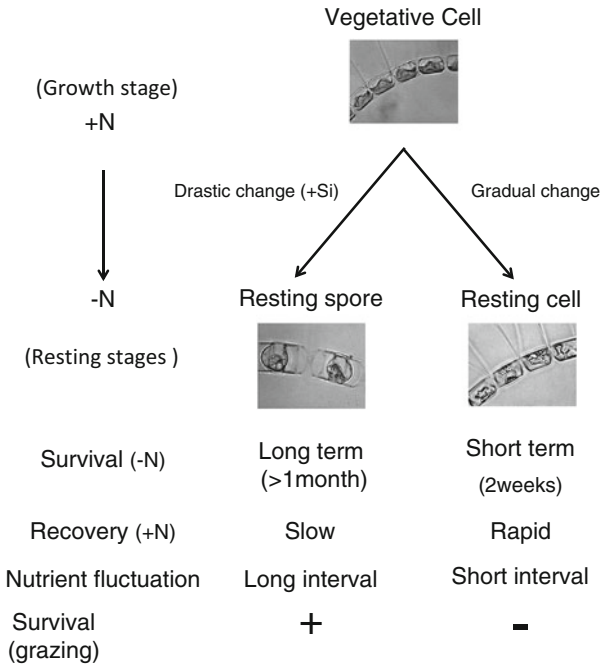

Fig. 10.4 Diagrammatic summary of nitrate conditions favoring growth or formation of either resting spores or resting cells, depending on whether nitrate and silicate availability is limiting and/or fluctuating, in a marine planktonic diatom, *Chaetoceros pseudocurvisetus* (photos taken by A. Kuwata)

and accumulation of glucan and/or neutral lipid as an energy store (Kuwata et al. 1993; Oku and Kamatani 1999). The result is that resting spores have a greater capacity to survive over extended periods, but resting cells can respond more rapidly when nitrogen becomes available again and so they can achieve active vegetative growth quicker (Kuwata and Takahashi 1999). Therefore, if nutrient supply is intermittent and only available for short time intervals, then resting cells represent a better strategy for the maintenance of the population, but if nutrient depletion lasts for longer periods, then the balance shifts in favor of resting spores. However, although resting cells do not normally survive estivation for as long as spores, they can do so in some specialized circumstances, such as in oxygenated freshwater sediments, where resting cells can last for up to 18 months of burial before survival starts to drop drastically (Jewson 1992; Jewson et al. 2006), or in marine sediments, where a correlation has been found between dark survival potential and the ability to accumulate nitrate intracellularly (Kamp et al. 2011). This can aid survival from months to decades in dark, anoxic conditions. In a later report, Kamp et al. (2013), showed that a possible survival mechanism used by the abundant oceanic species *Thalassiosira weissflogii* was the ability to use its intracellular nitrate pool for dissimilatory nitrate reduction to ammonium after sudden shifts to darkness and anoxia. Also, in stable oligotrophic

conditions, such as subtropical regions and the surface layers of stratified temperate pelagic regions in summer, some diatoms have developed more adaptable approaches, including symbiosis with nitrogen-fixing cyanobacteria (Fig. 10.3b), which are discussed further below; see also Chap. 19 by Decelle et al. (2015).

10.2.4 Physiological Response to Stress

Resting spores are rarely found in species that live in the open oceans, because of the heavy loss factor (e.g., Rynearson et al. 2013). Instead, alternative survival strategies have evolved that make use of changes in cell physiology to allow more plastic responses, which can then make it possible for cells to stay in suspension during stressful conditions. For example, Van Mooy et al. (2009) showed that in phosphorus-limited cultures of the diatom *T. pseudonana*, non-phosphorus "betaine" lipids were substituted for phosphatidylcholine betaine lipids. This substitution saved them 16 ± 8 % of their total phosphorus demand compared with cells grown under phosphorus-replete conditions. Another marine diatom in the study, *Chaetoceros affinis*, was found to also use betaine lipids.

Although diatoms are not unique in this, it does show how in regions of oligotrophic ocean, where phosphate is scarce, cells have the option to reduce their cellular phosphorus requirements by substituting non-phosphorus membrane lipids for phospholipids. Further, Dyhrman et al. (2012) showed that when *T. pseudonana* was grown under phosphorus deficiency, a number of changes occurred in cellular phosphorus allocation, including polyphosphate production, increased phosphorus transport, a switch to utilization of dissolved organic phosphorus through increased production of metalloenzymes, and a remodeling of the cell surface through production of sulfolipids. So, *T. pseudonana* has evolved a sophisticated response to phosphorus deficiency that involves multiple biochemical processes.

10.2.5 Iron Limitation

Another important research area producing examples of diatom adaptability is the response to low concentrations of iron. It is estimated that availability of iron limits primary productivity in 30–40 % of the world's oceans, particularly in regions of the Southern Ocean, equatorial Pacific Ocean and north Pacific Ocean (Moore et al. 2002; Denman 2008). These regions have relatively high concentrations of nutrients, such as nitrate, phosphate and silicate, but support only low concentrations of algal biomass, because concentrations of iron are exceedingly low. In these conditions, diatoms reduce their iron requirements (Sunda et al. 1991; Sunda 2001; Marchetti et al. 2006). Open-ocean-centric species, such as some *Thalassiosira*,

seem to have permanently modified their photosynthetic apparatus to require less iron (Strzepek and Harrison 2004), replacing iron-requiring electron-transport proteins with equivalent ones that need copper (Peers and Price 2006). The downside of this adaptation is that it seems to reduce their ability to cope with rapidly fluctuating light fields, which are more characteristic of coastal environments (Strzepek and Harrison 2004). Raphid diatoms seem to have evolved a different, more flexible approach that enables them to greatly reduce their iron requirements (Kustka et al. 2007). When starved of iron, *Phaeodactylum tricornutum* reduces the activity of those processes that require a lot of iron, such as photosynthesis, mitochondrial electron transport and nitrate assimilation (Allen et al. 2008), while also restructuring its proteome and increasing its use of alternative pathways for dealing with oxidative stress and iron uptake (Allen et al. 2008). Yet another adaptation linked to survival in exceedingly low iron availability was described by Thamatrakoln et al. (2012), who found that a specific protein in the coastal diatom *Thalassiosira pseudonana* localizes to the plastid and this enhances growth at subsaturating light intensities by increasing the photosynthetic efficiency of carbon fixation. In an earlier study, Marchetti and Cassar (2009) showed that diatoms responded to iron limitation with a decrease in cell size. They suggested that the associated increase in the surface area to volume ratio and the decrease in diffusive boundary layer thickness might improve nutrient uptake kinetics. They also suggested that it might be possible to use the valve morphometrics of pennates as a diagnostic tool for iron-limited diatom growth and relative changes in the Si:N (as well as Si:C) ratios in both extant diatom communities and fossil assemblages preserved in sediments. Other studies have also provided insights into survival in iron-limited oceans. Marchetti et al. (2009) found that the bloom-forming pennate diatoms *Pseudo-nitzschia* and *Fragilariopsis* use the iron-concentrating protein, ferritin, to store iron. Enhanced iron storage with ferritin gives the oceanic diatom *Pseudo-nitzschia granii* an advantage by allowing it to undergo several more cell divisions in the absence of iron than the comparably sized, oceanic-centric diatom *Thalassiosira oceanica*. Durkin et al. (2012) looked at frustule-related gene transcription in a microcosm study in the northeast subarctic Pacific Ocean and found evidence that community composition could influence silicon cycling in iron-limited environments. Another particularly good example arising from the iron limitation story and the identification of the role of individual genes is shown by a recent genetic study of two closely related diatom species by Lommer et al. (2010, 2012), who compared the oceanic diatom *Thalassiosira oceanica*, which is highly tolerant to iron limitation, with its coastal relative, *Thalassiosira pseudonana*. The results pointed to a recent transfer of an iron-regulated gene from the plastid to the nuclear genome, which allows the oceanic diatom to survive in a chronically iron-limited habitat. In a different study of proteins, Nunn et al. (2013) showed that *T. pseudonana* could acclimate physiologically to either low or high Fe concentrations within ten divisions. Studies like these have wider implications in helping us to better understand the general ecology of diatoms and, eventually, the factors driving their speciation.

10.2.6 Genomics and the Urea Cycle

However, one of the most striking recent discoveries was that diatoms have a ornithine–urea cycle (Armbrust et al. 2004; Allen et al. 2011; Bromke et al. 2013), which serves as a physiological distribution and repackaging hub for inorganic carbon and nitrogen, and contributes significantly to the metabolic response of diatoms to episodic nitrogen availability, enabling them to respond quickly and recover from prolonged periods of nutrient deprivation, so that they can rapidly proliferate after events such as upwellings. The presence of this cycle has many important implications for the evolutionary history of diatoms (see Allen et al. 2011). As we learn more about diatoms, it becomes apparent that their success is due to a wide range of adaptations that allow them to occupy so many different niches. In a review of the origin and evolution of diatoms, Kooistra et al. (2007) identified a number of factors contributing to the success of planktonic diatoms, including being well adapted to grow in deeply mixed and turbulent water columns, where cells are only intermittently exposed to high light levels (Mitchell and Holm-Hansen 1991; Falkowski and Raven 2007), the presence of a central vacuole that can store nutrients when plentiful for later use, a light harvesting system that is able to protect itself rapidly against high light intensity, CO_2 uptake mechanisms that are highly efficient, and formation of resting stages to overcome periods of stress. With the remarkable developments in diatom research in the last 5 years, we can now add to the list the numerous examples showing their physiological adaptability.

However, one cautionary note is that the model diatoms mainly used for genomic study, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, are not common in the ocean and are difficult to identify under the light microscope (Hasle and Syvertsen 1997). *T. pseudonana* is found in fresh, brackish and coastal water and is most likely descended from a freshwater ancestor (Alverson et al. 2011). The main habitat of *P. tricornutum* is intertidal rock pools (Hasle and Syvertsen 1997). Thus, caution is needed because the physiological traits identified from genomic information on these diatoms might differ from those of common oceanic species. Therefore, more genomic information is needed from oceanic taxa to help us understand more precisely their role in global processes.

10.2.7 Sinking and Staying in Suspension

A major contributor to diatom success has been the range of adaptations that allow them to remain in suspension or to sink to avoid stressful conditions (e.g., Smayda 1970; Smetacek 1985; Reynolds 1984). In plankton, size is an important factor (see Sarthou et al. 2005), with different niches requiring different balances between a whole range of processes from light absorption to nutrient uptake, growth rate and reducing the impact of grazing. It has often been thought that having a dense silica wall, and the resulting negative buoyancy, was a disadvantage for diatoms but it brings a number of advantages (Reynolds 1984; Raven and

Waite 2004), such as being able to sink to avoid light inhibition or nutrient limitation and also to protect against grazing by zooplankton (Hamm et al. 2003). According to Stokes' law (Hutchinson 1967; Miklasz and Denny 2010), the sinking rate increases with the square of the radius for spherical objects. Options to take advantage of or moderate this effect include changes in shape. Reynolds (1984) reviewed the literature on how changes from a spherical shape to plates or cylinders can reduce the sinking rate by 2–5 times. However, in marine conditions, there is also the possibility of changing cell density by altering the composition of the cytoplasm compared with the surrounding medium. In the largest diatoms, such as *Ethmodiscus rex* and several *Rhizoselenia* species, regulating their density can allow them to become positively buoyant (Smayda 1970; Villareal 1988, 1992; Moore and Villareal 1996; Villareal et al. 1996; Waite et al. 1997; Sarthou et al. 2005). Waite et al. (1992, 1997) showed that positive buoyancy requires energy expenditure, but that at other times, Stokes' law applies. Other diatom adaptations to give more subtle control of the sinking rate include the use of chitin threads secreted by the cell through strutted processes (fultoportulae) located in the cell wall. They can form long fibers (up to several times the diameter of the cell). Some species may have many of these, such as in the genus *Thalassiosira* and *Cyclotella* (McLachlan and Craigie 1966a, b; Herth and Schnepf 1982; Round et al. 1990; Kaczmarska et al. 2005). The role of these fibers is largely overlooked in terms of ecology, but there is now an improvement in our understanding of the genetics of chitin production (Durkin et al. 2009).

10.2.8 Life History

One adaptation widely used by diatoms is a size reduction and size restitution cycle to time the length of their life cycle, which enables them to switch cell resources from asexual cell division to sexual reproduction under optimum conditions (see Drebes 1977; Lewis 1983, 1984; Jewson 1992; Edlund and Stoermer 1997; Chepurnov et al. 2004; Mann 2011; Shirokawa and Shimada 2013; Kaczmarska et al. 2013). The importance of the role of sexual reproduction in population dynamics in marine planktonic diatoms is becoming recognized (e.g., Crawford 1995; D'Alelio et al. 2010). However, one of the diatom puzzles still to be solved is how cells determine when they are below the size threshold for switching from mitosis to meiosis.

10.2.9 Diatoms and Bacteria

One other exciting area that has developed out of genetic sequencing has been the identification of interactions between diatoms and bacteria (e.g., Amin et al. 2012). For example, symbiosis of diazotrophic cyanobacteria has been found inside the

frustules of *Hemiaulus hauckii*, and *Rhizosolenia clevei*, and on the spines of open ocean diatoms such as *Chaetoceros* spp. (Foster and Zehr 2006; Foster and O'Mullan 2008). Foster et al. (2011) reported the transport of nitrogen fixed in cyanobacterial heterocysts to their symbiotic diatom hosts. Other bacteria have been found in a range of close associations with diatoms (Carpenter and Janson 2000; Morris et al. 2006), including within the plastids of a freshwater Pinnularia diatom (Schmid 2003). Evidence is also being discovered on the role of diatom viruses as agents of horizontal gene transfer (Nagasaki 2008 and see also Chap. 20 in this book on Marine protist viruses by Nagasaki and Tomaru 2015, this volume).

10.2.10 Toxicity

Toxins produced by marine phytoplankton are causing increasing concern (Bates and Trainer 2006; Trainer et al. 2012). One worry has been an apparent increase found in sediments of *Pseudo-nitzschia* diatoms in coastal waters associated with increased eutrophication (Parsons et al. 2002). *Pseudo-nitzschia* produce the neurotoxin domoic acid and evidence from a widespread survey in the Pacific found it in both natural areas and areas that had been iron enriched (Silver et al. 2010). The toxin can produce problems for organisms higher up the food chain (Miralto et al. 1999) and may be produced in response to grazing, such as by copepods (Lauritano et al. 2011, 2012). More recently a non-protein amino acid, β-*N*-methylamino-L-alanine (BMAA), has been discovered to be produced by six diatom species (Jiang et al. 2014); previously, this compound, which causes a neurological disorder (Amyotrophic lateral sclerosis, ALS), was thought to be produced only by cyanobacteria.

10.2.11 Palaeoecology

Palaeoecological study of diatoms in marine sediments is going through an exciting period (see Smol and Stoermer 2010; Jordan and Stickley 2010; Leventer et al. 2010; Stickley et al. 2013). This is because evidence is accumulating that shows the benefits of integrating fossil data with present-day ecological theory. It is helping us to understand evolutionary processes as well as the effects of climate change on the functioning of marine ecosystems in the past, present and future (see Cermeño et al. 2013).

Diatoms are particularly useful in evaluating the degree of climate change because of their dominance in global processes and because they leave a fossil record. Much recent research has concentrated on the high latitudes of Antarctica and the Arctic, with studies combining palaeoecological and contemporary investigations (Pike et al. 2008, 2013). In particular, the southern high latitudes make a good case study, as they have been shown to play a critical role in the global cli-

mate system (Stickley et al. 2013). These latitudes have high nitrate but low algal and iron concentrations, as described above. Diatoms contribute up to 75 % of Southern Ocean production, most of which sinks as sediment to the seafloor, making it the world's main sink for biogenic silica for at least 4.5 million years (Cortese et al. 2004).

One of the main focuses of recent marine research has been establishing the response of the Antarctic ice sheets during the rapid climatic change that accompanied the last deglaciation (see Swann et al. 2013). This is important for understanding the susceptibility of these regions to future warming. Palaeoecological reconstruction from sediment cores in this area is helped by the presence of high-resolution seasonally laminated layers, in which diatom frustules are preserved (Pike and Stickley 2013). In one study (Pike et al. 2009) a relationship was found between changes in the Antarctic coastal diatoms *Thalassiosira antarctica* and *Porosira glacialis* and sea ice changes during the late Quaternary. This was confirmed by later studies investigating the last glacial and interglacial sea ice cover in the SW Atlantic, which is important in terms of the potential role in global deglaciation (Allen CS et al. 2011). Using a novel combination of diatom abundance stratigraphy with relative geomagnetic paleointensity data, and down-core magnetic susceptibility and ice core dust correlation, Collins et al. (2012) showed it was to possible to reconstruct high-resolution sea ice cover and correct earlier climate models that had suggested the sea ice extent potentially regulated the exchange of CO_2 release between the ocean and atmosphere during glacials. In another example of the use of diatoms, Pike et al. (2013) investigated causes for rising temperatures along the Antarctic Peninsula during the late Holocene, using the oxygen isotope composition of marine diatoms from Palmer Deep along the west Antarctic Peninsula continental margin. They found that two processes of atmospheric forcing (an increasing occurrence of La Niña events and rising levels of summer insolation) had a stronger influence during the late Holocene than oceanic processes driven by southern westerly winds and upwelling of the upper Circumpolar Deep water.

Further advances in reconstructing past environmental conditions will benefit from a greater understanding of the ecology of the diatoms. The Southern Ocean is a good example. In a study of three key species (*Fragilariopsis cylindrus*, *Chaetoceros simplex* and *Pseudo-nitzschia subcurvata*), Sackett et al. (2013) found that each species exhibited phenotypic plasticity in response to the salinity and temperature regimes that they experienced during the seasonal formation and decay of sea ice. The degree of plasticity (in terms of changes in macromolecular composition) was highly species specific and consistent with each species' known distribution and abundance in sea ice, meltwater and pelagic habitats. This suggests that phenotypic plasticity may have been selected for by the extreme variability of the polar marine environment. Sackett et al. (2013) argued that it is possible that changes in diatom macromolecular composition and shifts in species dominance occur in response to a changing climate and that this has the potential to alter nutrient and energy fluxes throughout the Southern Ocean ecosystem.

10.2.12 Evolution

A number of excellent recent reviews have covered the history of diatom evolution, using both fossil and genetic information (Round et al. 1990; Medlin et al. 1996; Armbrust et al. 2004; Sims et al. 2006; Kooistra et al. 2007; Bowler et al. 2008, 2010; Armbrust 2009; Medlin 2011). From the genetics, there is evidence that it is possible that the diatom lineage may go back to between 180 to 250 Myr (Medlin 2011), but the first siliceous fossils that have been found are marine and from about 110 Myr ago in the early Cretaceous (Gesonde and Harwood 1990; Harwood and Gesonde 1990; Suzuki and Oba 2015, this volume). There are only about 50 described species from this time, and many of these early forms are reminiscent of resting stages. They lived in coastal seas and they were radially symmetrical, a feature still typical of today's "centric" species. Possible earlier fossils have been described from the Jurassic, but these are pyritized and probably terrestrial (see Harwood and Nikolaev 1995; Nikolaev et al 2001). By the Late Cretaceous, diatoms had undergone extensive adaptive radiation and most of the early forms had disappeared (Harwood and Nikolaev 1995). Cell wall morphology was more typical of oceanic conditions, with specialized features already developed that are traceable to today's species (Jordan and Ito 2002). There were multiple species in several of the genera, such as *Hemiaulus* and *Triceratium,* which had similar basic morphologies but each had their own distinctive subtle variations in structure (e.g., Hajós and Stradner 1975). This is characteristic of conditions where speciation is occurring rapidly, because niches are available to be exploited. One good example is the benefits that developed from chain formation (see Crawford and Sims, 2008). The Late Cretaceous also sees the increasing importance of bipolar diatoms (e.g. Hemiaulales) and the first araphids (Harwood and Nikolaev 1995). At the end of the Cretaceous, about 65 Myr ago, diatoms survived the mass extinction that saw the loss of about 85 % of all species, including substantial reductions in the diversity of marine dinoflagellates and coccolithophorids. By 50 Myr ago, atmospheric O_2 concentrations had stabilized to today's levels, and atmospheric CO_2 concentrations continued to decline to near current levels (Guidry et al. 2007). This was followed in the middle Eocene (about 30 Myr ago) by the appearance of another major group of diatoms, the raphids, which are characterized by the presence of a highly specialized structure, the raphe, that gave the cell the ability to glide when in touch with a surface, and this opened up even more possibilities for adaptation and colonization of a wider range of habitats, especially benthic ones. Some species from this group remained or recolonized the plankton. One of these was the raphid species *Fragilariopsis kerguelensis*, which now dominates the diatom community in the Southern Ocean, which is the largest region of diatom-based carbon export (Zielinski and Gersonde 1997). Another is the toxic diatom genus *Pseudo-nitzschia*, discussed above.

10.3 Parmales

10.3.1 An Insight into Diatom Ancestry?

The exact origins of the diatoms has yet to be established, and it is still the subject of much debate, but the chances were greatly improved by the culturing of Parmales, which then made it possible to study their genetics (Ichinomiya et al. 2011). The order Parmales (Heterokonta) is a marine phytoplankton group that has small solitary cells, which are generally 2–5 μm in diameter with silicified cell walls composed of five or eight plates (Booth and Marchant 1987). They are widely distributed, but they are especially abundant in polar and subpolar waters (approximately 10^5–10^6 cells l^{-1}), where they are potentially important primary producers. However, until recently, parmalean algae had been described only from environmental samples examined by electron microscopy and, without information on their genomics, their phylogeny and evolutionary relationship with diatoms remained unresolved. Based on similarities in their intracellular ultrastructure and silicified cell wall, Parmales had been tentatively placed in the Chrysophyceae; therefore, a possible close association was proposed with diatoms (Mann and Marchant 1989).

One drawback of the study of Parmales was that it had been difficult to detect their cells in field samples due to their small size, but Ichinomiya et al. (2011) successfully identified cells and established a culture from the Oyashio region in the western subarctic Pacific by using a fluorescent dye, PDMPO [2-(4-pyridyl)-5-([4-(2-dimethylaminoethylaminocarbamoyl)-methoxy]phenyl)oxazole], which is co-deposited with incorporated silicon (Shimizu et al. 2001). SEM observation revealed that cells were surrounded by three shield plates, one dorsal plate, one ventral plate, and three girdle plates with a single wing (Fig. 10.1b), suggesting a similarity to *Triparma laevis* (Booth and Marchant 1987). Transmission electron microscopic (TEM) observation showed the typical ultrastructure of photosynthetic heterokontophytes, with two chloroplast endoplasmic reticulate membranes, three thylakoid lamellae and a mitochondrion with tubular cristae. HPLC analysis detected chlorophyll *a*, chlorophyll *c*, fucoxanthin and diadinoxanthin as major photosynthetic pigments, a composition shared with some other heterokont algae including diatoms.

Molecular phylogenetic analyses based on 18S rDNA and *rbc*L sequences surprisingly indicated that this early isolate was not closely related to Chrysophyceae but was included within the bolidophycean clade. Bolidophycean algae are small flagellates without siliceous cell wall structures and have been recognized as a sister group of diatoms based on their molecular phylogeny (Guillou et al. 1999a). Although parmalean algae are clearly distinguishable from bolidophycean algae by the presence of siliceous cell walls and the absence of flagella, their close relationship is supported by the similarity of their intracellular ultrastructures and photosynthetic pigment composition (Guillou et al. 1999a; Ichinomiya et al. 2011). It can be hypothesized that parmalean and bolidophycean algae (or their common ancestor) have a life cycle that switches between silicified non-flagellated and naked flag-

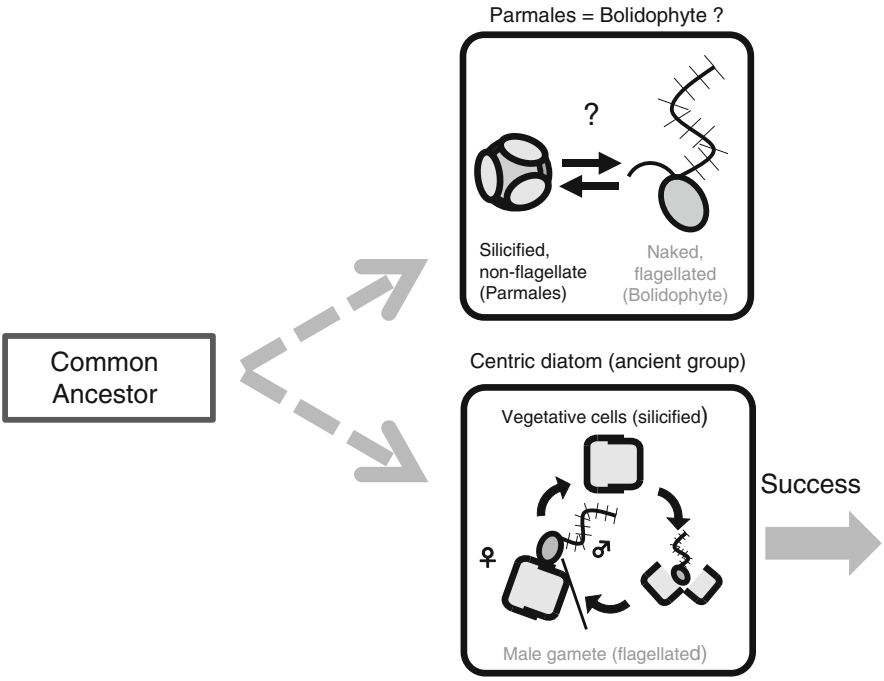

Fig. 10.5 Hypothetical summary of the possible evolutionary relationship of diatoms, Parmales and Bolidophytes

ellate stages, although such a morphological change within the isolated laboratory cultures has not been observed in either alga yet. This hypothetical life cycle would be similar to that of "centric" diatoms, which have a silicified, vegetative stage that switches to production of naked flagellated male gametes (sperm) for sexual reproduction (Fig. 10.5). Understanding how the life cycle of such a hypothetical ancestor of Parmales and Bolidophyceae might relate to that of diatoms is a key question. In a recent genomic study, transcriptome data of a *Bolidomonas* strain showed no heterozygous alleles in the coding regions and this suggests haploidity in *Bolidomonas* (Kessenich et al. 2014).

10.3.2 Effects of Silicon Limitation on Growth and Morphology of Parmales

We still know very little about the physiology of Parmales but, in a recent laboratory study, the effects of silicon limitation on growth and morphogenesis of plates was studied using the first established strain of *Triparma laevis*. The cells of *T. laevis* were surrounded by eight plates when grown with sufficient silicon. However, plate formation became incomplete when cells were cultured in a medium containing low

silicate (approximately <10 μM). Cells finally lost almost all their plates in a medium containing silicate concentrations lower than approximately 1 μM. However, silicon limitation did not affect the growth rate; cells continued to divide without changing their growth rate, even after all their plates were lost. Loss of plates was reversible; when cells without plates were transferred to a medium containing sufficient silicate, regeneration of shield and ventral plates was followed by the formation of girdle and triradiate plates. The results indicate that the response to silicon limitation in *T. laevis* is much more plastic (Yamada et al. 2014) than in most diatoms, where cell division usually ceases once silica becomes unavailable.

10.3.3 Ecology

Ecological studies of Parmales are at an early stage, but it is beginning to emerge that while Parmales is closely related to bolidophytes and diatoms, its growth characteristics appear different (Ichinomiya et al. 2013). *Bolidomonas pacifica*, which has been isolated from the tropical equatorial Pacific and the Mediterranean Sea (Guillou et al. 1999b), prefers warm conditions and has a high growth rate of 0.9 d^{-1} at 20 °C (Jacquet et al. 2001). Also, bloom-forming diatom species in the Oyashio region, such as *Thalassiosira nordenskioeldii* and *Chaetoceros debilis*, have much higher maximum growth rates (over 1.3 d^{-1}), with a broad temperature range of 0–15 °C, than that of *T. laevis* (Kuwata unpublished data). In this region, diatoms also form a dense bloom, reaching up to 10^5–10^6 cells l^{-1} in spring (Isada et al. 2009; Ichinomiya et al. 2010; Suzuki et al. 2011). This would be expected to give them an advantage throughout the euphotic zone in spring in the Oyashio region. In contrast, *T. laevis*, with its lower maximum growth rate (0.5 d^{-1}) and narrower temperature range (0–10 °C), was restricted in its development to the surface layer during the winter to spring period, but to the subsurface layer in summer, with a maximum abundance of 10^5 cells l^{-1} (Ichinomiya et al. 2013). This probably explains the relatively low abundances of *T. laevis* compared with diatoms.

Diatoms grow faster in the spring and are grazed by mesozooplankton, but they mostly sink out at the end of spring (Takahashi et al. 1990, 2008). In contrast, Parmales can remain in suspension in the subsurface layer due to their small cell size. Only a small fraction of Parmales sink out to below the euphotic zone in the northwestern Pacific (Komuro et al. 2005). These cells in the subsurface layer are probably grazed by nano- and micro-sized heterotrophic protozoans (Taniguchi et al. 1995) and incorporated into the microbial loop rather than the grazing food chain. However, we have no information on their buoyancy regulation, sinking rates or grazing. Many other aspects of their ecological role in marine ecosystems are still unclear; therefore, we need to learn more about what controls their temporal and spatial distributions, using combined laboratory and interdisciplinary field studies.

Also, future studies should include more genomic studies of diatoms and Parmales, to improve our understanding of their origin, evolutionary relationships and success in the modern ocean. If ecologically important species were selected

for this, and the derived information was used in conjunction with traditional marine programs, it could lead to a major step forward in our understanding of oceanic processes.

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Chapter 11
Planktonic Ciliates: Diverse Ecological Function in Seawater

Takashi Kamiyama

Abstract Planktonic ciliates are a representative group of microzooplankton, which play important roles in planktonic food webs. Based on results in previous various studies, quantitative information on roles as consumers of pico- and nanoplankton, as nutrient regenerators and as prey for various marine organisms were reviewed. The role as grazers of bacteria may be limited in small ciliates while autotrophic picoplankton is an essential food source for ciliates and can sustain populations of ciliates in oligotrophic waters. The grazing impact of ciliates is considered to greatly influence population dynamics of nanoplankton. Feeding responses of meso- and macrozooplankton suggested that ciliates become an essential food source for them. These findings show that ciliates play an important role in intermediation of energy flow from pico- and nanoplankton to larger plankton feeders. In addition, nutrient regeneration by ciliates is one of the important resources to support the production system not only in oligotrophic waters but also in other various waters. Furthermore, ciliates play roles as grazers for some harmful algal bloom species; the grazing impact may influence development and decay of the blooms. Since ciliates can also become food sources for bivalves and for polyp and ephyra stages of the moon jellyfish *Aurelia aurita*, they possibly contribute to production of bivalve aquaculture and occurrence of jellyfish blooms. These findings support that ciliates have diverse ecological functions in planktonic food webs.

Keywords Ciliate • Microzooplankton • Grazing impact • Nutrient regeneration • Harmful algal bloom • Jellyfish bloom • Bivalve aquaculture • Ecological role

T. Kamiyama (✉)
Tohoku National Fisheries Research Institute, Fisheries Research Agency,
3-27-5, Shinhama, Shiogama, Miyagi 985-0001, Japan
e-mail: kamiyama@affrc.go.jp

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11.1 Introduction

Since abundant microzooplankton passing through plankton nets have been found in the ocean, information on the taxonomy and ecophysiology has been accumulated based on various approaches. The predominant group in the microzooplankton community is free-living ciliates. Since the middle of 1900s, the abundance and biomass of ciliates have been clarified in various seawaters of the world, based on development or improvement of sampling and fixation methods (e.g., Capriulo 1990; Pierce and Turner 1992). Simultaneously, development of cultivation of ciliates enabled researchers to clarify high growth and feeding activities (Paranjape and Gold 1982; Gifford 1985). Furthermore, important roles of ciliates as food sources for meso- and macrozooplankton have been evaluated (Stoecker and Capuzzo 1990; Fessenden and Cowles 1994; Pérez et al. 1997). Results of these vigorous studies have suggested that ciliates play a role in intermediating trophic energy of picoplankton (including bacteria) and nanoplankton to higher trophic organisms through planktonic food webs. Information on the above topics has been reviewed in Taniguchi (1978), Fenchel (1987), Capriulo (1990), Laybourn-Parry (1992), Pierce and Turner (1992). Comprehensive information on tintinnid ciliates has been summarized (Dolan et al. 2013).

Here, among such ecological information on planktonic ciliates, I pick up quantitative information on ecological function in planktonic food webs according to the energy flow illustrated in Fig. 11.1, in comparison with the other nano- or microzooplankers (heterotrophic nanoflagellates, heterotrophic dinoflagellates). Nutrient

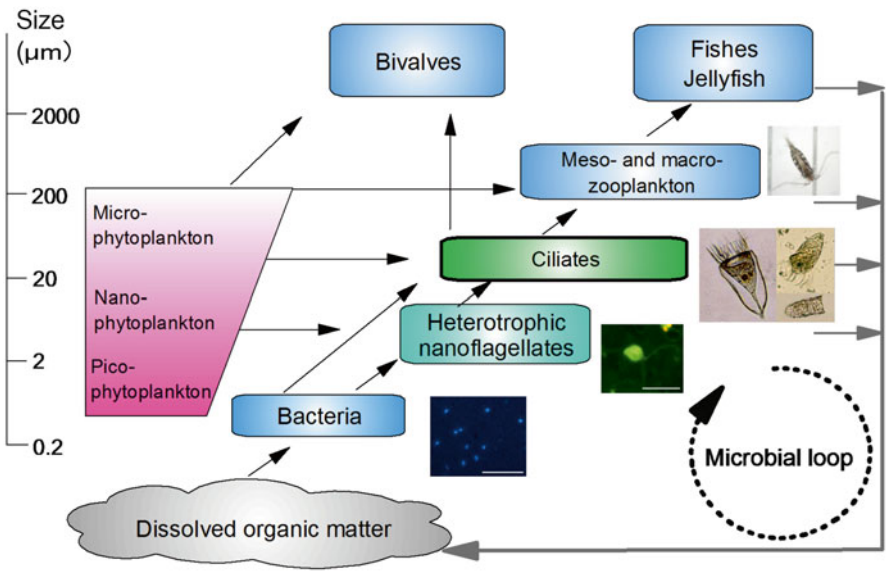

Fig. 11.1 Schematic energy flows of plankton food webs and the position of ciliates

regeneration caused by ubiquitous and abundant occurrences and high metabolic activities of ciliates will be mentioned as another function of ciliates, which contributes to primary production in various seawaters. Also, I want to highlight the function of ciliates in planktonic food webs by introducing specific roles of ciliates, which has not been focused on.

11.2 Potential Growth Influencing Grazing Impact of Ciliates

Individual feeding activities of ciliates on prey organisms are far lower than those of meso- and macrozooplankton, but community grazing impacts of ciliates generally surpass the others due to high growth ability of ciliates. There are many reviews on the potential growth ability of ciliates, which generally shows 1–2 divisions per day (e.g., Pierce and Turner 1992; Pérez et al. 1997). This level means that herbivorous ciliates can grow as faster as or faster than prey organisms (phytoplankton). The growth rates depend on the cell volume of ciliates and are associated with temperature, quality and quantity of the prey. Although the potential growth rates of ciliates cannot always be applied to natural population growth (Montagnes 2013), estimation of species-specific growth rates from environmental factors such as temperature and the cell volume is an effective method to understand population dynamics of the ciliate species (e.g., Müller and Gellar 1993), and the estimated values generally correspond to the values measured from the frequency of dividing cells (FDC) method under not food limitation conditions in temperate areas (Ota 2006). The growth ability of ciliates is generally higher than that of heterotrophic dinoflagellates, the same sized organisms of microzooplankton (Hansen et al. 1997). The specific growth rates of aloricate ciliates standardized at 15 °C was summarized to be 0.68–1.54 d^{-1} (Pérez et al. 1997), while the values of dinoflagellates are estimated to be 0.08–0.98 d^{-1} at the same temperature level based on reviewed data in Jeong (1999) and $Q_{10}=2.8$ (Hansen et al. 1997). Higher growth rates of ciliates do not always show predominant status of the microzooplankton community because heterotrophic dinoflagellates have a different survival strategy from ciliates as discussed in 11.3.4.

11.3 Grazing on Picoplankton and Nanoplankton

Almost all ciliate species are filter feeders and the main size range of the natural population is generally in the order magnitude of 10 μm. Also, the prey size is generally one eighth of ciliate body size (Hansen et al. 1994). Hence, picoplankton and nanoplankton are regarded as important prey sources for ciliates and ciliates sometimes control their population dynamics by grazing. Here I will evaluate feeding activities of ciliates and grazing impact on both prey sources.

11.3.1 Ciliates as Picoplankton Feeder

The concept of a microbial loop originating from seminal papers by Pomeroy (1974) and Azam et al. (1983) suggested that picoplankters mainly consisting of bacteria (heterotrophic bacteria), cyanobacteria and eukaryotic <2 μm phytoplankton are important primary producers. Among them, bacteria have been focused on as a food source for ciliates, and feeding activities of ciliates on bacteria have been clarified using various methods such as capture speeds of latex beads (Jonsson 1986) and fluorescent-labeled bacteria (FLB) (Sherr et al. 1987), the radio isotope tracer method (Hollibaugh et al. 1980), and the growth kinetic method (Rivier et al. 1985). In particular, the FLB method is an excellent approach to measure feeding rates on bacteria by natural ciliate assemblages and other protists, although immobility of surrogate prey particles possibly causes underestimation of the rates (e.g., Sherr and Sherr 1994). Practically, application of this method to various seawater thus has allowed us to know natural bacterivory activities of ciliates in each seawater systems. Feeding rates of ciliates depend on the target ciliates and experimental conditions, and the maximum clearance and ingestion rates of ciliates on bacteria prey are within the range in the order magnitude of 10^1–10^3 nl ind^{-1} h^{-1} and 10^1–10^3 cells ind^{-1} h^{-1}, respectively (Table 11.1). The volume-specific feeding rates on bacteria based on these previous data are in the same range as those on nanoplankton (Kamiyama 1999). Regardless of these feeding activities, results from many studies on bacterivory of ciliates suggested that ciliates cannot get enough food energy for their growth under the natural concentration levels of bacteria (e.g., Fenchel 1980; Rassoulzadegan and Etienne 1981; Capriulo and Carpenter 1983; Jonsson 1986; Fenchel and Jonsson 1988). This notion implied that bacteria would not be the main prey source for ciliates. However, after the existence of a lot of ciliates with nanoplankton size were discovered in the ocean using epifluorescence microscopy (Sherr et al. 1986), it was proved that the bacteria concentration in coastal waters can fill the energy demand of the small ciliates for growth (Sherr et al. 1989).

Utilization of bacteria by ciliates gave rise to our question as to how much impact ciliates can have on the bacterial community. The grazing impacts by ciliates in previous studies largely varied from 0 to 90 % of the standing stock of bacteria (Table 11.1). High values were observed in estuaries such as tidal creek water (Sherr et al. 1987) and embayments where ciliates were very abundant (>100 ind ml^{-1}, Seong et al. 2006), while the grazing impact in the oceanic water system seems to be not important because the values are only under 5 % of the bacterial standing stock per day. Grazing impact by the alternative bacteria feeder, heterotrophic nanoflagellates (HNF), estimated in the same study shows narrower ranges than values of ciliates (Fig. 11.2), and the importance seems to relatively increase in oceanic waters. Hence, the main consumer for bacteria is generally HNF in oceanic waters whereas the role of ciliates is probably limited in coastal waters. Furthermore, ciliates selectively feed on larger, actively growing and dividing cells in bacterioplankton assemblage as well as HNF (Sherr et al. 1992), implying that grazing impact of ciliates works a specific part of the bacterial assemblages, and limitedly influences changes in the bacteria community in coastal waters.

Table 11.1 Feeding activities of ciliates on bacteria in various seawater of the world

Area	Clearance rate on bacteria (nl ind^{-1} h^{-1})	Ingestion rate on bacteria (bacteria ind^{-1} h^{-1})	Grazing impact on standing stock of bacteria (% d^{-1})	Source
	Mean (range)			
Sapelo Island, tidal creek, USA	(140–260)		90	Sherr et al. (1987)
Sapelo Island, marsh floc, USA		(68–1802)		Albright et al. (1987)
Sapelo Island, marsh floc, USA		(148–4000)		Albright et al. (1987)
Sapelo Island, tidal creek, USA	168		45.3	Sherr et al. (1989)
Sapelo Island, open sound, USA	138		18.9	Sherr et al. (1989)
A salt marsh estuary	(140–270)			Sherr and Sherr (1987)
A salt marsh estuary	(49–406)			Gonzalez et al. (1990)
Savin Hill, Cove embayment, USA	90	224	6	Epstein and Shiaris (1992)
Korean waters	46 (4–385)	175 (15–713)	10.3 (0–59.6)	Seong et al. (2006)
Uchiumi Bay, Japan		(10–31)	(0.05–0.63)	Ichinotsuka et al. (2006)
Off South Island, NZ	(50–580)		(0.5–3.3)	James et al. (1996)
Northwest Mediterranean	(57–413)	(380–1095)		Sherr et al. (1988)
Northwest Mediterranean	(14–308)	(6–630)	1.6 (0.1–4.3)	Sherr et al. (1989)
Northwest Atlantic	296 (51–1408)	296 (24–1295)	(0.1–1.9)	Karayanni et al. (2008)
Takapoto Atoll	15	27.7	0.1	Sakka et al. (2000)
All range of the maximum value in each study	(15–1408)	(28–4000)	(0–90)	

Alternative autotrophic picoplankton (picophytoplankton) such as cyanobacteria are significant contributors to primary production in oligotrophic waters (e.g., Sherr and Sherr 1994). This group has a larger cell size than bacteria and has autofluorescent pigments. These characteristics allowed researchers to find these organisms within food vacuoles of small ciliates (Sherr et al. 1986; Kuosa 1990; Bernard and Rassoulzadegan 1993). The contribution of picophytoplankton to the food vacuole contents reached up to 72 % for small ciliates <30 μm (Rassoulzadegan et al. 1988) and could reach 100 % for three natural tintinnid ciliates (*Tintinnopsis beroidea*, *Rhabdonella* sp. *Dictyocysta elegans*) (Bernard and Rassoulzadegan

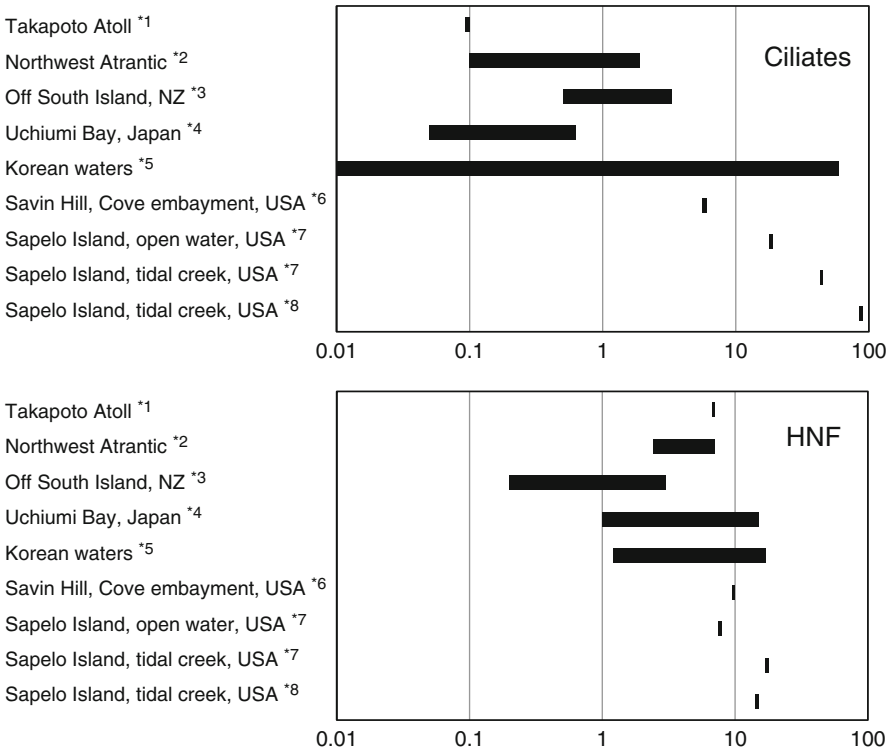

Fig. 11.2 Ranges of estimated daily consumption of ciliates and heterotrophic nanoflagellates (HNF) on the standing stock of bacteria in each water system in the same study. *1, Sakka et al. (2000); *2, Karayanni et al. (2008); *3, James et al. (1996); *4, Ichinotsuka et al. (2006); *5, Seong et al. (2006); *6, Epstein and Shiaris (1992); *7, Sherr et al. (1989); *8, Sherr et al. (1987)

1993). Also, growth of ciliates more sensitively responded to addition of cyanobacteria than that of bacteria (Pérez et al. 1996). These findings support that picophytoplankton are important food sources for ciliates, especially in oligotrophic waters. Also, in such seawater systems, grazing by ciliates can cause a significant impact on picophytoplankton as well as HNF. Small ciliates (<20 μm) could have consumed on average 17 % (1–35 %) of the cyanobacteria standing stock, and this value was evaluated to be at least equivalent to that of nanoflagellates in temperate nearshore waters of the Gulf of St. Lawrence, Canada (Tamigneaux et al. 1997). Estimated consumption rates of ciliates in the western, central and eastern Mediterranean Sea were 17–36 % of picophytoplankton production (Pitta et al. 2001). Grazing mortality of cyanobacteria by nanozooplankton nearly reached the level of their specific growth rates in oceanic surface waters off Japan, and one third of the value was estimated to be due to consumption of HNF and two thirds of the value due to ciliates (Kudoh et al. 1990). Importance of top-down effects of picophytoplankton by ciliates probably depends on contributions of small ciliates in the heterotrophic protist community.

11.3.2 Ciliates as Nanoplankton Feeders

The role of microplankton-sized ciliates as consumers of nanoplankton is generally more essential than that as consumers of picoplankton, considering the efficiency of energy intake from prey. Results of many studies in 1980s and 1990s showed that the size of prey observed in food vacuoles of ciliates is within the size category of nanoplankton (Bernard and Rassoulzadegan 1993) and the high feeding activity is exerted on prey size between 2 and 10 μm (e.g., Jonsson 1986; Kivi and Setälä 1995). Among nanoplankton, autotrophic ones (nanophytoplankton) have been used as a prey source to clarify feeding activities of ciliates in laboratory culture experiments, because they are easily cultivable by providing inorganic nutrients and light conditions without prey sources. Feeding activities on nanoplankton have been quantified using two major methods: decrease of prey organisms in environmental waters (e.g., Heinbokel 1978; Rassoulzadegan and Etienne 1981) and capture rates of surrogate prey such as fluorescent-labeled algae (FLA) (Rublee and Gallegos 1989). The former method has been used to measure the species-specific feeding rates at different prey concentrations and environmental factors, and the latter has often been used to measure the rates in the natural plankton community under natural environmental conditions. The maximum clearance and ingestion rates on nanoplankton obtained in these methods show a wide range as observed on bacteria; in the order of magnitude of 10^{0}–10^{2} μl ind^{-1} h^{-1} and 10^{-1}–10^{1} ngC ind^{-1} h^{-1}, respectively (Table 11.2). In general, volume-specific clearance rates and carbon-specific ingestion rates (ration in body carbon) are in the order of magnitude of 10^{4}–10^{5} h^{-1} and 10^{-2}–10^{0} d^{-1}, respectively, and maximum values reaching 1.7×10^{6} h^{-1} and 12.9 d^{-1} are reported, respectively (Table 11.2). This means that in general ciliates can clear the prey particles in the water of 3–30 times the cell volume per second and ingest the carbon of 0.24–24 times their own cells per day. There is no doubt ciliates are potentially important grazers on nanophytoplankton.

Grazing impact by ciliates are also very variable, but the daily values generally accounted for up to 60 % of the biomass of nanophytoplankton in coastal seawaters and up to 100 % of primary production due to mostly nanophytoplankton in various waters worldwide (Table 11.3). Although additional factors such as prey selectivity of ciliates under mixed prey conditions remains to be considered for the evaluations, it is certain that grazing impact of ciliates on phytoplankton community can strongly influence population dynamics of nanoplankton communities in various water systems.

The dilution method (Landry and Hassett 1982) and the modified methods (e.g., Gallegos 1989; Landry et al. 1995) have been widely used in various seawaters worldwide to evaluate grazing impact of microzooplankton community on phytoplankton. Results summarized in each region of world ocean showed that estimated daily consumption generally ranges from 20 to 80 % of the standing stock of phytoplankton (Chlorophyll-*a*) and from 59 to 75 % of the primary production (Calbet and Landry 2004). The values equal to and over 100 % of primary production were observed in several studies (Sherr and Sherr 2002). These values partly include the

Table 11.2 Feeding activities of marine ciliates on nanoplankton

	Prey		Clearance rate (µl ind^{-1} h^{-1})	Ingestion rate (ngC ind^{-1} h^{-1})	Volume specific clearance rate (×10^3 h^{-1})	Ration in body carbon (d^{-1})	
Ciliate species	Species/group	ESD (µm)	Mean (range)				Source
Balanion comatum	*Rhodomonas* sp.	10.3	2.8	2	1700	0.15	Jakobsen and Hansen (1997)
Balanion sp.	*Heterocapsa triquetra*	12.7		1.2*			Stoecker (1988)
Eutintinnus pectinis	Nanophytoplankton			(0.02–0.18)			Heinbokel (1978)
Favella azorica	*Heterocapsa circularisquama*	7.7	(4.1–27.5)	(0.38–7.20)		(0.03–0.55)	Kamiyama (1997)
F. taraikaensis	*Heterocapsa circularisquama*	7.7	(0.9–22.1)	(0.23–3.26)		(0.02–0.25)	Kamiyama (1997)
F. taraikaensis	*Prorocentrum minimum*	15–17	(0.4–14.5)	(0.01–7.12)		(0.01–12.9)	Taniguchi and Kawakami (1985)
Favella sp.	*Cryptomonas* sp., *Pyrenomonas salina*					3.8**	Strom and Morello (1998)
Favella sp.	Dinoflagellates		28.8	3.8*			Stoecker and Guillard (1982)
Favella sp.	*Heterocapsa triquetra*	12.7	(15–120)	1.9*			Stoecker (1988)
Helicostomella subulata	Nanophytoplankton		(0.3–4.8)	(0.01–0.33)			Heinbokel (1978)
H. subulata	Natural particles		(10–18)	(0.75–1.75)			Capriulo (1982)
Helicostomella spp.	Natural nanoplankton		0.35		50.1		Neuer and Cowles (1995)
Lohmanniella spiralis	Natural particles	1.5–18.9	(2.26–8.88)		(34.8–137)	(0.07–0.24)	Rassoulzadegan (1982)
Stenosemella olive	Natural particles		7	0.92			Capriulo (1982)

Stenosemella sp.	Natural particles	1.3–27		(0.10–0.88)		(0.43–2.23)	Rassoulzadegan and Etienne (1981)
Stenosemella spp.	Natural nanoplankton		(4.0–4.2)		(105–171)		Neuer and Cowles (1995)
Strobilidium spiralis	Nanophytoplankton		(3.0–4.0)		175	(0.13–0.15)	Verity (1991)
Strombidinopsis acuminatum	*Prorocentrum minimum*					5.4**	Strom and Morello (1998)
S. jeokjo	*Pfiesteria piscicida, Stoeckeria algicida*	13.5–13.9	(0.27–0.63)	(1.83–2.05)		(2.2–2.4)	Jeong et al. (2007)
S. jeokjo	*Oxyrrhis marina, Gyrodinium dominans*	15.6–20	(0.56–0.6)	(3.63–4.5)		(4.3–5.3)	Jeong et al. (2004)
Strombidinopsis sp.	*Protodinium simplex*	8.3		3.46		3.3	Montagnes and Lessard (1999)
Strombidinopsis sp.	*Prorocentrum minimum*	12.1		11.13		13	Jeong et al. (1999)
Strombidinopsis sp.	*Gymnodinium aureolum*	19.5		2.92		3.4	Yoo et al. (2010)
Strombidium sp.	*Pavlova lutheri*	2.2	(0.08–1.44)	(0.4–2.6)	(0.2–3.7)	(0.21–1.27)	Scott (1985)
Strombidium sp.	Nanophytoplankton			(0.63–0.82)			Verity (1988)
Tiarina spp.	Natural nanoplankton		3.85		100		Neuer and Cowles (1995)
Tintinnopsis acuminata	Nanophytoplankton		(1.4–2.6)	(0.04–0.38)			Heinbokel (1978)
T. acuminata	Natural particles		2	0.13			Capriulo (1982)

(continued)

Table 11.2 (continued)

Ciliate species	Prey: Species/group	Prey: ESD (μm)	Clearance rate (μl ind^{-1} h^{-1})	Ingestion rate (ngC ind^{-1} h^{-1})	Volume specific clearance rate (×10^3 h^{-1})	Ration in body carbon (d^{-1})	Source
			Mean (range)				
T. acuminata	*Dicrateria inornata*		(1.7–2.6)	0.18 (0.10–0.26)		(0.11–0.24)	Verity (1985)
T. dadayi	Nanophytoplankton			(0.13–0.30)			Verity (1988)
T. dadayi	Nanophytoplankton		(8–11)		114	(0.09–0.13)	Verity (1991)
T. fluviatile	Natural particles		(4–20)	(0.33–2.00)			Capriulo (1982)
T. parva	Natural particles		38	2.83			Capriulo (1982)
T. vasculum	*Dicrateria inornata*		(4.3–7.5)	(0.35–0.81)		(0.04–0.1)	Verity (1985)
T. vasculum	Natural particles		8	0.17			Capriulo (1982)
Uronema sp.	*Isochrysis galbana, Pyrenomonas salina*					5.9**	Strom and Morello (1998)
Natural ciliates	*Nannochloris*/*Chlorella*/*Thalasiossira*	1.9–5.3	2.5 (0.24–8.3)			(0.02–7.5)	Sherr et al. (1991)
All range of the maximum value in each study			(0.6–120)	(0.1–11)	(3.7–1700)	(0.1–12.9)	

Note that data using non-planktonic particles (beads and starch particles) were excluded

ESD equivalent spherical diameter

*Carbon contents of prey were estimated by each cell volume assumed as an elliptical sphere and a conversion factor of 0.36 pgC μm^{-3} for 10^1 μm^3 cells, 0.24 pgC μm^{-3} for 10^2 μm^3 cells or 0.16 pgC μm^{-3} for 10^3 μm^{3}cells (Verity et al. 1992)

**Ration in body volume (d^{-1})

consumption of protists other than ciliates and other small metazoans. However, since the maximum impact on primary production is similar to the values due to ciliate assemblages (Table 11.3), ciliates probably contribute to consumption of phytoplankton as the main component of microzooplankton.

Since HNF is the main grazer of bacteria, quantitative information on the feeding response of ciliates to HNF is important to evaluate the role of ciliates for linkage of the energy flow from bacteria to higher trophic level organisms, but there is still not a lot. Active growth and feeding responses of ciliates on a diet of HNF observed in laboratory experiments suggested that potentially HNF is an available food source for ciliates (Fenchel and Jonsson 1988; Verity 1991). Specific growth rates and yields of *Strombidium* sp. were observed to be 3–4 times higher when HNF was included as prey (Ohman and Synder 1991). Furthermore, the role as a consumer of HNF by microzooplankton has been directly evaluated in microbial food webs

Table 11.3 Grazing impact of ciliates on nanophytoplankton estimated in various waters of the world

	Grazing impact on nanophytoplankton		
	Standing stock (% d^{-1})	Production (% d^{-1})	
Area	Mean/range		Source
Long Island Sound, USA	12–41		Capriulo and Carpenter (1980)
Mediterranean	59		Rassoulzadegan and Etienne (1981)
Hylsfjord, Norway	6–37		Havskum and Hansen (1997)
Balsfjord, Norway	4–9		Archer et al. (2000)
Ullsfjord, Norway	3–9		Archer et al. (2000)
W Mediterranean, coastal		9–52	Rassoulzadegan et al. (1988)
Southern California Bight		4–20	Heinbokel and Beers (1979)
Solent, UK		60	Burkill (1982)
Central Long Island Sound		27	Capriulo and Carpenter (1983)
Subarctic Pacific, offshore		1–77	Strom et al. (1993)
W Mediterranean, offshore		25–40	Dolan and Marrasé (1995)
Antarctic, offshore		4–56	Klaas (1997)
Mediterranean, offshore		4–45	Dolan et al. (1999)
Ligurian Sea, Mediterranean		8–40	Pérez et al. (2000)
Mediterranean, offshore		26–70	Pitta et al. (2001)
Itata river plume, central Chile		0.2–99	Vargas and Martínez (2009)
Off Coliumo Bay, central Chile		64–90	Vargas and Martínez (2009)
Rhone River plume, NW Mediterranean		10–50	Christaki et al. (2009)
East China Sea		14–86	Ota and Taniguchi (2003)
All range	9–59	0.2–99	

using the dilution method with size fractionated seawater or with identification of each prey group. Safi et al. (2002) found that microzooplankton dominated by ciliates consumed 63 % of HNF production per day, which was higher than the value of chlorophyll-*a* production with the 5–22 μm size class (37 % d^{-1}). Umani and Beran (2003) showed that microzooplankton consumed more than 100 % d^{-1} of HNF production and 50–100 % d^{-1} of the initial standing stock in the northern Adriatic Sea. Because microzooplankton in both studies were dominated by ciliates when high consumption rates for HNF production were recorded, it is considered that strong trophic linkage from HNF to ciliates was formed.

11.3.3 Factors to Influence Feeding Activities of Ciliates

Feeding activities of ciliates on picoplankton and nanoplankton are influenced by spacial matching between ciliate and prey organisms, quality and quantity of prey items, and environmental factors. Ciliates have the ability of size selectivity for their prey. For tintinnid ciliates, 43–45 % and approximately 20 % of lorica diameter for the maximum and preferable prey size, respectively, were reported (reviewed in Montagnes 2013). The equivalent spherical diameter ratio between oligotrich ciliates and prey is considered to be 8:1 (Hansen et al. 1994). Hence, the size of prey may be one of the primary factors to decide which species dominate the ciliate population. Also, the upper limit of prey size for ciliates is lower than prey for heterotrophic dinoflagellates, which often ingest larger prey than their size. This may also be a key factor to determine which group dominated in microzooplankton in response to phytoplankton bloom (See Sect. 11.3.4).

Even if the size range of prey is suitable for ciliates, ciliate populations do not always develop and work their ecological function. Quality of prey is one of the essential factors to sensitively influence the feeding of ciliates. In the case of tintinnid ciliates, clear prey selection has been observed probably due to chemosensory behavior (Fenchel and Jonsson 1988; Stoecker 1988; Buskey and Stoecker 1989; Christaki et al. 1998). However, the chemical characteristics of substances inducing the chemosensory behavior have not been clarified.

Prey concentration is another important factor to influence their feeding rates. Changes in ingestion rates with prey concentrations is represented as a functional response, which is often fitted to the rectangular nonlinear model (e.g., Montagnes 2013). The half saturation constant, the prey concentration causing half of the maximum ingestion rate, is used as an indicator of ingestion response to prey concentration. If actual prey concentration mostly exceeds this value, ciliates would be able to use the potential feeding activity regardless of the prey concentration, and if not, ingestion rates would be sensitively influenced by prey concentration. In the case of marine ciliates, the half saturation constant is approximately 0.7 ppm, which is not influenced by ciliate cell size (Hansen et al. 1997). This level corresponds to the values under eutrophic seawater conditions (Sheldon et al. 1972). Hence, feeding of ciliates is strongly influenced by prey concentration except in eutrophic seawater even if all prey organisms were suitable for the ciliates.

As abiotic environmental factors, temperature, light, pH, and trace metals are considered to be effective factors influencing ecophysiological response of ciliates (Montagnes 2013). Increase of temperature promotes the growth and ingestion of ciliates; this relationship is often fitted to the Q_{10} model; the value for ciliates is often used as 2.8 (Hansen et al. 1997). However, the relation on the growth rate of ciliates is more realistic to be fitted to a positive linear function of 0.07 d^{-1} $°C^{-1}$ (Montagnes et al. 2003). In the natural ciliate community, change in temperature probably cause succession of predominant species as well as changes in growth and feeding activities because ciliate species have different suitable temperatures for appearance in seawater from sediment due to excystment (e.g., Kamiyama and Aizawa 1992; Kim and Taniguchi 1995). Ciliates showed positive phototactic behavior based on the data in Jonsson (1989) and Storm (2001). This behavior is available for staying in good prey conditions because many autotrophic flagellates have the same reaction. Pedersen and Hansen (2003) reported that growth of some of ciliates was influenced at pH 8.8–8.9, and inhibited at more than pH 9, which is lower than the limit of phytoplankton (Schmidt and Hansen 2001). The pH tolerance was different among the ciliate and dinoflagellate species. There results suggested that phytoplankton blooms leading to high pH in seawater potentially inhibit feeding and growth responses of some ciliate species and then may cause changes in the ciliate community and their grazing impact. Trace metal concentration in culture medium for ciliates influenced growth of oligotrich ciliate (Gifford 1985), and the effect is more sensitive than phytoplankton. If responses to dissolved chemical substances are different among ciliate species, this possibly become a factor causing succession of predominant species of the ciliate community, and may lead to changes in grazing impact on phytoplankton.

11.3.4 Comparison with the Heterotrophic Dinoflagellates

Heterotrophic dinoflagellates are one of the main members of microzooplankton as well as ciliates. Their ecological function as consumers of primary production is similar to ciliates. However, there are some characteristics different from ciliates. First, relatively lower feeding activities cause lower growth rates of dinoflagellates than ciliates (e.g., Hansen et al. 1997). This may be due to lower efficiency of raptorial feeding of most dinoflagellates than that of typical filter feeding of ciliates (Sherr and Sherr 2007; Jeong et al. 2010). This means that dinoflagellates lose the "competition" for development of populations between dinoflagellate and ciliates under sufficient food conditions. However, ciliates have a threshold concentration of prey to stop feeding on prey and die out within a few days when the prey concentration decreased below the threshold level (Fenchel 1990; Montagnes 1996; Jakobsen and Hansen 1997). This ecological weak point is in contrast to the capability of dinoflagellates to survive for over 1 month without food (Strom 1991; Menden-Deuer et al. 2005), and the difference suggests that dinoflagellates keep their densities higher than ciliates in seawaters with low prey density. Menden-Deuer et al. (2005) demonstrated that dinoflagellates can store a part of food energy

within the cells while ciliates mostly use the food energy for their growth. Secondly, the ability of dinoflagellates to utilize larger prey particles than their body scale together with nanoplankton sized prey (Jeong et al. 2010) is largely different from ciliates. For example, development of dinoflagellate populations after a diatom bloom in an Fe fertilization experiment in HNLC waters (Saito et al. 2005) may be realized by the survival strategy of dinoflagellates in sparse prey environments and the ability to utilize larger prey particles than their body scale (Jeong et al. 2010). The difference in survival strategy of both organisms may have resulted in the prosperity of both organisms in the microzooplankton community.

11.4 Prey for Meso- and Macrozooplankton

Evaluation of food values for meso- and macrozooplankton is one of the key points to consider the role of ciliates intermediating between microbial food energy and higher trophic levels. Gut content analyses have been carried out to elucidate the available food for meso and macrozooplankton, and tintinnid ciliates with a species-specific hard part (lorica) have been focused on (Pierce and Turner 1992). Stoecker (2013) showed that protists, copepods, euphausiids, mysids, penaeids, amphipods, gelatinous plankton, rotifers, ostracods, cladocerans, a variety of larval fish, and benthic invertebrates become predators of tintinnid ciliates. This section will especially point out quantitative information on predation of meso- and macrozooplankton on ciliates.

Copepods, the predominant mesozooplankton, have been focused on as consumers of ciliates, and then a lot of quantitative information about the feeding activities has been accumulated in feeding experiments during the past three decades (Stoecker and Capuzzo 1990; Stoecker 2013). The maximum clearance and ingestion rates on ciliates generally range from 10 to 1000 ml ind^{-1} d^{-1} and from 0.1 to 6 μgC ind^{-1} d^{-1} (Table 11.4). Daily carbon-specific feeding rates (ration in body carbon) of copepods on ciliates show a wide range (1–360 % d^{-1}), depending on the species and size of prey organisms. The values of large copepods in subarctic areas of the northern Pacific Ocean is low (less than 3 % d^{-1}) but small and meso-sized copepods in coastal or temperate areas indicated relatively high values exceeding 100 % d^{-1}. Prey energy captured from only ciliates sometimes can exceed the carbon demand of the copepods estimated from the respiration. Also, selective feeding and higher feeding rates of copepods on ciliates rather than phytoplankton have been clarified in several studies (Stoecker 2013). This is considered to be due to the larger size of ciliates than phytoplankton and the specific swimming behavior and/or chemical materials favorable for copepods (Stoecker and Capuzzo 1990). However, in natural mixed prey conditions, it is realistic that copepod feeding on ciliates is strongly influenced by other dominant prey (blooming phytoplankton). Interestingly, Fessenden and Cowles (1994) clearly showed this notion in that *Calanus pacificus* revealed significant feeding rates on only ciliates at the low chlorophyll-*a* concentration and on only chlorophyll-*a* fraction at a phytoplankton bloom. In addition, the

Table 11.4 Feeding activities of various copepod species on a diet of ciliates

Copepods	Clearance rate (ml ind^{-1} d^{-1})	Ingestion rate (μgC ind^{-1} d^{-1})	Ration in metabolic required carbon (% d^{-1})	Ration in consumed prey carbon (% d^{-1})	Ration in body carbon (% d^{-1})	Source
	Mean/range					
Acartia clause	7.4–21.5					Ayukai (1987)
A. clause		2.87–4.81			55–92	Dutz and Peters (2008)
A. tonsa	24–168	0.16–1.22		3–41	5.7–84.5	Gifford and Dagg (1988)
A. tonsa			48–422		40.8–360	Robertson (1983)
A. tonsa			68		6.5–58.6	Robertson (1983)
A. tonsa	5–290	0.1–0.4				Vargas and Gonzalez (2004)
A. tonsa nauplius	0.72–23.3					Dolan (1991)
Calanus australis	467–1078	0.08–2.73		1–27		Sánchez et al. (2011)
C. helgolandicus	66.5					Vincent and Hartmann (2001)
C. pacificus	298–778	0–6.5	0–100	0–100		Fessenden and Cowles (1994)
Centropages abdominalis	28.8–170	0.2–0.9	18–82	16.4–100		Fessenden and Cowles (1994)
C. brachiatus	150–290	0.1–0.3				Vargas and Gonzalez (2004)
C. chierhiae	104					Vincent and Hartmann (2001)
C. typicus	76–90	0.17–1.51			9–29	Broglio et al. (2004)
Clausocalanus spp.	37–110	0.06–0.18			0–2	Broglio et al. (2004)
Eucalanus pilcatu	110–146	1.52–2.64		19–30		Verity and Paffenhofer (1996)

(continued)

Table 11.4 (continued)

Copepods	Clearance rate (ml ind^{-1} d^{-1})	Ingestion rate (μgC ind^{-1} d^{-1})	Ration in metabolic required carbon (% d^{-1})	Ration in consumed prey carbon (% d^{-1})	Ration in body carbon (% d^{-1})	Source
	Mean/range					
Euterpina acutifrons	4–21	0.05–0.12			8–17	Broglio et al. (2004)
Metrida spp.	197–231				8–16	Lonsdale et al. (2000)
Neocalanus cristatus	370–800	1.31–4.22			0–0.7	Gifford (1993)
N. plumchrus	170–940	0.91–3.91			1–5	Gifford (1993)
Oithona similis	0–12.7	0–0.06				Castellani et al. (2005)
O. similis	27.1				78	Lonsdale et al. (2000)
O. similis	95–100	0.0–0.1				Vargas and Gonzalez (2004)
Oithona spp.	5–26	0.02–0.16			3–39	Broglio et al. (2004)
Paracalanus parvus	23–73	0.06–0.68			3–30	Broglio et al. (2004)
P. parvus	0–220	0.05–0.2				Vargas and Gonzalez (2004)
Pseudocalanus sp.	115–178	4.6		0.48		Fessenden and Cowles (1994)
Temora longicornis	77					Vincent and Hartmann (2001)
T. stylifera	24–103	0.6–0.13			1–4	Broglio et al. (2004)
All range of the maximum value in each study	13–1078	0.1–6.5	68–422	0.5–100	0.7–360	

biochemical makeup of heterotrophic protists can differ from algae, making them an essential nutritional ingredient in a copepod's diet (Tang and Taal 2005). In fact, ciliates are important sources to provide nitrogen to copepods and are also rich in polyunsaturated fatty acids and eicosapentaenoic acid, which is effective for reproduction of copepods (Dutz and Peters 2008). In a plankton community dominated by pico- and nanosized organisms, active utilization of ciliates by copepods means effective transportation of microbial energy sources to higher trophic levels.

The feeding impact of copepods on ciliate assemblage depends on the abundance of ciliates, phytoplankton biomass and its size composition. In oligotrophic waters (e.g., the Mediterranean Sea) and oceanic areas around the Arctic and Antarctic areas, in general, the predation pressure of copepods and other metazoans on the standing stock of ciliates is low (Table 11.5). However, in coastal areas and upwelling areas, predation by copepods often has a significant impact on ciliate assemblages (Fessenden and Cowles 1994; Nakamura and Turner 1997). Calbet and Saiz (2005) evaluated top-down effects of copepods on ciliates in a normal situation based on global averages of ciliate abundance and copepod ingestion rates. As a result, they considered that high copepod density with more than 7 ind l^{-1} is necessary to reduce more than 50 % of ciliate standing stocks. This situation seldom occurs in common oceanic waters but may often appear in a transition phase of the plankton community such as the case that abundances of ciliates and copepods are both high in a low concentration of phytoplankton at the end of the bloom or in phytoplankton assemblages dominated by pico- or nanophytoplankton (Stoecker 2013). In this case, strong predation by copepods on ciliates can induce trophic cascades such as release of top-down effects on phytoplankton by ciliates (e.g., Stoecker and Sanders 1985; Calbet and Saiz 2005).

The qualitative information on other crustacean feeding on ciliates is not a lot compared with copepods. Cladocera is another crustacean zooplankton to consume ciliates as well. The ingestion rates of the genus *Evadne*, *Penilia* and *Podon* in natural seawater in the NW Mediterranean Sea ranged from 0.01 to 0.70 μgC ind^{-1} d^{-1}, and carbon-specific ingestion ranged from 1 to 70 % d^{-1} (Broglio et al. 2004). Ingestion rates and clearance rates on ciliates by *Podon leuckarti* collected in the Comau Fjord, Chile, were reported to be 0.02–0.17μgC ind^{-1} d^{-1} and 133–367 ml ind^{-1} d^{-1}, respectively, and the values on ciliates were constituted as 2–7 % of ingestion on total prey organisms (Sánchez et al. 2011). *Euphausia pacifica*, an important krill in the north Pacific Ocean, was able to feed on protozoa and phytoplankton as well as small crustacea (Atkinson and Synder 1997). In a feeding experiment, ingestion rates on a diet of cultured aloricate ciliate *Strombidium conicum* were reported to be 1.01–3.24 μgC ind h^{-1} (Nakagawa et al. 2004). The daily ration accounted for 0.05–2.3 % of body carbon, which was close to daily carbon demand for their growth, respiration, and reproduction (2–4 %, Ross 1982). It is likely that ciliates become compensatory prey for survival of these crustacean zooplankton, especially during the period when phytoplankton is not abundant.

Gelatinous zooplankton is another candidate to utilize ciliates, because many groups are effective filter feeders to be able to feed on small zooplankton in seawater. Quantitative information on feeding of scyphomedusae and ctenophores is

Table 11.5 Feeding impact of crustacean zooplankton on ciliates and dinoflagellates of the standing stock and production of the prey in various areas of the world

Predator	Prey	Area	Grazing impact on		Source
			Standing stock (% d^{-1})	Production (% d^{-1})	
Acartia hongi	Ciliates + dinoflagellates	Gyeonggi Bay, Yellow Sea		13.7	Yang et al. (2010)
Acartia tonsa nauplius	Ciliates	Chesapeake Bay	34–200		Dolan (1991)
Calanus australis	Ciliates	Comau Fjord, Chile	27–73		Sanchez et al. (2011)
Neocalanus plumchrus	Ciliates + dinoflagellates	Gulf of Mexico, subarctic North Pacific	11–16		Gifford and Dagg (1991)
Oithona similis	Ciliates + dinoflagellates	Buzzards Bay, USA	8–46		Nakamura and Turner (1997)
O. similis	Ciliates	Ross Sea, Antarctica	0.3–4.8		Lonsdale et al. (2000)
O. similis	Ciliates + dinoflagellates	Oyashio region	0.3–1.5		Nishibe et al. (2010)
Copepod community	Ciliates	Southern Kattegat Denmark	53		Nielsen and Kiørboe (1991)
Copepod community	Ciliates	Oregon, USA	25–45		Fessenden and Cowles (1994)
Copepod community	Ciliates	North of South Georgia, Antarctica	0.057		Atkinson (1996)
Copepod community	Ciliates + dinoflagellates	NW coast, Spain	0.9–3.3	2.4–51.4	Batten et al. (2001)
Copepod community	Ciliates	NW Mediterranean	0.02–3.7		Broglio et al. (2004)
Copepod community	Ciliates	Irish Sea	1–47		Figueiredo et al. (2009)
Crustacean community	Ciliates	NW Mediterranean	0.8–6.9		Broglio et al. (2004)
Mesozooplankton	Ciliates	Alboran Sea SW Mediterranean Sea	0.3–3.5		Calbet et al. (2002)
Mesozooplankton	Ciliates	East New Zealand	6.4	21.3	Zeldis et al. (2002)
All range			0.3–200	2.4–51	

limited, but microzooplankton including ciliates may directly and indirectly be concerned with the recent increase of dense swarms of these jellyfish in various areas of the world (Purcell et al. 2007). The representative species of both groups are *Aurelia aurita* s.l. and *Mnemiopsis leidyi*, respectively. Feeding responses of *A. aurita* to ciliates will be described in Sect. 11.6.2. Volume-specific feeding rates of small *M. leidyi* on ciliates (>20 μm) in a laboratory experiment were measured to be 0.84–1.67 l cm^{-3} d^{-1}, which is about equal in magnitude to those on copepods (Stoecker et al. 1987b). The importance of ciliates in the diets of *M. leidyi* is variable in proportional to the relative biomass of ciliates and crustaceans in the plankton. Sullivan and Gifford (2004) evaluated that larval *M. leidyi*, if abundant, potentially exert a significant predation impact on their prey populations consisting of microplankton in the water column. In fact, top-down control of *M. leidyi* on microzooplankton ciliates in field seawater was confirmed by McNamara et al. (2013). However, the role of sole ciliates is still unknown.

11.5 Nutrient Regeneration

As an implication of the microbial loop concept, the main players operating the function of nutrient regeneration in the planktonic ecosystem are probably micro- and nanozooplankton, because of their high metabolic activities per body and high densities. However, to date the role of ciliates as nutrient regenerators has been hardly clarified quantitatively. This function is essential in oceanic waters under nutrient-poor conditions. On the other hand, regenerated nutrients have not attracted the attentions of researchers under nutrient-rich conditions observed in the upwelling or coastal areas. However, since the nutrient depletion is seasonally observed even in subarctic waters and coastal waters, the function of nutrient regeneration by microzooplankton including ciliates cannot be possibly negligible. Ota and Taniguchi (2001) evaluated potential regenerated nitrogen (NH_4-N) from ciliate assemblages based on growth rates, primary production, and gross growth efficiency cited from other reports in subtropical waters and subarctic waters. The ratio of regenerated NH_4-N to nutrient demand for primary production ranged from 14 to 17 % in the former regions and from 56 to 66 % in the latter regions. Furthermore, Ota and Taniguchi (2003) reported that NH_4-N excretion rates of ciliates were estimated to be 0.1–63.8 mgN $m^{-2}d^{-1}$, equivalent to 0.1–93.8 % of the nitrogen requirement by primary producers in summer and autumn in the East China Sea. These results suggest that even in nutrient rich areas, nitrogen regenerated by ciliates could be a significant contribution to sustainable primary productivity during periods of nutrient depletion. Data from laboratory experiments indicated that excretion rates of nitrogen such as NH_4-N from ciliates are generally in the range of 0.3–10 μgN dry-mg^{-1} h^{-1} (Caron and Goldman 1990). However, problems of data precision due to artifact errors in the experimental procedure and adsorption of regenerated nutrients by phytoplankton in experimental bottles were also pointed out. Accumulation and validation of the potential activities of nutrient regeneration by ciliates remain

in the future. On the other hand, the ^{15}N isotope dilution method (Paasche and Kristiansen 1982) and modified dilution methods (Andersen et al. 1991; Neuer and Franks 1993) have been developed to estimate the nutrient regeneration by microzooplankton under natural environmental conditions. These methods may be powerful to measure the regeneration activities of the microzooplankton community in field waters, and applicable to evaluate the role of ciliate community, which dominated microzooplankton assemblages. Further studies to evaluate the role of ciliates as nutrient regenerators in various field seawaters would be expected in future.

11.6 Specific Functions Through Prey–Predator Relationships

Among the diverse ecological function of marine ciliates, some are directly concerned with some ecological events in seawater, which often influence human activities. This section will focus on roles of ciliates as regulators of harmful algal blooms (HAB) and as contributors to jellyfish blooms; both events cause negative impact on fishery industries. Also, I will show the role as prey for bivalves, which may partly sustain bivalve aquaculture industries.

11.6.1 Role of Grazing on HAB Species

Almost all HAB species causing nuisance effects on marine organisms consist of phytoplankton. Hence, ciliates are candidates for grazers on them and the grazing impact by ciliates potentially plays a role to regulate development of the bloom and to terminate the bloom. However, many HAB species have a function to protect themselves from grazing of zooplankton: formation of a colony such as *Phaeocystis poucheri* (Jakobsen and Tang 2002) and production of toxic or nuisance substances by *Heterosigma akashiwo* (Taniguchi and Takeda 1988; Verity and Stoecker 1982), *Chrysochromulina polylepis* (Carlsson et al. 1990; Nielsen et al. 1990), *Alexandrium tamarense* (Stoecker et al. 1981; Hansen 1989), *Alexandrium ostenfeldii* (Hansen et al. 1992), *Gymnodinium aureolum* (Hansen 1995), and the exopolymer secretion (EPS) layer surrounding *Aureoumbra lagunensis* (Liu and Buskey 2000). These defense mechanisms surely support the development of the species-specific bloom. However, grazer plankton does not show no power against the HAB species. There are some reports showing feeding of ciliates on HAB species in the same rates as on common phytoplankton (reviewed by Kamiyama 1999 and Montagnes 2013). The following instances suggest that such active feeding of ciliates can influence bloom dynamics of the harmful algae.

Blooms of haptophycea *Phaeocystis* spp. often occur in coastal waters of north Europe. The cell size of this species (3–8 μm) is potentially suitable for ciliates as a

food source. However, they form colonies which reach approximately 10 cm in size as the bloom develops. Once the colonies are formed, the grazing impact due to ciliate assemblages would be inhibited. Positive feeding responses of ciliates to this species (Hansen et al. 1993; Weisse and Scheffel-Möser 1990; Admiraal and Venekamp 1986; Grattepanche et al. 2011) suggest that *Phaeocystis* sp. has no harmful chemical substances inhibiting feeding of herbivorous ciliates. Ingestion rates of a natural ciliate on a single-cell population of *Phaeocystis* accounted for 1–11 % of the ciliate body carbon per hour at the early stage of the bloom, and it was considered that the ciliate population fed heavily on the abundant single cells although the grazing pressure was not perfectly able to control development of the single cell population (Weisse and Scheffel-Möser 1990).

Heterocapsa circularisquama is an HAB species inducing mass mortality of only bivalves under the bloom situation. Active feeding of *Favella* spp. on this species was observed at the algal concentration of less than 1,000 cells mL^{-1} (Kamiyama 1997). When the suspension of *H. circularisquama* viably stained by fluorescent dye was supplied to natural seawater, stained *H. circularisquama* were observed in food vacuoles of 19 ciliate species and the mean ingestion rate of each ciliate species was estimated to be 0.2–14.5 cells ind^{-1} h^{-1} (Kamiyama et al. 2001). Then, these species-specific feeding activities were applied to estimate the sequential daily grazing impact on *H. circularisquama* population during 2 weeks in the course of the bloom. As a result, the maximum daily consumption by ciliate feeding accounted for 75 % of the concentration of *H. circularisquama* at the concentration in the order of magnitude of 10^2 cells mL^{-1} (Kamiyama and Matsuyama 2005). It is necessary to confirm whether these grazing impacts can be generalized but the estimation of grazing loss by ciliate species in the early stage of a bloom of *H. circularisquama* may improve the precision to predict the variation of this algal concentration.

Several *Alexandrium* species causing paralytic shellfish poisoning have been targeted as ciliate prey. However, growth and feeding responses of ciliates on this algal group have not shown consistent results; tintinnid ciliate *Favella* spp. can feed on *Alexandrium tamarense* (Stoecker et al. 1981; Hansen 1989; Kamiyama et al. 2005) and *Alexandrium ostenferdii* (Hansen et al. 1992), although abnormal swimming behavior, swelling and lysis of the cell occurred at the high concentration of these *Alexandrium* species (Hansen et al. 1992). These harmful effects are considered to be due to PSP toxin and the toxicity varied according to growth phase, physiological conditions and strain (Hansen 1989), and other unknown extracellular substances also cause negative responses of ciliates to *A. tamarense* (Colin and Dam 2003). In field environments, development of ciliate populations sometime occurs during the decline of the *Alexandrium* populations (Watras et al. 1985). Kamiyama et al. (2005) found that *Favella taraikaensis*, the culture strain that was established from seawater containing *A. tamarense* cells, could actively ingest *A. tamarense* and grow with similar rates on the other nontoxic prey. Specific strains of *F. taraikaensis* with tolerance for *A. tamarense* are possibly able to grow in the course of the *Alexandrium* bloom and the grazing impact by the developed ciliate population may influence the population dynamics of *Alexandrium* species.

Pfiesteria piscicida is a mixotrophic dinoflagellate linked to toxic events and fish kills along the Atlantic coast of the USA (e.g., Burkholder and Glasgow 1997). Stoecker et al. (2000) measured the potential grazing pressure of natural assemblages of microzooplankton including ciliates on cultured nontoxic zoospores (NTZ) of *P. piscicida*. As a result, instantaneous grazing rates of microzooplankton dominant by large tintinnids and oligotrichous ciliates ranged from 0 to 10.2 d^{-1} and higher grazing rates than the maximum instantaneous growth of NTZ (2 d^{-1}) were observed on many occasions. Intervals of low grazing pressure may give windows of opportunity for growth of *P. piscicida* NTZ, and grazing pressures by microzooplankton can prevent the net growth of NTZ (Stoecker et al. 2000). Based on the results of predator–prey interactions between *Strombidium* sp. and *P. piscicida* observed in laboratory experiments, Setälä et al. (2005) demonstrated that nontoxic *Pfiesteria* is under relatively strict grazing control, and that the formation of blooms requires periods of low grazing pressures or the means to escape grazing.

Cochlodinium polykrikoides is an important red tide species causing mass mortality of fish in aquaculture. *Strombidinopsis* sp. can actively feed on this species at the maximum ingestion rates of 504 cells ind^{-1} h^{-1} and grow well at the specific growth rate of 1.48 d^{-1} (Jeong et al. 1999). Furthermore, the potential control capability of *Strombidinopsis jeokjo* on *C. polykrikoides* bloom was evaluated in mesocosm enclosure experiments. Results suggested the possibility to control occurrence of the red tide in small field areas using large-scale cultures of *C. polykrikoides* (Jeong et al. 2008).

Effective grazing responses of ciliates to harmful algae as described above often depend on the concentration of harmful algae. Once the concentration of the algae exceeds certain levels, the negative effects of the algae occurs on feeding activities of predators. Hence, relationships between the abundance of predators and the target harmful algae at the early stage of the bloom are probably critical (Caron et al. 1989). Such a concentration is considered to be 10^6 cells ml^{-1} for *Aureococcus anophagefferens* (Caron et al. 1989; Lonsdale et al. 1996), 10^3 cells ml^{-1} for *Heterocapsa circularisquama* (Kamiyama and Arima 1997), and 10^4 cells ml^{-1} and 3×10^4 cells ml^{-1} for *Prorocentrum minimum* and *Premnesium parvum*, respectively (Rosetta and McManus 2003).

11.6.2 Role as Food Source for Aurelia aurita

An unprecedented number of jellyfish outbreaks have been reported in the 1990s and 2000s (reviewed by Purcell et al. 2007). In particular, *Aurelia aurita* populations have apparently increased in the Seto Inland Sea, Japan, since the 1980s, and most dramatically in the last 10 years (Uye and Ueta 2004), and in other Japanese embayments and Korean coastal waters as well (Kang and Park 2003; Uye and Shimauchi 2005). One of the causes inducing such increases is changes in lower trophic levels of the food web structure towards domination of small plankton such as flagellates. This change induces size reduction of the zooplankton community,

representing domination of flagellate eaters such as *Oithona* spp. and microzooplankton including ciliates. Small zooplankton is unsuitable as a prey source for fish, most of which are visual predators that prefer large zooplankton. This situation is beneficial for jellyfish, which are able to consume zooplankton with a wide size range (Purcell et al. 2007). Hence, it would be reasonable if increase of ciliates contributed to increases in the food source for *A. aurita*. However, ciliate cannot play an essential role as a food source for the medusa stage of *A. aurita*, as follows. If clearance rates of *A. aurita* medusa (Diameter 11.6 cm) on ciliates were reported to be 134 l ind^{-1} d^{-1} and ciliate biomass is 5 mgC m^{-3} (the maximum value in the Seto Inland Sea of Japan, Fig. 4 in Uye et al. 1996), energy gained from ciliates is estimated to be 0.67 mg ind^{-1} d^{-1}. This value is only 24 % of the minimum food requirement of medusa (2.7 $mg^{-1}ind^{-1}d^{-1}$ at 20 °C) calculated with parameters in Uye and Shimauchi (2005). Also, the insignificant grazing impact of medusa on the ciliate community is also evaluated from relatively low density of medusa and high potential growth rates of ciliates (Stoecker et al. 1987a). Increase of dense swarms of gelatinous plankton may enhance top-down control of mesozooplankton rather than on ciliates. This probably induces increases of ciliates and other microzooplankton by reducing predation of mesozooplankton on them due to trophic cascading (Stoecker et al. 1987a)

However, the food value of ciliates for the larval stages (polyp and ephyra) is different from that for medusa. Polyp and ephyra are far tinier compared with medusa, and have no or little mobile ability. These characteristics suggest that high densities of small prey are important for polyps and ephyra rather than sparse large prey. The density of mesozooplankton (copepods), generally in the 10 ind l^{-1} order of magnitude, is far lower than that of microzooplankton (ciliates) with a 10^3 ind l^{-1} order of magnitude in coastal embayments (e.g., Uye et al. 1996; Uye and Shimazu 1997). Ciliates are important candidates of prey organisms for polyps and ephyra. In the experiments providing cultured ciliates for polyps of *A. aurita*, the polyp recognized the ciliates attached on the tentacles of the polyp and transferred the captured ciliates into its mouth (Kamiyama 2012). The feeding rates changed in response to the densities of ciliates and the maximum value was estimated to be 0.33 μgC ind^{-1} h^{-1} (Kamiyama 2011b). If the carbon content of the polyp was 38.1 μgC ind^{-1}, daily carbon-specific feeding rates accounted for 21 % of the value. Furthermore, active growth (asexual reproduction) of polyps on a diet of ciliates was observed in laboratory experiments, and the increase in the growth rates responding to an increase of ciliate density was higher than that on the diet of *Artemia* nauplii (Kamiyama 2012). Hence, it is likely that asexual production of the polyp is more strongly promoted by higher densities of ciliates than by increases of crustacean mesozooplankton represented by *Artemia* nauplii. After *Aurelia aurita* transform from polyps to ephyra, they still actively feed on ciliates. The disc diameter of ephyra feeding on ciliates clearly increased with incubation time during 5 days and the daily relative increase of the disc diameter linearly increased with carbon-specific feeding rates until about 5 μgC ind^{-1} d^{-1} and reached about 20 % (Kamiyama unpublished data). The increase rate of the growth rates responding to feeding rates on ciliate prey was not significantly different from the value on *Artemia*

prey. On the other hand, the disc diameter of metephyra (larval medusa) did not increase on a diet of ciliates during 5 days of incubation, even if the consumption of prey ciliates was observed. These results suggest that planktonic ciliates are an available food source for growth of the ephyra stage of *A. aurita* as well as metazoans but the food value of ciliates probably decreases at and after the metephyra stage to be insufficient for their growth.

Utilization of different prey sources by polyp and ephyra stages from metephyra and medusa may have ecological advantages to promote jellyfish blooms. If metephyra and medusa mainly feed on meso- and macrozooplankton and reduce their abundances, population of microzooplankton such as ciliates will be developed because top-down effects by mesozooplankton are weakened. High density of microzooplankton probably promotes asexual reproduction of polyps and survival of ephyra under good prey conditions. Then, an increase of the ephyra will cause an increase of medusa inducing the jellyfish blooms and then matured medusa would produce planura by sexual reproduction to produce the next generation of *A. aurita*. In this scenario, the jellyfish bloom may be strongly associated with increases of microzooplankton and decreases of mesozooplankton; the ratio of microplankton to mesozooplankton may be a sign of the condition in which the jellyfish bloom occurs frequently.

11.6.3 Role as Food for Bivalves

Bivalves are representative benthic suspension feeders and can use benthic energy flow but also use pelagic energy flow. Phytoplankton dominating the plankton biomass is the main food for bivalves, especially for bivalves in suspension culture systems. Ciliates also play an essential role as food sources for bivalves in seawater where nano- or microphytoplankton biomass is temporary depleted or cannot dominate the plankton community. In laboratory experiments, feeding rates of the Pacific oyster *Crassostrea gigas* on planktonic ciliates were measured and similar values to autotrophic flagellates were confirmed (Duppuy et al. 1999; Kamiyama 2011a) and assimilation of ciliate prey into oyster tissue was also clarified (Kamiyama 2011a). Results of gut content analysis of the oyster showed that the number of loricae of tintinnids in the gut of the oyster generally depends on the abundance of tintinnids in seawater but the number of loricae decreased when chlorophyll-*a* concentration increased in the phytoplankton bloom (Kamiyama 2011a). This suggests that the food value of ciliates for cultured oysters is raised during the period when phytoplankton is not abundant. However, in areas where plankton assemblages were dominated by picoplankton, this selection of an abundant food source is not realized because the oyster cannot efficiently utilize picoplankton (e.g., Langdon and Newell 1990). In this situation, ciliates possibly play an important role in energy transfer from picoplankton to bivalves (Le Gal et al. 1997). The importance of heterotrophic plankton including ciliates was pointed out in a mussel culture environment in a lagoon on Magdalen Island, Canada (Trotte et al. 2007).

11.7 Conclusion and Future Subjects

Understanding the ecological function of microzooplankton has been proceeding based on the results of recent field research in various seawater systems. Significant roles of ciliates as consumers of pico- and nanoplankton and as prey for higher trophic level organisms in pelagic food webs are probably ubiquitous worldwide. The role of nutrient regeneration may be more important in wider seawater systems than we guess, although the effect depends on the locality and season. Such ecological roles directly or indirectly influence occurrences of HAB and jellyfish blooms and may contribute to production of bivalve aquaculture as supplemental food.

However, to date, we have understood only a few parts of the function of ciliates. To clearly understand their role as consumers of prey organisms in field waters, it is necessary to consider combinations of various prey organisms in water systems. For the role of a nutrient regenerator, further basic information on the ability of ciliates remains to be accumulated by laboratory and field experiments. I believe that ciliates play roles as prey for more various organisms that we have found out. Hence, quantitative information on predation by other protists and metazoans other than copepods should be further accumulated. It is also necessary to understand detailed mechanisms in prey–predator interaction in field seawater such as the trophic cascade. The ecological function of ciliates is dynamically changeable with changes in the abundance and community composition of ciliates. Hence, we need to investigate dynamics of food web components including organisms other than ciliates and interactions among the organisms. For that, application of quantitative identification techniques by molecular information would be expected in the future.

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Chapter 12
Biology and Paleontology of Coccolithophores (Haptophytes)

Kyoko Hagino and Jeremy R. Young

Abstract Coccolithophores are marine unicellular haptophytes that possess calcified scales called coccoliths. They are classified primarily on the morphology and crystallography of the coccoliths. This classification is well supported by molecular genetic results; however, a significant complication occurs in that coccolithophores have a haplodiplontic life cycle with asexual reproduction by binary fission in both the diploid and haploid phases and with very different types of coccoliths being produced in the two phases. Coccolithophores have very extensive fossil records, making them an attractive group for study of microevolutionary patterns through combined study of molecular genetics, the morphology of living species and the fossil record. The molecular studies showed that many conventional coccolithophore morpho-species consist of multiple discrete species often only differing from each other in minor morphological features or the size range of coccoliths. Paleontological studies showed that many fossil lineages had evolutionary size-increasing events in their history, and the size ranges of some fossil species are useful as index fossils for dating of marine sediments. Stratigraphic variations of the composition of fossil species and organic compounds (alkenones) produced by the Isochrysidales are useful for reconstruction of sea surface water condition and of sea surface temperature in the geological past. It is possible that ongoing global warming and ocean acidification may affect the distribution of coccolithophores and the composition of genotypes/species.

Keywords Haptophytes • Coccolithophores • Coccolith • Calcareous nannoplankton • Calcareous nannofossil • Microfossils

K. Hagino (✉)
Research and Education Faculty, Natural Sciences Cluster, Sciences Unit, Kochi University, Akebono-cho 2-5-1, Kochi, Kochi Prefecture 780-8520, Japan
e-mail: kyokohagino@frontier.hokudai.ac.jp

J.R. Young
Department of Earth Sciences, University College London, Gower Street, London WC1E 6BT, UK
e-mail: jeremy.young@ucl.ac.uk

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12.1 Introduction

The Haptophyta is a division of predominantly marine eukaryotic phytoplankton. They are primarily characterized by a thread-like organelle called the haptonema, which is inserted between two flagella. The haptonema differs from flagella in its microtubular structure and in its use. The haptonema is capable of coiling, bending, and adhesion to external substrata (Inouye and Kawachi 1994), and is used for handling of food by mixotrophic species (e.g., Kawachi et al. 1991). The haptophytes are primarily autotrophic algae that possess chloroplasts containing chlorophylls a and c (red alga lineage) and various photosynthetic pigments (e.g., Lenning et al. 2004), although mixotrophy is suspected to be widespread in the group (e.g., Unrein et al. 2014). The relationships of the haptophytes to other eukaryotic groups are not well understood, but recent study suggests that they are close to the SAR (Stramenopiles, Alveolates, and Rhizaria) group (Burki et al. 2012).

The haptophytes are subdivided into two classes: the Pavlovophyceae and Prymnesiophyceae (Fig. 12.1). The Pavlovophyceae comprises the single order Pavlovales. The Pavlovales are typically characterized by unequal flagella, knob scales covering the longer flagellum, a non-coiling rudimentary haptonema, fibrous hairs, paramylon-like granules, and lack of body scale and ultrastructure of the flagellar apparatus (e.g., Green and Jordan 1994; Green and Hori 1994). The Prymnesiophyceae differ from the Pavlovophyceae in having equal or subequal length smooth flagella, and usually a well-developed coiling haptonema (not in the Isochrysidales); they also have a covering of organic and/or mineralized body scales (Green and Jordan 1994). Organic and/or mineralized body scales of the Prymnesiophyceae are produced in Golgi-derived vesicles, and subsequently extruded onto the cell surface (e.g., Leadbeater 1994; Billard and Inouye 2004).

The Prymnesiophyceae vary in terms of haptonematal features and body scales. Classification of the Prymnesiophyceae is not entirely consistent among authors, and the following six orders are currently known: Phaeocystales, Prymnesiales, Isochrysidales, Syracosphaerales, Zygodiscales, and Coccolithales (Fig. 12.1) (e.g., Young et al. 2003; Edvardsen et al. 2011). Most extant coccolith-bearing families fall within these orders but there are some families incertae sedis whose phylogenetic position is unclear from morphology and for which molecular genetic data are not yet available (Young et al. 2005). There are also many further fossil taxa (e.g., Bown 1998). Furthermore, studies of environmental DNA suggested the presence of undescribed pico-haptophytes in the open ocean (e.g., Liu et al. 2009; Bittner et al. 2013). Members of the Prymnesiophyceae have long haptonema, except for the Isochrysidales, which have reduced haptonema or lack any trace of a haptonema (e.g., Green and Jordan 1994). Phaeocystales and nearly all species of Prymnesiales only have unmineralized body scales (Edvardsen et al. 2011), although one species, *Hyalolithus neolepis* (Prymnesiales), produces both organic and siliceous scales (Yoshida et al. 2006). Zygodiscales, Syracosphaerales, Coccolithales, and Noëlaerhabdaceae (Isochrysidales) produce both organic and calcified scales. Calcified scales produced by the Prymnesiophyceae are called

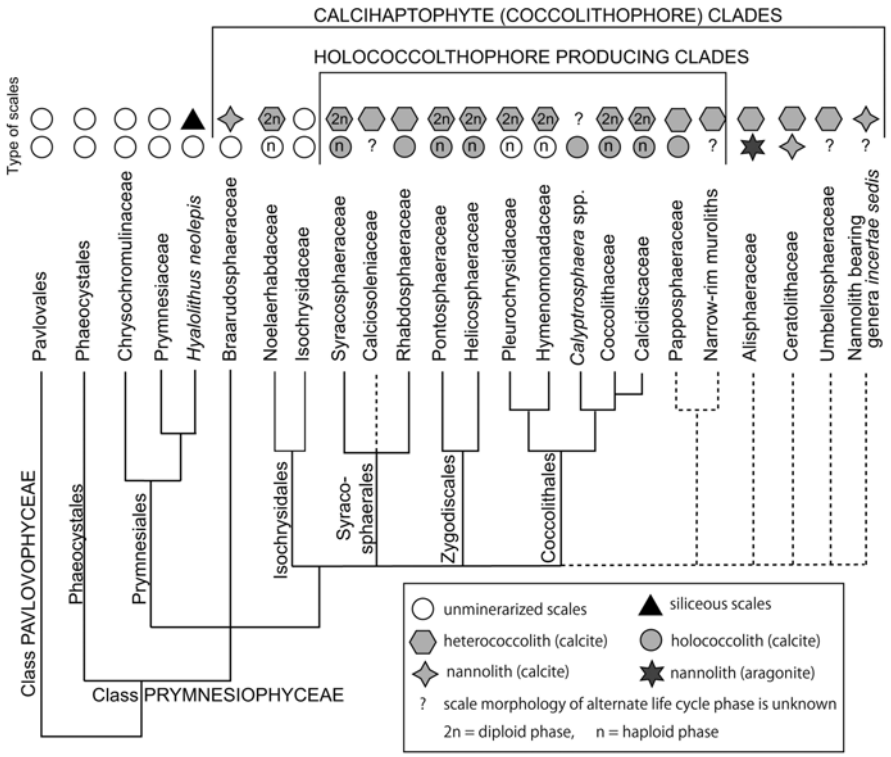

Fig. 12.1 Schematic consensus phylogeny of the haptophytes in relation to scale morphology. *Solid lines* indicate relationships based on molecular genetic data, and *dashed lines* relationships inferred from morphological data. The taxonomy and phylogeny of Phaeocystales and Prymnesiales follow Edvardsen et al. (2011). The phylogenetic positions of *Hyalolithus neolepis* and the Braarudosphaeraceae follow Yoshida et al. (2006) and Hagino et al. (2013), respectively. The phylogeny of the Calcihaptophyte clade, except the Braarudosphaeraceae, follows Hagino et al. (2009)

coccoliths (Young et al. 2003). These prymnesiophyte species characterized by coccoliths are collectively called coccolithophores. The clade including all coccolithophores only contains a few non-calcifying species, in the family Isochrysidaceae, order Isochrysidales, and is sometimes referred to as the subclass Calcihaptophycidae (de Vargas et al. 2007).

Coccolithophores are common and widespread components of the marine plankton. They play important roles in the oceanic carbon cycle by both calcification of coccoliths and photosynthesis (e.g., Rost and Riebesell 2004). Coccolithophores, together with other phytoplankton, form the base of the marine food chain. Cell contents of coccolithophores preyed on by zooplankton are digested and absorbed, but the calcareous coccoliths are typically excreted and incorporated in fecal pellets. The coccoliths packed in these fecal pellet sink to the sea floor quickly; in addition loose coccoliths and coccospheres commonly aggregate with other plankton debris to form marine snow (e.g., Honjo 1976). These two mechanisms limit the dissolution

of coccoliths in the water column, and greatly increase the sinking velocity of coccoliths. Loose coccoliths sink less than 1 m per day; on the other hand, fecal pellets sink 150–570 m per day in the water column (Honjo 1976; Steinmetz 1994; Fischer and Karakas 2009). As a result of these two mechanisms, coccoliths are quickly transported to the sea floor and form one of the main components of marine sediments, above the calcite compensation depth (CCD). Nonetheless significant dissolution and assemblage transformation do occur as shown by the fact that out of ~280 coccolithophore types known from plankton, only about 60 are known from the fossil record (Young et al. 2005). The effect of these processes on assemblage composition is discussed further in Andruleit et al. (2004). The evolutionary history of coccolithophores has been studied based on fossilized coccoliths from the marine sediment and from the morphology and molecular data of living species by micropaleontologists and biologists, respectively. Recently, the results from the two fields have been integrated and new findings on the evolution of coccolithophores have begun to appear. In this chapter, such new findings are summarized.

12.2 Life Cycle, Biomineralization and Molecular Phylogeny

Coccolithophores are classified primarily based on the morphology and crystallography of the coccoliths. Coccoliths are generally divided into three groups: heterococcoliths, holococcoliths, and nannoliths. Heterococcoliths are formed of a radial array of complex crystal units, while holococcoliths are formed of numerous minute euhedral crystals. Calcareous structures, which do not conform to either pattern, are called nannoliths (Fig. 12.2) (Young et al. 1999). Based on the morphology of coccoliths, about 180 heterococcolith-bearing species, 90 holococcolith-bearing species, and 10 nannolith-bearing species were described as ‘discrete species’ of living coccolithophores (e.g., Young et al. 2003, 2005).

The haptophytes have haploid–diploid life cycles, in which both phases are capable of independent asexual reproduction and have distinct patterns of scale morphology (e.g., Billard 1994). Like other haptophytes, coccolithophores reproduce asexually by binary fission in both the diploid and haploid phases (Fig. 12.3) (Billard and Inouye 2004). Several culture studies have revealed that the morphology of coccoliths drastically changes between the life cycle phases (e.g., Parke and Adams 1960). Culture studies combined with flow-cytometric analysis of the ploidy state have shown definitively that members of *Coccolithus* and *Calcidiscus* (Coccolithales) and *Coronosphaera* (Syracosphaerales) produce heterococcoliths and holococcoliths in their diploid and haploid phases, respectively (Figs. 12.1 and 12.3) (Houdan et al. 2004). In addition, combination coccospheres, with both heterococcoliths and holococcoliths, are occasionally observed in natural seawater samples recording life cycle transitions in these species (Fig. 12.3c). Combination coccospheres have been observed in some species of *Helicosphaera*, and *Pontosphaera* (Zygodiscales), *Syracosphaera* (Syracosphaerales), *Alisphaera* (Alisphaeraceae, family incertae sedis) *Papposphaera* and *Pappomonas* (Papposphaeraceae, family incertae sedis)

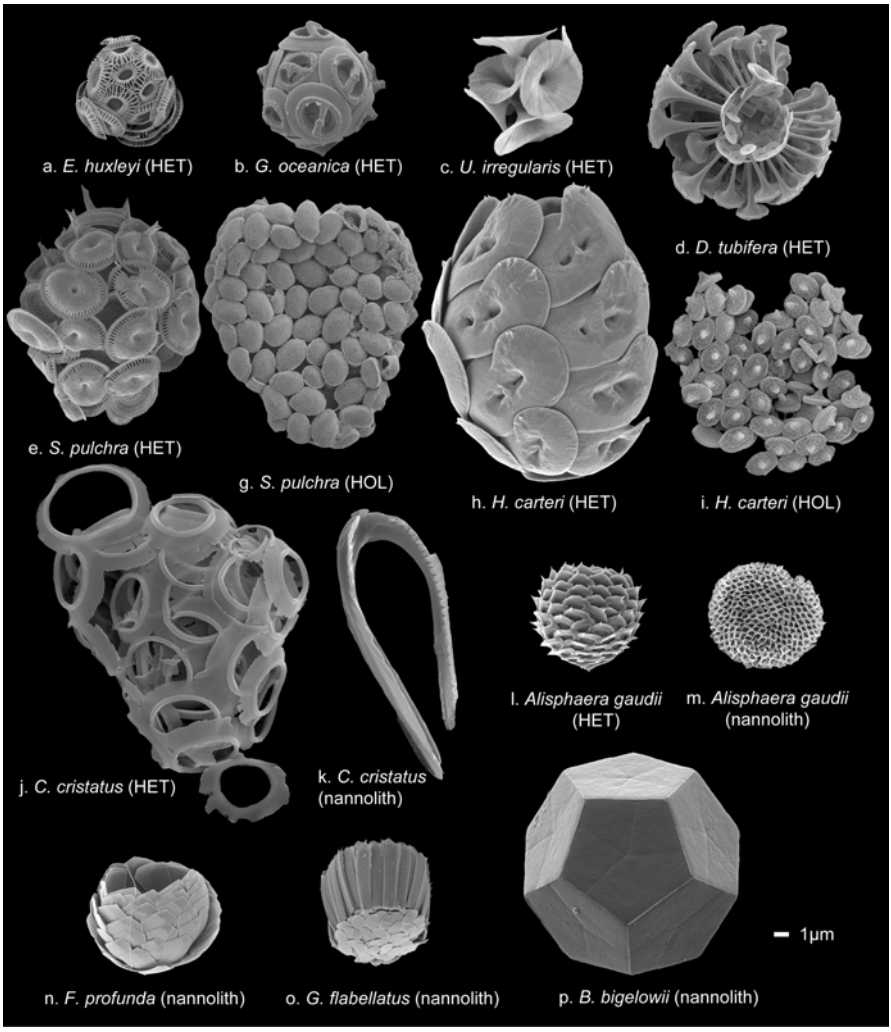

Fig. 12.2 SEM images of heterococcolith (HET)-, holococcolith (HOL)- and nannolith-bearing cells of coccolithophores: (**a**) *Emiliania huxleyi*, (**b**) *Gephyrocapsa oceanica*, (**c**) *Syracosphaera molischii* (HET), (**d**) *Umbellosphaera irregularis* (HET), (**e**) *Discosphaera tubifera* (HET), (**f**) *Syracosphaera pulchra* (HET), (**g**) *Syracosphaera pulchra* (HOL), (**h**) *Helicosphaera carteri* (HET), (**i**) *Helicosphaera carteri* (HOL), (**j**) *Ceratolithus cristatus* (HET), (**k**) *Ceratolithus cristatus* (nannolith), (**l**) *Alisphaera gaudii* (HET), (**m**) *Alisphaera gaudii* (nannolith), (**n**) *Florisphaera profunda* (nannolith), (**o**) *Gladiolithus flabellatus* (nannolith), and (**p**) *Braarudosphaera bigelowii* (nannolith)

produce holococcoliths in their alternate life cycle (e.g., Kamptner 1941; Thomsen et al. 1991; Cros and Fortuño 2002; Geisen et al. 2002; Frada et al. 2009). These results suggest that holococcolith-bearing 'species' are not discrete species, but rather are alternate life cycle stages of other heterococcolith-bearing species.

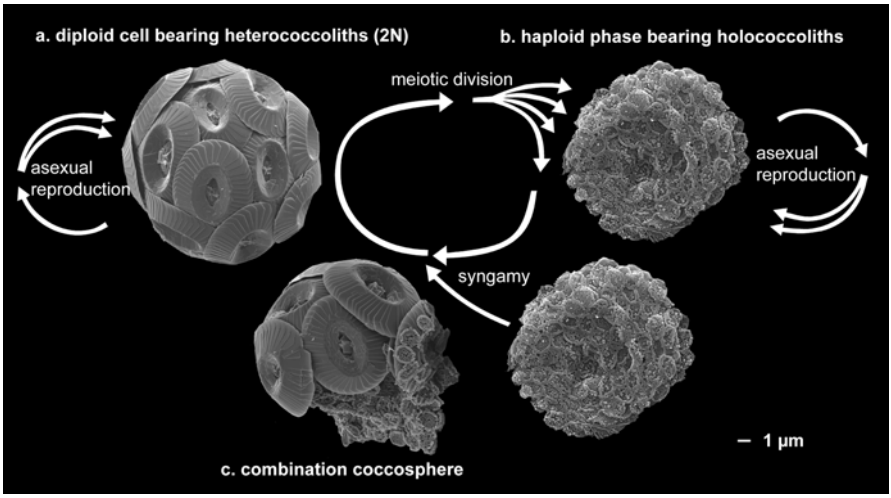

Fig. 12.3 Life cycle of *Coccolithus pelagicus*, showing the two different coccosphere types produced in the diploid and haploid phases and a combination coccosphere (*bottom left*) with both coccolith types as seen immediately after a syngamy

Nonetheless the life cycle associations of numerous described heterococcolith- and holococcolith-bearing taxa are still unclear, and inevitably taxonomic revisions will be needed as further associations are established.

Heterococcolith formation occurs intracellularly, in the Golgi cisternae (e.g., Drescher et al. 2012). By contrast the calcification site for holococcolith formation has not yet been determined. Holococcoliths are underlain by a Golgi-derived scale but have never been observed intracellularly. Parke and Adams (1960) and Rowson et al. (1986) artificially removed holococcoliths from cells of *Coccolithus pelagicus*, and observed that holococcoliths appear around the flagellar opening. Therefore it is thought that calcification of the holococcoliths may occur on/near the cell surface near the flagellar opening. The Nöelaerhabdaceae (Isochrysidales) form calcite heterococcoliths in a special vacuolar system of the endoplasmic reticulum directly connected to the nuclear membrane (Westbroek et al. 1989). They do not make calcified scales in their haploid phase. From combination coccospheres observed from natural samples, it is thought that *Alisphaera* (Family incertae sedis) forms aragonitic quadrate nannolith, originally described as *Polycrater,* in their alternate life cycle stage (Manton and Oates 1980; Cros and Fortuño 2002). Neither *Alisphaera* nor nannolith-bearing species such as the members of the Braarudosphaeraceae and Ceratolithaceae have been maintained in laboratory culture, and there is no information on the calcification system of the various nannoliths they form. It is possible that a range of different biomineralization processes occurs in them.

The molecular phylogeny of coccolithophores has been examined using Rubisco and 18S rRNA genes, mainly obtained from culturable species (e.g., Fujiwara et al. 2001; Medlin et al. 2008). Phylogenetic positions of many unculturable taxa are still

uncertain, but *Braarudosphaera bigelowii* was examined using the sequences obtained from isolates from natural seawater based on the single-cell PCR technique (e.g., Takano et al. 2006). In the phylogenetic trees, the Isochrysidales clade is consistently separated from other coccolithophore taxa (e.g., de Vargas et al. 2007; Liu et al. 2009). It is known that the Isochrysidales differ from other coccolithophores in that they produce alkenones (a unique series of long-chain, unsaturated ketones), lack a haptonema, and do not form coccoliths in the haploid phase (e.g., Saéz et al. 2004; de Vargas et al. 2007). The Coccolithales make a clade with multiple holococcolith-bearing taxa (e.g., *Calyptrosphaera* spp.) whose diploid phase form was not determined (Fig. 12.1) (e.g., de Vargas et al. 2007). The clades of Zygodiscales and Syracosphaerales are usually separated from the base of the Coccolithales clade, but the branching order of the coccolithophore orders are uncertain due to low supporting values (e.g., Hagino et al. 2009). *B. bigelowii*, nannolith bearing taxa makes a clade with Prymnesiaceae sp. The phylogenetic position of the clade containing *B. bigelowii* in the prymnesiophyte tree changes depending on the analyses (e.g., Takano et al. 2006; Hagino et al. 2013). Currently it is unknown whether the Braarudosphaeraceae separated from the common ancestor of other coccolithophores before the starting of calcification, and so how many times calcification occurred in the Prymnesiales.

Many conventional coccolithophore 'species' show subtle variation in the size and morphology of coccoliths. Traditionally such subtle variations were regarded as intra-specific variation, although they were sometimes tentatively used as criteria for subdivision of the 'species' into subspecies, variety, or morphotypes (e.g., Young and Westbroek 1991; Bollmann 1997; Knappertsbusch 1997). However, recent molecular studies have repeatedly found genetic differences between such subspecies, morphotypes or varieties (Sáez et al. 2003; Geisen et al. 2004). Saéz et al. (2003) found differences in 18S rDNA and/or tuf-A sequences among the subspecies/varieties of *Coccolithus pelagicus, Umbilicosphaera sibogae, Helicosphaera carteri* and *Pleurochrysis carterae*, and raised the subspecies/varieties to the species rank. They concluded that the subtle morphological/size changes of coccoliths are a result of pseudo-cryptic speciation, and suggested that strong stabilizing selection acts on the phenotype of many coccolithophores. In some coccolithophore lineages the size of scales/cells is the only key to the recognition of speciation from appearance. For example, *B. bigelowii* is a species complex consisting of more than five discrete species with identical morphology but which can be separated from each other based on the size of the pentalith (pentagonal nannolith) and 18S rDNA sequences. The size of the pentaliths of *B. bigelowii* increases corresponding to the branching order (Hagino et al. 2009). Thus, the size range of the coccolith is important for the classification of coccolithophores at species level.

At least in some cases, this pseudo-cryptic variation can help explain the remarkably wide distribution of some near ubiquitous species. For example, *Emiliania huxleyi* is the most abundant living coccolithophore and is distributed through the entire photic zone from tropical to polar waters. *E. huxleyi* has been classified into six morphotypes (A, B, B/C, C, O, R) and a variety *corona* (e.g., Young et al. 2003; Hagino et al. 2011), although classification of morphotypes is not entirely consistent

among authors. The composition of morphotypes differs between warm (tropical–temperate) and cold (subpolar–polar) surface waters, and between the upper and lower photic zones in the warm water regime (Table 12.1) (e.g., Hagino et al. 2000, 2005). The morphotypes A, B, B/C and R were distinguished from each other by genetic markers discovered from GPA or *tuf*A regions (Schroeder et al. 2005; Cook et al. 2011), and the strains from cold and warm water masses differ from each other in *cox* 1b sequences (Hagino et al. 2011). A pan-genome study revealed that *E. huxleyi* has extensive genomic variability that reflects different metabolic repertories (Read et al. 2013). Genetic variation in *E. huxleyi* is regarded as intra-specific variation, and it is thought that the genetic variability within *E. huxleyi* underpins its capacity to adapt to the tropical–polar waters, and to form massive blooms under a

Table 12.1 Composition of coccolithophore flora shown in Fig. 12.4

Coccolithophore flora	Upper photic zone		Middle photic zone	Lower photic zone
	Dominant taxa	Accompanying taxa		
I-a	*E. huxleyi* (Type A)	*U. tenuis*	*E. huxleyi* (Type C)	*F. profunda*
		C. leptoporus		
		G. ericsonii	*G. oceanica*	*Gladiolithus* spp.
I-b	*E. huxleyi* (Type A)	*R. parvula*		
		G. ericsonii	*A. oryza*	*E. huxleyi* (Type C)
		G. oceanica		
		C. leptoporus	*O. antillarum*	
II-a	*G. oceanica*	*O. antillarum*		
		C. leptoporus		
		E. huxleyi (Type A)		
		G. ericsonii		
		U. hulburtiana		
II-b	*G. oceanica*	*E. huxleyi* (Type A)		
		G. ericsonii		
		Syracosphaera spp.		
III	*U. irregularis*	*U. tenuis*		
		D. tubifera		
		R. clavigera		
		E. huxleyi (Type A)		
		G. oceanica		
		U. sibogae		
IV	*E. huxleyi* (Types A and C)	*G. oceanica*		
		C. leptoporus *Syracosphaera* spp.		
V-a	*E. huxleyi* (Types B, C, D, or O)	*G. mullerae*	Barren	
		C. pelagicus		
		C. leptoporus		
V-b	*E. huxleyi* (Types B, C, D, or O)	*C. leptoporus* *Papposphaera* spp.		
		Wigwamma spp.		

wide range of environmental conditions (Read et al. 2013). Morphotypes related to habitat environment were also reported from *G. oceanica*, a sibling species of *E. huxleyi* (Bollmann 1997; Hagino et al. 2000). Like *E. huxleyi*, *G. oceanica* has great intra-specific genetic diversity (Bendif et al. 2014). Since sexual-crossing experiments of coccolithophores have not yet been carried out successfully, the real taxonomic status of many species/morphotypes/varieties of coccolithophores based on morphology is still uncertain.

12.3 Biogeography and Seasonality

Taxonomic work on coccolithophores started in the late nineteenth century. Many major coccolithophore species were described from nearshore seawaters by observing seawater samples using light microscope or TEM by biologists from the 1870s to the 1960s (e.g., Wallich 1877; Lohmann 1902). In the early 1960s, new methods for the study of living coccolithophores were introduced by micropaleontologists who required information on living coccolithophores from the open ocean for the interpretation of oceanic fossil assemblages. These paleontologists used membrane filters to collect coccolithophores from known volumes of water and studied them under cross-polarized light microscope, TEM, and/or SEM. This method enabled quick onboard preparation of samples for morphological studies of mineralized species. McIntyre and Bé (1967) studied living coccolithophores using filter samples as well as fossil coccoliths preserved in the sea surface sediments in the Atlantic Ocean extensively. They showed that the Atlantic coccolithophore flora can be classified into five latitudinally aligned assemblages corresponding to the water masses, and that fossil coccolith assemblages from the sea surface sediments reflect living assemblages overlying (Fig. 12.4). Based on the several regional studies, subsequent studies suggested that the coccolithophore assemblages in the Pacific Ocean have latitudinal zonal distributions similar to the Atlantic assemblages (e.g., Winter and Siesser 1994). Increasing regional studies in the last two decades, however, showed that the distribution and composition of coccolithophores are not always comparable between the oceans (Fig. 12.4). The equatorial Atlantic Ocean was characterized by *E. huxleyi* dominant assemblages (I-a), while the coccolithophore assemblages in the equatorial Pacific change longitudinally corresponding to water oligotrophication (Table 12.1, Fig. 12.4) (Hagino and Okada 2004). *G. oceanica*–dominated assemblages (II-a, –b of Fig. 12.4) occur from the central equatorial Pacific, central–eastern equatorial Indian Ocean, and western coastal waters of the Pacific, although they have not been reported from the Atlantic Ocean. The cause of floral differences between the Atlantic and Indian–Pacific Oceans are unknown. *G. oceanica* common assemblages from the central equatorial Pacific (II-a) are different from the assemblages from the Indian Ocean (II-b) in common occurrence of *O. antillarum* and resemble the middle photic zone assemblages in the Atlantic, Indian, and Pacific Oceans (Table 12.1, Fig. 12.4). The *U. irregularis* common assemblages (III) were restricted in oligotrophic subtropical gyres in the Atlantic Ocean, but

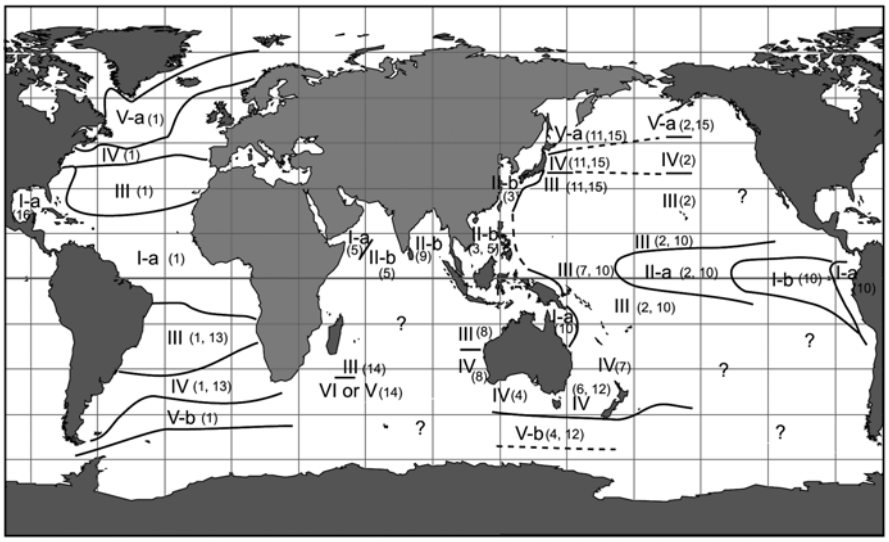

Fig. 12.4 Distribution of coccolithophore flora (I-a, I-b, II-a, II-b, III, IV, V-a, and V-b) in the sea surface level. The composition of each type of flora is shown in Table 12.1. Numerals within the parenthesis show the reference of each floral data; 1 = McIntyre and Bé (1967), 2 = Okada and Honjo (1973), 3 = Okada and Honjo (1975), 4 = Nishida (1986), 5 = Kleijne (1993), 6 = Findlay and Giraudeau (2000), 7 = Hagino et al. (2000), 8 = Takahashi and Okada (2000), 9 = Andruleit et al. (2003), 10 = Hagino and Okada (2004), 11 = Hagino et al. (2005), 12 = Cubillos et al. (2007), 13 = Boeckel and Baumann (2008), 14 = Mohan et al. (2008), 15 = Hagino et al. (2011), and 16 = Baumann and Boeckel (2013)

distributed in the oligotrophic subtropical and western equatorial Pacific (Fig. 12.4). These assemblages yield many holococcolith-bearing 'species' (Winter et al. 1994). This suggests that nutrient level have some effect on the life cycle of the coccolithophore. Temperate to polar waters of all oceans are dominated by *E. huxleyi*. The compositions of morphotypes of *E. huxleyi* and of the accompanying taxa differ between temperate, subpolar, and polar waters (Table 12.1). Both arctic and antarctic assemblages are dominated by *E. huxleyi* types B, B/C, C, or O, but the antarctic assemblage (V-b) differs from the arctic assemblage (V-a) in the rare occurrence of *C. pelagicus* and the common occurrence of *C. leptoporus* (Table 12.1). In both the Atlantic and Pacific Oceans, the lower photic zones of tropical–temperate waters were dominated by *Florisphaera profunda* and were characterized by the occurrence of members of *Algirosphaera* spp. and *Gladiolithus* spp. The lower photic zones of subpolar–polar waters are barren in coccolithophores, probably due to low light intensity (e.g., Okada and Honjo 1973).

The composition of coccolithophores is relatively stable through a year in the equatorial waters, while it changes seasonally from subtropical to subpolar waters (Okada and McIntyre 1979; Hagino and Okada 2004). In the subtropical–subpolar Atlantic, *E. huxleyi* is the one of the most abundant species throughout the year except in mid-summer (Okada and McIntyre 1979). *E. huxleyi* make massive

blooms in early summer (June) following the spring diatom bloom in the North Atlantic Ocean (e.g., Tyrrell and Merico 2004), and occasionally in summer (July–August) in the East Bering Sea (e.g., Murata and Takizawa 2002). *G. oceanica* occasionally makes a massive bloom in spring in the coastal waters of the temperate Pacific (e.g., Blackburn and Cresswell 1993; Kai et al. 1999). The seasonal life cycle phase change was only revealed for *C. pelagicus*. Holococcolith-bearing cells of *C. pelagicus* (haploid phase) are abundant from March to May, and heterococcolith-bearing cells of *C. pelagicus* (diploid phase) increase in abundance from June to August in the North Atlantic Ocean (Okada and McIntyre 1979). Combination coccospheres of *C. pelagicus*, it seems immediately after the syngamy, were found from the Northwest Pacific Ocean in June (Fig. 12.3c).

12.4 Coccolithophores in the Geological Past

Fossils of coccolithophores and other calcareous microfossils, associated with coccolithophores but whose phylogenetic position has not been confirmed, are referred to collectively as calcareous nannofossils. Fossils of heterococcoliths and holococcoliths first appeared in the Upper Triassic (~225 Ma) and in the Lower Jurassic (~188 Ma), respectively (Bown et al. 2004). Following an extinction event at the Triassic/Jurassic boundary (ca. 205 Ma), calcareous nannofossils gradually increased in the number of species through the Jurassic–Cretaceous despite some extinction and turnover events, notably at the Jurassic/Cretaceous boundary and in the Albian, associated with the Oceanic Anoxic Event. Peak diversity is recorded in sediments from the Campanian and Maastrichtian (80–68 Ma). This is followed by abrupt loss of about 90 % of the species at the K/Pg Mass extinction event triggered by the meteorite impact (e.g., Bown et al. 2004). Paleontological data strongly indicate that the extinction was very rapid (Pospichal 1996; MacLeod et al. 1997; Schulte et al. 2010), that all the common oceanic taxa were eliminated and that the few survivor taxa, which are mostly rare before the event, were largely coastal taxa (Bown 2005). The mechanism of extinction of coccolithophores at the event is still under debate; however, recent studies (e.g., Alegret et al. 2012; Ohno et al. 2014) have suggested that vaporization of anhydrite at the impact site may have produced sulfuric acid aerosol and severe ocean acidification leading to selective extinction of marine calcareous plankton, including coccolithophores. This hypothesis fits the coccolithophore record well, since they appear to be much more severely affected than non-calcifying phytoplankton such as diatoms, dinoflagellates and even non-calcifying haptophytes (Medlin et al. 2008).

The few survivor species flourished immediately after the mass extinction; however, new Cenozoic taxa rapidly appeared and replaced the survivors within 50–500 kyr from the extinction (Pospichal 1996; Bown et al. 2004). The diversity of calcareous nannofossils increased in the Late Paleocene to Early Eocene, but rapidly declined from the Middle Eocene to the Oligocene. Diversity recovered in the Miocene, but declined again from the Pliocene to the Pleistocene.

Calcareous nannofossils are invaluable as index fossils for dating of marine sediments since they provide reliable age markers and can be prepared from even very small samples. As a result they are widely used in both academic research and petroleum geology and their taxonomy and stratigraphic succession have been studied extensively over the past 50 years. One intriguing result of this research is that size-increase trends are frequently seen in the group. Figure 12.5 shows changes in the size range of coccoliths of *Gephyrocapsa* in the last 1.83 my, as an example of this type of size-increase trend, with three successive cycles of gradual size increase, separated by two abrupt episodes of loss of the larger specimens. Intriguingly, the enlarged specimens within each event resemble each other, but there are slight differences between the enlarged specimens from different events. The enlarged *Gephyrocapsa* specimens from 1.67 to 1.24 Ma had diagonal bridges and lacked a central collar, the enlarged specimens from ca. 1.04 Ma had a horizontal bridge and a well-developed collar, and the specimens enlarged in the last ≥ 0.3 Ma had diagonal bridges and well-developed collars (Fig. 12.5) (Matsuoka and Okada 1990). Since these enlargement events occurred concurrently at various remote sites, the size ranges of *Gephyrocapsa* coccoliths are widely used for dating of marine sediments (Fig. 12.5) (e.g., Hine and Weaver 1998; Raffi et al. 2006). Similarly, large specimens of fossils of *Calcidiscus*, *Coccolithus*, and *Reticulofenestra* are widely

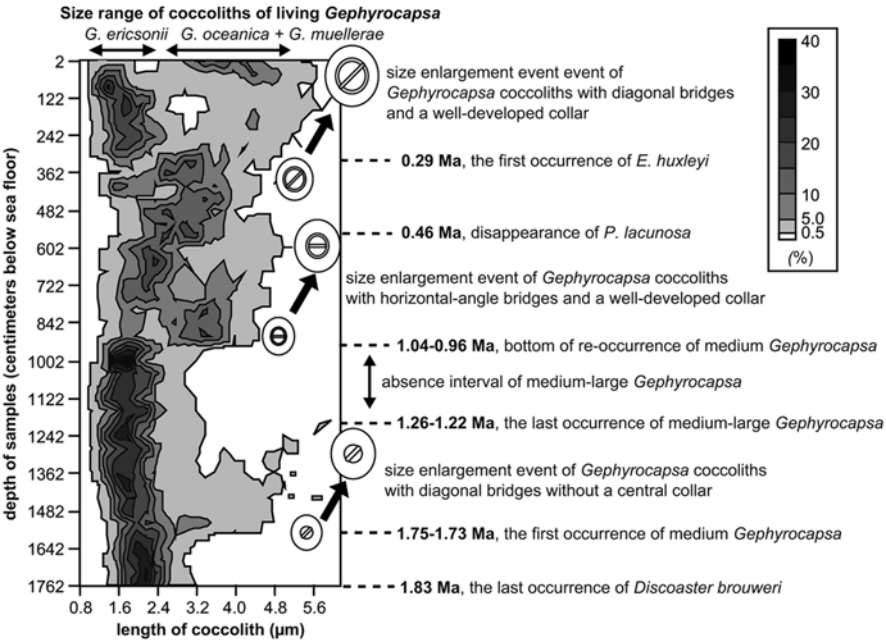

Fig. 12.5 Size variation of coccoliths of *Gephyrocapsa* spp. in the last 1.83 Ma, and fossil events recorded from the same core samples. Note that this figure was produced using morphometric data obtained by Matsuoka and Okada (1990). The age of the fossil events was emended following Raffi et al. (2006)

used for dating of Miocene–Pleistocene marine sediments (e.g., Hine and Weaver 1998; Raffi et al. 2006; Young 1998; de Kaenel et al. 1999).

Calcareous nannofossils are also used for reconstruction of paleoceanographic conditions. The horizontal distribution of water mass changes corresponding to the global climate changes such as glacial–interglacial cycles. The composition of coccolithophores in the sea surface water differs between the water masses, and calcareous nannofossil assemblages in the sea surface sediments reflect the living assemblage (McIntyre and Bé 1967). Therefore, stratigraphic changes in calcareous nannofossil assemblages can be interpreted as changes in overlying water masses. Based on stratigraphic changes in calcareous nannofossil assemblages, changes in the distribution of sea surface water masses in geological ages were reconstructed in the various sites of oceans (e.g., Baumann et al. 1999; Kameo and Sato 2000; Chiyonobu et al. 2012). The composition of morphotypes of *E. huxleyi* differs between cold and warm water masses (Table 12.1), and the morphotype B of *E. huxleyi* with a large (>4.5 μm) coccolith is constrained in the cold-water regime (Young et al. 2003). Therefore, fossil coccoliths of *E. huxleyi* > 4.5 μm are useful for reconstruction of the boundary of cold and warm water masses in geological ages (Colmenero-Hidalgo et al. 2002). The ratio of unsaturated alkenones produced by the members of the Isochrysidales reflects the water temperature where the organism (Isochrysidales) dwelled (Prahl and Wakeham 1987). Alkenones are resistant to diagenesis, and can be recovered from marine sediments up to 105 million years old (Farrimond et al. 1986). Thus, alkenones recovered from marine core sediments are useful for reconstruction of paleo sea surface temperatures (e.g., Sachs et al. 2000; Ternois et al. 2000). It is thought that light intensity is one of the most important limiting factors of the occurrence of lower photic zone dwellers such as *F. profunda*. The intensity of light in the lower photic zone depends on light transmission through the upper photic zone, and so on the abundance of upper photic zone dwellers. The number of upper photic zone dwellers is regulated by the nutrient concentration and so the intensity of water stratification. Therefore, changes in the relative abundance of fossils of lower photic zone dwellers in the total calcareous nannofossil assemblages in marine sediments reflect changes in surface water stratification in the geological past (e.g., Molfino and McIntyre 1990; Okada and Matsuoka 1996; Beaufort et al. 2001).

12.5 The Future of Coccolithophores

Emission of anthropogenic greenhouse gases affects not only global climate but also seawater chemistry. There is a growing concern that the combined increase of sea temperature and changes in marine chemistry may have widespread impacts on the marine ecosystem including on productivity and calcification of coccolithophores. Comparison of biogeographic studies of coccolithophores from subantarctic–antarctic regions revealed that *E. huxleyi* expanded their habitat polarwards in the last 25 years (Cubillos et al. 2007; Winter et al. 2013). *E. huxleyi*

only occurred to the north (equator-ward) of the Antarctic Polar Front (APF) before 1990; subsequently, however, it expanded to the south of the APF. It is assumed that rising of the surface temperature and/or oligotrophication (water stratification) of polar waters may have enabled migration of *E. huxleyi* into the polar water (Winter et al. 2013).

About 30 % of the anthropogenic CO_2 released into the atmosphere since the Industrial Revolution has been absorbed into the ocean (Sabine et al. 2004). This has led to a decline in the pH of sea surface water from 8.18 to 8.06 units, and it is predicted that the pH will decrease by another 0.3–0.4 units by the end of the century (IPCC 2007). It is thought that the further oceanic acidification (OA) will affect the calcification of marine organisms with calcareous skeletons such as corals and foraminifera (e.g., Gattuso et al. 1998; Barke and Elderfield 2002). Possible effects of this ongoing OA on coccolithophores are still under debate, since the results from culture and observational studies are not always consistent. Riebesell et al. (2000) studied the responses of clonal culture strains of *E. huxleyi* and *G. oceanica* to various pCO_2 conditions, and showed decline of calcification and/or increase of malformation in higher pCO_2 conditions. Recent culture studies, however, revealed that the responses of calcification to the higher pCO_2 condition differ between species and even strains (e.g., Langer et al. 2006, 2009; Iglesias-Rodríguez et al. 2008). Both *E. huxleyi* and *G. oceanica* have great intra-specific genetic diversities (e.g., Read et al. 2013; Bendif et al. 2014), and *cox* 1b genotypes of *E. huxleyi* were related to the ecological differences (temperature) (Hagino et al. 2011). Currently the genotypes that can be related to high tolerance of low-pH conditions have not yet been characterized; however, the differences in sensitivity to acidification among culture strains is considered to have arisen from genetic predisposition (Langer et al. 2009).

Field observations, however, provide evidence that carbonate chemistry may have a strong effect on coccolithophore biogeography. Using a large data set of paired shipboard observations of carbonate chemistry and coccolith mass, Beaufort et al. (2011) showed that the coccolith mass of Noëlaerhabdaceae (i.e., the group including *Emiliania* and *Gephyrocapsa*) from field samples is usually positively correlated with pH, and *E. huxleyi* strains established from low-pH areas possess highly calcified coccoliths. They also showed that this effect was seen in fossil samples from the Last Glacial Maximum to the near-present. Therefore there is a possibility that ongoing OA may induce changes in the composition of genotypes/morphotypes/species from the community adapted to the current-pH condition to a new community relatively resistant to low-pH conditions. The possible effect of OA on coccolithophores in the future is thus still uncertain. In order to estimate the effect, it is important to develop a comprehensive understanding of the physiology and genetic diversity of living coccolithophores, and of the response of total coccolithophore assemblages to global environmental changes in the geological past.

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Chapter 13
Diversity and Ecology of Thraustochytrid Protists in the Marine Environment

Ryosuke Nakai and Takeshi Naganuma

Abstract Thraustochytrids are heterotrophic estuarine/marine protists belonging to the class Labyrinthulomycetes within the stramenopile lineage. Thraustochytrid protists have been a neglected agent of the microbial food chain; however, they occur in detectable amounts in seawater, sediment, and algal and animal tissues. They have the ability to degrade a wide variety of organic substrates, including refractory substrates, by means of extracellular enzymes. Their wide distribution and degradation capability exhibit their ecological significance as decomposers. In particular, thraustochytrids may grow on terrestrial refractory matter in riverine input, and play a role in enhancing carbon cycling in estuarine and coastal areas. Additionally, they produce high amounts of long-chain polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), which are essential fatty acids for marine animals. Furthermore, some members are known to be pathogens of marine mollusks. These distinctive characteristics mean that thraustochytrid protists play a number of important roles in marine environments.

Keywords Thraustochytrid • Labyrinthulomycetes • Fungoid protist • Abundance • Biomass • Diversity • Food chain • Polyunsaturated fatty acids

13.1 Introduction

Thraustochytrids are heterotrophic fungus-like protists that belong to the eukaryotic stramenopile lineage (Cavalier-Smith et al. 1994; Honda et al. 1999; Tsui et al. 2009; Adl et al. 2012). Fungi and fungoid protists are decomposers in ecosystems, and their importance has long been recognized and investigated in terrestrial

R. Nakai
Genetic Strains Research Center, National Institute of Genetics,
1111 Yata, Mishima 411-8540, Japan

Superlative Postdoctoral Research Fellow of the Japan Society
for the Promotion of Science, Chiyoda-ku, Tokyo 102-8471, Japan

T. Naganuma (✉)
Graduate School of Biosphere Science, Hiroshima University,
1-4-4 Kagamiyama, Higashi-hiroshima 739-8528, Japan
e-mail: takn@hiroshima-u.ac.jp

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habitats. In contrast, fungoid protists in marine environments have generally been considered less important than bacteria in terms of their abundance, biomass and ecological role. Thraustochytrids can thus be considered part of the marine food chain that has been overlooked. However, they occur in detectable amounts in estuarine, marine and deep-sea waters throughout the world (Raghukumar and Schaumann 1993; Naganuma et al. 1998; López-Garcia et al. 2001). The abundance of planktonic thraustochytrids has been estimated to range from 10^3 to 10^5 l^{-1} using epifluorescence microscopic direct counts (reviewed in Raghukumar 2002). Although these abundances are small when compared with the numbers of bacterioplankton in general surface water, which have been estimated at 10^8–10^9 l^{-1} (Ducklow 2000), the cellular biovolume of thraustochytrids could reach ~10^3 times greater than that of bacterioplankton (Naganuma et al. 1998). Thraustochytrid cellular biomass has also been calculated to be ~10^4 times greater than the bacterioplankton biomass (Kimura et al. 1999). The relatively low abundance of thraustochytrids is thus offset by their high biovolume and biomass. Additionally, thraustochytrids are capable of producing extracellular cellulases and xylanases that decompose plant materials such as algal tissue and mangrove leaf litter (Sathe-Pathak et al. 1993; Bremer 1995; Nagano et al. 2011). They are also known to produce large amounts of omega-3 PUFAs such as DHA and DPA (Nakahara et al. 1996; Yokoyama et al. 2007), thus they have the potential to be food resources for organisms in higher trophic levels in the marine environment. Furthermore, some species of thraustochytrids have been implicated in pathogens affecting mollusks (Jones and O'Dor 1983; Stokes et al. 2002). These distinctive features mean that thraustochytrids play a number of important roles in marine ecosystems.

The thraustochytrid was first described in 1936 (Sparrow 1936) and from the late 1930s to the 1960s their isolation and characterization were reported (Sparrow 1943; Vishniac 1956; Goldstein 1963). By the 1990s, their occurrence in various habitats and their physiological requirements had been extensively studied (Perkins 1973; Bremer 1976; Jones and Harrison 1976; Bahnweg 1979; Gaertner 1979; Ulken et al. 1985; Moss 1986; Porter 1986). During the 1990s and 2000s, the abundance of thraustochytrids in the water columns and sediments was measured by direct microscopic enumeration methods (Raghukumar and Schaumann 1993; Naganuma et al. 1998; Raghukumar et al. 2001; Bongiorni et al. 2005a). At roughly the same time the development of molecular biological approaches led to taxonomic reinterpretations of thraustochytrids based on 18S rRNA and other gene sequences (Honda et al. 1999; Leander and Porter 2000; Yokoyama and Honda 2007; Yokoyama et al. 2007; Tsui et al. 2009). Subsequently, novel molecular techniques for detecting thraustochytrids were developed (Takao et al. 2007; Damare and Raghukumar 2010; Nakai et al. 2013). Garcia-Vedrenne et al. (2013) recently characterized a pathogenic thraustochytrid species using genomic and transcriptomic analyses. The biotechnological significance of thraustochytrids has also stimulated a number of studies on the PUFA yields of different thraustochytrids (reviewed in Raghukumar 2008; Lee Chang et al. 2014).

This chapter focuses on information related to the diversity and ecology of thraustochytrids in estuarine and marine environments, and also reviews methodologies for detecting thraustochytrids.

13.2 Taxonomy, Morphology and Molecular Phylogeny

Thraustochytrids are classified in the class Labyrinthulomycetes (also known as Labyrinthulea or Labyrinthulomycota) (Porter 1989; Cavalier-Smith et al. 1994; Honda et al. 1999; Dick 2001; Cavalier-Smith and Chao 2005). According to the current classification of eukaryotes, the Labyrinthulomycetes belong to the stramenopiles within the Stramenopiles, Alveolata, and Rhizaria (SAR) clade (Patterson 1989; Adl et al. 2012). The stramenopiles group is phylogenetically diverse and is composed of photosynthetic ochrophytes including brown algae and diatoms, coupled with non-photosynthetic bicoeceans and oomycetes (Leipe et al. 1994; Keeling et al. 2005; Tsui et al. 2009). The Labyrinthulomycetes have several stramenopile-specific characteristics, including tripartite tubular hairs on the flagellum of the zoospore and tubular mitochondrial cristae (Chamberlain and Moss 1988; Patterson 1989). The class Labyrinthulomycetes consists of two well-accepted families, the Thraustochytriaceae and the Labyrinthulaceae (Honda et al. 1999; Leander and Porter 2001), that largely correspond to the thraustochytrids and labyrinthulids, respectively. These two families have ectoplasmic nets ejected from a unique organelle at the cell surface known as a bothrosome (Porter 1969). This bothrosome is sometimes referred to as a sagenogenetosome (Perkins 1972) or sagenogen (Olive 1975). Additionally, they have cell walls composing of thin Golgi-derived non-cellulosic scales, instead of cellulose fibrils as in the Oomycetes (Darley et al. 1973; Ulken et al. 1985). The cell walls contain sulfated polysaccharides (Bahnweg and Jäckle 1986).

The thraustochytrid was first observed on the surfaces of marine algae from Woods Hole, MA, USA, and described as *Thraustochytrium proliferum* (Sparrow 1936). Although *Thraustochytrium* was originally reported as a chytridiaceous fungus, it was reclassified as a member of the Saprolegniales in the Oomycetes based on the observation of its biflagellate zoospores (Sparrow 1943; Dick 1973). However, because the sagenogenetosome ultrastructure of thraustochytrids was homologous to that of the labyrinthulids as described below, the thraustochytrids and labyrinthulids were subsequently merged into a single group (Perkins 1972; Olive 1975). Thraustochytrid cells are usually oval or spherical (Fig. 13.1) and in most cases a sagenogenetosome is present at a single site on the cell surface (Moss 1986). The cells are not fully enveloped or covered by their ectoplasmic nets. These nets are not used for motility, but contain extracellular enzymes that allow the penetration of solid surfaces and the digestion of organic compounds for adsorption and the transfer of nutrients (Bremer 1976; Coleman and Vestal 1987). The vegetative stages of thraustochytrids are composed of single cells measuring 4–20 μm in diameter, and

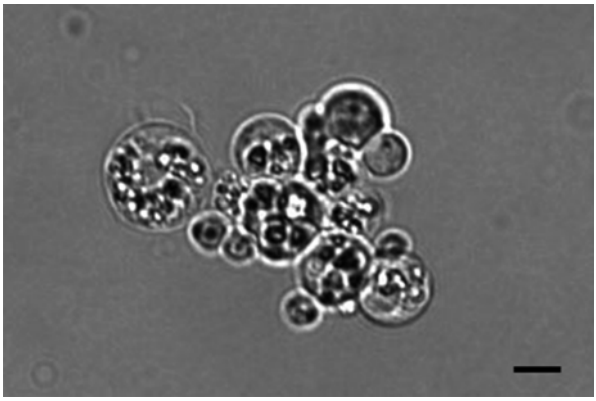

Fig. 13.1 Micrograph of a thraustochytrid strain, *Schizochytrium* sp. KR-5, isolated from the Seto Inland Sea, Japan. (Scale bar, 5 μm)

most thraustochytrids reproduce by means of zoospores (Raghukumar 2002). While many of the vegetative cells directly transform into zoosporangia, some differentiate into an amoeboid cell, which then transforms into a zoospore (Gaertner 1977). The mode of vegetative cells and other morphological characteristics vary between the thraustochytrid genera (Porter 1989; Yokoyama et al. 2007).

The family Thraustochytriaceae was originally composed of the following seven genera on the basis of their morphological features (Porter 1989; Moss 1991): *Althornia* Jones and Alderman, *Aplanochytrium* Bahnweg and Sparrow, *Japonochytrium* Kobayasi and Ookubo, *Labyrinthuloides* Perkins, *Schizochytrium* Goldstein and Belsky emend. Booth and Miller, *Thraustochytrium* Sparrow emend. Johnson, and *Ulkenia* Gaertner. However, comprehensive works by Honda et al. (1999) showed that these genus-level classifications were not consistent with molecular phylogenies based on 18S rRNA gene sequences. Using a combination of morphological and molecular phylogenetic characters, Leander and Porter (2000) concluded that *Aplanochytrium* and *Labyrinthuloides* are synonymous, and therefore emended the definition of the genus *Aplanochytrium*, which included all *Labyrinthuloides* species. Additionally, the species within the genus *Schizochytrium* were rearranged into three distinct genera, *Schizochytrium* sensu stricto, *Aurantiochytrium* and *Oblongichytrium* gen. nov. based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Yokoyama and Honda 2007). Furthermore, the genus *Ulkenia* was also separated into four genera containing three new genera, *Ulkenia* sensu stricto, *Botryochytrium*, *Parietichytrium* and *Sicyoidochytrium* gen. nov. (Yokoyama et al. 2007). During their taxonomic rearrangement of the genus *Ulkenia*, Yokoyama et al. (2007) identified several independent clusters in the phylogenetic tree that were formed by strains in the genus *Thraustochytrium* and unidentified species. These clusters are clearly visible in a tree based on labyrinthulomycete rRNA gene sequences determined in previous investigations (Fig. 13.2). The unidentified species includes a thraustochytrid pathogen known as Quahog Parasite Unknown (QPX) (Fig. 13.2), a pathogen of a bivalve mollusk (Whyte et al. 1994; Stokes et al. 2002). It is necessary to establish the taxonomy of these thraustochytrids based on both phenotypic and molecular phylogenetic analyses.

The remaining family within the class Labyrinthulomycetes is the Labyrinthulaceae. Cienkowski (1867) first described the genus *Labyrinthula* with two species, isolated from algae growing on rotted wooden pilings. The labyrinthulids consist of a single genus, *Labyrinthula* Cienkowski. Labyrinthulid cells are

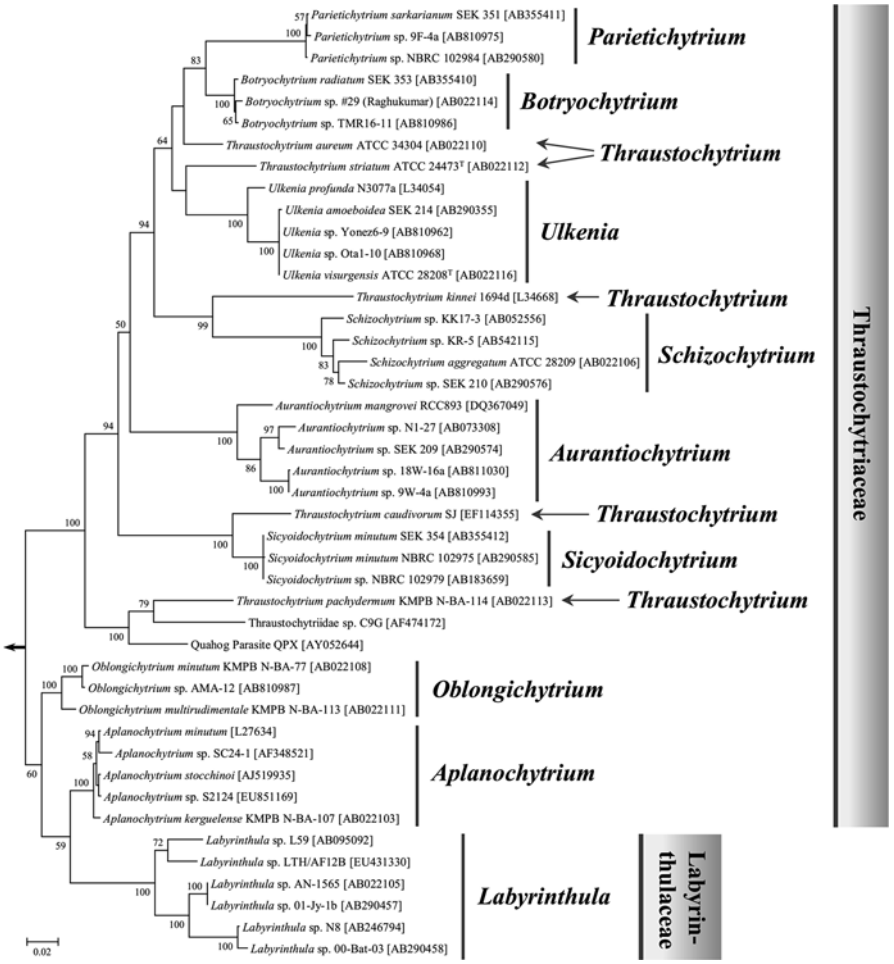

Fig. 13.2 Molecular phylogenetic tree of thraustochytrid and labyrinthulid protists constructed using 18S rRNA gene sequences obtained from previous investigations. The evolutionary history was inferred using the maximum likelihood method based on the Hasegawa–Kishino–Yano model (Hasegawa et al. 1985). There were a total of 1333 positions in the final dataset. Multiple sequence alignments and evolutionary analyses were conducted in CLUSTAL W (Thompson et al. 1994) and in MEGA6 (Tamura et al. 2013), respectively. The 18S rRNA gene sequences of the *Skeletonema costatum* CCAP 1077/3 (X85395) and *Thalassiosira weissflogii* CCAP1085/1 (FJ600728) were used as outgroups but were pruned from the tree. Scientific names on the tree are according to Yokoyama and Honda (2007) and Yokoyama et al. (2007). Accession numbers of nucleotide sequences recorded in DDBJ/EMBL/GenBank databases are shown in square brackets. Bootstrap values (percentages of 100 replications) >50 % are shown at nodes (Scar bar, 0.02 nucleotide substitutions per site)

spindle-shaped, and, unlike the thraustochytrids, grow colonially and glide inside ectoplasmic nets (Porter 1989). Some *Labyrinthula* species are thought to be the cause of wasting disease in eelgrass (Muehlstein 1992) and thus have attracted the attention of scientists in many fields. Tsui et al. (2009) showed that the Labyrinthulomycetes were divided into two sister clades A and B by multilocus phylogenetic analysis using the rRNA, actin, beta-tubulin, and elongation factor 1-alpha gene sequences. Clade A contains only thraustochytrids, whereas clade B includes labyrinthulids along with two thraustochytrid genera *Aplanochytrium* and *Oblongichytrium* (Tsui et al. 2009). Interestingly, in clade B, all of the gliding *Aplanochytrium* and *Labyrinthula* were phylogenetically nested among the non-gliding thraustochytrids. This phylogeny suggested that labyrinthulids that glide inside ectoplasmic nets evolved from thraustochytrids that had utilized the ectoplasm mainly for anchorage and nutrient assimilation (Tsui et al. 2009). Thus, molecular phylogenetic analyses have provided new insights into not only phylogenetic relationships within the Labyrinthulomycetes but also the evolutionary gain of ectoplasmic gliding. Additional data from more unstudied species will contribute to the validation of these insights. In particular, the following genera belonging to the Labyrinthulomycetes were recently reported: *Amphifila* (Anderson and Cavalier-Smith 2012), *Amphitrema* (Gomaa et al. 2013), *Archerella* (Gomaa et al. 2013), and *Diplophrys* sensu stricto (Takahashi et al. 2014). A comparison of these genera with known thraustochytrids and labyrinthulids will be important when considering labyrinthulomycete evolution.

13.3 Methodologies for Detecting Thraustochytrids

Knowledge regarding the abundance of organisms is imperative for understanding their ecological roles and distributions. Methods for detecting thraustochytrids are summarized in Table 13.1. In early studies, the abundance of thraustochytrids was determined by the culture-based most probable number (MPN) technique modified using pine pollen as an adsorption substratum (Gaertner 1968). The MPN approach based on adequate dilutions and replicates provides reliable data regarding thraustochytrid density fluctuations. Although this technique has revealed the relative abundance of thraustochytrids in different habitats (Ulken 1986; Bongiorni and Dini 2002), it has the following limitations: not all species may grow under the culture media and conditions used, and cells may die or multiply during the long sample processing time typically required (Raghukumar and Schaumann 1993).

The lack of rapid direct detection methods for surveying thraustochytrids initially hampered detailed studies of their distributions in various habitats. However, a detection technique using the fluorogenic acriflavine dye has been developed and applied for the enumeration of particle-bound thraustochytrids (Raghukumar and Schaumann 1993). This technique relies on the fact that thraustochytrid cell walls and cell contents fluoresce red and blue-green under blue-light excitation, respectively. This dual fluorescence distinguishes thraustochytrids from other protists and

Table 13.1 Development and application of methods for ecological studies on thraustochytrid protists

Samples	Objectives	Methods	References
Seawater and sediment	Culture-based enumeration of thraustochytrid abundance	MPN	Gaertner (1968)
Phytoplankton aggregate	Direct detection and enumeration of particle-bounded thraustochytrids	Acriflavine direct detection	Raghukumar and Schaumann (1993)
Seawater	Direct enumeration of planktonic thraustochytrids	Acriflavine direct detection	Naganuma et al. (1998)
Sediment	Direct enumeration of benthic thraustochytrids	Acriflavine direct detection	Santangelo et al. (2000)
Cultured strain	Direct visualization of thraustochytrids	FISH	Takao et al. (2007)
Zooplankton	Direct visualization of *Aplanochytrium kerguelensis*	ISH	Damare and Raghukumar (2010)
Seawater and sediment	Culture-independent analysis of labyrinthulomycete diversity	PCR, cloning, and sequencing	Collado-Mercado et al. (2010); Li et al. (2013)
Clam	Molecular detection and phylogenetic analysis of a thraustochytrid pathogen QPX	PCR, cloning, and sequencing	Stokes et al. (2002)
Clam	Molecular detection and quantification of QPX	qPCR	Lyons et al. (2006); Liu et al. (2009)
Seawater	Genus-specific enumeration of 7 thraustochytrid genera (*Aurantiochytrium*, *Botryochytrium*, *Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia*)	qPCR	Nakai et al. (2013)

Scientific names are according to Yokoyama and Honda (2007) and Yokoyama et al. (2007)

detritus. Naganuma et al. (1998) used this technique to estimate the numbers of planktonic thraustochytrids in the Seto Inland Sea, Japan. Detailed reviews of the numbers of thraustochytrids in seawater at various locations were made by Raghukumar and Damare (2011). Additionally, Santangelo et al. (2000) simultaneously enumerated the thraustochytrids and ciliates present in sandy sediments, using a combination of acriflavine technique for thraustochytrids (Raghukumar and Schaumann 1993) and separation method of nano-, micro-, and meiobenthic organisms from sediment by centrifugation in a non-linear silica gel gradient (Epstein 1995). However, even acriflavine counts have limitations as they may include protozoan cysts and exclude thraustochytrid zoospores and young vegetative cells lacking cell walls (Raghukumar and Schaumann 1993; Kimura et al. 2001). The extent of over- or underestimation should be evaluated in future studies by comparing results with more specific techniques.

To address this issue, molecular biological techniques for the detection of thraustochytrids have recently been proposed by many groups. Molecular approaches allow direct investigation of the diversity and phylogeny of organisms in almost any environment. For example, a fluorescence in situ hybridization (FISH) technique using a fluorescently labeled oligonucleotide marker (probe) complementary to unique gene sites is useful for specifically detecting individual target cells in microbial assemblages under a fluorescence microscope. Takao et al. (2007) established a FISH protocol for the detection of thraustochytrids that is particularly well adapted for the Thraustochytrid Phylogenetic Group (TPG), which includes the genera *Schizochytrium* sensu lato including *Aurantiochytrium*, *Thraustochytrium* and *Ulkenia* sensu lato (Honda et al. 1999; Yokoyama and Honda 2007). Because this protocol does not need any cultivation step, it is expected to overcome the disadvantages of culture-based MPN estimation. Additionally, Damare and Raghukumar (2010) also developed an in situ hybridization (ISH) assay to detect *Aplanochytrium kerguelensis* using a bright-field microscopy-based biotinylated probe, instead of a FISH probe. They successfully detected the presence of *Aplanochytrium* cells within the tissues of chaetognaths. This ISH method is a powerful tool for specific species within individual cells in tissue sections, providing insights into the potential associations between thraustochytrids and other organisms.

As thraustochytrid ecological function and abundance may vary among species, there was a need for taxon-specific quantification using molecular markers. Lyons et al. (2006) and Liu et al. (2009) established a quantitative polymerase chain reaction (qPCR) to detect QPX pathogen. Quantification by qPCR relies on detection of the increase in fluorescence from exponentially amplified DNA by a PCR involving an oligonucleotide marker (primer) set or a fluorochrome-labeled probe designed to bind to the desired DNA locus. qPCR-based quantification provides a highly sensitive and specific system for the identification of target organisms. This method is increasingly being used in marine microbiological studies, such as in the detection of dinoflagellates (e.g. Moorthi et al. 2006). Nakai et al. (2013) recently developed a new qPCR system for thraustochytrids with seven genus-specific DNA markers. They filtered coastal and open seawater samples collected around Japan through a membrane filter, and then used the qPCR system for DNA extracted directly from the filters. Their results showed the presence of *Aurantiochytrium* (ranging from 1.12×10^4 to 1.31×10^4 cells l^{-1}) and *Oblongichytrium* (ranging from 1.02×10^4 to 3.14×10^4 cells l^{-1}) in the samples. Genus-specific enumerations such as these will increase our understanding of thraustochytrid distributions in marine ecosystems.

There have been increasing reports of the existence of novel marine stramenopiles that are likely to include novel labyrinthulomycete lineages from various habitats (Massana et al. 2004, 2014). Using a culture-independent method with a labyrinthulomycete-specific PCR primer, Collado-Mercado et al. (2010) investigated the diversity of labyrinthulomycetes in DNA isolated from seawater and sediment samples collected around Long Island, NY, USA. They sequenced 68 near-full-length 18S rRNA gene sequences, and although 15 of the 68 sequences belonged to the thraustochytrid group, none of these 15 were related to the sequences of cultivated strains. Additionally, the remaining 53 sequences belonged either to

the labyrinthulid group or to other undescribed groups. Li et al. (2013) also found deep-branching unclassified labyrinthulomycete sequences, along with *Aplanochytrium*-related sequences, from Hawaiian waters. These studies have revealed higher-than-expected diversity of thraustochytrids and labyrinthulids in the marine environment. In the future, accurate molecular detection that takes such novel labyrinthulomycete members into consideration will be required.

To quantify and describe the abundance and dynamics of thraustochytrid taxa in the marine environment, there is a continuing need to improve the molecular approaches described above. However, in principle, the DNA markers used in these approaches are designed based on 'known' thraustochytrid gene sequences. Thus it is possible that as a result these approaches overlook 'unknown' thraustochytrids in the environment. This highlights the need to carefully review the gene sequences of labyrinthulomycetes obtained from DNA-marker-independent methods such as metagenomic sequencing, which directly analyzes environmental DNA by high-throughput sequencing (Hugenholtz and Tyson 2008).

13.4 Ecological Roles of Thraustochytrid Protists

Using the detection methods for thraustochytrids described above, previous studies have detected their presence in estuarine, marine and deep-sea environments (Raghukumar and Schaumann 1993; Naganuma et al. 1998; Santangelo et al. 2000; López-Garcia et al. 2001). Thraustochytrids in the water column are mostly between 3.5 and 20 μm in diameter (Raghukumar and Schaumann 1993; Naganuma et al. 1998) and larger than bacterioplankton (<1 μm; Naganuma and Miura 1997). In terms of size, planktonic thraustochytrids are thought to serve as suitable food sources for 100 μm-sized protozoans, heterotrophic dinoflagellates, and filter-feeding bivalves. Additionally, the carbon/nitrogen (C/N) ratio of the cultured thraustochytrid cells was determined to be 10.5 (Kimura et al. 1999), which was higher than that the ratio of coastal (5.9) and oceanic (6.8) bacteria (Fukuda et al. 1998). It has been suggested that thraustochytrids have a greater impact per cell on carbon cycling than bacterioplankton cells. Additionally, assuming an average thraustochytrid size of 10 μm, their biovolume and biomass were found to be on average 524 μm^3 $cell^{-1}$ and 1.65×10^{-4} μg C $cell^{-1}$, respectively (Naganuma et al. 1998; Kimura et al. 1999), much greater than those of bacterioplankton (0.1 μm^3 $cell^{-1}$ and 2.4×10^{-8} μg C $cell^{-1}$, respectively) (Naganuma and Miura 1997). The relatively low abundance of thraustochytrids is thus offset by their high cellular C/N ratio, high biovolume (10^3 of bacterioplankton), and high biomass (10^4 of bacterioplankton). Raghukumar et al. (2001) reported that maximum thraustochytrid biomass could reach values ranging from 36 to 217 % of bacterial biomass in the water column of the Arabian Sea. Thus, thraustochytrids are strong competitors with bacterioplankton and important contributors to ecosystem-level material cycling. Marine pelagic ecosystems are sustained by two types of food chains, the classical grazing food chain and the microbial food chain (e.g. Valiela 1995). The

microbial food chain, also known as the microbial loop, salvages organic drop-outs such as phytoplankton exudates and phyto- and zoodetritus from the grazing food chain (Pomeroy and Wiebe 1993). This chain is a pathway of particle-size increments: from μm-sized bacteria to flagellates, to ciliates, and to mm-sized zooplankton, involving four trophic levels. Assuming the chain starts with 10 μm-sized thraustochytrids, only three trophic levels are involved, and the whole transfer efficiency would thus be ten times higher than the chain starting from bacterioplankton. Another important aspect of their ecological role in the microbial food chain is their ability to decompose refractory organic substrates that are readily utilized by bacterioplankton. Thraustochytrids often occur in association with algal tissues (Sathe-Pathak et al. 1993), and they can degrade such refractory plant materials using extracellular cellulase (Bremer 1995; Nagano et al. 2011). Because terrigenous organic matter often contains refractory organic substrates such as cellulosic and phenolic compounds (Ittekkot 1988), it is hypothesized that the planktonic thraustochytrids may grow on terrestrial refractory matter contained in river water. This hypothesis is yet to be validated; however, circumstantial but supportive evidence has been reported (Kimura and Naganuma 2001).

Additionally, the nutrient value of thraustochytrid protists in marine food webs may be significant. Thraustochytrids contain high levels of omega-3 long-chain PUFAs such as DHA, DPA, and eicosapentaenoic acid (EPA) (Nakahara et al. 1996; Yokoyama et al. 2007; Lee Chang et al. 2014). These compounds often occur in marine animals but they are not synthesized de novo by the animals themselves. They have to be obtained as a diet from PUFA producers including thraustochytrids (Fell and Newell 1998). In particular, it seems likely that thraustochytrids are an important alternative food source for fish larvae that possess high concentrations of DHA such as sardines. This process is another important aspect of the contribution of thraustochytrids to marine food chains and carbon cycling. To investigate the biotechnological potential to use thraustochytrids for PUFA production, the effects of various culture conditions on DHA productivity have been investigated. For instance, *Aurantiochytrium* (formerly *Schizochytrium*) *limacinum* ATCC MYA-1381 has been shown to produce more than 4 g DHA l^{-1} using 9 % glucose or 12 % glycerol as a carbon source (Yokochi et al. 1998). The PUFA profiles of a number of thraustochytrid taxa were recently investigated and compared (Yokoyama et al. 2007; Lee Chang et al. 2014). The genetic transformation system for thraustochytrids was also developed with the objective of generating superior mutants for the production of PUFAs (Sakaguchi et al. 2012). Thraustochytrids should be thus viewed as a promising source for PUFA production.

Some of the distinctive features of thraustochytrid protists mean that they could potentially have significant impacts on benthic ecosystems. Thraustochytrids are reported to be particularly abundant in organic-rich seagrass and mangrove forest sediments (Bongiorni et al. 2005a; Wong et al. 2005). Their numbers in such sediments have been estimated to be in the order of 10^3–10^4 cells g^{-1} using acriflavine staining methods (reviewed in Bongiorni 2012). Bongiorni et al. (2005a) showed that, in seagrass beds characterized by the presence of highly refractory structural carbohydrates, thraustochytrids accounted for up to 50 % of total protist

abundance (the sum of ciliates, nanoflagellates and thraustochytrids). Additionally, thraustochytrid biomass (0.124 μg C g^{-1}) was almost three times larger than that of nanoflagellates (0.044 μg C g^{-1}). One of the reasons that thraustochytrids largely dominate these types of vegetated sediments is considered to be their capability to degrade refractory materials. In fact, sediment isolates exhibit a wide variety of enzymatic activities related to the degradation of carbohydrates, lipids and proteins under laboratory conditions (Bongiorni et al. 2005b). However, as these data were obtained from culturable thraustochytrids, the degradation capability of other uncultured thraustochytrids might be more than expected.

Several thraustochytrids have been regarded as pathogens. QPX is a thraustochytrid parasite that causes disease and morality in the hard clam, *Mercenaria mercenaria* (Whyte et al. 1994; Stokes et al. 2002). Because hard clams are benthic suspension feeders, they obtain particles, such as marine aggregates, from large volumes of seawater. Lyons et al. (2005) reported high densities of QPX in marine aggregates collected from coastal embayments in Cape Cod, MA, USA, where QPX outbreaks have occurred. They pointed out that such pathogen-laden aggregates might serve as lethal marine snow contributing to the spread and survival of the pathogen. Histological observations indicated that QPX infections typically occur in the clam gill, mantle, and siphon tissues (e.g. Smolowitz et al. 1998; Lyons et al. 2006). QPX physiology has been extensively studied, and in vitro studies have demonstrated that mucus production by QPX is temperature dependent and is highest at 24 °C and is lower below 16 °C and above 32 °C (Brothers et al. 2000). Mucus secretion by QPX related to inflammatory responses in the clam host is considered one of the primary virulence mechanisms. Using RNA-seq to analyze gene expression patterns, it was recently reported that genes related to proteolytic and glycolytic enzymes, such as metalloproteases and enolase, were expressed at a higher levels at 21 °C (Garcia-Vedrenne et al. 2013). It is thus possible that the enzymes involved in host tissue invasion are included in the mucus secreted by QPX. Another thraustochytrid pathogenic to the marine flatworm *Macrostomum lignano* has been named *Thraustochytrium caudivorum* (Schärer et al. 2007); however, its physiological and genomic characteristics are currently poorly known.

13.5 Conclusions and Future Perspectives

The taxonomic relationships of thraustochytrid species have been arranged and rearranged based on their morphology, chemotaxonomy and molecular phylogeny. Additionally, recent culture-independent studies have revealed that thraustochytrids in the marine environment are more phylogenetically diverse than anticipated. This finding emphasizes the need for novel cultivation techniques and conditions to grow previously uncultured thraustochytrids. One option for optimizing culture conditions is to use physiological characteristics inferred from genomic/metagenomic information, which is obtained by single-cell genomic or metagenomic sequencing. Currently, the genomes of three thraustochytrids, *Aplanochytrium kerguelense*,

Aurantiochytrium (formerly *Schizochytrium*) *limacinum* ATCC MYA-1381, and *Schizochytrium aggregatum* ATCC 28209 are being published by the US Department of Energy Joint Genome Institute (http://genome.jgi.doe.gov/). Further accumulation of genomic information will provide specific insights into the genetic basis for physiological differences between thraustochytrid taxa. The genome data will also reveal their biotechnological potential, other than the production of PUFAs.

A number of ecological studies on thraustochytrids, focused on their abundance, biovolume and biomass, have been undertaken. Further work is needed to estimate their growth and production rates in natural waters. Additionally, the answers to the following key questions are still unclear: (1) Which species of zooplankton prey on thraustochytrids? (2) Do fish and shellfish use the PUFA that they synthesize in the marine environment? (3) If they decompose terrestrial-derived organic matter present in river water, how does their scavenging impact biological production in the coastal zone? (4) What are the differences between the ecological roles of the different taxa? Thus, the dynamics of thraustochytrids and their interactions with other organisms in the environment are unclear and require further study.

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Chapter 14
Ecology and Distribution of Protists in Brackish Water Lakes

Hideki Ishida

Abstract A brackish water area means water that is a little salty, indicating salinity between fresh and marine waters. While it is known as the habitat for many diversified organisms, it is a harsh environment due to changes in salinity. Although it has been speculated for many years that biodiversity is lowered at a salinity of 5–8‰ within the salinity gradient in the brackish water area, planktons such as planktonic protists, in particular, have been recently found to rather increase their diversity in this salinity range. Planktonic protists are not likely to create new species easily that inhabit only specified areas, because their small size makes it easy for them to expand their habitat areas and less likely for them to be extinguished. In reality, endemic species are found in many places including brackish water areas, but we do not have a clear explanation for this discrepancy. Lake Shinji and Lake Nakaumi are the prominent brackish water lakes in Japan. In these lakes, a halocline is formed, as in many other brackish water lakes. This chapter provides an overview of the survey of protist diversity in these lakes, with an attempt to determine the environmental factors influencing the composition of species in the brackish water area.

Keywords Brackish water • Estuary • Halocline • Horohalinicum • Protist • Remane's concept

14.1 Brackish Water Area

Brackish water is water with a level of salinity between those of freshwater and seawater, generally defined by salinity from 5 to 35‰. A brackish water area is the area occupied by brackish water, of which river mouths or estuaries are the prime examples. Coastal lagoons are formed by sand bars at the mouths of streams separating them from the adjacent ocean, and they are often brackish water areas. Semi-enclosed inner bay areas sometime become brackish, just like the entire area of the

H. Ishida (✉)
Faculty of Life and Environmental Science, Department of Biological Sciences, Shimane University, Matsue, Japan
e-mail: h-ishida@life.shimane-u.ac.jp

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Baltic Sea, which is a well-known example. Inland saline lakes also sometimes become brackish, as seen in the Aral Sea. The characteristics of brackish water area are as follows: it is a semi-enclosed environment; it has relatively free contact points with the outer ocean; marine water is diluted by freshwater from a river; and it has a dynamic environment with marine water coming in and going out with the tide.

At the mouth of a river, both fresh and marine waters are naturally mixed up to become brackish water. Since marine water regularly moves up and down according to the ebb and flow of the tide, marine water comes deep into the river mouth at high tide, while freshwater flows to the river mouth at low tide. Although the salinity of the marine water is relatively stable, the volume of fresh water flowing from the river changes irregularly depending on changes in rainfall in water catchment areas. As a result, the salinity at the river mouth keeps changing at every moment. Since coastal lagoon lakes are often linked to the outside, the salinity of the lakes is influenced by changes in the surface of the sea as at the mouth of a river.

In a brackish water area, a double-layer structure is sometimes observed, with freshwater that has flowed from the upstream river on top and higher-salinity marine water below. Particularly, in areas along the coast with little difference between high and low tides, marine and fresh waters do not mix easily with each other due to the difference in density. If we look at the vertical cross-section of a brackish water area, the border of the layers is clear, showing the formulation of the so-called "halocline". The halocline has a large impact on the ecosystem during the summer time. As a result of the warming up of the upper layer, it becomes more difficult for these water layers to be mixed, and the bottom layer becomes oxygen deficient with extremely little dissolved oxygen.

For example, let us take a look at Japan's most prominent brackish water lakes, Lake Nakaumi and Lake Shinji (Fig. 14.1). In Lake Nakaumi, inflows of fresh low-salinity water from the Ohashi River and marine water from the Sakai Channel mix together to form brackish water. However, they do not mix easily. There is a tendency that the high-density marine water remains in the lower layer, while low-density freshwater has its place in the upper layer. Under the influence of wind and temperature, substantial movement of the water mass takes place even during a day. For example, in Lake Nakaumi, when a weak wind blows, the lake water does not show any significant movement. During moderate wind conditions, the lake water is mixed on the surface, forming a halocline between the mixed part and the lower stationary part. At the time of strong winds, as Fischer and others have reported (Fischer et al. 1979; Tokuoka et al. 1996, 2001), the low-salinity water mass in the upper layer moves to the leeward side, while the high-salinity layer compensatingly moves to the windward side. As a result, the high salinity layer is exposed on the surface at the edge of the lake in the windward area (Fig. 14.2).

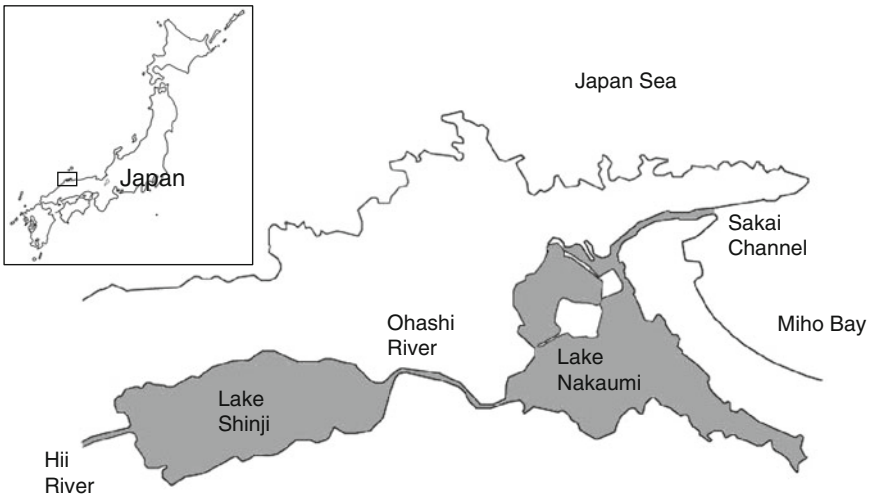

Fig. 14.1 Location of Lake Nakaumi and the Lake Shinji. Lake Nakaumi is one of the prominent brackish water lakes in Japan. Lake Shinji, located downstream of the Hii River, is connected by the Ohashi River with Lake Nakaumi, which is linked to the Sea of Japan by the Sakai Channel

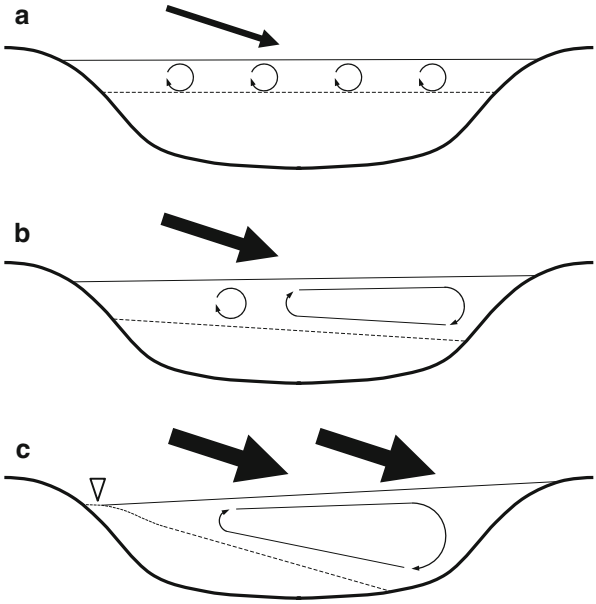

Fig. 14.2 Movements of haloclines in Lake Nakaumi in response to wind effects. At the time of weak wind (**a**), the lake water does not show a large movement. In moderate wind (**b**), the lake water is mixed on the surface, forming a halocline at the border between the mixed and lower parts. When strong wind blows (**c**), the high-salinity layer compensatingly moves windward, exposing the layer at the edge of the lake at the windward side (*arrowhead*)

14.2 Organisms in the Brackish Water Area

In general, the current velocity becomes slower at the mouth of the river, where accumulation of organic substances occurs. When the nutrient salt concentration becomes higher with the decomposition of organic substances, the water can sustain

more organisms. In other words, brackish water areas usually have higher primary production of organisms than other water areas. Diversified organisms are living in the brackish water area, because of the following reasons: it has a high primary production due to the presence of abundant nutrients; its salinity environment is different from either the adjacent ocean or the river upstream; its environment frequently and rapidly changes rapidly, and so on. Such an unstable environment also contributes to the occurrence of a number of organisms in the brackish water area in a way that some species usually living in freshwater or seawater, or pelagic species, which appear to have no relation to the brackish water area, sometimes spend part of their life there.

In a brackish water area, the region near the river mouth has low salinity, while the greater the distance from the river, the higher the salinity becomes. As the influences of fresh and marine waters differ depending on the distance from the river mouth and the depth of the water, the salinity environment shows a laminar distribution; therefore, organism distribution often becomes a zonal one. The brackish water area usually maintains a unique biota with organisms tolerant of the changing salinity—i.e., species that can live there even if the salinity changes (Remane and Schlieper 1971; Cognetti and Maltagliati 2000). Some organisms in the brackish water area have adapted to live in a very wide range of salinity, and they are called euryhaline organisms. On the other hand, those species specialized in living in a specific environment and seen in a limited area of distribution are called stenohaline organisms.

Even in brackish water, the diversity of species is reported to be different depending on salinity. Remane (1934) and others studied the relationship between salinity and the diversity of species based on their research conducted in the Baltic Sea, and found that the abundance of species declined to the minimum level (the Artenminimum) at salinity between 5 and 8‰ (Remane 1934; Fig. 14.3). By examining the ion composition of the diluted marine water, Khlebovich (1969) concluded

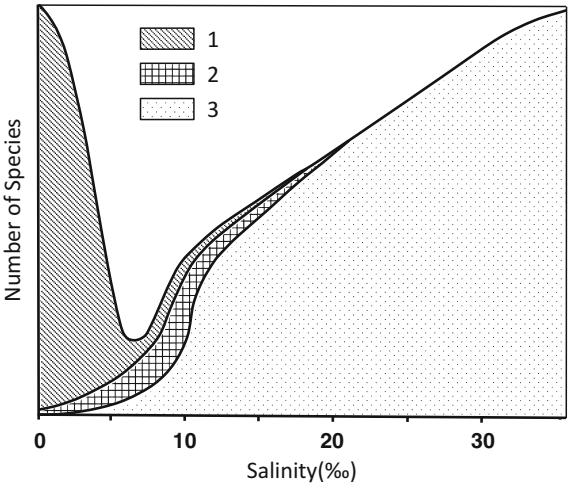

Fig. 14.3 Number of benthic species in response to changes in salinity. The graph shows the species richness of freshwater (*1*), brackish water (*2*), and marine species (*3*) in relation to water salinity. In the salinity range of 5–8‰, the total number of species becomes a minimum (Data adopted from Remane 1934)

that a radical change in the ion composition, which occurs at salinity between 5 and 8‰, is the cause of the Artenminimum. Kinne (1971) named this critical salinity as the "horohalinicum"—i.e., the region in which diversity lowers in the high-diversity brackish water area. However, Deaton and Greenberg (1986) found that the ion composition of the dilute marine water changed only a little in salinity between 5 and 8‰, while at salinity of 2‰ or lower, changes in the ion ratios were very large. This suggests that the Artenminimum cannot be explained by changes in the composition of ions in the brackish water. Deaton and Greenberg (1986) partly explained the phenomenon of decreasing diversity of species in the brackish water by pointing out that brackish water species need to evolve physiological mechanisms necessary for life in an environment of changing salinity, and brackish water and euryhaline species have lower rates of speciation compared with stenohaline, marine and freshwater counterparts.

14.3 Protists in the Brackish Water Area

When discussing the distribution of brackish water protists, two points need to be taken into account: one is what Beijerinck (1913) claimed, that "in micro-organisms, everything is everywhere, the environment selects"; and the other is the concept of the "Artenminimum" advocated by Remane (1934).

As the result of observing one pond for a long period of time, Finlay and Esteban (1998) reported that they were able to observe all the species of protists eventually. They said these species had not emerged simultaneously but continued changing while showing totally different compositions of species in response to changes in the environment. As protists are very small in size and sometimes take the form of dormant buds which facilitate dispersal, they can spread to wide areas rapidly. As a result, it is rather rare to find a specific protist living only in these limited areas, but we can see the same organism everywhere in the world. On the other hand, Azovsky (2000) and Hausmann et al. (2003) do not support the idea that "everything is everywhere, the environment selects," by showing, based on much research, that there are more than a few species that are seen only in specific areas. Even if opportunities for ectopic speciation are not many due to the high diffusibility of protists and a low extinction rate (Fenchel 1993; Wilkinson 2001; Finlay 2002; Finlay and Fenchel 2001), given unicellular protists are claimed to have had a sufficiently longer period of time to accumulate diversity than multicellular organisms (Foissner 2006).

While the concept of the Artenminimum advocated by Remane (1934) was for species centering on zoobenthos, it has been supported by many researchers as the one that can be applicable to many other species. In recent years, however, some zoobenthos (Boesch et al. 1976; Attrill 2002), plankton (Laprise and Dodson 1994), ciliates (Dolan and Gallegos 2001, Telesh and Heerkloss 2004) and bacteria (Crump et al. 1999; Hewson and Fuhrman 2004) have been found to be exceptions to Remane's model. In addition, it has been shown not only that the diversity of planktonic organisms does not become a minimum in the brackish water area in many

cases, but also that it will sometimes become a maximum. In particular, the diversity of ciliates was found to become a maximum near the horohalinicum area at the salinity of 5–8‰ (Fig. 14.4), showing that Remane's model cannot be applied to many planktonic micro-organisms.

The relationship between the diversity of species and environmental factors in the aquatic ecosystem is known as the "paradox of the plankton" (Hutchinson 1961). Naturally, in the condition of limited resources, multiple species in the same niche cannot stably co-exist, and therefore, the competitive exclusion principle is thought to be at work, whereby a small number of species exclude others (Gause 1934). In reality, however, when planktonic protists are collected, more than the expected species are observed, contradicting the competitive exclusion principle. Huisman and Weissing (1999) have shown that the competitive exclusion principle works in relatively stable conditions, and that when various environmental factors oscillate and change randomly as in a brackish water area, many species are able to co-exist. Basically, this concept is also applicable to protists, and quite a few protists are observed in brackish water areas.

The composition of protistan species in each area differs widely depending on the season as well. The major cause is temperature, but it is also greatly influenced by seasonal changes in salinity, the amount of light, and changes in food organisms (mainly bacteria). Moreover, protists themselves influence each other sometimes. For example, increases or decreases in planktonic ciliates are influenced by same conditions as benthic ciliates (Ekaterina et al. 2012).

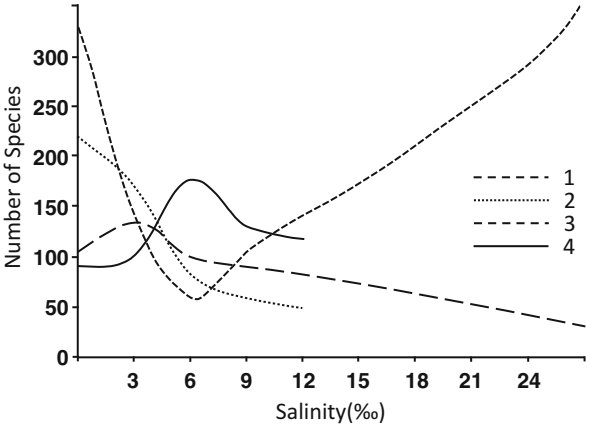

Fig. 14.4 Diversity of inhabitant zooplanktons in response to changes in salinity. The concept of the Artenminimum advocated by Remane was that the model for species centering on zoobenthos can also be applicable to many other species. However, some zoobenthos, plankton, ciliates and bacteria are found to be exceptions to Remane's model. Rotifera and Crustacea decrease in diversity in the horohalinicum area, while ciliates increase in numbers of species (*1*: Renema's curve; *2*: Rotifera; *3*: Crustacea; *4*: Ciliophora). The diversity of planktonic organisms does not become a minimum in the brackish water area in many cases but it will rather become a maximum (Data from Telesh and Heerkloss (2002, 2004), Telesh et al. (2009), Lindley and Batten (2002), and Telesh (2011))

14.4 Genuine Brackish Water Species

When we collect and observe protists inhabiting a brackish water area, we find many species that are classified as freshwater and marine species. Many researchers are asking "Are there genuine brackish water species?" "Genuine brackish water species" means species that do not inhabit fresh or marine waters, and can only inhabit a brackish water area. While Remane (1934) concluded that species in the Artenminimum are genuine brackish water species, Wolff (1973) conducted research on benthic organisms and found that even if some organisms only inhabit a brackish water area, the factors limiting their habitat are those other than salinity in many cases. As for planktonic organisms as well, long-term research and reanalysis of past data etc. have found inconsistencies with Remane's model (Telesh 2004; Telesh and Heerloss 2002; Telesh et al. 2008, 2011).

Some marine or freshwater species have evolved their tolerance for salinity or salinity changes in the brackish water area, making them capable of inhabiting the brackish water area. A salinity gradient lower than the horohalinicum of between 5 and 8‰ causes large changes of ion compositions, and is said to prevent biodiversity, placing a large obstacle for freshwater species to go beyond this concentration range into the brackish water (Khlebovich 1969; Kinne 1971). Among protists, however, especially ciliates have acquired the powerful ability to respond to salinity stress by developing a mechanism of unique cellular osmoregulation by contractile vacuoles (Stock et al. 2002). Moreover, some ciliates (and other protists) have acquired the ability to respond to changes in salinity by forming a cyst, which is also the cause of expansion and diversification of habitats. As many of the brackish water areas are formed as a mixture of fresh and marine waters, salinity changes in gradation. In other words, marine and fresh waters are not directly adjacent to each other. The change from freshwater to brackish water takes a very short period of time compared to that required for evolution of organisms; therefore, if there are genuine brackish water species evolved to live only in a brackish water environment, they should make a drastic change in such a short period of time. Rather, it can be thought that protists inhabiting fresh or marine water have evolved to become capable of responding to a wide range of salinity, and as a result, they have come to inhabit the brackish water area as well (Stock et al. 2002; Micronova et al. 2009). In that sense, it is reasonable to assume that "genuine brackish water species" do not exist, but that freshwater or marine species have taken a long time to expand their habitat areas.

14.5 Protists in Lake Shinji and Lake Nakaumi

Lake Nakaumi is one of the prominent brackish water lakes in Japan. Lake Shinji, located downstream of the Hii River, is connected by the Ohashi River to Lake Nakaumi, which is linked to the Sea of Japan by the Sakai Channel. The influence

of the marine water reaches up to Lake Shinji through the Ohashi River, making changes in water quality more complicated than in a landform that simply links a coast and a river. The salinity of Lake Shinji is on an annual average basis about one-tenth that of the marine water, while that of Lake Nakaumi is about half that of the marine water. In Lakes Shinji and Nakaumi, a halocline is formed, as in many other brackish water lakes. In particular, Lake Nakaumi has clear haloclines, maintaining its salinity at 10–20‰ in the upper layer and about 30‰ in the lower layer. In Lake Nakaumi, eutrophication is proceeding, with water deoxygenation in the lake bottom happening during the high-water-temperature period. The deoxygenated water has an impact not only on benthic organisms but also on planktonic protists, and in the deoxygenated water areas, the number of protistan species and the density of inhabitants substantially decrease. On the other hand, Lake Shinji has a salinity gradient in the range of 1–10‰, including the critical range of 5–8‰ that was shown to maximize the density of planktonic protists (Fig. 14.4).

The number of protistan species was 256, according to the survey conducted in Lake Shinji from 2003 to 2004 (Ishida and Ishibashi 2006). While *Gymnodinium paradoxum* and *Strobilidium gyrans* were observed at a wide range of water temperature and salinity conditions throughout the year, some species were observed only in the high water-temperature and low salinity-gradient areas, including a ciliate, *Tintinnopsis nitida*. Looking at the number of species as a whole, although the influences of the environment were presumed as evidenced by seasonality in the emergence of the species, their causes were often not clearly defined. Although this survey also measured environmental factors, pH, DO, COD, phosphorus, and nitrogen, in addition to water temperature and salinity, only a few factors showed clear relevance to the occurrence of the species, and determining factors were not clear for most of the species. Moreover, if we take the year 2008 as an example (Fig. 14.5), the water temperature was lowest in February and highest in August. The salinity was lowest in April and highest in January. On the other hand, the species richness was lowest in February and highest in October. While we have examined multiple years including 2008 for the relationship between environmental factors and the number of detected species, they fluctuate widely every year, and we have not found environmental factors other than salinity and water temperature to account for the species diversity. Some species of marine protozoa were observed in the fresh water. The opposite situation was also found, in which a protist species generally considered a freshwater dweller was observed in the brackish water. Therefore, it is necessary in future research to carefully consider how this kind of situation might affect the determination of habitation area and species composition.

A few environmental factors, which clearly determine what the species composition of protists will be in a particular brackish water area, have been identified. Similarly, environmental factors, which determine the species composition of protists inhabiting Lakes Shinji and Nakaumi, are not yet clear, nor is it clear whether the diversity of species is high in the horohalinicum concentration range. Continuing research is needed to find factors that can explain the composition of species in the brackish water area.

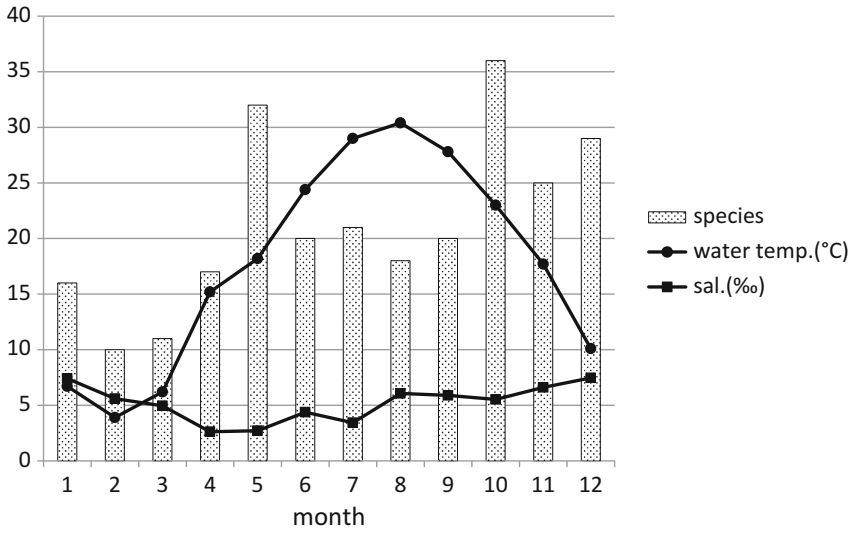

Fig. 14.5 Relationship between the number of ciliate species and environmental factors in Lake Shinji. The water temperature was lowest in February and highest in August. The salinity was lowest in April and highest in January. On the other hand, the species richness was lowest in February and highest in October. At the present time, there is little relationship between species diversity and environmental factors other than salinity and water temperature (Data from Ishida and Ishibashi 2006)

14.6 Conclusions

Due to their body size, protists spread to wide areas; therefore, speciation is not likely to be common in nature. On the other hand, it appears that the environment, which determines the distribution of protists in a brackish water area, changes with great complexity, while mechanisms are at work for diversified species to co-exist as well. This suggests that artificial changes in environments have serious impacts on the diversity of protists in brackish water areas. For protists, it is generally regarded that at least 30 % of species are not yet recorded, which requires our urgent attention to preserve the records and environment of these organisms.

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Chapter 15
Oldest Fossil Records of Marine Protists and the Geologic History Toward the Establishment of the Modern-Type Marine Protist World

Noritoshi Suzuki and Masahiro Oba

Abstract The oldest marine protist fossil goes back 1.8 Ga (Statherian, Paleoproterozoic), and the oldest dinosterane biomarkers 1.6 Ga (Calymmian, Mesoproterozoic). The probable heterotrophic agglutinated microfossil appeared when marine metazoans appeared in the Ediacaran. Multichambered foraminifers appeared around the start of biomineralization in Small Shelly Fossils in the early Cambrian. The first fossilizable radiolarian polycystine is likely to have appeared in the period of the Cambrian Explosion. After the initial appearance period, the emergence of fossilizable skeleton formative ability was concentrated in five short geological time intervals: (1) the Middle to Late Devonian for calcareous benthic foraminifers; (2) the Carnian to the Rhaetian (Triassic) for the "switching on" of fossilizable dinoflagellate cysts, nannoliths, coccoliths and calcareous cysts, and probably the molecular appearance of diatoms; (3) the Toarcian–Aalenian Ages for diversified dinoflagellates and coccolithophores, the establishment of symbiosis in radiolarian Acantharia and the appearance of planktic lifestyle in foraminifers; (4) the Albian–Maastrichtian Ages for the rapid accumulation of coccolithophores, the start of skeletogenesis both in silicoflagellates and marine centric diatoms, molecular appearance of both araphid and raphid diatoms, and the appearance of fossilizable araphid diatoms; and (5) the middle to late Eocene for the appearance of fossilizable raphid diatoms and radiolarian colonial collodarians and the continuous occurrences of ebridians. The establishment of the modern-type marine protist world was concluded in the late Eocene by the appearance of collodarians, the continuous occurrences of ebridians, and the substituted silicon precipitation marine protists as diatoms.

N. Suzuki (✉)
Institute of Geology and Paleontology, Graduate School of Science,
Tohoku University, Sendai 980-8578, Miyagi, Japan
e-mail: norinori@m.tohoku.ac.jp; suzuki.noritoshi@nifty.com

M. Oba
The Center for Academic Resources and Archives, Tohoku University Museum,
Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan
e-mail: vin3594@yahoo.co.jp

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Keywords Acritarch • Coccolithophores • Diatom • Dinoflagellate • Foraminifera • Molecular fossil • Oldest fossil records • Phaeodaria • Radiolaria • Silicoflagellate

15.1 Introduction

The origin of life on the Earth is the major issue for human beings, and so many efforts have long been made to unlock this question. Although the oldest record of life has still been in dispute from the point of view of identified reasons (Brasier et al. 2002; Porter 2006; Schopf 2006; De Gregorio et al. 2009; Pang et al. 2013), the most reliable evidence of the oldest life on the Earth is known as biogenic graphite from the 3.7-Ga metasedimentary rocks of the Isua Supracrustal Belt, Western Greenland (Ohtomo et al. 2014). If one fully believes the authors' intension, the oldest observable microfossil is string-like carbonaceous matter from the 3.5-Ga Apex Basalt in Western Australia (De Gregorio et al. 2009) or thread-like filaments from the 3.4-Ga Strelley Pool Formation in the Pilbara Craton, Western Australia (Sugitani et al. 2013). The oldest marine protist microfossil is an acritarch, which is recognized by organic-walled vesicular microfossils from the shales and silty shales of the 1.8-Ga Changzhougou Formation, the lowermost Changcheng Group in the Pangjiapu Region of North China (Lamb et al. 2009). This age of the oldest marine protist microfossil (1.8 Ga) is apparently 400 Myr younger than that of the oldest record on land (2.2 Ga). *Diskagma buttonii* Retallack, an urn-shaped fossil, occurred from the paleosol of the 2.2-Ga Hekpoort Formation near Waterval Onder, South Africa (Retallack et al. 2013). These may be interpreted as incomplete records in marine fossils or the precedent appearance of soil organisms.

Evidence of marine organisms is not only reliant on observable fossils but also "invisible" fossils. For example, a biomarker defined as organic geochemical remains, which are derived or are altered from organisms, is a powerful tool to detect organisms without observable fossils. The records of the oldest eukaryotic biomarkers used to be considered as steranes from the 2.7-Ga Fortescue Group in Australia (Brocks et al. 1999) and dinosterane indicative of dinoflagellates from the 2.78- to 2.45-Ga Mount Bruce Supergroup, Australia (Brocks et al. 2003), but these are now doubted as contaminations (Porter 2006; Rasmussen et al. 2008). At this time, the oldest steranes are known from the 2.46-Ga Transvaal Supergroup (Waldbauer et al. 2009). According to Javaux (2011), the oldest biomarker record of marine protists is dinosterane at ~1.6 Ga.

The topic of the early eukaryote records, including marine protists, is repeatedly reviewed and often updated (Porter 2004, 2006; Cavalier-Smith 2006; Huntley et al. 2006; Schopf 2006; Javaux 2007, 2011; Blank 2013; Knoll 2014; Rochette et al. 2014).

15.2 Geologic Record Types of Marine Protists

The geologic records of marine protists have been studied in micropaleontology and "biomarker" fields. These two fields contribute to not only the paleontological aspects but also paleobiology, paleoceanography, paleogeography and paleoclimatology. Well-known marine protist microfossils are vegetative and resting cells of diatoms and resting spores, dinoflagellate cysts, ebridians, foraminifers, calcareous nannofossils, polycystine radiolarians and silicoflagellates (Table 15.1). Test-bearing tintinnids and phaeodarians are rarely found in sedimentary rocks. Acritarchs are commonly found between the Mesoproterozoic and the middle Paleozoic as well. In consideration with the relative completeness of fossil records at higher taxonomic criteria, marine protist micropaleontology largely concerns the geologic history of Dictyophiceae, Diatomaea, Retaria, and Prymnesiophyceae of Adl et al. (2012). Dinophyceae and Thecofilosea are also traceable to an extent in fossil records.

One may strongly doubt the completeness of marine protist microfossil records due to dissolution and deformation in the process of lithification. This problem has long been carefully examined by many micropaleontologists for two centuries in order to know the true diversity. Exceptionally preserved diversified microfossils relevant to those from sea-floor surface sediments at the present are occasionally recovered from sedimentary rocks, named as Lagerstätte. Famous Ediacara and Burges Shale faunas are of Lagerstätte. Nodules or concretions composed of phosphorous, calcareous or manganese carbonate matter are Lagerstätte for siliceous and organic microfossils. Fossiliferous nodules are present as far back as the middle Cambrian (e.g., Won and Below 1999). Amber is also known as Lagerstätte, which confines arthropods and plant fragments on land and preserves marine protists in some exceptional cases (Girard et al. 2008). Although the oldest amber is found from the Upper Carboniferous (Pennsylvanian) Tradewater Formation, Illinois (Bray and Anderson 2009), amber becomes common since the Late Cretaceous in relation to the evolutionary history of land plants. It is noted that almost all the occurrence reports of non-marine protists in amber have been denied as "protist-like inclusions" (Schmidt et al. 2010; Girard et al. 2011), and then one should carefully evaluate if marine protists without tests were found in amber.

15.3 Marine Protists as a Major Component of Pelagic Sediments

Voluminous skeletal remains of diatoms, calcareous nannofossils, planktic foraminifers and radiolarians are widely and excellently preserved on/in the wide area of the ocean floor (Fig. 15.1). This sediment is said to be biogenetic sediment, and is further classified into foraminiferal ooze (or globigerina ooze), nannofossil ooze (or nanno ooze), diatom ooze, and radiolarian ooze based on the major components of

Table 15.1 The taxonomic group known as fossils

Taxonomic rank based on Adl et al. (2012)						
Supergroup	1st rank	2nd rank	3rd rank	4th rank	5th rank	General fossil names
SAR	Stramenopiles	Dictyophiceae	Dictyochales			Silicoflagellates
			Pedinellales			
		Diatomaea	Coscinodiscophytina			Diatoms
			Bacillariphytina			
		Dictyophiceae	Dinophyceae			Dinoflagellate cysts
		Ciliophora	Intramacronucleata	Spirotrichea	Choreotrichia	Tintinnids
	Rhizaria	Cercozoa	Thecofilosea	Phaeodaria		Phaeodarians (used to be radiolarians)
				Ebriacea		Ebridians
		Retaria		Tubothalamea		Benthic foraminifers
				Globothalamea		Planktic foraminifers
			Polycystina			Polycystines (or radiolarians)
Incertae sedis	Haptophyta	Prymnesiophyceae	Isochrysidales			Nannofossils, coccolithophores
			Coccolithales			

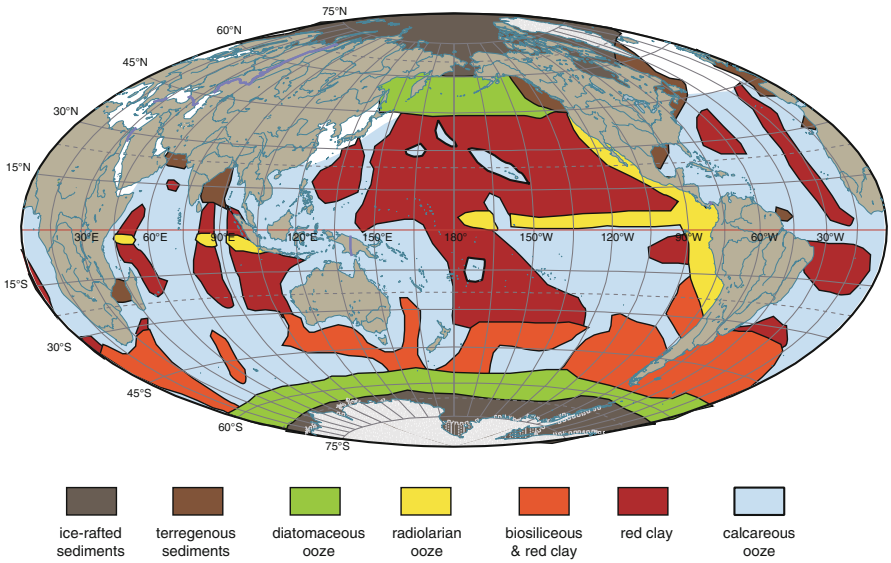


Fig. 15.1 Simplified distribution maps of ocean-floor sediments. (Compiled from Ocean University Course Team 1989, Kennett 1996, Wheeler and Stadnitskaia 2011). Note that the major component of ocean-floor sediment is skeletal remains of diatoms, nannofossils, planktic foraminifers and radiolarian polycystines

skeletal remains. The term "ooze" is defined as pelagic sediment consisting of at least 30 % skeletal remains of pelagic organisms, the rest being clay minerals (Neuendorf et al. 2012). The terms "siliceous ooze" (or "biosiliceous ooze") and "calcareous ooze" are also used for sediments containing at least 30 % siliceous skeletal remains for the former or 30 % calcareous skeletal remains for the latter. The composition of sediments is decided by the relative occupied dimension of each component in thinly pasted sediments on a slide glass. The wide area of the ocean floor below the pelagic ocean is covered with such biogenetic sediments 50–250 m thick (Fig. 15.2). In several high-productive regions like the high-latitudinal Southern Ocean and upwelling regions along the equatorial East Pacific, biogenetic sediments reach 1,000 m in thickness. This suggests a major record of marine protists in the past oceans, despite the small contributions of planktic foraminifers and radiolarians to the biomass in the living planktic organism community. The sedimentation rates of pelagic sediments have been estimated in 1,000 locations worldwide by international ocean drilling programs since the 1960s (the Deep Sea Drilling Project 1968–1984; Ocean Drilling Program 1985–2002; Integrated Ocean Drilling Program 2003–2013; International Ocean Discovery Program 2014–onwards). For instance, average sedimentation rates in the North Atlantic are estimated at 3–8 cm/Kyr in the early Pliocene and 1–2 cm/Kyr in the latest Pliocene (Expedition Scientists 2005), and those in the equatorial East Pacific are ~1.5 cm/Kyr in the late Miocene–Holocene (Lyle et al. 2009).

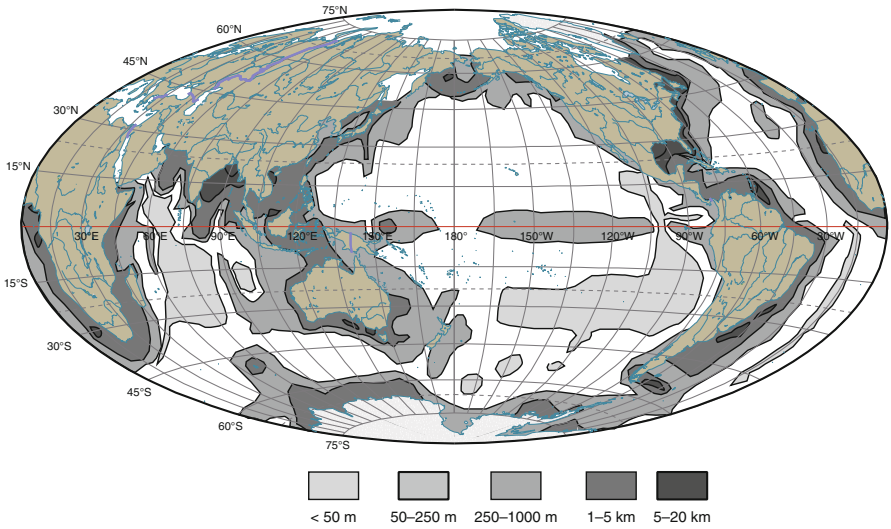


Fig. 15.2 Simplified map showing the total thickness of sediments on the ocean floor (Compiled from Yamamoto 1987, Kennett 1996, Wittaker et al. 2013)

Biogenetic ooze changes into biogenetic rock by diagenesis, which implies all the chemical, physical, and biologic changes undergone by sediment after its initial deposition, and during and after its lithification (Neuendorf et al. 2012). Calcareous ooze changes into carbonaceous rocks such as chalk and limestone, and siliceous ooze changes into chert, diatomite, radiolarite, and then these sediments with rich marine organic fossils alter into a source of oil and gas, in black shales. These rocks occupy substantial volumes of land. Although the numerical volume of these rocks composed with marine protist skeletal remains have not been estimated, it is enough for us to identify voluminous rocks sourced from marine protist rocks. The chalk on the White Cliffs of Dover between Great Britain and France is composed with almost pure Cretaceous coccolith remains (calcareous nannofossil remains). Neogene diatomite is widely distributed in Monterey to the Gulf of California as well as the Japan Sea coast of the Japanese Islands. Mesozoic cherts are commonly found in the accretionary prism that is a set of tear sediments and oceanic plates by subducting plate in the trench as a part of plate tectonic processes. Well-known accretionary prisms with chert and radiolarite are distributed in the Alps, the Alaska Range to the Coast Mountains in North America, Cordillera de la Costa in Chile, the south Great Dividing Range in east Australia, the Japanese Islands, and New Zealand. Rare but important lower to middle Paleozoic cherts are known in Scotland and the east coast between New York and Philadelphia in North America.

15.4 The Oldest Fossil Records of Marine Protists

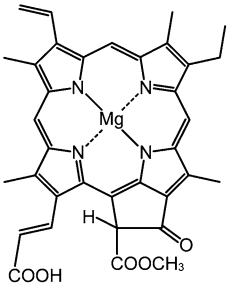
The fossil records of calcareous nannofossils, diatoms and dinoflagellates play an important role for reconstruction of primary productivity in the geological age. On the other hand, dominant heterotrophic protists such as ciliates are nearly completely missing in fossil records. Instead, highly fruitful fossil records are known for radiolarian polycystines and foraminifers through the Phanerozoic. The fossil records of calcareous nannofossils become abundant since the Cretaceous whereas those of diatoms became dominant from the upper Eocene to the present. Dinoflagellate cysts are also well preserved in Mesozoic and Cenozoic organic marine rocks. As micropaleontologists and molecular phylogeny researchers agree, the estimated age of the origin of these marine protists is significantly greater than the oldest age of observable fossil records. This may be due to skeletonless ancestors, a small fossilized opportunity due to a small biomass in the early time, drastic change in morphology, or mixture of these multiple reasons. This question could be answered with the existence of Lagerstätte to an extent. The oldest records of polycystines, diatoms, silicoflagellates and ebridians are probably related to the acquisition age of skeletogenesis ability because siliceous microfossils continuously occur from as old as the Neoproterozoic normal sedimentary rocks and Lagerstätte. On the other hand, the oldest records of calcareous nannofossils and foraminifers seem to be controlled by both the preservation effect and the small biomass in the early time. For instance, the oldest record of calcareous nannofossils occur from the Upper Triassic but the fossil records between the Upper Triassic and the Upper Jurassic are very sporadic, compared with those from the Lower Cretaceous and the upper. As the geologic history of marine microfossil records has been thoroughly documented by Hüneke and Henrich (2011), this chapter focuses on the essential points of marine microfossils for marine biologists.

15.5 Biomarker Evidence About Invisible Marine Protists

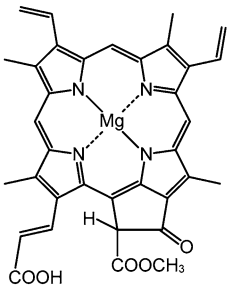
A biomarker is a powerful tool to reveal the geologic effects of invisible organisms. In particular, it is practically the only evidence to detect picoplankton and bacteria from sedimentary rock. In this section, the general knowledge of biomarkers is briefly summarized. As is generally accepted, the biomass of picoplankton and bacteria significantly contributes to the biochemical cycle of pelagic oceans. Limited groups of non-fossilized marine protists can be detected by biomarkers. The biomarkers specific to the whole of marine protists are not known yet. Like bacteria and archaea, cell membranes of marine protists naturally consist of lipids. Most membrane lipids of eukaryota and bacteria are acyl ester lipids. Archaea and some bacteria (such as sulfate-reducing bacteria) biosynthesize ether lipids as a membrane lipid. In particular, the ether lipids of archaea are definitely distinguished from the membrane lipids of eukaryota and bacteria because of the unique *sn*-2,3 rather than

sn-1,2 stereochemistry of the glycerol moieties (Koga et al. 1998a, b) and because archaea biosynthesize ether lipids with isoprenoidal carbon skeletons rather than ester-linked alkyl lipids (de Rosa and Gambacorta 1986). Archaeol is the most common and ubiquitous compound among the archaeal ether lipids (for a review, see Koga et al. 1998a, b). Furthermore, hydroxyarchaeol has the same core structure as archaeol but contains an additional hydroxyl group on the third carbon of the phytanyl moiety. Since this unique ether lipid has been observed almost exclusively in cultures of methanogenic archaea (Sprott et al. 1990; Koga et al. 1998a, b) or in consortia of methanotrophic archaea and sulfate-reducing bacteria performing anaerobic oxidation of methane (Blumenberg et al. 2004), hydroxyarchaeol is a good biomarker for methanogenic or methanotrophic archaea. But the major constituents of the membrane lipids of marine protists are ester lipids, which are common and ubiquitous lipids among eukaryota and bacteria, although some groups in marine protists have each diagnostic biomarkers as stated in Sects. 15.6.4 and 15.6.5.

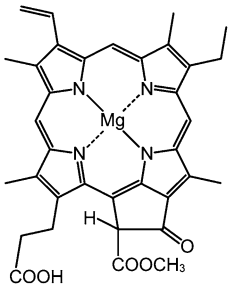
Chlorophylls *a*, *b*, *c* and *f* leave part of their molecules as biomarkers, although the source of them is unable to be clarified only from the biomarkers. Needless to say, chlorophylls (Chl) play an essential role in converting the energy of light to chemical energy stored in the products of photosynthesis. These compounds are classified into Chl *a*, *b*, *c*, *d* and *f* in the order of discovery. Chl *a* is the most common and ubiquitous pigment of almost oxygenic phototrophs. Higher plants, some green algae (such as euglenids and chlorarachniophytes) and Type II cyanobacteria (prochlorophytes) contain Chl *b*. Chl *d* has been identified as the major pigment in the cyanobacterium *Acaryochloris marina* (Miyashita et al. 1996). Recently, Chl *f* was isolated from red-light enrichment cultures obtained from stromatolite (Chen et al. 2010). It was revealed that this new Chl derives from filamentous cyanobacteria (Chen et al. 2012). Chl *c* is mainly derived from phototrophic protists, which are referred to collectively as "chromophytes" (Bacillariophyceae, Chrysophyceae, Phaeophyceae, Raphidophyceae, Xanthophyceae, Cryptophyta, Dinophyta and Haptophyta) (Fig. 15.3). Furthermore, the Chl *c* pigment family consists of 11 compounds, Chl c_1, Chl c_2, Chl c_3, Chl $c_{CS\text{-}170}$, Chl c_2-like pigment, [3,8-divinyl] protochlorophyllide, a nonpolar Chl *c*-like pigment, [3-vinyl-8-ethyl] Chl c_3, Chl c_1-like pigment, Chl c_2-MGDG (18:4/14:0) and Chl c_2-MGDG (14:0/14:0). Miyashita et al. (1997) reported that *Acaryochloris marina* (cyanobacterium) also contains [3,8-divinyl] protochlorophyllide. The distribution of Chl *c* pigments in chromophytes and cyanobacteria have been compiled by Zapata et al. (2006). As with other Chls, Chl *c* pigments change by diagenesis in sediments. The early diagenetic pathways and products of Chl *c* remain unknown. As molecular fossils derived from Chl *c* pigments, 17-nor-DPEP and porphyrins with an isocyclic five-membered ring attached to carbons 15 and 17 (MeCPP and CarboxylMeCPP) are known. 17-nor-DPEP was detected from Moroccan oil shales from the Maastrichtian Age (71–66 Ma) (Verne-Mismer et al. 1988). Me CPP and Carboxyl MeCPP were isolated from Messel oil shale (Eocene, 56–34 Ma) (Ocampo et al. 1992).



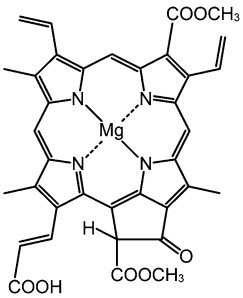
Chlorophyll c_1



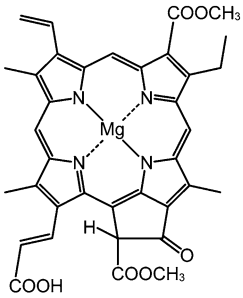
Chlorophyll c_2



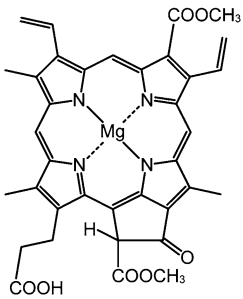
Divinyl protochlorophyllide



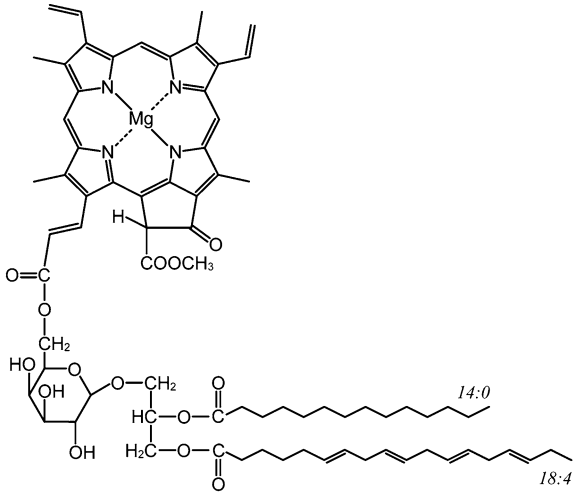
Chlorophyll c_3



Monovinyl chlorophyll c_3



Chlorophyll $c_{3\ (CS\text{-}170)}$



Chlorophyll c_2 monogalactosyldiacylglycerol (18:4/14:0)

Fig. 15.3 Chemical structure of chlorophyll *c*

15.6 The Oldest Fossil Records

15.6.1 Acritarchs and Dinoflagellates

Acritarchs are an artificial group of all indefinite organic microfossils. Some acritarchs are considered as dinoflagellate cysts, embryos of unknown origin, and/or extinct marine protists. The oldest reliable fossil record of acritarchs goes back to the 1.8-Ga Changhougou Formation, North China (Lamb et al. 2009). Although the origin of these acritarch fossils is unable to be identified and some doubts remain (Javaux 2011), this age is roughly concordant with the oldest records of dinosterane at ~1.6 Ga. Despite the absence of direct fossil evidence, dinoflagellate cysts are a major origin of pre-Triassic acritarchs because the abundance changes in the dinosteroid biomarker are concordant with those in acritarchs (Moldowan et al. 1996). In the early Cambrian case, dominant dinosteranes and other steranes commonly associated with dinoflagellates were detected with dominant acritarchs named *Globosphaeridium*, *Skiagia*, *Comasphaeridium* and *Lophosphaeridium*, which suggests a close relationship between these acritarchs and dinoflagellates (Talyzina et al. 2000).

Dinoflagellates are typical organic microfossils. These cysts have an organic, calcareous, or rarely siliceous wall, and dinoflagellate cysts with organic walls can be fossilized. Fossils of dinoflagellate cysts are abundantly preserved in organic shale, organic mudstone and source rocks of oil and gas. The dinoflagellate fossils at the motile stage are very rarely found in amber, as Lagerstätte (Masure et al. 2013). The dinosterane records imply the presence of dinoflagellate and/or other marine protists that can produce dinosterane. However, "true" dinoflagellate cysts can be determined only by the presence of both cellulosic plates arranged in particular patterns (tabulation) and excystment apertures (archeopyles) (Fensome et al. 1999), and the oldest "true" dinoflagellate cysts are found from the Carnian Stage (Middle Triassic) North West Shelf off Western Australia (Nicoll and Foster 1999). The oldest fossil record of dinoflagellate cysts used to be considered as the upper Silurian *Arpylorus antiquus* in southern Tunisia (Calandra 1964), but re-examination of complete specimens of this species in consideration with the morphological artifact effect and chemical analysis concluded that the fragments were eurypterid or arthropod in origin (Le Herisse et al. 2012).

The oldest record of observable fossils is apparently younger than that of dinosterane. This may be interpreted by the fact that characteristic tabulation and/or archeopyles first appeared in dinoflagellate lineages since the Middle Triassic (Le Herisse et al. 2012) or the "switching on" of preservable cyst-forming capability (Medlin and Fensome 2013). After the Middle Triassic, fossil records of dinoflagellate cysts become rich and continued (MacRae et al. 1996; Fensome et al. 1999).

15.6.2 Foraminifers

Foraminifera are unicellular protists with calcareous test forms in a large number of their members. Almost all the foraminiferal species live as benthos in marine conditions, but planktic foraminiferal species are widely distributed in abundance in the open oceans. Planktic foraminifers are rarely but surely observed in the southern oceans and the Arctic. In brackish environments, only benthic foraminifers live. Foraminifers exist in both soil and freshwater based on molecular data (Holzmann et al. 2003; Lejzerowicz et al. 2010), but such foraminifers are likely quite few and lack any hard test. The oldest foraminifers in molecular phylogenetic trees possess organic walls, and they (e.g., *Conicotheca nigrans*, *Nodellum aculeata*, *Resigella laevis*, *Resigella bilocularis*) have been discovered in the Challenger Deep, the deepest trench of the world's oceans (Gooday et al. 2008).

The evolutionary history of Foraminifera and its taxonomy have been well studied with both rich fossil records and molecular phylogenetic analysis (Loeblich and Tappan 1988; Kaminski et al. 2008; Vachard et al. 2010; Mikhalevich 2013; Pawlowski et al. 2013; Kaminski 2014). Morphological suprageneric classification system of Foraminifera is mainly based on the texture and wall composition such as calcareous and agglutinated walls (Loeblich and Tappan 1988). On the other hand, molecular studies revealed that single-chambered foraminifers are grouped into paraphyletic Monothalamana while multichambered foraminifers are divided into two classes, namely Globothalamana with globular chambers and Tubothalamea with typically tubular chambers (Pawlowski et al. 2013; Kaminski 2014). Differing from the morphological taxonomic system, molecular phylogenetic analysis interprets that calcareous walls and agglutinated walls are decisively evolved from non-consanguineous lineages in Monothalamana, Tubothalamea and Globothalamana, respectively. This strongly raises an ineludible requirement to revise the traditional evolutionary hypothesis of Loeblich and Tappan (1988) that Foraminifera evolved from organic-walled through agglutinated and further to calcareous lineages. Thus, the appearance age of single-, tubular- and globular-chambered foraminifers in fossil records are important to compare molecular phylogenetic trees with fossil records.

The oldest skeleton-bearing foraminifers seem to be a single-chambered form (Kaminski et al. 2008), but differentiation between testae amoebae and foraminifers is still in dispute. The oldest discovery of test-bearing foraminifers, regardless of a putative or reliable one, is single-chambered, tubular or vase-shaped agglutinated microfossils from the Cryogenian Rashof Formation (717–662 Ma), northern Namibia (Dalton et al. 2013), or those identified as *Titanotheka coimbrae* from the Vendian Yerbal Formation (635–541 Ma) of the Arroyo del Soldado Group in Uruguay (Gaucher and Sprechmann 1999). The tubular fossil, the genus *Platysolenites* (Hippocrepinacoidea) from the Fortunian formations (the lower Cambrian; 541–529 Ma) in Avalonia and Baltica (McIlroy et al. 2001), was regarded as the oldest fossil with a proloculus, a spherical initial chamber in foraminifers. This, however, remains very controversial because it can be interpreted as tubes of polychate worms (Finger et al. 2008; Vachard et al. 2010).

The undoubted benthic foraminifer with a planispiral tubular chamber is found from the Foungon Member of the Nandoumari Formation, southeastern Senegal (Culver 1991). The depositional age of the Foungon Member is presumed in the late Terreneuvian (525–521 Ma in the Cambrian) by occurrence of gastropod *Aldanella attleborensis* specimens (Shields et al. 2007; Dzki and Mazurek 2013). The foraminifers reported by Culver (1991) are of particular importance because these fossils are the oldest fossils belonging to Tubothalamea. The oldest multichambered foraminifers (trochospiral, planispiral and planispiral/uniserial revolution) probably belonging to Globothalamana were recovered from the Tancock Member of the Goldenville Group in Nova Scotia, Canada, assigned to the early Cambrian (548–510 Ma) (Scott et al. 2003; White 2009).

The oldest records of calcareous foraminifers have been attributed to the Ordovician Period (Loeblich and Tappan 1964; Schallreuter 1983; Sabirov and Gushchin 2006), but this has not been accepted up to now (Loeblich and Tappan 1988; Vachard et al. 2010; Nestell et al. 2011). According to these references, these "Ordovician calcareous foraminifers" are now considered as Devonian foraminifer fossils or are derived from polycystine radiolarians replaced by calcareous minerals. The most reliable oldest record of calcareous foraminifers dates to the Eifelian (393–388 Ma: Middle Devonian) when *Semitextularia* and *Paratikhinella* appeared (Mouravieff and Bultynck 1967; Vachard et al. 2010). As these two genera belonging to the superfamily Semitextularioidea (Order Earlandiida, Subclass Afusulinana, Class Fusulinata) and Fusulinata are an extinct foraminiferal group, the oldest record of calcareous foraminifers may not be recorded in the molecular phylogeny.

Planktic foraminifers are morphotaxonomically divided into two groups, Globigerinina and Heterhelicina (Boudagher-Fadel 2013), although their taxonomic rank differs in the literature (Order level in Boudagher-Fadel 2013, Suborders in Simmons et al. 1997, lower than Order in Pawlowski et al. 2013). The oldest records of planktic Globigerinina are *Conoglobigerina avariformis*, *C. balakhmatovae*, *C. dagestanica* and possibly *C. avarcia* (Superfamily Favusellaceoidea, Family Conoglobigerinidae) from the upper Bajocian (169–168 Ma: Middle Jurassic) (Simmons et al. 1997; Boudagher-Fadel 2013). Although their most probable ancestors are considered as benthic foraminiferal genera *Praegubkinella* or *Oberhauserella* (e.g., Clémence and Hart 2013), a missing link in the fossil records is present between the last occurrence of these two benthic foraminifers in the Toarcian (182–174 Ma) and the first occurrence of planktic *Conoglobigerina* in the upper Bajocian (169–168 Ma) (Simmons et al. 1997). Hart et al. (2003) suggest that planktic foraminifers evolved from a partially planktic (meroplanktic) form around the Toarcian Anoxic Events (182–174 Ma). Pawlowski et al. (2013) commented that Globigerinina have nothing to be considered as polyphyletic groups. Since Globigerinina have experienced overall faunal turnover twice in association with serious extinction events across the Cretaceous–Paleogene and Oligocene–Miocene boundaries (Vincent and Berger 1981; Caron and Homewood 1983), it is not necessary for all planktic foraminifers to be phylogenetically connected with *Conoglobigerina*.

Heterhelicina are so biserial to multiserial in shape that they look like benthic foraminifera. The oldest fossil data of Heterhelicina goes back to the late Albian (Boudagher-Fadel 2013).

Any biomarker specific to foraminifera is not known yet. In general, heterotrophs such as foraminifera have no diagnostic biomarker.

15.6.3 Radiolarian Polycystines

Radiolaria are divided into Polycystina and Spasmaria based on molecular phylogenetic studies (Sierra et al. 2013). Polycystina are a radiolarian group with an opal skeleton, and their extant groups are classified into Orders Collodaria, Nassellaria and Spumellaria (Suzuki and Aita 2011). Spasmaria comprise Acantharia and Taxopodia, and this group has never been found as fossils due to their easily dissolved skeletons. In addition to these extant radiolarian orders, three extinct orders (Archaeospicularia, Albaillellaria, Latentifistularia) in Polycystina are mainly found from the Paleozoic strata. The remaining order, Entactinaria, is one of the major polycystine group in the Paleozoic. Entactinaria have presumably survived in the present ocean (De Wever et al. 2001), but no clade relevant to Entactinaria has been depicted in molecular phylogenetic trees (Pernice et al. 2013). Polycystina are exclusively marine holoplanktic protists throughout the Phanerozoic. The oldest fossil records of polycystines are important to know the early evolution of Radiolaria in relation to Foraminifera because their molecular phylogenetic relationships remain controversial. Nikolaev et al. (2004) illustrated a sister group of Foraminifera and Radiolaria in Rhizaria, and Pawlowski and Burki (2009) separated Foraminifera from a sister group of Radiolaria. The sister relationships between Foraminifera and Radiolaria are now widely accepted by studies with cDNA libraries (Sierra et al. 2013) and polyubiquitin insertion (Ishitani et al. 2011), but the position of Foraminifera has been unstable. For instance, the Foraminifera clade comprises a sister group with Polycystina and Spasmaria (a group of Acantharia and Taxopodia) in a combined 18S and 28S rDNA alignment (Krabberød et al. 2011). In this chapter, fossil records of polycystines are only documented.

The oldest reliable fossil record of polycystines is the approximate 515-Ma upper lower Cambrian Shashkunar Formation, Altai Mountains, southern Siberia, Russia (Obut and Iwata 2000; Pouille et al. 2011; Korovnikov et al. 2013). Mineralized skeletons of some polycystines in the Cambrian and Ordovician comprise aggregates of isolated spicules (e.g., Malez 2011) so they are occasionally difficult to distinguish from remains of siliceous sponge spicules. The oldest polycystine is recognized with the presence of a hollow space for protoplasm inside a spherical test. Three species (*Altaisphaera sparsispinosa*, *Alt. acanthophora* and *Atl. muricata*) belonging to the Order Archaeospicularia were recovered from chert. The presence of chert suggests sufficient biogenic productivity at that time. The Order Nassellaria are a major polycystine group in the present ocean, but the timing of their origin is still controversial. Continuous fossil records of Nassellaria date to the earliest Triassic (252 Ma) (Kamata et al. 2007) while sporadic occurrences of nassellarian-like polycystines are known from the Upper Devonian (372–382 Ma) (Cheng 1986; Isakova and Nazarov 1986; Takemura et al. 2009). Thus, caution is needed with regard to interpolating the molecular clock tie-point of Nassellaria. The

fossil record of Collodaria is also sporadic because all the collodarian species except for the family Collosphaeridae lack siliceous skeletons or are simply surrounded with isolated siliceous spicules. The oldest fossil records of Collodaria have been attribute to the Eocene (De Wever et al. 2001), but this is doubtful without published evidence. The Order Spumellaria is the largest polycystine group in both species number and abundance, but it is not yet possible to clearly identify the oldest records because many spumellarians and superficially similar Entactinaria are still undescribed.

As with foraminifera, there are no diagnostic biomarkers for Radiolaria.

15.6.4 Diatoms

The diatom test, known as a frustule, is a disk, flat or pennate in superficial appearance and with a space for protoplasm inside the frustules. The traditional higher classification system for diatoms is a division of Coscinodiscophycea, Bacillariophyceae and Fragilariophycea (Round et al. 1990; Williams and Kociolek 2011). By contrast, the higher classification system on the basis of the combination of molecular phylogenetic analysis, cytological features and reproduction patterns is Coscinodiscophyceae, Mediophyceae, and Bacillariophyceae (Medlin and Kaczmarska 2004; Medlin 2011). The terms "Coscinophycea" and "Coscinodiscophyceae" look very similar but have different taxonomic definitions from each other (Medlin 2011; Williams and Kociolek 2011). As precise unification between these two classification systems is still in progress (e.g., Theriot et al. 2011), morphological groups such as centric, raphid and araphid diatoms are practically used (Williams and Kociolek 2011). Coscinodiscophycea, Bacillariophyceae and Fragilariophycea are referred to "radial centrics," "bi- or multipolar centrics" and "bipolar, pennate diatoms," respectively. Pennate diatoms are classified into raphid and araphid diatoms based on the presence or absence of a slit on the frustule, respectively. Raphid pennate diatoms are considered as a valid monophyletic taxon, Mediophyceae, whereas the remaining diatoms classified into araphid pennate and centric diatoms have different phylogenetic taxa (e.g., Sorhannus 2007). Mediophyceae are often depicted as "bi- (multi-) polar centrics."

The fossil records of diatoms are well summarized in several papers (Sims et al. 2006; Kooistra et al. 2007; Medlin 2011). The widely accepted oldest diatom fossils are resting spores of freshwater diatoms named *Calyptosporium* from the uppermost Jurassic–lowermost Cretaceous Dabokni Formation in Korea (155–130 Ma) (Chang and Park 2008). This age assignation is supported by the large amount of knowledge of the Late Jurassic–Late Cretaceous tectonic history in China, Korea and Japan (e.g., Chang et al. 2003; Suzuki et al. 2007; Sha et al. 2012). The undoubted oldest marine diatoms were recovered from the subsurface clayey radiolarian diatomite deposited in ~500 m water depths nearshore (Gersonde and Harwood 1990; Ling and Lazarus 1990) on the Dronning Maud Land margin, eastern Weddell Sea. These well-preserved flora and fauna comprise diversified diatom resting spores and centric diatoms as well as many radiolarians and they are assignable around the Aptian–

Albian boundary. Lagerstätte that contains extremely well-preserved polycystines with very fragile siliceous skeletons are known from the Jurassic to the Berriasian (early Cretaceous: 145–139.8 Ma) (Ogawa et al. 1996; Matsuoka 1998; Kiessling et al. 1999; Suzuki and Ogane 2004), but no Lagerstätte prior to the Aptian–Albian boundary (113 Ma) has yielded any signatures of marine diatom fossils. This implies that fossilized marine diatoms appeared at some point (140–113 Ma) in the Early Cretaceous. Marine centric diatoms and resting spores prior to the late Albian (115–110 Ma) include colonial planktic genera in shallow coastal waters (Girard et al. 2009) but pelagic planktic marine diatoms have never been reported from the sediments at and prior to this age. The marine diatoms from shallow to distal shelf environments were first recorded from the Santonian–Campanian (86.3–72.1 Ma) Kanguk Formation in the Canadian High Arc (Witkowski et al. 2011a). This flora contains the oldest substantial diversified araphid diatoms (Witkowski et al. 2011a, b). The oldest records of raphid diatoms are documented as Paleogene "*Navicula simbirskiana*," *Lyrella* species ("*Navicula hennedyi*" and "*Navicula praetexta*") from Ulyanovsk (previous geographic name: Simbirsk), Russia, according to Sims et al. (2006). These diatoms, however, seem not to have been found from this region since the first description (e.g., Oreshkina and Aleksandrova 2007). As no raphid diatoms are contained in any other Paleocene deposits with abundant diatoms, the reliable oldest record of raphid diatoms is the lower Lutetian (lowest middle Eocene: ca 46–47 Ma) (e.g., Gladenkov 2012).

Molecular phylogenetic dating for diatoms indicates a considerably older age than the oldest fossil records (Sorhannus 2007). The first diatom lineage in molecular phylogeny appeared between 183 and 250 Ma (the mean age is 217 Ma), though the oldest fossil records are from the approximate Aptian–Albian boundary (113 Ma). Raphid and araphid diatoms evolved from bi- (or multi-) polar centric diatoms around 96.5 Ma and from araphid diatoms around 93.8 Ma, regardless of the Santonian–Campanian (86.3–72.1 Ma) for the oldest araphid diatom fossil records and the lower Lutetian (46–47 Ma) for the oldest raphid diatom fossil records, respectively. The time gap between the oldest fossil records and molecular dating in given diatom phylogeny is called a "ghost range" (Sorhannus 2007). The "diatom" older than the oldest diatom fossil records is named as a "pre-diatom" or "Ur-diatom," which probably developed scales and lived in shallow marine environments (Sims et al. 2006).

The origin of marine diatoms has also still been under discussion (Sims et al. 2006; Medlin 2011), even though fossil and molecular data are referred to. In addition, the evolutionary history of marine diatoms is very complex because marine diatoms multiply invaded freshwater environments and *vice versa* (Kaczmarsk et al. 2005; Sims et al. 2006; Alverson et al. 2007). Once marine diatoms appeared in saline water conditions, their distribution was still limited in the nearshore environment until the Late Cretaceous, albeit that there was benthic or planktic life. Their distribution extended toward the pelagic ocean after the Santonian–Campanian at the latest. Marine diatoms become dominant in the Late Cretaceous, in the uppermost Paleocene, and then continuously occurred and diversified in higher latitudes since the late Eocene (35 Ma). Instead of dominated diatoms in the late Eocene, polycystines, which also precipitate rich dissolved silica in seawater, reduced in test size and the weight of the siliceous skeleton (e.g., Lazarus et al. 2009).

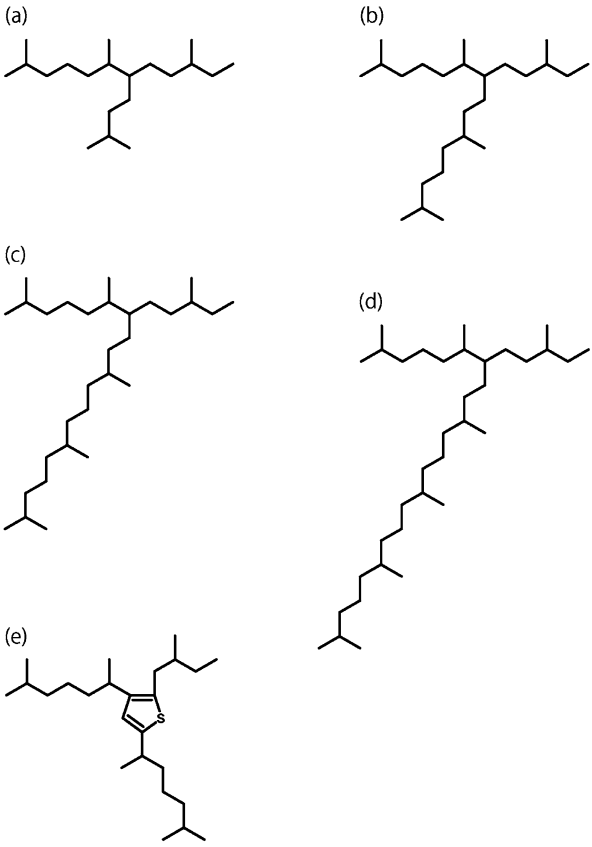


Fig. 15.4 Structures of the highly branched isoprenoid (HBI) and those derivative: (**a**) C_{20} HBI skeleton, (**b**) C_{25} HBI skeleton, (**c**) C_{30} HBI skeleton, (**d**) C_{35} HBI skeleton, and (**e**) a skeleton of C_{25} HBI thiophene. C_{20}, C_{25}, C_{30} and C_{35} HBI alkenes have one or more double bonds within the carbon chain

Specific biomarkers for diatoms are found not only from living diatoms but also from sedimentary rocks. C_{20}, C_{25}, C_{30}, and C_{35} highly branched isoprenoid (HBI) hydrocarbons and those derivatives are generally accepted to be the specific biomarkers of few diatom taxa (Fig. 15.4). C_{25} HBI alkenes have been detected in cultures of *Haslea* (Volkman et al. 1994; Wraige et al. 1997; Allard et al. 2001), *Navicula* (Belt et al. 2001a), *Pleurosigma* (Belt et al. 2000, 2001b) and various *Rhizosolenia* sp. (Sinninghe Damsté et al. 1999; Rowland et al. 2001). C_{30} HBI alkenes have been found from cultures of various *Rhizosolenia* sp. (Volkman et al. 1994; Rowland et al. 2001). Biological precursors of C_{20} and C_{35} HBI alkenes have not yet been found, but organic geochemists think that those HBIs also derive from diatoms which are not analyzed for their lipid constitution.

HBI alkenes are widely distributed in modern sediments worldwide, especially, C_{25} HBI alkenes are the most commonly reported (Rowland and Robson 1990; Belt et al. 2000). Recently, there has been some interest in the C_{25} mono-unsaturated HBI isomer (IP_{25}) as a proxy for past sea ice presence in the Arctic (Belt et al. 2007). HBI alkenes are hydrogenated to produce HBI alkanes in the sediments during early diagenesis. If sediments are sulfur rich, HBI alkenes are prone to sulfurization during early diagenesis (Kohnen et al. 1990; Werne et al. 2000). C_{20} and C_{25} HBI thio-

phenes were identified in a variety of sulfur-rich oil shales and oils (Sinninghe Damsté et al. 1989). Katsumata and Shimoyama (2001) detected C_{25} HBI thiophenes in Miocene to Pliocene sedimentary rocks in Shinjo Basin, Japan.

To determine the geological timing of the origin of the HBI biosynthetic pathway, Sinninghe Damsté et al. (2004) analyzed the occurrences of C_{25} and C_{30} HBIs in over 400 sediment samples that ranged from 570 to 0.002 Ma. The first occurrence of C_{25} HBIs in sediment is noted in the upper Turonian sediment (~91 Ma) from Guyana Basin (French Guyana) in the Canje Formation. Before this era, no C_{25} HBIs occur, whereas after this first occurrence, C_{25} HBIs have a widespread occurrence in marine sediments. Since the evidence of fossil silica skeletons, C_{30} HBIs were only detected in 6 out of over 400 sediment samples from the last 3 Ma, suggesting that C_{30} HBI biosynthesis is a relatively young adaptation of the HBI biosynthetic pathway (Sinninghe Damsté et al. 2004).

15.6.5 Coccolithophores and Calcareous Nannofossils

Coccolithophores are marine, or rarely brackish, unicellular phytoplankton with haptonema and belong to Class Prymnesiophyceae in Phylum Haptophyta (Adl et al. 2012). Fossil coccolithophores are customarily referred to as "calcareous nannofossils," which is a collective paleontological term for fossil discoasters and coccoliths whose size is smaller than approximate 30 μm in diameter (Bown and Young 1998; Neuendorf et al. 2012). As several similar but confusable terms are used for this group, related similar terminology such as "coccoliths," "coccospheres," "coccolithophores" and "nannoliths" should be clearly clarified. The following explanation is mainly based on Gardin et al. (2012). The term fossil "coccolithophores" are preferred to "calcareous nannofossils" because any fossil remains and fragments of some calcareous organisms can be referred to by this term. A similar term "nannoplankton" is an informal group including coccolithophores and chrysophytes, but excluding the bacterial picoplankton. This term is used for living forms but not for fossil forms. Haptophyta precipitate organic or calcareous scales in some groups and these scales loosely or tightly surround a haptophyte cell to form a sphere. The sphere comprised of calcareous scales is called a coccolithophore, and each of the isolated scales is called a coccolith. "Coccoliths" are a general term applied to various microscopic calcareous structural elements or button-like plates averaging 3 μm (rarely up to 35 μm) in diameter, constructed of minute calcite and aragonite crystals (Neuendorf et al. 2012). Coccolithophores form two types of coccolith, namely heterococcoliths and holococcoliths. The former is made of crystal units of variable sizes and shapes, while the latter is of a single type of minute crystallites (Young et al. 2004). In addition to coccoliths, anomalous calcareous structures lacking the typical features of hetero- or holococcoliths are called nannoliths (Billard and Inouye 2004). This is formed by unknown phylogenetic affinity because of lack of living analogues, although it can be produced by calcareous dinoflagellates (Gardin et al. 2012).

Fossil records of calcareous nannofossils are roughly relevant to haptophytes forming coccoliths or unknown algae forming non-dinoflagellate nannoliths. The reliable oldest records of coccoliths are known just below the Norian–Rhaetian boundary (~205 Ma; the Upper Triassic) and the oldest identifiable coccolithophore species is *Crucirhabdus minutus* in the Rhaetian (205–201.3 Ma). On the other hand, calcispheres probably identified as calcareous dinocysts first appeared in the late Carnian (235–221 Ma) (Preto et al. 2013). Non-dinoflagellate nannoliths (*Priniosphaera triassica* and *Thoracosphaera*? *wombatensis*) and calcareous cysts (*Orthopithonella* sp. and *Obliquipithonella* sp.) are known from the mid- to late Norian (215–205 Ma) (Gardin et al. 2012). The Norian occurrence data for coccolithophore are now regarded as Rhaetian under the significantly improved Triassic timescale (Lucas 2013), and occurrences of coccoliths have not been supported by any evidence so far, according to Gardin et al. (2012).

The evolutionary origin of Haptophyta is inevitably older than the oldest fossil records of coccoliths and nannoliths based on molecular data (Liu et al. 2010). The environmental genetics of Haptophyta with four genes (*SSU*, *LSU*, *tufA,* and *rbcL*) dates the origin of Haptophyta at 824 Ma in the Cryogenian Period of the Neoproterozoic. Genetic calcifying ability evolved in ~310 Ma (329–291 Ma), which corresponds to the middle Moscovian (middle late Carboniferous). The emergence of calcifying ability in haptophytes is interpreted as the transition from mixotrophy to autotrophy according to Liu et al. (2010). A habitat change from coastal/neritic waters to the pelagic realms by the divergence of coccolithophores and Prymnesiales occurred at ~267 Ma (291–243 Ma), between the lower Cisuralian (lower Permian) and the Anisian (early Middle Triassic). This means non-fossilized coccolithophores with calcifying ability had already appeared by the Anisian, and fossilized coccoliths started to develop since the Rhaetian.

Biomarkers specific to haptophytes are well studied as a paleo-temperature index, so the oldest records of these biomarkers are also well documented. A series of long-chain (C_{37}–C_{39}) di-, tri- and tetraunsaturated methyl and ethyl ketones (alkenones: Fig. 15.5) appear to be present only in haptophycean algae such as Gephyrocapsaceae (Volkman et al. 1980; Marlowe et al. 1984). These alkenones were first observed as abundant components of Miocene to Pleistocene sediments from the Walvis Ridge off West Africa (Boon et al. 1978) and of the present sediment from the Black Sea (de Leeuw et al. 1980). *Emiliania huxleyi* and other haptophytes have been found to increase the degree of unsaturation of their alkenones as temperature decreases (Brassell et al. 1986). It is thought that the lowering of the melting points of these compounds enables maintenance of their cellular fluidity and function in cold climates. Hence, many studies have used as a proxy paleo sea surface temperatures from sedimentary alkenone distributions (for a review, see Brassell 1993). Alkenones are well detected in many marine sediments dating back to the mid-Eocene (~45 Ma), which corresponded with the emergence of many genera belonging to the family Gephyrocapsaceae (Marlowe et al. 1990). However, Farrimond et al. (1986) reported the occurrence of alkenones in Cenomanian (~95 Ma) and mid-Albian (~105 Ma) sediments. It may be presumed to reflect contributions from ancestors of the Gephyrocapsaceae.

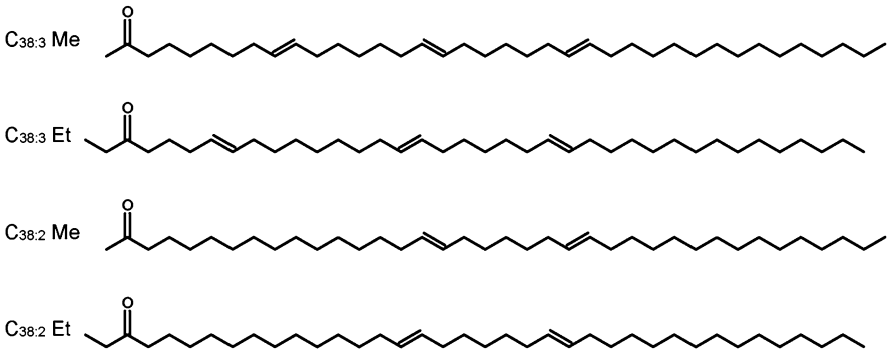


Fig. 15.5 Structures of the C_{38} alkenones. Abbreviations ($C_{38:3}$ Me) refer to carbon number (38), number of double bonds in the carbon chain (3), and whether the alkenone is methyl (Me; alken-2-one) or ethyl (Et; alken-3-one)

15.6.6 Silicoflagellates (Dictyophyceae), Ebridians (Ebriacea), Phaeodaria and Others

Silicoflagellates and ebridians are single-celled marine holoplanktic picoplankton (<20–30 μm in size) which are commonly encountered in high-latitudinal Neogene samples, and have been studied together because they co-occur and their microscopic slides are prepared with the same laboratory procedures. The term "silicoflagellates" has been used as a formal taxonomic Order Silicoflagellata, but this group is now regarded as Order Dictyochales (Dictyochophyceae, Stramenopiles) (Adl et al. 2012). Dictyochophyceae are characterized by a silica skeleton being present during at least one life stage. The silicoflagellate skeleton is marked by a simple geometric framework that is comprised of a basal ring with spiny apical structures. The fossil records of silicoflagellates are well documented mainly from both high-latitudinal regions. Biostratigraphies for the Neogene and Quaternary (Kobayashi 1988; Tsoy 2011), Paleogene (Ling 1981; Perch-Nielsen 1985) and Upper Cretaceous ones (McCartney et al. 2011) have been well established.

Fossil records of silicoflagellates have been well reviewed by McCartney (2013). The oldest silicoflagellate fossils are two *Variramus* species (*V. aculeifera* and *V. loperi*) and *Vallacerta hannai* which have been found from the lower Albian (113–105 Ma) of the Lower Cretaceous, the Dronning Maud Land margin, eastern Weddell Sea (McCartney et al. 1990). This was dated by co-occurring polycystines (Ling and Lazarus 1990). Silicoflagellate fossils abruptly became dominant since the Santonian (86.3–83.6 Ma) in the Arctic regions, and become common in the Campanian (83.6–72.1 Ma) (McCartney et al. 2010; McCartney 2013). Eight silicoflagellate genera (*Arctyocha*, *Corbisema*, *Cornua*, *Lyramula*, *Schulzyocha*, *Umpicoha*, *Vallacerta*, and *Variramus*) are known from the Cretaceous, but only one genus, *Corbisema*, survived through the Cretaceous–Paleogene Mass Extinction Event. Thus, all the Cenozoic silicoflagellates evolved from *Corbisema*. According to Brown and Sorhannus (2010), molecular phylogenetic data suggest that

Dictyophyceae is divergent from Pelagophyceae, and the origin age of silicoflagellates by a molecular genetic timescale is estimated at 397 Ma (95 % probability = 520–279 Ma) or 382 Ma (95 % probability = 497–264 Ma), the Early to Middle Devonian.

Ebridians are a phagotrophic unicellular flagellate in marine coastal waters. Ebridians are classified as Ebriacea (Order Cryomonadia, Class Thecofilosea, Cercozoa, Rhizaria) and are characterized by two sub-apically inserted cilia, with an open internal siliceous skeleton, and are phagotrophic without plastids (Hoppenrath and Leander 2006; Howe et al. 2011; Adl et al. 2012). Several fossil genera and species have been described (e.g., Loeblich et al. 1968; Ling 1985; Onodera and Takahashi 2009); only two extant species are known. One species, *Ebria tripartita*, is widely distributed in coastal oceans and feeds extensively on diatoms, while the other species, *Hermesinum adriaticum*, is restricted to warmer, hypoxic or anoxic seawaters and is mixotrophic or autotrophic with numerous *Synechococcus*-like endosymbiotic cyanobacteria (Hargraves 2002). Fossil records of ebridians are little known although several ebridians have been described (Loeblich et al. 1968). Ebridian fossils are found from the upper middle Eocene (42.0–33.7 Ma) to the Lower Oligocene in both high-latitudinal regions (e.g., Bohaty and Harwood 2000; Onodera and Takahashi 2009), but are sporadically reported as putative ebridians from older marine sediments. An ebridian species, *Ammodochium danicum*? is reported from the lower Paleocene from Seymour Island, eastern Antarctic Peninsula (Harwood 1988). Bignot (1985) noted that ebridian fossils appear at the end of the Cretaceous, but little (or nothing) is known about the end-Cretaceous to Paleocene ebridians in literature.

Phaeodaria (Class Theocofilosea, Cercozoa, Rhizaria) (Howe et al. 2011) are a unicellular testate marine protist which are commonly found in the mesopelagic and deeper depths down to 8,000 m (Nakamura and Suzuki 2015). The phaeodarian skeleton is too fragile to dissolve during sinking in water columns (Takahashi and Honjo 1981), and consequently fossil records are quite rare. The oldest fossil record of Phaeodaria is from the Rhaetian (205–201 Ma: Upper Triassic) deep-water sediments in Japan (Hori et al. 2009). The Rhaetian species (*Medusetta japonica* and *Triassiphaeodina niyodoensis*) are classified into the family Medusettidae. Fossil records are quite limited but almost all the known fossil genera in the Mesozoic belong to the extant genera.

Other small siliceous microfossils called archaeomonads, a siliceous cyst of marine chrysomonadins, occurred from the Lower Cretaceous through the Miocene and possibly Pliocene (Perch-Nielsen 1978).

15.7 Geologic History of Unfossilized and Fossilizable Phases (Fig. 15.6)

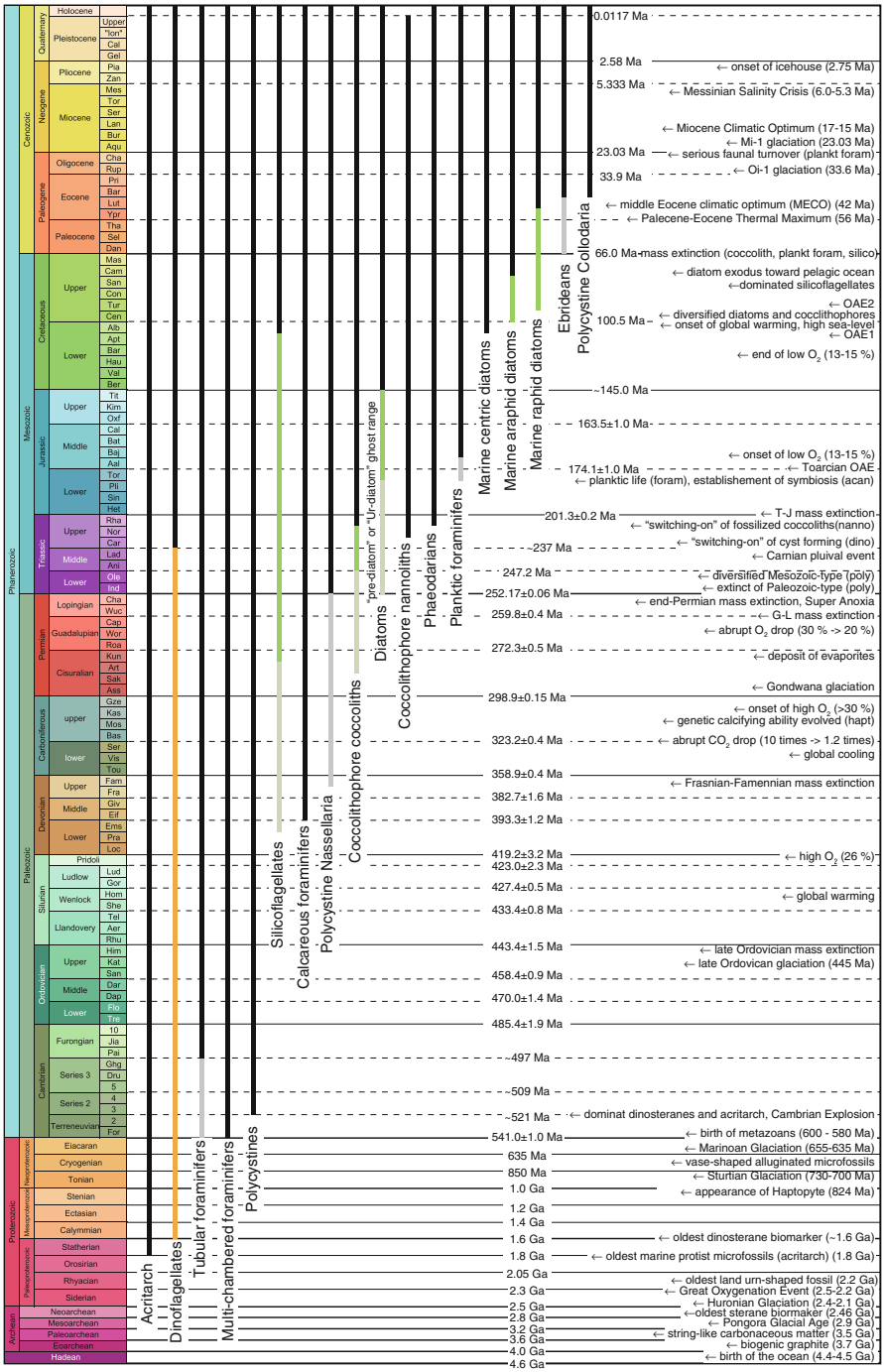


Fig. 15.6 The geologic history of fossilizable marine protists with major geologic events. The color and age of the geologic timescale is followed by the standard geologic timescale of the International Commission on Stratigraphy (2014). *Black vertical line*: maximum range of fossil records for the selected taxa. *Orange vertical line*: reliable range of biomarkers. *Green vertical line*: molecular phylogenetic range. *Light green vertical line*: error range of the molecular phylogenetic range. *Gray vertical lines*: doubtful or intermittent ranges of the fossils

15.7.1 General Trends in Fossilizable Marine Protists

The start of skeletal formation in marine protists is important for the implication and function of its existence. It is generally considered that the skeleton has functions of protection against predators, the storage of bioessential elements like Ca for depletion, and sinking weight for abrupt escape from predators and for reproduction in deeper waters. Marine protists are traditionally divided into skeletonless protists and skeleton-bearing protists, but this is too simple to discuss skeletons in marine protists. Protists are dividable into "true" non-biomineralized protists, non-skeletal biomineralized protists, fragile / easily soluble skeletal protists, and fossilizable skeletal protists. These four categories are also applicable as "phases" for different life stages such as motile and immotile stages, for example. The importance of non-skeletal biomineralization was already focused on by Raven and Knoll (2010). Non-skeletal biomineralized protists are indicative of protists that precipitate mineralized particles within intracellular vesicles in a cell under tight enzymatic control (Raven and Knoll 2010). Fragile/easily soluble skeletal protists are the motile dinoflagellates, loricate tintinnid ciliates, and parmales, for example. Phaeodaria are also regarded as fragile/easily soluble skeletal protists. The same taxonomic group can be divided into these four categories. For instance, the vegetative stage of diatom *Chaetoceros* is rarely preserved as fossils while the cyst of this genus is well preserved in marine sediments, and subsequently the former is fragile/easily soluble skeletal protists and the latter is fossilized skeletal protists.

This section briefly reviews the oldest fossil records of fossilized skeletal protists and their reliability for acritarchs and dinoflagellates, foraminifers, polycystines, diatoms, coccolithophores, silicoflagellates, and ebridians. This review reaches a general conclusion that the oldest records of the fossilized skeletal phase of these protists are universally younger than their probable originating geologic time, on the basis of molecular phylogenetic analysis. Once some protists have been preserved as fossils, the divergent timing of fossil records in a lower taxonomic rank is expected to be the same with its molecular phylogenetic timing. Although this expectation is not tested in this chapter, this test will succeed in foraminifers and polycystines because of their generous continuous fossil records as fossilized skeletal protists since the Cambrian. Diatoms consist of both fragile/easily soluble skeletal and fossilized skeletal protists, so the appearance ages of araphid and raphid pennate diatoms are different between fossil data and molecular phylogenetic data. This gives us a warning in estimating the molecular clock at the nodes in molecular phylogenetic trees. In addition, all in all, the molecular clock still needs to improve its precision for specifying related geological events because the geological event is precisely dated in recent years. Although we well recognize this uncertainty in the molecular clock, we try to find referable geological events by using the most probable age of the molecular clock in this section.

15.7.2 Hadean, Archean and Proterozoic

Since the ocean was born at 4.4–4.5 Ga (the early Hadean), no marine protists existed until 1.8 Ga (the start of the Statherian). At that time, atmospheric free oxygen was enough to shield organisms from ultraviolet sunlight due to the formation of the ozone layer and the geomagnetosphere after the Great Oxidation Event (GOE: 2.32–2.45 Ga in the Siderian; Canfield 2005). Acritarchs and the oldest marine protists producing dinosterane appeared at 1.8 Ga (the start of the Statherian) and at 1.6 Ga (the start of the Calymmian, namely that of the Mesoproterozoic), although no expected triggers for the birth of these two organic marine protists have been specified. As haptophytes appeared around 824 Ma (in the Cryogenian), probable oxygen-producing marine organisms consisted of cyanobacteria, acritarchs, dinosterane-forming marine protists and haptophytes just before the Sturtian glaciation in the Snowball Earth Period (730–700 Ma), suggesting contributions to multiplication of free oxygen. From the Cryogenian to the Ediacaran, probable heterotroph agglutinated microfossils appeared in the fossil record. The Ediacaran Period is the important period for the appearance of marine metazoans without mineralized skeletons and for finding trace fossils as evidence of epifaunal predators. As no organisms developed the ability to form skeletons until the start of the Cambrian, marine protists might have protected themselves from predators by agglutinated tests.

15.7.3 Paleozoic

The start of the Cambrian is the start of biomineralization by the appearance of Small Shelly Fossils (SSF). No marine protists acquired fossilizable biomineralization ability at that time; instead, foraminifers started to form multi-chambers by agglutination. From the fossil and molecular phylogenetic records, only radiolarians are known to have had a planktic lifestyle in the late Neoproterozoic and the Cambrian, and the skeleton-bearing radiolarians first appeared just after the end of the Terreneuvian. The Terreneuvian is the period of the Cambrian Explosion when almost all the metazoan groups appeared in the geological record. At nearly the same time, acritarchs and dinosterane-producing marine protists, and protoconodont animals (a nektonic ancestor of conodonts) dominated, and predation pressure and the food chain for microplanktons like radiolarians were presumed to have been drastically changed.

It is interesting that extant fossilizable marine protists never appeared from the Cambrian Explosion Period to the Early Devonian, except for tubular foraminifers with agglutinated tests. The appearance of such tubular foraminifers in the Paibian (late Cambrian) marked the final fossilized benthic marine protists in the Earth's history. Benthic foraminifers are so diverse in both fossil and extant marine protists that they are the most successful benthic marine protists. The major diversified marine organisms which probably exerted predation pressure on planktic marine

protists were conodonts that appeared in the Ordovician. Reliable fish appeared in the Dapingian (early middle Ordovician). The top-level predator changed from *Anomalocaris* to Eurypterids in the Silurian, and then from Eurypterids to Osteichthyes in the Devonian. This change, however, did not affect the "creation" and extinction of any marine protists. Significant changes such as faunal and floral turnovers and the evolution and extinction happened merely in the lower taxonomic ranks in each group between Series 2 of the Cambrian and the Early Devonian.

One of the significant events for fossilizable marine protists was achievement of calcifying ability by benthic foraminifers in the Eifelian (the early Middle Devonian). These benthic foraminifers lived in shallow warm neritic environments such as a continental platform. Fusulinids, a larger benthic foraminifer, became dominant as a successor from the Moscovian (the late Carboniferous) to the end of the Capitanian (the end of the Guadalupian, in the Permian). The Moscovian Stage was also the most probable age for Haptophyta to acquire genetic calcifying ability at ~310 Ma. In pelagic oceans, unfossilized silicoflagellates might have originated at 397 Ma (the middle Emsian, the late Early Devonian) or 382 Ma (the early Givetian, the late Middle Devonian). Although Osteichthyes and Paleozoic-type ammonoids as higher predators flourished in the pelagic ocean in the Early to Middle Devonian, less is known about possible reasons to interpret the appearance of silicoflagellates. At the Frasnian–Famennian boundary (in the Late Devonian), marine organisms suffered from the Frasnian–Famennian Mass Extinction Event, but this event was only related to the appearance of nassellarian-like polycystines in the Famennian. The appearance of nassellarian-like polycystines is important because such a body plan is unique among marine protists.

The Carboniferous Period was characterized by an abrupt CO_2 drop from 10 times to 1.2 times around the Bashkirian (the start of the late Carboniferous), global cooling, and the onset of a high-O_2 atmosphere (>30 %) from the Kasimovian–Gzhelian (in the middle–late late Carboniferous). These changes were not related to the origination of new fossilizable marine protist groups. After the Gondwana glaciation in the Asselian (in the Cisuralian Epoch of the Permian), the global oceanic environments drastically changed from the Kungurian (the late Cisuralian) to the end-Permian in that voluminous evaporates such as halite and gypsum were deposited. As the Gondwana glaciation also ended by the Kungurian, the salinity of the sea waters considerably attenuated. Atmospheric oxygen was reduced from ~30 to 20 % at the Wordian–Capitanian boundary (in the late Guadalupian). Coccolithophores were divergent from Prymnesiales at ~267 Ma (in the Wordian). The Guadalupian–Lopingian Mass Extinction, in association with the faunal turnover from the larger fusulinids to the smaller ones, occurred on a global scale in the Lopingian, and then the end-Permian Mass Extinction occurred by double-extinctions. As the severe conditions reached the chlorophyll-a maximum water depths, pelagic phytoplankton were inevitably damaged in the end-Permian Mass Extinction.

15.7.4 Mesozoic

The end-Permian Mass Extinction has been hypothesized as resulting in the molecular phylogenetic appearance of coccolithophores and diatoms, but it may not be related to their evolution if we simply refer to the best probable age of the molecular clock. As already documented above, the divergence of coccolithophores was possibly related to the sharp drop of atmospheric oxygen and attenuated seawater salinities. The appearance of diatoms has a mean age of 217 Ma with a range of 183–250 Ma, indicating the middle Norian (in the middle Late Triassic). This mean age estimation may be meaningful because the geologic interval from the Norian to the Rhaetian (in the late Late Triassic) saw the appearance of fossilizable non-dinoflagellate nannoliths and calcareous cysts of unknown marine protists (in the mid- to late Norian), with "switching on" of fossilizable coccoliths (in the Rhaetian). In addition, "switching on" of fossilizable dinoflagellate cysts dates to the Carnian, prior to the Norian. This is like "the Middle–Late Triassic phytoplankton evolutionary explosion."

The next milestone evolution in marine protists is concentrated around the Toarcian (the Early Jurassic) and the Aalenian (the Middle Jurassic). In the Toarcian, a well-known global anoxic event occurred worldwide and was probably related to the onset of the low atmospheric oxygen period (13–15 %) which continued until the middle Early Cretaceous. In the interval between the Toarcian and Aalenian, dinoflagellates and coccolithophores diversified in species as well as in abundance, the firm symbiosis being completed between radiolarian Acantharia and *Phaeocystis* lineages (Prymnesiales), and planktic foraminifers appearing. Little is known about why the atmospheric oxygen was low regardless of diversified dinoflagellates and coccolithophores.

The oldest records of fossil silicoflagellates and marine centric diatoms date to the Aptian–Albian boundary, but this spontaneous appearance may not be the "true" oldest appearance of these marine protists because they are found in the exact same locality. Regardless of this uncertainty, it is worthy of consideration because the low atmospheric oxygen period ended before the Aptian–Albian boundary, the intermittent but global oxygen anoxic events occurred between the Aptian and Cenomanian, and the Radiolarian Optimum Event (ROE) with abundant polycystines in sediments since the latest Carboniferous ended in the Aptian. The demise of the ROE may have enabled silicoflagellates and centric diatoms to precipitate siliceous skeletons. After the Aptian–Albian boundary, a gradual change in siliceous marine phytoplankton occurred, with molecular phylogenetic appearance of both araphid and raphid diatoms in the early Late Cretaceous, appearance of fossilizable araphid diatoms and the diatom exodus toward pelagic oceans in the Santonian–Campanian, dominant silicoflagellates in the Campanian, and the fossil record congestion of Phaeodaria between the Campanian and the Maastrichtian. In conclusion, the Late Cretaceous is marked not only by the rapid accumulation of coccolithophores but also by various developments of siliceous phytoplanktons.

15.7.5 Cenozoic

The Cretaceous–Paleogene Mass Extinction caused serious damage to coccolithophores, dinoflagellates and planktic foraminifers but only small effects on polycystines. In addition, all the silicoflagellate genera, except for a single genus, were extinct at this boundary. After this mass extinction event, ebridians appeared as new fossilizable mixotrophic or heterotrophic marine protists.

The latest appearance events of fossilizable marine protists involved raphid diatoms and radiolarian collodarians, in association with continuous occurrences of ebridians in the middle to late Eocene. The Earth gradually cooled after the Middle Eocene Climatic Optimum Event (MECO) at 40.4–39.9 Ma. The substitution of silicon precipitation marine protists from polycystines to diatoms followed these events. The emergence of radiolarian collodarians was not a simple evolution in Radiolaria because Collodaria acquired dinoflagellate symbionts and were the only radiolarian group with a colonial lifestyle.

15.8 Establishment of the Modern-Type Marine Protists (Fig. 15.7)

The establishment timing of the "modern-type protists" is closely related to the composition of phytoplankton groups as primary productivity producers and the geologic history of marine environments. According to Knoll et al. (2007), the marine protist contributors to primary production changed twice in geologic history. The first period was the Paleoproterozoic (2.5–1.6 Ga) and Mesoproterozoic (1.6–1.0 Ga) when cyanobacteria and other photosynthetic bacteria dominated. The second period started from the latest Neoproterozoic (~600 Ma) or the Cambrian (541.0–485.4 Ma) in the enrolment of green algae with cyanobacteria and other photosynthetic bacteria. The third period is presumed to begin at the Triassic in which cyanobacteria played a relatively minor role in continental shelf production.

The establishment of the modern-type primary producers is considered to be the Mesozoic with the replacement of red algal lineages from green photosynthetic phytoplanktons (Falkowski et al. 2004). Approximately 90 % of marine invertebrates became extinct in an interval of 60 ± 48 Kyr between 251.941 ± 0.037 Ma and 251.880 ± 0.031 Ma, just prior to the Permian–Triassic boundary (251.902 ± 0.024 Ma) (Burgess et al. 2014). The Permian–Triassic boundary is formally defined by the first occurrence horizon of a conodont *Hindeodus parvus* in the Meishan D section, South China (Yin et al. 2001). In this interval, extraordinary marine ecosystems developed by global warming, ocean acidification, depleted dissolved and free oxygen in association with Siberian volcanic activity. This event led to an abrupt decline in eukaryotic marine phytoplankton, proliferation of prasinophyte and acritarchs ("disaster taxa") and a general increase in the abundance of green sulfur bacteria and cyanobacteria in the oceans (Algeo et al. 2013). Falkowski et al. (2004)

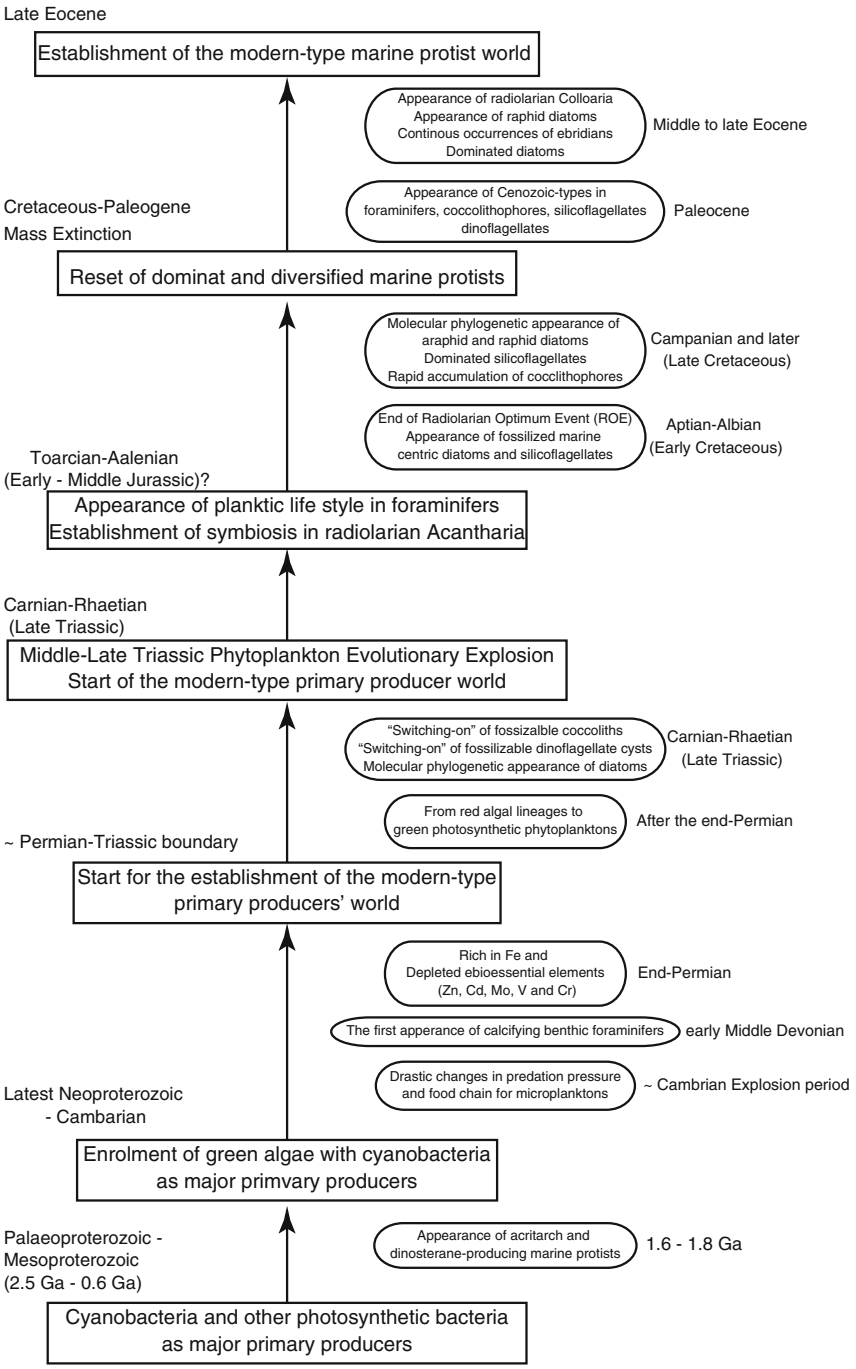


Fig. 15.7 Major events of marine protists toward the establishment of the modern-type marine protists

explained the dominated red algae lineages in this interval by the Fe-rich and Zn- and Cd-depleted sea waters around the end-Permian Mass Extinctions. Furthermore, the bioessential elements depleted from the sea water are known to be Mo, V and Cr, and probably both Zn and Cd, by the geologic data (Takahashi et al. 2014). A notable establishment of the modern-type primary producers is algal symbionts. The order Acantharia, a radiolarian group, established symbiosis with *Phaeocystis* lineages around the early Jurassic, probably in the Toarcian (Decelle et al. 2012). Planktic foraminifera also appeared around the Toarcian, which may be related to the establishment of symbiosis at that time. Fossil records of calcareous nannofossils, diatoms and dinoflagellate cysts became abundant in the middle–late Eocene, rather than the Mesozoic. In conclusion, the establishment of the modern-type primary producers dates to the late Eocene.

The reset of dominant and diversified taxa within a marine protist group was different. The foraminifers, coccolithophores and silicoflagellates were mostly replaced by the Cenozoic-type fauna after the Cretaceous–Paleogene Mass Extinction. All the Polycystines except for the Order Collodaria were largely replaced by the Orders Nassellaria and Spumellaria at the Permian–Triassic boundary, and these orders were not strongly affected by the Cretaceous–Paleogene Mass Extinction. However, the Order Collodaria, a dominant polycystine group in the pelagic modern ocean, first appeared in the late Eocene. Ebridians are a minor component in the fossil records as well as the modern marine protist world, but they are commonly found from the upper middle Eocene. In diatoms, the late Eocene time is the appearance period of raphid diatoms. As these records are comprehensively considered, the modern-type marine protist world started in the late Eocene when collodarian polycystines, raphid diatoms, and ebridians appeared. This late Eocene establishment of the modern-type marine protist world should be tested in consideration with the remaining major unfossilized marine protists such as ciliates.

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Part II
Symbiosis

Chapter 16
Diversity and Phylogeny of Marine Parasitic Dinoflagellates

Takeo Horiguchi

Abstract The dinoflagellates are an assemblage of autotrophic, myxotrophic and heterotrophic species and play important roles in marine ecosystems. Most of them are free-living, but several are known as symbionts of marine invertebrates or as parasites of aquatic animals and protists. Molecular phylogenetic studies on parasitic dinoflagellates reveal that the parasitism has evolved polyphyletically appearing several times independently within the division Dinophyta. The Dinophyta consists of "core" dinoflagellates (typical dinoflagellates) and "basal" dinoflagellates. Within the basal dinoflagellates, the parasitic clades of Perkinsea, Ellobiophyceae and Syndiniophyceae are recognized. A large portion of environmental clone sequences recovered from various parts of the oceanic environments are thought to be members of the parasitic order Syndiniales (Syndiniophyceae). Therefore, the ecological function of parasitic dinoflagellates in the ocean is now better understood. The members of parasitic genera such as *Parvilucifera* (Perkinsea) and *Amoebophrya* (Syndiniophyceae) infect other dinoflagellates; therefore, much attention has been paid, because of their possible ability to control harmful algal blooms (HABs). The parasitism has also evolved within the "core" dinoflagellates (Dinophyceae) a number of times. These include *Blastodinium*, *Chytriodinium*, *Dissodinium*, *Haplozoon*, *Oodinium*, *Paulsenella* and *Tintinnophagus.* Because there are already several reviews on diversity of parasitic dinoflagellates, in this volume, only recent developments in phylogenetic studies on parasitic marine dinoflagellates are mainly reviewed here.

Keywords Alveolata • Environmental clone sequences • Parasitic dinoflagellates • Phylogeny

T. Horiguchi (✉)
Department of Biological Sciences, Faculty of Science,
Hokkaido University, Sapporo 060-0810, Hokkaido, Japan
e-mail: horig@mail.sci.hokudai.ac.jp

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16.1 Introduction

The dinoflagellates are a group of protists that inhabit a wide variety of habitats, from freshwater to open ocean. Although they are basically unicellular organisms, the morphological variations that they exhibit are spectacularly diverse. Furthermore, their mode of lifestyle is also extremely complex and diverse. Nearly half of the 2500 species of extant dinoflagellate species are photosynthetic species and the rests are heterotrophic forms. Most of the photosynthetic species are free-living, while some of them are known as symbionts of marine invertebrates, generally termed zooxanthellae (see Chap. 17) and even several photosynthetic species are regarded as parasitic dinoflagellates. The heterotrophic dinoflagellates display a wide range of trophic strategies and various types of feeding methods (Gaines and Elbrächter 1987). Once again, many of the heterotrophic dinoflagellates are free-living, but some representatives are known as parasitic forms.

There are approximately 150 parasitic dinoflagellate species (Coats 1999) representing about 35 genera. The host range of parasitic dinoflagellates is wide and includes other protists (including dinoflagellates), appendicularias, annelids, siphonophores, radiolarians, marine crustaceans and their eggs, and fish and their eggs. In some cases, the parasitic dinoflagellates have large impacts on host populations. The parasitism of dinoflagellates affects the health condition, survival, and reproduction of its host, and furthermore influences the total biomass of the host population. *Hematodinium* spp. are parasites of crustaceans, including commercially important lobsters and crabs, and they make their hosts commercially unsuitable. Therefore, these species that affect fishery activities are thought to be serious pathogens for marine crustacean fisheries (Stentiford and Shields 2005). Some of the parasitic dinoflagellates have received much attention because of their possible effect of controlling development of so-called harmful algal blooms (HABs). These dinoflagellates include *Parvilucifera* and *Amoebophrya* (e.g., Norén et al. 1999; Park et al. 2013). Although not dinoflagellates, recently the diatom bloom control by nanoflagellate parasites was documented by using the Imaging FlowCytobot device (Peacock et al. 2014).

Historically, studies on parasitic dinoflagellates have started mainly in Europe and the authors of early studies include Edouard Chatton (e.g., Chatton 1910, 1912, 1920; Chatton and Poisson 1931), Jean and Monique Cachon (e.g., Cachon 1964; Cachon and Cachon 1987) and German protistologists, such as Valetin Dogiel, Gerhard Drebes and Eberhard Schnepf (e.g., Dogiel 1906; Drebes and Schnepf 1982), For the historical aspects of studies on parasitic dinoflagellate studies, see Coats (1999).

Since there are already reviews on biodiversity of parasitic dinoflagellates (e.g., Chatton 1920; Cachon and Cachon 1987; Shields 1994; Coats 1999; Skovgaard 2014), in this Chapter I will focus on recent developments concerning parasitic dinoflagellates on which molecular data are available.

16.2 Phylogenetic Affinities of Parasitic Marine Dinoflagellates

The phylum Dinophyta (Dinozoa), a member of the Alveolata, consists of "core" dinoflagellates and "basal" dinoflagellates (see Chap. 2). The "core" dinoflagellates, i.e., equivalent to the class Dinophyceae, are characterized by possession of typical dinokont motile cells, i.e., typically the cell has a transverse girdle, called the "cingulum," and a longitudinal furrow, termed the "sulcus." In addition, the members of the Dinophyceae possess a special type of nucleus, the dinokaryon, which is characterized by permanently condensed chromosomes, lack of nucleosomes and involvement of an extranuclear spindle during nuclear division. The chromosomes are known to contain both eukaryotic and prokaryotic proteins (Shoguchi et al. 2013).

A number of molecular phylogenic studies on parasitic dinoflagellates have been published so far (e.g., Bachvaroff et al. 2014; Norén et al. 1999; Gómez et al. 2009; Harada et al. 2007; Saldarriaga et al. 2004; Skovgaard et al. 2012). Furthermore,

in relation to parasitic dinoflagellates, the studies on environmental DNA clone analyses should be mentioned. One way to unveil the protozoan biodiversity by a culture-independent method is known as environmental clone analysis and one of the surprising early outcomes of this method was discovery of two novel lineages within the Alveolata (López-García et al. 2001; Moon-van der Staay et al. 2001). These clones were obtained from the DNA extracted from the 0.2–5 μm fraction size of the seawater samples (López-García et al. 2001). At the time of the discovery, because each clade consisted of only environmental clones and no known organisms were included, each clade was named Marine Alveolate (MALV) Group I and MALV Group II, respectively. Later, the members of MALV Group I and Group II were detected from various marine environments, including pelagic waters, neritic waters, bottom sediments, hydrothermal vents and permanent anoxic deep waters, and these "organisms" usually represent the most abundant eukaryotic lineages in environmental genetic libraries (e.g., Groisillier et al. 2006). It was soon realized that the parasitic genera, such as *Amoebophrya*, *Hematodinium* and *Syndinium*, belong to MALV Group II (Skovgaard et al. 2005). Later, Dolven et al. (2007) discovered that possible intracellular parasites of radiolarians belong to MALV Group I and Harada et al. (2007) demonstrated that parasitic protists, *Duboscquella* spp. (now *Euduboscquella*) infecting tintinnid ciliates, belong to MALV Group I. The fish egg parasite, *Ichthyodinium*, was also demonstrated to be a member of MALV Group I (Skovgaard et al. 2009). Large-scale analyses of environmental clones revealed that many of the environmental sequences form a large clade, including the above-mentioned MALV Groups I and II, and this large clade comes to a sister position of the core dinoflagellates (Guillou et al. 2008). This large clade was regarded to correspond to the order Syndiniales of the Dinophyceae (Guillou et al. 2008). Guillou et al. (2008) recognized five major clades (Syndiniales Groups I–V) within this large clade. MALV Group I was renamed as Syndiniales Group I and it was shown that eight well supported clades existed in this group, while MALV Group II was renamed as Syndiniales Group II and 44 subclades were

recognized. Syndiniales Group I includes members of *Euduboscquella* and *Ichtyodinium*, while Group II contains *Amoebophrya* spp. together with a large number of unidentified environmental sequences. Groups III and V consist only of environmental clones, while Group IV includes *Hematodinium* and *Syndinium*. It is possible that all these unknown (environmental) sequences represent syndinialean parasitic dinoflagellates, which have not been identified. Because most of the environmental clone sequences were obtained from pico- to nanosized fractions, it is highly likely that these sequences originated from unicellular dinospores of the parasitic dinoflagellates. Care must be taken, however, when interpreting DNA sequences from small-sized fractions, because the possibility of the presence of extracellular DNA in the environments cannot be ruled out (Not et al. 2009).

16.3 Parasitic "Basal" Dinoflagellates

Figure 16.1 shows phylogenetic affinities of dinoflagellates based on SSU rDNA and the tree indicates that the parasitic dinoflagellates can be divided into two major groups from the phylogenetic point of view, i.e., the first group consists of the organisms belonging to the "core" dinoflagellates, while the other group belongs to the "basal" dinoflagellates, such as Perkinsea, Ellobiophyceae, and Syndiniophyceae (in this analysis, non-parasitic dinoflagellates, such as *Oxyrrhis* and *Psammosa* were excluded). Although each group forms a well-supported clade (Fig. 16.1), the branching order among these clades was ambiguous. The character evolution within the basal dinoflagellates in relation to both Apicomplexa and core dinoflagellates, such as the presence or absence of an apical complex (conoid/pseudoconoid) etc., is an intriguing question, but this is beyond the scope of this chapter; for details, see Leander and Keeling (2003), Okamoto et al. (2012) and Chap. 2 of this volume. As the representatives of basal dinoflagellates, the members of Perkinsea, Ellobiophyceae and Syndiniophyceae will be discussed here.

16.3.1 Perkinsea

The class Perkinsea includes the genera, *Perkinsus*, *Parvilucifera*, *Rastrimonas* and *Phagodinium*. The former two genera are mostly marine, but the latter two are freshwater protists. Members of the genus *Perkinsus* are important pathogen of mollusks, distributed widely worldwide from temperate to tropical coastal waters (Villalba et al. 2004). Currently, seven species are recognized in the genus *Perkinsus*. Of these, *Perkinsus marinus* is well known as a pathogen (Perkinsosis) of oysters and is often associated with mass mortality (Villalba et al. 2004, and also Chap. 26 of this volume). In 1999, the perkinsid protist that infests toxic dinoflagellates was described as a new genus, *Parvilucifera* (Norén et al. 1999). The type species of *Parvilucifera*, *P. infectans*, was described as a parasite of the toxic dinoflagellate

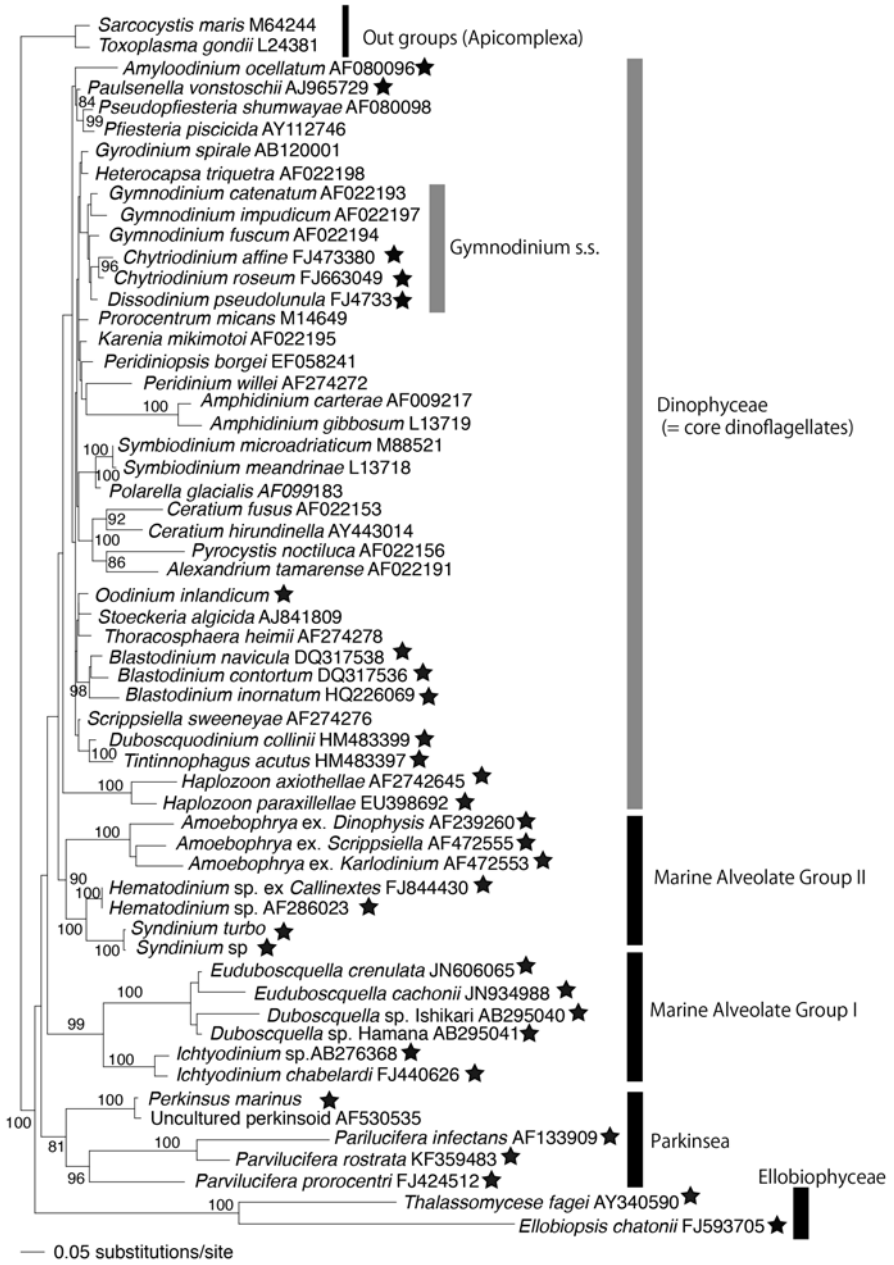

Fig. 16.1 Phylogenetic affinities of parasitic dinoflagellates inferred from SSU rDNA sequences (maximum likelihood tree). The mode used selected by Akaike Information Criteria was GTR+I+G. A heuristic search was performed with a TBR branch-swapping algorithm, and the starting tree was obtained by the NJ method. The parameters in these analyses were: assumed nucleotide frequencies A=0.2540, C=0.1887, G=0.2551 and T=0.2620; substitution rate matrix with A↔C=1.1776, A↔G=2.7863, A↔T=1.2891, C↔G=0.9384, C↔T=5.1843 and G↔T=1.0000; proportion of sites assumed to follow a gamma distribution with shape parameter=0.622; and number of rate categories=4. Bootstrap analysis for ML was calculated for 100 replicates. The bootstrap values >80 are indicated at nodes. The taxa indicated by a *star* represent parasitic dinoflagellates. A *vertical gray line* indicates a clade of "core" dinoflagellates, while *black lines* represent "basal" dinoflagellates

Dinophysis norvegica. The life cycle of *P. infectans* starts from the infection of motile cells (zooids) and after successive infection, the parasite appears as a round opaque vacuole-like structure. The cytoplasm of the host dinoflagellate degrades gradually during sporogeny and this results in the death of the host cell. The matured sporangium is spherical and possesses a cell wall which is ornamented with more or less regularly arranged warts. Zooids are formed within the sporangium and many zooids (500–700 zooids in a medium sized sporongium) are produced (Norén et al. 1999). It was also demonstrated that *P. infectans* can infect other dinoflagellates such as *Alexandrium* spp. In addition to the type species, three more species have been described in the genus *Parvilucifera* to date, i.e., *P. prorocentri* (Leander and Hoppenrath 2008) (Fig. 16.2a), *P. sinerae* (Figueroa et al. 2008) and *P. rostrata* (Lepelletier et al. 2014). The host of *P. prorocentri* is so far restricted to the benthic dinoflagellate *Prorocentrum fukuyoi*, but *P. sinerae* was found to infect toxic planktonic dinoflagellates, such as *Alexandrium* spp., *Karenia brevis* and *Gymnodinium catenatum. P. rostrata* is also known to parasitize toxic dinoflagellates (e.g., *Alexandrium* spp.) and non-toxic dinoflagellates (e.g., *Heterocapsa triquetra* and *Kryptoperidinium foliaceum*) (Lepelletier et al. 2014). Because these host dinoflagellates listed above are known as HAB species, *Parvilucifera* spp. are regarded as possible agents for biological control of these bloom-forming organisms (Norén et al. 1999).

Because *Perkinsus* possesses an apical complex, the organism was once placed in the Apicomplexa (Levine 1978). In the recent molecular phylogenetic studies, however, it has been shown that the Perkinsea occupies the earliest branching position within the Dinophyta (Dinozoa) (e.g., Bachvaroff et al. 2014) (Fig. 16.1).

16.3.2 Ellobiophyceae

The Ellobiophyceae (Ellobiopsida) has long been regarded as an enigmatic group of parasites with unknown phylogenetic affinities (Whisler 1990). It consists of five genera: *Ellobiopsis*, *Thalassomyces*, *Parallobiopsis*, *Ellobiocystis* and *Rhizellobiopsis*. These five genera are distinguished based on the presence or absence of a root system, the type of root, and the number of trophomeres and gonomeres (Kane 1964). The first four genera are parasites of crustaceans, but *Rhizellobiopsis* is known as a parasite of polychaete warms (Zachs 1923). Whisler (1990) classified the group into three categories: (1) the genus *Ellobiopsis*; (2) the

Fig. 16.2 (continued) (**d**) 24 min; (**e**) 74 min; (**f**) 172 min (From Harada et al. 2007 with permission). (**g**) *Amoebophrya* sp. infecting *Dinophysis norvegica*, showing a typical beehive stage (*arrow*). (**h**) Sequential photographs of release of a vermiform stage of *Amoebophrya* sp. Note that most of the cytoplasm of *Dinophysis* is missing (From Nagasawa 2004 with permission), (**i**) *Syndinium* sp. infecting a copepod, *Paracalanus parvus* s. l. (From Horiguchi et al. 2006 with permission) (**i**) (*inset*) shows TEM of a spore of *Syndinium* sp. (*scale bar* = 1 μm), showing typical syndinialean chromosomes

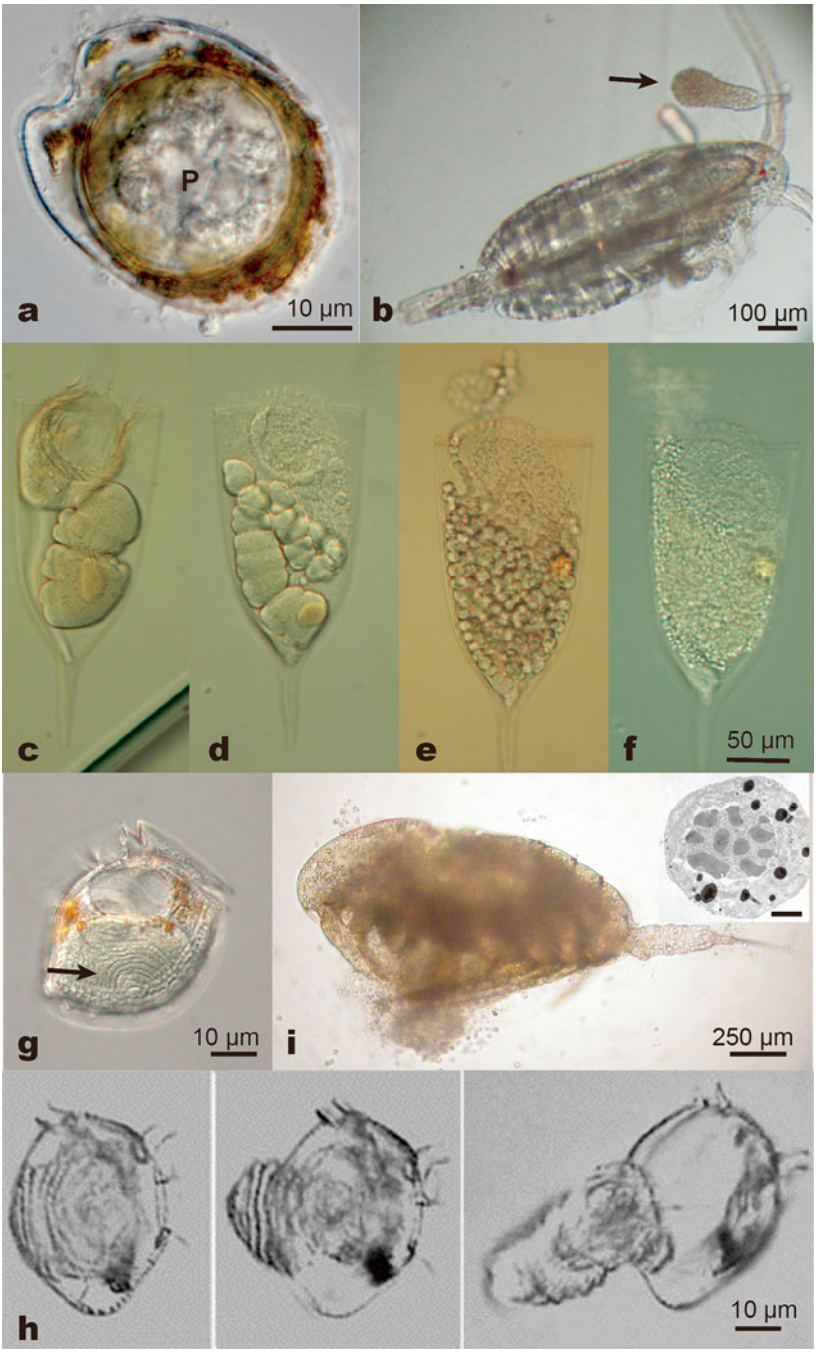

Fig. 16.2 Parasitic dinoflagellates belonging to "basal" dinoflagellates. (**a**) *Parvilucifera prorocentri* (P) infecting *Prorocentrum fukuyoi* (Photograph courtesy of Dr. M. Hoppenrath). (**b**) *Ellobiopsis chattoni* attaching to *Bestiolina* sp. (Photograph courtesy of Dr. S. Ohtsuka). (**c–f**) Different stage of maturation of *Euduboscquella* sp: (**c**) time of starting observation (0 min);

genus *Thalassomyces*; and (3) the genera *Parallobiopsis*, *Ellobiocystis* and *Rhizellobiopsis* which are tentatively regarded as members of the Ellobiophyceae based on external morphological resemblance, but their true affinities must be tested (Whisler 1990; Silberman et al. 2004). The genus *Ellobiopsis* was established by Caullery (1910) based on the type species, *E. chattoni*, and the members of this genus parasitize various species of copepods (Fig. 16.2b). Currently, three species are recognized in the genus (Gómez 2012). The body of *Ellobiopsis* consists of a trophomere with a stalk by which the cell penetrates the host body and one or two gonomeres are formed by segmentation of the distal portion of the cell body. The sporulation occurs as the gonomere becomes constricted from the trophomere, and groups of dividing cells are released from the remains of the gonomere. The released cells further divide and form nonflagellated spores (Shields 1994). The members of the genus *Thalassomyces* are regarded as mesoparasites, i.e., part of the body inside the host and part of the body outside the host, and infect various groups of crustaceans, including mysids, shrimps, euphausiids and amphipods (Shields 1994). The genus is characterized by having branched trophomeres and with a ramifying root system, which penetrates the host tissue (Kane 1964), and with one or more gonomeres at the distal end of each trophomere.

Silberman et al. (2004), using SSU rDNA sequences, revealed that one of the genera of Ellobiophyceae, *Thalassomyces*, belonged to the Alveolata. Gómez et al. (2009) obtained the sequence of *Ellobiopsis* and demonstrated that *Thalassomyces* and *Ellobiopsis* formed a clade and the clade belongs to the Alveolata, showing possible phylogenetic affinities with MALV Group I, although such a relationship has not been demonstrated in our genetic analysis (Fig. 16.1).

16.3.3 Syndiniophyceae

The order Syndiniales (Syndiniophyceae) is distinguished from the core dinoflagellates with the following characteristics (Loeblich III 1976): (1) low chromosome numbers (4–10) (Fig.16.2i, inset); (2) V-shaped chromosomes; (3) chromosome containing enough quantity of histochemically detectable basic proteins; (4) possession of centrioles associated with mitosis; (5) intracellular parasitism as a mode of nutrition; and (6) lack of cell cover containing thecal plates. The Syndiniopyceae almost corresponds to the clade consisting of MALV Groups I and II (or Syndiniales Groups I–V). Briåte et al. (2010) demonstrated that the intracellular parasites (symbionts) of radiolarians are phylogenetically diverse and they are divided into four distinct clades, RAS (Radiolarian Associated Sequences) 1–4. RAS 1 and 2 belong to MALV Group I, while RAS 3 and 4 belong to MALV Group II. They also noted that the parasitic members of the MALV Group cluster according to their host types, such as ciliates, fish, copepods, dinoflagellates; so far, they have never intermixed (Briåte et al. 2010).

The genus *Eudubscquella* was established to include most of the species formerly accommodated in the genus *Duboscquella* (Coats et al. 2012). In the revision

of the family Duboscquellidae, Coats et al. (2012) pointed out that the type species of the genus *Duboscquella*, *D. tintinnicola*, possesses an apparent dinokont type of swarmers (with cingulum and sulcus); therefore, the organisms should belong to the core dinoflagellates (Dinophyceae) and not to the Syndiniales (Syndiniophyceae). Subsequently, another species in this genus was moved to a new genus, *Euduboscquella*. The genus *Euduboscquella* (the former *Duboscquella*) has been known as a parasite infecting mainly tintinnid ciliates, although other types of hosts are known, i.e., a dinoflagellate, *Noctiluca* (Cachon and Cachon 1987). In most cases, *Euduboscquella* are lethal to their host organisms, even having a significant impact on entire populations. This is the case for *Euduboscquella cachoni* and the ciliate *Eutintinnus pectinis* in Chesapeake Bay, USA (Coats and Heisler 1989). The genus *Euduboscquella* has a characteristic pattern of sporogenesis, i. e. successive nuclear and cytoplasmic division without interruption which is called "palintomy" (Fig. 16.2c–f), through which numerous biflagellate spores are produced. Two types of dinospores have been reported, i.e., macrospores and microspores. Both types of spore may be formed by the same species, but only one type is released from a given host (Coats 1988). The ciliates *Favella ehrenbergii, Tintinnopsis campanula,* and *Codonella galea* were the first organisms that had been reported to be the hosts of *Duboscquella tintinnicola* (Chatton 1920). The second species, *D. anisospora*, was characterized by its unequal dinospore size and unique trophont morphology (Chatton 1920). Cachon (1964) described five new species. These were distinguished from each other in trophont morphology and the hosts of these new species include non-tintinnid ciliates as well as dinoflagellates. Coats (1988) examined the cytology and life history of parasite in tintinnids and described a new species, *D. cachoni*, which differs from other members of the genus by the structure of the trophont, the pattern of sporogenesis, and spore morphology. The life cycle of *Duboscquella* has been known in detail. Three phases can be recognized, i.e., the feeding phase known as a trophont, the reproductive phase in sporogenesis, and the transmission phase as a motile dinospore. Establishment of infection is due to accidental ingestion of dinospores by susceptible hosts (Cachon 1964). In the host cytoplasm, the flagella, girdle, and sulcus of the dinospore vanish and the trophont grows in the host cytoplasm. Finally, the trophont engulfs all or part of its host cytoplasm by phagocytosis, and escapes from the host. The trophont phase is followed by a sporogenesis phase. Coats et al. (2012) reported a sexual process in *Euduboscquella crenulata*. The detail of syngamy of a spherical nonmotile female spore with an actively swimming male spore and subsequent formation of a zygote and meiosis were reported (Coats et al. 2012). Genetic variations within the genus *Euduboscquella* were examined and all of the species, together with environmental clone sequences, belong to clade 4 of Group I of Guillou et al. (2008) (Bachvaroff et al. 2012). For the details of *Euduboscquella* and other parasitic species infecting tintinnid ciliates, refer to the review by Coats and Bachvaroff (2013).

The members of the genus *Syndinium* parasitize marine planktonic copepods and radiolarians (Skovgaard et al. 2005). Within the host body, the parasite develops into a multicellular plasmodium. The plasmodium enlarges and fills almost the entire body of the host and the host is eventually killed. Then, sporulation takes

place and the swarmers (dinospores) are produced (Fig. 16.2i). The dinospore possesses a typical syndinialean nucleus and trichocysts (Fig. 16.2i, inset). The swarmers are released from the split of the exoskeleton of the dead host. The swarmer possesses two flagella, and it may contribute to dispersal. Three types of swarmers (microspores, macrospores and rostrate spores) that are morphologically distinct from each other are produced by the parasite infecting the copepod *Paracalanus parvus* but only one type of swarmer is always released from a single individual of the host (Skovgaard et al. 2005). Skovgaard et al. (2005) demonstrated the phylogenetic position of this species using a molecular technique. According their phylogenetic analysis, *Syndinium* was placed as a sister taxon to *Hematodinium* spp. and both parasites were affiliated with MALV Group II (or Syndenean Group IV of Guillou et al. 2008).

The *Hematodinium* spp. are parasitic dinoflagellates that infect the haemolymph of marine decapod crustaceans. An outbreak of *Hematodinium* spp. causes serious problems, because they damage the stock of commercially important crustaceans, such as Norway lobsters (*Nephrops norvegicus*), snow crabs (*Chionoecetes opilio*), Tanner crabs (*C. bairdi*), American blue crabs (*Callinectes sapidus*) and velvet swimming crabs (*Necora puber*) (Stentiford and Shields 2005). Infections by *Hematodinium* spp. have been reported from various parts of the world (Stentiford and Shields 2005), but within the genus, there are only two named species, i.e., *H. perezi* (type species, Chatton and Poisson 1931) and *H. australi*s (Hudson and Shields 1994). The difficulty in specific identification has been due to lack of morphological differences between the parasites from different hosts (Hudson and Shields 1994), but more importantly, because of lack of detailed information concerning the morphology of swarmers, ultrastructure and molecular data on the type species, *H. perezi* (Hudson and Shields 1994; Small et al. 2012). Recently, Small et al. (2012) were able to characterize the type species collected from one of the type hosts, a portunid crab, *Liocarcinus depurator*, collected from a similar geographical location to that of the type description. Then it was possible to compare the DNA sequences that have been reported from various parts of the world and they found that *Hematodinium* infecting different types of crabs from the USA and China represents different genotypes of *H. perezi*. Based on the culture study on *Hematodinium* sp. from the Norway lobster, Appleton and Vickerman (1988) demonstrated the presence of eight life cycle stages. The presence of a free-living *Hematodinium* stage associated with the blue crab in water samples was suggested by PCR. The exposure of healthy *Hematodinium*-free crabs to water that contained presumptive *Hematodinium* dinospores led to infection after two days and the crabs died within 4 days (Frischer et al. 2006). The PCR assay is a sensitive method to detect the presence of *Hematodinium* spp. within the host (e.g., Gruebl et al. 2002), but more recently another type of detection method was developed (Gornik et al. 2013). In the latter method, the immunological approach was developed against a newly discovered dinoflagellate-specific nuclear protein, i.e., dinoflagellate/viral nucleoprotein (DVNP) (Gornik et al. 2012). Both immunofluorescence assay and Western blot methods were shown to be very sensitive to detect the presence of the parasite (e.g., 25 parasites per milliliter of crustacean haemolymph) (Gornik et al. 2013).

The genus *Amoebophrya* is unique among parasitic dinoflagellates, because it parasitizes other dinoflagellates. In addition to dinoflagellates, however, *Amoebophrya* also infects ciliates, rhizopods, acantharias, and multicellular animals, such as siphonophores and chaetognaths (Cachon and Cachon 1987). *Amoebophrya* spp. have been reported to distribute worldwide, including North America, Europe, Mediterranean, Asia and Australia (Park et al. 2013). The genus *Amoebophrya* has a specialized life cycle divided into three distinct phases, i.e., a free-living reproductive phase (the sporont stage), an intracellular parasitic phase (the trophont stage) (beehive stage) (Fig. 16.2g) and vermiform (Fig. 16.2h). In the sporont stage, a motile spore (= dinospore) has a girdle with a half turn. The dinospore perforates the host membrane with its pointed end and squeezes itself into the host cytoplasm. The parasites develops themselves either in the host cytoplasm or in the host nucleus. In the host cytoplasm or nucleus the dinospore becomes a trophont, and the parasitic phase begins. The trophont rapidly grows and the mature trophont occupies much of the host cell; it looks like a typical beehive. When the trophont growth finishes, the flagella begin to beat, and the parasite everts itself. A long multinucleate, coiled body, termed the vermiform, emerges from the host and swims off in a spiral manner with all its flagella beating (Fig. 16.2h). The vermiform can produce 60–400 dinospores (Chambouvet et al. 2008) and these dinospores seek out new hosts (Cachon and Cachon 1987). The ultrastructure of the developmental stages of *Amoebophrya ceratii* was demonstrated by Fritz and Nass (1992). More recently, the detailed ultrastructure of the dinospores, infection process and developmental stage of trophonts of *Amoebophyra* sp. parasitizing a dinoflagellate, *Akashiwo sanguinea*, has been illustrated (Miller et al. 2012). Of the species in *Amoebophrya, A. ceratii* and *A. leptidisci* parasitize free-living dinoflagellates, and *A. leptidisci* is a parasite specific to the heterotrophic noctilucalean dinoflagellate *Leptodiscus medusoides* (*Pratjetella medusoides*). *A. ceratii*, on the other hand, is believed to parasitize many different dinoflagellate species, thus it seems to lack host specificity. However, Janson et al. (2000) indicated that "*Amoebophrya ceratii"* contains host-specific multi species on the basis of a comparison of SSU rDNA sequences from *Amoebophrya* strains, infecting *Dinophysis norvegica* and *Akashiwo sanguinea (= Gymnodinium sanguineum)*. In the study by Gunderson et al. (2002) it was revealed that species of the genus *Amoebophrya* in Chesapeake Bay which infected different dinoflagellates did not all belong to the same species. Salomon et al. (2003) also suggested that the same host, *Dinophysis norvegica*, was infected by different species of *Amoebophrya* in the North Sea and Baltic Sea. Recently, much attention has been paid to members of the genus *Amoebophrya*, because a high prevalence of these parasites may contribute to the termination of HABs (e.g., Chambouvet et al. 2008; Park et al. 2013). Chambouvet et al. (2008) demonstrated that in a marine coastal Penezé estuary in northern Brittany, France, the decline of four species of dinoflagellate blooms, i.e., *Heterocapsa rotundata*, *Scrippsiella trochoidea*, *Alexandrium minutum* and *Heterocapsa triquetra* was correlated with the release of dinospores of parasites (= *Amoebophrya* spp.). The large number of released dinospores of particular parasite "species" did not prevent growth of other species of dinoflagellates, suggesting the presence of host–parasite specificity

(Chambouvet et al. 2008). In the Thau lagoon (Mediterranean Sea, France), the locally existing *Amoebophyra* species did not infect newly introduced *Alexandrium catenella/tamarense*, but parasitized only other co-occurring dinoflagellates species (Chambouvet et al. 2011). Cross-infection experiments between *A. catenalla/tamarense* strains from the Thau lagoon and a strain of *Amoebphyrya* sp. from North America showed that all the strains were strongly infected by the parasite from the USA. The result of the crossing experiment seems to support the hypothesis called the "enemy release hypothesis (ERH)" that the exotic species can become invasive when they experience less regulation by natural enemies than the native species (Chambouvet et al. 2008). Kim et al. (2008) showed that *Amoebophrya* strains from various host dinoflagellates and various parts of the world formed a strongly supported monophyletic group and are subdivided into three clades (Groups I to III). They also found that the strains of the parasites do not seem to be linked with either the phylogeny of their dinoflagellate hosts or their geographic origin, i.e., the strain infecting *Prorocentrum minimum* belonging to Group I is more closely related to the strain infecting *Kalrodinium veneficum* (Group I) than to the parasite infecting the same genus of the dinoflagellate *Prorocentrum micans* (Group II) (Kim et al. 2008). They also discovered that the strains of Group II tend to infect the cytoplasm of the host, while members of other groups tend to infect the nucleus of the host dinoflagellates. The quantitative distributional patterns of free-living stage (dinospore) and infected stages of Amoebophrydae along the transect of the Mediterranean Sea from coastal to open ocean oligotrophic locations were investigated using fluorescent in situ hybridization technique (FISH) (Siano et al. 2011). In this investigation, these authors enumerated (1) total eukaryotic cells; (2) Amoebophyridae dinospores; and (3) infected hosts and prevalences. Siano et al. (2011) found that the dinospores were more abundant at a coastal station (maximum 1.5×10^3 cells/ml) than in oligotrophic waters (maximum 51 ± 16.3 cells/ml), where they represented 10.3–34.9% of the total eukaryotic community at 40 and 30 m depth. They also demonstrated a positive correlation between dinospore occurrence and higher concentration of $NO_3 + NO_2$ at the coastal station.

The genus *Ichtyodinium* is known as an endoparasite, infecting eggs of pelagic marine fish and formally a single species, *I. chabelardi*, is known (Hollande and Cachon 1952). Skovgaard et al. (2009) observed development of *I. chabelardi* infecting sardines (*Sardina pilchardus*). The early stage of infection was recognized as the presence of one or more spherical, almost transparent structures inside the yolk sac of embryos. The yolk sac is then filled with the parasite's cell mass. Finally, the *Ichthyodinium* cells are released from the yolk sack of hatched larvae. Skovgaard et al. (2009) demonstrated the production of actively swimming cells after release. The motile cells show S-shaped dinokont morphology with a twisted cingulum. Based on the molecular data, genetically identical parasite species can infect several different fish eggs (Skovgaard et al. 2009). Although two genotypes have been found between the parasites from different regions, i.e., Atlantic region and Pacific region (South East Asia), the sequence differences of SSU rDNA between these two genotypes are rather small (98 % identity) and whether these two genotypes represent distinct species or not is not clear (Sørensen et al. 2014) .

16.4 Parasitic Dinoflagellates Belonging to the "Core" Dinoflagellates

The members of the class Dinophyceae consist of both photosynthetic and heterotrophic representatives. Most of the heterotrophic dinoflagellates are free-living members, such as *Protoperidinium* and its related genera, members of the Dinophysiales (some have kleptochloroplasts or extracellular cyanobacterial symbionts) and sand-dwelling *Amphidiniopsis* spp., etc. However, there are parasitic dinoflagellates belonging to the core dinoflagellates. The parasitic genera that have been confirmed to belong to the core dinoflagellates by molecular means include *Amyloodinium*, *Blastodinium*, *Chytriodinium*, *Dissodinium*, *Haplozoon*, *Oodinium*, *Paulsenella* and *Tintinnophagus* (Fig. 16.1).

The genus *Blastodinium* live as parasite in copepods. *Blastodinium* spp. can commonly be detected in living copepods as large greenish or brownish bodies in their gut (Fig. 16.3a) (only one species, *B. hyalinum*, is known to be colorless) (Fig. 16.3b). So far, 13 recognized species and one subspecies in *Blastodinium* have been recognized, but the presence of more (hitherto unknown) species diversity within the genus has been suggested (Skovgaard et al. 2012). The shape of *Blastodinium* in a copepod gut generally looks spindle-shaped, and the species of this genus have been divided into three groups based on the shape of the parasite body (Chatton 1920). The key characters to identify the species of this genus are the host species, the size and shape of parasites, and the pattern of division during sporocyte production. The number of sporocytes produced by each trophocyte is also a taxonomic character. A pathway of infection is not clear, but it is believed that dinospores are ingested and settle themselves inside the gut of their host (Chatton 1920). *Blastodinium* does not attach to the host body, but the anterior beak of young trophocytes and/or the spinules of the helicoidal crests may function as anchoring devices (Skovgaard et al. 2012). Infected copepods look like they are in good health, although generally infection leads to a size reduction of the hosts. Infection with *Balstodinium* induces castration of infected female hosts (Skovgaard et al. 2012). Experimental transplantation was not successful (e.g., Skovgaard 2005), but portions of the life cycle have been revealed from observations in living hosts. The initial parasitic cell (trophocytes) is binucleate, pelliculate and an elongated oval in shape (Fig. 16.3c). In the host gut, the trophocyte may divide directly to produce equal daughter trophocytes, and each trophocyte may undergo palisporogenesis within its original wall and produce sporocytes (Fig. 16.3c). After repeated division of the sporocytes, they are expelled from the host body. Initially, expelled sporocytes are nonmotile, but later they become dinospores (Chatton 1920; Skovgaard et al. 2012). The detail of ultrastructure of *Blastodinium* has been reported by Skovgaard et al. (2012). The nucleus of *Blastodinium* shows characteristics of dinomitosis, but the appearance of chromosomes seems to be different between the life cycle stages. In the trophocyte the chromosomes are decondensed with a large amount of granular contents (ribosomal subunits) around; progressive condensation takes place during sporogenesis and the chromosomes look like that of a typical

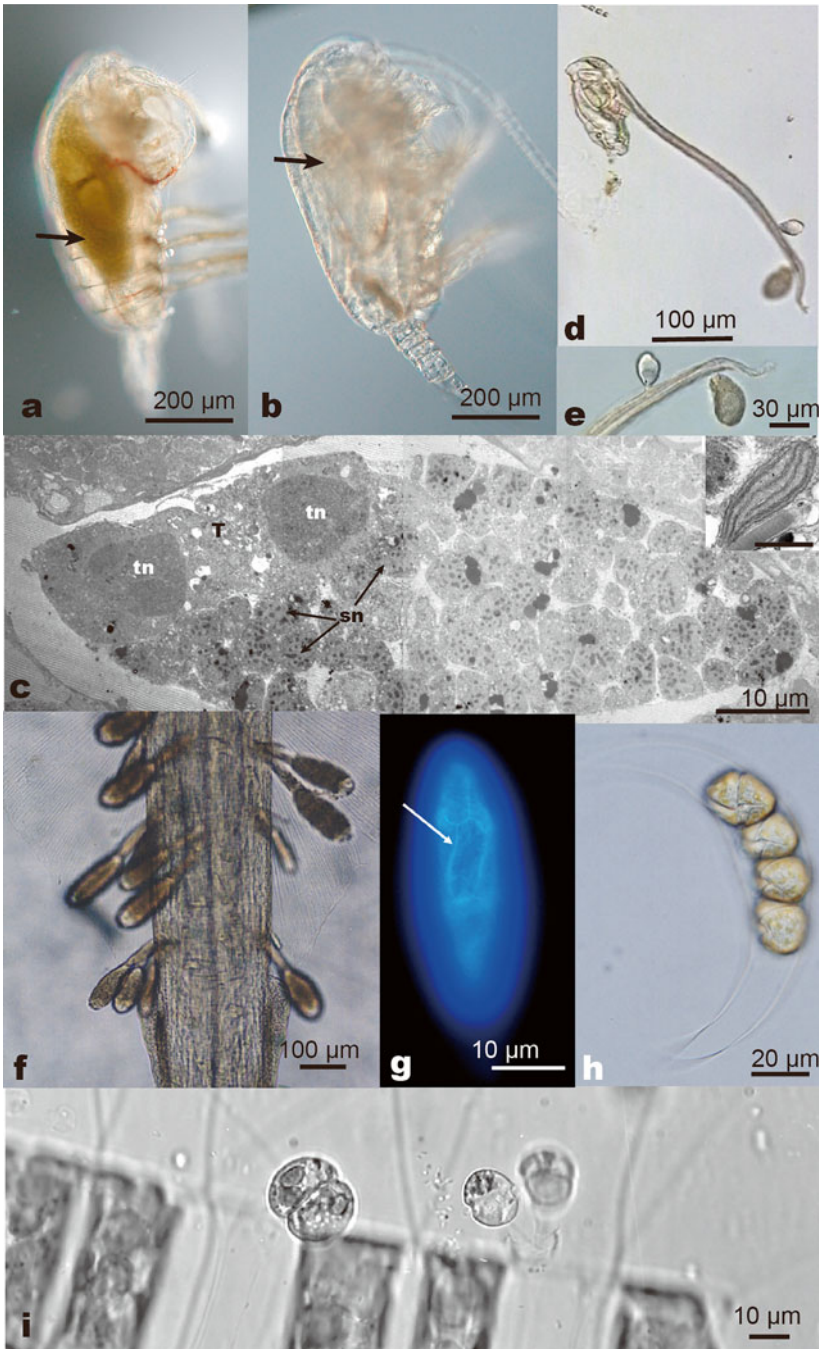

Fig. 16.3 Parasitic dinoflagellates belonging to "core" dinoflagellates. (**a**) *Blastodinium inornatum* infecting a copepod, *Paracalanus parvus* s. l. (**b**) *Blastodinium hyalinum* in *Paracalanus parvus* s. l. (**c**) Longitudinal section of *Blastodinium navicula*, showing two large trophocyte nuclei (tn) in a trophycyte (T) and surrounding many sporocytes. In the sporocyte, a typical dinoflagellate

dinokaryon (Soyer 1971; Skovgaard et al.. 2012) (Fig. 16.3c). Many of the parasitic dinoflagellates are known to be devoid of chloroplasts, but in *Blastodinium*, well-developed chloroplasts are present. However, under the TEM, the chloroplasts in the trophocyte are not well developed and are reminiscent of etioplasts (Soyer 1970), but in the sporocytes, the chloroplasts (Fig. 16.3c, inset) are well developed and peripherally located (Skovgaard et al. 2012). Photosynthetic activity of these chloroplasts has been examined, and it was revealed that these chloroplasts partly contribute the parasite's energy resource (Pasternak et al. 1984). Later, Skovgaard et al. (2012) demonstrated that photosynthetic activity of *B.* cf. *mangini* is less than half of that of free-living, mixotrophic dinoflagellates and suggested that the estimate by Pasternak et al. (1984) may be a comparatively high estimate and that a substantial part of energy that the dinoflagellate requires is obtained from the host (Skovgaard et al. 2012). The shape of the matured dinospores is typical of thecate peridinioid dinoflagellates and the plate arrangement has been reported (Skovgaard et al. 2007, 2009, 2012). The recent knowledge on taxonomy, phylogeny, ecology and distribution have been reviewed by Skovgaard et al. (2012). As the morphology of the dinopore suggests, the molecular phylogenetic studies showed that the members of *Blastodinium* belong to the "core" dinoflagellates (Fig. 16.1). In the phylogenetic analyses, the genus was shown to be monophyletic with relatively low support (Coats et al. 2008; Skovgaard and Salomonsen 2009), but other works did not support its monophyly (e.g., Skovgaard et al. 2007; Alves-de-Souza et al. 2011).

The genus *Oodinium* is an ectoparasitic dinoflagellate, belonging to the family Oodiniaceae, order Blastodiniales, class Blastodiniphyceae (Fensome et al. 1993). Its hosts include annelida, appendicularia, acantharia, chaetognaths and Thaliacea (Cachon and Cachon 1987). The genus was established based on the type species, *O. puchetii*, by Chatton (1912) as a parasite of the appendicularia, *Oikopleura*. The members of the genus *Oodinium* possess a typical dinokaryotic nucleus in the dinospores stage as well as in the young trophont stage only (Fig. 16.3c, d). As the trophont matures, the chromosomes become diffused and the typical dinokaryotic nature is lost (Cachon and Cachon 1977, 1987) (Fig. 16.3e, f). The dinospores of *Oodinium* show typical dinospores morphology with an apparent cingulum (Chatton 1912), thus supporting the inclusion of this genus in the core dinoflagellates. The most characteristic structure of the *Oodinium* cell is the presence of a distinct stalk, which is an anchoring organelle on the host body surface and may act as a feeding apparatus. Other allied genera, *Amyloodinium, Crepidoodinium,* and *Piscinoodinium*, also have a similar anchoring apparatus. However these genera are distinguished by

Fig. 16.3 (continued) nucleus (sn) and chloroplast profile (**c**, *inset*, *scale bar* = 500 nm) can be seen. (**d**) *Oodinium pouchetii* infecting *Oikopleura* sp. (From Nagasawa 2004 with permission). (**e**) Close-up photograph of *Oodinium pouchetii*. (**f**) *Oodinium inlandicum* heavily infecting a chaetognath, *Sagitta crassa* (From Horiguchi and Ohtsuka 2001 with permission). (**g**) Calcofluor-stained *O. inlandicum*, showing thecal plates (From Horiguchi and Ohtsuka 2001 with permission). (**h**) Secondary cyst of *Dissodinium pseudolunula* (Photograph courtesy of Dr. Y. Takano). (**i**) *Paulsenella chaetoceratis* cells attaching setae of a diatom *Chaetoceros. Left*: two secondary cysts (Photograph courtesy of Dr. G. Drebes)

the following. The genus *Amyloodinium* is distinguished from *Oodinium* by the difference in the shape of the anchoring apparatus, and by production of starch grains in the former (Brown and Hovasse 1946). The genera *Crepidoodinium* and *Piscinoodinium* are characterized by the possession of chloroplasts and the lack of thecal plates (Lom 1981). The pattern of sporogenesis is palintomy. Detaching of a sufficiently matured *Oodinium* from the host induces mitosis and cytokinesis after 7–8 h and subsequently produces smaller sporocytes. Usually the rupture of the stalk precedes sporogenesis (Cachon and Cachon 1987). At least some of the species of the genus, such as *O. pouchetii*, *O. dogieli*, *O. jordani* and *O. inlandicum* (Fig. 16.3h, i), have been shown to possess thecal plates even during the trophont stage, similar to those of peridinialean free-living dinoflagellates, although the accurate thecal plate arrangement has never been analyzed (Hovasse 1935; Cachon and Cachon 1977; McLean and Nielsen 1989; Horiguchi and Ohtsuka 2001). The genetic sequences of members of the genus *Oodinium* have not been deposited in the GenBank, so far. Here we were able to sequence 18S rDNA of *O. inlandicum* (Fig. 16.1). As the tree shows, *O. inlandicum* is included in the "core" dinoflagellate lineage, but showed no clear affinities with other dinoflagellates currently known in the genetic sequences (Fig. 16.1).

Other parasitic dinoflagellate genera belonging to the core dinoflagellates (class Dinophyceae) include *Duboscquella*, *Duboscquodinium*, *Tintinnophagus*, *Paulsenella*, and *Haplozoon.* The first three genera are known to parasitize tintinnid ciliates (Coats et al. 2010, 2012; Coats and Bachvaroff 2013). As mentioned above, Coats et al. (2012) noted that the type species of the genus *Duboscquella*, *D. tintinnicola*, produces a typical dinoflagellate type of dinospores with a distinct cingulum and sulcus, and they decided to reclassify *D. tintinnicola* to the family Dubosquellidae in the class Dinophyceae. The genus *Duboscquodinium* was described based on the type species *D. collini*, which is known as a parasite of the ciliate *Eutintinnus fraknoii* (Chatton 1952). *D. collini* is apparently different from syndinialean parasites of ciliates, such as *Euduboscquella*, in having a dinokaryotic nucleus with condensed chromosomes. The genus *Tintinnophagus* was recently established based on the type species *T. acutus*, which is an ectoparasite of the ciliate *Tintinnopsis cylindrical*. The dinospores of *T. acutus* show typical morphology of a dinokont, with acute apex and rounded antapex. Interestingly, although no chloroplasts are present, a prominent eyespot is seen in the sulcal region (Coats et al. 2010). The sequences of *D. tintinnicola* are not available at this stage, but the molecular phylogenetic trees indicate that both *T. acutus* and *D. collini* are included in the clade consisting of core dinoflagellates (Coats et al. 2010; Coats and Bachvaroff 2013) (Fig. 16.1), but the phylogenetic affinities with these two species and with other dinoflagellates are not consistent from tree to tree.

The genus *Dissodinium* is an ectoparasite of copepod eggs. Currently, the genus contains two species, *D. pseudolunula* and *D. pseudocalan*i (Gómez et al. 2009). Elbrächter and Drebes (1978) and Gómez et al. (2009) described life cycle stages of *D. pseudulunula.* The dinospore attaches to the copepod egg and sucks up content

from the egg myzocytotically using the structure called a peduncle. The dinospore becomes bigger and finally reaches the size of the copepod egg. After feeding, the dinoflagellate forms the primary (spherical) cyst wall. Inside the primary cyst, 4–16 crescent shaped or ovoid secondary cysts are formed. Each secondary cyst forms 4–16 *Gymnodinium*-like dinospores, which possess yellowish chloroplasts (Fig. 16.3j) (Elbrächter and Drebes 1978). On the other hand, *D. pseudocalani* is devoid of chloroplasts (Drebes 1969). The genus *Cytriodinium* is an ectoparasite of copepod and krill eggs (Gómez-Gutiérrez et al. 2009). Gómez et al. (2009) reported mode of life cycle of *C. affine* in some detail. They also investigated phylogenetic affinities of *C. affine, C. roseum* and *D. pseudolunul*a and found that all three species formed a moderately supported clade within the *Gymnodinium sensu stricto* clade (Daugbjerg et al. 2000) (Fig. 16.1).

The members of the genus *Paulsenella* are colorless ectoparasites of marine diatoms (Fig. 16.3i). Three species are known so far. The colorless motile dinospores, which possesses typical dinokont morphology, are attracted to diatoms by a chemotactic response (Schnepf and Drebes 1986). Although the motile cells of *Paulsenella* have been regarded to be unarmored (= *Gymnodinium*-like) (Drebes and Schnepf 1988), based on its phylogenetic position—i.e., *Paulsenella* formed a clade with *Amyloodinium* and *Pfiesteria piscicida* and their allies and all the latter dinoflagellates possess thin peridinioid thecal plates—Kühn and Medlin (2005) suggested that the dinospores of *Paulsenella* must have thin thecal plates. After attachment, the dinospore forms a peduncle and feeds on the content of the diatom cell by myzocytosis. After feeding, the trophont (primary cyst) begins binary fission inside the cyst wall. The cell division takes place within the primary cyst and two daughter cells are formed (Fig. 16.3i). Each daughter cell develops a cyst wall and thus becomes a secondary cyst. Sometimes, the secondary cysts further divide to form tertiary cysts (and rarely even quaternary cysts are formed). The final cyst stage functions as a dinosporangium and forms motile dinospores (Drebes and Schnepf 1988).

The members of the genus *Haplozoon* are parasites, infecting intestines of polychaete worms. *Haplozoon* spp. possess very unusual morphology. The superficial multicellular body of *Haplozoon* consists of three parts, i.e., an anterior trophocyte with a retractable stylet that penetrates the gut surface of the host animal, one or multiple rows of rectangular gonocytes and posterior rows of sporocytes (Rueckert and Leander 2008). The study by Leander et al. (2002) revealed that the body of the haplozoan is not truly multicellular or colonial, but is enveloped by a single, continuous membrane and compartmentalized by internal sheets of alveoli. Early ultrastructural study revealed that *H. axiothellae* possesses a typical dinokaryon (Siebert and West 1974). The studies by Leander et al. (2002) and Rueckert and Leander (2008) characterized features of surface morphology of the haplozoans in detail. Shumway (1924) reported production of typical dinospores. Rueckert and Leander (2008) showed a clade of *Haplozoon* spp. positioned at the base of "core" dinoflagellates (Dinophyceae) and similar topology was obtained in this study (Fig. 16.1).

16.5 Perspectives

Because of discovery of syndinialean (environmental) sequences from all over the marine environments, the importance of parasitic dinoflagellates in the marine ecosystems is apparent, but at the same time, it is realized that we have understood only minor portions of the true biodiversity of these parasitic life-forms. To understand the ecological and evolutionary role of syndinialean parasites in the oceanic environments, unveiling of the species biodiversity of these parasites will be required. In addition, the species diversity of parasites other than syndinialean dinoflagellates should also be studied, because molecular work suggests the presence of further diversity of parasitic dinoflagellates. Although many molecular phylogenetic studies on these parasitic dinoflagellates have been published, as seen in this chapter, the phylogenetic affinities of these dinoflagellates and other (free-living) dinoflagellates are still partly ambiguous; therefore, further studies using other molecular markers will be required. Because the parasitic dinoflagellates can have a large impact on other organisms and on human activities, such as fisheries, various types of research projects to investigate life cycles, detection methods, host–parasite specificity, biological controlling mechanisms and geographic distributions, etc., should be actively carried out.

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Chapter 17
Biology of Symbiotic Dinoflagellates (*Symbiodinium*) in Corals

Hiroshi Yamashita and Kazuhiko Koike

Abstract The chapter summarizes the diversity and ecology of zooxanthellae, especially focusing on the dinoflagellate genus *Symbiodinium. Symbiodinium* spp. are known to engage in mutual symbioses with a wide variety of marine invertebrates (e.g., corals) and protists, in both tropical and temperate waters. Because of an increasing awareness of "coral bleaching" (death of corals due to loss of symbiotic algae), there have been many investigations into *Symbiodinium* and coral interactions. In this chapter, the taxonomy and general ecology of *Symbiodinium* are initially described, followed by recent genetic classification systems, which have contributed to a clearer understanding of *Symbiodinium* diversity and its host specificity. Finally, mechanisms of both symbiotic initiation and breakdown in association with corals are highlighted, to predict the future of coral reef ecosystems in the changing ocean environments.

Keywords Zooxanthella • *Symbiodinium* • Dinoflagellate • Coral reef • Coral bleaching • Climate change • Symbiosis

17.1 The Basic Nature of *Symbiodinium*

17.1.1 Zooxanthella and Symbiodinium

Coral reefs are often referred to as "rainforests of the sea" because they are one of the most biologically diverse and ecologically important habitats in the oceans. Scleractinian corals are primarily responsible for building the reef structure, and they coexist with symbiotic microalgae commonly referred to as "zooxanthellae". This partnership combining a heterotroph (animal host) with an autotroph (algal

H. Yamashita
Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Fisheries Research Agency, 148-446 Fukai-Ohta, Ishigaki, Okinawa 907-0451, Japan

K. Koike (✉)
Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan
e-mail: kazkoike@hiroshima-u.ac.jp

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symbiont) into a single functional unit is known as a "holobiont", and they substantially contribute to coral reef productivity.

The term "zooxanthella" (plural: "zooxanthellae") was first recognized as a symbiont genus (originally described as *Zooxanthella nutricula*) in a radiolarian (Brandt 1881), but is often used broadly to indicate an assortment of golden-brown microalgae living in various host animals in marine environments (Blank and Trench 1986). This colloquial term "zooxanthellae", which is without taxonomic significance, includes a high phyletic diversity of microalgae, with members belonging to Bacillariophyceae, Cryptophyceae, Rhodophyceae, and Dinophyceae (reviewed in Blank and Trench 1986; Trench 1993; Rowan 1998). Based on the pioneering work of Taylor (1971) who identified dinoflagellate symbionts by virtue of their systematic affinities with free-living dinoflagellates, both gymnodinioid and amphidinioid dinoflagellates were recognized to be prominent among zooxanthellae within invertebrates. Among these dinoflagellates, the amphidinioid dinoflagellates, *Amphidinium* spp., are probably only a minor component found within a limited group of animals, i.e., flatworms and radiolarians. *Symbiodinium*, recognized as a gymnodinioid dinoflagellate, is a major group of zooxanthellae in invertebrates and protozoan symbiosis, and is the most well studied. Of note, the Acoelomorph worm, *Waminoa litus*, harbors both *Amphidinium* and *Symbiodinium* simultaneously (Hikosaka-Katayama et al. 2012).

17.1.2 Symbiodinium *Taxonomy*

Kawaguti (1944) was the first to observe that symbiotic zooxanthellae of the reef coral *Acropora corymbosa* produce motile gymnodinioid-like swarmers when isolated from their host coral, confirming those symbionts as having dinoflagellate affinities. After that, this gymnodinioid-like dinoflagellate was formally described as a new genus, *Symbiodinium*, and a new species, *Symbiodinium microadriaticum* (Freudenthal 1962). It was once classified within the order Gymnodiniales, and as a taxonomic synonym *Gymnodinium microadriaticum* (Frendenthal) Taylor, but now has been classified within a distinct order, Suessiales. Other *Symbiodinium* "species" with formal species descriptions are *Symbiodinium pilosum* Trench and Blank 1987 ex. Trench 2000, *Symbiodinium goreaui* Trench and Blank 1987 ex. Trench 2000, *Symbiodinium kawagutii* Trench and Blank 1987 ex. Trench 2000, *Symbiodinium microadriaticum* subsp. *condylactis* Blank and Huss 1989, *Symbiodinium* (=*Gymnodinium*) *linucheae* (Trench and Thinh 1995) LaJeunesse 2001, *Symbiodinium natans* Hansen and Daugbjerg 2009, *Symbiodinium minutum* LaJeunesse et al. 2012, *Symbiodinium psygmophilum* LaJeunesse et al. 2012, and *Symbiodinium voratum* Jeong et al. 2014. There are several additional binomials that appear in some literature, but these are still without formal descriptions (i.e., *nomina nuda*).

17.1.3 Symbiodinium *Cell Cycle and Architecture*

Under cultured conditions, *Symbiodinium* species exhibit diel morphological alterations between a motile phase during the daytime and a coccoid phase at night (e.g., Fitt et al. 1981). Cells in the motile phase possess transverse and longitudinal flagella, like other planktonic dinoflagellates, and swim in a circular motion (e.g., Freudenthal 1962; Steele 1975). Toward evening, cells discard the flagella and transform into a motionless spherical form (Fig. 17.1a, b). Cell surface structure is the most prominent distinction between the phases: the motile cells are covered with dinoflagellate characteristic "amphiesmal vesicles," which contain thin cellulosic thecal plates, whereas the coccoid cells have a thick "pellicle layer" (e.g., Loeblich III and Sherley 1979; Schoenberg and Trench 1980b) (Fig. 17.1c, d). The thecal plate tabulation and the surface structure of the pellicle layer are criteria for

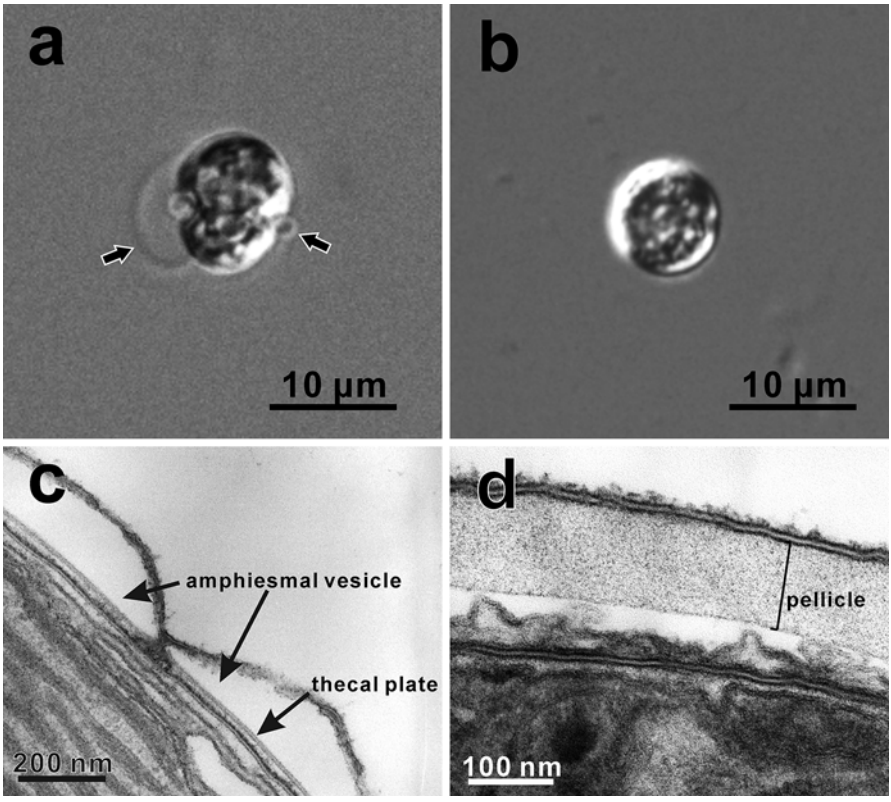

Fig. 17.1 Light micrographs of cultured *Symbiodinium* cell during the daytime (**a**: motile stage cell), and nighttime (**b**: coccoid stage cell). The motile stage cells have two flagella (*arrows*), while coccoid stage cells have no flagella and are spherical in form. Transmission electron micrographs of cell surface of motile cell (**c**) and cocoid cell (**d**). The motile cells are covered with amphiesmal vesicles which contain thecal plates, whereas coccoid stage cells are limited by a pellicular layer

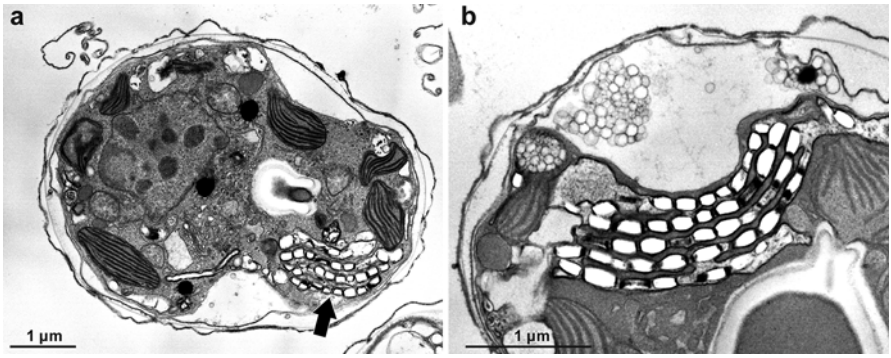

Fig. 17.2 Transmission electron micrographs of a cultured *Symbiodinium* cell during the daytime. An eyespot locates near the sulcal region of the cell (**a**: *arrow*). An eyespot consists of several layers of crystalline deposits of uric acid (**b**). The original figures were in Yamashita et al. (2009)

Symbiodinium identification (Trench and Blank 1987; Hansen and Daugbjerg 2009; Jeong et al. 2014). The coccoid cells in culture are similar to the symbiotic phase in host tissues, although the pellicle layer is thinner in the latter phase (Schoenberg and Trench 1980b). Interestingly, the motile cell possesses an eyespot (Yamashita et al. 2009; Hansen and Daugbjerg 2009; Jeong et al. 2014), which disappears in the nocturnal coccoid phase. The eyespot consists of crystalline deposits of uric acid (Clode et al. 2009) clustered in several rows, and is located near the sulcus region (Fig. 17.2). The crystalline clusters refract and polarize light in the manner of amorphous material. The eyespot also deflects ultraviolet, blue and green light (Yamashita et al. 2009).

17.1.4 Contributions to Host Animals

Symbiodinium have been best documented as symbionts of scleractinian corals; however, they can be found within a variety of other invertebrate hosts. For example, some of the members of Cnidaria (Anthozoa, Scyphozoa, Hydrozoa), Mollusca (Bivalvia, Gastropoda), Porifera, Foraminifera, and also Heterotrichea ciliates harbor *Symbiodinium* (Hill and Wilcox 1998; Carlos et al. 1999; Pawlowski et al. 2001; Lobban et al. 2002; also reviewed in Trench 1993; Baker 2003). Most of these host animals, including corals, harbor *Symbiodinium* in the endodermal cell layer (i.e., intracellular symbiosis, e.g., Taylor 1968; Hirose et al. 2008). Some of the molluscan hosts (giant clams, e.g., genus *Tridacna* and *Hippopus*) harbor them in "zooxanthellal tubes" which arise from the clam's stomach and extend into the mantle (i.e., intercellular symbiosis) (Norton et al. 1992).

Symbiodinium cells are known to release a significant portion of their photosynthetic products. The released products are usually small molecular weight metabolites such as glycerol and glucose (e.g., Muscatine 1967; Ishikura et al. 1999).

Although there are varying degrees of dependence, the animal hosts utilize these photosynthetic products for their nutrition. For example, in the case of giant clams, more than 50 % (Trench et al. 1981) or "most" (Klumpp and Lucas 1994) of the required organic substances were supplied by photosynthetic products of the symbionts, or through direct digestion of newly divided *Symbiodinium* cells (Maruyama and Heslinga 1997). In the case of coral hosts, Tanaka et al. (2006) reported that >86 % of photosynthetically fixed carbon, and >70 % of organic nitrogen synthesized by *Symbiodinium* was translocated and accumulated in the *Acropora* coral tissues. Similarly, Tremblay et al. (2012) estimated that 78 % of photosynthetically derived carbon was translocated to a *Stylophora* coral. Corals constantly release organic matter as a result of their daily metabolism (Crossland 1987; Ferrier-Pagès et al. 1998; Tanaka et al. 2009), and 28 % of the carbon fixed by *Symbiodinium* was released from *Stylophora* coral as dissolved organic carbon (Tremblay et al. 2012). This organic matter released from corals could be readily utilized by heterotrophic microbes (Nakajima et al. 2009), and thus plays an important role as an energy carrier in coral reef ecosystems (Wild et al. 2004). *Symbiodinium* significantly contribute to coral growth and stimulates coral skeleton calcification (e.g., Barnes and Chalker 1990), and coral reef production.

17.1.5 Free-Living Symbiodinium

Survival of the host animals is entirely or partially dependent on *Symbiodinium*, and *Symbiodinium* also needs to reside in the hosts to acquire nutrients. However, many *Symbiodinium* can live independently without host animals. This can be easily understood by the fact that *Symbiodinium* can be cultured in media, although there certainly are unculturable types. A monophyletic "free-living specialized" group, which probably does not engage in symbiosis, has been found (Yamashita and Koike 2013; Fig. 17.3). This group consists of at least *S. pilosum*, *S. natans*, and *S. voratum,* and it may have evolved as a free-living lineage. They were found in a diverse array of environments, such as sand, tide pools, macroalgal surfaces, and the water column (e.g., Hirose et al. 2008; Reimer et al. 2010; Yamashita and Koike 2013; Jeong et al. 2014). They are rarely found in host tissue (e.g., LaJeunesse 2002) and were not acquired by cnidarian hosts in infection experiments—or even if they were acquired, they did not persist as symbionts (Coffroth et al. 2006; LaJeunesse 2001; Yamashita et al. 2014). Interestingly, *S. voratum* possesses a well-developed peduncle (an organelle for prey capture) used to devour bacteria and other eukaryotic microalgae (Jeong et al. 2012), and the species is the solitary exception that exhibits nocturnal motility (Jeong et al. 2014). These characteristics suggest that this species is capable of subsisting in the open environment (Jeong et al. 2014). These free-living specialists are unique among *Symbiodinium* and are a key group in understanding the evolution of symbiosis.

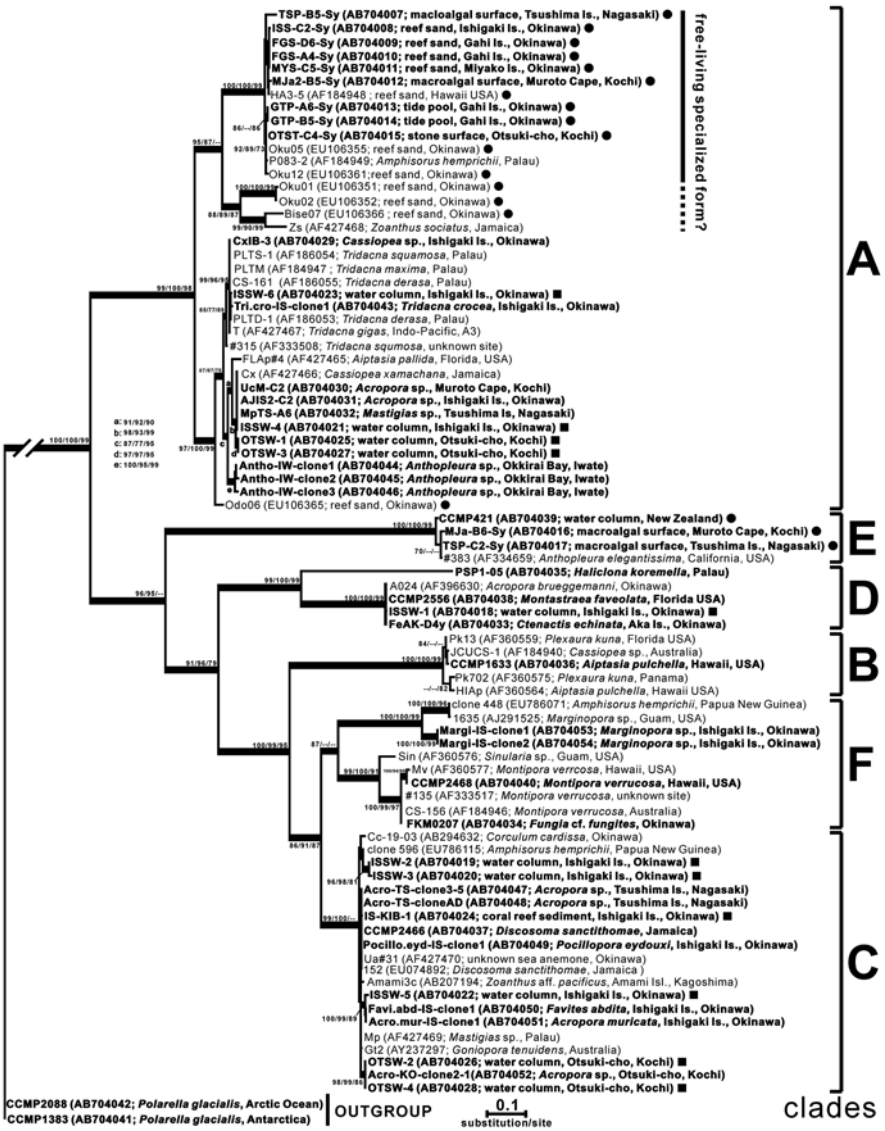

Fig. 17.3 ML phylogenetic tree of *Symbiodinium* based on internal transcribed spacer −1, −2 and nuclear 5.8S rRNA gene. Bold-typed taxa were determined by Yamashita and Koike (2013). *Solid circles* and *boxes* indicate free-living strains and environmental clones, respectively. Bootstrap values (>70 %) are shown by the *branches* (ML/MP/NJ), and Bayesian posterior probabilities (>0.95) are highlighted by *thick branches*. For more detail, see Yamashita and Koike (2013)

17.2 Genetic Diversity of *Symbiodinium*

Until the 1970s, species radiation in *Symbiodinium* was thought to be very low and rather conspecific (Freudenthal 1962; Taylor 1974). This was due to the lack of readily discernible morphological features, especially in the spherical coccoid form, based on light microscopic observations (LaJeunesse 2001). However subsequent detailed investigations—including biochemical, behavioral, infectivity, physiology, and ultrastructure characteristics—on several culture strains isolated from various host animals revealed significant differences within the genus *Symbiodinium* (Schoenberg and Trench 1980a, b, c). Nevertheless, taxonomic studies of *Symbiodinium* have been hindered by lack of coherent identification/classification schemes. Rowan and Powers (1991a, b) applied molecular genetic methods to *Symbiodinium* identification. Based on RFLP (restriction fragment length polymorphism) analysis using the small subunit ribosomal RNA gene (18S rDNA), they showed that *Symbiodinium* consisted of genetically diverse assemblages and could be divided into several genetic groups (now recognized as clades A, B and C). There have been varieties of genetic markers used for *Symbiodinium* clade identifications—for example, the nuclear ribosomal RNA gene (Rowan and Powers 1991a, b; Wilcox 1998; LaJeunesse 2001), plastid-encoded genes (Santos et al. 2002a; Takishita et al. 2003), and mitochondrial genes (Takabayashi et al. 2004). As a result, nine clades (A–I) are currently recognized (Pochon and Gates 2010). The concept of dividing *Symbiodinium* assemblages into several genetic clades, not into morphological taxonomic species, has been broadly accepted since phylotypes belonging to different clades have been shown to exhibit similar patterns of sensitivity to light and temperature (e.g., Rowan et al. 1997; Kinzie et al. 2001; Rowan 2004).

Each clade is further divided into numerous small groups usually referred to as types or subclades. LaJeunesse and Trench (2000) applied denaturing gradient gel electrophoresis (DGGE) analyses using a region encompassing the nuclear internal transcribed spacer 2 (ITS2) and a partial region of the 5.8S rRNA gene to examine the variation of *Symbiodinium* within a sea anemone *Anthopleura elegantissima*. ITS regions are potentially good markers for interspecific comparisons between *Symbiodinium* taxa (Hunter et al. 1997), and LaJeunesse (2001) identified 6, 5, 2, and 2 ITS types within clades A, B, C, and F. *Symbiodinium* classifications have been gradually shifting from the cladal level to the type level, because even closely related sister types within a clade often exhibit different patterns of sensitivity to stress (Tchernov et al. 2004; Frade et al. 2008; Sampayo et al. 2008; Hennige et al. 2009).

The types are usually represented as a combination of a letter designating the clade (A, B, C, etc) and a number (e.g., 1, 2, etc.; thus types are represented as type A1, A2, etc.). The types within a clade have been identified by a variety of molecular screening techniques (reviewed in Baker 2003; Coffroth and Santos 2005). These techniques include DGGE using the ITS2 region (LaJeunesse and Trench 2000), temperature gradient gel electrophoresis (TGGE) targeting 18S rDNA

(Carlos et al. 2000), single-strand conformational polymorphism (SSCP) targeting the ITS1 and flanking region of rDNA (van Oppen et al. 2001), and length heteroplasmy of the chloroplast large subunit (cp23S)-rDNA (Santos et al. 2002b). The DGGE and SSCP methods are used in many studies, and more than 400 ITS types are recorded (Franklin et al. 2012: GeoSymbio; https://sites.google.com/site/geosymbio/). For more fine-scale classifications, microsatellite loci have often been used. Although microsatellites are powerful markers for identifying *Symbiodinium* populations on a finer scale, it is still limited in use for certain clades. For instance, microsatellite markers for some of the members of clade A (Pinzón et al. 2011), clade B (Santos and Coffroth 2003), and clade C (Bay et al. 2009; Wham et al. 2013) have been developed.

Although these genetic classifications have been widely adopted, there is little consensus among them. The alphanumeric "type" identification schemes differ among research communities and generate additional taxonomic confusion. For example, ITS2 type B1 and cp23S type B184 are the same *Symbiodinium* type (Santos et al. 2004) and the same species (LaJeunesse et al. 2012). Therefore nomenclatural clarity and taxonomic stabilities are needed (LaJeunesse et al. 2012).

The number of formally described "species" is far smaller than the number of alphanumeric types. Although clade E seems to comprise a single species, *S. voratum* (Jeong et al. 2014), other *Symbiodinium* clades probably consist of multiple species (Finney et al. 2010; LaJeunesse et al. 2004a, b, 2010, 2012; Sampayo et al. 2009; Thornhill et al. 2014). For example, in clade A, *S. microadriaticum*, *S. microadriaticum* subsp. *condylactis*, *S. pilosum*, *S. natans*, and *S. linucheae* are represented by ITS type A1, A1.1, A2, A2 relative (closely related A2), and A4, respectively (LaJeunesse 2001; Yamashita and Koike 2013). Furthermore, *S. minutum* and *S. psygmophilum* are represented by ITS type B1 and B2, respectively (LaJeunesse et al. 2012). Other *Symbiodinium* species, *S. goreaui* and *S. kawagutii*, belong to clades C and F respectively (LaJeunesse 2001); however, many additional species within these clades are expected to be found.

The previously mentioned rDNA and mitochondrial cytochrome oxidase 1 analyses imply that the degree of separation between clade radiations is similar to different genera, families, and even orders in other dinoflagellates (Rowan and Powers 1992; Stern et al. 2010). Future taxonomic revision of this genus is required, and many of the clades will probably be reclassified into distinct genera (LaJeunesse et al. 2012).

17.3 Mechanisms of Symbiosis

17.3.1 Symbiosis Establishment

In the initial stage of symbiosis, new generations of host animals must acquire *Symbiodinium* by one of two plausible modes: vertical transmission (acquisition by maternal inheritance, i.e., a "closed system") or horizontal transmission (acquisition

from the environment: i.e., an "open system") (Trench 1987). In vertical transmission, *Symbiodinium* cells are directly inherited via the egg (Hirose and Hidaka 2006; Hikosaka-Katayama et al. 2012), therefore this acquisition mode guarantees inheritance of maternal-compatible symbionts. However, horizontal transmission is rather common among animals. About 80 % of spawn-gamete type corals acquire *Symbiodinium* from environments (Baird et al. 2009). This fact may be attributed to an assumption that horizontal transmission offers an opportunity to new generations of host animals to acquire *Symbiodinium* adapted to their surrounding environments. There is still scant evidence supporting this assumption (Coffroth and Santos 2005).

The acroporid corals, the most common and abundant reef corals, also acquire their own symbionts via horizontal transmission. *Symbiodinium* genetic compositions in these acroporid corals are often different between the larvae/juvenile stage and the adults. In Pacific coral reefs, adult acroporid corals mainly harbor clade C *Symbiodinium* (LaJeunesse et al. 2004a), although background levels of other *Symbiodinium*, for instance clade D, are sometime detected (Mieog et al. 2007; Correa et al. 2009; Silverstein et al. 2012). However, in the juvenile stage, they seem to be more flexible in acquiring other clades, and the clades can shift during growth. On the Great Barrier Reef, 83-day-old *Acropora longicyathus* juveniles harbored clades A, C, and D, although 10-day-old juveniles associated with clade A (Gómez-Cabrera et al. 2008). Abrego et al. (2009a, b) demonstrated that clade D is the main symbiont type in 1-month-old *Acropora tenuis* and *Acropora millepora* juveniles on the Great Barrier Reef. In these field observations, clade A and/or clade D seem to be initially acquired by *Acropora* juveniles. This might be attributed to the tough and opportunistic ("weedy") characteristics of clade A and/or D (Rowan 1998; Toller et al. 2001; Baker 2001, 2003). Previous laboratory infection tests revealed that acroporid coral larvae can acquire a wide variety of *Symbiodinium* clades (A, B, C, D, and F) (see Cumbo et al. 2013). These results may indicate that symbiont recognition systems are inactive or underdeveloped in the larvae (Cumbo et al. 2013), and thus the symbionts of juvenile acroporid corals are dominated by highly infectious and potentially opportunistic symbionts (Abrego et al. 2009a), like clade A and/or clade D. This flexibility for symbiont preference in the juvenile does not simply reflect the environmental composition and/or the abundance of *Symbiodinium* clades. Yamashita et al. (2013) quantified environmental *Symbiodinium* cell densities for about 1 year using quantitative real-time PCR, and found that *Symbiodinium* in the water column at Urasoko Bay, Okinawa, Japan, mainly consisted of clade C *Symbiodinium* with far smaller numbers of clades A and D. If the coral recruits randomly acquire symbionts, dominant clade C should be detected, however, naturally settled *Acropora* recruits in this bay usually harbored clade A and/or clade D, as observed on the Great Barrier Reef (Yamashita et al. 2013). A laboratory experiment also showed apparent preference of particular *Symbiodinium* clades or types in coral juveniles at the initial infectious stage (Yamashita et al. 2014). The *Symbiodinium* populations in the sediments are also important for the initial symbiont uptake. Littman et al. (2008) reported a greater density of *Symbiodinium* in the sediment compared with that in the water column of the Great Barrier Reef, and the

Symbiodinium compositions of reef sediments are more diverse compared with that of the water column (Takabayashi et al. 2011; but see Pochon et al. 2010). Indeed, laboratory experiments have shown that acroporid coral larvae can acquire *Symbiodinium* after contact with sediments (Adams et al. 2009; Cumbo et al. 2013).

Even putting aside cladal preference, corals must acquire *Symbiodinium* from the environment anyway. It is thus likely that corals possess some mechanism for discriminating *Symbiodinium* from other planktonic particles and for selecting them from the environment. Mechanisms underlying this process are not still clear; however, lectin-mediated recognition has been proposed. Lectins, which are multivalent sugar-binding proteins, are known as pattern recognition receptors, and have been shown to play important roles in the innate immune response in invertebrates. A lectin, identified from octocoral *Sinularia lochmodes*, was densely distributed on the surface of *Symbiodinium* cells in the host tissue (Jimbo et al. 2000), and was revealed to induce a morphological change in *Symbiodinium* cells from the flagellated swimming stage into the nonmotile coccoid stage, the latter similar to the form in the hosts, while maintaining *Symbiodinium* growth. Interestingly, the lectin had an adverse effect on the other non-symbiotic microalgae; it destroyed them or disturbed their growth (Koike et al. 2004). Jimbo et al. (2013) found that the ability of the lectin to induce a morphological change in *Symbiodinium* cells can be attenuated by pre-treating the cells with glycosidases (enzymes to digest cell surface glycoconjugates) or by adding the lectin binding inhibitor (N-acetyl-D-galactosamine). These results suggest that glycoconjugates on the *Symbiodinium* cell surface are the key ligand through which the lectin induces morphological transformation. Similar mechanisms have also been found in scleractinian corals (Wood-Charlson et al. 2006; Jimbo et al. 2010). Not only important in the initial infection, lectins might be involved in maintaining the symbionts and so a breakdown of this function leads to coral breaching. Vidal-Dupiol et al. (2009) demonstrated a significant decrease in gene transcription of a lectin during coral bleaching induced by raising the water temperature.

17.3.2 Symbiosis Collapse

In recent decades, a phenomenon known as “coral bleaching” has become a serious issue and has resulted in the mass mortality of corals (Brown 1997). Coral reefs have been declining year after year, and it is estimated that almost 19 % of the world’s coral reefs have disappeared since 1950 (Wilkinson 2008). “Coral bleaching” refers to the coral skeleton’s white color becoming apparent (Fig. 17.4) because of loss of *Symbiodinium* or/and loss of their photosynthetic pigments, finally resulting in coral death (Brown 1997).

Many environmental triggers are known to lead to coral bleaching, such as elevated seawater temperatures (Jaap 1979; Hoegh-Guldberg and Smith 1989; Gates et al. 1992; Brown et al. 1995), high light intensity (Hoegh-Guldberg and Smith 1989; Banaszak and Trench 1995), salinity stress (Goreau 1964; Egaña and DiSalvo

Fig. 17.4 Coral bleaching observed at Urasoko Bay, Okinawa, Japan in 2007 (Photos taken by Koike K)

1982), cold shock (Steen and Muscatine 1987; Kobluk and Lysenko 1994), and disease (Kushmaro et al. 1996). Elevated seawater temperature is thought to be one of the main factors leading to coral bleaching. An enormous worldwide coral bleaching event was observed in 1998, due to elevated seawater temperatures caused by El Niño (e.g., Hoegh-Guldberg 1999). How elevated temperatures deteriorate the symbiosis between corals and *Symbiodinium*, and which components and mechanisms result in a breakdown, are still unclear.

Before and after the events of coral bleaching, the cladal composition of some corals shifted from clade C to clade D (Baker et al. 2004; Jones et al. 2008). Although *Symbiodinium* has many genetic types within a clade, it is known that some of the corals with clade D symbionts are more resistant to thermal stress than those with clade C symbionts (Glynn et al. 2001; Rowan 2004). Buddemeier and Fautin (1993) proposed the "adaptive bleaching hypothesis" (ABH), which states that stressed corals bleach to change the symbiont clade according to environmental conditions. The hypothesis includes two plausible modes: "switching" and "shuffling" (Baker 2003). Switching can be attributed to an uptake of exogenous symbionts (Kinzie et al. 2001; Lewis and Coffroth 2004). Shuffling can be a shift from background endogenous symbionts to a major one, which is a phenomenon that is assumed to occur in scleractinian corals (Correa et al. 2009; Jones et al. 2008; Mieog et al. 2007). Symbiont shuffling is considered to incur a risk of death during the period of shuffling (Mieog et al. 2007). Yamashita et al. (2011) observed a possible process in the "shuffling": they set a trap to coral branches in the field and collected expelled *Symbiodinium*. Even in their highly sensitive quantitative PCR analysis, only clade C was detected within the expelled population, while clade D was retained in the coral tissue of *Pocillopora eydouxi*.

Corals expel *Symbiodinium* even under non-stressful steady-state conditions (e.g., Koike et al. 2007). Titlyanov et al. (1996) reported that corals exocytose excess *Symbiodinium* populations in the gastroderm cells into the body cavity and digest them by phagocytosis at the mesenterial filaments, and finally expel them outside. They also found that the numbers of both dividing and degraded cell were

similar, and concluded that corals regulate *Symbiodinium* density by digesting and expelling excess symbiont cells. This process is a "healthy" function in corals to maintain the symbiosis, and is clearly separated from "symbiosis breakdown" under stress conditions. During this process, the expelled population is dominated by degraded and photosynthetically inactive cells. This phenomenon has been found in many scleractinian corals (Fujise et al. 2014). Under stress, corals release more "healthy-looking" *Symbiodinium*, rather than the degraded form. This is obvious under harsh thermal stress (i.e., 32 °C), and a large number of *Symbiodinium* are probably expelled due to host cell detachment (e.g., Gates et al. 1992), not through the proper digestion process. However, because many natural coral bleaching events can occur under moderate thermal stress conditions with 1–2 °C higher than usual seawater temperature for a longer duration (Goreau and Hayes 1994; Podestá and Glynn 1997, etc.), observations of the phenomena under moderate but prolonged thermal stress events are needed. Bhagooli and Hidaka (2004) and Hill and Ralph (2007) observed *Symbiodinium* expulsion phenomena under several thermal conditions, and at 30 °C in the early stages (within 24 h), the expelled populations were mainly composed of normal cells, but the degraded population became greater with prolonged exposure to 30 °C. Fujise et al. (2015) reported two *Acropora* corals actively digested and expelled damaged symbiont cells under 30 °C, but this function may not fully keep up with accumulation of the damaged cells along with prolonged exposure to the heat stress, and the corals eventually expelled normal-morphology but photosynthetically incompetent cells. This response may be an adaptive strategy to moderate thermal stress to prevent accumulation of damaged symbionts, which causes subsequent coral deterioration.

17.4 Conclusion

In this chapter, we have summarized recent progress in understanding *Symbiodinium* biology, especially focusing on its animal engagements. *Symbiodinium* comprises a genetically diverse group, which may extend to multiple genera or even families, and each might be assigned to its own specific physiology and host specificity. Conformation of characteristic physiological groups is a matter of vital importance for hosts to adapt to environments. Therefore, host animals, especially corals, seem to try many possible means to switch or shuffle more appropriate symbionts during growth and for survival under exposed stresses. *Symbiodinium* are also dependent on symbiosis, otherwise they would not grow so densely (e.g., hundreds of thousands of cells cm^{-2} of coral surfaces) in oligotrophic waters. Even considering its long relationship with corals from the mid-Triassic Period (Trench 1997), many *Symbiodinium* still maintain a free-living mode, with flagella and an eyespot, and, as a result, most animals must acquire them horizontally. The fact that the totally free-living types or species are not distantly related to other symbiotic species is also interesting in understanding the evolution of symbiosis.

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Chapter 18
Biology of Symbiotic Apostome Ciliates: Their Diversity and Importance in the Aquatic Ecosystems

Susumu Ohtsuka, Toshinobu Suzaki, Atsushi Kanazawa, and Motonori Ando

Abstract Recently apostome ciliates have been paid more attention in the aquatic ecosystems, since the host population dynamics are more highly influenced by these protists than previously expected. Apostomes are all symbiotic, associated mainly with a wide variety of planktonic and benthic crustaceans and other invertebrates such as cnidarians and chaetognaths. They can also be found within cysts of other apostomes. Some taxa require two distinct hosts.

The life cycles of apostomes are complicated, essentially consisting of four morphologically and functionally different stages: quiescent, encysted phoronts; feeding trophonts; divisional, encysted tomonts; and infective tomites, with several substages in some taxa. Each metamorphosis accompanies reformation of kineties and organelles. Excystation from phoronts to trophonts is triggered by cues such as molting, injury and predation of hosts. A cell within a phoront is furnished with cilia ready to hatch, and with specialized, membranous organelles related to rapid expansion of food vacuoles. Trophonts with or without a cytostome take nutrients through phagocytosis or pinocytosis, respectively. Some taxa such as *Gymnodinoides* are exuviotrophic and harmless to the host, while genera such as *Vampyrophrya* are regarded as parasitoids rather than parasites. Proliferation is mainly due to palintomy to produce numerous tomites within a tomont, whose duration seems to be

S. Ohtsuka (✉)
Takehara Marine Science Station, Setouchi Field Science Center,
Graduate School of Biosphere Science, Hiroshima University,
5-8-1 Minato-machi, Takehara, Hiroshima 725-0024, Japan
e-mail: ohtsuka@hiroshima-u.ac.jp

T. Suzaki
Graduate School of Science, Kobe University,
Kobe, Hyogo, Japan

A. Kanazawa
Graduate School of Science, Kobe University,
1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

M. Ando
Graduate School of Education, Okayama University,
3-1-1 Tsushimanaka, Kita-ku, Okayama 700-8530, Japan

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most greatly influenced by water temperature in the life cycle of apostomes. Tomites actively search for a new host and then transform into phoronts on it.

The present paper briefly reviews previous studies concerning apostomes, and our original data on the histotrophic species *Vampyrophrya pelagica* infecting copepods.

Keywords Apostomes • Crustaceans • Exuviotrophy • Histophagy • Host • Parasite • Parasitoid • Symbiosis

18.1 Introduction

Symbiosis has recently been paid much more attention by aquatic biologists, partly because symbiotic relationships are poorly understood in aquatic ecosystems in contrast to terrestrial ones (cf. Rohdes 2005), and partly because some protist parasitoids such as dinoflagellates and ciliates have been revealed to make a great impact on the population dynamics of their hosts (cf. Ianora et al. 1987; Kimmerer and McKinnon 1990; Gómez-Gutiérrez et al. 2003; Ohtsuka et al. 2004, 2007, 2010).

All apostome ciliates are symbiotic mainly to marine planktonic and benthic crustaceans (Chatton and Lwoff 1935; Landers 1991b; Bradbury et al. 1996; Kudo 1966; Bradbury 1974b; Gómez-Gutiérrez et al. 2003, 2012), and rarely to cnidarians (Kudo 1966; Bradbury 1974b), ctenophores (Kudo 1966; Skovgaad 2014), echinoderms (Bradbury 1974b), annelids (Kudo 1966), chaetognaths (Skovgaad 2014), ophiuroids (Bradbury 1974b) and even apostomes (Kudo 1966). Life cycles are complex, but typically consist of four morphologically and functionally different forms, namely, phoronts, trophonts, tomonts, and tomites, with some intermediate stages in some taxa (Chatton and Lwoff 1935; Kudo 1966; Bradbury et al. 1974; Bradbury 1996; Landers 2010) or without forming reproductive cysts (=tomonts) in *Polyspira delagei* (Chatton and Lwoff 1935; Bradbury 1974b). The phoront is a quiescent, encysted stage on the body surface of the host; the trophont excysted from the phoront is a feeding stage that may consume host tissues or exuvial fluids; a fully grown trophont changes into a tomont, another encysted stage in which tomites are produced by palintomy; the tomite is a free-living, infective stage searching for a new host (Bradbury 1996). The life cycle of apostomes specifically infecting the crustacean endocuticle is greatly modified with a mouthless trophont and additional stages with different cell surface structures (Bradbury et al. 1974). An intermediate condition between the trophont and the tomont (orthotomont) is sometimes called a protomont, which ceases feeding and is furnished with kineties different from those of the trophont; before being released from the tomont, cells may be called protomites. Hatching of the phoront is the critical point in the life cycle, which may be triggered by molting of the host or predation of the host by predators (Chatton and Lwoff 1935; Trager 1957; Grimes and Bradbury 1992; Landers 2004; Ohtsuka et al. 2004).

In addition to their unique life cycles, apostome ciliates are morphologically characterized by (1) the presence of a rosette-like structure in the tomites and trophonts, whose function is still unknown; (2) spiral ciliary rows in the trophonts,

with short adoral ones (Fig. 18.1a–d); (3) an oval- to band-shaped macronucleus; (4) one micronucleus; and (5) a single contractile vacuole (cf. Chatton and Lwoff 1935; Bradbury 1966a, b; Kudo 1966; Capriulo and Small 1986; Landers et al. 1996). Although apostomes have been surmised to possess an inconspicuous cytostome, histotrophic apostomes such as *Vampyrophrya pelagica* are considered to possess a cytostome, which enlarges in size to engulf partially digested host tissues (Grimes and Bradbury 1992; Ohtsuka et al 2010).

Most apostomes are exuviotrophic and harmless to their hosts, while some species are histotrophic or chymotrophic and seriously pathogenic to them (Bradbury et al. 1974; Bradbury 1996). The former includes *Gymnodinioides* (Fig. 18.1b) *Hyalophysa, Terebrospira,* and *Polyspira*, which parasitize a variety of benthic crustaceans such as crabs, shrimps and barnacles, feeding on exuvial fluid (Bradbury 1996). The following four taxa are regarded as pathogenic (Bradbury 1996). *V. pelagica* (Fig. 18.1a), infecting marine planktonic copepods, kills injured copepods and also seem to greatly influence the population dynamics of invertebrate

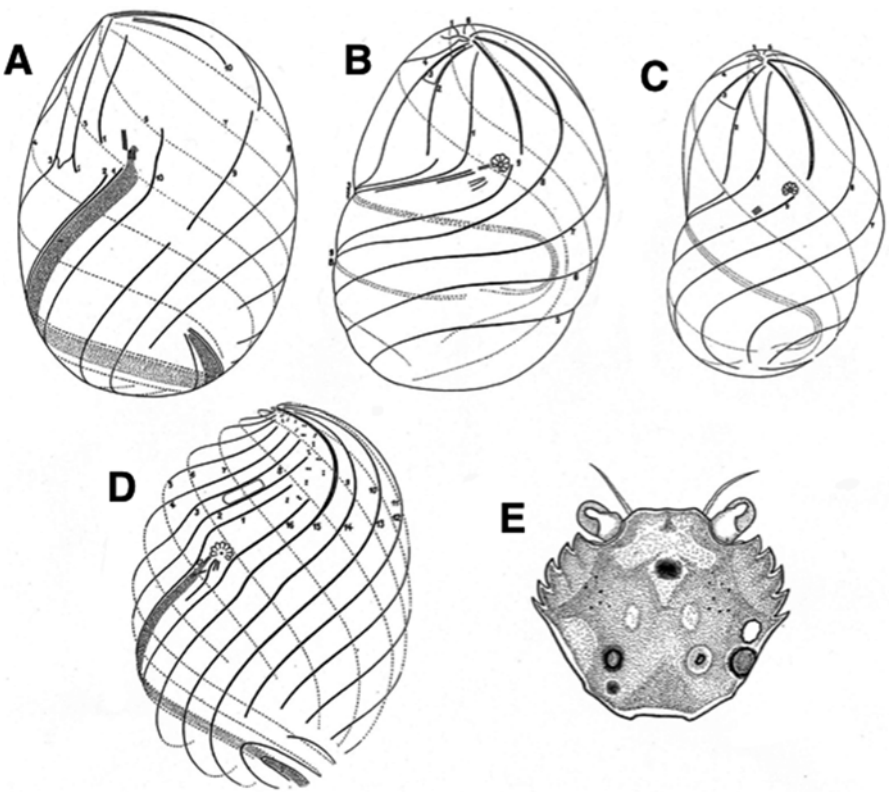

Fig. 18.1 Apostome ciliates cited from Chatton and Lwoff (1935). (**a**–**b**) Infraciliature of trophonts (**a** *Vampyrophrya pelagica*; **b** *Gymnodinioides inkystans*). (**c**) *Synophrya hypertrophica*. (**d**) *Spirophrya subparasitica*). (**e**) Melanization of crab carapace caused by feeding of *Synophrya hypertrophica* (citation permitted by Station Biologique de Roscoff)

copepod predators such as chaetognaths, ctenophores and medusae, because tissues of ingested prey copepods are consumed earlier by the trophonts rather than by these predators (Grimes and Bradbury 1992; Bradbury 1996; Ohtsuka et al. 2004). *Collinia* and *Pseudocollinia* are parasitoids infecting krill and amphipods, some of which show high prevalence of up to 98 % and cause mass mortality of the host (Capriulo and Small 1986; Capriulo et al. 1991; Bradbury 1996; Gómez-Gutiérrez et al., 2003, 2012). *Synophrya* (Fig. 18.1c) causes melanization of the cuticle of the host decapod crustaceans (Fig. 18.1e) and may be lethal to small decapods (Chatton and Lwoff 1935; Johnson and Bradbury 1976; Bradbury 1996).

Apostome ciliates are found not only on marine but also on freshwater crustaceans (Chatton and Lwoff 1935; Sewell 1951; Ohtsuka et al. 2004). For example, *V. pelagica* parasitizes shallow-water copepods in the world oceans with relatively low host specificity and high prevalence during warm-water seasons (Chatton and Lwoff 1935; Grimes and Bradbury 1992; Ohtsuka et al. 2004). Presumably phoronts of oceanic forms are frequently found on the bodies of planktonic copepods and krill (Shields 1994; Ohtsuka et al. 2003; Ohtsuka and Boxshall 2004), although their exact identity is not confirmed yet. *Gymnodinioides caridinae* and two species of *Hyalophysa* infect freshwater shrimps (Miyashita 1933; Bradbury and Clamp 1973; Landers et al. 1996).

Chatton and Lwoff (1935) have made a supreme contribution to the study of the morphology, taxonomy and life cycles of apostomes ciliates, which have been followed by Bradbury (1966a, b, 1973, 1974a, b, 1989, 1996) and her groups (1967a, b, 1973, 1974, 1976, 1987, 1992, 1996, 1997, 2008). These works have clarified the taxonomy and life cycles of many types of apostome ciliates and the cytological features of each stage. Yet, ecological surveys on apostomes are rarely carried out. The only exceptional cases are *V. pelagica*, *Hyalophysa* spp. and *Pseudocollinia brintoni* in which the life cycles, host specificities, and seasonal and distributional occurrences of phoronts on host crustaceans have been clarified (Bradbury 1966a, b, 1996; Grimes 1976; Landers 1986; Grimes and Bradbury 1992; Ohtsuka et al. 2004; Gómez-Gutiérrez et al. 2012).

Parasitological terminology essentially follows Bush et al. (2001).

18.2 Host Specificities and Site Preference

Host specificities of apostome ciliates are shown in Table 18.1. Some apostomes exhibit differentiation in attachment for phoronts and food source for trophonts. For example, *V. pelagica* and *Spirophrya subparasitica* (Fig. 18.1d) need two hosts: copepods for phoronts and predatory invertebrates such as chaetognaths and jellyfish for trophonts (Chatton and Lwoff 1935; Grimes and Bradbury 1992; Ohtsuka et al. 2004). The exuviotrophic genera such as *Hyalophysa* and *Gymnodinioides* utilize a wide variety of freshwater and marine decapods. Hosts of *Hyalophysa* have been summarized by Landers et al. (1996). In *H. chattoni* over 18 species of decapods are known as hosts, which are distributed from freshwater to marine environments (salinity: 0–30 ppt). In crabs and shrimps in Florida, USA, distinct host specificities were

Table 18.1 Hosts and feeding strategies of apostome ciliates

Taxon	Habitat[a]	Trophic mode	Host	Remark	Source
Colliniidae					
Collinia	F, M	Osmotrophic	Trophont, tomont and protomites in haemocoel of krill, isopods and amphipods	Parasitoid ciliates infecting krill	Puytorac and Grain (1975)
Pseudocolliniidae					
Fusiforma	M		Ciliates found in amphipods		Chantangsi et al. (2013)
Pseudocollinia	M	Osmotrophic	Trophont, tomont and protomites in cephalothorax and abdomen of krill; tomites form filaments in water	Blood-infecting parasitoid ciliates; astomatous at all stages of the cell cycle	Capriulo and Small (1986) and Gómez-Gutiérrez et al. (2006)
Foettingeriidae					
Calospira	M		Phoront on integuments of harpacticoid copepods; trophont in their carcasses; tomont and tomite in water		Kudo (1966)
Cyrtocaryum	M		No encystment; trophont in digestive tube of polychaetes	Trophont, astomous	Kudo (1966)
Foettingeria	M	Exuviotrophic	Phoront on copepods, ostracods, amphipods, isopods and decapods; trophont in gastrovascular cavity of actinozoans; tomont on outer surface of actinozoans	Requires dual hosts for encystment of phoronts, but feeds only on the chime of actinozoans, not on crustacean carcasses	Chatton and Lwoff (1935) and Bradbury (1974a, b)

(continued)

Table 18.1 (continued)

Taxon	Habitat[a]	Trophic mode	Host	Remark	Source
Gymnodinioides	F, M	Exuviotrophic	Phoront on integuments of decapods and krill; trophont in their molts	Food uptake by pinocytosis	Kudo (1966), Bradbury et al. (1996), and Landers et al. (2007)
Hyalophysa	M	Exuviotrophic	Phoront on integuments of decapods; trophont in their molts; tomont in molt or substrate	Abundant membrane organelles in trophonts	Bradbury (1973)
Ophiurespira	M	Exuviotrophic	Trophont in guts of ophiuroids	Abundant membrane organelles in trophonts	Chatton and Lwoff (1935) and Bradbury (1974b)
Pericaryon	M		Trophont in gastrovascular cavity of ctenophores		Kudo (1966)
Phoretrophrya	M		Phoront and tomont on appendages of phyllocarids; trophont in their molts		Kudo (1966)
Photorophrya	M		Phoront, trophont and tomite in encysted stages of other apostomes		Chatton and Lwoff (1935) and Kudo (1966)
Polyspira	M	Exuviotrophic	Phoront on gills of crabs; trophont in their molts	Food uptake by micropinocytosis; abundant membrane organelles in trophonts	Bradbury (1974b)

Spirophrya	M	Histotrophic	Phoront on crustaceans such as copepods; trophont in crustaceans when eaten by *Cladonema*; protomont leaving *Cladonema* and then tomont formed on outer surface of *Cladonema*	Requires two hosts for encystment of phoronts	Chatton and Lwoff (1935)
Synophrya	M	Histoexuviotrophic[b]	Phoront in branchial lamellae of crabs; hypertrophont in their tissue; trophont in their molts	Astomatous at hypertrophont stage; membrane organelles absent	Chatton and Lwoff (1935) and Landers (2010)
Terebrospira	F, M	Osmotrophic	Phoront on cuticle of shrimps; trophont within cuticle of shrimps; tomont on cuticle of shrimps or substrate	Astomatous at trophont stage; food uptake by micropinocytosis; membrane organelles present	Bradbury (1974b)
Traumatiophtora	M		Trophont in carcasses of marine copepods		Chatton and Lwoff (1935) and Kudo (1966)
Vampyrophrya	M	Histotrophic	Phoront on integuments of copepods; trophont in copepods or in guts of invertebrate predators feeding on copepods; tomont in copepod fed or on substrate	Requires two hosts for encystment of phoronts (not obilgatory); a large cytostome appears in trophonts; membrane organelles present	Chatton and Lwoff (1935), Grimes and Bradbury (1992), and Ohtsuka et al. (2004)

[a]*F* freshwater, *M* marine

[b]Two feeding strategies in one cell cycle (histotrophic phase in hypertrophonts and exuviotrophic phase in trophonts)

found between species of *Hyalophysa* and *Gymnodinioides* (Landers 2004): penaeid shrimps, *Farfantepenaeus* spp. were infected with both genera, while *Palaemon floridanus* and *Hippolyte zostericola* were infected only by *Hyalophysa* spp. and *Gymnodinioides* spp., respectively. The endoparasitoid genus *Collinia* exclusively utilizes isopods, amphipods and krill as hosts (Capriulo and Small 1986). Members of the genus *Photorophrya* are exceptionally ecto- or endoparasites of phoronts of the exuviotrophic apostome *Gymnodinioides*.

The host specificities and developmental stage specificities of the histotrophic *V. pelagica* infecting planktonic copepods have been well surveyed (Grimes and Bradbury 1992; Ohtsuka et al. 2004). Although its host specificity is relatively low, it tends to prefer calanoids to cyclopoids. However, some species of the cyclopoid genus *Oithona* and the harpacticoid *Microsetella norvegica* are totally rejected by this apostome (Ohtsuka et al. 2004). In contrast, another harpacticoid *Euterpina acutifrons* is prevalently parasitized by the apostome (Ohtsuka et al. 2004). Tomites have been observed to crawl around the body of these unpreferable copepods for a moment to seek attachment sites, but then to finally swim away into the water (our unpublished data). Some physiochemical cues from copepods may be needed for the attachment of the tomite. Not only adults but also naupliar and copepodid stages of copepods are highly infected by this apostome (Ohtsuka et al. 2004, unpublished data). However it is supposed that phoronts are cast off after molting of non-adult copepods without completion of its life cycle, because only exuvial fluids seem to be insufficient for growth.

Aggregation of phoronts on a particular part of their hosts' body is frequently observed. For example, phoronts of the exuviotrophic apostome *H. chattoni* have a concentrated distribution on the antennal scales, around the eyestalks, under the carapace and under the telson of the host shrimp (Landers 1986). As for gregarious phenomena, Landers (1986) suggested that a tomite may release some chemicals from the membrane-bounded canals on a settling site of the host, by which other tomites subsequently recognize the forerunner's settlement. Ohtsuka et al. (2004) found a distinct tendency for phoronts of *V. pelagica* to aggregate almost exclusively on the prosomal ventral side and/or appendages of planktonic copepods. The feeding and swimming currents of the host seem to influence its attachment site.

18.3 Life Cycle

The life cycles of some apostomes are complex. Essentially, four stages—phoronts, trophonts, tomonts and tomites—are recognized irrespective of species with different feeding habits (Figs. 18.2 and 18.3). Modifications of the essential life cycle are found in some taxa.

The life cycle of the exuviotrophic apostome *Hyalophysa* has been summarized by Bradbury (1966a), Bradbury and Clamp (1973), Grimes (1976), and Landers (1986). It is considered the most primitive pattern among those of apostomes (Chatton and Lwoff 1935; Bradbury 1974b; Johnson and Bradbury 1976). The phoront (ovoid-shaped, approximately 45–79 μm on its long axis) is attached on the lamellar folds of

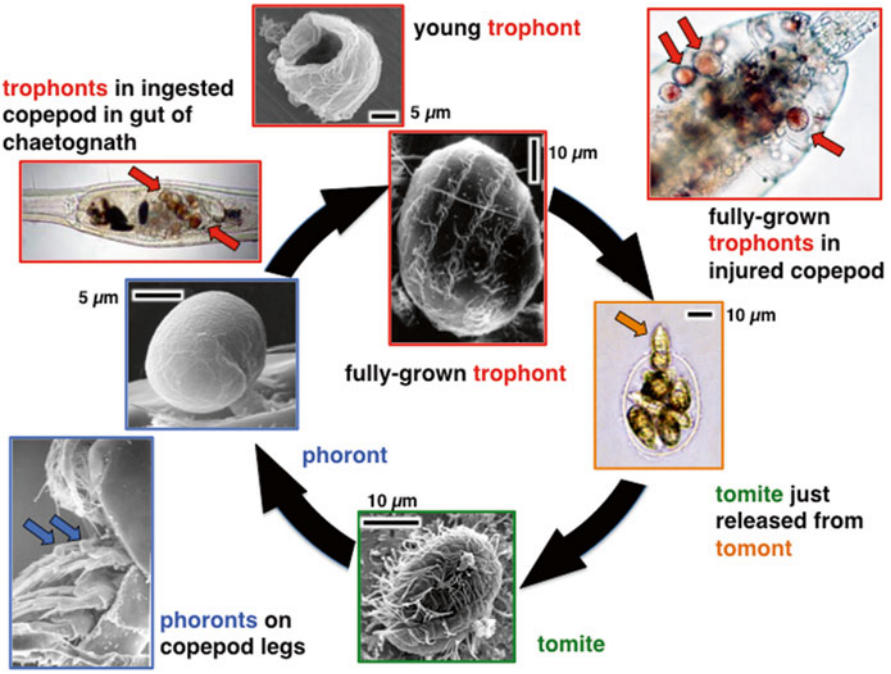

Fig. 18.2 Schematic illustration of the life cycle of *Vampyrophrya pelagica* in the Seto Inland Sea, western Japan. Hatching of the phoronts is triggered either by predation of invertebrate predators on infected copepods or by injury of infected copepods. Tomonts are usually attached to fecal pellets of predators (= secondary host) or to inside carcasses of digested copepods

the gills or on the carapace of decapods by a short peduncle. *Hyalophysa* spends most of its life (1 to over 3 weeks) on the crustacean hosts as a phoront. The trophont (ovoid, ranging from 38–50 to 180–200 μm long) excysts from a phoront just before molting of host crabs, and then actively feeds on exuvial fluids remaining in the cast-off exoskeleton. After a few and up to 24 h the fully mature trophont (protomont) swims out of the exoskeleton and settles on the substrate or remains within the exuviae to metamorphose to an encysted tomont. Palintomy occurs within the tomont to produce infective tomites (protomites). Released tomites (46×24 μm in *H. chattoni*) search for a new host, and then encyst on the host.

The life cycle of the histotrophic apostome *V. pelagica* is shown in Figs. 18.2 and 18.3. This is basically similar to that of the exuviotrophic apostome *Hyalophysa*, but differs in triggers for hatching from phoronts to trophonts and foods for the trophonts. The oval phoronts (13–25×9–18 μm) are attached mainly on the ventral side and appendages of the prosome of planktonic copepods by a short stalk of approximately 5 μm. These excyst in two ways: physical injury or predation on copepods infected by the apostome from invertebrate predators such as medusae, ctenophores and chaetognaths (Chatton and Lwoff 1935; Grimes and Bradbury 1992; Ohtsuka et al. 2004). The former and latter are called one-host and two-host life cycles, respectively. In both ways the trigger for hatching must be oozing of the body fluid of copepods. The trophonts (soon after excystation 16×14 μm; fully grown cell

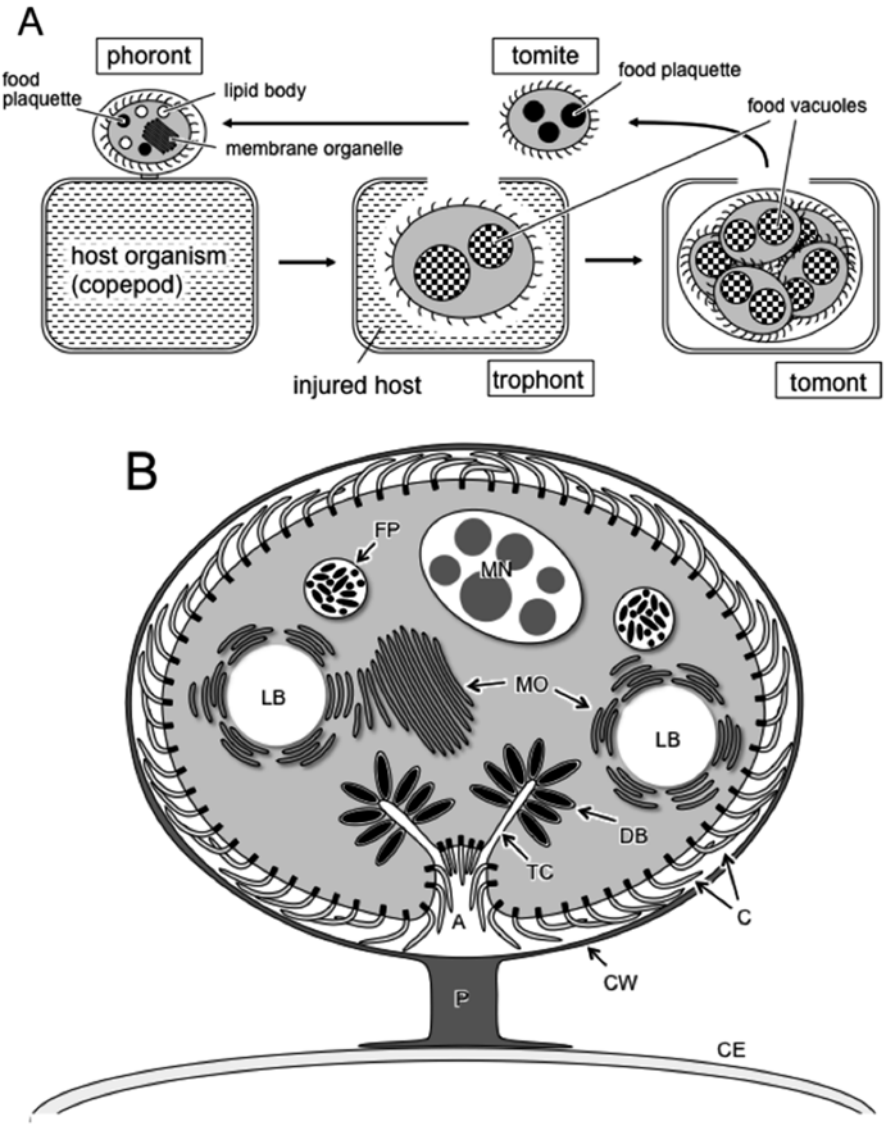

Fig. 18.3 (**a**) Schematic drawing showing the appearance of unique cytoplasmic structures in *Vampyrophrya pelagica* as observed in a stage-dependent manner. In fully grown trophonts, many food vacuoles are formed, which frequently fuse with each other to form a large mass. In this stage, no other characteristic organelles (membrane organelles, lipid bodies and food plaquettes) are present in the cytoplasm. In tomonts, contents of the food vacuoles are gradually digested, and each food vacuole reduces in size. In tomites, food vacuoles containing partially digested food materials are observed as smaller food plaquettes. At the phoront stage, many lipid bodies appear

50–90 × 30–50 μm) start devouring copepod tissues once they enter the inside through fissures. In the one-host life cycle, injured copepods are finally killed by the trophonts, suggesting that this apostome can be regarded as a parasitoid rather than a parasite. In the two-host life cycle, the trophonts can be resistant to digestion in the guts of the invertebrate predators. However, they seem to be unable to complete the cycle after predation by fish. They were easily digested by larvae of *Plecoglossus altivelis altivelis* in our laboratory experiment. Finally the diameters of fully grown trophonts become about three times as large as the initial cells. They settle inside the totally consumed copepod cuticle and metamorphose into tomonts. A tomont (48–70 × 33–59 μm) produces 2 to 32 tomites. The tomites (18–37 × 7–23 μm) are released from a pore of the cyst, and then seek a new host. The life cycle of another histotrophic apostome, *Spirophrya subparasitica* (Fig. 18.1d), is similar to that of *V. pelagica* in that it utilizes two hosts, i.e., copepods and their predators (cnidarian polyps) as the first and second host, respectively. However, it differs from the latter in tomonts attached to the second host (Chatton and Lwoff 1935).

The life cycle of the endoparasitoid *Pseudocollinia*, causing mass mortalities of krill, has been well addressed by Gómez-Gutiérrez et al. (2012), although the infection mechanism is still unknown. After host tissues are rapidly consumed by trophonts, host bodies are ruptured, and then filamentous structures comprising colonial phoronts and bacteria are released. Infection experiments using the filaments were unsuccessful in the laboratory (Gómez-Gutiérrez et al. 2012).

The life cycle of *Synophrya hypertrophica* infecting the gill lamellae and carapace of decapods is highly modified from those of the above-mentioned three taxa (Chatton and Lwoff 1935; Johnson and Bradbury 1976; Bradbury 1996). This is unique in having a double life cycle with utilization of two different food sources from a host and

Fig. 18.3 (continued) and degradation of the food plaquettes further proceeds. Membrane organelles are constructed in phoronts as a membrane reservoir for forming food vacuoles in the following trophont stage. (**b**) Schematic drawing of the cytoplasmic structures in the partially-grown phoront cell. In this stage of the life cycle, a peduncle (*P*) is formed near the atrium (*A*), by which the phoront is firmly attached to the copepod exoskeleton (*CE*). The atrium is a ventral invagination of the plasma membrane with cilia located inside the depression, and is continuous to finger-like tubular extensions called canaliculi (*TC*). Many dense bodies (*DB*) surround the tips of canaliculi, which are implicated in the process of secretion of materials for the formation of the peduncle that is continuous to the cyst wall (*CW*). The atrium, tubular canaliculi, and dense bodies are present in tomites and early-stage phoronts, but disappears when the peduncle is fully constructed. Lipid bodies (*LB*) and food plaquettes (*FP*) are also characteristic structures in trophonts. Lipid bodies are gradually formed in tomites and phoronts, and disappear in trophonts. Membrane organelles (*MO*) are frequently observed to be associated with the surface of the lipid bodies, finally forming a large mass of stacked disks in the fully-grown phoronts. In phoronts, large chromatin bodies are observed in the macronucleus (*MN*). Cilia (*C*) on the surface of phoronts are not degenerated, ensuring quick release of young trophonts from the cyst

in proliferation of trophonts (for details, refer to Fig. 5 in Bradbury 1996). After a phoront on the host gill metamorphoses to a trophont, it enters the blood sinus of the gill through a pore of the host exoskeleton as an endoparasite and then encysts just below it. Absorbing blood by osmotrophy, the encysted trophont is widely expanded and finally transforms into a tomont that produces a hundred trophonts due to palintomy when detecting impending molt in the host blood. Using a macrostome, trophonts released from the host molt feed on exuvial fluid as do ordinary exuviotrophic apostomes such as *Hyalophys*, and then encyst on the substrate as tomonts. The remaining process is similar to the life cycles of the exuviotrophs.

Another apostome ciliate that can penetrate the cuticle of decapod crustaceans is *Terebrospira*. The life cycle of this apostome is highly complex as well (Bradbury et al. 1974). This genus accommodates only two species whose life cycles differ remarkably from each other. However, that of the first described species, *T. lenticularis*, seems to be incompletely understood (see Bradbury et al. 1974, p. 684). That of *T. chattoni* on the exoskeleton of the estuarine shrimp *Palaemonetes pugio* is briefly explained herein. There are two different types of life cycle: the development of the protomonts occurs either on the new exoskeleton of the same host or free of the host. In the former development, the orthotomont is compartmented on the exoskeleton of the host. The number of daughter cells in a compartment depends on the size of protomont, ranging from 3 to 7 cells based on figures by Bradbury et al. (1974). Long before host ecdysis, each trophont exits from its compartment, and commences digging and dissolving the host endocuticle to form a tubular feeding scar called a "gallery". The gallery approximately ranges from 200 to 1000 μm, depending on the thickness of the endocuticle. The trophont is mouthless, absorbing dissolved cuticle material all over the cell through pinocytosis (Bradbury 1974b). A few hours before host molt, the trophont metamorphoses to the protomont. The protomont slips into the exuvial space via a pore at the end of the gallery, and settles on the new exoskeleton to dedifferentiate to the encysted orthotomont (50–100 μm). Within the orthotomont (cyst), palintomy occurs to produce daughter cells, each of which is subsequently compartmentalized. In the development of the protomont free of the host, there are two potential sub-pathways. Firstly the protomont remains at the "chamber" located at the gallery end connected to the exuvial space until immediately after ecdysis. When the molt is discarded, it swims in the water and then settles on the substrate and encysts. Within the encysted tomont, numerous tomites are formed. Each tomite settles on a new host and forms a single-compartmented cyst or a phoront. This is in contrast to the above-mentioned pathway. Another sub-pathway is as follows. The trophont may metamorphose to the orthotomont in the chamber. The latter divides several times to form small daughter cells that swim freely into the water when the old exoskeleton is cast off. Bradbury et al. (1974) did not mention how these swimming daughter cells behave.

Palintomy is essential to proliferation of apostome ciliates. Sexual reproduction is little known in apostomes (Bradbury et al. 1974). However, conjugation has been observed to occur between paired trophonts or protomontin a variety of apostomes such as *Collinia*, *Hyalophysa*, *Polyspira*, *Terebrospira* and *Vampyrophrya* (Bradbury 1966a; Bradbury et al. 1974; Capriulo and Small 1986; Grimes and Bradbury 1992; Ohtsuka et al. 2004).

18.4 Cytological Features

Metamorphosis of apostome ciliates occurs in accordance with different functions. Bradbury (1966b, 1974a) compared the ultrastructures of tomites and phoronts of the exuviotrophic apostome *H. chattoni*: the phoront is essentially similar to the preceding stage tomite, but differs in the presence of a microtubular core in the macronucleus, and the absence of trichocysts paralleling the kineties and of a ventral tuft of cilia. The microtubular core in the macronucleus might function in either changing the morphology of the macronucleus from phoronts to trophonts or positioning the macronucleus in the cytoplasm (Bradbury 1974a). The phoront cysts are composed mainly of fibrous substances, lacking separable layers, and are similar to loricae of some suctorian ciliates (Bradbury 1974a). Bradbury and Trager (1967a) have found out that the infraciliature of phoronts of *H. chattoni* differentiates to that of trophonts without an intervening dedifferentiation by movement of the cytoplasm.

Mouthless trophonts of *T. chattoni* and *Synophrya* sp. are suggested to feed on dissolved endocuticle of host crustaceans by means of pinocytosis from all over the cell surface (Bradbury 1974b; Bradbury et al. 1974; Landers 2010). The chitinivorous ciliate *Ascophrys rodor*, an ectosymbiont of the shrimp *Palaemon serratus*, forms an unusual ingesting apparatus, which is present only when the ciliate is in its feeding stage (Bradbury et al. 1987). In other apostomes such as *Vampyrophrya* and *Hyalophysa*, a macrostome appears at the feeding stage, which is capable of ingesting a large amount of liquid or semiliquid food in a short period of time (Bradbury 1996; Landers 1991b).

Bradbury (1974b) has revealed distinct relationships between feeding habits and cytoplasmic membrane organelles of trophonts of four genera and species of foettingeriid apostomes. The membrane organelles were observed in two forms, cup-shaped and flattened, but the relationship between these two forms is not understood. These structures are considered as a membrane reservoir, able to release membranes to form cell membranes and food vacuole membranes at the trophont stage (Bradbury 1974b; Ohtsuka et al. 2004). Therefore, rapid-growing and larger-sized trophonts of apostomes tend to bear a larger number of membrane organelles (Bradbury 1974b). Similar vesicles of varying appearance have been documented in the buccal regions of many ciliates (Fischer-Defoy and Hausmann 1981). The discoid vesicles in *Paramecium* are empty (Allen 1974), while similar vesicles in other ciliates such as *Climacostomum* (Fischer-Defoy and Hausmann 1981), *Pseudomicrothorax* (Hausmann and Peck 1979) and *Trichodina* (Hausmann and Hausmann 1981) contain diffuse electron-dense materials inside, which are regarded as digestive enzymes such as lysosomal hydrolases (Peck and Hausmann 1980).

In *V. pelagica,* the membrane organelle is seen as a flattened sac of approximately 50 nm in thickness, and is found exclusively in the cytoplasm of the phoronts and young trophonts. These structures totally disappear in mature trophonts, suggesting that they are precursors of food vacuole membranes (Ohtsuka et al. 2004). The membrane organelle of *V. pelagica* contains electron-dense materials with a homogeneous appearance (Fig. 18.4a, f), while those in *Polyspira delagei* (Bradbury 1974b) and *H. chattoni* (Bradbury 1973) show banding patterns inside the vesicles

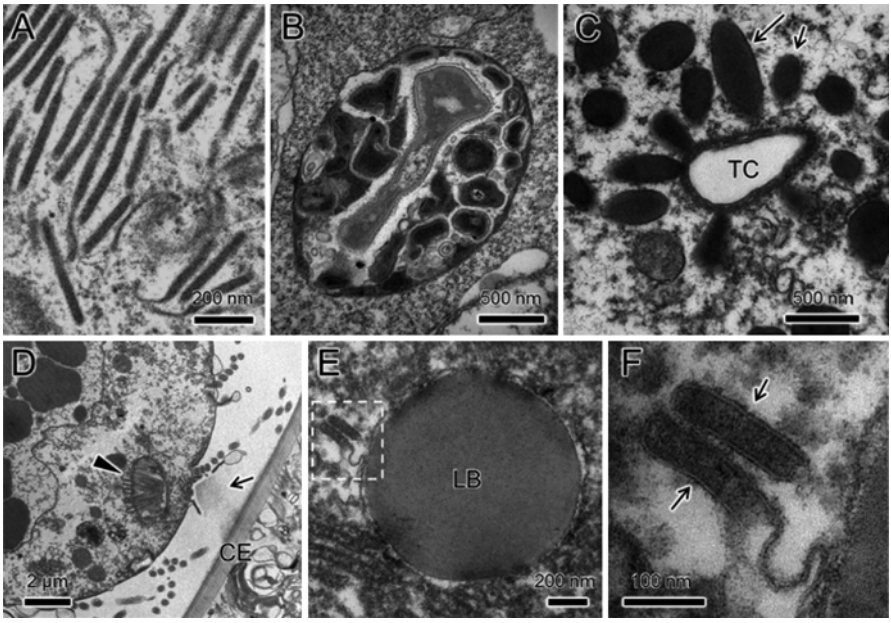

Fig. 18.4 Unique organelles of *Vampyrophrya pelagica*. (**a**) Membrane organelles in the phoront stage, which are usually seen as stacked flat sacs with or without containing electron-dense materials. (**b**) A food plaque in a tomite cell, in which partially digested copepod tissues are contained. (**c**) A tubular canaliculus (TC) and attached rod-like structures (*arrows*) observed at the early phoront stage. (**d**) A rosette structure (*triangle*) containing a bundle of cilia, and a freshly forming peduncle (*arrow*) seen as amorphous material in the very vicinity of the copepod exoskeleton (CE). (**e**, **f**) Lipid body (LB) and membrane organelles at the early phoront stage; the lipid body is frequently observed as partially surrounded by a single membrane layer, which is continuous to the membrane organelles (*arrows*); an enlargement of the area in **f** shown by the *square* in **e**

with regularly arranged materials, resembling cup-shaped coated vesicles observed in peritrich ciliates (McKanna 1973). In young phoronts, membrane organelles are frequently observed in close vicinity to lipid bodies (Figs. 18.3b and 18.4e), being continuous with the membrane that partially covers the lipid bodies (Fig. 18.4f).

In the exuviotrophic apostome *H. chattoni* infecting grass shrimp, non-feeding migratory tomites bear trichocysts, which are digested in autophagic vacuoles soon after metamorphosis to phoronts (Landers 1991a). Although the function of trichocysts remains unknown, these may interfere with the rapid expansion of the cell during the trophont stage. Landers (1991b) also observed the attachment and cyst formation of the tomite. The attachment is composed of three processes: peduncle secretion, and formation of inner and outer cyst walls. The attachment peduncle is formed from the release of secretory dense bodies that are located in the cytoplasm and surround the specialized areas on the ventral invaginations of the pellicle. Two types of dense bodies are observed: bipolar and homogenous types. Only the latter is involved in secretion, while the former may simply represent an immature stage. These dense bodies are reduced by 75 % when tomites crawl on the host, and by 95 % in the cell encysted for 1.5 h. Figure 18.4c shows a cluster of dense bodies in

V. pelagica surrounding a membrane tubule called a "canaliculus," which is continuous to an invagination of the plasma membrane (atrium). These vesicles, together with the membrane structures (the atrium and canaliculi) finally disappear concomitantly with the appearance of the peduncle, suggesting that the dense bodies are supplying materials for constructing the peduncle in *H. chattoni* (Landers 1991b) and also in *V. pelagica* (Fig. 18.4d). These structures are schematically depicted in Fig. 18.3b. The outer cyst wall is secreted from the pellicle (possibly from the exocystosis of membrane-bounded vesicles originating from the subkinetal rough endoplasmic reticulum), but the exact mechanism of secretion is still unknown. The inner cyst wall is formed by secretion across the entire cell surface by non-exocytic process. Although the cyst wall is composed mainly of chitin, the chemical component of the outer and inner cyst walls is different, with the presence of carbohydrates in the inner wall but not in the outer wall.

Food plaquettes (Fig. 18.3b) and lipid bodies (Figs. 18.3 and 18.4e, f) are also organelles characteristic of apostome ciliates. In *H. chattoni*, the digestion process of food plaquettes was traced throughout the cell cycle (Landers et al. 2001). By using nigrosine and polystyrene microspheres as tracers, they found that food plaquettes transformed to late-stage digestive vesicles, and were further retained in the cytoplasm of phoronts and trophonts, sometimes even after encystment to form tomonts. In *V. pelagica*, food plaquettes are usually digested and disappear in phoronts, and immature phoronts with many undigested plaquettes did not have a capacity to excyst (Grimes and Bradbury 1992). A food plaquette of *V. pelagica* in the early phoront stage is shown in Fig. 18.4b, in which partially digested copepod tissues are contained. Lipid bodies appear following the formation of food vacuoles, suggesting that lipid droplets are formed by rapid digestion of solubilized nutrients in the food vacuole (Grimes and Bradbury 1992). In *H. chattoni*, membrane organelles were observed to be continuously associated with vacuoles that resemble lipid droplets in *V. pelagica* (Bradbury 1973).

18.5 Ecology

Grimes (1976) examined the host specificity and prevalence of exuviotrophic apostomes, three species of *Hyalophysa* and *Gymnodinioides inkystanus*, on decapods and other crustaceans from freshwater to marine environments in North Carolina, USA. According to her, the three species of *Hyalophysa* were never sympatric: *H. lwoffi* was found exclusively on freshwater shrimp and crayfish (prevalence: 20–100 %); *H. trageri* was found only on semiterrestrial crabs (60–100 %); *H. chattoni* showed low host specificity on totally aquatic or mud-dwelling decapods inhabiting sea-waters with salinities ranging from 10 to 28 ‰ (50–100 %). On the other hand, *G. inkystanus* co-occurred with *H. chattoni* and *H. trageri*, but exhibited host specificity different from these two species; e.g., aquatic gammarids were parasitized only by *G. inkystanus*, and not by *H. chattoni*. Not only decapods but also barnacles and other crustaceans were infected by *G. inkystanus* (Grimes 1976).

Johnson and Bradbury (1976) reported that 30 % of decapod specimens (shrimp and crabs) collected from the offshore waters (35–36‰) of the southeastern USA

were infected by *Synophrya hypertrophica*. However the incidence was much lower in low-salinity inshore (2 ‰) and estuarine (0 ‰) waters than in offshore waters.

Landers (1986) observed relationships between morphological changes from tomites to pre-hatching trophonts of *H. chattoni* and the molt cycle of the host shrimp *Palaemonetes pugio*. He recognized an intermediate (meridional) stage between free-swimming tomites and pre-hatching trophonts, at which the ciliates are waiting for a molting cue from the host, and he found that encysted tomites changed to the meridional stage within 48 h of settling on the host. Metamorphosis from the meridional stage to the pre-hatching trophont more frequently occurred in late pre-molt stages of the host than an early one.

The seasonal occurrence of phoronts of the histotrophic apostome *V. pelagica* on the body surface of a variety of planktonic copepods in the Seto Inland Sea, western Japan, was well investigated in consideration of relationships between environmental factors and occurrences of both copepods as the first host and predatory chaetognaths as the second host (Ohtsuka et al. 2004). The host specificity was so low that the infection was widely found in most calanoids, some species of cyclopoids (*Corycaeus affinis* and *Oithona* spp.) and a harpacticoid (*Euterpina acutifrons*) from July to January (rarely February) (Figs. 18.5 and 18.6). Prevalence of the phoronts on both sexes of the most abundant species *Paracalanus parvus* s.l. was high (up to 100 %) during August to January, while there was no occurrence from March

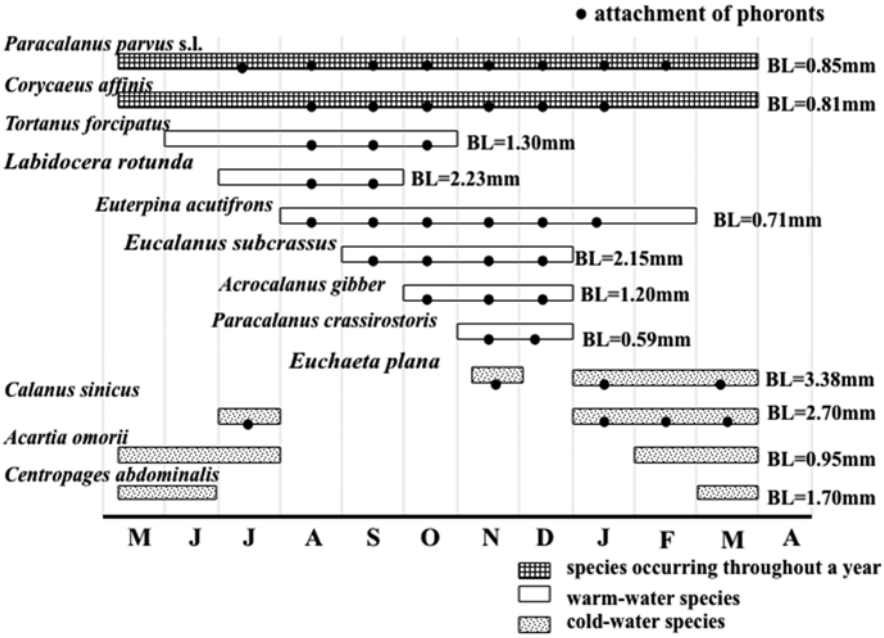

Fig. 18.5 Relationships between seasonal succession of pelagic copepod communities and attachment of *phoronts of Vampyrophrya pelagica* on the host copepods in the Seto Inland Sea, western Japan, from May 1999 to April 2000 (After Ohtsuka et al. 2004). Note that large-sized species (approximately 3 mm in body length) were exclusively infected during cold seasons

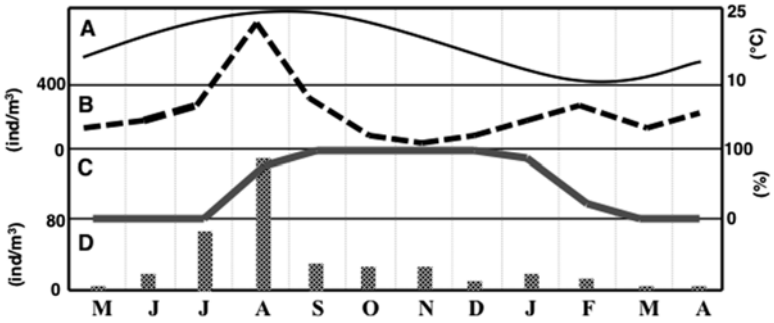

Fig. 18.6 Seasonal changes in relationships among *Vampyrophrya pelagica* and the first and second hosts based on a survey from May 1999 to April 2000 in the Seto Inland Sea, western Japan (after Ohtsuka et al. 2004). (**a**) Water temperature. (**b**) Abundance of dominant copepod *Paracalanus parvus* s.l. as the first host. (**c**) Prevalence of the phoronts on adult females of *Paracalanus parvus* s.l. (**d**) Abundance of pelagic chaetognaths as the second host. Note the rapid decrease in abundance of chaetognaths in September when the prevalence reached up to approximately 100 %

Table 18.2 Average duration (h) of each stage of the cell cycle in *Vampyrophrya pelagica* at 25, 20, 15, and 10 °C after artificial injury of infected copepods

Water temperature (°C)	Phoront	Trophont	Tomont	Total
25	1.3	2.1	13.8	17.2
20	1.0	17.5	16.9	19.4
15	1.1	27.0	38.3	31.4
10	1.6	31.7	250.7	284.0

to June with intermediate conditions between these seasons. During February to June the occurrence of the phoronts was found only in large-sized calanoids such as *Calanus sinicus* (body length of female 2.7 mm long) and *Euchaeta plana* (3.4 mm long), although small calanoids such as *Acartia omorii* (1.0 mm long) and *Centropages abdominalis* (1.7 mm long) were predominant. This distinct seasonality can be explained by a combination of the turnover of the life cycle of *V. pelagica*, relatively low abundance of copepods during cold-water seasons, and the longevity of copepods. The turnover at the in situ lowest water temperature (approximately 10 °C) was approximately 16 times slower than at the highest (approximately 25 °C) (Table 18.2). The duration of phoronts was nearly constant (1.0–1.6 h) irrespective of water temperature. The lowest water temperature in February and early March (around 10 °C) is likely to cause little production of infective tomites, because the duration of tomonts is highly extended by low water temperature of all stages (13.8 h at 25 °C vs 250.7 h at 10 °C).

Not only less production of tomites but also relatively low abundance of planktonic copepod hosts seem to cause no prevalence of phoronts during cold seasons. Since large-sized calanoids usually have longer longevity than smaller ones (estimated longevity for *C. sinicus*: over 140 days at around 10 °C), they seem to have

been infected by tomites before active production of the infective stage ceased with low temperature, and to have survived during cold seasons with phoronts attached on the body.

A schematic illustration of relationships between water temperature, abundances of copepods and chaetognaths, and prevalence of the phoronts on the most dominant copepod host *P. parvus* s.l. is shown in Fig. 18.6 based on our field survey during May 1999 to April 2000. The incidence of the phoronts on the copepods suddenly increased with an increase in water temperature in August, partly because of an increase in the abundance of the first host copepods and the second host chaetognaths, and partly because of activated production of the infective stage and high predation pressure of chaetognaths on the copepod under high water temperature. However a sudden drop in the abundance of both *P. parvus* s.l. and chaetognaths occurred after September, when the water temperature was almost the same as in August. This pattern could have been caused by activation of the two-host life cycle: high predation pressure of chaetognaths on the highly infected copepods and subsequent starvation of the predators. High prevalence during summer to early winter may be explained mainly by high abundance of warm-water copepods other than *P. parvus* s.l. during which other copepod predators such as medusae and ctenophores were also abundant as the second host in addition to chaetognaths. These predators were confirmed to play a role as the second host of *V. pelagica* (Grimes and Bradbury 1992; Ohtsuka et al. 2004).

18.6 Pathogenic Influence

Bradbury (1996) listed three groups of apostomes as pathogens: *V. pelagica*, *S. hypertrophica*, and *Collina* spp. In addition, *Pseudocollinia* spp. must be newly added as pathogenic taxa on the basis of the recent studies. *Vampyrophrya pelagica* parasitizes coastal planktonic copepods and is distributed in the world oceans (Chatton and Lwoff 1935; Grimes and Bradbury 1992; Ohtsuka et al. 2004). Phoronts are attached mainly on the ventral surface of the host prosome, waiting for predation of the copepods by predators such as ctenophores, cnidarians, and chaetognaths; predation induces excystation of the phoront, and ingested copepods are consumed by hatching trophonts which are resistant to predators' enzymes (Grimes and Bradbury 1992; Bradbury 1996; Ohtsuka et al. 2004). This phenomenon means that ingested copepods are not nutritious to the invertebrate predators. Actually a rapid decrease in the abundance of chaetognaths seems to be associated with a rapid increase of the prevalence of *Vampyrophrya* on copepods in the Seto Inland Sea, Japan, during warm seasons (see Figs. 18.5 and 18.6) (Ohtsuka et al. 2004). However, when parasitized copepods are fed by fish larvae, the phoronts seem to be digested with the hosts. In addition, physically wounded copepods also induce excystation of the phoronts, and then are totally fed on by the trophonts (Grimes and Bradbury 1992; Bradbury 1996; Ohtsuka et al. 2004). Considering these, *V. pelagica* can be

regarded as a parasitoid rather than a parasite. The negative impacts of this parasite on the hosts and their predators have not been estimated exactly.

Synophrya hypertrophica infects the gills of a wide variety of crabs (Chatton and Lwoff 1935; Johnson and Bradbury 1976). Forty-four percent of decapod species collected from the southeastern USA suffered from lesions on the carapace (see Fig. 18.1e) and gill lamellae, which were caused by the apostome (Johnson and Bradbury 1976). Trophonts invade the blood sinus of the host gill, feeding on blood with osmotrophy (Bradbury 1996). The host reacts against the parasite by covering with its exoskeleton and by surrounding with a dense ring of secreted melanin and necrotic cells (Johnson and Bradbury 1976). Most crabs with melanized gill spots have no living parasite cells due to the strong defensive reaction of the host (Bradbury 1996). However, young crabs that are heavily infected by the parasite seem to be killed (Bradbury 1996).

Collinia and *Pseudocollinia* are an endoparasite of freshwater and marine crustaceans such as amphipods, isopods, and krill (Capriulo and Small 1986; Gómez-Gutiérrez et al. 2003, 2012). *Pseudocollinia beringensis* infects the haemocoel cavity of the krill *Thysanoessa inermis* in the Bering Sea with the highest prevalence of 98 % in its subadults and adults, possibly leading to reduction in growth and reproduction and even to death (Capriulo et al. 1991). Recently a mass mortality of krill caused by *Pseudocollinia oregonensis* was observed off Oregon, USA, using a remotely operated vehicle (Gómez-Gutiérrez et al. 2003, 2012). Heaps of krill carcasses were found on the bottom, ranging from 220 to 550 m deep along a 1.5 km transect. This parasitoid kills the host within 40 h of infection, because the trophonts devour all the tissues of the host and the tomites rupture the host cephalothorax. They suggested that the swarming behavior of krill makes infection more feasible to cause mass mortality.

18.7 Evolutionary Trends

Apostome ciliates are considered to have evolved from ancestral ciliates feeding on exuvial fluids of crustaceans (Chatton and Lwoff 1935; Bradbury 1974b; Johnson and Bradbury 1976). Apostomes have morphological traits that have characterized them as members of the taxa that are now classified as the Class Phyllopharyngea (Chatton and Lwoff 1935; Corliss 1979), as apostome species such as *H. chattoni* and *V. pelagica* have a ventrally located cytostome and gland-like organelles (atrium and canaliculi) that resemble those in some phyllopharyngean ciliates. By contrast, Small and Lynn (1985), Landers (1986), and Bradbury (1989) favored placing apostomes within the Class Oligohymenophorea, because of the morphological similarities in somatic kinetids and the ultrastructure of the postciliary microtubular ribbons. To discriminate these possibilities, a molecular phylogeny was inferred from SSU rRNA genes isolated from *Gymnodinioides*, *Hyalophysa* and *Vampyrophrya* (Clamp et al. 2008). The analysis clearly supported assignment of apostomes to the Class Oligohymenophorea.

Based on the morphology of the cytostome and infraciliature and the symbiotic life cycle involving microstome–macrostome transformation, Bradbury (1989) pointed out a close relationship of apostomes to histotrophic hymenostomes, and proposed an idea that apostomes are classified as one of the suborders within the order Hymenostomatida in the subclass Hymenostomatia (Class Oligohymenophorea). However, the phylogenetic analysis by Clamp et al. (2008) pointed out that these apostomes should be placed as a different subclass rather than placing them within the hymenostomes. The apostome species examined by Clamp et al. (2008) belong to the Family Foettingeriidae in the Order Apostomatida, all sharing unique cytoplasmic structures including membrane organelles, dense bodies, and tubular canaliculi (Bradbury 1973; Ohtsuka et al 2004). Two other orders, Astomatophorida and Pilisuctorida, are included in the same subclass, and the phylogenetic assignment of these apostomes remains to be clarified. Phylogenetic analyses were also performed for two species of *Pseudocollinia* (Gómez-Gutiérrez et al. 2012) and *Fusiforma themisticola* (Chantangsi et al. 2013) by using SSU rRNA and cox1 gene sequences, which also placed these species in a well-supported monophyletic clade with other apostomes.

18.8 Future Perspectives

Apostome ciliates have been intensively investigated mainly in Europe and USA. In contrast, fewer studies have been carried out in other areas except for studies of the life cycle of *V. pelagica* in Japan (Ohtsuka et al. 2004) and the potentially high diversity of apostomes on copepods in Chinese waters (Guo et al. 2012). The species diversity in these waters must be investigated by modern methods. In particular, deep-sea forms have never been studied, although phoront-like cells are frequently observed to parasitize deep-sea zooplankters (Sewell 1951; Ohtsuka et al. 2003; Ohtsuka and Boxshall 2004). Relationships between cellular metamorphoses (rearrangements of organelles) and functions should be surveyed in apostomes of different feeding types. Studies on the life cycle and negative impacts of parasitoid apostomes on hosts are an urgent matter in marine ecology.

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Chapter 19
Photosymbiosis in Marine Planktonic Protists

Johan Decelle, Sébastien Colin, and Rachel A. Foster

Abstract Some of the most enigmatic components in the plankton are the diverse eukaryotic protists that live in close association with one or more partners. Mutualistic and commensal planktonic interactions are most commonly encountered in the oligotrophic open ocean at tropical and subtropical latitudes. They are functionally and ecologically distinct, and involve a great taxonomic diversity of single-celled partners. Protists like Foraminifera, Radiolaria, dinoflagellates and diatoms can all harbor microalgal symbionts of eukaryotic and prokaryotic origin inside (endosymbiosis) and/or outside (ectosymbiosis) their cytoplasm. Such symbioses (photosymbioses) combine phototrophy, heterotrophy and sometimes dinitrogen (N_2) fixation (reduction of N_2 to ammonium). Symbiotic microorganisms therefore represent an important component of marine ecosystems and play a role in the food web and biogeochemical cycling (e.g., carbon and nitrogen). Despite their important ecological function and early recognition in the late nineteenth century, our knowledge about the diversity, distribution, and metabolic exchanges for many of the photosymbioses remains rudimentary compared with the other marine and terrestrial symbioses. Recent technical advances in single-cell genomics and imaging have greatly improved our understanding about planktonic symbioses. This review aims to present and compare many eukaryote–eukaryote and eukaryote–prokaryote photosymbioses described so far in the open ocean with an emphasis on their ecology and potential function in the ecosystem.

Keywords Symbiosis • Plankton • Protists • Open ocean • Mutualism • Single-cell • Cyanobiont • N_2 fixation

J. Decelle (✉)
Sorbonne Universités, UPMC Univ Paris 06, UMR 7144,
Station Biologique de Roscoff, 29680 Roscoff, France
e-mail: johandecelle@yahoo.fr

S. Colin
CNRS, UMR 7144, Station Biologique de Roscoff, 29680 Roscoff, France
e-mail: colin@sb-roscoff.fr

R.A. Foster
Department of Ecology, Environment and Plant Sciences, Stockholm University,
Stockholm, Sweden
e-mail: rachel.foster@su.se

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19.1 Introduction

From cells to communities, all life entities live in an interconnected network of complex interactions with non-relatives (Thompson 1999). Symbiosis, defined as the living together of differently named organisms, is one of the most intimate and sophisticated interactions (de Bary 1878). The original definition broadly encompasses mutualistic interactions (both partners benefit), commensal interactions (one partner benefits without affecting the other) and parasitic interactions (one partner is exploited) between physically associated organisms. Symbiosis has been integral in the evolution of life (Margulis 1970), is common across all domains and occurs in all habitats. In the plankton, mutualistic partnerships like photosymbioses (involving a photosynthetic partner) are ubiquitous in the vast oceanic regions where the surface waters are deprived of nutrients (Taylor 1982). Oligotrophy is probably a major driving force in the development of such planktonic symbioses. The combination of two or more cells and their metabolisms allow the associated partners to flourish in apparently less-than-optimal environments.

Observations of photosymbiotic consortia in the plankton date back to the 1850s, and were concurrent with descriptions of terrestrial symbioses in plants and lichens (Schwendener 1868; Frank 1877). The English naturalist, Thomas Henry Huxley, was the first to observe and describe minute yellow cells residing in colonies of protists (Radiolaria) during an expedition to the Pacific Ocean (Huxley 1851). These cells were also observed years later in other planktonic taxa by the German biologists Johannes Müller (1858) and Ernst Haeckel (1887). However, since these earlier observations in the world's oceans, much less attention has focused on the planktonic photosymbioses compared with other marine symbioses, e.g., corals, sponges, and deep-sea invertebrates.

In the ocean, there are various modes of photosymbiosis that are functionally and ecologically distinct, involving a wide taxonomic diversity of microbial partners (Gast et al. 2009; Stoecker et al. 2009; Anderson 2012). The planktonic photosymbioses can be divided into two broad groups of associations: (1) a prokaryotic symbiont, i.e., cyanobacteria, bacteria, and a single-celled eukaryotic host; and (2) two single-celled eukaryotic partners. These planktonic systems are unique since they are between two unicellular partners, and are therefore a more "simple" model compared with the better-studied terrestrial (and other marine) systems involving multicellular organisms (i.e., plants, invertebrates). In the plankton, there are also several examples where there are more than two symbiotic partners: a single host cell can accommodate mixed symbiotic populations of bacteria and/or cyanobacteria, or in some cases the mixed populations are of eukaryotic and prokaryotic origins. The partnerships often can be mixotrophic by combining heterotrophy and autotrophy, and therefore short-cut the traditional microbial food web. Forming photosymbioses therefore provides a significant competitive advantage in a nutritionally demanding habitat like the dilute open ocean. However, many of them are inconspicuous, and often the role or function of one or more of the partners is unknown and presumed. Recently, several advancements in single-cell technology and imaging have been

combined with culture-independent approaches and have allowed new planktonic photosymbioses to be identified and visualized, and have allowed the metabolite exchanges between partners to be quantified.

This review describes the diversity of known planktonic photosymbioses involving protists. We consider hereafter a symbiosis as a stable and physical partnership with intact symbiotic cells that are recurrently observed. Kleptoplastidy (sequestration of undigested chloroplasts from algal preys) is not considered in this review, although this is a common planktonic lifestyle (Stoecker et al. 2009; Hansen 2011; Johnson 2011). We largely focus on marine photosymbioses in Radiolaria, Foraminifera and heterotrophic dinoflagellates as these are the best studied, are widespread, and are therefore of ecological significance. We also present new methods and technologies adapted for studying symbioses and conclude with a broader outlook on the field of symbioses.

19.2 Symbiotic Partnerships in Protists

19.2.1 Rhizaria

Foraminifera and Radiolaria (supergroup Rhizaria) are important components of open-ocean planktonic communities due to their abundance, predation, and contribution to the vertical flux of organic matter, and as primary producers, as some taxa form conspicuous symbiotic associations with microalgae (Swanberg and Caron 1991; Caron et al. 1995; Lampitt et al. 2009). Both Foraminifera and Radiolaria synthesize mineral skeletons composed of long spines (up to 1 mm long), and possess amoeboid cytoplasmic strands, which extend outward into the environment. Their highly dynamic strands constitute an effective apparatus of endocytosis to engulf preys or symbionts. In addition, the spines and strands offer a very large surface area and protective microhabitat for hosting both ecto- and endosymbiotic residents (Fig. 19.1a–h).

Symbiotic Foraminifera and Radiolaria are ubiquitously distributed in the upper surface oligotrophic waters. They are relatively scarce, ranging from tens to hundreds per liter (Michaels et al. 1995). Higher abundances generally occur near the surface during periods of calm and warm conditions when the water column is strongly stratified (Michaels 1991). Some host taxa also penetrate deeper in the photic zone residing in the deep chlorophyll maximum (DCM), where higher concentrations of food and nutrients can be obtained (Fairbanks and Wiebe 1980). Some photosymbioses are also abundant in cold and highly productive waters (Henjes et al. 2007; Decelle et al. 2012a). The latter observations in different environmental conditions emphasize the broad distribution and physiological capacity of photosymbiosis in Radiolaria and Foraminifera.

The large cells of Foraminifera and Radiolaria concentrate high numbers ($10–1 \times 10^6$ cells) of actively photosynthesizing microalgae, and therefore represent

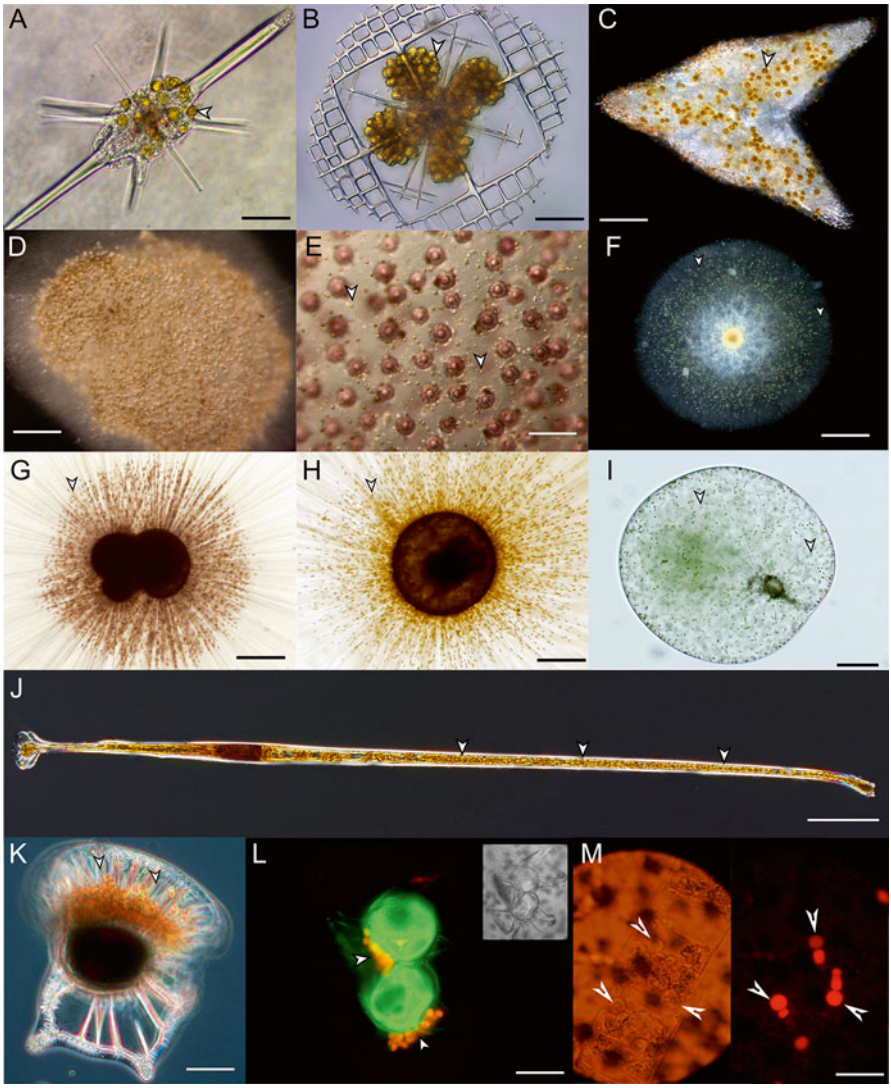

Fig. 19.1 A panel of different mutualistic symbioses in marine planktonic protists (symbionts are indicated by *arrows*). (**a**, **b**) Acantharia (Radiolaria) host symbiotic cells of *Phaeocystis* (Haptophytes); *scale bar* = 50 μm. (**c**) A spumellaria (Radiolaria) with unidentified dinoflagellates; *scale bar* = 40 μm. (**d**, **e**) Collodaria (Radiolaria) with the dinoflagellate *Brandtodinium nutricula*; *scale bar* = 100 μm (**d**) and 20 μm (**e**). (**f**) The Collodaria *Thalassolampe margarodes* living with the green algae *Pedinomonas symbiotica*; *scale bar* = 200 μm (Image courtesy of Christian Sardet). (**g**, **h**) The Foraminifera *Globigerinoides sacculifer* and *Orbulina universa* living with the dinoflagellate *Pelagodinium béii*; *scale bar* = 50 μm (Image courtesy of Katsunori Kimoto). (**i**) The dinoflagellate *Noctiluca scintillans* with the green algae *Pedinomonas noctilucae*; *scale bar* = 100 μm (Image courtesy of Ken Furuya). (**j**) The dinoflagellate *Amphisolenia* sp. hosts pelagophyte endosymbionts along its cell; *scale bar* = 50 μm (Image courtesy of John Dolan). (**k**, **l**) The dinoflagellate *Ornithocercus magnificus* harbors symbiotic cyanobacteria in its girdle, which can be vertically transmitted during the host division (**l**); *scale bar* = 30 μm (**l** Image courtesy of John Dolan). (**m**) Light and epifluoresence micrographs show a chain of the diatom *Hemiaulus* sp. with the cyanobacteria *Richelia* inside the frustule; *scale bar* = 30 μm

a significant source of net primary productivity in the ocean (Caron and Swanberg 1990). The rates of primary production derived from foraminiferal and radiolarian cells generally exceed rates of primary production in equivalent volumes of the surrounding seawater by more than four orders of magnitude. The total production of symbiotic microalgae typically contributes to approximately 1 % of the total primary production in surface waters, and occasionally accounts for up to 20 % during periods of high host cell densities (Michaels 1988; Caron et al. 1995).

19.2.1.1 Planktonic Foraminifera

About 50 extant morphospecies of planktonic Foraminifera are recognized today (Hemleben et al. 1989), of which five species have been regularly observed with intracellular symbiotic microalgae: *Globigerinella siphonifera*, *Globigerinoides ruber*, *Globigerinoides conglobatus*, *Globigerinoides sacculifer* and *Orbulina universa* (Fig. 19.1g, h). The earliest observations of symbiotic microalgae in Foraminifera occurred in the nineteenth century during the circumnavigation of the H.M.S. Challenger (Tizard et al. 1885; Murray 1897). Between 50 and 90 % of the foraminiferal cells found in surface waters at tropical and subtropical latitudes are symbiotic all year round (Caron et al. 1995; Stoecker et al. 1996), while symbiont-barren species are numerically more abundant at deeper depths (Bé et al. 1982). The five symbiotic host species possess spines extending from their tests, and form a monophyletic clade in the 18S rRNA phylogeny (Aurahs et al. 2009). The monophyletic nature indicates that endosymbiosis with microalgae has a single evolutionary origin in planktonic Foraminifera. The large foraminiferal host cell harbors 500–20,000 microalgal cells, which are enclosed in host vacuoles attached to a complex network of rhizopods. During the day, the host cell spreads the microalgae with its cytoplasmic strands outside its calcareous test along the spines, and brings them back at night to the inner cytoplasm (Spero 1987). Thus, hosts combine both behavioral and morphological adaptations for maximum photosynthetic activity of their symbiotic partners.

Compared with their benthic counterparts, planktonic Foraminifera associate with a lower taxonomic diversity of symbiotic microalgae (Lee 2006). The four hosts *G. ruber*, *G. conglobatus*, *G. sacculifer* and *O. universa* all establish a symbiosis with the photosynthetic dinoflagellate *Pelagodinium béii* in the photic layer (Table 19.1, Fig. 19.1g, h: Shaked and de Vargas 2006; Siano et al. 2010). This association has been particularly well studied worldwide. The flagellated microalgal symbiont was first described as *Gymnodinium béii* in the East Pacific Ocean (Spero 1987), and then taxonomically re-defined as *P. béii* by rRNA sequencing and ultrastructural characters (Siano et al. 2010). In the 18S and 28S rRNA phylogeny, *Pelagodinium* is a sister group to the genus *Symbiodinium*, the iconic symbiont of many benthic invertebrates and protists of reef ecosystems, including benthic Foraminifera (Pochon and Gates 2010). Both *Pelagodinium* and *Symbiodinium* genera belong to the order Suessiales that are dinoflagellates, characterized by the absence of armored (thecal) plates. Other members of the Suessiales were found in

Table 19.1 Summary of described planktonic symbioses found in the open ocean

Host	Genetic data of the host	Symbiont	Genetic data of the symbiont	Symbiont ultrastructure (*in hospite*)	Symbiont culture	Type of symbiosis	References
Foraminifera							
Globigerinoides ruber, Globigerinoides conglobatus, Globigerinoides sacculifer, Orbulina universa	18S rRNA	*Pelagodinium béii* (dinoflagellates, Suessiales)	18S and 28S rRNA, ITS	Yes	Yes	Photosymbiosis, mutualistic endosymbiosis	Shaked and de Vargas (2006), Siano et al. (2010), and Gast and Caron (1996)
Globigerinella siphonifera	18S rRNA	Type I: *Chrysochromulina* sp. (haptophytes, clade B); type II unidentified	18S rRNA	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Faber et al. (1988) and Gast et al. (2000)
Globigerina bulloides, Globigerina falconensis, Turborotalita quinqueloba	No	Unidentified dinoflagellates	No	No	No	Ectosymbiosis, commensalism	Alldredge and Jones (1973) and Spero and Angel (1991)
Hastigerina pelagica	No	*Pyrocystis* sp. and *Dissodinium* sp. (dinoflagellates, Pyrocystales)	No	No	No	Ectosymbiosis, commensalism	Alldredge and Jones (1973) and Hemleben and Spindler (1983)

Radiolaria							
Acantharia (Arthracanthida and Symphiacanthida; clades E and F)	18S and 28S rRNA	*Phaeocystis* (haptophytes)	18S and 28S rRNA, plastidial 16S rRNA, rbcL and psbA	Yes	Yes (but uncultivable after the symbiotic stage)	Photosymbiosis, mutualistic endosymbiosis	Decelle et al. (2012c) and Febvre and Febvre-Chevalier (1979)
Acantharia (*Acanthochiasma* sp.; clade B)	18S and 28S rRNA	*Chrysochromulina* (haptophytes); *Azadinium* sp., *Pelagodinium* sp., *Heterocapsa* sp. (dinoflagellates)	18S rRNA and ITS	No	Yes (except for *Chrysochromulina*)	Photosymbiosis, mutualistic endosymbiosis	Decelle et al. (2012b)
Collodaria (*Thalassolampe margarodes)*	No	*Pedinomonas symbiotica* (chlorophytes)	No	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Cachon and Caram (1979)
Collodaria (*Collozoum* sp., *Thalassicola* sp.)	No	*Brandtodinium nutricula* (dinoflagellates, Peridiniales)	18S and 28S rRNA (clades B1 and B2)	Yes	Yes	Photosymbiosis, mutualistic endosymbiosis	Probert et al. (2014) and Gast and Caron (1996)
Spumellaria (*Spongostaurus* sp.)	No	*Brandtodinium nutriculum* (dinoflagellates, Peridiniales)	18S rRNA	No	Yes	Photosymbiosis, mutualistic endosymbiosis	Gast and Caron (1996)
Spumellaria (*Spongostaurus* sp.)	No	Cyanobacteria (*Synechococcus* sp. and *Prochlorococcus* sp.)	16S rRNA	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Foster et al. (2006a, b)
Spumellaria (*Spongodrymus* sp.)	No	*Tetraselmis sp.* (chlorophytes)	18S rRNA	No	Yes	Photosymbiosis, mutualistic endosymbiosis	Gast et al. (2000) and Gast and Caron (2001)

(continued)

Table 19.1 (continued)

Host	Genetic data of the host	Symbiont	Genetic data of the symbiont	Symbiont ultrastructure (*in hospite*)	Symbiont culture	Type of symbiosis	References
Spumellaria (*Spongodrymus* sp.)	No	Haptophytes	No	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Anderson et al. (1983a)
Spumellaria (*Dictyocoryne truncatum, Dictyocoryne profunda*)	No	Cyanobacteria (*Synechococcus* sp. and *Prochlorococcus* sp.)	16S rRNA	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Yuasa et al. (2012) and Foster et al. (2006a, b)
Nassellaria (unidentified)	No	*Brandtodinium nutriculum* (dinoflagellates, Peridiniales)	18S rRNA	No	Yes	Photosymbiosis, mutualistic endosymbiosis	Probert et al. (2014)
Dinoflagellates							
Amphisolenia sp.	No	Cyanobacteria (*Synechococcus* sp. and *Prochlorococcus* sp.) and pelagophytes (stramenopiles)	16 s rRNA and nifH (cyanobacteria) and 18S rRNA (pelagophytes)	Yes	No	Photosymbiosis, mutualistic endosymbiosis +ectosymbiosis	Daugbjerg et al. (2013), Farnelid et al. (2010), and Lucas (1991)
Ornithocercus sp., *Citharistes* sp., *Histioneis* sp.	No	Cyanobacteria (*Synechococcus* sp. and *Prochlorococcus* sp.) and N_2-fixing cyanobacteria	16 s rRNA and nifH	Yes	No	Photosymbiosis, mutualistic ectosymbiosis	Farnelid et al. (2010) and Foster et al. (2006a, b)

Noctiluca scintillans	No	*Pedinomonas noctilucae* (chlorophyte)	No	Yes	Yes	Photosymbiosis, mutualistic endosymbiosis	Sweeney (1976)
Podolampas bipes, Podolampas reticulata	No	Dictyochophytes (stramenopiles)	No	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Schweikert and Elbrächter (2004)
The Peridiniales *Durinskia, Kryptoperidinium, Peridinium and Galeidinium* (called dinotoms)	Yes	Pennate and centric diatoms	Cytochrome c oxidase (cox) 1, 2 and 3; cytochrome b (cob); LSUrRNA	No	No	Tertiary endosymbiosis	Imanian et al. (2010) and Imanian and Keeling (2007)
Haptophytes							
Unidentified prymnesiophyte (presumably *Braarudosphaera bigelowii* sp.)	Yes	Cyanobacteria UCYN-A	16S rRNA + genome	Yes	No	N-fixing symbiosis	Thompson et al. (2012) and Hagino et al. (2013)
Ciliates							
Mesodinium rubrum	No	Cryptophyte	No	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Hansen and Fenchel (2006)
Oligotrichida	No	Prasinophyte	No	Yes	No	Photosymbiosis, mutualistic endosymbiosis	Stoecker et al. (1988–1989)

(continued)

Table 19.1 (continued)

Host	Genetic data of the host	Symbiont	Genetic data of the symbiont	Symbiont ultrastructure (*in hospite*)	Symbiont culture	Type of symbiosis	References
Codonella sp. (tintinnid)	No	Cyanobacteria (*Synechococcus* sp.)	16S rRNA	No	No	Photosymbiosis, mutualistic endosymbiosis	Carpenter and Foster (2002) and Foster et al. (2006a, b)
Eutintinnus apertus, Eutintinnus pinguis	No	Diatoms (*Fragilariopsis*, *Planktoniella*, *Chaetoceros*, *Hemiaulus*)	No	No	No	Ectosymbiosis, commensalism?	Gómez (2007) and Balech (1962)
Diatoms							
Rhizosolenia sp., *Hemiaulus* sp., *Chaetoceros* sp.	No	Cyanobacteria *Richelia intracellularis* and *Calothrix rhizosoleniae*	16S rRNA, genomes	Yes	No/yes	N-fixing	See review in Foster and O'Mullan (2008)
Leptocylindrus mediterraneus (often as empty frustules)	No	*Solenica setigera* (stramenopiles MAST3) and cyanobacteria (*Synechococcus* sp.)	18S rRNA (*Solenica*) and no sequences for Cyanobacteria	Yes	No	Parasitism or ectosymbiosis?	Gómez et al. (2010) and Buck and Bentham (1998)
Climacodium frauenfeldianum (pennate)	No	Cyanobacteria (close to *Cyanothece* sp.)	16S rRNA	No	No		Carpenter and Janson (2000)
Centric diatoms (*Thalassiosira* sp.)	No	The coccolithophore *Reticulofenestra sessilis* (haptophytes)	No	No (only SEM)	No	Ectosymbiosis?	Frada et al. (2010)

symbiosis with freshwater sponges in the oligotrophic Lake Baikal (Annenkova et al. 2011), indicating that this order has a high propensity for symbioses.

In the Foraminifera *Globigerinella siphonifera* (previously named as *G. aequilateralis*), two types of microalgal symbionts have been identified, but they never occur concomitantly in the same host cell (Faber et al. 1988). Ultrastructure observations and rRNA sequencing showed that type I belongs to non-calcifying haptophytes (Faber et al. 1988; Gast et al. 2000). But the description of this partnership is much less complete than that of *P. béii* as it is only based on a few host individuals collected from a small geographic region (Sargasso Sea, Jamaica and Barbados). Symbionts of type II were ultrastructurally distinct from type I, but it was not possible to distinguish them as haptophytes or chrysophytes (Faber et al. 1988). Recently, a study obtained the 18S rDNA sequence of the symbiont from hosts collected in the North Pacific and showed that the symbiont of type II is related to the genus *Pelagomonas* (Fujiki et al. 2014).

Other related planktonic foraminiferans, such as *Globigerina bulloides*, *G. falconensis*, and *Turborotalita quinqueloba*, do not establish endosymbiotic relationships but offer a suitable microhabitat for free-swimming microalgae, presumably belonging to dinoflagellates. Foraminiferal cells that are gently hand-collected by divers in the natural environment can contain more than 200 microalgal cells in their rhizopodial network (Alldredge and Jones 1973; Spero and Angel 1991). After host isolation, the swimming microalgal symbionts are not ingested by their hosts, do not disperse in the medium, and can increase their abundance, and therefore the relationship is considered a facultative ectosymbiosis. In this case, the microalgae are considered as commensals since they do not transfer any carbon source to the host but they can utilize host-derived metabolites or food debris as nutrients (Hemleben et al. 1989). Another commensal relationship occurs between the planktonic Foraminifera, *Hastigerina pelagica*, and photosynthetic dinoflagellates of the order Pyrocystales (*Pyrocystis* sp. and *Dissodinium* sp.), which regularly attach to the surface of the host capsule (Alldredge and Jones 1973; Hemleben and Spindler 1983). Abundances of 5–79 *Dissodinium* cells associate with *H. pelagica*, and typically increase in number with the surface area of the host capsule. The *Pyrocystis* sp. symbiont is too large to increase its population density on an individual *H. pelagica*, and commonly occurs as a single individual symbiont.

19.2.1.2 Radiolaria

Compared with planktonic Foraminifera, Radiolaria encompass a larger taxonomic diversity with four main orders: Spumellaria, Nassellaria, Collodaria and Acantharia (Chap. 7, this volume). Radiolarian taxa are able to biomineralize silica (Spumellaria and Nassellaria) and strontium sulfate (Acantharia) to build complex and delicate skeletons (Figs. 19.1a–c and 19.2v). These planktonic protists are primarily heterotrophs but some species are also known to maintain few to thousands of endosymbiotic microalgae and/or bacterial symbionts (Table 19.1). Recent studies have

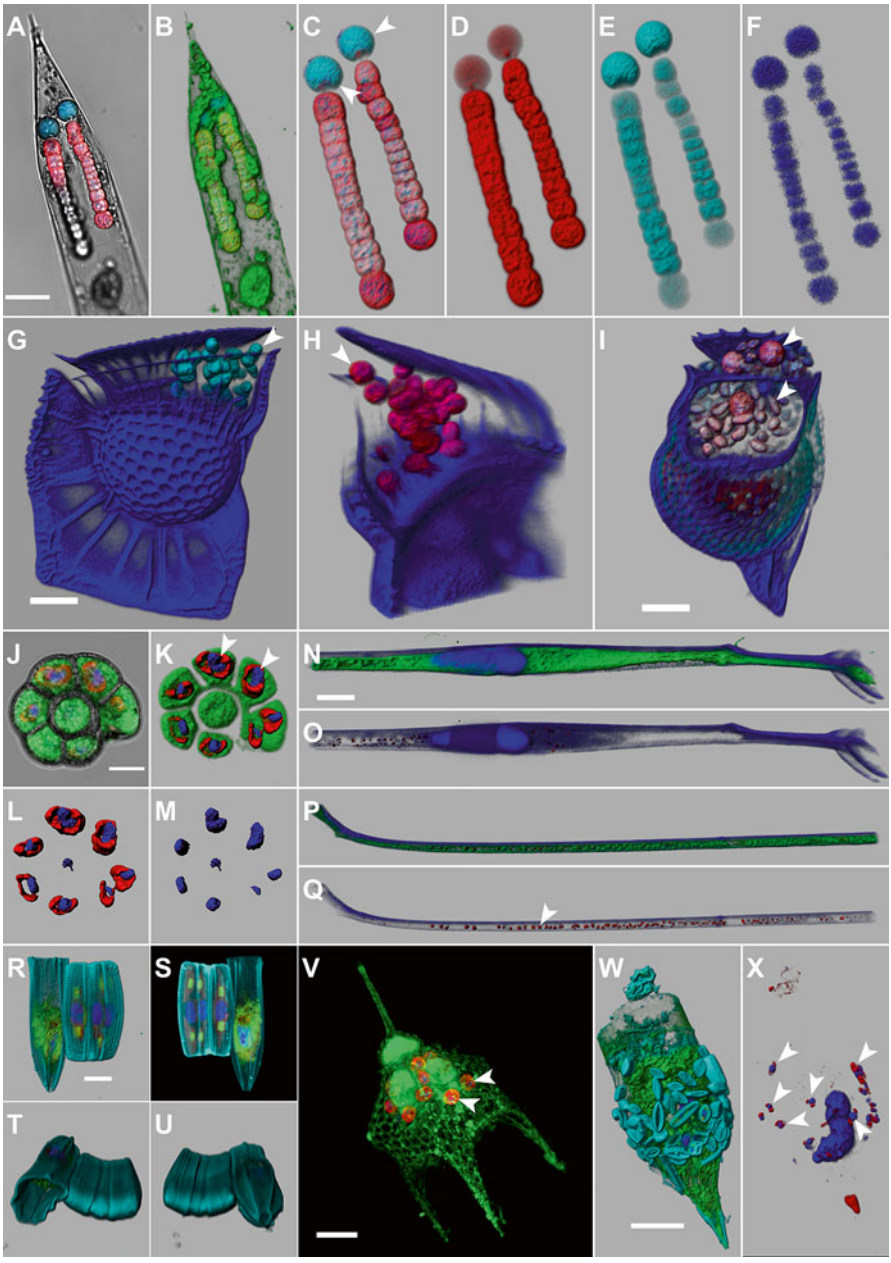

Fig. 19.2 Morphological investigations about symbiotic consortia through a multi-staining strategy and confocal laser scanning microscopy. Planktonic cells have been sampled and fixed with formaldehyde from the *Tara-Oceans* expedition. (**a**–**f**) A diatom *Rhizosolenia* sp. associates with the cyanobiont *Richelia* sp. in the North Indian Ocean (station 42). Two trichomes are visible with their heterocysts (*arrows*). *Blue*, DNA; *green*, membranes (mb); *red*, chlorophyll (Chl); *cyan*, phycoerythrin (PE); *scale bar* = 20 µm. (**g**, **h**) A Dinoflagellate *Ornithocercus* sp. associates with cyanobacteria (*arrows*) in the North Atlantic Ocean (station 151). *Blue*, DNA and Theca; *red*, Chl; *cyan*, PE; *scale bar* = 20 µm. (**i**) The phaeosome chamber of the dinoflagellate *Histioneis* sp. displays two phenotypes of cyanobacteria (*arrows*) in the North Atlantic Ocean (station 151). *Blue*, DNA and Theca; *red*, Chl; *cyan*, PE; *scale bar* = 10 µm. (**j**–**m**) A Foraminifera with endosymbiotic

reported on both the identity of the microalgal symbionts, and also on the specificity and biogeography of the radiolarian symbioses.

The majority of Collodaria, some Spumellaria and Nassellaria radiolarians live with the dinoflagellate *Brandtodinium nutricula* in the upper euphotic layer worldwide (Fig. 19.1d, e: Gast and Caron 1996; Probert et al. 2014). The *B. nutricula* symbionts are 10–15 µm in cell diameter and were formally named *Zooxanthella nutricula* in the nineteenth century (Brandt 1881). Their taxonomic status has been recently revised based on the sequencing of ribosomal markers and morphological descriptions from clonal cultures and the *in hospite* stage (Probert et al. 2014). Contrary to the naked dinoflagellates *Pelagodinium* and *Symbiodinium*, *Brandtodinium* possesses thecal plates, a distinguishing morphological feature of the Peridiniales order. In the ribosomal gene phylogeny, *Brandtodinium* comprises two subclades (B1 and B2), in which the 18S and 28S rRNA sequences are identical irrespective of host taxon and geographic origin. Interestingly, a sequence that corresponds to the symbiont of the pelagic hydrozoan jellyfish *Velella velella* clusters with subclade B2, suggesting that the microalga *Brandtodinium* is a generalist symbiont not only among Radiolaria but more broadly in plankton (Gast and Caron 2001; Probert et al. 2014).

The collodarian species *Thalassolampe margarodes* associates with the green algae prasinophyte *Pedinomonas symbiotica*, a symbiotic partnership that occurs year round in the Mediterranean Sea (Fig. 19.1f; Cachon and Caram 1979). Hundreds of 4–5 µm flagellated cells are scattered in the host jelly, and are motile in the spring and summer seasons. During fall and winter, symbionts lose their flagella. Another prasinophyte is symbiotic with spongiose Spumellaria *Spongodrymus* sp. in the Sargasso Sea (Gast et al. 2000; Gast and Caron 2001). Recent phylogenetic analyses reveal that the latter symbiont is related to *Tetraselmis*, a genus already described in symbiosis with acoel flatworms from benthic coastal waters (Parke and Manton 1967; Decelle unpubl). The same host genus (*Spongodrymus* sp.) collected in the Sargasso Sea also harbors 50–100 haptophyte cells in its rhizopodial and axopodial rhizopodial network (Anderson et al. 1983a).

Spumellaria radiolarians can also associate with prokaryotic symbionts. Using light microscopy (LM) more than 100 yellow-green pigmented microalgal cells were found in the Spumellaria *Euchitonia elegans* (Takahashi et al. 2003). Using epifluorescence microscopy (EFM) and DAPI-staining, Takahashi et al. (2003) also

Fig. 19.2 (continued) dinoflagellates in the North Indian Ocean (station 42). Two of them are dividing (*arrows*). *Blue*, DNA; *green*, mb; *red*, Chl; *scale bar* = 10 µm. (**n**–**q**) A dinoflagellate *Amphisolenia* sp. associates with many small symbiotic microalgae of Pelagophyceae (*red spots*, *arrow*), in the North Indian Ocean (station 42). *Blue*, DNA; *green*, mb; *red*, Chl; *cyan*, PE; *scale bar* = 20 µm. (**r**–**u**) A diatom chain of *Fragilariopsis doliolus* bound to the lorica of a tintinnid ciliate in the Mid-Pacific Ocean (station 106). 3D models allow several viewing angles. *Blue*, DNA; *green*, mb; *red*, Chl; *cyan*, surface staining; *scale bar* = 10 µm. (**v**) A Nassellaria radiolarian harbors a few red dinoflagellate endosymbionts in the North Indian Ocean (station 42). *Blue*, DNA; *green*, mb; *red*, Chl; *scale bar* = 20 µm. (**w**, **x**) The lorica of the ciliate *Codonellopsis* sp. aggregates epiphyte pennate diatoms, which were still alive prior to fixation (chloroplast and nuclei are visible, *arrows*) in the South Atlantic Ocean (station 84). *Blue*, DNA; *green*, mb; *red*, Chl; *cyan*, surface staining; *scale bar* = 20 µm

reported viable microalgal symbionts in *Dictyocoryne truncatum* collected in Japanese waters. Similar sized algal symbionts were also observed within the intrashell cytoplasmic region of *D. truncatum* in the tropical North Atlantic (Anderson and Matsuoka 1992). Anderson and Matsuoka (1992) also reported co-existing bacterial symbionts, and later work by Foster et al. (2006a, b) described the endosymbionts of *D. truncatum* and another radiolarian, *Spongostaurus* sp., collected in the Pacific and Atlantic Oceans, as similar to cyanobacteria (*Synechococcus* sp. and *Prochlorococcus* sp.) in both 16S rDNA sequence and cell morphology (e.g., size, thylakoid distribution). Most recently, Yuasa et al. (2012) identified *Synechococcus* cells (clade II) in individual *D. profunda* cells collected in the East China Sea. The symbionts of *D. profunda* were similar in sequence similarity (i.e., *Synechococcus-like*) to the symbionts sequenced and visualized by Foster et al. (2006a) in both spumellarian genera (*D. truncatum* and *Spongostaurus* sp.). Although the spumellarian Radiolaria occur worldwide with seemingly similar bacterial and cyanobacterial partners, the host diversity is virtually unknown and little studied.

Acantharia, characterized by their star-shaped skeleton of 10 or 20 spicules made of celestite, generally outnumber their rhizarian counterparts in the open ocean with abundances ranging from 10 to 40 cells per liter (Caron and Swanberg 1990; Michaels et al. 1995). Often in spring, symbiont-bearing Acantharia can form bloom-like events, reaching densities of up to 500,000 individuals m^{-2} (Massera Bottazzi and Andreoli 1981, Decelle, pers. observations in Gulf of Naples). About 150 extant species of Acantharia are described (Schewiakoff 1926), and were recently classified into eight clades using the 18S and 28S rRNA phylogeny (I, III and A-F in Decelle et al. 2012c). The great majority of symbiotic Acantharia belong to clades E and F (Fig. 19.1a, b). These acantharians have a more complex and robust mineralized skeleton compared with their non-symbiotic relatives, a phenotypic complexity that could be an adaptive process of living in symbiosis with microalgae (Moran 2007). Contrary to their radiolarian counterparts where symbionts remain in the rhizopodial network surrounding the central capsule, Acantharia hold their microalgae within the central capsule, representing a higher degree of symbiont integration. Depending on the host taxonomy, ten to hundreds of non-motile symbiotic cells of 5–10 μm in cell diameter can be observed. Three decades ago, *in hospite* ultrastructural observations described the symbionts as haptophytes (Febvre and Febvre-Chevalier 1979). More recently, ribosomal and plastidial genetic markers obtained from several host isolates identified the symbionts as the haptophyte *Phaeocystis* (Decelle et al. 2012a). The microalga *Phaeocystis* has been extensively studied due to its widespread distribution, high abundance and harmful blooms in coastal areas but its symbiotic lifestyle was unknown (Verity and Smetacek 1996; Schoemann et al. 2005). Sequences of the symbiotic *Phaeocystis* do not form distinct clades (except for those from the West Pacific Ocean), but are genetically identical to the cultured free-living strains isolated worldwide. Genetic relatedness and specificity of the symbiont was dependent on geographic location whereby all acantharian host species live with the local species of *Phaeocystis* in a given oceanic region (Decelle et al. 2012a).

Other microalgal cells have also been reported as symbionts in another acantharian host species, *Acanthochiasma* sp., belonging to the early diverging clade B (Schewiakoff 1926; Decelle et al. 2012b). The *Acanthochiasma* sp. hosts were isolated from one site in the Mediterranean Sea for two consecutive years and multiple algal partners, including distantly related dinoflagellates (*Heterocapsa* sp., *Pelagodinium* sp., *Azadinium* sp. and *Scrippsiella* sp.) as well as a haptophyte (*Chrysochromulina* sp.), were found in association. The microalgal symbionts can be present in the same host cell simultaneously either along the spines or internal in the cytoplasm.

19.2.2 Dinoflagellate Hosts

Dinoflagellates are an important group of protists in marine and fresh waters with tremendous morphological and trophic diversity. They have a high propensity for symbiotic partnerships as both symbionts and hosts. As mentioned before, photosynthetic dinoflagellates can be mutualistic symbionts to a rich diversity of invertebrates and other single-celled eukaryotic partners. Heterotrophic dinoflagellates can be hosts by associating with microalgal and bacterial cells (Table 19.1). Some of these dinoflagellates live with eukaryotic microalgae, while others carry mixed populations of unicellular cyanobacteria and bacteria in their pouch-like girdles and one group also takes up mixed assemblages of eukaryotic and prokaryotic symbiotic populations.

The earliest descriptions of symbioses involving dinoflagellate hosts date back to over 100 years ago when the unicellular phototrophic partners were observed by microscopy as colored bodies and were called ‘phaeosomes’ (Schütt 1895). Today, these particular pigmented symbionts are identified as cyanobacteria and are more commonly referred to as cyanobionts (Taylor 1982). The majority of dinoflagellate hosts are planktonic with the exception of the benthic *Sinophysis* genus (Escalera et al. 2011). The dinoflagellate-hosting cyanobionts and bacteria are widely distributed in all oceanic basins, more particularly in the sunlit layer of oligotrophic waters (above 100 m). They often occur at low to background densities (1–100 cells L^{-1}) at temperatures between 20 and 30 °C.

The heterotrophic dinoflagellate hosts belong to two families: Amphisoleniaceae (*Amphisolenia, Triposolenia*) and Dinophysaceae (*Ornithocercus*, *Citharistes*, *Histioneis Parahistiones*) in the order Dinophysiales (Gómez et al. 2011). The cyanobacterial and bacterial symbionts of the Dinophysaceae reside externally in the girdle of the host dinoflagellates (Figs. 19.1k, l and 19.2g–i). The associations are quite fragile such that if the cells are disturbed by gently tapping on the slide holding the cells, often the symbionts are released from the girdle (Foster pers. observations). There is an observable complexity to the degree to which the host holds its external symbionts. In the most advanced form in *Citharistes* spp., the girdle has been reduced to a small chamber with an opening that corresponds to the size of the symbiont’s cell diameter (Lucas 1991). Taylor (1982) referred to the chamber of

Citharistes as a "greenhouse" since it contained so many cyanobacterial symbionts. Moreover, a size selectivity was suggested in the symbiotic *Citharistes* spp. since sequences (16S rRNA) derived from the cyanobionts formed a unique subclade (Foster et al. 2006b). The other genera of dinoflagellate hosts (*Ornithocercus*, *Histioneis, Parahistioneis*) have large and broad girdles with less symbiont specificity, often found with mixed cell sizes of cyanobacterial (and bacterial) populations. The function of the symbionts for their hosts are unknown; however, given the large percentage of the host cell volume taken up by its symbiotic partners and the observable adaptations to house their partners it seems that the relationships are important and/or necessary.

Based on several observations (Taylor 1982; Lucas 1991; Foster et al. 2006a) we can summarize the cyanobacterial and bacteria cell characters. The cyanobionts of the dinoflagellates are described as oblong, ellipsoid/rod, cylindrical or coccoid in shape, and reported cell lengths and widths range from 0.6 to 10 µm and from 0.3 to 6 µm, respectively (Fig. 19.2g–i). Thylakoid distributions in the cyanobionts are scattered, transverse, peripheral, irregular, or stacked and in layers of 2–3 or 3–4 or 4–6. Sheaths, carboxysomes, and various storage granules (cyanophycin, starch) have also been reported for the cyanobacterial symbionts. For the bacterial symbionts, cells are typically coccoid or rod shaped, with cell length and diameter ranging from 1 to 6 µm (Tarangkoon et al. 2010).

The molecular diversity of the cyanobacterial and bacterial symbionts has been identified in a limited number of studies and based on two gene markers: the 16S rRNA and *nifH* (encoding for the N_2-fixing enzyme nitrogenase) (Table 19.1). A majority of 16S rRNA sequences obtained from the hosts *Ornithocercus, Citharistes, and Histioneis* were most similar to pico-cyanobacteria *Synechococcus* and *Prochlorococcus* and a small subset of sequences had low sequence identity (94 %) to other cyanobacteria, including known N_2-fixing cyanobacteria (Foster et al. 2006a, b). Two to three divergent sequence types were also identified in several host individuals suggesting a mixed symbiont population, including bacteria. Mixed populations of cyanobacteria and bacteria were also corroborated in parallel TEM-immunocytochemistry study by Foster et al. (2006a) and a later study by Farnelid et al. (2010) that found a predominance of bacterial *nifH* gene sequences most similar to alpha, beta and gamma-proteobacteria. Given that the dinoflagellate hosts (Dinophysiales) are non-photosynthetic and occur in nutrient deplete surface waters, it can be hypothesized that the cyanobacterial symbiont supplies fixed carbon and the bacterial partners potentially fixed N_2 for their host.

The symbiotic microalgae of *Amphisolenia* spp. (Amphisoleniaceae) are both eukaryotic and prokaryotic in origin (Figs. 19.1j and 19.2n–q: Lucas 1991; Farnelid et al. 2010). Several 16S rRNA sequences retrieved from individual *A. bidentata* and *A. globifera* were most similar to pico- and filamentous cyanobacteria and a few sequences with low similarity (<92 % identical) to 16S rRNA sequences from eukaryotic plastids (Foster et al. 2006b). In addition, *nifH* gene sequences similar to cluster I (alpha-, gamma-, beta-proteobacteria) and cluster III have also been reported from other *Amphisolenia* spp. (Farnelid et al. 2010). The identity of the eukaryotic microalgae (approximately 100 cells located in the cytoplasm) has been

recently revealed by 18S and 28S rRNA sequences as closely related to members of the Pelagophyceae (stramenopiles), presumably representing a new genus (Daugbjerg et al. 2013).

The large heterotrophic dinoflagellate *Noctiluca scintillans*, renowned as producing spectacular blue-green bioluminescence, can possess up to 10,000 eukaryotic microalgal cells of 2–6 μm in cell diameter. These symbiotic microalgae were identified by morphology as the prasinophyte *Pedinomonas noctilucae* (Fig. 19.1i: Sweeney 1976). The original depiction described flagellated photosynthetic symbionts free-swimming in the large host vacuole, a compartment that was also called a "greenhouse" (Hansen et al. 2004). The microalga *Pedinomonas* sp. is also found in symbiosis in radiolarian taxa (see above), but the absence of molecular data for both symbioses does not allow the symbiont's identity to be confirmed. The *Noctiluca–Pedinomonas* association is regularly observed in the tropical and subtropical areas of Southeast Asia (e.g., Thailand, India, Philippines, New Guinea) in the Indian and Pacific Oceans, and in the Red Sea (Harrison et al. 2011). In these regions, the *Noctiluca* symbiosis can have a significant ecological impact since it regularly forms extensive blooms, called "green" tides, where densities can reach up to 5×10^6 cells L^{-1} (Harrison et al. 2011; Madhu et al. 2012). *N. scintillans* can also remain strictly heterotrophic, feeding on a large variety of prey with its tentacle, and can reach bloom densities in temperate, tropical and polar waters (Elbrächter and Qi 1998). The latter is commonly referred to as "red" *Noctiluca*, while the photosymbiotic form is called "green" *Noctiluca*. Although molecular data is lacking, the "green" and "red" *Noctiluca* are considered to be the same morphospecies (Sweeney 1978). The "red" *Noctiluca* has a significantly wider distribution than the "green" one (Harrison et al. 2011), but can also occur in the same region at different seasonal periods. In the Arabian Sea, the "green" *Noctiluca* occurs during the winter season when productivity is high while the "red" one appears in summer when water temperature and oligotrophy increase (Harrison et al. 2011).

Some species of the heterotrophic dinoflagellate *Podolampas* spp. (*P. bipes* and *P. reticulata* of the order Peridiniales) have multiple plastid-containing cells belonging to Dictyochophytes (stramenopiles). The *Podolampas* symbiosis is widely distributed in the Atlantic and Pacific Oceans and also in the Mediterranean Sea, but the exact phylogenetic diversity of the symbionts remain unknown (Sournia 1986; Schweikert and Elbrächter 2004; Gómez et al. 2010).

Other members of Peridiniales, like the genera *Durinskia, Kryptoperidinium, Peridinium and Galeidinium*, harbor pennate and centric diatoms in their cytoplasm, and are referred to as "dinotoms" (Table 19.1: Imanian and Keeling 2007; Imanian et al. 2010). These dinoflagellates can be planktonic or benthic, and are very diverse in their morphologies and habitats (freshwater and marine habitats). Despite the loss of the cell wall, the diatom endosymbionts are relatively intact as they retain a large nucleus, a large volume of cytoplasm and mitochondria, and complete nuclear and mitochondrial genomes (Imanian et al. 2010). In addition, the symbiont is integrated within its dinoflagellate host at all different stages of the life cycle including cell division, sexual reproduction, and cyst formation (Chesnick and Cox 1987; Figueroa et al. 2009).

Some heterotrophic dinoflagellates like *Amphidinium poechilochroum* and *Gymnodinium gracilentum* are known to sequester chloroplasts (kleptoplastidy) of cryptophyte and haptophyte origins (Gast et al. 2007; Jakobsen et al. 2000; Hansen 2011). However, most morphological descriptions in TEM have shown that these hosts can also maintain symbiont nuclei, cytoplasm and mitochondria, along with the plastid (Hansen 2011). Thus, these cases of temporary acquired phototrophy may not represent kleptoplastidy *sensu stricto* and rather encompass different degrees of symbiont reduction. Further morphological investigation over resolutive temporal scales is therefore needed to follow the fate of ingested preys/organelles and define the exact nature of the partnership.

19.2.3 Haptophyte Hosts

There have been a few recent studies reporting a novel symbiosis between a haptophyte host (from the class Prymnesiophyceae) and an uncultivated N_2-fixing unicellular cyanobacterium (Thompson et al. 2012; Hagino et al. 2013; Krupke et al. 2013). The unicellular cyanobacterium, commonly referred to as UCYN-A (formerly group A), was first identified in the subtropical Pacific Ocean by its *nifH* gene sequence and was considered a widespread free-living population (Zehr et al. 2001; Moisander et al. 2010). Within a decade of genetic identification, the genome of the UCYN-A cells was sequenced, revealing some unusual gene deletions like the oxygen-evolving photosystem II, ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCo) for CO_2 fixation, and tricarboxylic acid (TCA), suggesting that it could be a host-dependent symbiont (Zehr et al. 2008; Tripp et al. 2010). Most recently, three independent studies from geographically distinct areas of the oceans (Atlantic, Pacific, and coastal Japan) found 1–2 UCYN-A cells associated with a haptophyte host (Thompson et al. 2012; Hagino et al. 2013; Krupke et al. 2013). The 18S rRNA sequence derived from the host was most closely related to a calcareous haptophyte, *Braarudosphaera bigelowii* (Thompson et al. 2012; Hagino et al. 2013). In two of the studies it was not possible to determine the location of the UCYN-A cells, and it was presumed to be external or loosely attached. However, in the cells collected by Hagino et al. (2013) from Tosa Bay, Japan, TEM images clearly showed 1–2 spheroid bodies with internal lamellae associated with the *B. bigelowii* cells. Although the observations for the UCYN-A symbiosis are quite limited so far, it is intriguing to consider the impact that the symbiosis has on both the global C and N cycles since the host potentially bears calcareous plates and UCYN-A is thought to be rather widespread in subtropical and tropical waters and important to the marine N cycle.

19.2.4 Ciliate Hosts

About 23 % of the planktonic ciliates from marine and freshwater habitats can acquire phototrophy by plastid retention and algal endosymbiosis (Foissner et al. 1999). Symbiosis between the freshwater ciliate *Paramecium bursaria* and the

microalga *Chlorella* sp. is probably the most studied interaction (Kodama and Fujishima 2010). In nutrient-poor marine environments, planktonic ciliates, mainly tintinnids, can hold symbiotic cyanobacteria (Foster et al. 2006a, b; Johnson 2011). The tropical tintinnid *Codonella* sp., collected from the North Atlantic and Pacific Oceans, has been described as hosting a mixed population of cyanobacteria (*Synechococcus*-like) and bacteria in the oral groove of the lorica by molecular analyses (16S rRNA) and TEM observations (Foster et al. 2006a, b). In the *Codonella*, the cyanobacterial symbionts were observed only in the upper lorica, and many were elongated and some in the process of cellular division; therefore it was concluded that the symbionts could be temporarily maintained and were not simply consumed by the heterotrophic hosts (Foster et al. 2006a). Moreover, although the nature of the partnership is unknown, the lorica of other tintinnids like *Eutintinnus apertus* and *E. pinguis* commonly harbors epiphytic diatoms like *Fragilariopsis*, *Planktoniella*, *Chaetoceros* and *Hemiaulus* (Fig. 19.2r–u, w, x: Rampi 1952; Balech 1962; Gómez 2007).

To date, an endosymbiosis with eukaryotic microalgae was never reported in open-ocean ciliates, contrary to reef and freshwater ecosystems (Lobban et al. 2002). In coastal waters, the ciliate *Mesodinium rubrum* houses a "permanent" cryptophyte endosymbiont (Hansen and Fenchel 2006), and the estuarine Oligotrichida would have small prasinophyte endosymbionts (Stoecker et al. 1988–1989). However, as in heterotrophic dinoflagellates, many ciliates (including *Mesodinium*) acquire phototrophy along a continuum of strategies from plastid retention (kleptoplastidy) to intact symbiont cells (photosymbiosis). But in most cases, it is not clear where the cursor lies along this continuum, and the nature of the relationships therefore remains unresolved.

19.2.5 Diatom Hosts

Recent attention, including a fairly extensive review (Foster and O'Mullan 2008), has largely focused on the symbioses between the diatoms *Rhizosolenia*, *Hemiaulus*, and *Chaetoceros* and their heterocystous (heterocyst, specialized cell for N_2 fixation) cyanobacterial partners, *Richelia intracellularis* and *Calothrix rhizosoleniae*, and are therefore covered here in limited detail with emphasis on the most recent discoveries (Figs. 19.1m and 19.2a–f). Note there is conflicting nomenclature in the literature for the diatom–heterocystous cyanobacteria symbioses, and for simplicity we distinguish *C. rhizosoleniae* as the symbiont of *Chaetoceros* spp. and *R. intracellularis* as the symbiont of *Hemiaulus* and *Rhizosolenia* spp. Recently, partial genomes for the symbiotic strains *R. intracellularis* and *C. rhizosoleniae* were sequenced where the location of the symbiont and its dependence on the host appeared linked to the evolution of the symbiont's genome (Hilton et al. 2013). For example, the internal *R. intracellularis* symbiont of the *H. hauckii* diatom was unusually small (3.2 Mb) and lacked the majority of genes for nitrogen (N) acquisition, while the genome of the external symbiont *C. rhizosoleniae* (6.0 Mb) appeared to be complete for all the N acquisition pathways and similar in size to free-living cyanobacteria.

An intriguing and little-studied cosmopolitan symbiosis from the open ocean and eastern Arabian Sea is a three-part partnership between a chain-forming centric diatom, *Leptocylindrus mediterraneus*, an aplastidic colonial protist, *Solenicola setigera* (belonging to the stramenopiles lineage MAST3), and the unicellular cyanobacterium *Synechococcus* sp. (Hasle 1975; Buck and Bentham 1998; Gómez 2007; Gómez et al. 2010; Padmakumar et al. 2012). In general, the basis of the partnership is unresolved; however, often the diatom is devoid of cellular content, thus the *Solenicola* may be parasitic on the diatoms or seek refuge, and in return the diatom may persist by exudations from the protist (Buck and Bentham 1998). The *L. mediterraneus* symbiosis occurs worldwide at usually low biomasses (<500 colonies L^{-1}), and can at times be a large component of the biomass (e.g., 5–31 μg C L^{-1}) (Buck and Bentham 1998). Another diatom symbioses common to the oligotrophic subtropical and tropical waters is between the large chain-forming pennate diatom *Climacodium frauenfeldianum* and a unicellular cyanobacterium similar in cell diameter and 16S rRNA sequence to N_2 fixer *Cyanothece* sp. ATCC51142 (Carpenter and Janson 2000). In the Gulf of Mexico and South Atlantic Ocean, centric diatoms (*Thalassiosira* sp.) have been observed associated with numerous cells of the coccolithophore *Reticulofenestra sessilis* (haptophytes) that are attached to the host frustule and maintain a nucleus (Frada et al. 2010; Colin pers. observations). Overall, the phylogenetic diversity of both partners remains unstudied in all the diatom symbioses, the benefit to the symbiont is unknown, and the function of the symbiont for the host has been limited in study (see below).

19.3 Life History of Partners and Symbiont Transmission

19.3.1 Host and Symbiont Dependence on Symbiosis

Our understanding about the life cycle of microalgae-bearing hosts is very limited, largely because many symbioses have evaded isolation and often reside in remote areas far away from laboratory facilities. Similar to many corals and lichens, a symbiotic partnership with microalgae is obligatory for the Foraminifera and Radiolaria hosts as they cannot survive without their endosymbionts (at least during the adult stage) (Bé et al. 1982; Caron and Swanberg 1990). Conversely, in the dinoflagellate order Dinophysiales, several of the hosts have been observed without symbionts, suggesting that the relationship is temporary and/or less obligatory. Given the heterotrophic nature of the dinoflagellate hosts, one could also argue on the side of a temporary partnership or simply that the hosts consume their prokaryotic partners. However, food vacuoles devoid of cyanobacteria and the presence of dividing symbiotic cells in the dinoflagellate girdles have been reported (Janson et al. 1995; Foster et al. 2006a), indicating that the cells are maintained for at least some time. In the diatom–*Richelia/Calothrix* symbioses, there are limited observations of hosts devoid of symbionts. Moreover when *Hemiaulus* spp. and *Rhizosolenia* spp. reach

bloom densities, they are always reported as symbiotic, suggesting the necessity of the symbiont for high growth rates. There has been one laboratory study of the *Rhizosolenia clevei–Richelia intracellularis* symbiosis that concluded the partnership was not obligate for the host diatom (Villareal 1990). In general, it seems that the host reliance on symbiosis is linked to the physical integration of their symbionts where in many partnerships identified as endosymbiotic, the relationships are more obligatory compared with ectosymbiosis, which tends to be more facultative and/or temporary.

The life cycle of symbiotic microalgae is considered equally or more enigmatic than that of their host partners, and remains largely understudied. In many partnerships, it is unknown whether symbionts alternate between free-living and symbiotic stages (facultative symbiont), or if they only live associated with a host (obligate symbiont). Detection of free-living symbionts in a natural sample is difficult, and often requires specific sampling, cultivation and molecular methods (see Sect. 19.5). In the vast majority of terrestrial and marine symbiotic associations described to date, including the coastal–benthic photosymbiosis in reef ecosystems, the symbionts are typically elusive outside the host, and can be members of the rare biosphere in some symbioses (Webster et al. 2010; Pedrós-Alió 2012). In plankton, no studies have specifically explored the free-living phase of mutualist symbionts, leading to an incomplete understanding on the functioning of the interaction. Some described microalgal symbionts are cultivable in traditional host-free media (e.g., *Phaeocystis*, *Brandtodinium*, *Pelagodinium*, *C. rhizosoleniae*: Table 19.1). This indicates that symbiosis is not essential for the symbionts' survival and that these symbionts may have an active free-living phase in the environment. Conversely, once isolated from its host, the symbiont *Pedinomonas noctilucae* only grows in a specific medium, close to the intracellular conditions of the host (low pH, nutrient-rich), and not in natural seawater or freshwater (Sweeney 1976; Okaichi et al. 1991). This suggests that *P. noctilucae* has a very limited or absent free-living phase in the natural environment. In Acantharia–*Phaeocystis* symbiosis, the free-living stage of the symbiont is indirectly known as it is genetically identical to the known free-living *Phaeocystis* species (Decelle et al. 2012a). The free-living population of *Phaeocystis* is very large, reaching up to several million cells per liter (Schoemann et al. 2005), whereas only a relatively small number of cells occur in symbiosis (10–100 microalgal cells per acantharian host cell; Decelle et al. 2012a). Likewise, some symbionts of Dinophysiales, Radiolaria and Foraminifera (*Synechococcus* sp. clade II, *Chrysochromulina* sp.) are phylogenetically identical or very close to members that are known to be abundant and widespread microalgae in marine ecosystems (Zwirglmaier et al. 2008; Yuasa et al. 2012). This mode of symbiosis where the symbiont seem to be abundant in free-living raises many questions and hypotheses about the exact nature/dependence of the partners, and warrants further investigation (Decelle 2013: see Sect. 19.5).

The dependence of symbionts on their hosts can be reflected by their location (e.g., internal, external) and their genome size and content. Genome streamlining is a common feature of internal symbionts that reside in a host over multiple generations and therefore have limited exposure to the environment, and/or when there is

a redundancy of genes and metabolic functions provided by the host (Moran 2003; Sachs et al. 2011). The loss of unnecessary genes and metabolic pathways through evolutionary time therefore creates a dependence on the host that can become essential for the symbiont's survival. Two such examples have been recently identified in the plankton: both the *R. intracellularis* and UCYN-A genomes are highly reduced, lacking several pathways necessary for an independent lifestyle, and are therefore interpreted as potentially obligatory partnerships (Tripp et al. 2010; Hilton et al. 2013).

19.3.2 Symbiont Transmission and Maintenance

To perpetuate the partnership to next generations, hosts can acquire their symbiont by two fundamentally different mechanisms: (1) vertical transmission where symbionts are inherited from parent to daughter cells in asexual division or transmitted through gametes; and (2) horizontal transmission where symbionts are acquired from the surrounding environment. Both mechanisms can also co-exist during the life cycle of a host; however, to the best of our knowledge this has not been documented in planktonic symbioses.

There are a few well-documented observations of vertical transmission. For example, in the association between the dinoflagellate *Podolampas* and the dictyochophyte microalgae, symbionts are transmitted to the daughter cells during mitotic division (Schweikert and Elbrächter 2004). Similarly, the cyanobacteria that are located in the girdle of Dinophysiales (Fig. 19.1l) and the green microalgae of the dinoflagellate *Noctiluca* are transmitted to the daughter cells when the host divides. In the diatom–cyanobacteria symbioses, vertical transmission has only been described in the *Rhizosolenia–Richelia* symbioses where the *Richelia* symbionts migrate to the opposite valves of the host cells prior to host binary fission (Taylor 1982; Villareal 1989, 1990). Interestingly, in laboratory culture experiments, the growth rates of the host *Rhizosolenia* diatom and *Richelia* symbiont were asynchronous leading eventually to asymbiotic hosts (Villareal 1989, 1990). In field collected *Hemiaulus–Richelia* symbioses, estimated growth rates were similar between the host and symbiont and in some the symbiont's growth was accelerated (Foster et al. 2011). Therefore it seems that various levels of control may be in place for the hosts to maintain their symbiotic population.

All cases of vertical transmission in the planktonic symbioses occur when the host asexually divides and no evidence of such transmission has been reported during sexual reproduction. Horizontal transmission is the main suggested mechanism of symbiont acquisition in many planktonic symbioses on the basis that aposymbiotic hosts are observed at the beginning and at the end of their life cycle. When the dinoflagellate *Noctiluca* undergoes gametogenesis, a large number of gametes (up to 1000) of about 10 μm in size are produced (Fukuda and Endoh 2006); however,

no microalgal symbionts have been observed in the gametes or in newly produced vegetative cells (pers. communication, K. Furuya). In Radiolaria and planktonic Foraminifera, hosts lose their symbiotic microalgae before gametogenesis, and many species sink deep into the water column (Michaels 1991). It is still difficult to say whether symbionts are lysed, digested and/or released, and this can vary depending on the host taxa. In Acantharia, just prior to gametogenesis, the lack of chlorophyll autofluorescence indicates the absence of microalgal cells at this stage (Decelle et al. 2012a), while in Collodaria, intact microalgae can persist (Yuasa and Takahashi 2014). In Foraminifera, symbionts are lysed but some are also shed outside the host cell (Hemleben et al. 1989). When reproductive maturity is reached, the host releases thousands of 2–4 μm diameter bi-flagellated swarmers into the environment. Swarmers are presumably reproductive gametes although neither a fusion nor their ploidy status has been identified. The small size of radiolarian and foraminiferal swarmers compared with their symbiotic microalgae (e.g., 5–6 μm for *Phaeocystis* sp., 8–11 μm for *Pelagodinium* sp., 10–15 μm for *Brandtodinium* sp.) precludes retention and acquisition of symbionts at this stage. The symbionts are thought to be acquired from the environment later during the early juvenile stages of the host. The frequency of this *de novo* symbiont acquisition is not known because the lifespan of host in natural conditions has not yet been determined. In Foraminifera and Radiolaria, symbiotic species would reproduce once or twice per month, a biological rhythm mediated by the synodic lunar cycle (Casey et al. 1971; Spindler et al. 1979; Bijma et al. 1990).

During the life cycle of Radiolaria, Foraminifera and other hosts, population of microalgal symbionts typically increases as the host cell ages. This may be the result of asexual division of symbionts since TEM observations in radiolarian cells, in the girdle of several Dinophysiales, and the lorica of the tintinnid *Codonella* have shown dividing and/or elongated symbiotic cells (Anderson 1983; Foster et al. 2006a; Farnelid et al. 2010; Tarangkoon et al. 2010). The population increase may also originate from a continuous acquisition during the life cycle where the host recurrently takes up new symbionts from the environment. However, this second mechanism has not been shown with limited evidence from reinfection experiments where adult Foraminifera cells maintain the capacity to acquire symbionts from the environment (Bé et al. 1982). The growing symbiotic population implies that the host must exert a control to maintain a stable extraneous population. Similar to symbiotic reef ciliates and corals (Johnson 2011), one presumed strategy in Radiolaria is the regular ingestion of their symbionts. For example, Collodaria can consume 4 % of their symbionts each day, which constitutes a supplemental energy source for the host (Anderson 1983). Taylor (1982) speculated that the cyanobionts of dinoflagellates were maintained for a period of time and at some stage harvested for food by their host; however, often the contents of the food vacuoles are too degraded to resolve the morphological identity of the material (Tarangkoon et al. 2010).

19.4 Metabolic Interactions and Physiological Adaptations

Since the early pioneering physiological experiments of the late nineteenth century (Geddes 1878; Brandt 1881), it has been largely recognized that microalgal symbionts are not parasitic but provide nutritional resources to their hosts. In return, the microalgal symbionts benefit from a nutrient-rich microenvironment relative to ambient seawater, and protection from predators, parasites and viruses (Taylor 1982). Given the apparent mutual benefits for the partners, symbioses with microalgae are typically considered mutualistic. However, our knowledge about the metabolic interactions between partners is limited; the types of metabolites as well as the exchange rates are poorly described in many of the planktonic symbioses.

19.4.1 Eukaryote–Eukaryote Photosymbioses

In Radiolaria, Foraminifera and the dinoflagellate *Noctiluca*, radiotracer experiments with ^{14}C- labeled bicarbonate ($H^{14}CO_3$) have shown a transfer of photosynthetic-derived carbon products from the symbiont to the host (Anderson et al. 1983b; Hansen et al. 2004). In Radiolaria, most of the ^{14}C was associated with low molecular weight compounds such as glycerol, probably constituting a storage pool for the host. But no research has focused on the identity of other transferred products, like glucose, maltose and organic acids as found in other reef and freshwater photosymbioses (Yellowlees et al. 2008). In addition, nothing is known about the metabolic products (presumably carbon and nitrogenous compounds) that are transferred from the host to the symbionts.

Eukaryotic microalgal symbionts typically contain starch granules and large vacuoles and have prominent plastids compared with their free-living forms (Febvre and Febvre-Chevalier 1979; Anderson et al. 1983a). These observations suggest that their photosynthetic capacity is accentuated, and presumably by the host. Moreover, several different studies have reported very high production rates from the symbionts that exceed the host and symbiont requirements for growth (Jørgensen et al. 1985; Lombard et al. 2009). Lombard et al. (2009) hypothesized that the carbon overproduction is transformed into exudates that chemically attract the host preys, like copepods. The opposite has also been proposed, stating that some photosynthetic products could act as chemical repellents against predators (Anderson 1983).

In any case, different studies came to the conclusion that the symbiotic microalgae of Radiolaria, Foraminifera and Dinoflagellate (*Noctiluca*) are not the only source of food for the host since it continues to eat different preys, such as bacteria, ciliates and copepods (Anderson 1983; Swanberg and Caron 1991; Hansen et al. 2004). In general, host heterotrophy appears to also be necessary to maintain the symbiotic relationship. Photosynthesis derived from the symbionts could therefore

represent a supplemental energy source, allowing the host to survive periods of low nutrient and/or prey concentrations.

In addition to host nourishment, symbionts of Foraminifera are also known to sustain and increase the calcification of the host test as in coral symbioses (Rink et al. 1998; Moya et al. 2006). A similar scenario probably occurs in other symbionts that live with biomineralizing hosts like Radiolaria and calcareous haptophytes. In Acantharia–*Phaeocystis* symbioses, the symbiont has been shown to produce a great quantity of sulfur compounds, such as dimethylsulfoniopropionate (DMSP), dimethylsulfide (DMS) and dimethyl sulfoxide (DMSO), that are presumably transferred to the host (Decelle et al. 2012a). Owing to their high scavenging capacity of oxidants (Sunda et al. 2002), these sulfur compounds would confer antioxidant protection to the host in order to cope with oxidants produced by the symbiont's photosynthesis and by the high irradiance of surface oligotrophic waters. It is interesting to note that these sulfur molecules were also detected in high concentrations in benthic photosymbioses (Van Bergeijk and Stal 2001; Yost and Mitchelmore 2009). UV photoprotection has also been reported in symbiotic cyanobacteria living within benthic organisms (*Prochloron* sp. with the ascidian host: Hirose et al. 2006). Photoprotection seems thus to be a key physiological adaptation in photosymbioses dwelling in well-illuminated environments, and has to be further investigated in plankton.

19.4.2 Eukaryote–Prokaryote Symbioses

Much less is known about the exact nature of the eukaryote–prokaryote symbioses but a few generalizations can be made. In dinoflagellates (Dinophysiales), the function of the cyanobionts for the hosts is unknown and largely presumed based on our understanding of free-living cyanobacteria. For example, all cyanobacteria are photosynthetic and some are diazotrophic (N_2 fixers), thus it is assumed that the cyanobionts transfer fixed photosynthetic products and/or fix N_2.

In several of the diatom–heterocystous cyanobacteria and diatom–unicellular symbioses, a nanoSIMS approach with isotopically labeled $^{15}N_2$ quantified the N_2 fixation in the individual symbiotic cells and showed transfer of fixed N_2 from the symbiont to the host diatom (Foster et al. 2010. See Sect. 19.5 for technical details). Growth and N_2 fixation rates were accelerated in the diatom–cyanobacterial symbioses compared with measurements on cyanobacterial cells which were living freely, suggesting that the hosts could regulate their symbiotic partners' growth and metabolism (Foster et al. 2011). Most recently, also using nanoSIMS, Thompson et al. (2012) demonstrated that the haptophyte microalgal hosts transfer fixed carbon to their UCYN-A symbionts in return for fixed N_2 by UCYN-A. The latter was the first demonstration of a mutualistic partnership in the eukaryote–prokaryote symbioses and also similar to terrestrial symbioses between plants and N_2-fixing cyanobacteria.

19.5 Methods for Studying Symbioses in Plankton

19.5.1 Sampling

Symbioses are difficult to isolate and cultivate and therefore often we must rely on sampling and culture-independent techniques (see below). A major difficulty in sampling planktonic symbioses is to do so without damaging or destroying the integrity of the partnership. In the field, traditional mesh plankton nets are employed; however, often the delicate host cells are damaged or destroyed (e.g., Acantharia, colonial Radiolaria), and partners easily detach from each other, especially in the case of ectosymbiosis and fragile symbioses. In addition, artificial associations can occur during net collection. More gentle collecting strategies have been adopted for collecting symbiotic associations. For example, gravity filtration directly from Niskin bottles onto membrane filters, followed by isolation using a micropipette under the microscope (Foster et al. 2006a, b), hand-collection into wide-mouthed bottles by diving, or towing a net while swimming, and more recently flow cytometric sorting was successful in the isolation and identification of new symbioses (Huber et al. 1996; Thompson et al. 2012; Decelle et al. 2012b).

19.5.2 Morphological Description of Symbiotic Associations

Transmitted light microscopy (LM) remains the most accessible method for observing symbiotic partnerships in plankton. Often the dense/dark cytoplasm or external skeleton of the host hinders the observation of endosymbionts, and some symbioses may be overlooked. Epifluorescence microscopy is a useful alternative (Takahashi et al. 2003). The cyanobacteria and cryptophyte phycobiliproteins (i.e., phycoerythrin, PE) emit yellow/orange fluorescence under blue/green excitation while chlorophyll a emits red under blue light excitation. Additionally, under blue excitation, green autofluorescence (GAF) is also widespread in diverse planktonic taxa (Tang and Dobbs 2007). The detection of both PE fluorescence and GAF is specifically useful to highlight the otherwise inconspicuous cyanobionts residing in the girdle of the Dinophysiales hosts (Fig. 19.1l) and diatoms.

A combination of fluorescent dyes can stain various organelles simultaneously, making visible the cell characters of the symbiotic partners. It can be applied to live or dead cells, resolving information about the viability of the cells at the time of collection or preservation (Zetsche and Meysman 2012). DNA dyes can enumerate the different nuclei/cells and also identify putative cellular divisions of each partner (Fig. 19.2j–m in Suzuki et al. 2009). Staining the cell membranes and cytoplasm resolves the core-cell boundaries, and can be used to distinguish between ecto- or endosymbiosis. Finally, an aspecific fluorescent painting of the external surface can be applied to delineate the entire shape of a symbiotic consortium (Colin, unpubl. See also Fig. 19.2). When conjugated with optical sectioning microscopy (e.g., confocal laser scanning microscopy), a suitable combination of fluorescent dyes con-

trasts the partner morphologies and highlights the whole 3D structure of the symbiotic assemblages (Fig. 19.2).

Traditional histological preparations, e.g., TEM and SEM, are still essential to describe the ultrastructure characters of the partner cells (Hagino et al. 2013), and in some cases coupled with immunostaining for resolving the presence/absence of biomarkers, i.e., nitrogenase, phycoerythrin (Janson et al. 1995; Foster et al. 2006a). Recently, atomic force microscopy (AFM) has emerged as a new imaging tool to visualize the 3D nature of microbial populations while also making measurements of cell characters (e.g., diameter, height) (Dufrêne 2002). AFM can be used to visualize and measure the cellular volumes of the symbiotic partners, and in particular for epibiotic symbiotic partners (Malfatti and Azam 2009). Finally, AFM can also be combined with secondary-ion mass spectrometry (SIMS) to describe both the topography and chemical composition of individual cells (Sheik et al. 2012).

19.5.3 Genetic Identification of Symbiotic Partners

Morphology-based identification is highly challenging, especially for the symbiont, since most diagnostic characters are lost in a symbiotic state, and consequently it provides only vague taxonomic assignments. On the contrary, obtaining phylogenetic markers from symbiotic partners can give a species-level identification, which is an essential primary step to study symbioses. Individual host cells containing endo- or ectosymbionts have to be isolated and carefully washed in sterile conditions. The individual symbiotic cells can be used directly as a template in polymerase chain reaction (PCR) without DNA extraction. The latter approach was used with individual planktonic partnerships and the assay sensitivity was greatly improved with an additional preliminary reverse transcription step to obtain more copies of rRNA (Foster et al. 2006b). Alternatively, DNA is extracted and multiple phylogenetic markers, such as 16S, 18S and 28S rRNA, targeting both the host and symbionts, can be obtained by PCR (Gast et al. 2000; Shaked and de Vargas 2006; Decelle et al. 2012a). Typically, 18S and 28S rRNAs are used in phylogenetic studies, while others have used in addition conserved functional genes (*nifH, narB, hetR, rbcL*), which detect the genetic potential for a particular partner's metabolism and also their diversity (Janson et al. 1999; Foster and Zehr 2006; Farnelid et al. 2010; Foster et al. 2010).

Genetic information of the interacting partners is necessary not only to identify them, but also to unveil the specificity and co-evolution of the relationship at a broad geographic scale (Decelle et al. 2012a). Molecular clocks can also be performed to estimate the timing of the symbiosis and the diversification pattern of each partner at the start of the interaction (Shaked and de Vargas 2006; Decelle et al. 2012a). Finally, the sequences obtained for the host and/or symbiont can also be used to study the distribution of the partnerships using quantitative PCR (qPCR) and environmental metabarcoding approaches. The latter assays can be combined with hydrographic condition measurements in order to identify if a particular environmental condition(s) promotes the presence/absence of a symbiosis.

19.5.4 Symbiont Cultivation

Symbiont isolation and cultivation in free-living can be useful to characterize its genetic identity and metabolic capacity. One approach is to mechanically crush the host cell and subsequently isolate the symbionts in an appropriate culture medium (Siano et al. 2010; Decelle et al. 2012b; Probert et al. 2014). Foster et al. (2010) picked several chains of the *Chaetoceros–Calothrix* symbiosis in the field under the microscope with a pulled micropipette and transferred the cells directly to nitrogen deplete growth media. Within several months the diatom host was gone; however, the symbiotic *Calothrix* persisted (see details Foster et al. 2010). In other systems, cultivating the symbiont has been less successful, probably because the host dependence for survival is too high, or the transition between the symbiotic and free-living state is too severe for the symbiont's physiology (e.g., osmoregulation), or not enough is known of the chemical conditions (e.g., medium recipe) that promote isolation. Different combinations of culture medium can be tested to help in establishing a free-living state (see the example of *Pedinomonas noctilucae*, Okaichi et al. 1991). Once the symbiont is growing, a wide array of physiological experiments and studies in genomics and transcriptomics is possible to decipher its metabolic capacity. In addition, some symbionts can regain their original morphological features in the free-living stage (thecae, frustules, flagella, scales etc.), which allow one to confirm the molecular identification and improve their taxonomic status (Probert et al. 2014; Siano et al. 2010).

19.5.5 Unveiling Metabolic Interactions Between Partners

It is now possible to visualize and measure the elemental composition of individual cells and to track the transfer of labeled substrates. The latter methodologies have been successfully applied to study symbiotic systems, including planktonic partnerships (e.g., Foster et al. 2011; Thompson et al. 2012). For example, in cells that were previously incubated with stable isotopically labeled substrates (i.e., ^{13}C-bicarbonate, $^{15}N_2$), the cellular location and transfer of fixed products were easily visualized and quantified using a nanoSIMS instrument (see review by Musat et al. 2011). Moreover, the same methodology has been combined with in situ hybridization assays using halogenated probes (e.g., HISH) to identify a particular sequence type while also quantifying the uptake of a particular substrate (Musat et al. 2008). The nanoSIMS approach has also been combined with a phylogenetic microarray (chip) where the community rRNA was hybridized to a pre-designed microarray and the isotope incorporation into the various "spots" was measured by the nanoSIMS instrument (CHIP-SIP: Mayali et al. 2012). If the genome for a particular symbiont and/or host was known, one could apply a similar CHIP-SIP approach to identify and quantify the coordination of cellular activity by the partners for a particular pathway, e.g., nitrogen acquisition.

19.6 Perspectives

Marine protists that have developed symbiotic associations are fundamental players in the food web and biogeochemical cycles of the open ocean. Many of these partnerships are highly complex and much remains to be studied regarding their identity, ecology, and metabolic interactions. In particular, research efforts should continue to describe the morphology and genetic diversity of both hosts and symbionts, in order to unveil novel symbioses and to identify the level of specificity in the partnerships. Some symbionts are considered generalists in plankton, living in association with a wide taxonomic spectrum of protistan and metazoan hosts (e.g., *Brandtodinium*, *Pelagodinium*, *Tetraselmis*), suggesting that they possess a symbiotic "competence", while in some of the symbioses, the hosts are highly specific and appear to select for a particular symbiont. Whether the selection or competence is linked to a particular life trait, metabolic capacity, or ecological fitness, it does, however warrant greater attention.

Given the prevalence of horizontal transmission for symbiont acquisition, one of the more intriguing and open questions revolves around the mechanism(s) by which host and symbionts can establish de novo their partnership with an appropriate partner, especially in the vast and microbiota-diluted open ocean. In some eukaryote–eukaryote symbioses, taking up an abundant free-living partner is considered strategic by increasing the partner encounter rate (Decelle 2013). Identifying other strategies at different stages of the symbiosis development, including host–symbiont communication and recognition, remains a challenging yet open area for research.

Moreover, in order to improve our understanding about the ecology and general functioning of each symbiosis, the life cycles of both partners need to be dually investigated. For most planktonic symbioses, it is unclear whether symbiosis is obligatory or facultative for each partner, and whether one partner is active or in dormancy, or is abundant or rare with and without the other partner. FISH assays are an attractive means to identify and quantify the presence of a free-living stage of the symbiont, as well as to investigate whether the targeted symbiont can occur in different host taxa (i.e., generalists). In parallel, specific fluorescent probes could target the host at different life stages (i.e., gametes–juveniles–adults), and when applied to time interval sampling could identify the timing of symbiont acquisition from the surrounding environment.

Another future challenge will be to properly quantify symbioses in environmental samples to assess their ecological importance, and their temporal and spatial dynamics. For this purpose, molecular methods like metabarcoding and qPCR can be used, but microscopy techniques are also a promising approach that have to be considered. For instance, in situ imaging instruments can detect and enumerate symbioses directly in the environment (Olson and Sosik 2007; Malkassian et al. 2011; Erickson et al. 2012), which can be particularly useful for rare and delicate planktonic symbioses. Automated light microscopy, including automated acquisition and classification by sophisticated algorithms, is another promising, not yet

applied approach to quantify symbiotic associations in the environment (Rodenacker et al. 2006; Zeder et al. 2011; Schulze et al. 2013). Coupling automated microscopy and FISH assays is a highly interesting combination to rapidly visualize and quantify free-living and associated forms of described partnerships.

Finally, the function of each partner has to be studied to better assess the role of the symbiosis in nutrient recycling and also to determine the nature of the interaction (e.g., parasitism or mutualism). Stable isotope probing (SIP), single-cell genomics, metabolomics, proteomics, and secondary-ion mass spectrometry (SIMS) are all emerging methodologies that can determine physiological and metabolic activities of each partner. Together with appropriate sampling, an interdisciplinary approach of integrating cutting-edge methodologies and technologies, improvements in our understanding of planktonic symbioses are on the near horizon.

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Chapter 20
Marine Protist Viruses

Yuji Tomaru, Kei Kimura, and Keizo Nagasaki

Abstract Viruses are ubiquitous in the sea and one of the important factors affecting the ecology and evolution of marine protists. Although the existence of "protist viruses" was recognized before the 1980s, the knowledge of their physiological, ecological and genetic features has been progressively accumulated during the last quarter of a century, especially for viruses infecting marine microalgae. These novel viruses are highly diverse, which has extended our understanding of the host–virus relationships in many aspects. In this chapter, we discuss what is currently known about viruses infecting marine protists, mainly focusing on microalgae.

Keywords Bloom • Death • Diatom • Infection • Microalgae • Mortality • Virus

20.1 Introduction

Since the existence of large numbers of virus-like particles (VLPs) in natural waters was reported for the first time, aquatic viruses have been intensively examined (Bergh et al. 1989; Wommack and Colwell 2000). Currently the viruses are regarded as one of the major biological factors that regulate carbon cycling, microbial biomass and the genetic diversity of protists (Fuhrman 1999; Brussaard 2004; Suttle 2005; Brussaard et al. 2008). The number of viruses in natural waters has been estimated to be approximately 10^6 particles ml^{-1} in oligotrophic waters and approximately 10^8 particles ml^{-1} in highly eutrophic areas; however, this may be underestimated due to insufficiencies of the virus detection methods (Suttle 2007; Tomaru and Nagasaki 2007; Holmfeldt et al. 2012; Steward et al. 2013; Mojica et al. 2014).

Y. Tomaru (✉)
National Research Institute of Fisheries and Environment of Inland Sea,
Fisheries Research Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan
e-mail: tomaruy@affrc.go.jp

K. Kimura
Institute of Lowland and Marine Research, Saga University,
1, Honjo-machi, Saga, Saga 840-8502, Japan

K. Nagasaki
Seikai National Fisheries Research Institute, Fisheries Research Agency,
1551-8 Taira-machi, Nagasaki, Nagasaki 851-2213, Japan

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Most of the virus particles in marine environments are considered to be bacteriophages because of the high abundance of their hosts in natural waters, and the protist-infecting viruses are considered to occupy a non-negligible portion of marine viruses. To date, more than 40 viruses infecting marine protists, most of them microalgae, have been isolated and examined. Viruses that harbor a large double-stranded DNA (dsDNA) genome, i.e., nucleocytoplasmic large DNA viruses (NCLDV), have been most intensively studied so far in this field. On the other hand, recent studies have shown that the genomes of marine protist virus species are highly diverse; i.e., dsDNA, single-stranded DNA (ssDNA), double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) (Hyman and Abedon 2012). Although the basic biological characteristics and functions of some viruses have been intensively studied, their taxonomic position is not sufficiently understood due to the poverty of genetic information on protist viruses in databases, in contrast to those of animals, plants and insects. This indicates that marine protist virus study is still one of the frontier fields within the aquatic biological sciences.

20.2 Taxonomy of Marine Protist Viruses

20.2.1 dsDNA Virus

All the known marine protist viruses harboring the dsDNA genome are commonly members of NCLDVs (nucleo-cytoplasmic large DNA viruses). Most of the viruses infecting marine microalgae were considered to belong to the family Phycodnaviridae, e.g., viruses infectious to *Emiliania huxleyi* (Coccolithophyceae), *Heterosigma akashiwo* (Raphidophyceae), *Micromonas pusilla* and *Ostreococcus tauri* (Mamiellophyceae). However, recent studies have revealed the diversity among NCLDVs; for example, "HcDNAV" is a virus infectious to the bivalve-killing bloom-forming dinoflagellate *Heterocapsa circularisquama* (Dinophyceae) and is assigned as an unclassified virus but might be grouped into the family Asfaviridae in which African swine fever virus is the single member (Ogata et al. 2009). Further, a virus infecting the harmful bloom-forming prymnesiophyte *Phaeocystis globosa* seems not to be a phycodnavirus, but to be another clade clustering the large-genome-sized viruses Megavirus, Mimivirus (both infecting *Acanthamoeba*) and *Cafeteria roenbergensis* virus (CroV) (see below) (Santini et al. 2013).

Two viruses infecting heterotrophic microorganisms have been isolated from marine environments. One is "CroV" which infects the small bacterivorous marine flagellate *Cafeteria roenbergensis* (Fischer et al. 2010). The other is "*Pandoravirus salinus*" which is infectious to *Acanthamoeba castellanii* (Douglas) Page (Philippe et al. 2013). The features worthy of special mention of the latter virus are its huge capsid's morphology (1 μm in length and 0.5 μm in diameter) and genome size (2.47 Mb encoding 2556 ORFs (open reading frames)); surprisingly, the ratio of putative protein-coding sequences without a recognizable homolog is as high as 93 % (Philippe et al. 2013). In addition, its capsid formation process significantly

differs from the other eukaryotic DNA viruses and phages; i.e., the viral tegument and internal compartment are synthesized simultaneously until the final sealing process. These outstanding features suggest the existence of the fourth domain of life, and it is expected that further survey on *Pandoravirus* may connect the missing link between viruses and cells (Philippe et al. 2013).

On the other hand, there are several partially characterized dsDNA viruses infecting protists. SmDNAV is an unique dsDNA virus infecting a marine fungoid protist thraustochytrid, *Sicyoidochytrium minutum* (Takao et al. 2007). Because *S. minutum* is considered to be a producer of polyunsaturated fatty acids and a pathogen to mollusks, the host–virus ecology is of much interest. The virion is "squashed ball-like" in shape (approximately 146 and 112 nm in length and width, respectively) and lacks a tail; thus, it is distinct from any other previously known viruses. Virions are formed in the cytoplasm of host cells. SmDNAV harbors a dsDNA genome approximately 200 kb in length, but it has not been sequenced yet.

A virus infecting *H. akashiwo* (OIs1) consisted of two distinct morphotypes (80 nm and 30 nm in diameter and 20 kb and 130 kb in genome size, respectively) which simultaneously replicated within the cytoplasm of infected cells (Lawrence et al. 2006).

TampV infectious to *Teleaulax amphioxeia*, a marine free-living cryptophyte is also a polyhedral large virus approximately 203 nm in diameter (Nagasaki et al. 2009). The host alga plays a role as an endosymbiont when fed by the bloom-forming ciliate *Myrionecta rubra*. This ciliate is a specific prey organism for the toxic dinoflagellate *Dinophysis* species. *Dinophysis* utilizes the protoplasts of *T. amphioxeia* in its cytoplasm (Park et al. 2006; Nagai et al. 2008; Nishitani et al. 2008). Because of the complicated microbial relationship, the ecology of TampV is also attractive. TampV propagates in its host's cytoplasm and is assumed to be a dsDNA virus (Nagasaki et al. 2009).

20.2.2 ssDNA Viruses

All of the marine protist viruses harboring an ssDNA genome that have been isolated so far are infectious to diatoms, either centric or pennate species (Tomaru and Nagasaki 2011; Tomaru et al. 2012). To date, eight different ssDNA diatom viruses have been reported and at least five species among them are considered to belong to the genus *Bacilladnavirus* based on phylogenetic analysis using deduced amino acid sequences of a replication-related protein (Fig. 20.1). They are icosahedral and 32–38 nm in diameter, lacking a tail and an outer membrane. The virions are accumulated in the host nucleus. In addition to the icosahedral virions, fibrous or rod-shaped structures 17–27 nm in width and 0.5–1.4 μm long are often observed in the host nuclei when diatoms are infected with ssDNA viruses (ClorDNAV, Csp05DNAV, Csp07DNAV, CsetDNAV). In most cases, the icosahedral virions are observed in a lysate by a negative staining method (Fig. 20.2), but the rod-shaped particles are not. The rod-shaped particles are thus considered to be precursors of mature

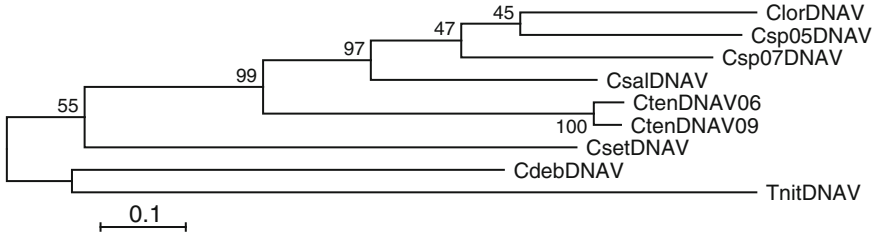

Fig. 20.1 Maximum likelihood (ML) trees calculated from confidently aligned regions of amino acid sequences of putative replication-related protein. ML bootstrap values (%) from 1000 samples are shown at the nodes. The ML distance scale bars are shown. The amino acid sequences that were used for comparison in the analyses are as follows, with their database accession numbers (these refer to the DDBJ database unless otherwise stated): CdebDNAV, AB504376; ClorDNAV, AB553581; CsalDNAV, AB193315; CsetDNAV, AB781089; CtenDNAV06, AB597949; CtenDNAV09, Y. Tomaru unpublished data; Csp05DNAV, AB647334; TnitDNAV, AB781284; *Chaetoceros* sp. SS628-11 DNA virus (Csp07DNAV), AB844272

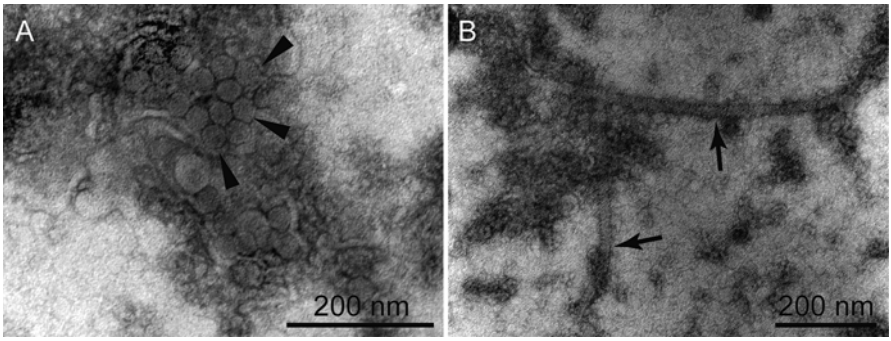

Fig. 20.2 Transmission electron micrographs of negatively stained Csp07DNAV particles. (**a**) Csp07DNAV particles in a *Chaetoceros* sp. stain SS628-11 culture lysate. *Arrow heads*: viral particles. (**b**) *Rod-shaped* particles in the culture lysate

icosahedral virions. Exceptionally, in the case of Csp07DNAV infectious to *Chaetoceros* sp. (Kimura and Tomaru 2013), many rod-shaped particles with the same morphological characters observed in the host nuclei were observed in the lysate (Fig. 20.2). However, the infectivity of the rod-shaped particles is unknown.

In some of the above-mentioned diatom viruses, the genome consists of 5–7 kb covalently closed circular ssDNA, as well as a segment of linear ssDNA <1 kb in length. The linear segment is complementary to a portion of the closed circle creating a partially double-stranded genome. In some cases, the genome lacks the segment of linear ssDNA (Tomaru et al. 2008b). In the case of CsetDNAV, infecting the bloom-forming diatom *Chaetoceros setoensis*, the genome is composed of a covalently closed circular ssDNA and eight different short complementary fragments, which are 67–145 nucleotides in length (Tomaru et al. 2013b). The segments may be primers, but their exact role has not been revealed yet. In any cases, the genome has two major ORFs, encoding putative replication-related protein and viral

structural protein. The replication proteins of the ssDNA diatom viruses showed low similarity to those of "rodent stool-associated circular genome virus" and "bat circovirus", which are both ssDNA viruses (family *Circoviridae*, genus *Circovirus*) (Todd et al. 2000), but not to any of the viruses infecting marine protists (Kimura and Tomaru 2013). This is presumably due to the insufficient database of ssDNA viruses infecting marine protists.

Two other reported *Chaetoceros* viruses, CspNIV (Bettarel et al. 2005) and CwNIV (Eissler et al. 2009), seem to be included in the genus *Bacilladnavirus* because their morphological and physiological characters are similar to those of the ssDNA diatom virus members mentioned above. Their genome type and sequence analysis, however, are essential for further phylogenetic studies among the ssDNA protist virus group.

20.2.3 dsRNA Virus

The isolation and characterization of a dsRNA virus infecting marine protists have been reported for only one species, *Micromonas pusilla* reovirus (MpRV) (Brussaard et al. 2004). MpRV harbors 11-segmented dsRNAs ranging between 741 and 5792 bp in length, with a total size of 25,563 bp, and is classified as a sole member of the genus *Mimoreovirus* in the family of *Reoviridae* based on the morphological and genomic characteristics (Attoui et al. 2006). The virus can coexist with a large-genome-sized dsDNA virus infecting the same *M. pusilla* strain (MpV). It has a narrow host range and a latent period of 36 h, and shows sensitivity to temperatures above 35 °C. The intact virus particles are 90–95 nm including outer-coat components. The subcore particles had a mean size of 50 nm and a smooth surface, indicating that MpRV belongs to the non-turreted *Reoviridae*.

20.2.4 ssRNA

The first report of marine protist ssRNA virus is HaRNAV infecting the harmful bloom-forming raphidophyte *H. akashiwo* (Tai et al. 2003). At least eight ssRNA viruses have been reported to date and the host organisms for seven species among them are stramenopiles: Raphidophyceae (Tai et al. 2003; Lang et al. 2004), Labyrinthulea (Takao et al. 2006) and Bacillariophyta (Tomaru and Nagasaki 2011; Tomaru et al. 2012, 2013a). The corresponding virus genera are assigned as *Marnavirus*, *Labyrnavirus* and *Bacillarnavirus*, respectively (Suttle 2011; Adams and Carstens 2012). The virus particles are icosahedral, are 22–35 nm in diameter, and accumulate in the host cytoplasm. The genomes are approximately 9 kb in length and commonly encode an RNA helicase, RNA-dependent RNA polymerase (RdRP) and capsid protein. However, they are diverse in genome organization. The genomes of *Marnavirus*, *Labyrnavirus* and *Bacillarnavirus* are composed of 1, 3 and 2 ORFs, respectively (Lang et al. 2008). Another virus HcRNAV infecting the

bloom-forming dinoflagellate *H. circularisquama* is also a small virus harboring a linear positive-sense ssRNA genome of 4.4 kb in length, containing two ORFs; the upstream ORF encodes a replication polyprotein and the downstream ORF codes for a single major capsid protein (MCP) (Tomaru et al. 2004a; Nagasaki et al. 2005). A cryo-electron microscopy study on HcRNAV particles revealed that the virus has a diameter of 34 nm and $T=3$ symmetry, and consists of 180 quasi-equivalent monomers (Miller et al. 2011). HcRNAV has been placed in a new genus *Dinornavirus* within the new class *Alvernaviridae* (Adams and Carstens 2012), because phylogenetic analysis of the RdRP showed HcRNAV was deeply branched and apparently distinct from other protist viruses (Fig. 20.3).

20.3 Ecology

Several microalgal host–virus systems in natural environments have been intensively studied, e.g., *E. huxleyi*, *P. globosa*, *M. pusilla*, *H. akashiwo* and *H. circularisquama* and their respective viruses from the viewpoint of ecology. In these relationships, the viruses contribute to the disintegration of the hosts' blooms and the succession of host clonal composition. Therefore, viral infection has not only a quantitative impact (reducing biomass) but also a qualitative impact (changing clonal composition) on microalgal host populations in natural environments.

20.3.1 Impact of Viral Infection on Protist Populations

Temporal changes of protistan host and its infectious virus abundances based on high-resolution field surveys demonstrated their intimate relationships in nature. In general, a typical increase in abundance of viruses is observed when its host forms a bloom, e.g., *E. huxleyi* and EhV and/or unidentified smaller viruses (Brussaard et al. 1996; Schroeder et al. 2003; Vardi et al. 2009, 2012), *P. globosa* and PgV (Baudoux et al. 2006), *H. akashiwo* and HaV (Tomaru et al. 2008a), *H. circularisquama* and its viruses (Nagasaki et al. 2004; Tomaru et al. 2007), and *Chaetoceros* spp. and their viruses (Tomaru et al. 2011a). Other than the microalgal host and virus systems, viruses infecting the heterotrophic flagellate *C. roenbergensis* are also believed to be important for its host's dynamics in nature (Massana et al. 2007).

There are several methods to estimate viral induced mortality, e.g. (1) the direct counting method, counting virally infected cells by transmission electron microscopy (TEM); (2) the theoretical calculation method, estimating cell lysis from the total number of viruses and burst size per cell; and (3) the modified dilution method, estimating the viral lysis of phytoplankton cells using the classical dilution approach originally developed to assess the grazing impact of marine microzooplankton (Landry and Hassett 1982). The direct counting method demonstrated that the proportion of cells harboring VLPs in *E. huxleyi*, *H. akashiwo* and *H. circularisquama*

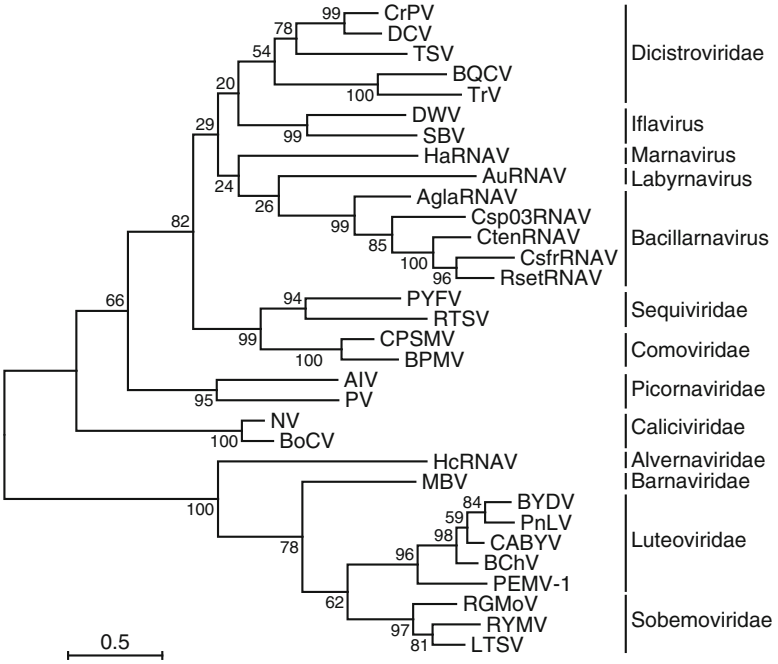

Fig. 20.3 Maximum likelihood (ML) trees calculated from confidently aligned regions of amino acid sequences of RNA-dependent RNA polymerase (RdRp) domain. ML bootstrap values (%) from 1000 samples are shown at the nodes. The ML distance scale bars are shown. Amino acid sequences used for comparison in the analyses are as follows with the organism's scientific names, with abbreviations in parentheses if necessary, and the database accession numbers (referring to the US National Center for Biotechnology Information (NCBI) unless otherwise stated): beet chlorosis virus (BChV), AAK49964; bovine enteric calicivirus (BoCV), AJ011099; bean pod mottle virus (BPMV), NC__003496; black queen cell virus (BQCV), NC__003784; barley yellow dwarf virus (BYDV), BAA01054; cucurbit aphid-borne yellows virus (CABYV), CAA54251; cowpea severe mosaic virus (CPSMV), M83830; cricket paralysis virus (CrPV), NC__003924; drosophila C virus (DCV), NC__001834; deformed wing virus (DWV), NC__004830; foot-and-mouth disease virus (FMDV), P03306; *Heterosigma akashiwo* RNA virus (HaRNAV), NC__005281; *Heterocapsa circularisquama* RNA virus 109 (HcRNAV), DDBJ accession number AB218609; lucerne transient streak virus (LTSV), NP__736596; mushroom bacilliform virus (MBV), NP_042510; Norwalk virus (NV), M87661; pea enation mosaic virus 1 (PEMV-1), AAA72297; poinsettia latent virus (PnLV), CAI34771; human poliovirus 1 Mahoney (PV), V01149; parsnip yellow fleck virus (PYFV), D14066; ryegrass mottle virus (RGMoV), NP__736587; *Rhizosolenia setigera* RNA virus (RsRNAV), DDBJ accession number: AB243297; rice turgo spherical virus (RTSV), AAA66056; rice yellow mottle virus (RYMV), CAE81345; sacbrood virus (SBV), NC_002066; *Aurantiochytrium* single-stranded RNA virus (AuRNAV), BAE47143; *Triatoma* virus (TrV), NC__003783; taura syndrome virus (TSV), NC__003005; *Asterionellopsis glacialis* RNA virus (AglaRNAV), unpublished data; *Chaetoceros socialis* f. *radians* RNA virus (CsfrRNAV), AB469874; *Chaetoceros* sp. number 03 RNA virus (Csp03RNAV), AB639040; *Chaetoceros tenuissimus* RNA virus (CtenRNAV), AB375474

bloom populations at termination stages in nature ranged from approximately 5–88 % to total cells (Nagasaki et al. 1994; Brussaard et al. 1996; Tomaru and Nagasaki 2004). The increases of viruses in a water column theoretically explained 25–100 % of the total net mortality in *E. huxleyi* blooms during a bloom decline stage (Bratbak et al. 1993; Jacquet et al. 2002). The modified dilution method showed that the virally induced mortality of *Micromonas* spp. populations in a mesocosm and *P. globosa* single cells in a field were estimated at 9–25 % and 35 % day^{-1}, respectively (Evans et al. 2003; Baudoux et al. 2006). Although each evaluation method has both technical merit and demerit (Short 2012), these data suggest that viral impact on phytoplankton cell mortality is significant in natural environments.

20.3.2 Effects of Environmental Factors on Viral Infection

The viral susceptibilities of the host cells and their interactions are changeable under different growth phases (Brussaard 2004). For example, in the case of a dsDNA virus "PpV" and its host *Phaeocystis pouchetii* (Coccolithophyceae), a decrease in burst size was observed when host cells in the stationary phase were exposed to a viral attack (Bratbak et al. 1998). The latent period and burst size of HcDNAV infectious to *H. circularisquama* are shorter and larger, respectively, accompanied by the higher host growth (Nagasaki et al. 2003). The sexual phase is also a key factor determining virus sensitivity; e.g., diploid-phase cells of *E. huxleyi* were susceptible to EhV but its haploid-phase cells were not (Frada et al. 2008). Planktonic diatom *Chaetoceros* species maintain logarithmic growth even when its infectious viruses coexist; however, the population crashes in the late stationary phase (Tomaru et al. 2011c). The virus infectivity of diatoms, therefore, might be related to their cell growth. These studies indicate that the physiological conditions of the host can have an important impact on the susceptibility to viral infection and viral proliferation upon infection. In contrast, Eissler et al. (2009) reported that lytic rates of CwNIV infectious to *Chaetoceros* cf. *wighamii* (Bacillariophyta) had no relationships with its host growth rates. This might explain that the relationship between the viral sensitivity and the host growth significantly differs among species and clones. Further analysis would be required to give a clear conclusion on this matter.

Besides, various environmental factors are assumed to affect host–virus systems in natural environments. In general, water temperature is one of the most important factors in determining viral infection efficiency and the fate of virally infected cells, because it is a critical factor in any biological processes. In the case of *H. akashiwo* and its virus HaV, a host strain shows virus sensitivity below 25 °C but not at 30 °C (Nagasaki and Yamaguchi 1998). Furthermore, its virus sensitivity pattern along with a water temperature change was shown to differ also at the clonal level (Nagasaki and Yamaguchi 1998). Bloom dynamics of a harmful algal species *P. globosa* are affected by several distinct groups of its infectious viruses, and their

interactions and potential dominance are considered to be strongly controlled by temperature (Baudoux and Brussaard 2005). Suitable temperatures for proliferation of two distinct viruses, CtenDNAV and CtenRNAV, both infecting *Chaetoceros tenuissimus* (Bacillariophyta), are considerably different; therefore, the diatom host-virus relationships might be also significantly regulated by water temperature (Tomaru et al. 2014).

20.3.3 Infection Specificities in Algal Host-Virus Systems

In general, viral infection against protists is host strain specific rather than species specific. This simultaneously implies that the virus susceptibilities of host strains are also highly diverse. The host strain specificity of HaV infecting *H. akashiwo* are varied. Tomaru et al. (2004b) isolated 90 *H. akashiwo* strains and 65 HaV strains using four *H. akashiwo* strains with different virus susceptibilities during a series of the same bloom. Then, they carried out a cross-reactivity test between the host–virus strains ($65 \times 90 = 5850$ combinations), and found they could be respectively divided into six host groups and three virus groups. This suggests the high complexity between the host and virus systems in natural environments.

In contrast to the above-mentioned case, the host strain specificities of HcRNAV infectious to *H. circularisquama* appear to be relatively simple and their relationships have been well studied. The key factor determining the diversity is considered to be a relationship between a viral receptor on a host cell surface and a viral capsid structure. HcRNAV clones are divided into two types (types UA and CY) based on their intraspecies host specificity patterns ($56 \times 107 = 5992$ combination cross-reactivity tests) (Tomaru et al. 2004a). Each type shows its own strain-specific infectivity that is complementary to each other; the *H. circularisquama* strains sensitive to HcRNAV type UA were resistant to HcRNAV type CY, and vice versa, showing that HcRNAV is not species specific but strain specific. These two HcRNAV types can coexist in natural water. The full sequences of typical HcRNAV clones for type UA and CY (HcRNAV34 and 109, respectively) are 97 % identical at the nucleotide sequence level to each other. Each genome has two ORFs. ORF-1 and ORF-2 encode a replication-related polyprotein and viral MCP, respectively (see Sect. 20.2.4). Genomic comparison suggests that the complementary host ranges of the two HcRNAV types are strongly related to the amino acid substitution patterns in ORF-2, i.e., viral capsid. The tertiary structure of the viral capsid indicated many of the amino acid substitutions were located in regions on the outside of the viral capsid proteins. This indicates that the intraspecies host specificity of HcRNAV is determined by structural differences of the viral capsid surface that may affect its binding affinity to the host cell and the uncoating process. The transfection experiment strengthened this idea: (1) the intraspecies host specificity of HcRNAV is determined by the upstream events of virus infection; and (2) the host intracellular condition is permissive for HcRNAV replication even in the case of incompatible host–virus combinations (Mizumoto et al. 2007).

By the cross-reactivity test, *H. circularisquama* clones are also divided into two types according to the sensitivity spectra to the two HcRNAV types (types UA and CY); however, the two host types are indistinguishable when comparing their morphology or the sequences of the internal spacer regions of the ribosomal RNA genes. These two host types coexist in natural water. Thus, there are at least two distinct host–virus systems between *H. circularisquama* and HcRNAV; that is, multiple types of host and virus coexist within natural blooms of *H. circularisquama* and their combinations are regulated with sophisticated molecular mechanisms. Further recent study reported the existence of another subtype of HcRNAV showing infectivity to a *H. circularisquama* that is resistant to both HcRNAV34 and 109 (Mizumoto et al. 2008; Nakayama et al. 2013); thus, the host–virus relationship is more complicated than first estimated by Tomaru et al. (2004a).

Other than the above cases, the intraspecies host strain specificities of viruses are observed for many microalgae and their virus systems, e.g., *M. pusilla* and MpV (Waters and Chan 1982; Sahlsten 1998), *H. akashiwo* and HaNIV (Lawrence et al. 2001) and several diatoms and their viruses (Tomaru and Nagasaki 2011).

20.3.4 Multiple Viral Infections

Several studies have revealed that multiple species of viruses affect a single host population in nature. Field studies indicated that two different sized VLPs (185–200 and 50–60 nm) coexisted in the same *E. huxleyi* cell (Brussaard et al. 1996). In the late stage of *H. circularisquama* blooms in western Japan, cells harboring HcDNAV- and HcRNAV-like particles were simultaneously detected in the same sample, although they were not found in the same cell (Fig. 20.4) (Tomaru and Nagasaki 2004). The viruses infecting *P. globosa* isolated from the southern North Sea were divided into at least two groups (Baudoux and Brussaard 2005). They are discriminated based on particle size (150 and 100 nm, respectively), genome size (466 and 177 kb, respectively), and structural protein composition. Similar results have also been reported in diatom viruses (Shirai et al. 2008; Tomaru et al. 2011b). In most cases, infection specificities of viruses and viral physiological characters are diverse (see the above paragraphs). These reports imply that multiple viral species play important roles in determining the dynamics and genetic diversity of their host community.

At least four different viruses infecting *H. akashiwo* have been reported: HaV, HaNIV, HaRNAV and OIs1 (Nagasaki and Yamaguchi 1997; Lawrence et al. 2001, 2002, 2006; Tai et al. 2003). During a field survey at Seto Inland Sea, Japan, in 2000, viruses other than HaV were not detected among the 65 viral isolates (Tomaru et al. 2004b). It is enigmatic why distribution of viruses showed such a geographic heterogeneity, considering that the other three viruses were isolated from British Columbia, Canada.

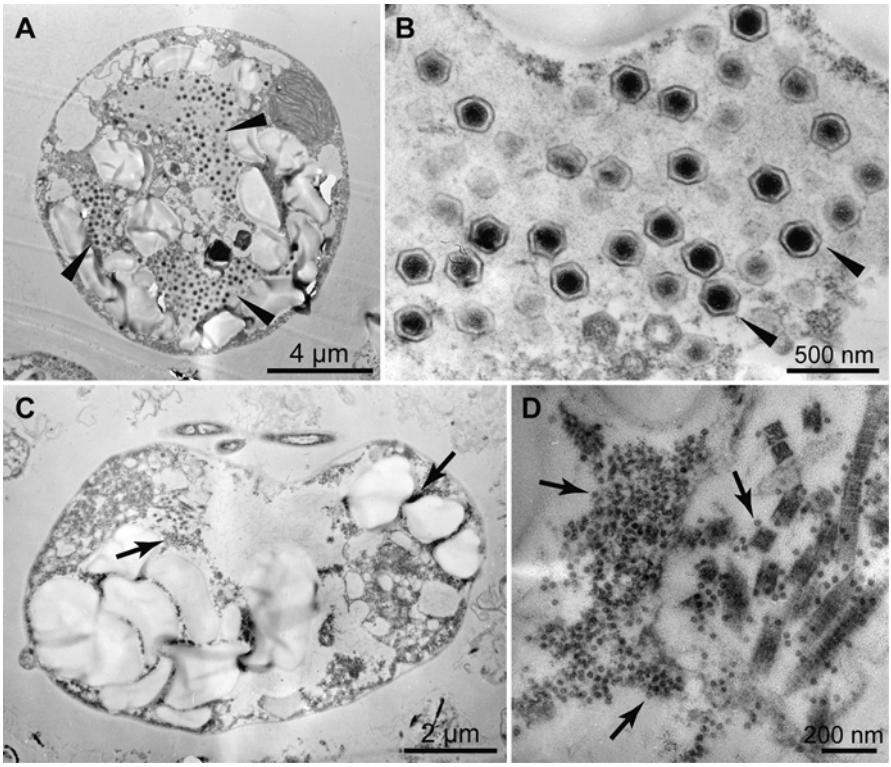

Fig. 20.4 Transmission electron micrographs of *Heterocapsa circularisquama* cells collected from Fukura Bay on August 29, 2001, during its bloom period. TEM preparation was conducted following incubation at 25 °C for 24 h. (**a**) *H. circularisquama* cell harboring large VLPs (*arrows*) showing degradation of cell organelles within the cytoplasm. (**b**) Higher magnification of the large VLPs within the *H. circularisquama* cytoplasm (*arrows*). (**c**) *H. circularisquama* cell harboring small VLPs (*arrows*) showing degradation of cell organelles within the cytoplasm. (**d**) Higher magnification of the small VLPs (*arrows*) (Modified from Tomaru and Nagasaki (2004) with permission from the Plankton Society of Japan)

20.3.5 Resistance to Viral Infection

Resistance to viral infections in marine microalgal hosts is considered to be primarily regulated by upstream events of viral infection, i.e., a viral adsorption process which affects the strain-specific host specificities of viruses (Nagasaki et al. 2005; Tarutani et al. 2006; Mizumoto et al. 2007). In several marine protistan host–virus systems, regrowth of host cells following viral lysis were reported: *H. circularisquama* and HcRNAV, *P. pouchetii* and PpV, *Pyramimonas orientalis* (Prasinophyceae) and PoV, *Chrysochromulina ericina* (Coccolithophyceae) and CeV, *E. huxleyi* and EhV, *Ostreococcus tauri* (Mamiellophyceae) and OtV, *Bathycoccus* sp. (Mamiellophyceae), BpV, *M. pusilla* and MpV, and *Aurantiochytrium* sp. and AuRNAV (Thyrhaug et al. 2003; Takao et al. 2005; Zingone et al. 2006; Tomaru

et al. 2009; Thomas et al. 2011). Thyrhaug et al. (2003) suggested that excess viral molecules released during cell lysis may compete with virus particles for receptor sites on the host cell surface, thus reducing infection, which contributes to stable coexistence of a microalgal host and its lytic virus. According to Zingone et al. (2006), it is difficult to explain the resistance mechanism of *M. pusilla* against MpV based on inhibition alone. There is the possibility that *M. pusilla* cells could mutate to become virus resistant at the cell surface; this resistance was sustained at least for several years in culture without virus addition (Waters and Chan 1982; Zingone et al. 2006), and no positive result supporting the possibilities of latent or lysogenic infections in *M. pusilla* was obtained (Zingone et al. 2006). Suppression of intracellular viral genome RNA replication has been observed in *H. circularisquama*, where the transfected HcRNAV genome was replicated only in susceptible cells but not in survivors through viral infections (Tomaru et al. 2009). *H. circularisquama* cells maintained this virus resistance under viral pressure (as long as fresh viruses were supplied to the culture); however, they could turn back to being susceptible to viral infections once the viral pressure was removed. Therefore, this viral-resistance mechanism is considered due not to mutation or lysogeny but to one of their (unidentified) physiological systems. Thomas et al. (2011) hypothesized that two mechanisms of resistance related to cellular physiology (especially to growth conditions) may operate in *O. tauri*: (1) viruses can attach to their host cells but no new particles develop; and (2) *O. tauri* acquires tolerance to its virus and releases these viruses consistently. Another unique virus resistance is observed in diatom *C. tenuissimus* and its RNA virus (Kimura and Tomaru 2014). In this system, the host cells completely crash due to viral infection under an axenic condition; in contrast, with coexistence of bacteria, they can survive through infection and show regrowth. This phenomenon suggests that bacteria might affect a significant effect on their systems in nature. This also indicates that the result of examinations on virus resistance or host sensitivity can be affected by bacterial coexistence; thus, they should be checked by using axenic cultures.

The mechanisms supporting the protist viruses' resistance seem to be diverse. The diversity may be important to sustain each host–virus system in natural environments. For instance, considering that the HcRNAV resistance of *H. circularisquama* is reversible (see above), host–virus coexistence in natural environments may be enabled through the mutual control between the host–virus populations, i.e., when a natural host population (viral susceptible cells) is infected by viruses, most cells are lysed but survivors increase as resistant cells even in high virus pressure; then the viral concentration decreases because there are no susceptible host cells; in the next step, the resistant cells are turned into susceptible cells under no virus pressure and increase. In a natural environment, they coexisted for over 2 months in Ago Bay in 2002 where their concentrations ranged from 10^0 to 10^2 host cells ml^{-1} and from 10^0 to 10^3 virus infectious units ml^{-1}; (Tomaru et al. 2007). Such observations support the idea that a mutual controlling system may prevent the host population from reaching high abundance in nature.

20.4 Implications

In the last two decades, numerous field and laboratory studies on marine protist viruses have revealed the significance of viruses for their host dynamics in nature. They simultaneously indicated the complex biological and ecological relationships in the host–virus systems, and there may be far more complicated interactions than we had predicted. Technological advancements such as analytical instruments, molecular biological methods and genomics have brought a number of new discoveries to marine protist virus ecology, typically concentrated in several limited species, e.g., *E. huxleyi* (Vardi et al. 2009, 2012) and *P. globosa* (Brussaard et al. 2005; Baudoux et al. 2006). In general, however, to understand a viral induced mortality rate in a host population in a natural environment is still laborious and time consuming even using an up-to-date method. Further technological innovations may be necessary to reveal the roles of diverse marine protist viruses in regulating their host populations in nature.

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Chapter 21
Biology of Parasitic Heterotrophic Nanoflagellates: Parasitoids of Diatoms

Michael Schweikert

Abstract Heterotrophic nanoflagellates are important consumers of bacteria in all environments. Nowadays, some few exceptions are known, termed parasitic nanoflagellates (PNF), feeding on much larger prey in marine waters. They are feeding on planktonic and benthic diatoms. Since these flagellates inevitably kill their host they are parasitoids rather than parasites despite the use of the term PNF. The name parasitic nanoflagellate refers to an ecological function rather than to taxonomic entities, since these organisms correspond to different taxonomic groups. It can be expected that the diversity of PNF is much greater than known so far, since reports of undescribed species can be found in the literature. Examples of different parasitoidic nanoflagellates are given here. Morphological features and taxonomic positions of these species, as well as their sophisticated modes of infection and food acquisition, will be described.

Keywords Heterotrophic parasitic nanoflagellates • HNF • Parasitic nanoflagellates • PNF *Pirsonia* • *Pseudaphelidium* • *Chytridium* • Fungi • Oomycetes • Infection

21.1 General Biology of Heterotrophic Parasitic Nanoflagellates

Intensive investigations of the marine environment for decades have resolved an enormously complex web of interactions on different trophic levels. While nutrients are delivered from lower trophic levels to higher trophic levels, from primary producers to consumers, food webs gain more complexity with parasitic and mutualistic interactions. Nowadays, it is well known that heterotrophic flagellates (HNF) serve as important bacterial consumers in freshwater and marine environments, transferring the amount of dissolved organic carbon (DOC) via consumption of bacterial into higher trophic levels. Only 2–10 μm in size, they are important

M. Schweikert (✉)
Department of Zoology, Institute for Biomaterials and Biomolecular Systems, University of Stuttgart, Pfaffenwaldring 57, D-70569 Stuttgart, Germany
e-mail: Michael.Schweikert@bio.uni-stuttgart.de

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members of the so called microbial loop, turning over the vast amount of fast-growing bacteria in the environment (e.g., Laybourn-Parry and Parry 2000). It is generally assumed that consumers are larger than their prey. However, some heterotrophic nanoflagellates rather break this rule. The term heterotrophic nanoflagellates in general does not refer to a distinct taxonomical clade; rather, it describes its morphology or appearance in the light microscope (transparent and small). These organisms are assigned to a diversity of taxonomical groups.

Initially, at the end of the last century, the first nanoflagellates were described, morphologically similar in size and appearance to HNF, feeding specifically on much larger primary producers, mainly diatoms, sized about 15 µm–2 mm. These so-called parasitic nanoflagellates were overlooked before, most probably because traditional sampling and cultivation methods were applied. Cells detach easily from host cells in plankton net samples and during chemical fixation. Cultivation is not trivial and always needs pre- and co-cultivation of the host diatom species in such an amount that there are enough cells for infection.

To reveal the life cycle or host specificity of a parasitic nanoflagellate, investigations of living cells are inevitable. The parasitic nanoflagellates were often recognized while in situations of the host diatom being the dominating species in the plankton community at that time. In environmental samples, about 90 % of a diatom population can be found to be infected (Kühn et al. 2004). In general, these infections are lethal for the diatoms, resulting in a rapid decrease in abundance of host diatoms, often from blooming to extinction within few days (Grahame 1976; Tillmann et al. 1999; Kühn et al. 2004). Their host specificity and their short life cycles make them key players in the control of population dynamics (Gachon et al. 2010). In the North Sea, almost all diatom species are assumed to be susceptible to parasitic nanoflagellate infections (Kühn et al. 2004).

Each species of parasitic nanoflagellates invades a few diatom species only or, in most cases, only one species specifically. They display a variety of specialized modes of infection, feeding structures and behavior.

Parasitic nanoflagellates of diatoms were attracted by the diatom cell from a distance of several 100 µm by substances released by the diatom into the environment (Held 1973; Kühn et al. 2004). This kind of long-distance attraction is rather nonspecific, since host diatom species can be replaced as an attractant by other diatom species, small pieces of cheese, ham or rice grains. After this nonspecific attraction, parasites attach to the cell surface, resembling a siliceous frustule in diatoms (Figs. 21.1 and 21.2). Besides silica, frustules can contain proteins (e.g., diatotepin) and sugar polymers (e.g., cellulose and chitin), additionally. Diatom frustules display species-specific morphology on the valves as well as on the girdle bands (Fig. 21.1). The valve surface is often structured by areolae and can display protrusions or elevations to the outside, like strutted or labiate processes or setae (Figs. 21.1 and 21.2). Strutted processes are known to guide filaments of chitin from the plasma membrane through the frustule to be elevated into the environment. After attachment, on the surface of the diatom cell wall many known species of nanoflagellate diatom parasites migrate over the frustule surface displaying a tapping movement of their anterior flagellum, and a trailing behavior of the posterior

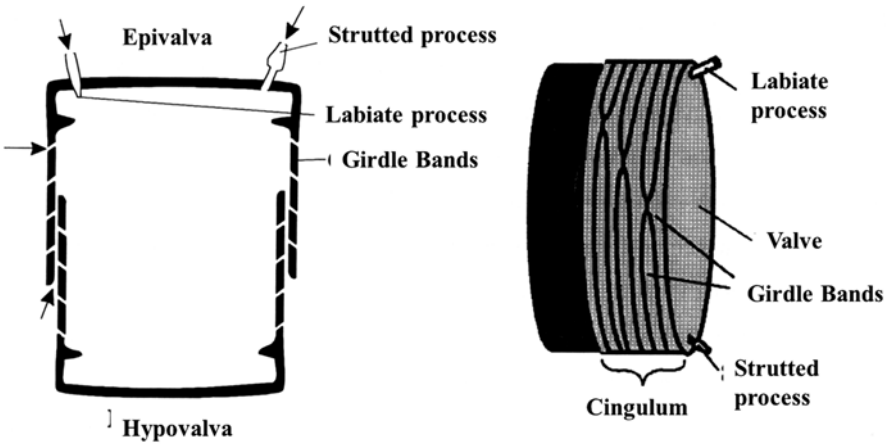

Fig. 21.1 Scheme of the diatom frustule. The valve can be discriminated into two halves, the larger Epivalva and the smaller Hypovalva. Besides structures on the valve surface, strutted processes and labiate processes can be present. Infection sites known to overcome the frustule are through both processes and through gaps between the different components of the valve, as girdle bands and overlapping valve. Possible infection sites are marked with *arrows*

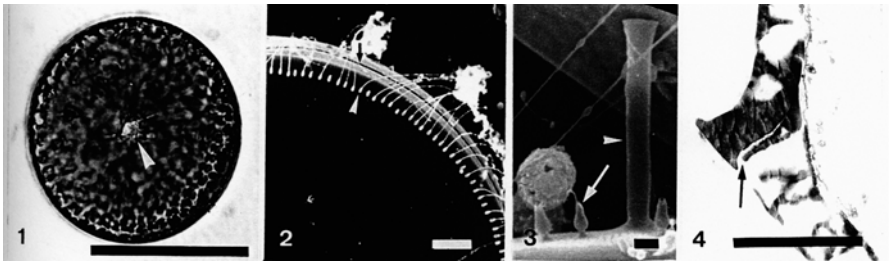

Fig. 21.2 Light and electron microscopy images of details on the frustule of the diatom *Thalassiosira punctigera*. (**a**) Light microscopy images of a living cell of *T. punctigera* embraced by a siliceous frustule. (**b**) Scanning electron micrograph of a valve of *T. punctigera* with numerous chitin fibers elevating from strutted processes (*white arrowhead*) and the overlapping of the Hypovalva (*front*) and Epivalva (*back*). The possible infection site is marked with a *black arrow*. (**c**) Scanning electron micrograph of the valve of *T. punctigera*. A strutted process (*white arrow*) and a labiate process (*white arrowhead*) are marked. A flagellate cell of *Pirsonia punctigera* is already attached to its specific infection site at an strutted process. (**d**) Transmission electron micrograph of a frustule of *T. punctigerae*. A fissure between the valve and the first girdle band as a possible infection site is marked (*black arrow*)

flagellum. It is rather speculative to conclude a morphology or chemical recognition on the frustule surface by these protists, but this behavior results in a diatom and parasite species-specific invasion of the diatom cell. This short-distance recognition guides the cell to its specific invasion site on the frustule surface where it settles. While some species can penetrate the frustule through all holes with a suitable size, some species prefer specific sites, like strutted processes, labiate processes, an

opening at the end of the setae, or gaps between girdle bands or less silicified parts, covered by polycarbonates only.

21.2 The Genus *Pirsonia/Pseudopirsonia*

The genus *Pirsonia* was described by Schnepf et al. 1990 for nanoflagellate parasitoids of diatoms in the North Sea. The genus comprises six species, namely *P. guinaridae* (Schnepf et al. 1990), *P. verrucosa* (Kühn et al. 1996), *P. formosa* (Kühn et al. 1996), *P. diadema* (Kühn et al. 1996; Kühn 1997, 1998; Kühn and Hofmann 1999; Schnepf and Schweikert 1996), *P. eucampiae* (Kühn et al. 1996) and *P. punctigerae* (Schweikert and Schnepf 1997a). Details of morphological characteristics for all *Pirsonia/Pseudopirsonia* species are given in Kühn et al. (2004, Table 21.1). Based on morphology, a seventh species, *P. mucosa* (Kühn et al. 1996), was assigned to this genus, but phylogenetic investigations have revealed its distinct relationship to all other monophyletic *Pirsonia* species and its transfer to the new genus *Pseudopirsonia* (Kühn et al. 2004) within Cercozoa was confessed. The closest relatives to *P. mucosa* known are *Hyphochytrium catenoides* and *Rhizidiomyces apophysatus* within Heterokonta. *Pirsonia* species are located within stramenopiles, which is a term that was introduced as a rankless, informal name for heterokont eukaryotes (Patterson 1989). This description was emended in the revised classification of Adl et al. (2012), Cavalier-Smith (1998); the stramenopiles were placed

Table 21.1 *Pirsonia/Pseudopirsonia* species and their respective infection sites and diversity of host diatom species

Pirsonia/Pseudopirsonia species	Host diatom	Infection site
P. guinardiae	*Guinardia flaccida*	Fissures between girdle bands
P. diadema	*Coscinodiscus granii*	Strutted processes (rimoportulae)
	Coscinodiscus wailesii	
P. eucampiae	*Eucampia zodiacus*	Fissures between girdle bands
P. formosa	*Eucampia zodiacus*	Fissures between girdle bands
	Guinardia delicatula	
	Leptocylindrus danicus	
	Rhizosolenia imbricata	
	Rhizosolenia setigera	
P. verrucosa	*Guinardia delicatula*	Fissures between girdle bands
P. punctigerae	*Thalassiosira punctigera*	Strutted processes
	Thalassiosira hendeyi	
Pseudopirsonia mucosa	*Rhizosolenia shrubsolei*	Fissures between girdle bands
	Rhizosolenia setigera	
	Rhizosolenia delicatula	
	Cerataulina pelagica	
	Eucampia zodiacus	

Adapted from: Kühn et al. (1996, 2004)

within the SAR clade together with the Alveolates and the Rhizaria. The anterior flagellum possess tripartite, tubular hair-like projections (mastigonemes) and tubular mitochondrial cristae with a central filament (Patterson 1989) Stramenopiles comprises a huge range of taxa that include, for example, *Phaeophyceae, Chrysophycea, Labyrinthulomycota* and *Bacillariophyceae*. Details in the ultrastructure of the flagellar basis support the molecular relationship to the Chrysophyceae.

The life cycle and feeding mode of the *Pirsonia* species is unique (Fig. 21.2). Comprehensive information on the host range of *Pirsonia/Pseudopirsonia* species is given in Kühn et al. 2000. Flagellates attach to the diatom frustule and squeeze a pseudopod through the frustule, either between the girdle bands or through the rimoportulae or labiate processes, which resemble minute tubular openings that enable penetration of the cell wall (Fig. 21.3: 1 + 2). The pseudopod then creates the trophosome, which performs phagocytosis and take up parts of the diatom protoplast and digests it in a central vacuole (Fig. 21.3: 2–6). Nutrients are transported

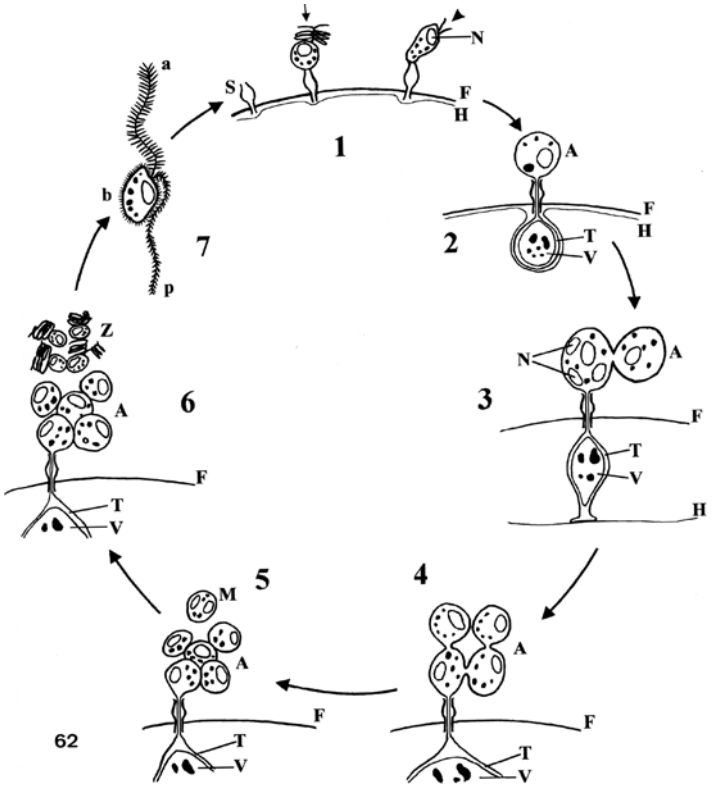

Fig. 21.3 Scheme of the life cycle of *Pirsonia punctigera*. Different steps in the life cycle of *Pirsonia punctigera* infecting the diatom *Thalassiosira punctigera*. *S* strutted process, *F* frustule, *H* host plasma membrane, *N* nucleus, *A* auxosome, *T* trophosome, *V* food vacuole, *M* flagellate mother cell, *Z* zoospores, *a* anterior flagellum with mastigonemes, *p* posterior flagellum with small mastigonemes (From Schweikert and Schnepf 1997b)

into the part of the cell remaining outside the frustule named the auxosome (Fig. 21.3: 2–6). The auxosome grows, divides and produces numerous offspring as long as the nutrient supply continues (Fig. 21.3: 3–6). The first cells that detach are named flagellate mother cells, after two divisions giving rise to motile flagellates (Fig. 21.3: 5–7). Depending on the species, from a single cell infection up to 60 flagellated cells may be generated in less than a day. Multiple infections can occur in the environment and are common under culture conditions, resulting in joint trophosomes of different individuals. Flagellates can infect new diatom cells immediately after detachment from the auxosome. Dormant stages have been reported for two *Pirsonia* species only, namely *P. guinardiae and P. formosa*. These cysts may enable the flagellate to overcome times of absence of host diatoms in the environment. *Pirsonia* species themselves can serve as food for other larger predators, e.g., the peduncle-feeding dinoflagellate *Cryptecodinium cohnii* (unpubl. data).

21.3 *Pseudaphelidium drebesii*

Pseudaphelidium drebesii is a pathogenic opisthokont protist of the centric marine diatom *Thalassiosira punctigera* (Schweikert and Schnepf 1996, 1997b). The cells are about 5 µm long and 3 µm wide with a single opisthokont flagellum about 15 µm long. The motile unikont zoospore attaches to a host cell wall, migrates on the frustule surface, attaches and encysts by means of fibrillar substances, most probably carbohydrates (Fig. 21.4 A–C). The encysted flagellate penetrates the frustule through a hole in its cyst wall placed directly over an opening of the frustule, like gaps between girdle bands or between valve and the adjacent girdle band. This is facilitated by a unique process and the involvement of a specialized cellular structure, the infection tube (Fig. 21.5). First, an increasing volume of a vacuole generates an intracellular pressure antagonistic to the already manifested cyst wall. Then, an infection tube, produced before by the flagellate, is everted into the fissure in the frustule, which allows the cell to be squeezed through by means of intracellular pressure (Fig. 21.5). The invaded cell gets into intimate contact with the diatom protoplast and starts phagocytosis. Outside empty cyst walls remain as an indication of infection. Food acquisition is achieved by embracement of parts of the diatom protoplast by the parasitic nanoflagellate and uptake by phagocytosis into a single food vacuole (Fig. 21.4 E–H). While growing, the flagellate develops into a plasmodium with a hundred nuclei. At the end of the trophic phase the plasmodium is a hollow sphere filling the frustule completely, replacing the diatom protoplast (Fig. 21.4 H). It cleaves to form amoeboid cells which are slowly motile and finally encyst after one or two cell divisions (Fig. 21.4 I+J). Cysts can rest or release up to four motile unikont cells immediately after excystment (Fig. 21.4 K+L). These flagellates are infectious immediately after excystment. The ultrastructural organization of the zoospores (Schweikert and Schnepf 1996, 1997b) as well as the infection tube indicates a close taxonomic relationship to the Plasmodiophoraceae, a family within opisthokonta. All members of Plasmodiophoraceae are parasites,

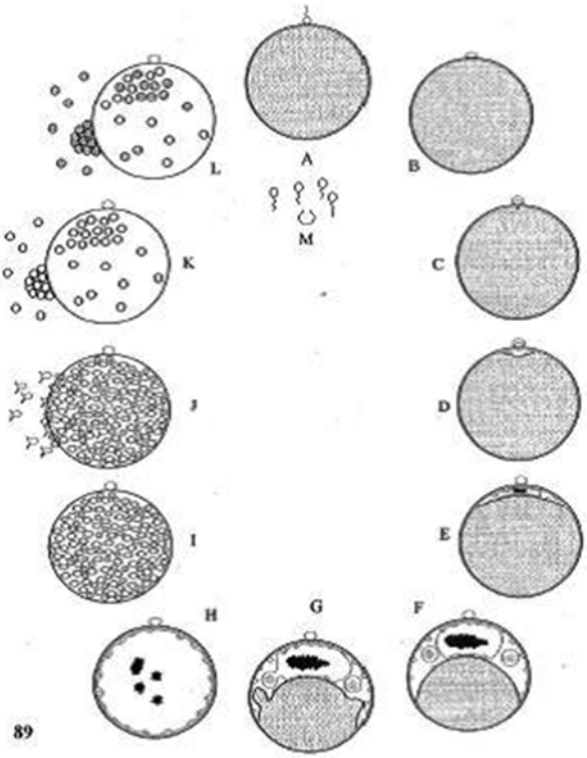

Fig. 21.4 Scheme of the life cycle of *Pseudaphelidium drebesii*. Different steps in the life cycle of *Pseudaphelidium drebesii* infecting the diatom *Thalassiosira punctigera* A–L (For details, see text in Sect. 21.3. From Schweikert and Schnepf 1996)

often causing hypertrophy in seed plants, and are relatives of the lichen fungi and Oomycetes.

Besides *Pseudaphelidium drebesii* there are two other nanoflagellate plasmodiophoromycete species known to infect diatoms, named *Phagomyxa belleochae* on the diatom *Bellerochea malleus* and *P. odontellae* on the diatom *Odontella sinensis* (Schnepf 1994, 1999; Schnepf et al. 2000; Bulman et al. 2001). Both species infect the cell via fissures within the frustule and enter the cell wall completely. They form plasmodia, like *Pseudaphelidium*, but then build a common envelope for zoospore development named zoosporangium. The zoosporangium releases numerous zoospores able to invade new host cells immediately.

21.4 Zoosporic Fungi

Zoosporic fungi are protists appearing like fungi but display flagellated zoospores during their life cycle. While Chytridiomycetes are unicellular true fungi with unikont opisthokont zoospores, Oomycetes are stramenopiles with heterokont flagellation. Chytrids display minute, round zoospores of about 1–2 µm in diameter and

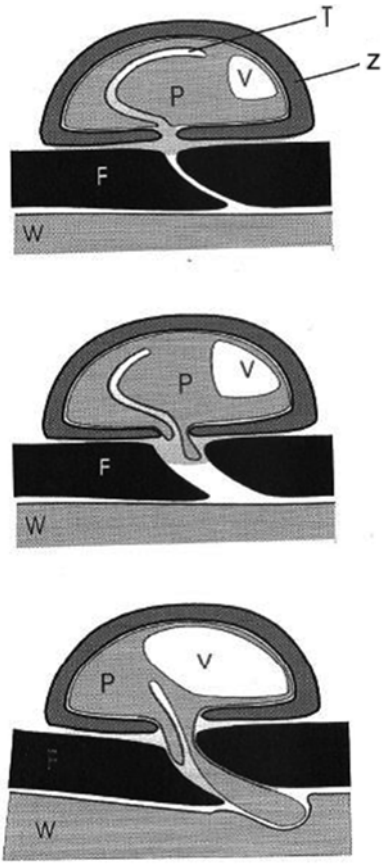

Fig. 21.5 Scheme of the infection process of *P. drebesii*. *Top*: attached zoospore with infection tube and small vacuole. *Middle*: beginning of infection, the vacuole increases pressing the cytoplasm into the fissure of the frustule. *Bottom*: *P. drebesii* passing the frustule with the aid of the pressure of the increasing vacuole and the infection tube. *T* infection tube, *P* cytoplasm of *P. drebesii*, *F* frustule, *W* Host diatom, *Z* zoospore cell wall (For details, see text in Sect. 21.3. From Schweikert and Schnepf 1996)

with one amastigote posteriorly oriented flagellum of about 15–20 µm in length. They are known in the majority of freshwater environments and decompose organic matter, but some feed on living diatoms (e.g.: Gärtner 1979). Most Chytrids infecting diatoms were reported from freshwater environments too (see Ibelings et al. 2004) but only three Chytridiomycota infecting diatoms in marine waters were taxonomically described, namely *Rhizophydium littoreum* Amon, *Thalassochytrium graciliopsis* Nyvall Pedersen et Longcore and *Chytridium polysiphoniae* Cohn (Gleason et al. 2010).

Drebes has shown a chytrid on a marine diatom of the genus *Chaetoceros* already. Additionally, the chain-forming diatom *Bellerochea malleus* is susceptible to chytrid infection (Fig. 21.6). Both chytrids are not described taxonomically so far. Zoospores of Chytridiomycota may be attracted by the host diatom by chemotaxis (Held 1973; Mühlenstein et al. 1988). They settle onto the surface and the flagellum retracts shortly after. Cells attach themselves by producing a rigid polymeric wall, most probably containing chitin (Fig. 21.6: 1). A haustorium is developed, penetrating the diatom frustule at sites that are less or not silicified (Fig. 21.6: 1 + 2). Some

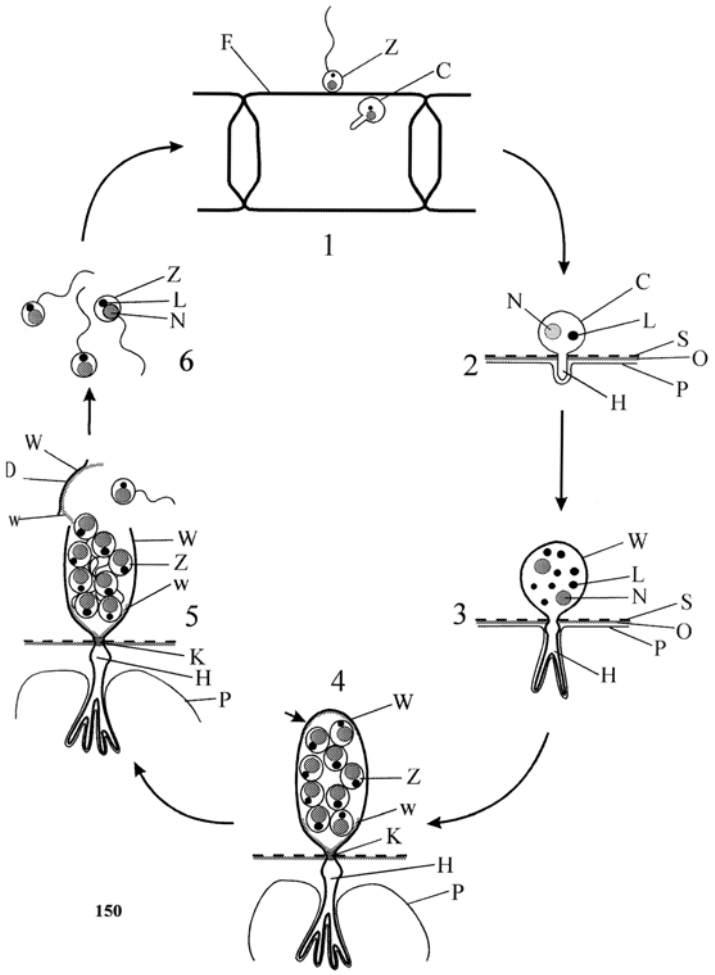

Fig. 21.6 Fig. 2: Scheme of the life cycle of *Chytridium spec.* Different steps in the life cycle of *Chytridium spec.* infecting the diatom *Bellerochea malleus 1–6. F* diatom frustule, *Z* zoospore, *C* cyst, *N* nucleus, *L* lipid droplet, *S* silicified component of the frustule, *O* organic component of the frustule, *P* diatom plasma membrane, *H* haustorium, *K* plug, *W* primary sporangium wall, *p* secondary sporangium wall, *D* Lid

zoospores may be unsuccessful with infection of the diatom when settling at sites that are more silicified or on diatom species with strongly silicified frustules. The haustorium gets into intimate contact with the diatom plasma membrane and start to branch (Fig. 21.6: 2 + 3). Nutrients are taken up osmotrophically with the aid of the haustorium and a sporangium develops from the zoospore outside the cell (Fig. 21.6: 3 + 4). Numerous zoospores were built inside the sporangium and were released after maturation through an operculum (Fig. 21.6: 4–6). In this chytrid, the operculum is closed by a lid before and later removed by a internal pressure, generated by the mature zoospores (Fig. 21.6: 5). Multiple infections of single diatom cells

occur and lead to smaller zoosporangia due to nutrient depletion. Chytrid-infecting *B. malleus* is a member of the genus *Chytridium* indicated by ultrastructural characters of the operculate oosporangium and the intimate arrangement of lipid droplet, ribosomes and nuclear membrane embracing the former two inside the zoospore (Longcore 1996).

Although belonging to the Chromista, two parasitic nanoflagellates were assigned to Zoosporic fungi in the literature. *Lagenisma coscinodiscii* (Oomycetes, Heterokonta, Chromista) infecting diatom species of the diatom genus *Coscinodiscus* (Drebes 1966, 1968) was investigated intensively and its complex life cycle was resolved by Schnepf and Drebes (1977), and Schnepf et al. (1978a, b). Like *Pseudaphelidium drebesii*, attached zoospores encyst and overcome the frustule through gaps between different components of the frustule by means of an infection tube. After osmotrophic feeding the whole organism forms into a zoosporangium built inside the frustule. The biflagellated zoospores escape the tube-shaped zoosporangium through an opening and have to pass two cyst stages to become infectious again for *Coscinodiscus* species (Hoppenrath et al. 2009). A further less investigated parasitic nanoflagellate found to infect marine diatoms is named *Ectrogella* (Oomycetes, Heterokonta) (Sparrow 1960). Although different species exist, no discrimination on species level due to host specificity or molecular phylogeny has been performed so far. The parasitic nanoflagellate was reported frequently by researchers to be present in plankton samples (Hanic et al. 2009; Li et al. 2010).

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Part III
Interactions Between Marine Protists and Humans

Chapter 22
Fish and Shellfish Diseases Caused by Marine Protists

Hiroshi Yokoyama, Naoki Itoh, and Kazuo Ogawa

Abstract Many protists specifically parasitize marine organisms, and some of them cause diseases in economically important fish, shellfish or other invertebrates. In this chapter, we give general accounts of considerable protistan parasites for the fishery industry with their impacts and control measures, if any. Parasites of fish include amoebae, ichthyosporea, flagellates, X-cells with an unknown taxonomic position, and ciliates, and those of invertebrates include *Marteilia* and *Perkinsus* for shellfish, and a kinetoplastid for edible ascidians.

Keywords Parasite • Pathogen • Infection • Disease • Fishery • Aquaculture • Fish • Shellfish

22.1 Introduction

In addition to free-living and symbiotic species, there are many parasitic protistan species in the marine environment. Generally they rarely pose detrimental problems for wild populations of host organisms; however, with the development of aquaculture, some of them have caused significant damage to aquaculture industries as infectious pathogens because high stocking density of fish in aquaculture farms facilitates replication and transmission of parasites, and commercial transfer of living fish causes spreading of parasites and provides opportunities for parasites to meet novel or naive host species.

H. Yokoyama • N. Itoh
Graduate School of Agricultural and Life Sciences, The University of Tokyo,
1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

K. Ogawa (✉)
Meguro Parasitological Museum, 4-1-1, Shimomeguro, Meguro-ku, Tokyo 153-0064, Japan
e-mail: ogawak@kiseichu.org

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This chapter lists parasitic protistan groups causing diseases in valuable marine fish and invertebrates, mainly depending on their economic importance, recent research progress or interest in other aspects.

22.2 Amoebae

Several species of amoebae (class Flabellinea, phylum Amoebozoa) are known to infect marine fish. Among them, *Neoparamoeba perurans* Young, Crosbie, Adams, Nowak et Morrison, 2007 is most problematic and a predominant agent of amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) and turbot (*Scophthalmus maximus*) cultured in marine environments in Europe, North America, Australia and Chile (Dyková 2008). Ayu sweetfish (*Plecoglossus altivelis*) is a diadromous fish, extensively cultured in Japan. Recently, artificially spawned juvenile ayu maintained in a sea water hatchery in Japan suffered mass mortality from *N. perurans* infection (Crosbie et al. 2010). This amoeba is basically free-living but infects fish, which show gross signs of excessive production of mucus, changes in the color of the gills, and microscopic signs of hypertrophy and hyperplasia of epithelium associated with the attachment of amoeba trophozoites (Fig. 22.1a). Neighboring secondary lamellae first adhere to one another and then fuse completely, either partly or focally (Dyková 2008). These pathological changes cause a decrease in the gill respiratory surface area. The most successful treatment of AGD in sea-caged Atlantic salmon is freshwater baths for 2–3 h, though this is not fully effective in killing amoebae. Repeated bathing is required, as infected fish acquire no immunity against reinfection. AGD seriously affects sea-cage culture of salmonids worldwide. Current treatment in Tasmania, Australia, amounts to a total of up to 14 % of production costs.

22.3 *Ichthyophonus*

The genus *Ichthyophonus* is now classified as a protozoan member of the class Ichthyosporea in the phylum Amoebidiozoa, though it has long been classified into the fungi (McVicar 2011). *Ichthyophonus hoferi* Plehn et Mulsow, 1911 shows no host specificity, recorded from 35 marine and 48 freshwater fish. The available literature suggests that *Ichthyophonus* is of marine origin, as many isolates from freshwater fish have been directly linked to the sea (McVicar 2011). The parasite has different life stages in fish, the terminology of each stage being followed by Kocan (2013). *Ichthyophonus* infects various tissues and organs of fish in the form of multinucleate schizonts surrounded by a thick wall of host origin, with a size ranging from 10 to >200 μm in diameter (Fig. 22.1b). Fish-to-fish transmission of the parasite is possible when infected fish are eaten by a predator or scavenger fish. In the host stomach, schizonts are liberated from the cyst wall and transform into

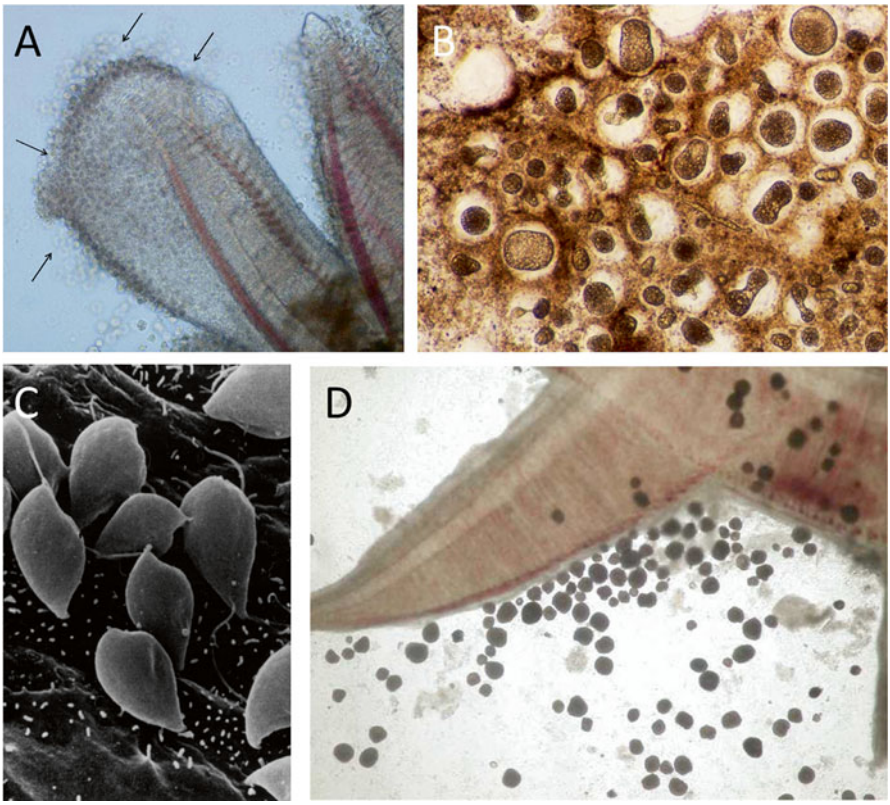

Fig. 22.1 Amoebae, amoebidiozoans and flagellates. (**a**) *Neoparamoeba perurans* on the gills of ayu sweetfish (*Plecoglossus altivelis*). *Arrows* show numerous trophozoites of the amoeba (This photo was provided by Mr. D. Nakano, Fukui Prefectural Fisheries Experimental Station, Japan). (**b**) Schizonts of *Ichthyophonus hoferi* in the skeletal muscle of rainbow trout (*Oncorhynchus mykiss*) (This photo was provided by Dr. K. Hatai, Nippon Veterinary and Life Science University). (**c**) SEM photo of *Ichthyobodo* sp. on the skin of bastard halibut (*Paralichthys olivaceus*) (This photo was provided by Dr. S. Urawa, Hokkaido National Fisheries Research Institute). (**d**) *Amyloodinium ocellatum* on the gills of Japanese pufferfish (*Takifugu rubripes*) (This photo was provided by Mr. S. Tanaka, Mie Prefecture Fisheries Research Institute)

two cell types, a larger multinucleate amoeboid cell and a smaller spherical cell. Both types of cells invade the host's stomach mucosa, are subsequently transported throughout the host's body via circulating blood and develop into a multinucleated schizont contained by a host cyst wall (Kocan et al. 2013). Water-borne transmission was demonstrated by Kocan et al. (2010), who showed that merozoites released into the water from schizonts near the host's skin surface were infectious to SPF fish. Epizootics in Atlantic herring (*Clupea harengus*) caused by *I. hoferi* infection led to mass mortality with an estimate of 50 % of mature Atlantic herring being destroyed in the 1950s (McVicar 2011). When rainbow trout (*Oncorhynchus mykiss*) cultured in fresh water were fed infected herring, 50–90 % mortality was recorded

in a US farm. *Ichthyophonus* infection also causes degeneration and necrosis of fish flesh and poor quality for smoking and pickling (McVicar 2011).

22.4 Flagellates

Flagellates are a polyphylectic group of unicellular organisms, many of which are known as parasites of marine fish. *Ichthyobodo necator* (Pinto, 1928), formerly known as the only species representing the genus, constitutes a species complex, many of which have not yet been given species names. *I. necator* sensu lato, pyriform and having two flagellae, attaches to the fish epithelium by a proboscis which protrudes into the host cell (Alvarez-Pellitero 2008). Infected fish show non-specific disease signs like excess mucus secretion and discoloration of the body. Histopathologically, the skin epithelium shows spongiosis, vacuolation and edema, followed by degeneration and sloughing of the epidermis (Alvarez-Pellitero 2008). Affected marine fish include bastard halibut (*Paralichthys olivaceus*) (Fig. 22.1c), Japanese pufferfish (*Takifugu rubripes*) and common dab (*Limanda limanda*). Fry and juvenile fish are more prone to suffer severe infections and even mortality (Alvarez-Pellitero 2008).

Amyloodinium ocellatum (Brawn, 1931) (Dinophyceae, Dinophyta) is an ectoparasitic flagellate, which attaches to the gills and skin of marine and brackish water fish with its attachment disc inserted into the host epithelium. When the trophont, an unpigmented, pear-shaped to ovoid cell, grows to a certain volume (up to 150 μm in size), it detaches from the host (Fig. 22.1d), falls to the bottom and transforms into a tomont, which secretes a wall forming a cyst. The tomont divides several times inside the cyst to transform into biflagellate dinospores, an infective stage to fish. Heavily infected fish suffer from anoxia, leading to mortality (Alvarez-Pellitero 2008). This flagellate is particularly dangerous to fish kept in aquariums and tanks with a low rate of water exchange.

Ichthyodinium chabelardi Hollande et Cachon, 1953 (class unknown in the phylum Dinophyta) is unique in that it infects the yolk sac of several marine fish in Europe, where it multiplies and replaces the yolk. The infection is fatal to the fry as the parasite rupture the yolk sac after hatching (Noga and Levy 2006). Development of the parasite is complicated. The schizont, 8–15 μm in diameter, as the earliest detectable stage in the yolk, undergoes multiple nuclear divisions to form a large (100–200 μm), multinucleate plasmodium, which repeat two more generations of schizonts to produce numerous forms similar to the earliest schizonts (Noga and Levy 2006). The yolk sac, replete with parasites, breaks open (Fig. 22.2a). The parasites divide once or twice in the water to produce biflagellate dinospores (Lom and Dyková 1992; Noga and Levy 2006). Their further fate is unknown. The infection was known only among wild marine fish, but recently it occurred in a fish hatchery. Mass mortality of fertilized eggs and hatched larvae of leopard coralgrouper (*Plectropomus leoparadus*) occurred, which was caused by *Ichthyodinium* sp. (Mori et al. 2007). When fertilized eggs of the host fish were exposed to rearing seawater from a broodstock tank with a disease outbreak, the eggs became infected. In contrast,

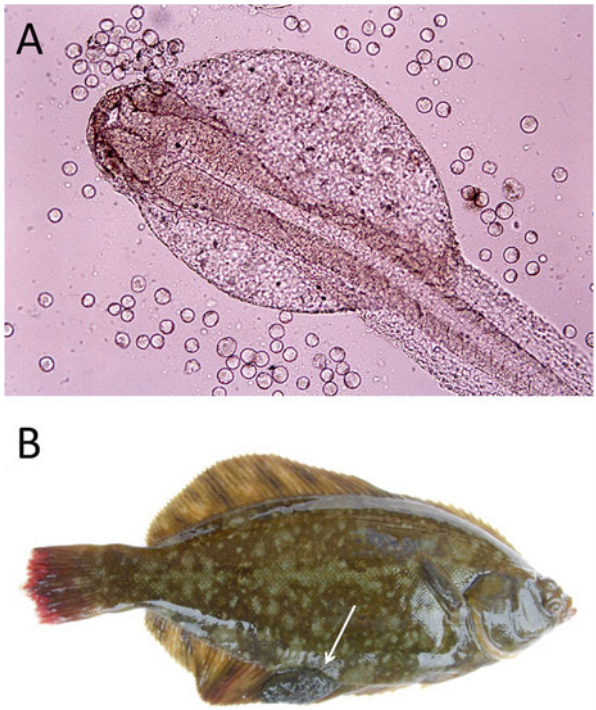

Fig. 22.2 *Ichthyodinium* (flagellate) and X-cells. (**a**) Schizonts of *Ichthyodinium* sp. released from the yolk of leopard coralgrouper (*Plectropomus leoparadus*). (**b**) Pseudotumor caused by X-cells on the fin (*arrow*) of a pleuronectid (*Pseudopleuronectes obscurus*)

no infection occurred when the broodstock and incubating fertilized eggs were kept in oxidant-treated seawater. These experimental data suggest horizontal transmission of the parasite to the eggs (Mori et al. 2007), though the infective stage has not yet been specified.

22.5 X-Cells

Tumor-like structures, or pseudotumors, caused by massive proliferation of X-cells, are found in such organs as the skin, fins (Fig. 22.2b), pseudobranch and gills of various demersal marine fish species from all over the world (Miwa et al. 2004; Freeman et al. 2011). X-cells are characterized by a centrally located, large nucleus with a prominent nucleolus (Miwa et al. 2004). The true identity of X-cells has long been unresolved. These lesions were once thought to be related to water pollution, whereas viral, protozoan or microsporidian infections were proposed by other scientists as causative agents (Miwa et al. 2004). In this century, molecular analyses confirmed that X-cells are protozoan parasites (Miwa et al. 2004). No robust support has been obtained on their placement within any established phylum, but they are currently thought to be members of the Alveolata (Freeman et al. 2011). Their life cycle is unknown.

22.6 Ciliates

Ciliates (phylum Ciliophora) are covered with cilia as locomotory or feeding organelles. They possess food vacuoles, contractile vacuoles and dimorphic nuclei, a large vegetative macronucleus and a small generative micronucleus (Lom and Dyková 1992). Most ciliates reproduce by binary fission, but some proliferate by budding or multiple cell divisions (palintomy). They have various lifestyles from free-living to commensal, opportunistic or obligate parasitism. Several species of ciliates have been recognized as serious pathogens in fisheries and aquaculture industries as below.

Cryptocaryon irritans Brown, 1951 is an obligate parasite and a cosmopolitan with a broad host range and high pathogenicity (Lom and Dyková 1992). Cryptocaryoniasis, also known as white spot disease of marine fish, causes mass mortality of fish in exhibition aquariums and aquaculture. The external sign of affected fish is characterized by numerous white spots on the body surface, which are easily recognized by the naked eye (Fig. 22.3a, b). This appearance resembles that of fish infected with *Ichthyophthirius multifiliis* Fouquet, 1876, also known as the causative agent of white spot disease of freshwater fish. However, the systematic position of *C. irritans* is considerably distant from that of *I. multifiliis.* The former

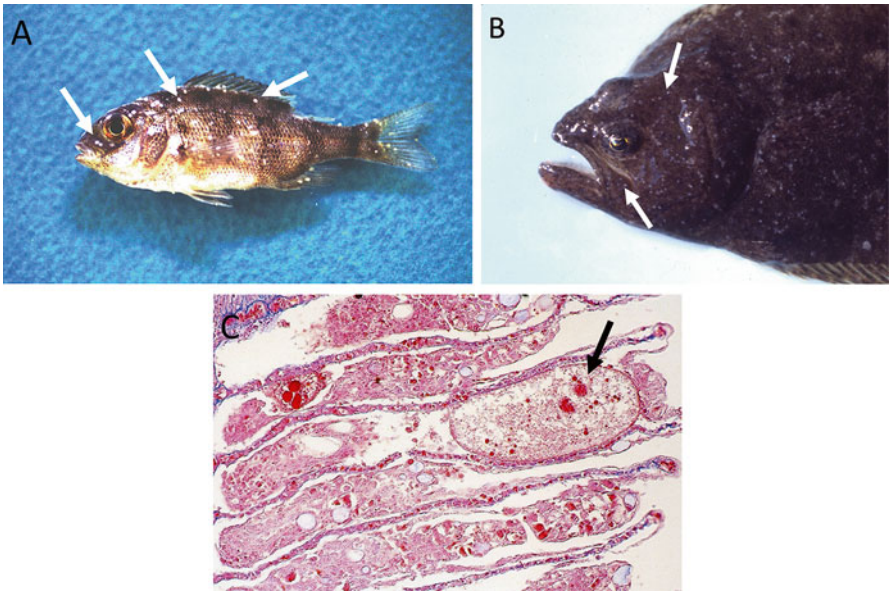

Fig. 22.3 *Cryptocaryon irritans*. (**a**) External signs of cryptocaryoniasis in red seabream (*Pagrus major*). (**b**) Bastard halibut (*Paralichthys olivaceus*). *Arrows* show numerous *white* spots on the body surface. (**c**) Histopathology of trophont (*arrow*) invading under the gill epithelium of Japanese pufferfish (*Takifugu rubripes*)

and the latter belong to the class Prostomatea and Oligohymenophorea, respectively (Wright and Colorni 2002).

The life cycle of *C. irritans* involves five developmental stages. The trophont is a parasitic stage, growing up to 450 μm in size, within the host epithelium of the skin and gill. It is characterized by the presence of four closely connected macronuclei. After maturation of the trophont, it leaves the host as a protomont, and then is encysted as a tomont at the bottom of the water. Within the tomont, palintomic cell divisions occur so that numerous tomites are produced. Then tomites are excysted as theronts, which are the infective stage for susceptible host fish. One to 3 weeks are required to complete the life cycle of *C. irritans*, although it depends on water temperature. The optimum temperature for the development of *C. irritans* is 25–31 °C.

Infection with *C. irritans* causes hyperplasia and necrosis of the epithelium (Fig. 22.3c), resulting in impairment of osmotic regulation and respiratory function in the host fish. Affected fish show restlessness and anorexia, and finally die. Kaige and Miyazaki (1985) reported that 1000 2-year-old fish of cultured bastard halibut (*Paralichthys olivaceus*) died of the disease within only 3 days in a land-based tank. There is a lack of effective treatment against cryptocaryoniasis of cultured food fish. Oral administration of lysozyme hydrochloride or bovine lactoferrin are partially efficacious against white spot disease of red seabream (Kakuta and Kurokura 1995). In a land-based facility, the life cycle of *C. irritans* can be interrupted by four consecutive transfers of fish into clean tanks at 3-day intervals to prevent reinfection (Colorni 1985). Acquired immunity of fish against *C. irritans* has been demonstrated, but development of vaccination requires further studies.

Scuticociliatid ciliates are characterized by the presence of the scutica, a hook-like field of kinetosomes associated with the paroral membrane appearing during binary fission of ciliates (Lom and Dyková 1992). *Miamiensis avidus* (=*Philasterides dicentrarchi*), *Pseudocohnilembus persalinus*, *Uronema marinum* and *U. nigricans* have been found in fish farms. Some other species of scuticociliates affect crustaceans and mollusks. The scuticociliates are usually free-living but opportunistically infest marine fish and shellfish. They infect the gills and skin of fish, causing dermal ulceration and hemorrhagic lesions in the skeletal muscle (Fig. 22.4). In advanced stage of infection, the ciliates may even penetrate the internal organs and the brain, resulting in severe encephalitis associated with softening and liquefaction of the brain tissue (Iglesias et al. 2001). Low-salinity conditions induce the propagation of *M. avidus* and outbreaks of scuticociliatosis in bastard halibut farms (Takagishi et al. 2009). This implies that avoiding the use of hyposaline seawater may reduce fish mortality in farms. Kasai et al. (2002) demonstrated that UV treatment of supplied water was effective in preventing scuticociliatosis in a farm. Considering the invasiveness of the ciliate, a bath treatment with drugs is unlikely to be efficacious against the disease. Scuticociliates are usually scavengers which feed on bacteria and dead tissues on the bottom, and invade only weakened fish. Thus the best way to prevent the disease is to keep a clean tank, such as with regular brushing of the tank wall and an increased exchange rate of rearing water.

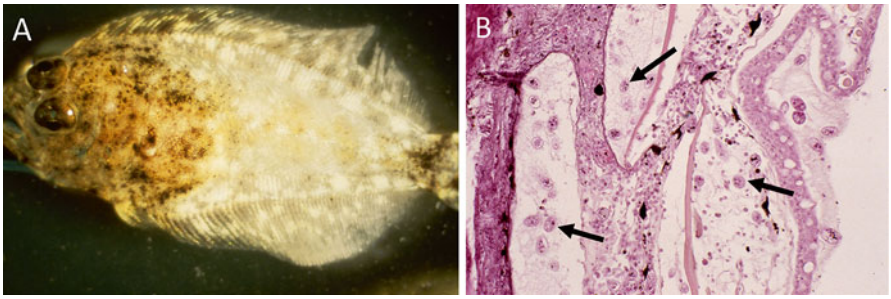

Fig. 22.4 *Miamiensis avidus*. (**a**) External appearance of infected bastard halibut (*Paralichthys olivaceus*). Note the partial bleaching of the epidermis. (**b**) Histopathology of the skin of infected bastard halibut. *Arrows* show numerous ciliates in the scalepocket of flounder skin (These photos were provided by Dr. M. Ototake, National Research Institute of Aquaculture)

Trichodinid ciliates belonging to the order Mobilina are ubiquitous and facultative ectoparasites of fish. Marine species include *Trichodina gobii*, *T. jadranica, T. murmanica* and *T. rectuncinata* (Lom and Dyková 1992). The ciliates are dome shaped and possess buccal ciliature and an adoral ciliary complex, which allows for adhesion to the host surface. The adoral ciliary complex consists of an adhesive disc and denticles which are observed using a silver impregnation technique. These structures are important for the taxonomy of the trichodinids. They attach predominantly to the gill and/or skin of fish and feed on suspended bacteria and tissue debris. The ciliates reproduce by binary fission on the host fish and transmit directly from fish to fish, facilitating rapid proliferation in a confinement situation. Although attachment of *Trichodina* spp. likely causes physical damage or irritation to the host tissue, serious pathology due to the infection was rarely observed. Khan (2004) documented that hatchery-reared Atlantic cod (*Gadus morhua*) infected with *T. murmanica* exhibited fin erosion, tail necrosis, and finally mass mortality. A chronic infection with *Trichodina* spp. may also result in growth inhibition of fish. Heavy trichodiniasis occurs only in stressed fish and small juveniles, often showing excessive mucus secretion. Poor water quality may also be responsible for the disease outbreak. Due to opportunistic occurrences of trichodiniasis, mitigation of fish stress and improvement of environmental conditions may control the disease.

22.7 *Perkinsus*

Although establishment of a phylum Perkinsozoa including three genera, *Perkinsus*, *Parvilucifera* and *Colpodella,* is proposed, it is widely accepted that the genus *Perkinsus* is placed at the base of the dinoflagellate clade within the alveolates (Villalba et al. 2004). As species related to *Perkinsus*, the genus *Parvilucifera* parasitizing dinoflagellates and an unidentified parasite in freshwater frog have been known, as well as species even from freshwater lake sediments (Bråte et al. 2010).

The presence of such diversified relatives reflects the important position of *Perkinsus* for understanding of the early evolution of dinoflagellates and apicomplexans.

So far, seven species have been described in this genus, and the representative species, *P. marinus* (Mackin et al.) Levine, 1978 is known to cause massive mortality in eastern oyster *Crassostrea virginica* along the east coasts of the United States and Gulf of Mexico. Mortality caused by the parasite usually exceeds 50 % and often reaches more than 90 %. In addition to socioeconomic impacts, ecological impacts such as degradation of oyster reefs and subsequent reduction of water clarity are also concerning.

P. olseni Lester and Davis, 1981 (Fig. 22.5a) was first reported as a pathogen of blacklip abalone (*Haliotis rubra*) in Australia. Separately, *P. atlanticus* was described from the grooved carpet shell (*Ruditapes decussatus*) in Portugal (Azevedo 1989), but later *P. atlanticus* was found to be a synonym of *P. olseni* (Murrell et al. 2002). Currently, *P. olseni* is distributed in the Pacific Ocean, Australia, the North Island of New Zealand, Vietnam, South Korea, Japan, the People's Republic of China, India, Thailand, Portugal, Spain, France, Italy, and Uruguay, but has not yet been reported from North America. The host species of *P. olseni* range extremely widely, and currently *P. olseni* has been found from gastropods (*Haliotis rubra*, *H. laevigata*, *H. cyclobates* and *H. scalaris*) and bivalves (pearl oyster (*Pinctada maxima*), clam species (*Ruditapes* spp. and *Paphia aurea*) and so on) (Villalba et al. 2004; Bower 2006). Currently, this parasite is considered

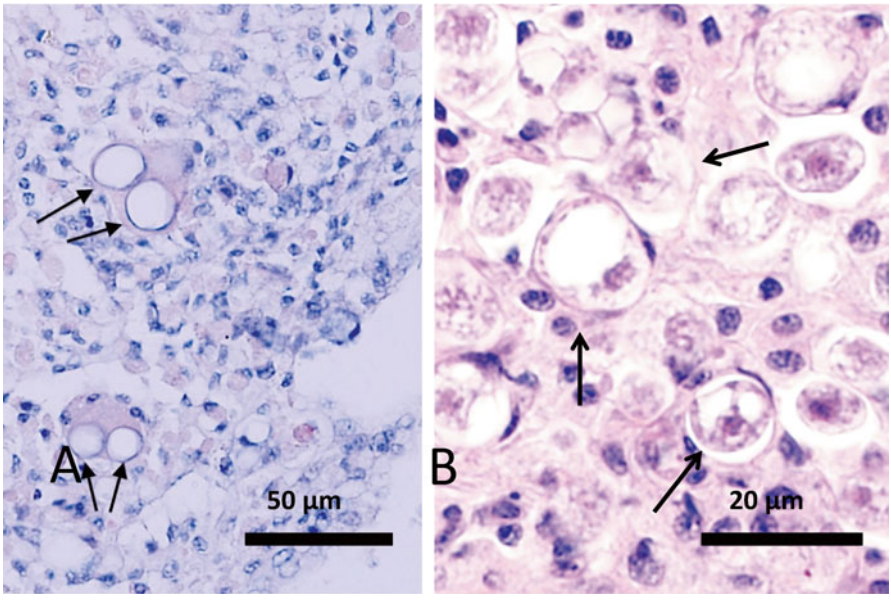

Fig. 22.5 Histological sections of (**a**) Manila clam (*Ruditapes philippinarum*) infected with *Perkinsus olseni* (*arrows*) and (**b**) Japanese scallop (*Patinopecten yessoensis*) infected with *P. qugwadi* (*arrows*)

to be involved in reduction of clam production due to higher pathogenicity on juveniles or interference of gonadal maturation (Choi and Park 2010; Waki and Yoshinaga 2013).

P. qugwadi Blackbourn, Bower et Mayer, 1998 (Fig. 22.5b) is known as a causative agent in the Japanese scallop (*Patinopecten yessoensis*) imported to British Columbia in western Canada. The impact on the host species is severe, and mortality reaches up to 98 % in juveniles and 60 % in adults (Bower et al. 1998). Since there has been no report of *Perkinsus* infection in the host's origin, Japan, and strict quarantine was conducted for introduction of the scallop to British Columbia, it is believed that *P. qugwadi* originally parasitized domestic bivalve species in the endemic area, expanded its host range and exhibited strong virulence toward the Japanese scallop.

Negative impacts have not been clearly determined in the other four *Perkinsus* species: *P. chesapeaki* (=*P. andrewsi* syn.) McLaughlin, Tall, Shaheen, Elsayed et Faisal, 2000 from the soft shell clam (*Mya arenaria*) in North America; *P. mediterraneus* Casas, Grau, Reece, Apakupakul, Azevedo et Villalba, 2004 in the flat oyster (*Ostrea edulis*) in Europe; *P. honshuensis* Dungan et Reece, 2008 from the Manila clam (*R. philippinarum*) in Japan; and *P. beihaiensis* Moss, Xiao, Dungan et Reece, 2008 from the Hong Kong oyster (*C. hongkongensis*) in China.

The life cycle of *Perkinsus marinus* is suggested as shown in Fig. 22.6 (Bower 2006). Within living hosts, a spherical cell (trophozoite) develops to a stage with a spherical nucleus and a vacuole which occupies most of the cytoplasm volume. Vegetative stage proliferation (palintomy) occurs and a shizont including 4–64 immature trophozoites is resultantly formed. When the shizont cell wall is ruptured, immature trophozoites are liberated and spread into the host body by the hemolymph, and then this asexual proliferation is repeated. When the host dies and the tissue becomes anoxic, the trophozoite enlarges and develops into a prezoosporangium (hypnospore) with a thick cell wall. In the marine environment, the prezoosporangium develops into a zoosporangium with a discharge tube, and then undergoes palintomy to produce a large number of biflagellated zoospores. Liberated zoospores appear to be an infective stage; however, experimentally, other stages are also infective (Villalba et al. 2004). Although the same developmental cycle is predicted for other species, *P. qugwadi* seems to have a different life cycle, since its zoospores are observed in living hosts and the prezoosporangium is not developed in RFTM (Blackbourn et al. 1998).

As countermeasures to Perkinsosis, improvement of management strategies, such as schematic transplantation and harvesting with epizootiological knowledge, may be able to reduce industrial damage. Also, selective breeding programs and development of resistant marker genes are being conducted (Villalba et al. 2004; Yu et al. 2011). Disinfectant compounds effective against *Perkinsus* cells have been known, and they can be applicable in closed systems for hatcheries and nurseries (Villalba et al. 2004).

Against *P. qugwadi* infection, surviving individuals and a hybrid strain between Japanese and local scallop species showed tolerance to the parasite (Bower et al. 1999), and they are mainly used in scallop culture in British Colombia with increase of production even in enzootic areas of *P. qugwadi*.

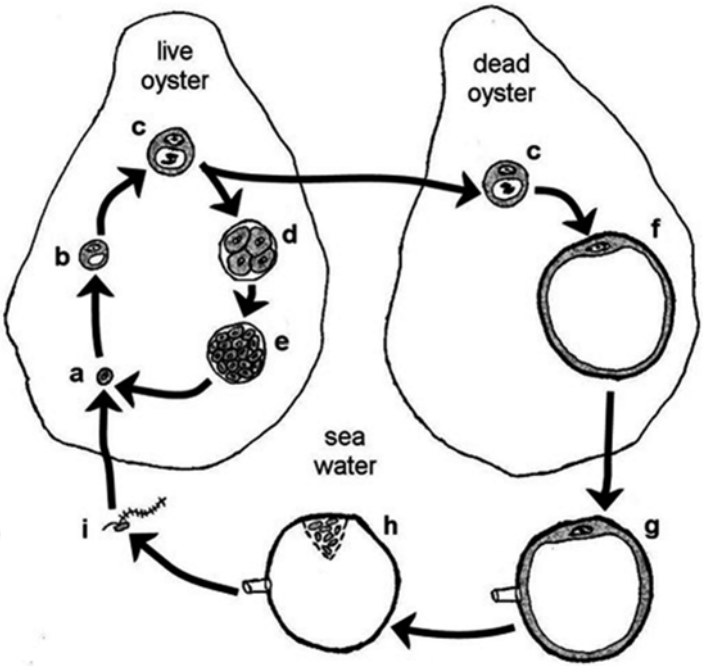

Fig. 22.6 Developmental life cycle of *Perkinsus marinus* within living and dead oyster (*Crassostrea virginica*) and in the marine environment: (**a**) immature trophozoite; (**b**) trophozoite with a cytoplasmic vacuole; (**c**) mature trophozoite; (**d**) early shizont containing four trophozoites; (**e**) shizont containing numerous immature trophozoites; (**f**) prezoosporangium (hypnospore); (**g**) prezoosporangium with a discharge tube; (**h**) zoosporangium containing zoospores; and (**i**) biflagellated zoospore (This diagram was provided by Dr. Susan Bower, Pacific Biological Station, Canada)

22.8 *Marteilia*

The genus *Marteilia* is classified in the order Paramyxida (phylum Cercozoa) which is characterized by sporulation through internal cleavages within an amoeboid stem cell. A secondary cell (sporont) produced in the stem cell contains tertiary cells (spores), and each spore is composed of multiple cells. Three genera are described in this order, and they are distinguished by the number of cells present in a spore: *Paramarteilia* for bicellular spores; *Marteilia* for tricellular spores and *Paramyxa* for tetracellular spores (Feist et al. 2009). All paramyxids are parasitic organisms of marine invertebrates. Although a relationship with haplosporidian species was suggested, any phylogenetic relationships with described species have been not confirmed yet (Berthe et al. 2000; Reece et al. 2004), leaving the phylogenetic position of paramyxids under controversy.

Important pathogenic species for bivalves are confined to the genus *Marteilia,* and they are classified into two types according to sporulation sites: a digestive type

and an ovary type. In general, digestive types inhibit the host's nutrient absorption and consequently cause higher mortality of bivalves (Moyer et al. 1993). In addition to lethal effects on host species, poor nutrition conditions lead to reduction of gametogenesis and delay of gonadal development. One of the most important species in these digestive types is *M. refringens* Grizel, Comps, Bonami, Cosserants et Duthoit, 1974 in the flat oyster (*Ostrea edulis*) in Europe. Because of its serious mortality, sometimes reaching 100 %, the flat oyster industry in France has been detrimentally damaged. In addition to the flat oyster, *M. refringens* is also found from other oysters and mussels (*Mytilus* spp.) (Berthe et al. 2004; Bower 2006), the razor clam (*Solen marginatus*) (López-Flores et al. 2008a) and the striped Venus clam (*Chamalea gallina*) (López-Flores et al. 2008b) in Europe.

As a related species, *M. sydneyi* Perkins et Wolf, 1976 is known as an industrially significant pathogen of the Sydney rock oyster (*Saccostrea glomerata*) in Australia, with high mortality, often exceeding 80 % (Bower 2006). Recently, *M. cochillia* Carrasco, Hine, Durfort, Andree, Malchus, Lacuesta, González, Roque, Rodgers et Furones, 2013 was described as a new species involved in mortality of the edible cockle (*Cerastoderma edule*) in Spain. Additionally, *M. lengehi* Comps, 1976 and *M. christenseni* Comps, 1986 have been described from a rock oyster (*Saccostrea cucullata*) and the peppery furrow shell (*Scrobicularia piperata*), respectively, while their pathogenicity in host species is unknown.

The life cycle of digestive *Marteilia* has been considered as follows. An unknown infective stage invades through body surface tissues, such as the mantle, the gills and the labial palps, and then infect the host hemocytes via phagocytosis at this early stage. Then, the parasites repeat division in the hemocytes and spread into the host body. Sporulation occurs within the epithelium of the digestive gland tubules, and produced spores are released in the lumen of the digestive tract (Kleeman et al. 2002). After releasing out from the host, *M. refringens* is ingested by copepods and migrates into host egg cells (Audemard et al. 2002). Infection from copepods to bivalves is not established (Audemard et al. 2002), and life cycle of *Marteilia* is not yet fully understood.

Basically, farm strategies are employed to reduce the risk of digestive *Marteilia*, by planting and harvesting oysters outside the infection season (Berthe et al. 2004). Recently, a strain of Sydney rock oyster resistant against *M. sydneyi* has been established and used for empirical research of aquaculture (Nell et al. 2000).

The only species of the ovary type is *M. chungmuensis* Comps, Park et Desportes, 1986 in the Pacific oyster (*C. gigas*). Oysters infected with the parasite continuously maintain ovaries and produce oocytes after the spawning season (summer to autumn), showing an abnormal appearance with irregular shape nodules (Fig. 22.7). Although the mortality caused by this disease is not extremely high (Tun et al. 2008), infected oysters with an abnormal appearance are unacceptable in the market. Infection of *M. chungmuensis* has also been reported from other oyster species (Limpanont et al. 2013), though abnormal appearance has never been reported in these host oysters. Similar ovarian *Marteilia* has been reported from the

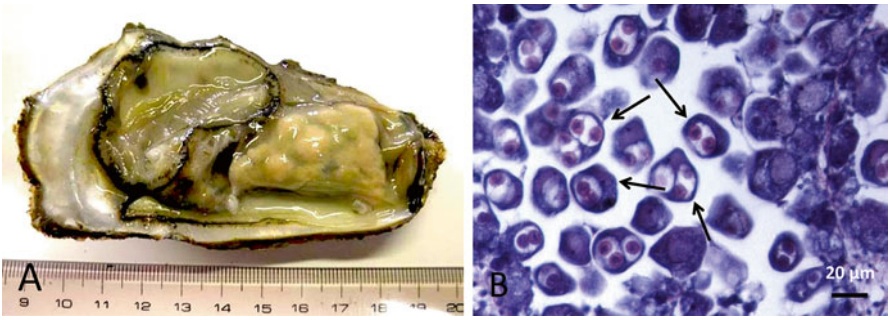

Fig. 22.7 Pacific oyster infected with abnormal enlargement of the ovary (**a**), and the causative agent, *Marteilia chungmuensis* (*arrows*), in host oocytes (**b**). Bar = 20 µm

Manila clam (*R. philippinarum*), the black lipped oyster (*C. echinata*) and *S. cucullata*, while parasites in these host bivalves have not been identified into species.

Except for its sporulation inside oocytes, the life cycle of *M. chungmuensis* resembles that of digestive types. Retained inside an oocyte, sporangium is released out from the host body. Since experimental transmission between oysters is not established, the presence of intermediate host(s) is hypothesized like *M. refringens* (Itoh et al. 2004).

It has been known that prevalence is lower in oysters with limited exposure to sea water, maybe because they have only limited opportunities for infection or the oysters are more differentiated into males in these low-nutrition conditions. However, their growth rate is significantly lower than that of control oysters (Tun et al. 2006); therefore, further revision of this management strategy is needed practically.

22.9 *Azumiobodo hoyamushi*

Azumiobodo hoyamushi Hirose, Nozawa, Kumagai et Kitamura, 2012 (class Kinetoplastea, phylum Euglenozoa) (Fig. 22.8) is distinct from other known polykinetoplastic neobodonids in the presence of globular bodies with electron-dense bands. Phylogenetically, this species is related to a free-living kinetoplastid, *Cruzella marina* (Hirose et al. 2012), but *A. hoyamushi* parasitizes the edible ascidian *Halocynthia roretzi* and causes soft tunic syndrome in which affected ascidians have thin and soft tunics (outer covers) and die when the tunic tears. Industrial damage from this disease has been serious in Korea and Japan, and, in particular, annual production of edible ascidians in Korea was decreased from 42,000 tons in 1994 to 4000 tons in 2004 (Kumagai et al. 2010).

Experimental transmission to ascidians was established by either cohabitation with diseased ascidians, immersion of affected tunics or immersion of *A. hoyamushi* cultured in a specific medium, while injection of hemolymph of diseased ascidians

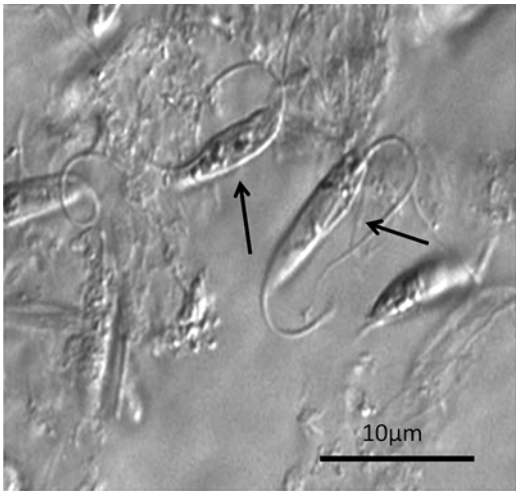

Fig. 22.8 *Azumiobodo hoyamushi* (*arrows*), the causative agent of soft tunic syndrome in the edible ascidian *Halocynthia roretzi*. (This picture was provided by Dr. Akira Kumagai, Miyagi Prefecture Fisheries Technology Institute, Japan)

did not develop the disease (Kumagai et al. 2010, 2011). Therefore, it seems that the parasite invades the host by water movement (Kumagai et al. 2010). The invading parasite is suspected to release cellulase and/or proteinase to collapse crosslink fibers of the host tunic (Hirose et al. 2012), and then parasites are released out from the softened tunics into sea water to transfer into their next hosts (Kumagai et al. 2011).

Park et al. (2013) found that several antiprotozoal drugs were effective in controlling *A. hoyamushi* without a negative impact on the ascidians, but there are many problems to be solved before its practical administration.

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Chapter 23
Taxonomy and Distribution of the Unarmored Dinoflagellates *Cochlodinium polykrikoides* and *C. fulvescens*

Mitsunori Iwataki, Haruyoshi Takayama, Kazuya Takahashi, and Kazumi Matsuoka

Abstract Red tides of the unarmored dinoflagellate *Cochlodinium polykrikoides* and related chain-forming *Cochlodinium* species have been responsible for the mass mortality of fish in coastal areas of East Asia, and in Central and North America over the past few decades. Since 2000, *C. polykrikoides* has been reported for the first time from Southeast Asia, India, the Middle East and the Mediterranean, so its distribution has seemingly expanded. Recent molecular phylogenetic works have detected intraspecific populations of *C. polykrikoides*, which presumably indicates their original distribution area. Reported occurrences of the chain-forming *Cochlodinium* spp., *Cochlodinium fulvescens* and *C. polykrikoides* are summarized with reference to their ribotypes, in order to consider the expansion of their distribution area and recent migration. The ultrastructure of *C. polykrikoides* was recently analyzed and showed the characteristic features seen in all other dinoflagellates. The apical groove structure of *C. fulvescens* is also provided in this chapter, for morphological comparison with *C. polykrikoides* and further characterization of chain-forming *Cochlodinium* spp. that produce fish-killing red tides.

Keywords Apical groove • *Cochlodinium polykrikoides* • *Cochlodinium fulvescens* • Dinoflagellate • Distribution • Harmful algal bloom (HAB) • Phylogeny • Red tide • Taxonomy

M. Iwataki (✉)
Asian Natural Environmental Science Center, The University of Tokyo,
1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
e-mail: iwataki@anesc.u-tokyo.ac.jp

H. Takayama
Hatami, Ondo, Hiroshima 737-1207, Japan

K. Takahashi
Graduate School of Science and Engineering, Yamagata University,
1-4-12 Kojirakawa, Yamagata 990-8560, Japan

K. Matsuoka
Institute for East China Sea Research, Nagasaki University,
1551-7 Taira-machi, Nagasaki 851-2213, Japan

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23.1 Introduction

The unarmored dinoflagellate *Cochlodinium polykrikoides* Margalef has formed red tides responsible for mass mortalities of fish and invertebrates, especially in coastal areas of Japan, Korea and the USA over the past few decades (Kudela et al. 2008; Matsuoka et al. 2008; Kudela and Gobler 2012). In Japan, the first fish mass mortality due to *C. polykrikoides* was recorded in 1979, and thereafter damage to fisheries has been documented from the western coast of Japan (Matsuoka and Iwataki 2004). In 1995, huge fisheries damage estimated at 76.4 billion KRW was reported from the southern coast of Korea, which was caused by this harmful species. A similar chain-forming *Cochlodinium*, *Cochlodinium fulvescens* Iwataki, Kawami et Matsuoka, is also known to be a harmful algal bloom (HAB) species, forming red tides in Canada and around the coast of California, USA (Whyte et al. 2001; Curtiss et al. 2008; Kudela and Gobler 2012; Howard et al. 2012). Red tides caused by *Cochlodinium* were reported from East Asia and Central and North America up until 1990; however, they were reported from India, the Middle East, Southeast Asia and the Mediterranean as recently as 2011 (Kudela and Gobler 2012). Since the harmful effects of *Cochlodinium* and continuous fisheries damages have attracted researchers' attention, knowledge on this causative species has been accumulating. Recent studies on the taxonomy, phylogeny and distribution of chain-forming *Cochlodinium* species have provided morphological characteristics for unambiguous species identification, and molecular identification based on rDNA sequences has verified the geographical distribution and recent expansion at the population level.

23.2 Taxonomy of *Cochlodinium polykrikoides* and Related Species

After the continuous reports of fish killing in coastal areas, *C. polykrikoides* was repeatedly cited and its various aspects, such as morphology, phylogeny, ecophysiology, life cycle and harmful effects, were examined (e.g, Jeong et al. 2004; Kim et al. 2002, 2004; Gobler et al. 2008; Iwataki et al. 2008, 2010; Ki and Han 2008; Tang and Gobler 2009a, 2012). However, the harmful species *C. polykrikoides* has been reported under several different species names such as *Cochlodinium catenatum* and *Cochlodinium heterolobatum* (e.g., Matsuoka et al. 2008). Taxonomic knowledge is therefore essential to consider the recent distribution and expansion. Table 23.1 shows the *Cochlodinium* species so far described with reference to the possession of chloroplasts.

Table 23.1 Photosynthetic and non-photosynthetic *Cochlodinium* spp. previously described

Photosynthetic species	Non-photosynthetic species	
C. catenatum Okamura (1916)	*C. achromaticum* Lebour (1925)	*C. flavum* Kofoid et Swezy (1921)
C. convolutum Kofoid et Swezy (1921)	*C. acutum* A. Cleve-Euler	*C. helicoides* Lebour (1925)
C. fulvescens Iwataki et al. (2007)	*C. adriaticum* Schiller (1933)	*C. lebourae* Kofoid et Swezy (1921)
C. helix (Pouchet) Lemmermann (1899)	*C. angustatum* Kofoid et Swezy (1921)	*C. longum* Lohmann (1908)
C. geminatum (Schütt) Schütt (1896)	*C. archimedes* (Pouchet) Lemmermann (1899)	*C. miniatum* Kofoid et Swezy (1921)
C. heterolobatum Silva (1967)	*C. atromaculatum* Kofoid et Swezy (1921)	*C. moniliforme* Margalef (1961)
C. pirum (Schütt) Lemmermann (1899)	*C. augustum* Kofoid et Swezy (1921)	*C. pellucidum* Lohmann (1908)
C. polykrikoides Margalef (1961)	*C. bahusiense* A. Cleve-Euler	*C. pulchellum* Lebour (1917)
	C. brandtii Wulff (1916)	*C. pupa* Lebour (1925)
	C. cavatum Kofoid et Swezy (1921)	*C. radiatum* Kofoid et Swezy (1921)
	C. cereum Kofoid et Swezy (1921)	*C. rosaceum* Kofoid et Swezy (1921)
	C. citron Kofoid et Swezy (1921)	*C. schuettii* Kofoid et Swezy (1921)
	C. clarissimum Kofoid et Swezy (1921)	*C. scintillans* Kofoid et Swezy (1921)
	C. cnidophorum Biecheler (1939)	*C. semistriatum* Elbrächter (1979)
	C. conspiratum Kofoid et Swezy (1921)	*C. strangulatum* (Schütt) Schütt (1896)*
	C. constrictum (Schütt) Lemmermann (1899)	*C. turbineum* Kofoid et Swezy (1921)
	C. distortum Kofoid et Swezy (1921)	*C. vinctum* Kofoid et Swezy (1921)
	C. elongatum Kofoid et Swezy (1921)	*C. virescens* Kofoid et Swezy (1921)
	C. faurei Kofoid et Swezy (1921)	*C. volutum* Kofoid et Swezy (1921)

* indicates the type species of the genus *Cochlodinium*

23.2.1 Taxonomic Background

The genus *Cochlodinium* was originally described by Schütt (1896), with the type species *Cochlodinium strangulatum*, and Kofoid and Swezy (1921) later proposing a generic description circumscribing unarmored dinoflagellates with a cingulum that encircles the cell more than one and a half times. Since then, more than 40 species have been described and assigned to *Cochlodinium* (Table 23.1). Given that most of the described *Cochlodinium* species are non-photosynthetic and they have rarely been reported since their original description, only several photosynthetic members can be compared for unambiguous identification of the harmful red tide species, *C. polykrikoides*.

Table 23.1 shows eight *Cochlodinium* species so far described as photosynthetic species. Of those species, *Cochlodinium convolutum* apparently differs from chain-forming species such as *C. polykrikoides* and *C. fulvescens*, because of the larger and elongated ovoid cell shape, roundly rectangular nucleus, circular apical groove, and the sulcus harboring the longitudinal flagellum (Matsuoka et al. 2008). Phylogenetic positions of *C. convolutum* and *Cochlodinium helix*, closely related to *Ceratoperidinium* and distant from *C. polykrikoides*, were more recently discovered (as *C.* cf. *convolutum* and *C.* cf. *helix* in Reñé et al. 2013b). Another species, *Cochlodinium pirum*, is also a non-chain-forming species closely resembling *C. convolutum* and *C. helix* (Schütt 1895).

The other five *Cochlodinium* species, *C. catenatum*, *C. fulvescens*, *Cochlodinium geminatum*, *C. heterolobatum* and *C. polykrikoides*, are therefore regarded as photosynthetic chain-forming *Cochlodinium* species in this paper. *Cochlodinium fulvescens* is a recently described species (Fig. 23.1), and its morphological differences and phylogenetic relationship to *C. polykrikoides* were verified in several reports (Iwataki et al. 2007; Matsuoka et al. 2008). The synonymy of *C. catenatum* and *C. heterolobatum* with *C. polykrikoides* were discussed in previous papers (e.g., Matsuoka et al. 2008), even though *C. catenatum* has not been detected from the type locality (Tokyo Bay, Japan) in recent years, and its identity remains a taxonomic problem. *Cochlodinium geminatum* is also a chain-forming species with rod-like elongated chloroplasts similar

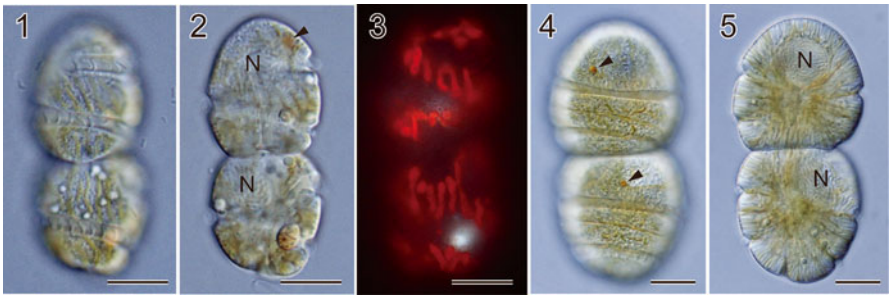

Fig. 23.1 Light microscopy of *Cochlodinium polykrikoides* (*1–3*) and *Cochlodinium fulvescens* (*4*, *5*), showing nucleus (*N*) and eyespot (*arrowheads*). Scales = 10 μm

to *C. polykrikoides* (Schütt 1895). Kofoid and Swezy (1921) reported *C. geminatum* from La Jolla, California, with an illustration of a single cell taken from the lower cell of a two-cell chain in Fig. 75.2 by Schütt (1895). According to Qiu et al. (2013), *C. geminatum* is related to an unarmored dinoflagellate *Polykrikos*, and distant from *C. polykrikoides*. However, this *C. geminatum* (as *Polykrikos geminatum*) from Zhuhai, China, somewhat differs from the original description of *C. geminatum* given by Schütt (1895), because the shallow sulcus located on the cell surface in *C. geminatum* (see Fig. 75 in Schütt 1895) was not observed from cells collected from China. Given these morphological differences, the *C. geminatum* reported from California, USA, and Zhuhai, China, is different from the *C. geminatum* originally reported by Schütt (1895). In the present study, reports of three *Cochlodinium* species, *C. catenatum*, *C. geminatum* (sensu Schütt 1895) and *C. heterolobatum*, are therefore considered as chain-forming *Cochlodinium* species related to *C. polykrikoides*.

23.2.2 Further Characterization of Chain-Forming Cochlodinium *spp.*

Although their phylogenetic position in dinoflagellates has not been revealed, the monophyly of *C. polykrikoides* and *C. fulvescens* is reliable and has been supported by several molecular phylogenetic works (e.g., Iwataki et al. 2008; Richlen et al. 2010; Reñé et al. 2013a). The morphology of these two species also suggests their close relationship, especially the combination of features, the chain colony formation and shallow sulcus without harboring the longitudinal flagellum (Fig. 23.1). These characteristics can be considered as synapomorphies of the phylogenetic cluster composed of *C. polykrikoides* and *C. fulvescens*. The difference in chloroplast shape, i.e., longitudinally elongated rod-like in *C. polykrikoides* and granular in *C. fulvescens*, seems to be a variable characteristic in the chain-forming *Cochlodinium* clade. Observations of the rod-like chloroplasts also from *C. catenatum* and *C. geminatum* are therefore interesting, as this implies their close affinity for *C. polykrikoides*, rather than *C. fulvescens*, in this clade.

The presence of an eyespot (type A, in Moestrup and Daugbjerg 2007) in *C. polykrikoides* was recently demonstrated by transmission electron microscopy (Iwataki et al. 2010). The eyespot, if present, is located in the sulcal region in many other dinoflagellates, and therefore its location on the dorsal side in the epicone is characteristic of *C. polykrikoides*. Moreover, the eyespot is located beside the invagination near the connection between the U-shaped apical groove and the sulcal extension on the dorsal side (Iwataki et al. 2010). A red-pigmented "eyespot" in the epicone can be seen under a light microscope (Fig. 23.1), and it was reported also from *C. fulvescens* (Iwataki et al. 2007). Figure 23.2 shows a scanning electron micrograph showing the apical groove of *C. fulvescens*. The groove is rather similar to that of *C. polykrikoides*, especially the connection of the sulcal extension on the dorsal side, and the presence of the invagination near the connection, which is related to the eyespot. Since the characteristic features of the apical groove have

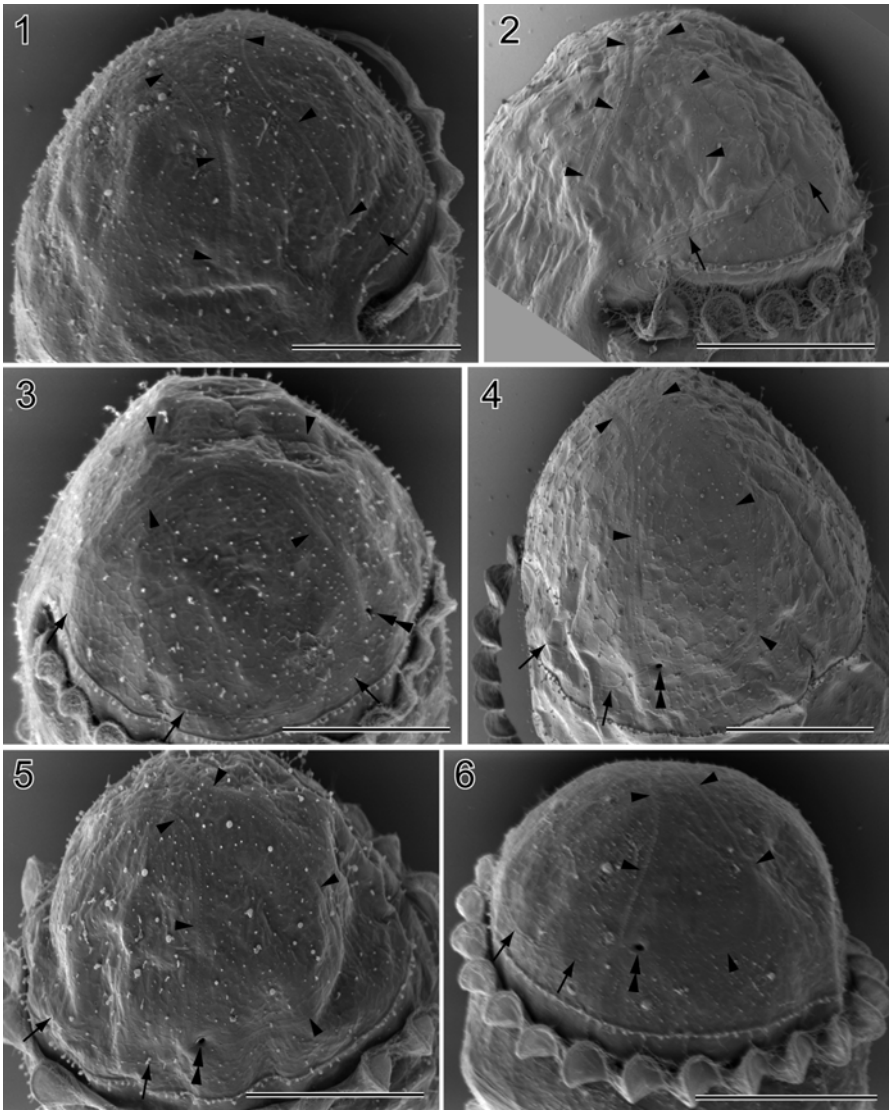

Fig. 23.2 Scanning electron microscopy of *Cochlodinium fulvescens*, showing the apical groove. *1* and *2*: ventral view. *3*: lateral view. *4–6*: dorsal view. Note the apical groove (*arrowheads*), dorsal pore (*double arrowheads*), and sulcal extension (*arrows*). Scales = 10 μm

never been reported from any other dinoflagellate, this type of apical groove characterizes the affinity of these two chain-forming *Cochlodinium* species. A small difference between the two species was found in the apical groove (Fig. 23.3). Around one-third from the ventral opening of the apical groove, the lines of the two grooves are constricted and form the narrow part in *C. fulvescens*, while the groove lines are almost parallel and form a U-shape in *C. polykrikoides*.

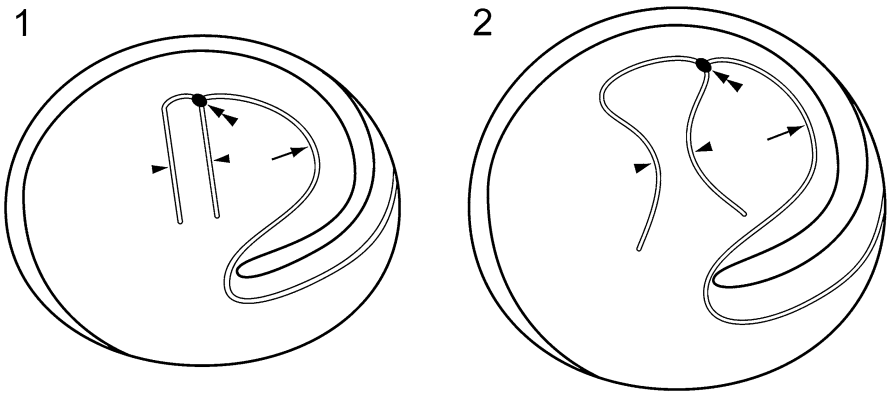

Fig. 23.3 Diagrammatic illustrations of apical groove. *1*: *Cochlodinium polykrikoides. 2*: *Cochlodinium fulvescens*. Labels indicate apical groove (*arrowheads*), dorsal pore (*double arrowheads*), and sulcal extension (*arrows*)

To our present knowledge, cells of the chain-forming *Cochlodinium* clade, composed of *C. fulvescens* and *C. polykrikoides*, are characterized by the following morphological features: (1) a spherical nucleus located in the anterior; (2) a shallow sulcus encircling the cell surface, which is distant from the longitudinal flagellar canal harboring the longitudinal flagellum; (3) a U-shaped or vase-like outlined apical groove connected with the sulcal extension on the dorsal side; and (4) an eyespot associated with the invagination located at the connection between the apical groove and the sulcal extension (on the dorsal side of the epicone), in addition to their chain colony formation.

23.2.3 Dinoflagellates Related to Chain-Forming Cochlodinium

The chain-forming *Cochlodinium* species, *C. fulvescens* and *C. polykrikoides*, are closely related and well characterized by their morphology, but their closest relative in the dinoflagellates remains unknown. Although the eyespot located in the epicone associated with the invagination on the dorsal side of the apical groove is diagnostic, it has never been seen in any other dinoflagellate. On the other hand, other morphological features are shared with some unarmored dinoflagellates. For example, the formation of a chain colony is known to occur in some unarmored dinoflagellates, e.g., *Gymnodinium catenatum* and *Gymnodinium impudicum*, the shallow sulcus without harboring the longitudinal flagellum is found in *Warnowia* spp., and the eyespot located on the dorsal side has been reported in *Amphidinium cupulatisquama* (Tamura et al. 2009). However, previous molecular works have not shown a relationship between chain-forming *Cochlodinium* and these dinoflagellates. In phylogenetic trees based on LSU rDNA sequences given by Hoppenrath

et al. (2009) and Moestrup et al. (2014), only a relationship between *C. polykrikoides*, *C. fulvescens* and *Akashiwo sanguinea* was found, but the support values were low. On the other hand, the ultrastructure provides an insight into related species of chain-forming *Cochlodinium*. The flagellar apparatus of *C. polykrikoides* was recently analyzed by TEM, and its striated ventral connective (vc) extended to the posterior along the longitudinal flagellar canal was distinctive from other unarmored dinoflagellates (Iwataki et al. 2010). The length and striations of the vc are distinctive, because the vc is considerably shorter in other unarmored dinoflagellates, e.g., *Gymnodinium aureolum* (Hansen 2001). A similar vc structure to *C. polykrikoides*, striated and extended toward the posterior, has so far been reported from *A. sanguinea* and *Levanderina fissa* (= *Gyrodinium instriatum*) (Roberts and Roberts 1991; Moestrup et al. 2014). Furthermore, some molecular and ultrastructural observations imply *Akashiwo* and *Levanderina* may be related to *C. polykrikoides*, but further evidence is required for discussion of their affinity.

23.3 Recent Expansion of *Cochlodinium polykrikoides*

The harmful red tide forming species *C. polykrikoides* was first reported from Puerto Rico (Margalef 1961). After the huge fisheries damages caused by this harmful dinoflagellate, many researchers reported the blooms especially from East and Southeast Asia, and from North and Central America (Kudela and Gobler 2012). The distribution of *Cochlodinium* was summarized in Matsuoka et al. (2008) and Kudela and Gobler (2012). The former compiled the occurrence information of *C. polykrikoides* and synonymous species, and the latter focused on the bloom events and showed the species geographic expansion. Fig. 23.4 shows the distribution of *Cochlodinium* spp., particularly of *C. polykrikoides* and *C. fulvescens*. Reports of the other photosynthetic chain-forming *Cochlodinium* spp. *C. catenatum* and *C. heterolobatum* were included in *C. polykrikoides* due to their morphological similarity, as discussed above.

Up until 1990, *C. polykrikoides* and similar chain-forming *Cochlodinium* species were reported from Puerto Rico (original description of *C. polykrikoides*), New Jersey (as *C. heterolobatum* Silva), Tokyo Bay and Costa Rica (as *C. catenatum* Okamura) (Okamura 1916; Margalef 1961; Silva 1967; Hargraves and Víquez 1981). Of the other *Cochlodinium* species, *C. geminatum* has features common to those of chain-forming *Cochlodinium* and might be related to *C. polykrikoides*. However, the type locality of *C. geminatum* is unknown. According to Kofoid and Swezy (1921), cells of *C. geminatum* were probably collected from the coast of Naples, Italy, or during the Atlantic expedition. Therefore the location of *C. geminatum* is not included in Fig. 23.4. Qi et al. (1993) reported fish mass mortality due to a bloom of *Cochlodinium* sp. from the Fujian coast, China, in 1990. Cells of the causative *Cochlodinium* species were spindle-shaped and 60–70 μm long, yellow or colorless, with a nucleus located posteriorly; these characters resemble *Gyrodinium* spp. In Queensland, Australia, a red tide *Cochlodinium* similar to *C. helix* was

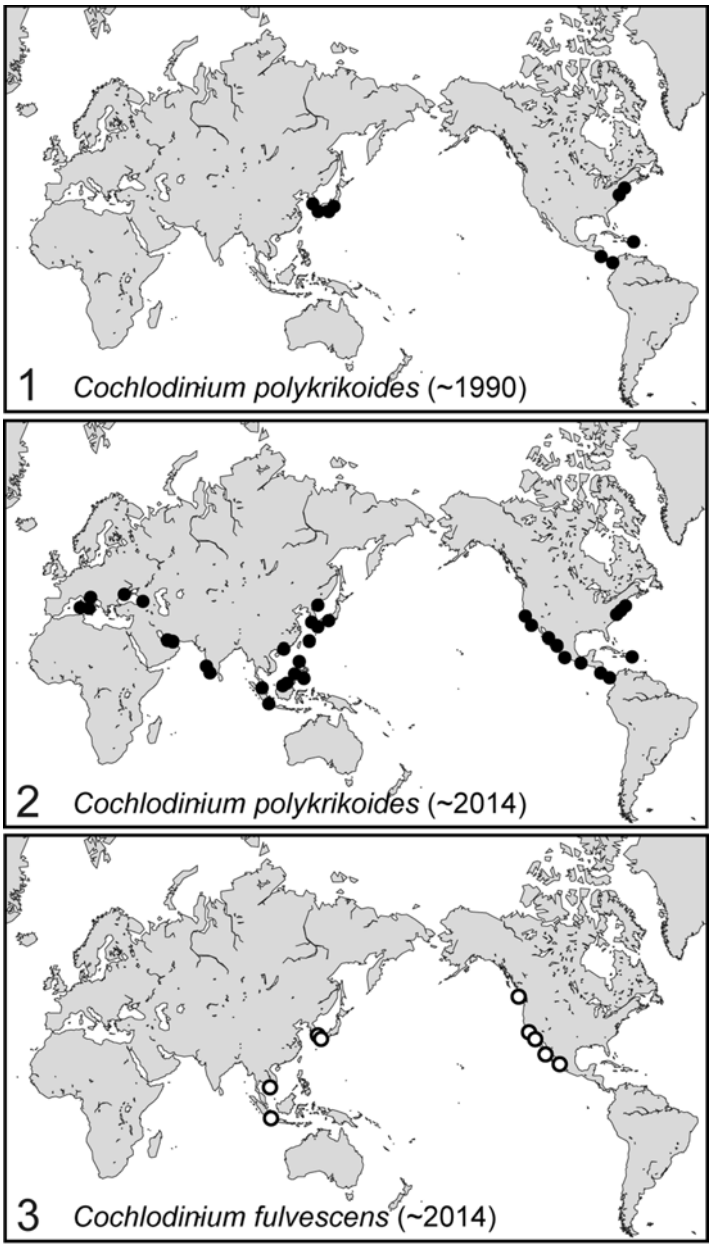

Fig. 23.4 Distribution of chain-forming *Cochlodinium* spp. Occurrences of *C. polykrikoides* until 1990 (*1*) and 2014 (*2*). Occurrences of *C. fulvescens* until 2014 (*3*)

reported (Hallegraeff 1992). *Cochlodinium helix* is an unarmored dinoflagellate related to *Ceratoperidinium* and is morphologically discernible from *C. polykrikoides* apparently. Chain-forming *Cochlodinium* spp. were distributed at least in Central and North America, East Asia, and Europe(?) until 1990, according to these reports.

After 1990, reports of *C. polykrikoides* have considerably increased. The species was reported from Japan, Korea and the USA repeatedly, Central America (Belize, Guatemala and Mexico) and from Italy. After 2000, *C. polykrikoides* was also reported from the Black Sea and the Mediterranean (Croatia, Italy, Spain and Ukraine), the Indian Ocean (India), the Middle East (Iran, Oman and UAE), and Southeast Asia (Indonesia, Malaysia and the Philippines). These reports imply the recent global expansion of *C. polykrikoides*; however, it is also difficult to demonstrate the absence of *C. polykrikoides* before 2000 in each region.

Figure 23.4 also shows the distribution of *C. fulvescens* discriminated from *C. polykrikoides. Cochlodinium fulvescens* was first reported from Indonesia and Japan, and then red tides occurring in Canada and on the coast of California were identified as being caused by this species (Iwataki et al. 2007, 2008). It was subsequently reported from Mexico and Pakistan. The present authors observed cells of *C. fulvescens* from the southern coast of Vietnam. This species is distributed at least along the Pacific coasts of Central and North America, and East and Southeast Asia.

23.4 Possibility of Recent Migration

Red tides of *C. polykrikoides* have basically been detected only from coastal waters; however, satellite image data also showed the movement of *Cochlodinium* red tides across the seas. For example, a red tide that occurred on the northern coast of Borneo, Malaysia, then moved to Palawan Island, the Philippines, in 2005, and a red tide on the southern coast of Korea reached the northern coast of Japan. Both of these red tides were detected by satellite imagery (Miyahara et al. 2004; Azanza et al. 2008). In the latter case, the genetic identity between Korean and Japanese cells was also demonstrated (Nishitani et al. 2008). This information suggests that red tides of *C. polykrikoides* can possible move at least toward adjacent regions via water currents. Moreover, recent molecular studies determined intraspecific relationships in *C. polykrikoides*, and demonstrated the original distribution and recent migration of *C. polykrikoides* at the population level.

23.4.1 Intraspecific Relationships in Cochlodinium fulvescens

DNA sequences of *C. fulvescens* so far analyzed show its phylogenetic position as a sister clade to *C. polykrikoides*. However, intraspecific relationships in the species have not fully been analyzed yet, because the submitted LSU rDNA sequences of the species were only from Canada, Japan, and the USA (Fig. 23.4).

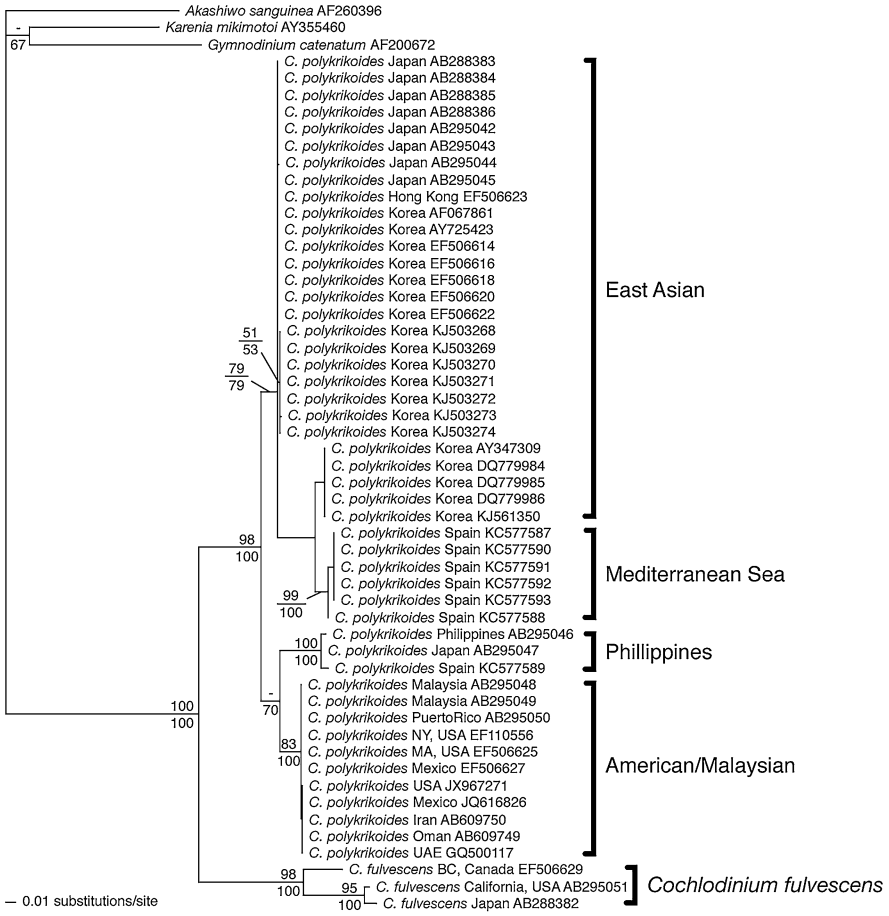

Fig. 23.5 Maximum likelihood tree of chain-forming *Cochlodinium* spp. based on LSU rDNA sequences

23.4.2 Intraspecific Relationships in Cochlodinium polykrikoides

Figure 23.5 shows the phylogenetic relationship of *C. polykrikoides* based on LSU rDNA sequences. The molecular phylogenetic data subdivided the species into four subclades of *C. polykrikoides*, and each ribotype may represent an original geographic distribution of the population (Iwataki et al. 2008; Richlen et al. 2010; Reñé et al. 2013a).

The ribotype "East Asian" is composed of isolates collected from coastal areas of Japan, Korea and Hong Kong. As yet, occurrence reports of this ribotype are restricted to East Asia. This ribotype is apparently responsible for the continuous red tides and fish mass mortalities in Japan and Korea.

The ribotype "Philippines" was first reported including samples collected from Omura Bay, Japan, in July 2003 and Manila Bay, the Philippines, in October 2004 (Iwataki et al. 2008). Cells of *C. polykrikoides* assigned to this ribotype were later reported from the southern coast of Korea, by specific primer sets of qPCR, and L'Ametlla Harbour, Spain (Reñé et al. 2013a; Park et al. 2014). Considering the occurrences in the Western Pacific and Mediterranean, the ribotype is likely to distribute widely, from tropical to temperate regions. Chain colonies were usually only two cells in culture, and red tides or fish killing have not been reported from this ribotype, interestingly. Azanza et al. (2008) reported a huge bloom of *Cochlodinium* in Palawan, the Philippines, in 2005, which originated on the northern coast of Borneo, Malaysia. Since all reported ribotypes in northern Borneo were assigned to "American/Malaysian", the *C. polykrikoides* that occurred in Palawan in 2005 is assumed not to belong to the "Philippines" ribotype (Iwataki et al. 2008).

All specimens of *C. polykrikoides* so far obtained from the Central and North American coasts have belonged to the ribotype "American/Malaysian", including a sample collected from the type locality (Puerto Rico) of *C. polykrikoides*. This ribotype is also responsible for harmful red tides (Tang and Gobler 2009a, b). When the ribotype was first reported, the same ribotype was detected also from Borneo, Malaysia (Iwataki et al. 2008). In Sabah on the northern coast of Borneo, Malaysia, a red tide of *C. polykrikoides* was first observed in 2005 (Anton et al. 2008). In 2008–2009, a red tide of *C. polykrikoides* suddenly emerged in the Arabian Gulf region in the Middle East, and the cells were also identified as belonging to the American/Malaysian ribotype (Richlen et al. 2010; Matsuoka et al. 2010). Before the red tide, *Cochlodinium* was observed also from Kuwaiti coastal waters (Richlen et al. 2010). The identical LSU rDNA sequences and detached range of distribution among *C. polykrikoides* from America, Malaysia and the Middle East strongly imply recent introduction of this ribotype from other regions. However, further evidence is required to understand the original distribution and recent migration of the American/Malaysian ribotype.

The "European" ribotype was recently detected from the Catalan coast in Spain, separated from three other ribotypes in *C. polykrikoides* (Reñé et al. 2013a). Around the Mediterranean and Black Sea, occurrences of *C. polykrikoides* were reported from Naples, Italy; the Adriatic Sea in the Mediterranean; Odessa Bay, Ukraine; and near the Caucasian coast, Russia, in the Black Sea. Although morphological information is limited for the European ribotype, it is interesting that the cells collected from the Catalan coast, Spain, formed a colony only by two cells (Reñé et al. 2013a). Another chain-forming *Cochlodinium*, *C. geminatum*, was first described by Schütt (1895), but the type locality of this species was not documented and was probably from Italy or the Atlantic, as mentioned above. If *C. geminatum* was collected from Naples and the ribotype "European" was detected only from the Mediterranean, this ribotype may have a limited distribution.

23.5 Conclusion

- Chain-forming *Cochlodinium* species resembling *Cochlodinium polykrikoides* have been described under several names, such as *C. catenatum*, *C. geminatum* and *C. heterolobatum*.
- Morphological features of the nucleus, sulcus, apical groove and eyespot characterize the chain-forming *Cochlodinium* species *C. fulvescens* and *C. polykrikoides*.
- Intraspecific rDNA-based ribotypes of *Cochlodinium polykrikoides*, "American/Malaysian", "East Asian" and "Europe", may represent regional populations of *C. polykrikoides*, while the distribution range of "Philippines" is unclear.
- At least two ribotypes, "American/Malaysian" and "East Asian", are responsible for harmful red tides killing fish and invertebrates.
- Another harmful *Cochlodinium*, *Cochlodinium fulvescens* is distributed in East and Southeast Asia and along the Pacific coasts of Central and North America.

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Chapter 24
Paralytic Shellfish Poison (PSP)–Producing Dinoflagellate and PSP-Infested Organisms

Manabu Asakawa, Gloria Gomez-Delan, Mercy Barte- Quilantang, and Katsutoshi Ito

Abstract Paralytic shellfish poison (PSP) is produced by numerous microalgae species, mainly toxic marine dinoflagellates species of the genera *Alexandrium*, *Gymnodinium,* and *Pyrodinium*, and is accumulated in many species of filter-feeding organisms such as bivalve mollusks through the food chain. Besides these toxic dinoflagellates, certain freshwater cyanobacteria such as *Anabaena circinarlis* and *Aphanizomenon flos-aquae* also produce PSP. PSP is transferred and bioaccumulates throughout aquatic food webs, and can be vectored to terrestrial biota. Fishery closures and food poisoning due to PSP have been documented in several vectors. Traditionally, only filter-feeding mollusks that concentrate these toxic algae are considered in monitoring programs for PSP; however, increasing attention is being paid to higher-order predators. Besides commercially important bivalves, other PSP-bearing organisms such as carnivorous gastropods and crustaceans have been reported. From the food hygiene point of view, it is important to summarize the role of marine organisms as vectors of PSP and discuss the need for surveillance to protect public health and ensure the quality of seafood. This chapter shows several case studies pertaining to management actions to prevent food-poisoning incidents by PSP accumulation in filter-feeding (traditional) and non-filter-feeding (non-traditional) vectors of PSP.

M. Asakawa (✉)
Graduate School of Biosphere Science, Department of Biofunctional Science and Technology, Hiroshima University, 1-4-4, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan
e-mail: asakawa@hiroshima-u.ac.jp

G. Gomez-Delan
College of Fisheries Technology, Cebu Technological University-Carmen, Cebu Campus, 6005, Cebu, Philippines

M.B.- Quilantang
College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao 5023, Iloilo, Philippines

K. Ito
National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Maruishi 2-17-5, Hatsukaichi, Hiroshima 739-0452, Japan

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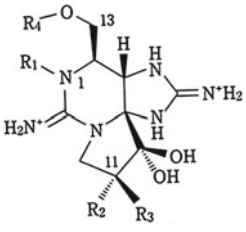
Keywords Paralytic shellfish poison • Toxic dinoflagellate • Filter-feeding • Bivalves • Mossworm • Gastropod • Saxitoxin • Gonyautoxin

24.1 Introduction

Paralytic shellfish poison (PSP), one of the most notorious and hazardous marine biotoxins known, is produced by numerous microalgae species, mainly toxic marine dinoflagellates species of the genera *Alexandrium*, *Gymnodinium,* and *Pyrodinium*, and is accumulated in many species of marine filter-feeding organisms such as bivalve mollusks through the food chain (Asakawa et al. 1993, 1995, 2005; Oshima et al. 1987, 1993; Gacutan et al. 1985; Azanza 1997). Shellfish toxicity related to the unarmored and chain-forming species *Gymnodinium catenatum* has been reported from many countries such as Galicia (Spain), Tasmania (Australia) and Kyushu (Japan). In Japan, the PSP-producing dinoflagellates *Alexandrium tamarense, A. catenella*, *A. tamiyavanichii* and *G. catenatum* occur and intoxicate many edible bivalves. *Alexandrium* spp. cause problems mainly in cold and temperate waters in the northern hemisphere, while the tropical species *Pyrodinium bahamense* var. *compressum* is found in Southeast Asia. The occurrence of harmful algal blooms (HAB) has been increasing globally throughout the twentieth century. HAB has a severe impact on the economy and human health, because phycotoxin transfer through the food chain, contaminating edible shellfish, resulting in intoxication incidents. In the Philippines, the first HAB or so-called toxic red tides of *Pyrodinium* species were recorded in June, 1983 where it was first observed in the Samar-Leyte areas, Central Philippines, which are important fishing areas. Blooms of this species occurred 135 times in 27 different coastal waters of the country between 1983 and 2005 with a total of 2161 reported PSP-poisoning cases and 123 fatalities. *Pyrodinium* blooms of red tide continue to invade many other coastal waters of Manila Bay and Bataan, Samar/Leyte, Cebu and Negros Islands and Balete Bay (Davao) in Luzon, Visayas and Mindanao, three major islands of the Philippines. Due to the continuous hazards to public health and the economy, the Philippine government, the Department of Agriculture (DA) through the Bureau of Fisheries and Aquatic Resources (BFAR) initiated a "Toxic Red Tide Monitoring Program" in different parts of the country in 1984 (Corrales and Gomez 1990; Bajarias et al. 2006). Besides these toxic dinoflagellates, certain freshwater cyanobacteria, also called blue-green algae, such as *Anabaena circinarlis* (Andrew and Jones 1995; Andrew et al. 1995) and *Aphanizomenon flos-aquae* (Pereira et al. 2000; Ferreira et al. 2001) also produce PSP. Recently, besides these species, certain filamentous cyanobacteria belonging to the genera *Cylindrospermopsis*, *Lyngbya* and *Planktothrix* have been found to produce PSP (Lagos et al. 1999; Carmichael et al. 1997; Pomati et al. 2000). In addition to these, the genus *Raphidiopsis* has also been described as a PSP producer (Katia et al. 2012; Plominsky et al. 2009; Yunes et al. 2009; Soto-Liebe et al. 2010). Blooms of cyanobacteria are becoming prevalent in

municipal water supplies, swimming areas and aquaculture zones, etc., and are responsible for sporadic episodes of animal poisonings from municipal and recreational water supplies. It is very interesting that the PSP composition and concentration of these species have been found to vary with geographic region and environmental factors.

PSP is a common seafood toxicity problem with worldwide distribution, and this poisoning is the foodborne illness associated with the consumption of seafood products of filter-feeding molluscan bivalves contaminated with PSP. Accumulation of phycotoxins by filter-feeding shellfish is a well-known global phenomenon. Contamination of bivalves with PSP produced by these species has posed a serious problem to the shellfish culture industry as well as to public health in various parts of the world. As PSP comprises a group of naturally occurring neurotoxic alkaloids produced among several genera of primarily marine dinoflagellates and freshwater cyanobacteria, PSP, once ingested by humans, acts as a sodium channel-blocking agent in mammals and causes paralysis and other symptoms, with frequent death. The first PSP toxin was isolated from the Alaska butter clam *Saxiomus giganteus* and it was designated saxitoxin (STX) later (Schantz et al. 1957; Schantz 1960). STX is a trialkyl tetrahydropurine that is extremely stable in physiological solutions unless it undergoes exposure to alkaline conditions. Subsequent studies showed that PSP is not composed of STX alone, but includes at least further 20 derivatives whose structures are closely related to each other, with a range of hydroxyl, carbamyl, and sulfate moieties at four sites on the backbone structure (Oshima et al. 1987, 1993). As shown in Fig. 24.1, these substitutions result in analogs varying more than three orders of magnitude in toxicity. The carbamate toxins have the highest toxicity, and they include STX, neoSTX, and gonyautoxins (GTX1-4). The decarbamoyl toxins (dcSTX, dcneoSTX, dcGTX1-4) have intermediate toxicity and are reported in bivalves, but are not commonly found in toxic dinoflagellates. The *N*-sulfocarbamoyl toxins (B1 [GTX5], B2 [GTX6] and C1-4) are less toxic. Within these PSP components, STX is a most powerful neurotoxin like tetrodotoxin (TTX), having an LD_{50} in mice (intraperitoneally) of 10 μg/kg (as compared with an LD_{50} for sodium cyanide of 10 mg/kg). It is also important as a pharmacological tool, as it is a highly selective sodium channel blocker, which binds to the voltage-dependent sodium channel in nerve cells, thereby preventing the passive influx of sodium ion into excitable cells, and restricting signal trans-



R_1	R_2	R_3	R_4: $CONH_2$	R_4: $CONHSO_3^-$	R_4: H
H	H	H	STX (2483)	GTX5 (160)	dcSTX (1274)
OH	H	H	neoSTX (2295)	GTX6 (180)	dcneoSTX (33)
OH	OSO_3^-	H	GTX1 (2468)	C3 (33)	
H	OSO_3^-	H	GTX2 (892)	C1 (15)	dcGTX2 (1617)
H	H	OSO_3^-	GTX3 (1584)	C2 (239)	dcGTX3 (1872)
OH	H	OSO_3^-	GTX4 (1803)	C4 (143)	

Fig. 24.1 Structures of PSP components and their specific toxicities (MU/μmol)

mission between neurons. Within 30 min, persons suffering PSP can experience symptoms including a burning sensation on the face; paresthesia and numbness, first around the lips and mouth and then involving the face and neck; muscular weakness; a sensation of lightness and floating; ataxia; motor incoordination; drowsiness; incoherence; and progressively decreasing ventilator efficiency. With high doses of PSP, the poisoning may be fatal, resulting from respiratory paralysis and asphyxiation if the patient is not treated with medical respiratory support. Currently, there is no antidote available. The minimum lethal dose (MLD) of PSP in humans is estimated to be 3000 mouse units (MU) based mainly on fatal cases induced by this toxin (Hashimoto and Noguchi 1989). One MU of PSP is defined as the amount of this toxin that can kill a 20 g ddY strain male mouse in 15 min, after intraperitoneal administration. In USA and Canada, the quarantine limit of PSP in bivalves for home consumption has been set at 80 μg (as STX)/100 g edible part. On the other hand, government quarantine limits to protect against PSP toxification by bivalves have been set in Japan. The official PSP determination method in Japan is essentially the same as that of the AOAC mouse bioassay method (Horwitz 1980; AOAC 1990). In converting the death time to the toxicity, it recommends use of the dose–death time curve for STX, desirably between 5 and 7 min. The quarantine limit is set at 4 MU/g edible part, a value that is essentially the same as that in the USA and Canada. Since these quarantine limits were set, successful management and monitoring programs have minimized PSP-poisoning cases and associated deaths. Much is known about the toxin sources, primarily certain dinoflagellate species, and there is extensive information on toxin transfer to the traditional vectors of filter-feeding molluscan bivalves. Traditionally, only filter-feeding mollusks that concentrate these toxic algae are considered in monitoring programs for PSP, diarrheic shellfish poison (DSP), neurotoxic shellfish poison (NSP) and amnesic shellfish poison (ASP); however, increasing attention is being paid to higher-order consumers. Besides commercially important bivalves, other organisms such as carnivorous gastropods and crustaceans have been reported.

This article summarizes the role of some marine organisms as vectors of PSP and discusses the need for surveillance to protect public health and ensure the quality of seafood products. We include several case studies pertaining to management actions to prevent food-poisoning incidents from PSP accumulation in filter-feeding (traditional) and non-filter-feeding (non-traditional) vectors of PSP.

24.2 Accumulation of PSP by Filter-Feeding (Traditional) Vectors

Various species of bivalves are toxified: scallop *Patinopecten yessoensis*, oyster *Crassostrea gigas*, mussel *Mytilus edulis*, “hiogi” scallop *Chlamys nobilis*, short-necked clam *Tapes japonica*, *etc*. In addition, the protochordate ascidian *Holocynthia roretzi* is sometimes toxified (Nagashima et al. 1984). Recently, an occurrence of

PSP-contaminated ascidian *Microcosmus vulgaris* (grooved sea squirt) from the West Istrian coast (Adriatic Sea, Croatia) was reported (Roje-Busatto and Ujevic 2014).

24.2.1 Occurrence of Toxic Dinoflagellate and PSP-Infested Bivalves in Seto Inland Sea, Japan

24.2.1.1 Case Study in Hiroshima Bay—Occurrence of *A. tamarense*

In April 1992, paralytic toxicity substantially exceeding the quarantine limit of 4 MU/g edible parts as PSP was detected in the cultured oyster *Crassostrea gigas*, the mussel *Mytilus edulis* and the short-necked clam *Tapes japonica* from Hiroshima Bay, Hiroshima Prefecture, concomitantly with the appearance of the toxic dinoflagellate *A. tamarense* (Fig. 24.2) (Asakawa et al. 1993, 1995). The toxicities were 31.4 MU/g for the oyster, 214.6 MU/g for the mussel and 20.3 MU/g for the short-necked clam on 22nd April. In GTX analysis of each toxin by high-performance liquid chromatography-fluorescent detection (HPLC-FLD), C1, 2, GTX4, 1, 3 and 2 were detected. In STX analysis, a small peak of STX was detected in the mussel and short-necked clam toxin, but not in the oyster toxin. Consequently, the toxin of the bivalves in Hiroshima Bay was found to be comprised of GTX1–4 as the major

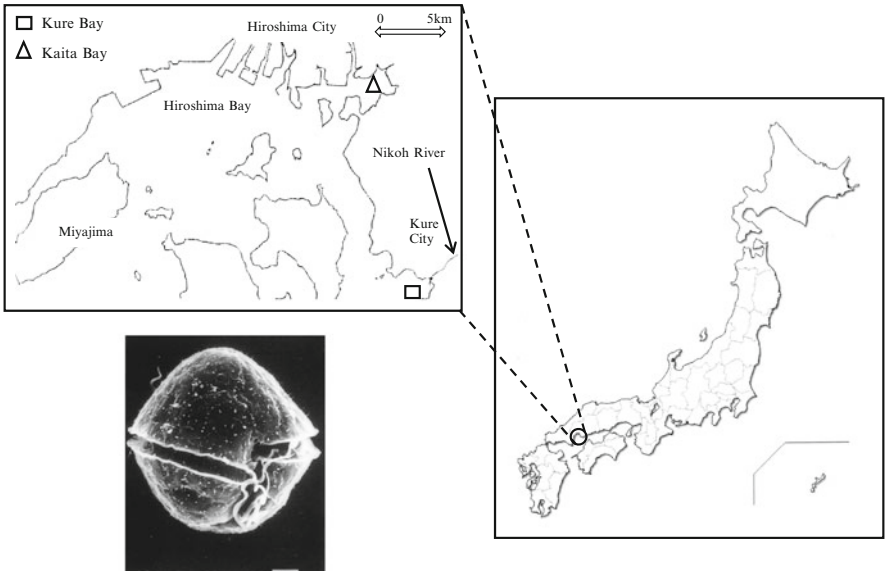


Fig. 24.2 Map showing sampling locations in Hiroshima Bay, and picture showing a scanning electron microscope image of *Alexandrium tamarense* isolated from Hiroshima Bay (scale bar: 5 μm)

components, which accounted for approximately 92–95 mol% of all components, with a trace of STX. In all cases, GTX1 was the major component (approximately 51–55 mol%). On the other hand, the content of C1, 2 was 1.6–4.5 mol% irrespective of the sample. It was concluded from these results that the toxin of the above bivalves collected in Hiroshima Bay in April 1992 consisted predominantly of PSP, possibly derived from the toxic plankton *A. tamarens*e detected there. Since the first infestation (in 1992) of shellfish with PSP in Hiroshima Bay, which is one of the largest oyster culture areas in Japan, *A. tamarense* has been periodically associated with episodes of PSP toxicity. Subsequent monitoring for toxins contained in commercial shellfish by mouse bioassay showed that short-necked clams, mussels and oysters were contaminated with PSP from the end of March to May in association with the appearance of *A. tamarense* in this bay. Fortunately, there have been no cases of food poisoning connected with the present PSP infestation of bivalves. In the 1993–2004 survey on the occurrence of PSP-producing dinoflagellates in Hiroshima Bay, maximum cell density and PSP compositions of *A. tamarense* in Kure Bay and Kaita Bay, which are located within Hiroshima Bay, were investigated (Fig. 24.3). Five strains of *A. tamarense* were isolated and their PSP profiles were investigated (Table 24.1). This investigation, developed as an extension of previous studies in which the authors participated, was undertaken to assess levels of shellfish intoxication by *A. tamarense*, potential health risks to human shellfish consumers and the possible need for regulatory intervention (Asakawa et al. 2005).

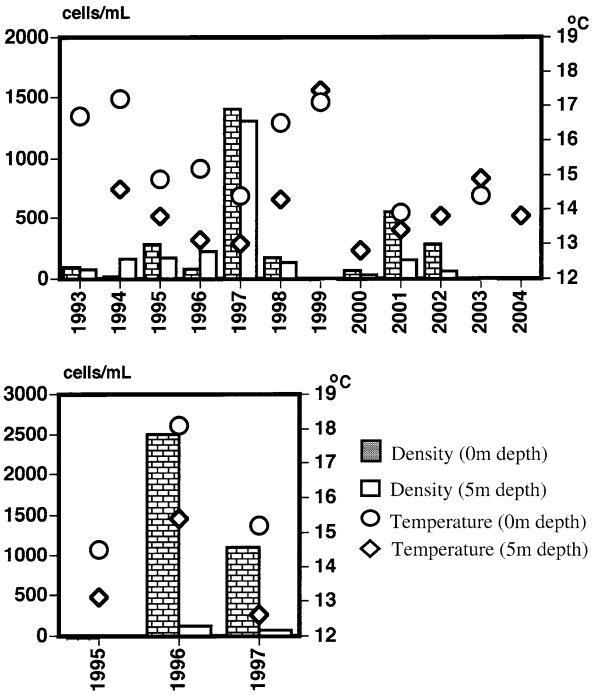


Fig. 24.3 Maximum cell density of *Alexandrium tamarense* in Kure Bay (**a**) and Kaita Bay (**b**), Hiroshima Prefecture

Table 24.1 Toxin profiles of *Alexandrium tamarense* isolated in Hiroshima Bay and toxic bivalves

	Kure Bay						Kaita Bay
PSP component	Dinoflagellate	Bivalves (5 m depth)		Dinoflagellate			
	ATKR-97	Oyster	Mussel	ATKR-94	ATKR-95	ATKR-01	ATKT-97
GTX1	8.6	15.5	52.5	7.1	12.3	1.0	2.5
GTX2	7.6	12.0	13.1	0.0	0.9	1.4	0.6
GTX3	0.7	11.7	9.3	1.8	27.1	6.0	4.4
GTX4	4.2	4.8	11.5	40.8	11.6	34.4	0.6
dcGTX2	0.3	4.0	0.9	0	0	0	0.3
dcGTX3	0	3.8	1.2	0	0	0	0.3
C1 (PX1)	12.4	28.4	2.6	1.4	0.9	3.6	7.8
C2 (PX2)	50.0	7.6	1.8	30.4	32.2	20.4	60.4
C3 (PX3)	0	0	2.7	0	0.4	0	0
C4 (PX4)	0	0	0.7	0	0	0	0
neoSTX	16.2	7.0	2.7	18.5	13.3	32.5	16.6
STX	0	5.2	1.0	0	1.3	0.7	6.5

All results are shown in mol%

24.2.1.2 Case Study in Bingo-Nada—Occurrence of *A. tamiyavanichii*

In Japan, a new appearance of toxic dinoflagellates and toxification of shellfish in the Seto Inland Sea were reported. In early December 1999, a bloom due to *A. tamiyavanichii* (formerly called *A. cohoticula*), which has not been reported so far as a causative plankton for PSP infestation of bivalves, occurred around the southeast coasts of the Seto Inland Sea, resulting in PSP toxification of wild bivalves (Hashimoto et al. 2002). The occurrence of the same species from October to November 2004 and toxification of bivalves in Harima-Nada of Seto Inland Sea were also reported (Sagara et al. 2010). This species was originally described by Balech (1967), who found it for the first time in phytoplankton samples from the Gulf of Mexico and described its morphological characteristics. Discoveries of *A. tamiyavanichii* as the causative organism of PSP in the Gulf of Thailand were reported (Phanichyakarn et al. 1993; Kodama et al. 1988). On the other hand, during surveillance of the distribution of PSP-producing dinoflagellates in 2003, 2004 and 2005 along the coastlines of the Seto Inland Sea, Hiroshima Prefecture, some species of toxic phytoplankton were isolated from the eastern coasts, Bingo-Nada, the central regions of the Seto Inland Sea (Beppu et al. 2008). It was rather unexpectedly revealed on the basis of the morphological characteristics that one of them was unambiguously identified as a long-chain-forming species of *A. tamiyavanichii*, which is a tropical species of dinoflagellate. Two isolated strains (ATY041106, ATY051018) of *A. tamiyavanichii* showed specific toxicity of 38.7×10^{-6} and 111.5×10^{-6} MU/cell, respectively. These values seemed to be several times or much higher than that of *A. catenella* (AC030816, AC040614), having a specific toxicity of 4.5×10^{-6} and 4.1×10^{-6} MU/cell, respectively, isolated in the same area. HPLC-FLD

analysis revealed that the toxins in ATY041106 exist almost exclusively as β-epimers (C2, GTX3, 4), which accounted for 72.7 mol%. The toxin profiles of this strain feature the presence of a large amount of GTX3 (59.1 mol%) and a small amount (20.6 mol%) of C1, 2 in comparison with the PSP compositions of *A. tamarense*, which is isolated as the main responsible species in Hiroshima Bay, a western part of the coastal sea in Hiroshima Prefecture. It was revealed that the toxin profiles of two strains (AC030816, AC040614) of *A. catenella* exist almost exclusively as β-epimers (C2, GTX3, 4), which accounted for 81.8 and 56.5 mol%, in the same manner. The toxin profiles of these two strains feature the presence of a large amount of C2 (80.5 and 46.3 mol%) in comparison with the PSP compositions of *A. tamiyavanichii*.

This is the first record to show the distribution and harmful influence of *A. tamiyavanichii* and *A. catenella* in Bingo-Nada in Hiroshima Prefecture. Not only detection of PSP-producing phytoplankton *A. tamiyavanichii* in Bingo-Nada, the eastern part of coastal sea in Hiroshima Prefecture, but also toxification of the bivalves in this area has not been reported so far. There is a possibility of contamination of bivalves during late fall to winter in this area with blooms of *A. tamiyavanichii* in the near future. This is a huge threat to a massive oyster culture area, as Hiroshima Bay belongs to the western coastal waters of Hiroshima Prefecture, where cultured oysters are harvested mainly during late fall to winter. Though contamination of bivalves with these PSP-producing planktons in this area has not occurred yet, attention should be paid to this species as well as the other causative dinoflagellates from the standpoint of public health and food hygiene.

24.2.2 Accumulation and Biotransformation of PSP in Short-Necked Clams Fed with the Toxic Dinoflagellate A. tamarense

As a part of studies on PSP accumulation kinetics in bivalves, the short-necked clam *Tapes japonica* was experimentally contaminated with PSP by feeding with *A. tamarense* for 2, 4, 6, 8 and 10 days, and the processes of PSP accumulation and bioconversion were investigated: the toxicity level was determined by a mouse bioassay and toxin components were identified by HPLC-FLD (Asakawa et al. 2005). In this feeding experiment, the strain of *A. tamarense* ATHS-92 possessed specific toxicity of 186.7 ± 81 (mean ± SD, $n = 5$) $\times 10^{-6}$ MU/cell. The total toxin concentration of this strain was 140.4 ± 61 (mean ± SD, $n = 5$) fmol/cell. Figure 24.4a shows the changes in toxicity and the PSP accumulation rate in short-necked clams fed with *A. tamarense*. In this figure, the accumulation rate is the ratio of total toxicity accumulated in the bivalves fed with this toxic dinoflagellate to the total toxicity of supplied cells in each feeding experiment. In the short-necked clams, toxicity levels reached 1.8 (0.9), 3.2 (1.6), 3.8 (2.0), 3.5 (1.9) and 4.6 (2.3) MU/g (nmol/g) shucked meat after 2, 4, 6, 8 and 10 days of feeding, respectively. The amounts of toxins of short-necked clams increased almost in parallel with the numbers of *A. tamarense* cells taken by them. However, the toxicity levels of these bivalves were extremely low in comparison with available toxicity during the exposure periods. It is generally

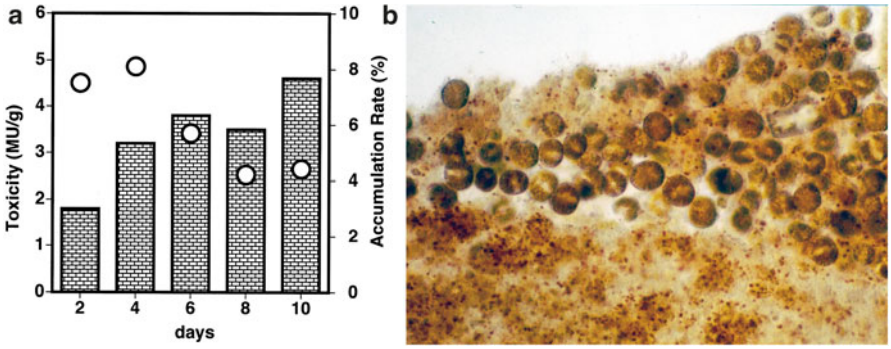


Fig. 24.4 Toxicity and PSP accumulation rate of short-necked clams fed with *Alexandrium tamarense* (**a**), and undigested cells of *Alexandrium tamarense* in the pseudofeces of short-necked clams (**b**)

accepted that filter-feeding activity becomes lower when bivalves are exposed to high densities of toxic dinoflagellates (Bricelj et al. 1990). In the present study, the calculated clearance rates were 78.3, 98.4, 99.2, 98.9 and 98.9 %, respectively. In all experimental groups, these rates were high. Therefore, the reason why the toxicity level of short-necked clams was low does not seem to be a decline of feeding activity. In this connection, the accumulation rates of PSP toxins were estimated to be 7.5, 8.1, 5.7, 4.2 and 4.4 % after 2, 4, 6, 8 and 10 days of feeding, respectively. The rate decreased as the toxicity of the clams increased. These data indicate that most of the toxins contained in *A. tamarense* cells were not accumulated in the short-necked clams. In other words, the low toxicity level of short-necked clams appeared to reflect a low accumulation rate. Both release of toxins from contaminated bivalves to the outside water and detoxification within bivalve tissue have been reported. These processes may partly explain the low accumulation rates in this feeding experiment. Interestingly, at the end of each exposure period, many undigested algal cells were found in pseudofeces under microscopic observation (Fig. 24.4b). This may be one of the major reasons why the accumulation rate of toxin was much lower than expected. The PSP profiles of the partially purified toxins from the cultured cells of *A. tamarense* and the short-necked clams fed with the plankton are summarized in Table 24.2. C2 and GTX4 were the dominant toxins in cultured *A. tamarense* cells. GTX1, 3, C1, 3 and neoSTX were also detected. Remarkable differences were observed in the relative abundance of the toxins between the responsible dinoflagellates and the contaminated shellfish. The most notable difference was the change in the relative amounts of C2, GTX1, 4 during the first two days exposure. In the toxic bivalves, the amount of C2, which was dominant in *A. tamarense*, decreased less than half a percent, suggesting rapid conversion of PSP after feeding. Subsequently, the amount of GTX1 in the shellfish meat reached 50.1 mol%, while that of GTX4 decreased to about half of that in *A. tamarense*.

As for the configuration of 11-hydroxysulfate, PSP components in ATHS-92 exist almost exclusively as β-epimers (GTX3, 4 and C2), accounting for 72.8 mol%

Table 24.2 Toxin profiles of *Alexandrium tamarense* and short-necked clams fed with *A. tamarense*

PSP component	*A. tamarense*	Short-necked clams fed with *A. tamarense*				
		2 days	4 days	6 days	8 days	10 days
GTX1	10.4	50.1	38.9	38.7	37.5	48.1
GTX2	0.0	0.6	0.5	0.8	1.1	1.9
GTX3	1.6	2.6	3.6	3.5	5.3	7.3
GTX4	34.5	17.0	18.3	14.0	15.1	13.6
C1 (PX1)	0.8	2.3	1.5	2.5	2.2	1.5
C2 (PX2)	36.7	6.2	11.9	13.3	16.4	7.6
C3 (PX3)	1.3	0.0	0.0	0.0	0.0	0.0
neoSTX	14.7	21.2	23.7	26.5	20.7	18.7
STX	0.0	0.0	1.6	0.7	1.7	1.3

All results are shown in mol%

of the total. The same tendency was observed in the strains from Hiroshima Bay in 1993, 1994 and 1995 (Asakawa et al. 1995, 2005). This contrasts with the case of the short-necked clams, where β-epimers represent 25.8, 33.8, 30.8, 36.8 and 28.5 mol% of the total after 2, 4, 6, 8 and 10 days of feeding, respectively. The contents of β-epimers in *A. tamarense* were approximately twice those in the bivalves fed with this dinoflagellate. A comparison of the toxin profiles between the causative dinoflagellate and the contaminated bivalves showed that PSP components exist in the bivalves in the form of the chemically more stable α-epimers at an early stage (within 2 days) after the feeding of *A. tamarense*, suggesting rapid conversion of PSP components after feeding.

Chen and Chou (2002) reported rapid transformation of GTX4 to GTX1 in the purple clams *Hiatula rostrata* fed with the toxic dinoflagellate *A. minutum*. Oshima et al. (1990, 1995) reported rapid degradation of *N*-1 hydroxy toxins and conversions of 11β-hydroxysulfate to 11α-epimer during the accumulation process in scallops, mussels and oysters. On the other hand, Ichimi et al. (2001) reported that there was a similar trend in the relative proportions of predominant toxins within *A. tamarense* and mussels experimentally contaminated with PSP by being fed with *A. tamarense*. Murakami et al. (1999a, b) reported that the ratio of α-epimer (GTX1) to β-epimer (GTX4) at C11 tended to increase up to the equilibrium point of 3: 1 in PSP-infested bivalves from Ibaraki Prefecture. Several reports have indicated that bivalves have higher proportions of carbamate toxins than the causative dinoflagellates. Onoue et al. (1981a, b, 1983) and Noguchi et al. (1983a, b) reported a possible bioconversion process from low-toxicity components such as C1, 2 to high-toxicity ones, based on the results of feeding experiments. C1, 2 in *A. catenella* were hardly detected in mussels, which attained a higher toxicity level than would have been expected from the uptake of *A. catenella* cells. In contrast, it was also reported that the toxin profiles of the Tasmanian dinoflagellate *G. catenatum* and infested shellfish were characterized by unusually high proportions of low-potency sulfocarbamoyl toxin, which comprised 98–99 % and 77–93 %, respectively, of total toxins (Oshima et al. 1987). The difference in toxin profiles between the

bivalves and dinoflagellates has been partly explained by the enzymatic and/or chemical transformation of toxins after accumulation in bivalve tissues (Shimizu and Yoshioka 1981; Sullivan et al. 1983; Asakawa et al. 1987; Oshima 1995; Murakami et al. 1999a, b). In this connection, a small amount of STX was detected in the short-necked clams after 4 days of feeding. There appear to be enzymatic processes leading to the transformation of GTXs to STX through the reductive elimination of the C-11 hydroxysulfate and *N*-1 hydroxyl moieties. The toxin profiles of the short-necked clams and *A. tamarense* were not significantly different, except for epimerization of toxins from β- to α-type, and the remarkable change in the relative amounts of C2 (Table 24.2). Interestingly, the total ratio of β- and α-epimers in short-necked clams sampled after 2 days of feeding experiments was almost constant. This indicates that equilibrium is reached rapidly after the uptake of toxins by short-necked clams.

24.2.3 Accumulation of PSP by a Marine Mossworm (Molluscoidea) as a Filter-Feeding Vector

To elucidate the mechanism of toxification of the scallop *Patinopecten yessoensis* with PSP in Funka Bay, Hokkaido, Japan, the lethal potency of marine plankton around the scallop farming area was investigated (Noguchi et al. 1990). During screening for paralytic potency in marine organisms, lethal potency was found in a brown seaweed and also in the shell of a scallop, both of which were fouled with organisms inclusive of mossworm. Figure. 24.5a shows a scanning electron microscope image of the mossworm on the brown algae.

Two lots (2.0 kg each) of dried product of fouled brown seaweed (unidentified) were obtained. They were collected from Funka Bay, Hokkaido, Japan, in July 1989 and 1990. In addition, raw fouled seaweed (5 kg), probably of the same species, was collected from the same bay in July 1990. With a knife, the mossworm was stripped off the dried alga harvested in summer of 1989 and showed a lethal score of 18 MU/g dry basis. In the summer of 1990, the mossworm from a raw alga exhibited a score of 2.4 MU/g, while dried ones had scores of 9.0 MU/g (Noguchi et al. 1991, 1992). About 15 g of mossworm was stripped off the brown alga by a knife in each year, and was homogenized with three volumes of 0.1 % acetic acid and centrifuged at 2000 × g for 20 min. The residue was extracted twice more in the same manner. The supernatants obtained were combined and concentrated under reduced pressure, as in the routine method for extraction of PSP. The extract was ultrafiltered through a Diaflo YM-2 membrane (Amicon) to cut off more than 1000 Da substances. The filtrate was adjusted to pH 5.5 with 1 M NaOH and passed successively through a column (φ1.0 × 7.0 cm) of activated charcoal equilibrated with a mixture of 1 % acetic acid and 20 % ethanol (1:1) and a cartridge column (φ1.1 × 1.3 cm) of Sep-Pak C18 (Waters, Milford, MA, US) equilibrated with 0.1 % acetic acid. The unadsorbed portion was combined, evaporated to dryness *in vacuo*, and lyophilized. A partially purified mossworm toxin from the 1989 sample was analyzed by HPLC-FLD. The toxin composed of GTX1, 2, 3, 4 and neoSTX, whose molar ratios were 4:3:9:1:6. GTX1-3 and neoSTX, were the main components with the 1990

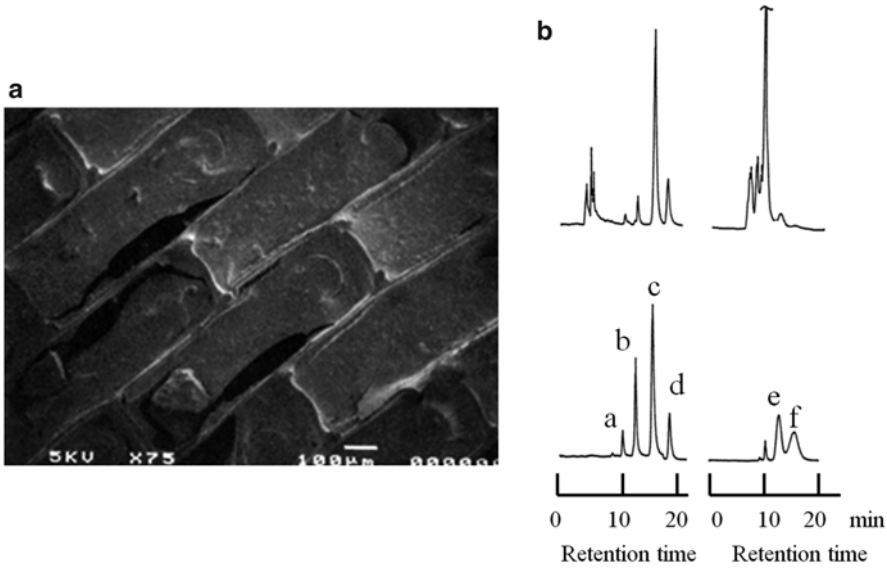


Fig. 24.5 Mossworms stripped off seaweed sample (dried brown algae) harvested in the summer of 1989, and their PSP composition. (**a**) Scanning electron microscope image of mossworm on the brown algae; scale bar 100 μm. (**b**) HPLC-FID pattern of GTX (*left*) and STX (*right*) groups from the mossworm sample, along with that of authentic PSP (*lower*). GTX standards: (*a–d*) GTX4, 1, 3, 2 STX standards: (*e, f*) neoSTX, STX

sample. The mossworm toxin was identified as PSP, which was composed mainly of GTX1, 3 and neoSTX (Fig. 24.5b). Such a toxin pattern resembled those of *A. tamarense* and the scallop collected from Funka Bay in the summer of 1989 and 1990 (Noguchi et al. unpublished data). Since the mossworm is a filter-feeding organism, this suggested that the mossworm toxin could have come from *A. tamarense* through the food chain. This is the first detection of PSP in the mossworm. It is generally accepted that bivalves are infested by ingesting PSP-producing toxic dinoflagellates. The present data, however, suggested that unknown organisms such as mossworms are involved in the toxification of bivalves and brown seaweed.

In relation to these episodes, toxicity and toxin composition in mossworms adherents to toxic bivalves from Fukue Island, Nagasaki, Japan, during 1995 and 1996 were reported (Takatani et al. 1997).

24.3 Accumulation of PSP by Non-Filter-Feeding (Non-traditional) Vectors

Bivalve mollusks are unquestionably the most serious vectors of shellfish toxins including PSP, DSP, ASP and NSP. Non-bivalve invertebrates have increasingly been documented as accumulating PSP and have been implicated in PSP incidents.

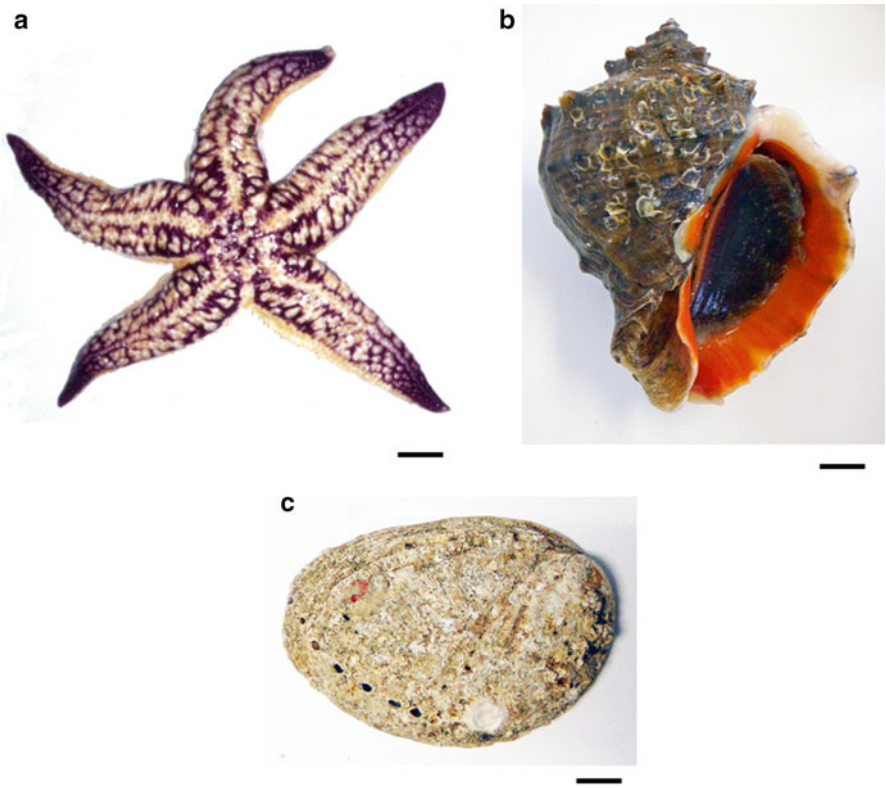


Fig. 24.6 Non-filter-feeding (non-traditional) vectors of PSP (scale bar: 1 cm). Carnivorous species: (**a**) starfish *Asterias amurensis*; (**b**) rapa whelk *Rapana venosa*. Herbivorous species: (**c**) ormer *Haliotis tuberculata*

Figure 24.6 shows marine organisms other than bivalves as vectors of PSP. On the other hand, occurrence of PSP such as dcSTX and STX in vertebrates of freshwater pufferfish as the non-filter-feeding (non-traditional) vectors of PSP was reported. In Bangladesh, two species of freshwater pufferfish, *Tetraodon cutcutia* and *Chelonodon patoca*, are known as PSP-bearing freshwater puffer. It was reported that the toxins from *T. cutcutia* were composed of STX, dcSTX, GTX2, 3, dcGTX2, 3, and three unidentified components which are possibly related to PSP (Zaman et al. 1997). They are sometimes caught mixed up with other freshwater fish, and are consumed by the local people who have little knowledge of the toxicity of pufferfish, resulting in sporadic occurrences of food-poisoning incidents, including some fatal cases. In this connection, a new component of PSP was isolated from a Bangladeshi freshwater puffer, *T. cutcutia*. Its structure was deduced to be carbamoyl-N-methylsaxitoxin based on some instrumental analyses (Zaman et al. 1998). In Cambodia, poisoning incidents from the consumption of PSP-bearing freshwater pufferfish collected from lakes and rivers are common and sometimes result in human fatalities (Ngy et al. 2008).

24.3.1 Echinoderms

The toxicity of starfish has been described in various ways for many years, but most of the reports can be explained by the presence of saponins. It has unexpectedly shown that starfish can also be toxified with PSP as described below. Sommer and Meyer (1937) reported that traces of paralytic toxin may have been present in several common invertebrates, such as starfish, limpets and chitons, which lived in close proximity to toxic mussel beds in the intertidal zone. However, no identification of PSP was made.

24.3.1.1 Case Study: Starfish *Asterias amurensis*: Fig. 24.6a

In May 1996, during surveillance on the toxicity of invertebrates such as bivalves inhabiting the coasts of Hiroshima Bay, the starfish *Asterias amurensis* collected in the estuary of the Nikoh River (Fig. 24.2) was found to contain toxins which showed strong paralytic action in mice; the maximum toxicity (as PSP) was 8.0 MU/g for the whole body and 28.7 MU/g for the viscera during the monitoring period, March to July 1996 (Fig. 24.7). Attempts were made to identify the paralytic toxins in the starfish. They were extracted with 80 % ethanol acidified with acetic acid, followed by defatting with dichloromethane. The aqueous layer obtained was treated with activated charcoal and then applied to a Sep-Pak C18 cartridge. The unbound toxic fraction was analyzed by HPLC-FLD techniques. The starfish toxin was rather unexpectedly identified as PSP. It comprised high-toxic components (GTX1, 2, 3, 4, decarbamoyl-GTX3; dcGTX3 and dcSTX) as the major components, which accounted for approximately 77 mol% of all components, along with C1-4. Of the high-toxic components, GTX1 was present in the largest amounts. It was concluded that the toxin of starfish collected in the estuary of Nikoh River in May 1996 consisted of PSP, which supposedly came via the food chain from toxic bivalves living in the same area (Asakawa et al. 1997). To our knowledge, this is the first report of the occurrence of PSP in the starfish. In addition to this, the PSP toxins contained in the starfish *A. pectinifera* were considered to be transferred from bivalves or detritus living in the same area, which were contaminated with PSP (Ito et al. 2003).

24.3.2 Gastropods

Molluscan gastropods accumulate PSP primarily acquired through predation (in many cases of PSP-infested bivalves). Besides bivalves, several carnivorous gastropods and crustaceans at high trophic levels of the food chain have been reported as PSP vectors in relation to toxic dinoflagellates blooms (Shumway 1995). Because gastropods are able to bioaccumulate high concentrations of PSP, they are

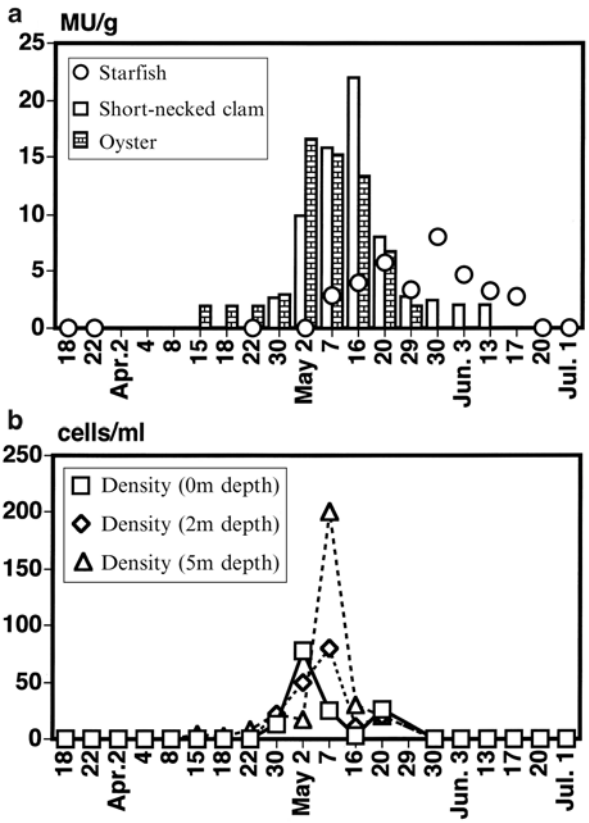


Fig. 24.7 Toxicities of the starfish *Asterias amurensis*, the short-necked clam and the oyster in the estuary of Nikoh River (**a**)* in 1996, along with maximum cell density of *Alexandrium tamarense* (**b**) in each layer (*refers to Fig. 24.2)

a significant risk to human consumers, and have been the cause of multiple fatalities. Accumulation of PSP in the bivalve and two species of gastropods in the southern part of Chile were reported (Compagnon et al. 1998). In gastropods, PSP are typically concentrated in the digestive gland but some species concentrate in the muscle tissue.

24.3.2.1 Carnivorous Gastropod

It is well known that whelks and other species of carnivorous snails prey on other mollusks, predominantly filter-feeding bivalves such as scallops, mussels, and clams. In regions where bivalves such as the scallop *Placopecten magellanicus* , surfclam *Spisula solidissima*, northern horsemussel *Modiolus modiolus* and ocean quahog *Arctica islandica* harbor PSP from the Georges Bank, secondary intoxication of carnivorous mollusks such as the northern moonsnail *Euspira heros* and the waved whelk *Buccinum undatum* poses an extra threat to public health (White et al. 1993).

24.3.2.2 Case Study: Rapa Whelk *Rapana venosa*: Fig. 24.6b

During surveillance on the toxicity of invertebrates such as bivalves inhabiting the coasts of Hiroshima Bay in 2001 and 2002, the carnivorous gastropod rapa whelk *Rapana venosa* collected in the estuary of Nikoh River was found to contain toxins which showed paralytic actions in mice; the maximum toxicities as PSP were 4.2 MU/g (May 2001) and 11.4 MU/g (April 2002). Their total toxicities were 224 and 206 MU/viscera of one specimen during the monitoring period. The viscera were extracted with 80 % ethanol acidified with acetic acid, followed by defatting with dichloromethane. The aqueous layer obtained was treated with activated charcoal and then applied to a Sep-Pak C18 cartridge. The purified gastropod toxin from the viscera analyzed by HPLC-FLD techniques was rather unexpectedly identified as PSP. It was comprised of high-toxic components (GTX3, 2, and STX) as the major components, which accounted for approximately 91 mol% of all components along with C1, 2 (Ito et al. 2004). Judging from their toxin patterns, it is suggested that the PSP toxification mechanism of the gastropod is that PSP toxins produced by phytoplankton such as *A. tamarense* are transferred to and accumulated in plankton feeders such as the short-necked clam, and are then transferred to this carnivorous rapa whelk *R. venosa* through predation.

24.3.2.3 Herbivorous Gastropod

Shellfish of Haliotidae such as abalone (other common names: ormer and perlemon) are of great commercial value and are widely cultivated in Japan and other countries. Unlike filter-feeding bivalve mollusks, these shellfish have not been supposed to be related to PSP outbreaks, because they are not plankton feeders but feed on plants that encrust rocks. So far, there has been no report on toxification of haliotis, except that an ormer, *Haliotis tuberculata*, from northwestern Spain in 1991 showed negligible toxicity of 0.78 ng STX/g meat on a mouse bioassay (Martinez et al. 1993). Subsequently, abalone in this region was affected by toxin concentrations high enough to enforce closure of the industry. In 1999, STXs were detected in abalone from two farms located along the west coast of South Africa (Picher et al. 2001).

24.3.2.4 *Case Study*: Ormer *H. tuberculata*: Fig. 24.6c

Paralytic toxicity in excess of the quarantine limit of 4 MU/g as PSP was detected in the ormer *H. tuberculata* imported from Vigo, Spain, to Japan, between January and April, 1994 (Nagashima et al. 1994, 1995). The ormer exhibited an unprecedented anatomical distribution of PSP toxicity. The anatomical distribution of PSP toxicity in six samples was examined. The foot was the most toxic in all specimens (32.9–106 MU/g), followed by the epipodium of the foot (14.3–30.9 MU/g) and mouth (11.4–33.2 MU/g), while the right shell muscle (3.2–8.2 MU/g), digestive

gland (2.0–3.7 MU/g), and viscera (4.2–15.6 MU/g) were less toxic. Ctenidium showed significant individual variation in toxicity (6.4–99.9 MU/g). The muscular tissues were highly toxic, in comparison with the visceral ones. PSPs were obtained from muscular portions of ten separate toxic specimens and analyzed by means of HPLC-FLD to compare the toxin compositions. HPLC-FLD analysis demonstrated that the ormer toxins apparently comprised members of the STX group, such as STX, neoSTX, and decarbamoylSTX (dcSTX). Regardless of the tissue, dcSTX was the principal component, accounting for 83 mol% (epipodium of foot) to 97 mol% (digestive gland) of all components. Electrospray ionization (ESI) mass spectral analysis confirmed that the principal toxin component from the Spanish ormer was dcSTX. The spectrum agreed well with that of dcSTX from a xanthid crab, *Z. aeneus* (Arakawa et al. 1994). Neither GTX group nor C toxins appeared in any of the tissues. No significant individual variation in PSP composition was observed. These results reveal toxification with PSP of a herbivorous marine gastropod, and suggest a unique metabolism of PSP in the ormer. DcSTX was the most abundant toxin reported in abalone, followed by low concentrations of STX. The source of these toxins remains unknown. The toxic dinoflagellates are the common PSP producers in this region; however, they do not display temporal or geographical distributions corresponding to that of abalone toxicity (Bravo et al. 2001). Also, the toxin profile of these potential sources differs from that of the abalone, although biotransformation may be responsible for this discrepancy. It is worth noting that no other PSP problems were reported for other mollusks or crustaceans in this region (Bravo et al. 1999).

24.3.3 Crustacea

Crabs are valued as popular seafood items and are widely consumed as many styles of human food such as boiled, steamed, or processed foods in various parts of the world. While most species are edible, some are toxic to humans and other mammals. Several cases of poisoning stemming from the ingestion of coral reef crabs and resulting in human fatalities have been reported (Alcala and Halstead 1970; Hashimoto 1979). Other than the filter feeders, several species of coral reef crabs have been known to be toxic with PSP.

In Japan, PSP-bearing toxic xanthid crabs mainly inhabit Nansei-shoto (southwestern islands off Kyushu and in the Okinawan archipelago), especially Ishigaki Island (Fig. 24.8), and they can harbor toxins in their tissues at concentrations that would be fatal to other animals. Among non-filter-feeding, non-molluscan species, STX analogs have been found most commonly in three xanthid crabs, *Zosimus aeneus* (Fig. 24.9a), *Atergatis floridus* (Fig. 24.9b) and *Platypodia granulosa*, inhabiting a tropical and subtropical area (Arakawa et al. 1994, 1995a, b; Daigo et al. 1985; Inoue et al. 1968; Koyama et al. 1981; Noguchi et al. 1969; Yasumoto et al. 1981; Sagara et al. 2009). Table 24.3 shows the toxicity of *Z. aeneus* and *A. floridus* from Ishigaki Island, Okinawa Prefecture, Japan, and/or Camotes Island,

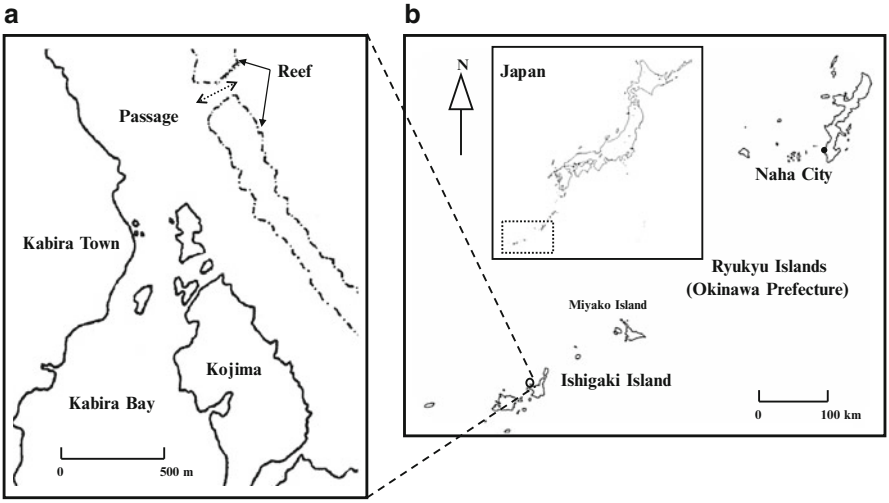


Fig. 24.8 Map showing the xanthid crab specimen collecting locations on the reef at the outside of Kabira Bay on Ishigaki Island. The location of Ishigaki Island in Okinawa Prefecture, Japan, is shown in the map to the *right* (**b**). The map on the *left* (**a**) shows an enlarged image of Kabira Bay to pinpoint the sampling location

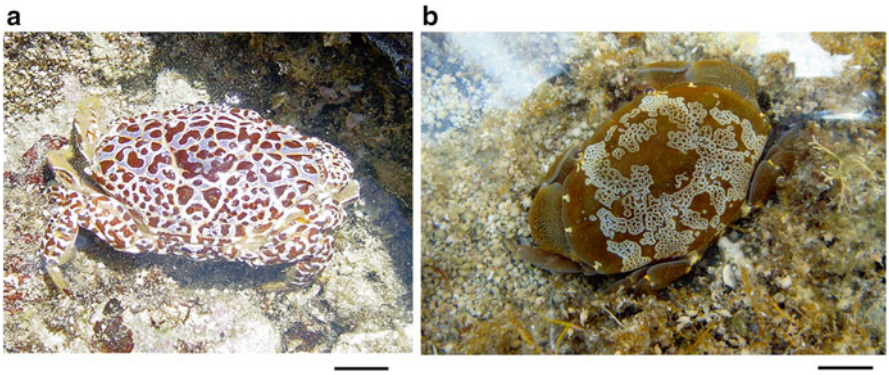


Fig. 24.9 Toxic species of xanthid crabs collected on the reef at the outside of Kabira Bay on Ishigaki Island (scale bar: 1 cm): (**a**) *Zosimus aeneus*; (**b**) *Atergatis floridus*

Central Visayas, the Philippines (Fig. 24.9; Asakawa et al. 2014). The highest average toxicity is 1777 MU/g muscle in *Z. aeneus* and 4641 MU/g muscle in *A. floridus*. These mean that only 2 g of such muscle is equivalent to more than 3000 MU estimated as the MLD of PSP in human.

In the Philippines, sporadic outbreaks of crab, cone shell, and fish poisoning are known to occur. In Negros Island, central Visayas, the Philippines, human fatalities resulting from ingestion of the xanthid crabs *Z. aeneus*, *A. floridus*, *Lophozozymus pictor*, *Demania toxica,* and *D. alcalai* (also known as a synonym of *D. cultripes*)

Table 24.3 Toxicity of two species of xanthid crab

Date of collection		Number of specimens	Body weight (g) (average ± SD)	Toxicity (MU/g): average ± SD			
				Carapace	Viscera	Appendage	Muscle
(A) *Zosimus aeneus* from Ishigaki Island in Okinawa Prefecture, Japan							
2007	June 2	3	62 ± 57	NT	62 ± 41	338 ± 189	NT
	June 3	3	13 ± 1	NT	89 ± 41	306 ± 36	NT
2009	June 18	4	49 ± 24	211 ± 130	574 ± 47	646 ± 533	1777 ± 1091
(B) *Atergatis floridus*							
b-1. Ishigaki Island in Okinawa Prefecture, Japan							
2007	June 2	3	30 ± 3	NT	253 ± 203	113 ± 115	NT
	June 3	3	18 ± 8	NT	64 ± 41	88 ± 40	NT
2009	June 18	3	20 ± 5	183 ± 47	484 ± 255	503 ± 179	1408 ± 404
	June 19	3	23 ± 4	807 ± 693	532 ± 200	856 ± 700	2538 ± 2077
2010	June 25	3	23 ± 1	332 ± 49	654 ± 137	1257 ± 607	4641 ± 972
b-2. Camotes Island in Central Visayas, the Philippines							
2009	August 10	5	11 ± 3	NT	106 ± 56	221 ± 189	719 ± 349

NT not tested, *Av* average, *SD* standard deviation

have been reported (Alcala 1983; Gonzales and Alcala 1977). In both *L. Pictor* and *D. alcalai*, when ingested, in Negros Island, the toxin was identified as palytoxin (PTX), a highly lethal toxin of the zoanthid species of the genus *Palythoa* (Yasumoto et al. 1986). In Camotes Island, human cases of crab intoxication have been reported (Carumbana et al. 1976).

24.3.3.1 Case Study in the Philippines

In April 2004, food-poisoning incidents with two deaths due to ingestion of unidentified crabs resembling the shape of *Demania* sp. in the town of Carmen, eastern coast in Cebu Island (Visayas region, Central Philippines) was reported by Professor G.G. Delan, one of our authors (personal communication). Relatives of victims and many fishermen in villages along the seashore provided information regarding these incidents. About 20 min after the meal, two men (aged 55 and 62 years) who ate the "crab" soup complained of dizziness, nausea, numbness of the tongue, anesthesia of the mouth, and vigorous vomiting. Subsequently, they fell down and died after 2 h, due to respiratory distress. Toxicity and associated toxins found in xanthid crabs that inhabit areas surrounding Cebu Island remain to be identified. Therefore, the crabs responsible for this food poisoning were collected and assessed for their toxicity. Figure 24.10 shows sampling locations in Cebu Island. In coral reefs along the town of Carmen, eastern coast in Cebu Island faced with the Camotes Sea, a total of seven xanthid crab specimens were collected by fishermen using crab cages during the period from February to August 2006. Specimens were identified as the xanthid crab *D. cultripes* by Dr. M. Shimomura of the Kitakyushu Museum of Natural

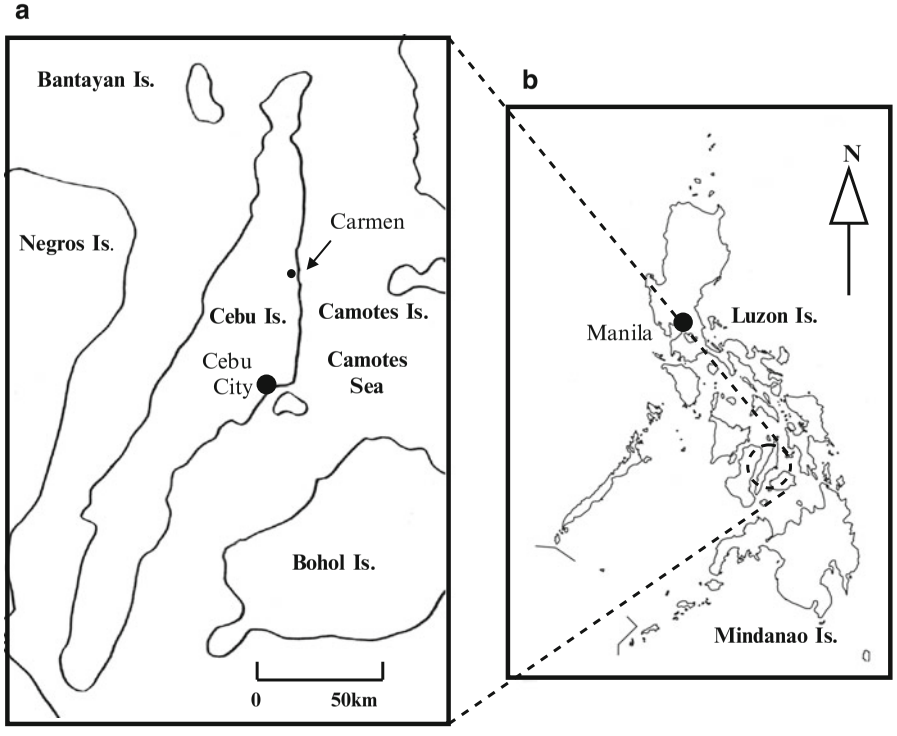


Fig. 24.10 Map showing the xanthid crab specimen collecting locations on Camotes Island and Camotes Sea near Cebu Island. The location of Cebu Island in the Philippines is shown on the map to the *right* (**b**). The map on the *left* (**a**) shows an enlarged image of the Central Visayas Region of the Philippines

History & Human History (Fig. 24.11). The genus *Demania*, which belongs to the Xanthidae family, currently contains 17 species (Ng et al. 2008). Distribution of four species, *D. cultripes* (synonym of *D. alcalai*), *D. reynaudii*, *D. scaberrima*, and *D. toxica*, in the Philippines is now confirmed. All seven crab specimens responsible for the food poisoning were identified as the xanthid crab *D. cultripes*. Following identification, specimens were dissected into viscera and appendages to determine the anatomical distribution of toxins. Screening test of crabs disclosed the presence of paralytic toxins in all seven specimens tested. The results of toxicity tests are summarized in Table 24.4. The average weight of a crab specimen was 135.1 ± 65.0 g (mean ± SD)/whole body. Symptoms observed in injected mice were characteristic of those due to toxicity of TTX or PSP. After 2–3 min, the respiration of mice injected intraperitoneally with a lethal dose of the toxin extract from *D. cultripes* became labored and irregular. The mice became inactive and showed ataxia within 10 min after injection. Death resulting from respiratory failure occurred in less than 25 min after injection. It became clear that all these seven specimens of *D. cultripes* tested in these experiments were toxic irrespective of their collection time. TTX toxicity scores for their viscera and appendages were 18.2 ± 16.0 and 4.4 ± 2.6 MU/g, respectively although there were individual variations in toxicity. The frequency of toxic samples was 100 %, and individual toxicities showed variations ranging from 2.7 to 52.1 MU/g. The highest recorded scores of TTX toxicity for viscera and appendages were 52.1 and 7.7 MU/g, respectively. It appeared that in the *D. cultripes*, the toxicity of the viscera is greater than that of the appendages. Our results indicate that *D. cultripes* from the Camotes Sea is a species with lower toxicity in comparison with those of other xanthid crab such as *Z. aeneus* and *A. floridus*, with the highest score of 16,500 MU/g as PSP being reported (Daigo et al. 1985; Koyama et al. 1981; Noguchi et al. 1969). Nevertheless, people in

Fig. 24.11 Toxic species of xanthid crab *Demania cultripes*, which contained PSP and TTX, collected from the Camotes Sea near Cebu Island (scale bar = 1.0 cm)

Table 24.4 Toxicity of xanthid crab *Demania cultripes* from Cebu Island in the Philippines (2006)

No.	Month of collection	Weight (g)	Toxicity (MU/g) as TTX	
			Viscera	Appendage
1	February	73.3	24.1	3.9
2	May	193.3	10.9	2.7
3	July	162.6	52.1	6.7
4	July	30.9	12.1	7.7
5	August	108.9	5.6	ND
6	August	180.0	11.9	5.2
7	August	197.0	10.6	4.5
Average ± SD		135 ± 65	18.2 ± 16.0	4.4 ± 2.6

ND not detected: less than 2 MU/g
Av. average, *SD* standard deviation

coastal areas should be warned of the potential hazard of this crab in order to prevent its intentional or accidental consumption. The toxin partially purified from viscera of *D. cultripes* showed a clear peak in HPLC-FLD chromatograms corresponding to the retention time of standard TTX and PSP. In HPLC analysis of TTX, the toxin revealed three peaks with retention times of 18, 20 and 22 min, which were similar to those of TTX, 4*epi*-TTX and 4, 9-anhydroTTX, respectively. Furthermore, the GC-MS method (Asakawa et al. 2000) revealed that this toxin has a C9 base, which is a common chemical basic structure of TTX and its analogs. Therefore, from symptoms observed in mice and the results of HPLC-FLD and GC-MS analysis, it can be concluded that *D. cultripes* toxin is a mixture of TTX and TTX derivatives. TTX was the main toxic principle, because its relative ratio to the total toxicity was as high as 90 %. On the other hand, the results of HPLC-FLD analysis for PSP components are shown in Fig. 24.11. For GTX analysis, the two main peaks were identified as GTX1 and GTX3. The other one small peak near GTX1 was regarded as GTX4. In addition to this, GTX2 was detected as a trace component on the chromatogram. Analysis of STXs showed one large peak for hydroxySTX (hySTX) with a retention time of about 35 min (Fig.24.12b). In all the chromatographic experiments, the toxins of *D. cultripes* from Cebu Island were indistinguishable from TTX and PSP. Judging from the symptoms of the patients and the results of instrumental analysis, it seems most probable that the causative food responsible for the poisoning with two deaths in April 2004, mentioned above, was *D. cultripes* containing TTX and PSP. From the food hygiene point of view, people in coastal areas should be warned of the potential hazard of this crab in order to prevent its intentional or accidental consumption. In addition to this, taking the estimated fatal minimum oral dosage of TTX for humans as being 10,000 MU (Noguchi and Ebesu 2001) and variation of the toxicity in the viscera into consideration, *D. cultripes* is a toxic species and must not be consumed.

It was previously reported that TTX is the major toxin, and GTX 1 and 3 are minor toxins in the xanthid crab *L. pictor* from Taiwan (Tsai et al. 1995). In contrast, in crabs from Negros Island, the Philippines, PTX was found to be the pre-

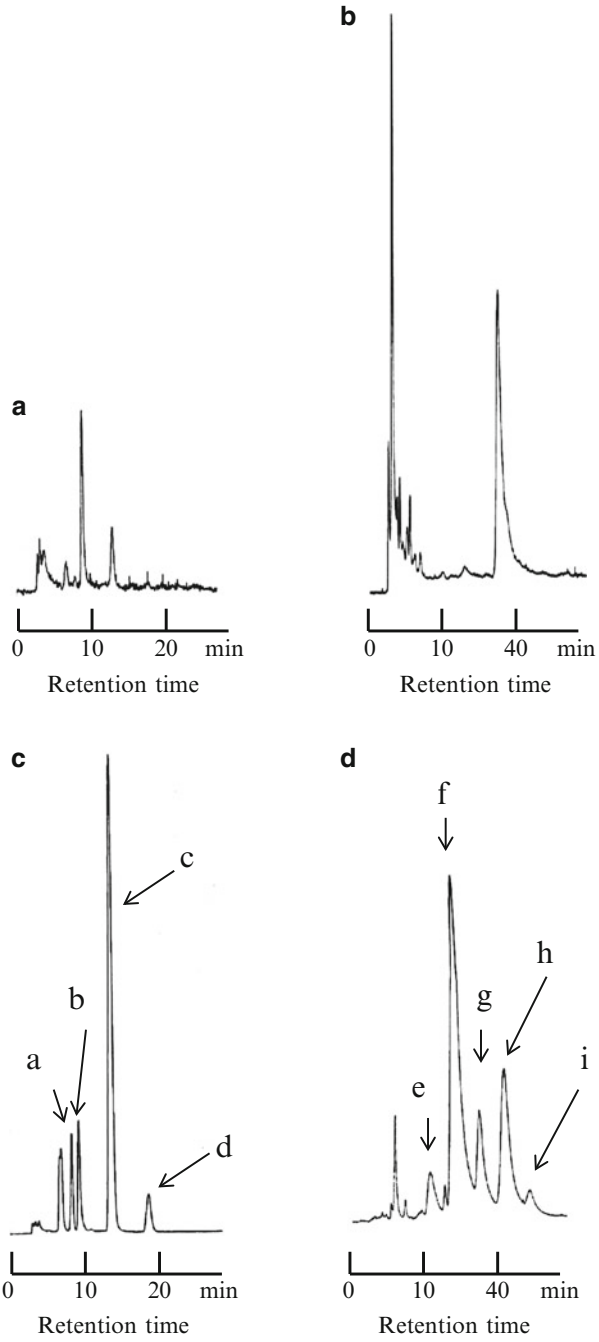


Fig. 24.12 HPLC-FLD analysis of PSP components contained in toxic species of the xanthid crab *Demania cultripes* (**a**). (**b**) GTXs and STXs analysis of viscera. (**c**) GTXs std (*a*: GTX4, *b*: GTX1, *c*: GTX3, *d*: GTX2). (**d**) STXs std. (*e*: hyneoSTX, *f*: neoSTX, *g*: hySTX, *h*: dcSTX, *i*: STX)

dominant toxin (Yasumoto et al. 1986). TTX and PSP are the major and minor toxins, respectively, in *A. floridus* inhabiting Miura Peninsula, Kanagawa Prefecture, Japan, which differs from crabs found in Okinawa, Japan (Noguchi et al. 1983c). The *A. floridus* specimen from Fiji Island was found to have STX and its derivatives as the major toxins (Raj et al. 1983).

It is not clear at present whether the toxin in our crab specimens is of endogenous or exogenous origin. Because crabs are generally detritus (and not planktonic) feeders, the most plausible explanation is that crab specimens accumulated the toxin by feeding on toxic marine organisms in sampling areas (Kotaki et al. 1983; Yasumoto et al. 1983). In order to elucidate the diet of *D. cultripes*, microscopic examination of stomach contents of the species is needed. Further studies are now in progress to elucidate the associated mechanism of toxicity. In addition, investigation of individual, local, and size-dependent variations in toxicity of *D. cultripes* is also needed. Because *D. cultripes* specimens are large enough to be regarded as food items, the potential danger of their consumption should be disseminated to the public to prevent future cases of food poisonings.

To our knowledge, this is the first evidence of occurrence of TTX and PSP in *D. cultripes* inhabiting the coast of Cebu Island and confirmation of its implication in human poisoning. In this connection, paralytic toxin profiles of the xanthid crab *A. floridus* collected on reefs of Ishigaki Island, Okinawa Prefecture, Japan, and Camotes Island, Cebu Province, the Philippines, were examined (Asakawa et al. 2014). On the other hand, it was also reported that PSP-containing *A. floridus* maintains a fairly high toxicity level for a long period when fed nontoxic diets (Noguchi et al. personal communication). This observation may suggest that PSP in this toxic crab mentioned above is endogenous. In addition to this, it may also suggest that the crabs have the PSP and/or TTX-specific binding substance. Through a complex system of trophic interrelationships, non-filter-feeding organisms can also be exposed to PSP and/or TTX and thus accumulate and play a role as vectors in marine food web. Oikawa et al. (2002, 2004, 2005) showed the presence of PSP toxins in the viscera of the edible shore crab *Telmessus acutidens*. It was also revealed that the total toxicity in the crab viscera increased linearly with the amount of toxic mussels the crabs ingested by feeding experiments. In this connection, secondary intoxication with PSP in New England rock crabs, *Cancer irroratus*, was reported (Thomas 1979).

24.4 Conclusion

PSP produced by toxic dinoflagellates is transferred and bioaccumulated throughout aquatic food webs, and can be vectored to terrestrial biota, including humans. PSP, which comprises STX and its related compounds, is responsible for the sometimes fatal toxic seafood related syndromes. PSP poisonings typically result from consumption of filter-feeding molluscan shellfish that concentrate toxins from bloom-forming microalgae—mainly marine dinoflagellates and freshwater or

brackish cyanobacteria. In spite of the wide spread of HAB and numerous poisoning cases of PSP, successful implementation of the monitoring programs for both PSP-producing microalgae and PSP contamination in shellfish in many countries has helped to minimize public health risks. As the occurrence of several new non-filter-feeding (non-traditional) vectors of PSP other than bivalves and secondary intoxication of edible gastropod are huge problems from the view point of food hygiene and public health as well as fisheries, legislation should be adjusted to extend the monitoring of marine biotoxins to a wider range of species besides commercially important edible bivalves. Actually, in April 2004, the Ministry of Health, Labour and Welfare in Japan set a regulation limit for carnivorous and scavenging species. Consequently, dealing in crabs over 4 MU/g in hepatopancreas was prohibited by the Food Sanitation Law in Japan. With the apparent expansion in PSP-producing microorganisms worldwide, an ever-increasing demand for seafood, and the emergence of seafood as an economic commodity for export, particularly in developing countries, more study is required on PSP sources, distribution, and fate in these non-traditional PSP vectors to ensure both public safety and consumer confidence on local, national, and international scales.

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Chapter 25
Interactions Between Harmful Algae and Algicidal and Growth-Inhibiting Bacteria Associated with Seaweeds and Seagrasses

Ichiro Imai

Abstract Phytoplankton and bacteria are the main components of marine ecosystems and hence comprise abundant numbers and biomass in the sea. There exist close and diverse interactions between phytoplankton and bacteria in marine ecosystems. Among these relationships, bacteria possessing algicidal and growth-inhibiting activities against phytoplankton have been increasingly gathering attention and are expected to control phytoplankton dynamics especially harmful algal blooms in coastal environments. Algicidal activities are divided into two types, i.e., the direct attack type, needing direct attachment to host algal cells, and the indirect attack type, producing algicidal matter. Some bacteria kill part of algal populations, and they are growth-inhibiting bacteria. In harmful algal blooms (HABs) of such as the raphidophytes *Heterosigma akashiwo* and *Chattonella* spp., bacteria play an important role in the bloom terminations. Most algicidal bacteria are particle-associated forms in coastal seas. A new finding is that huge numbers of algicidal bacteria against HAB species (dinoflagellates and raphidophytes) were attached to the surface of seaweeds such as the green alga *Ulva pertusa,* the red alga *Gelidium* spp. and the brown algae *Sargassum muticum* and *S. thunbergii*. The densities of these algicidal bacteria reached as many as 10^6 g^{-1} wet weight. Further, algicidal bacteria were found in the biofilm of the seagrass *Zostera marina* with high densities of 10^7 g^{-1} wet blade or more. In the case of the toxic dinoflagellate *Alexandrium tamarense*, growth-inhibiting bacteria with strong activities were isolated from the blade of *Z. marina*. In developed countries, seagrass- and seaweed beds have been lost by reclamation for increasing land of commercial uses. We here propose restoration of seagrass- and seaweed beds, not only for increasing the nursery grounds of various marine lives but also for creating preventative strategies for HAB occurrences. This is a kind of activity in conformity with the Sato-Umi concept recently proposed.

I. Imai (✉)
Graduate School of Fisheries Sciences, Hokkaido University,
3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan
e-mail: imai1ro@fish.hokudai.ac.jp

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Abbreviation

AB	Algicidal bacteria
FLB	Free-living bacteria
GIB	Growth-inhibiting bacteria
HA	Harmful algae
HAB	Harmful algal bloom
MPN	Most probable number
PAB	Particle-associated bacteria

25.1 Introduction

In marine ecosystems, planktonic microalgae (phytoplankton) and bacteria are the most numerous and abundant components. Bacteria may stimulate or inhibit phytoplankton growth through nutrient regeneration, endosymbiosis, and production of stimulatory or inhibitory substances (Cole 1982; Riquelme and Ishida 1989), and may kill in extreme cases (Ishio et al. 1989; Sakata 1990; Furuki and Kobayashi 1991; Sakata et al. 1991; Imai et al. 1991, 1993, 1995; Fukami et al. 1992). Since harmful algal blooms (HABs) have occurred and caused huge fishery damage to aquaculture industries through mass mortality of caged fish by noxious red tides and toxin contamination of bivalves by toxic blooms, especially in temperate coastal waters, those bacteria possessing killing activities against phytoplankton have gathered attention as possible terminators of HABs (Ishida 1994; Imai et al. 1998a; Doucette et al. 1998; Skerratt et al. 2002; Mayali and Azam 2004; Sohn et al. 2004; Nakashima et al. 2006; Imai and Kimura 2008; Liu et al. 2008; Mayali et al. 2008; Roth et al. 2008; Kim et al. 2009; Park et al. 2010; Bai et al. 2011; Inaba et al. 2014; Onishi et al. 2014).

In freshwater ecosystems, algal-lysing agents (viruses, bacteria, protists, etc.) have traditionally been studied by using a soft-agar overlayer technique, utilizing microalgae to make a lawn on agar plates (Safferman and Morris 1963; Daft et al. 1975; Yamamoto 1978; Manage et al. 2000; Kim et al. 2008a; Yang et al. 2013), and their roles have been discussed. On the other hand, there are few species of marine phytoplankton, especially those of harmful flagellates, capable of growing and making a lawn on agar plates, and algal-lysing or killing microorganisms have not been investigated as well as freshwater microalgae. After the development of a method for detecting microorganisms that kill and/or inhibit the growth of marine

HAB species which cannot grow on agar plates by applying the most probable number method (MPN method), many strains of bacteria showing activities of killing and/or growth inhibition of host microalgae have been isolated and reported. These bacteria active against microalgae had no distinct names (Baker and Herson 1978; Ishio et al. 1989; Furuki and Kobayashi 1991) before addressing those bacteria as algicidal bacteria (Sakata 1990; Sakata et al. 1991; Imai et al. 1991, 1993). The term "algicidal bacteria" is widely used in related papers published nowadays.

Papers on algicidal bacteria and growth-inhibiting bacteria have increasingly been published in recent years, and the definition of algicidal bacteria (and growth-inhibiting bacteria) in those papers has sometimes appeared to be rather obscure. For example, the bacterium *Pseudoalteromonas* strain SP48 barely killed the toxic dinoflagellate *Alexandrium tamarense* with the initial addition of 9×10^6 cells ml^{-1} or more (Su et al. 2007), and the growth of the killer dinoflagellate *Pfiesteria piscicida* was reported to be inhibited with the inoculation of a bacterial density of about 10^8 cells ml^{-1} (Hare et al. 2005). Added bacterial densities are unrealistic in coastal sea environments where the total bacterial concentrations are usually between 10^6 and 10^7 cells ml^{-1} (van Es and Meyer-Reil 1982; Imai 1989), and the algicidal activities shown at these impractically high bacterial densities need reconsideration as artifact phenomena. I would like to define that algicidal bacteria kill phytoplankton with inoculation cell densities realistic in the sea and grow using the organic matter derived from killed phytoplankton cells.

25.2 Characteristics of Algicidal Bacteria

Algal-lysing agents such as viruses and bacteria have been well investigated in freshwater ecosystems (lakes and ponds) since the 1960s or earlier, and it is strongly suggested that these agents play an important role in terminating blooms of cyanobacteria and others (Safferman and Morris 1963; Daft et al. 1975). Therefore, it is easily supposed that similar microbial agents exist in marine coastal ecosystems and that those agents also terminate harmful algal blooms at the final stage. The Fisheries Agency of Japan supported these investigations during the 1990s for about 10 years, and rather many algicidal bacteria (AB) and growth-inhibiting bacteria (GIB) were actually isolated from Japanese coastal waters. Analyses of small subunit ribosomal DNA (SSU rDNA) revealed that common ABs and GIBs are usually Gram-negatives belonging to the alpha- and gamma-proteobacteria (mainly the genera *Alteromonas*, *Pseudoalteromonas, Pseudomonas* and *Vibrio*) or the phylum Bacteroides (mainly the genera *Cytophaga* and *Saprospira*) (Yoshinaga et al. 1998; Salomon and Imai 2006; Imai 2011).

Figure 25.1 shows an example of algicidal activities against three phytoplankton species by the bacterium *Alteromonsa* sp. strain S (Imai et al. 1995). Co-culture was made for 3 days after the addition of bacterial cells with densities of about 10^3 cells ml^{-1}, and all the phytoplankton cells were entirely killed. Cells of naked species of

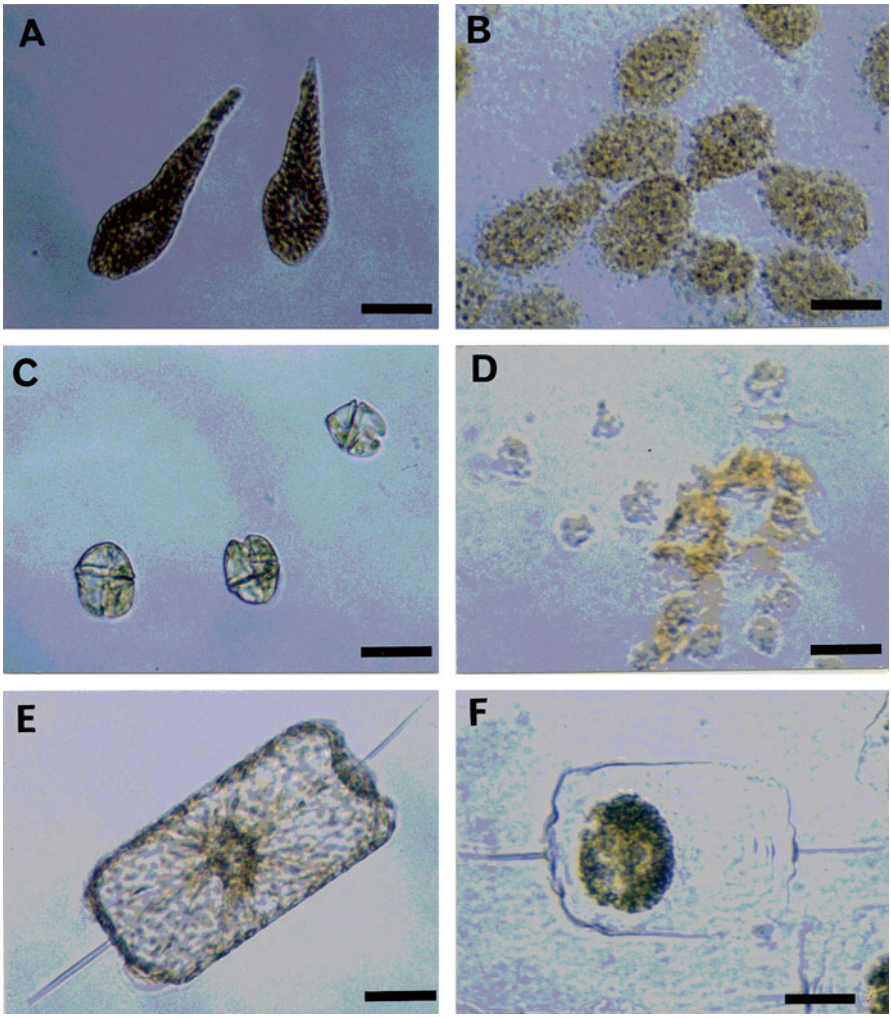

Fig. 25.1 Microscopic observations on the algicidal activities of the bacterium *Alteromonas* strain S against three marine phytoplankton species (Imai et al. 1995). Co-culture was made for three days. *Bars*: 30 μm. (**a**) Live cells of *Chattonella antiqua* (Raphidophyceae). (**b**) Burst cells of *C. antiqua* killed by the bacterium. (**c**) Live cells of *Karenia mikimotoi* (Dinophyceae). (**d**) Dead and burst cells of *K. mikimotoi* killed by the bacterium. (**e**) Live cell of *Ditylum brightwellii* (Bacillariophyceae). (**f**) Dead cell of *D. brightwellii* killed by the bacterium

the raphidophyte *Chattonella antiqua* and the dinoflagellate *Karenia mikimotoi* were destroyed and burst. The bacterium also killed the diatom *Ditylum brightwellii,* but it could not lyse frustules. In the case of the activities of GIBs against *C. antiqua*, two types of growth-inhibiting activities were observed (Inaba et al. 2014). The first is a change of the cells into a roundish form from the normal spindle-shaped

vegetative cell morphology of *C. antiqua*. This altered morphology resulted in a loss of motility and sinking of those cells to the bottom of culture vessels within a week. This eventually caused the death of *C. antiqua*. The second type of inhibition was an elongation of the vegetative cells that was caused by the delay of cell division and also reduced its motility. Elongated *C. antiqua* cells lost their ability to swim straight and rotated around at the bottom of culture plate wells.

The gliding bacteria such as the genera *Cytophaga* and *Saprospira* are generally direct attack type, and those of alpha- and gamma-proteobacteria and firmicutes are the types for extracellular production of algicidal matter (Sakata et al. 1991; Imai et al. 1993, 1995; Kondo and Imai 2001; Skerratt et al. 2002). Concerning the prey specificity, some bacteria kill only one algal species, others kill multiple algal species, and still others kill different algal species from several groups (Mayali and Azam 2004; Park et al. 2010; Inaba et al. 2014). Direct attack-type bacteria tend to prey upon algal species of wide range.

An interesting role of dissolved organic matter was demonstrated in *Alteromonas* E401, the killer of the harmful dinoflagellate *Karenia mikimotoi* (Yoshinaga et al. 1995). The bacterium strain E401 produced a high molecular weight (>10 kD) heat-labile compound showing algicidal activity. This killing substance was specifically produced in response to excreted organic matter (EOM) from *K. mikimotoi*. The algicidal activity was restricted to *K. mikimotoi* and *Gymnodinium catenatum*, but gave no effects on other dinoflagellates, diatoms or raphidophytes examined. This is also a case for experiments using raphidophyte *Heterosigma akashiwo*. If this is frequent in the sea, species-specific algicidal activity against blooming algal species is a common phenomenon induced by the EOM from each microalgal species.

Ectoenzymes, particularly ectoproteases, are the likely candidates for algicidal organic matter excreted by algicidal bacteria. Mitsutani et al. (2001) found that a stationary culture cell extract of *Pseudoalteromonas* A25 showed both algicidal and high protease activities. At least some algicidal bacteria might kill prey algae by using some kinds of proteases. As other algicidal substances, Isatin and red pigments of prodigiosins were identified from marine algicidal bacteria (Nakashima et al. 2006; Kim et al. 2008b; Sakata et al. 2011).

Skerratt et al. (2002) showed that Gram-negative algicidal bacteria appear to use the AI-2 mechanism (quorum sensing) at the mid- to late stage of the log growth phase. The AI-2 mechanism has been thought to be for bacteria communicating between species rather than acetylated homoserinelactones (AHLs). There is a metabolic benefit for these bioactive mechanisms to be activated simultaneously rather than individually.

Imai et al. (1995) observed the swarming of the bacterium *Alteromonas* S to the valve face of the diatom *Ditylum brightwellii* in the early stage of algicidal attack (Fig. 25.2). The swarming capacity to prey algal cells is thought to be an advantageous strategy for algicidal bacteria. Skerratt et al. (2002) also reported the swarming of algicidal bacteria. Swarming is presumably a result of chemotaxis. It is of interest to reveal whether algicidal activities relate to swarming and quorum sensing.

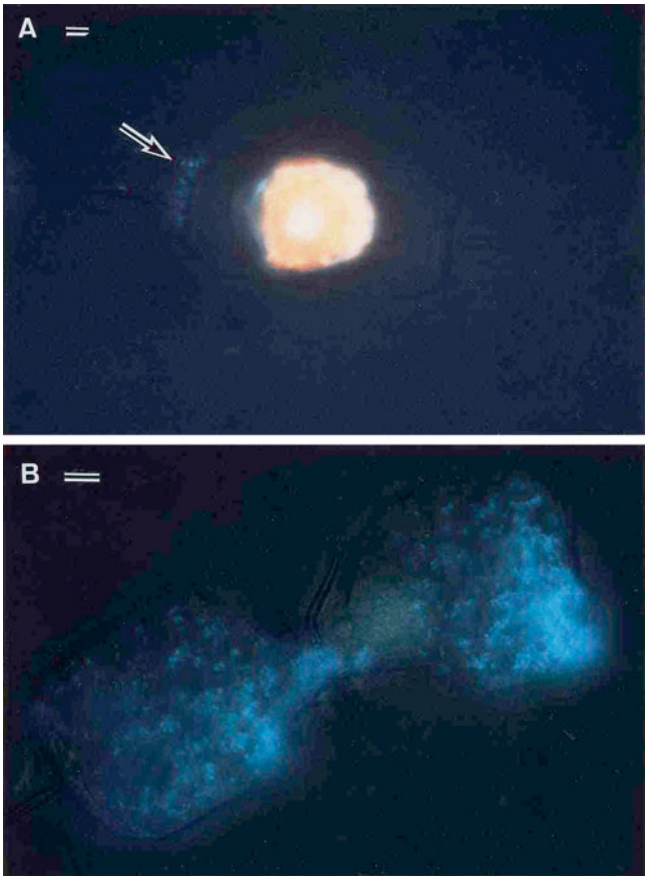

Fig. 25.2 Epifluorescence micrographs of the bacterium *Alteromonas* strain S attacking the diatom *Ditylum brightwellii* (Imai et al. 1995). Bacterial cells are seen as fluorescing spots. *Bars*: 10 μm. (**a**) Cell of *D. brightwellii* and the bacterial cells (*indicated by an arrow*) gathering to a valve face of the diatom. Co-culture was made for 1 day. (**b**) Killed diatom cells attacked by the bacterium. Numerous bacterial cells are seen inside the diatom cell. Co-culture was made for 2 days

25.3 Ecological Relationship Between Algicidal Bacteria and Harmful Algae in Coastal Seas

In northern Hiroshima Bay, the western Seto Inland Sea, the dynamics of the *Heterosigma akashiwo* (Raphidophyceae) killer bacteria revealed a close relationship with that of *H. akashiwo* populations (Imai et al. 1998a; Kim et al. 1998). *H. akashiwo* killers followed the increase of *H. akashiwo* cells, reached a maximal level after the beginning of decline of *H. akashiwo*, maintained a high level for at least 1 week after the crash of the bloom, and then decreased (Fig. 25.3). Similar results were reported between the dynamics of the raphidophytes *Fibrocapsa japonica* and *Chattonella*

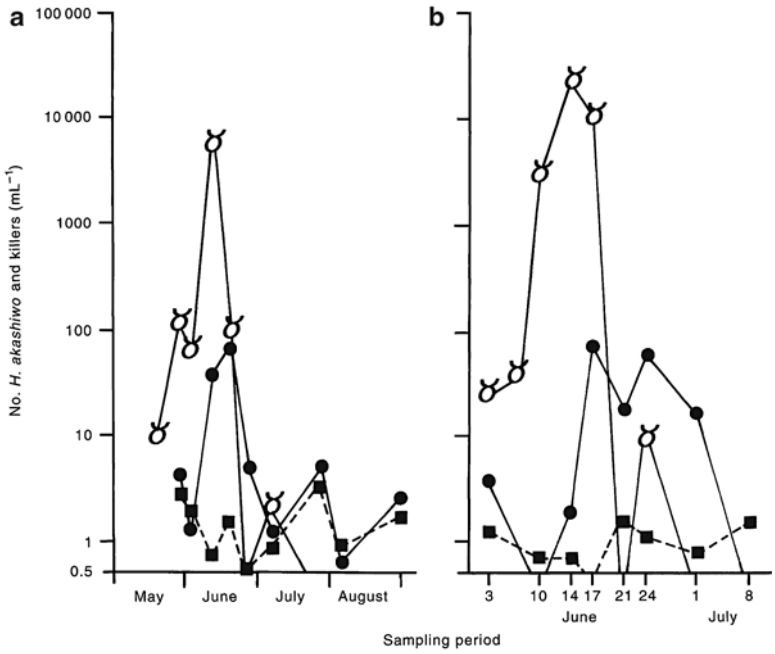

Fig. 25.3 Fluctuations in densities of *Heterosigma akashiwo*, H-killers (*closed circles*: algicidal micro-organisms against *H. akashiwo*) and C-killers (*closed squares*: algicidal micro-organisms against *Chattonella antiqua*) in the surface waters collected at a station in northern Hiroshima Bay (**a**) from May 28 to August 31, 1992; and (**b**) from June 3 to July 8, 1993 (Imai et al. 1998a)

subsalsa and that of the killers of *F. japonica* and *C. subsalsa* in shallow brackish detention ponds in South Carolina, USA (Liu et al. 2008).

The population structure of algicidal bacteria was also determined by restriction fragment length polymorphism (RFLP) analysis of the bacterial 16S rRNA genes during *H. akashiwo* red tides there (Yoshinaga et al. 1998). Bacteria belonging to gamma-proteobacteria were the dominant algicidal agents during the termination period of red tides followed by the strains of the phylum Bacteroides in 1994 and 1995. This result clearly indicates the specific group of algicidal bacteria (gamma-proteobacteria) associated with the termination of *H. akashiwo* red tides in the coastal areas such as Hiroshima Bay.

In Harima-Nada, the eastern Seto Inland Sea, the cell density of algicidal *Cytophaga* sp. J18/M01 (originally isolated from Harima-Nada) increased just after the peak of a small bloom of *Chattonella* spp. in the summers of 1997 (Fig. 25.4). This bacterium has a wide prey range (Imai et al. 1993) and also showed a close relationship with the change of the total microalgal biomass including diatoms and others (Imai et al. 2001). *Cytophaga* sp. J18/M01 (originally isolated from Harima-Nada in the summer of 1990) is the same species as *Cytophaga* sp. AA8-2 and AA8-3, which were originally isolated from Ago Bay in the summer of 1995 (Imai et al. 1999; Kondo et al. 1999). The algicidal bacterium *Kordia algicida* OT-1 with strong activity was also isolated from Korean costal water (Sohn et al. 2004), and this bacterial

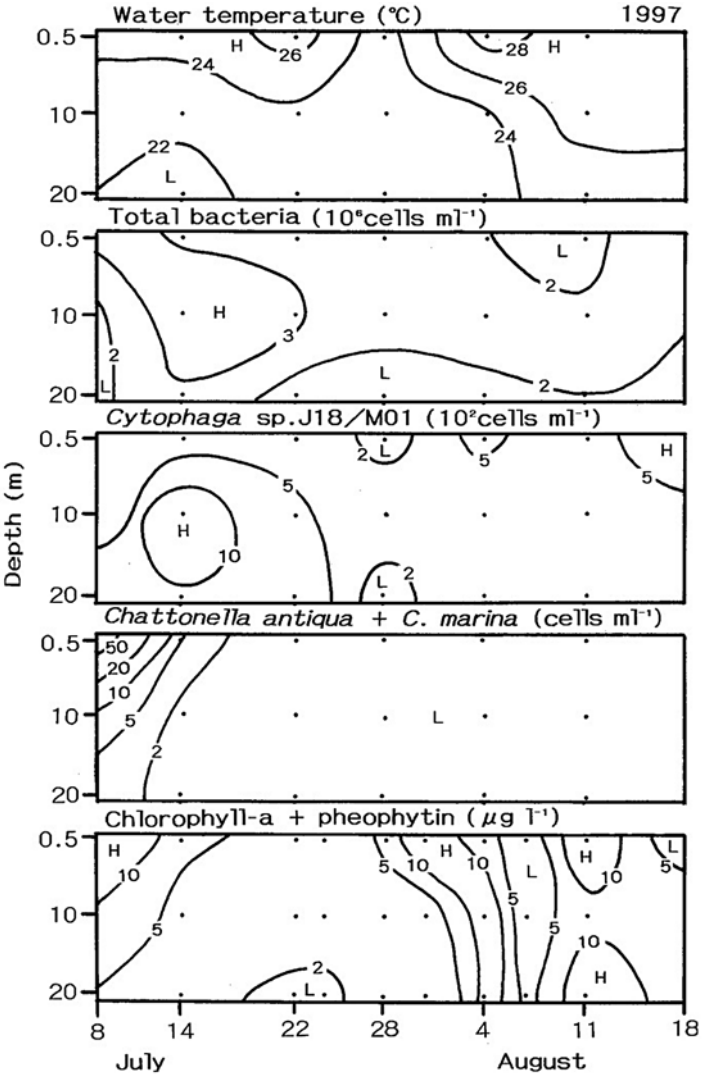

Fig. 25.4 Changes in vertical profiles of water temperature, total bacteria, algicidal bacterium *Cytophaga* sp. J18/M01, *Chattonella* spp. (*C. antiqua* and *C. marina*), and microalgal biomass (chlorophyll a and pheophytin) at the NH3 station in northern Harima-Nada, the eastern Seto Inland Sea, during the summer of 1997 (Imai et al. 2001)

strain has the same base-pair sequence of a 16 s rRNA gene to *Cytophaga* sp. AA8-2 and AA8-3 (Mayali 2007). Hence this algicidal *Cytophaga* species shows wide distribution in coastal areas of Japan and Korea. Yooseph et al. (2010) investigated the sequences of 197 diverse marine genomes collected from around the world along with previously published marine prokaryotic genomes in the context of marine

metagenomic data. It is interesting to notice that the bacterium *K. algicidal* (hence *Cytophaga* sp. AA8-2 and AA8-3) was detected from almost all samples from the global ocean. The algicidal bacterium *Cytophaga* sp. J18/M01 and the same species clones are consequently thought to be globally distributed both in the ocean and in the coastal seas of the world. It is hence considered that algicidal processes by bacteria are not peculiar phenomena in marine environments as previously expected.

Bacterial populations were fractionated into two groups (PABs and FLBs) by the filtration of water samples through a 3 μm pore filter, and algicidal activities and/or growth-inhibiting activities against HAB species were checked for each isolated bacterial strain of both fractions collected in the Yatsushiro Sea and Harima-Nada. The results clearly demonstrated that algicidal bacteria were more abundantly found in the fraction of PAB (particle-associated bacteria) (Imai et al. 2009a; Park et al. 2010; Inaba et al. 2014).

In general, macroaggregates are heavily colonized by bacteria as compared with the surrounding water, and are important for biochemical processes as "hot spots" (Simon et al. 2002; Azam and Malfatti 2007). It is interesting to note that the *Cytophaga* and gamma-proteobacteria groups are among the dominant bacterial flora attached to macroaggregates or marine snow (Doucette et al. 1998; Azam and Malfatti 2007). Swarming was observed in the algicidal process by *Alteromonas* sp. and *Cytophaga* sp. (Imai et al. 1995; Skerratt et al. 2002). When a small number of algicidal bacteria cells aggregated around a single microalgal cell or on a macroaggregate, such microscale patchiness could create an algicidal hot spot in the sea. These hot spots are thought to be important in the ecology of algicidal bacteria (Doucette et al. 1998; Imai et al. 1998a, b; Mayali and Azam 2004).

25.4 Seaweed Beds as Prevention Strategies for Red Tides

A new aspect of the ecology of algicidal bacteria is the discovery that huge numbers of algicidal bacteria attach onto the surface of seaweeds such as *Ulva* sp. (Chlorophyceae), *Gelidium* sp. (Rhodophyceae), *Sargassum muticum* and *Sargassum thunbergii* (both Phaeophyceae) (Imai et al. 2002). The abundance of algicidal bacteria on these macroalgae was determined with the MPN method (Imai et al. 1998b) on the coast of Osaka Bay in the Seto Inland Sea, and the maximum numbers of about 10^5–10^6 g^{-1} (wet weight) were detected for *Karenia mikimotoi, Fibrocapsa japonica* (Raphidophyceae), and *Heterosigma akashiwo*. Algicidal bacteria were also abundant (occasionally > 10^3 MPN ml^{-1}) in seawater at seaweed beds regardless of the occurrences of the relevant HAB species. Strains of algicidal bacteria were isolated from *Ulva* sp., *Gelidium* sp. and seawater at the seaweed bed, and these strains were identified on the basis of the analyses of 16S rDNA genes. These bacteria also belonged to gamma-proteobacteria and the phylum Bacteroides, and the strains belonging to alpha-proteobacteria were newly identified (Imai et al. 2006a). Table 25.1 shows the prey range of algicidal bacteria isolated from the surface of seaweeds and seawater in the seaweed bed of Misaki-kouen of Osaka Bay in 1999 (Imai et al. 2006a). The dinoflagellate *K. mikimotoi* was the most susceptible

Table 25.1 Prey range of algicidal bacteria isolated from the surface of seaweeds (*Ulva* sp. and *Gelidium* sp.) and seawater in seaweed bed (Imai et al. 2006a)

	Prey algae							
Bacterial strain (origin)	*Chattonella antiqua*	*C. marina*	*C . ovata*	*Fibrocapsa japonica*	*Heterosigma akashiwo* (893)	*H. akashiwo* (IWA)	*Karenia mikimotoi*	*Heterocapsa circularisquama*
Pseudoalteromonas sp. 46 (*Ulva* sp.)	–	–	–	++	–	++	++	–
Pseudoalteromonas sp. 47 (*Ulva* sp.)	–	–	–	++	–	++	++	–
Octadecabacter sp. 49 (*Ulva* sp.)	++	–	–	–	–	–	–	–
Pseudoalteromonas sp. 53 (*Ulva* sp.)	–	–	–	++	–	++	++	–
Rhodobacteraceae 63 (seawater in seaweed bed)	–	–	+	+	+	–	++	–
Alteromonas sp. 57 (*Gelidium* sp.)	–	+	++	–	+	+	–	–
Vibrio sp. 55 (*Ulva* sp.)	–	–	++	–	–	+	++	–
Vibrio sp. 58 (*Gelidium* sp.)	–	–	++	–	–	+	++	–

++ Decrease below initial cell density; + lower growth than control (no addition of bacteria); – no effects

species, followed by the raphidophyte *F. japonica*, *H. akashiwo* (IWA) and *C. ovata*. The armored dinoflagellate *Heterocapsa circularisquama* was not killed by any of the algicidal bacteria examined. Each strain tended to show a rather limited prey range, causing mortality in at most three microalgal species of the eight strains of seven red tide species. *Vibrio* sp. 55 (isolated from *Ulva*) and *Vibrio* sp. 58 (isolated from *Gelidium*) were the same species based on the same 16S rDNA sequences, and revealed the same pattern of algicidal efficiency (Imai et al. 2006a). This fact implies that some kinds of algicidal bacteria freely attach to the surface of seaweeds regardless of the seaweed species common to their habitat.

Ulva pertusa was cultivated with red sea bream (*Pagrus major*) in a pen cage in Shimo-Haya Bay, Wakayama Prefecture, and algicidal bacteria were investigated for the dinoflagellates *K. mikimotoi* and *H. circularisquama*, and the raphidophytes *C. antiqua*, *H. akashiwo* and *F. japonica* (Imai et al. 2012a). ABs were enumerated with co-culturing experiments using bacterial strains forming colonies on nutrient agar plates, considering the indication of underestimation of ABs with the MPN method (Imai et al. 1998a, b). Table 25.2 shows densities of ABs on the surface of *U. pertusa*. *K. mikimotoi* killers were most abundant, followed by *F. japonica* killers. The detected densities of ABs were in the range of 10^4–10^6 cells g^{-1} wet weight, and the total algicidal bacteria (number of bacteria that killed at least one HAB species examined with co-culture experiments) were 2.46×10^5–9.12×10^5 cells g^{-1} wet weight occupying the ratio of 33–80 % of the total number of colony-forming bacteria on the examined leaves of *U. pertusa* (Table 25.2). Figure 25.5 represents the numbers of ABs enumerated with a co-culturing experiment in seawater samples collected at the *U. pertusa* cage (station 1), coastal point (station 2) and 1-km offshore point (station 3). Killers for *K. mikimotoi* and *F. japonica* tended to be more abundant than those for other HAB species, and killers tended to be more abundant at cage and coastal point than offshore. There is a possibility that ABs were supplied from seaweed areas to offshore areas.

Based on these studies, we can propose a new prevention strategy for red tides by using seaweeds in aquaculture areas (Fig. 25.6). Co-culturing of *Gelidium* sp. or *Ulva* sp. and finfish such as red sea bream or yellowtail is proposed to be effective

Table 25.2 Fluctuations of algicidal bacteria ($\times 10^5$ cells g^{-1} wet weight) on the surface of the green alga *Ulva pertusa* cultivated in a cage at station 1 in Shimo-Haya Bay during the period of April to August 2003 (Imai et al. 2012a). Enumeration was made with a co-culturing experiment using bacterial strains forming colonies on nutrient agar plate and five species of red tide plankton

Target red tide plankton species	April 24	June 13	August 28
Karenia mikimotoi	3.51	7.98	1.13
Heterocapsa circularisquama	1.87	<0.28	0.57
Chattonella antiqua	0.94	0.86	0.38
Heterosigma akashiwo	0.47	<0.28	0.19
Fibrocapsa japonica	1.64	2.28	1.70
[a]Total algicidal bacteria	4.91	9.12	2.46
Total colony-forming bacteria	7.01	11.4	7.56

[a] Number of bacteria that killed at least one species of red tide phytoplankton. Some bacteria killed two or more plankton species

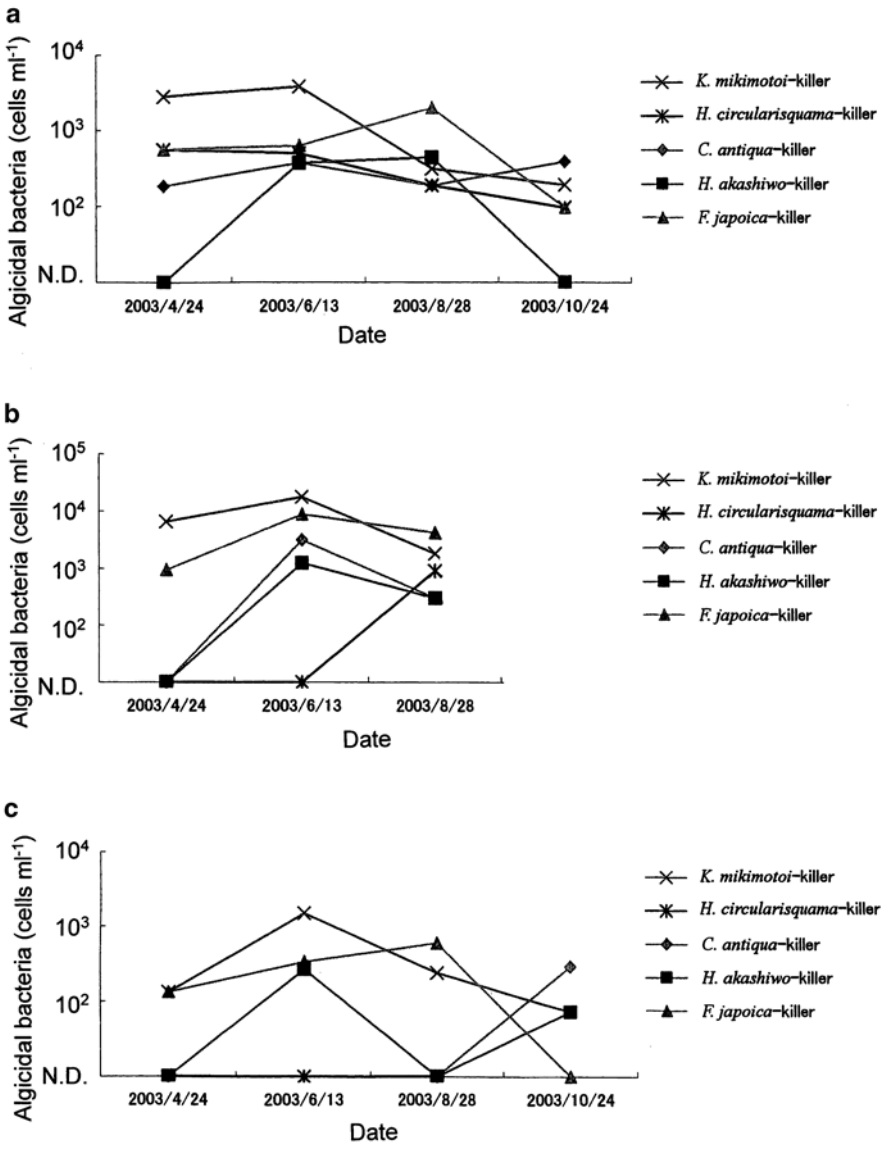

Fig. 25.5 Fluctuations of algicidal bacteria in surface seawater at the stations in Shimo-Haya Bay during the period of April to October 2003 (Imai et al. 2012a). Enumerations were made with co-culturing experiments using bacterial strains forming colonies on agar plates and HAB species. (**a**) Station 1, cage of culture of *Ulva pertusa* with red sea bream. (**b**) Station 2, coastal water. (**c**) Station 3, about 1 km offshore from station B

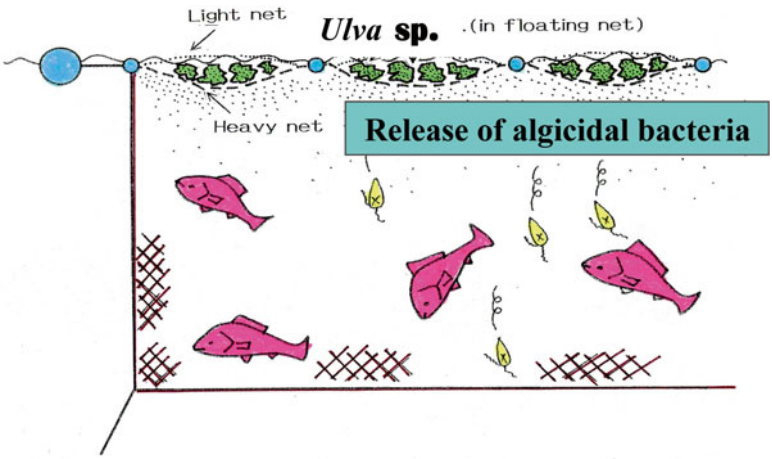

Fig. 25.6 Possible prevention strategy against noxious red tides by use of seaweeds such as *Ulva pertusa* that release algicidal bacteria into the surrounding seawater in aquaculture areas. Co-culture of seaweeds and fishes is proposed in the same cages and/or adjacent water (Imai et al. 2002)

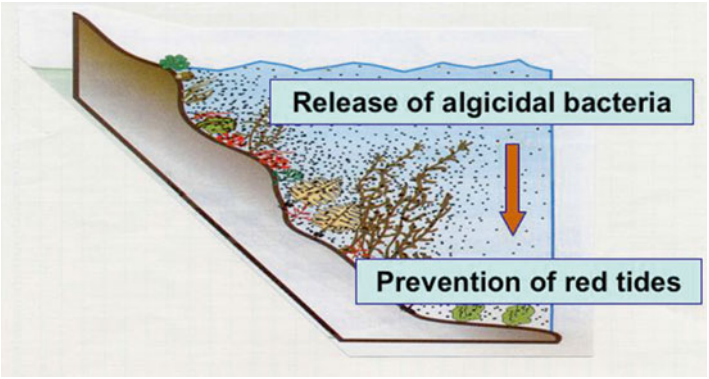

Fig. 25.7 Seaweed beds as sources of algicidal bacteria that help prevent the occurrence of red tides in coastal areas (Imai et al. 2006b)

in cage cultures (Imai et al. 2002). Many algicidal bacteria would be continually released from the surface of seaweeds to the surrounding seawater, and would contribute to reduce cell densities of HA species. Consequently, these bacteria probably play an important role in preventing HAB occurrences. This strategy is thought to be effective in enclosed and small-scale inlets.

When we artificially develop and restore the natural seaweed beds under the large-scale plan, these newly recovered seaweed beds would presumably function as tools to prevent occurrences of HABs by virtue of the continuously released algicidal bacteria (Fig. 25.7). This is regarded as a kind of bioremediation (biostimulation and bioaugmentation, simultaneously). Further, seaweed beds also serve as nursery grounds for important fishery resources such as fish and invertebrates for commercial use.

25.5 Seagrass Beds as Prevention Strategies for Harmful Algal Blooms

Seagrass beds have an important function in coastal ecosystems to maintain biodiversity and to provide feeding, housing, and spawning grounds for marine life (Nakaoka and Aioi 2001; Williams and Heck 2001). As an interesting feature, the seagrass *Zostera marina* and *Z. noltii* possess growth-inhibiting activity against phytoplankton through allelopathy (Harrison and Chan 1980; Wit et al. 2012). For example, an extract from *Z. marina* was lethal at a concentration of 0.25 mg dry leaf/mL to eight species of examined microalgae, i.e., the pennate diatoms *Cylindrotheca fusiformis, Nitzschia angularis, N. frustulum, N. longissima*, the centric diatom *Skeletonema costaum*, the dinoflagellates *Gonyaulax polyedrum*, *Protogonyaulax tamarensis* (*Alexandrium tamarense*), and the green alga *Platymonas* sp. (Harrison and Chan 1980). The growth of phytoplankton was delayed by the addition of *Z. noltii* in mesocosm experiments (Wit et al. 2012). However, since highly diverse microorganisms possessing various activities live in seagrass beds, it is expected that there exist various kinds of algicidal and/or growth-inhibiting bacteria against phytoplankton. Interestingly, algicidal bacteria against red tide-causing flagellates were actually discovered with high densities in the biofilm on the surface of *Z. marina* blades (Imai et al. 2009b). The densities of those algicidal bacteria were 9.19×10^6–64.3×10^6 cell g^{-1} wet weight for the raphidophyte *Chattonella antiqua* and the dinoflagellates *Heterocapsa circularisquama*, *Karenia mikimotoi* and *Cochlodinium polykrikoides* (Table 25.3). Consequently, it is considered that seagrasses are favorite habitats for algicidal bacteria, and they have an ability to autonomously proliferate via algicidal activity and utilization of resultant organic matter. Hence it is expected that it will be possible to autonomously exterminate and/or prevent occurrences of red tides. Future studies are essential to determine whether allelopathy or algicidal bacteria are more effective to kill or inhibit the growth of phytoplankton.

Concerning toxic species such as paralytic shellfish poisoning (PSP)-causative *Alexandrium tamarense*, growth-inhibiting bacteria possessing strong activity were

Table 25.3 Abundance of algicidal bacteria against five species of harmful algal bloom (HABs) detected from the surface of seagrass (Zostera marina) leaf and from the seawater of the seagrass bed (particle-associated [PAB] and free-living [FLB] fractions) collected on July 13, 2006 (Imai et al. 2009b)

	Algicidal bacteria		
	Zostera leaf	Sea water ($\times 10^3$/ml)	
Target HAB species	($\times 10^6$/g wet leaf)	PAB	FLB
Chattonella antiqua	9.19	4.8	0
Heterosigma akashiwo	0	2.4	0
Heterocapsa circularisquama	9.19	0	0
Karenia mikimotoi	64.3	2.4	0
Cochlodinium polykrikoides	27.6	2.4	0

discovered from the surface of blades of *Zostera marina* (Onishi et al. 2014). The isolated bacterial strain (E9) markedly inhibited the growth of *A. tamarense* even with an initial inoculum size as small as 2.9 cells ml^{-1} (Fig. 25.8). The variety of the morphology affected by the bacterium was shown in Fig. 25.9. After addition of the cells of inhibiting bacterial strain E9, thecal plates were often detached from the algal cell after around 3 days, and spherical cells, presumably temporary cysts, were also frequently observed at the same time in experimental vessels. These naked cells were eventually killed by the bacterium (Fig. 25.9). Small subunit ribosomal DNA

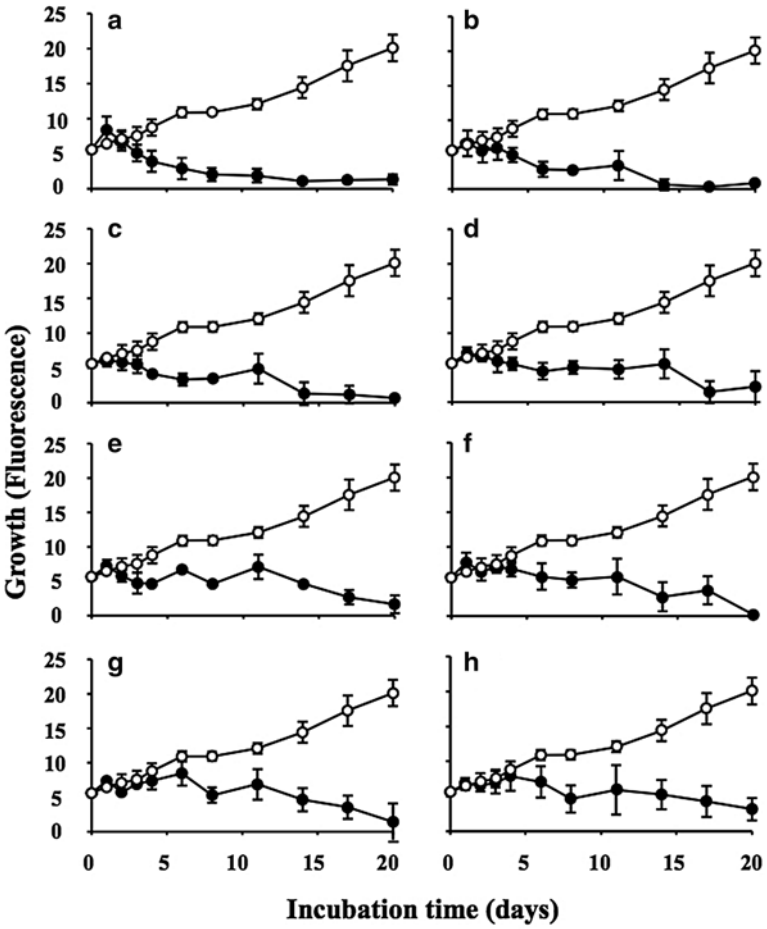

Fig. 25.8 Effects of growth-inhibiting bacterial strain E9 with different inoculum sizes on *Alexandrium tamarense* in the modified SWM-3 medium (Onishi et al. 2014). The initial cell density of *A. tamarense* was 3.6×10^2 cells ml^{-1}. The initial bacterial densities (cell ml^{-1}) were (**a**) 2.9×10^7, (**b**) 2.9×10^6, (**c**) 2.9×10^5, (**d**) 2.9×10^4, (**e**) 2.9×10^3, (**f**) 2.9×10^2, (**g**) 2.9×10^1, and (**h**) 2.9×10^0. Control (*open circle*) indicates the growth of *A. tamarense* with no addition of bacterial cells (axenic culture)

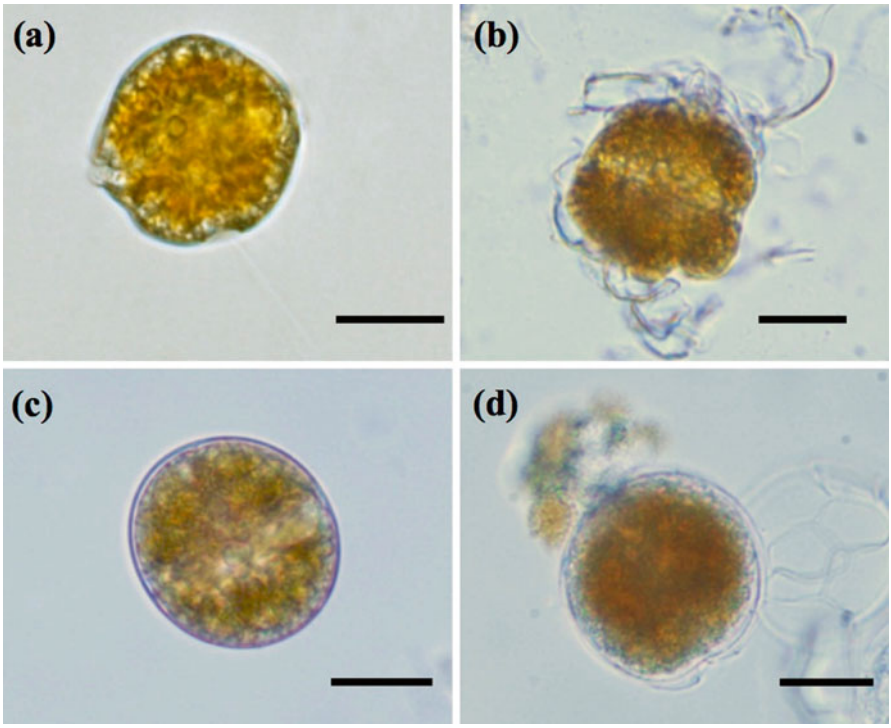

Fig. 25.9 Effects of bacterial strain E9 on morphology of *Alexandrium tamarense*: (**a**) control, no addition of bacterial cells; (**b**) *A. tamarense* cell with detached thecal plates after 3 days' incubation; (**c**) spherical cell of suspected temporary cyst observed after 3 days' incubation; (**d**) disrupted cell releasing cell contents observed on day 7 after addition of bacterial cells (Onishi et al. 2014). Scale bar 20 μm

(SSU rDNA) sequencing analysis demonstrated that the most probable affiliation of this strain (E9) was a member of the genus *Flavobacterium* belonging to the phylum Bacteroides, and proved that another inhibitory bacterial strain (E8) against *A. tamarense* showed the same sequence as the strain E9, indicating the same species (Fig. 25.10). Two other bacterial strains (E4-2 and E10) isolated from the same seagrass sample, showing a different colony color from E9, revealed no growth-inhibiting activity against *A. tamarense*. Interestingly, the strain E4-2 revealed the same sequence of SSU rDNA as E8 and E9 (100 %), and the strain E10 matched E8 and E9 with 99.8 % similarity. In conclusion, it is considered that seagrass beds have the potential to prevent occurrences of not only harmful red tides (Imai et al. 2009b; Imai and Yamaguchi 2012), but also toxic dinoflagellate blooms by virtue of the association of strong growth-inhibiting bacteria (Onishi et al. 2014).

Seagrass beds have been rapidly disappearing at a rate of 110 km^2 $year^{-1}$ in the world since 1980, and 29 % of the initial area has disappeared since 1879, when seagrass areas were first approximately estimated (Waycott et al. 2009). On the

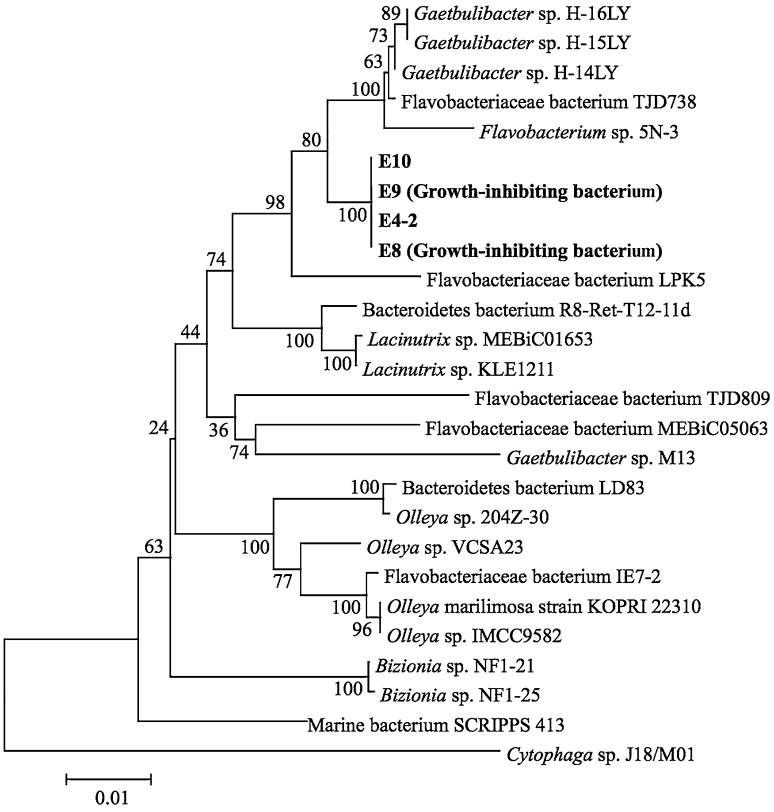

Fig. 25.10 Phylogenetic tree including the growth-inhibiting bacteria E8 and E9 and two closely related bacterial strains (E10 and E4-2) based on 16S rRNA gene sequences (Onishi et al. 2014). The tree was constructed using the neighbor-joining method and maximum likelihood method (NJ/ML)

other hand, the scale and frequency of occurrences of harmful algal blooms have been increasing globally (Hallegraeff 1993). There is an interesting report from the Mediterranean coast that the large-scale decline of seagrass beds was accompanied by increasing frequency of the toxic dinoflagellate blooms of *Alexandrium minutum* (Abdenadher et al. 2012). Also, on the coast of the Seto Inland Sea, Japan, incidents of HABs markedly increased (from <50 to the maximum of 299 in 1976) during the 1960s and 1970s, and seagrass beds had dramatically been reduced to one quarter (from 22,635 ha in 1960 to 6381 ha in 1989) during the same period (Imai and Yamaguchi 2012). This is thought to be a case of catastrophic shifts (Scheffer et al. 2001) with regard to the function of controlling phytoplankton by seagrass beds in coastal ecosystems. Therefore, it is proposed that restoration and/or creation of seagrass beds is potentially and urgently important to prevent HABs.

25.6 Perspectives

Algal blooms produce much abundant organic matter. After the rapid bloom termination by algicidal bacteria, killed algal cells must be decomposed rapidly and enter into the food web mainly through microbial loops (Kamiyama et al. 2000) without delay. If not, bloom terminations directly contribute to the deterioration of coastal environments such as anoxia. Seagrass beds and seaweed beds are expected to be hot spots of microbial processes such as algicidal activity, decomposition of excessively produced organic matter, and hence the function of microbial loops. More extensive studies are needed on the effects of algal blooms and decay on food webs in these hot spot areas in the future.

In freshwater ecosystems, one of the most dramatic state shifts is the sudden loss of transparency and vegetation in shallow lakes subject to human-induced eutrophication (Scheffer et al. 1993, 2001). Experimental study suggested that water plants increased water clarity, thereby enhancing their own growing conditions. The reduction of phytoplankton biomass and turbidity by water plants involves various mechanisms, i.e., reduction of nutrients, protection of phytoplankton grazers (*Daphnia* etc.) against fish predation, and prevention of sediment resuspension. A real example of an increase in vegetation and accompanying transparency was observed in Lake Biwa in the 1990s (Haga and Ohtsuka 2008).

Similarly, in marine coastal ecosystems, seagrass (*Zostera marina*) beds also occasionally disappear due to dense red tides such as the raphidophyte *Heterosigma akashiwo* (Lee et al. 2007). Seagrass (*Z. marina*) bed can recover by itself through new shoot recruitment from the seed bank under sufficient light conditions. Since seagrass harbors huge numbers of algicidal bacteria against HAB species, restoration and/or creation of seagrass beds should be effective for keeping the phytoplankton biomass in an adequately low range and for preventing occurrences of HABs in adjacent waters.

Sato-Umi is a newly proposed concept for sustainable fisheries, identified as "high productivity and biodiversity in the coastal sea with human interaction" (Yanagi 2008). As mentioned above, the creation and/or restoration of seagrass beds and seaweed beds are considered to contribute to preventing HAB occurrences by virtue of the activities of algicidal bacteria released from seaweeds and seagrasses to adjacent coastal waters (Fig. 25.11). These strategies are ultimately environment friendly, and restoration and maintenance of seaweed and seagrass beds are important to maintain the health of the coastal sea, preventing the occurrence of HABs (Imai et al. 2006b; Imai and Yamaguchi 2012). This is a kind of harmony between humankind and nature in conformity with the concept of "Sato-Umi" (Yanagi 2008; Imai and Yamaguchi 2012; Onishi et al. 2014).

The Sato-Umi concept would be also applicable to freshwater ecosystems. An algicidal bacterium (*Agrobacterium vitis*) active against the toxic cyanobacterium *Microcystis aeruginosa* was actually isolated from the surface of the water plant *Egeria densa* (Imai et al. 2012b). Therefore, creating an adequate scale of water-plant zone is expected to keep lake conditions in a healthy state with lower

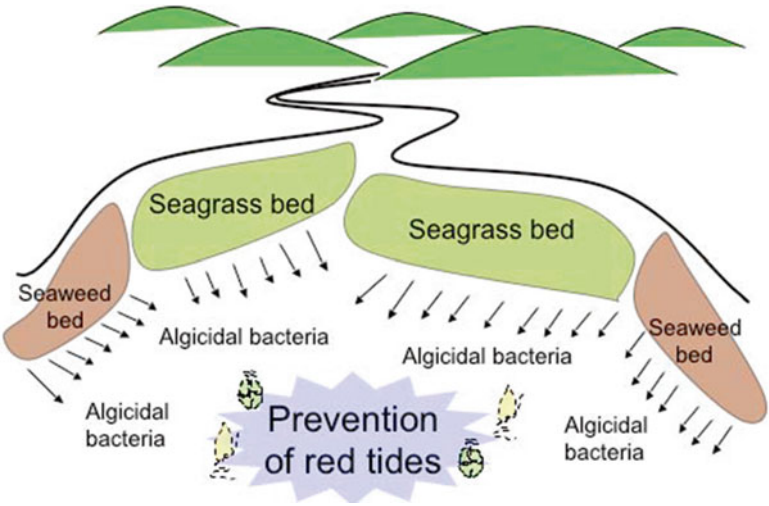

Fig. 25.11 Schematic representation of strategies for preventing red tide (harmful algal bloom) occurrences by restoration and/or development of seaweed- and seagrass beds in coastal areas (Imai et al. 2009b). Algicidal bacteria would be supplied to seawater and prevent red tide occurrences by virtue of controlling phytoplankton populations within some moderate levels

frequencies of toxic cyanobacterial blooms by virtue of algicidal bacteria (Imai et al. 2012b) and with a clear state (Scheffer et al. 2001). Studies on interrelationships among higher plants, seaweeds, phytoplankton, and microorganisms such as bacteria and viruses promise to solve environmental problems in aquatic (both marine and freshwater) ecosystems in the future.

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Chapter 26
Relationships Between Aquatic Protists and Humans

Susumu Ohtsuka, Toshinobu Suzaki, Noritoshi Suzuki, Takeo Horiguchi, and Kengo Suzuki

Abstract Protists exhibit a tremendously wide variety of lifestyles and metabolic functions. Considering predicted shortages of natural resources and foods in the near future, much more attention has to be paid to these potentialities of protists from applied and industrial points of view. We provide a general overview of their potentialities, some of which have already been industrialized. On the other hand, our health and economics have long been damaged by other potentialities of protists such as harmful algal blooms and paralytic shellfish poisoning. We have been intensively searching for effective countermeasures to avoid or reduce these detrimental impacts. Wise use of protistan beneficial potentialities is a key issue to respond adequately to rapidly and drastically ongoing changes of global environments. In addition we have to exactly consider what is now happening to the protistan communities due to anthropogenic impacts.

Keywords Anthropogenic impact • Disease • Environment • Food • Health • Human society • Natural resources • Red tide

S. Ohtsuka (✉)
Takehara Marine Science Station, Setouchi Field Science Center, Graduate School of Biosphere Science, Hiroshima University, 5-8-1 Minato-machi, Takehara, Hiroshima 725-0024, Japan
e-mail: ohtsuka@hiroshima-u.ac.jp

T. Suzaki
Graduate School of Science, Kobe University,
Kobe, Hyogo, Japan

N. Suzuki
Institute of Geology and Paleontology, Graduate School of Science,
Tohoku University, Sendai 980-8578, Miyagi, Japan

T. Horiguchi
Department of Biological Sciences, Faculty of Science,
Hokkaido University, Sapporo 060-0810, Hokkaido, Japan

K. Suzuki
euglena Co., Ltd, 5-33-1 Shiba, Minato-ku, Tokyo 108-0014, Japan

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26.1 Overviews

We have already understood that protists exhibit a tremendously wide variety of lifestyles and functions. This is partly due to the unicellular nature of protists to perform all metabolic procedures. More attention has recently been focused on protists for applied scientific and industrial purposes. This chapter briefly summarizes modern issues concerning aquatic protists with an emphasis on their direct and indirect relationships to human societies. As far as photosynthetic protists are concerned, "wise use" of these is well documented by some comprehensive textbooks (Inouye 2007; Watanabe 2012; Kurahashi and Oyaizu 2013).

Protists have been intensively investigated with modern techniques, leading to exploitation for basic and applied sciences. Ultrastructural and molecular genetic analyses of protists have drastically shown us major taxonomic and phylogenetic revisions. To our surprise, malaria parasites, ciliates and dinoflagellates consist of a monophyletic clade called "Alveolata". Myxozoa is already regarded as an extremely reduced multicellular animal to adapt itself to the parasitic mode of life. Complete genome sequences are also available for many pathogenic and model protists. Endosymbiosis of organelles is still a hot spot in evolutionary protistology (e.g., Nowack and Melkonian 2010). Since many free-living protists are readily cultured in vitro and for mass culture, we have noticed their immense potentialities. As cellular and genetic functions of protists are newly discovered, we will be able to enjoy their benefits much more. For example, although we have still been consuming limited fossil fuels made of ancient marine protists, we are now making efforts to innovate substitutes produced by living protists such as *Botryococcus braunii* to reduce emission of CO_2 gas. Many other protists and their metabolic products are also used as foods and medicines for humans, foods for aquaculture, fuels and fertilizers. These taxa belong to red and green algae, Chrysophyseae, Phaeophyceae and Labyrinthulea. Mass culture of the green alga *Dunaliella* is industrialized to produce β-carotene in Australia, Israel and China. Diatomite or fossilized diatoms are widely used for filtration, fillers, building materials and absorbents. Such direct industrial utilizations of protists have been getting extensively in progress. On the other hand, molecular infectious mechanisms of severely pathogenic protists such as malaria parasites and *Perkinsus* are also intensively investigated to prevent pandemic diseases.

Indirect influences of protists on human societies are also of paramount importance for environmental stability and production of foods. Autotrophic protists such as diatoms, dinoflagellates and coccolithophoids play great roles as primary producers in the aquatic ecosystems to sustain zooplankters and fish. The biomass and total amount of primary production of these marine autotrophs are estimated at 1 billion ton C and 40–50 billion ton C/year, respectively (Inouye 2007). The annual production of terrestrial plants is nearly equal to that of marine algae, although the former's biomass is much higher by 500–600 times. Biological-pumping photosynthesis of marine phytoplankton is a key issue to understand global energy circulation. DMS (dimethylsulfide) originating from DMSP (dimethylsulfoniopropionate) produced

by marine Haptophyta easily becomes aerosolized to form clouds, and is closely related to precipitation and climate changes. Endosymbiotic protists in digestive organs of termites and ruminants function in decomposition of plants, and are indirectly related to circulations of matters and energies, although such mutualistic protists seem to be rarely known in the aquatic ecosystems. An exception is a highly derived apicomplexan *Nephromyces* found in the renal sac of phylogenetically advanced molgulid tunicates, in which metabolic mutual aids between the apicomplexan, its symbiotic bacteria and the host are supposed (Saffo et al. 2010). Ciliates and heterotrophic flagellates function as consumers and decomposers in microbial loops and interstices, which are regarded as natural purifying facilities. Endosymbiotic zooxanthelles in hexacorals primarily support many kinds of production in tropical and subtropical shallow waters. So we can enjoy SCUBA diving and other marine sports in Phuket, Hawaii, and other resorts. Some freshwater unicellular algae are utilized for bioassay called WET (whole effluent toxicity) in the world.

On the other hand we have long been suffering from pathogenic and poisonous protists. A wide variety of protists cause serious diseases for human beings, livestock and other organisms. In 1990s *Pfiesteria* (Dinophyceae) concerned us as a killer, although the truth has not come out yet. Apicomplexa, Microspora (Enterocytozoon) and Ascetospora are all parasitic, causing diseases of human and aquatic animals. Some species of Myxozoa and Apicomplexa are lethal to cultured fish and shellfish. Aquaculture of American oyster *Crassostrea virginica* was heavily devastated by the perkinsid *Perkinsus marinus*. Planktonic and benthic dinoflagellates produce toxins to cause serious symptoms (PSP (paralytic shellfish poisoning), DSP (diarrhetic shellfish poisoning), NSP (neurotoxic shellfish poisoning), ASP (amnesic shellfish poisoning) for human beings and cultured fish/shellfish. Surprisingly some diatoms also produce toxins to induce abnormalities in planktonic copepods. Red tides caused by coastal phytoplankters such as diatoms, dinoflagellates, Haptophyceae and Raphidophyceae have frequently caused enormous economic losses for coastal fish and shellfish farms. Recently these toxic and harmful phytoplankters have been spreading all over the world via ship ballast water.

Human populations have been rapidly expanding for a few decades and are estimated to exceed 10 billion around 2100. Modern issues such as shortage of foods, waters and energies and prevalence of diseases have been getting more and more serious. In particular we have to solve the energy problems as soon as possible, because we have experienced the great tragedy of the Fukushima nuclear power plant since 11 March 2011. In addition, anthropological activities have been causing drastic environmental changes such as global warming, depletion of the ozone layer, contaminations and losses of biodiversity. Seeing our outrages against nature, some famous scientists pessimistically predict that the lord of creation, *Homo sapiens*, will not be so long lived. However, we are sensible enough to avoid the worst situation through strategic "wise use" of nature and biodiversity. Marine protists are of pivotal importance to innovate the real sustainable development of human societies. Some hot topics on aquatic protists closely related to human societies and economics, and vice versa, are provided below.

26.2 Materials for Basic Biology and Medicine

Marine protists are important resources that have a wide range of biological and medical applications (see review in Varfolomeev and Wasserman 2011; Pignolet et al. 2013). Due to its relatively easy and low-cost cultivation, *Dunaliella* (Chlorophyta) has been extensively used as a material for research on carotenoid and fatty acid metabolism (Fu et al. 2013; Lamers et al. 2012) and basic nutriology (Harari et al. 2013), and for studying signal transduction and salinity tolerance (Anila et al. 2013; Ramos et al. 2011). Recently, *Dunaliella* has attracted much interest due to its potential use as a feedstock for biofuel (Tang et al. 2011; Davidi et al. 2012; Chen et al. 2011) and expolymers (Mishra et al. 2012). In terms of environmental investigations, *Dunaliella* has been used as a test organism to examine the toxicity effects of zinc and silver nanoparticles (Manzo et al. 2013; Oukarroum et al. 2012), carbon nanotubes (Wei et al. 2010), and chemical pesticides (Chen et al. 2011). *Dunaliella* is also used for bioremediation of heavy metals (Imani et al. 2011) and naphthenic acids in crude oils (Quesnel et al. 2011). Since microalgae are a major food source for marine heterotrophic protists (Bernard and Rassoulzadegan 1990), small invertebrates such as copepods, and larvae of various marine organisms (Conceição et al. 2009), *Dunaliella* has been widely used as an experimental food for these organisms.

The unicellular red alga *Porphyridium* (Rhodophyta) is a source of several important products for application in basic biology and medicine (Razaghi et al. 2014). The products include fatty acids and lipids, pigments, and cell-wall polysaccharides (Pignolet et al. 2013). Phycoerythrin (B-phycoerythrin) is a red-fluorescent, multi-subunit pigment protein of *Porphyridium* that can be conjugated to other molecules for making fluorescent probes, mainly used as an indicator for immunofluorescence microscopy. B-phycoerythrin was also shown to have antitumor activity by inducing apoptosis in tumor cells (Minkova et al. 2011). *Porphyridium* is encapsulated within a sulfated polysaccharide gelatinous layer that protects the microalgae against desiccation or changes in salinity. This polysaccharide has applications in various industries such as cosmetics and health food markets (Pignolet et al. 2013), and also in medicine because of its antiviral and antitumor activities (Singh et al. 2000; Sun et al. 2012) and its effect in reducing blood cholesterol levels (Ibañez and Cifuentes 2012).

Dinoflagellates produce various types of phycotoxins. These natural metabolites are accumulated in the food chain in different marine organisms, thereby causing human poisoning due to ingestion of contaminated seafood (see Chap. 24, this volume, and also Rossini and Hess 2010). Some of the toxins (okadaic acid, gonyautoxin, and yessotoxin) are used as experimental tools in cell biology and medicine, especially to investigate neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Rossini and Hess 2010). Okadaic acid is a polyether toxin that was originally derived from the marine dinoflagellate *Prorocentrum*. It is a reversible and selective inhibitor of two serine threonine protein phosphatases, and is used to identify biological processes that are mediated through the reversible

phosphorylation of these proteins (Cohen et al. 1990). Paralytic shellfish poisons, including saxitoxin, neosaxitoxin, and gonyautoxin, are alkaloids that are potent sodium channel blockers in nerve axons which cause progressive paralysis and death from respiratory failure. By inactivating sodium channels, saxitoxin has an ability to induce anesthesia, which deserves further investigation (Wiese et al. 2010). Yessotoxin is produced by a number of dinoflagellate species, such as *Lingulodinium polyedrum, Gonyaulax spinifera* and *Protoceratium reticulatum*. Apoptosis can be induced in different cell systems through activation of the mitochondrial pathways, and the potential use of yessotoxin as an antitumor drug has been under consideration in therapeutic applications (Korsnes and Espenes 2011).

26.3 Foods

Recently algal products and their metabolic activities have been widely utilized or potentially considered for production of foods for humans and cultured animals, medicines, fuels and industrial materials, and microbial concentration of rare metals (Inouye 2007); Watanabe 2012; Kurahashi and Oyaizu 2013). Productions of carotenoids by green algae such as *Dunaliella* and *Haematococcus* have already been industrialized in the USA, Israel, China, India, Sweden and Japan (Watanabe 2012). Although industrial production of shale gas and oil has recently been established in the USA and Canada, these materials cannot be permanently utilized for human societies.

Potential utilizations of protists should be continuously studied for the real sustainable society in the future. In addition, symbiotic interactions among protists, bacteria and viruses will be more industrially utilized in the future. For example, endosymbioses between *Chlorella* and phycodnavirus can effectively produce polysaccharides such as hyaluronan and chitin (Yamada 2010). As discussed in Chap. 20, phage therapy may be widely applied to avoid harmful algal blooming and diseases in aquaculture. We will briefly introduce one example of successful utilization of euglenoids in the venture company euglena Co., Ltd., Japan.

Shall we take a look at one of the most successful examples of utilization of aquatic protists efficiently for human societies? The protist is *Euglena*. The autotrophic flagellate *Euglena* and its related taxa are a small-sized phytoplankter of around 50 μm in cell size, and are abundantly distributed in freshwaters such as marshes, ponds and rice paddies, and also in brackish and sea waters. The microalgae were first observed by Antony von Leeuwenhoek in the seventeenth century. Photosynthesis of the microalgae is suggested to have been conducted by secondarily obtained green algae. Recently much more attention has been paid to these unicellular autotrophs as food supplements since 1990, due to their high nourishment. euglena Co., Ltd. first succeeded in mass culture of *Euglena gracilis* (Fig. 26.1a), and has produced a variety of commodities made of *Euglena* since 2005. The main factory is located in Ishigaki Island, Okinawa, subtropical Japan, to process *Euglena* powder. The powder is utilized to enhance nutrition in cookies, castella (a Japanese

sponge cake), Chinese noodles, hamburger and drinks (Fig. 26.1b). The annual production of *Euglena* powder by the factory is about 60 tons.

Euglena powder is nutritionally characteristic of two unique features. The first is rich nutrients and easy digestibility. *Euglena* powder contains 59 nutritional components such as vitamins, provitamins, minerals, amino acids and unsaturated fatty acids. Easy digestibility is based on the absence of a cell wall in *Euglena* cells. Surprisingly, an extraordinarily high digestion rate or 93% was observed in laboratory experiments of digestion in mice fed the contained proteins. Another feature is its intracellular "paramylon", which exclusively comprises crystals of β-1,3 glucan. Paramylon is highly crystallized and differs from other β glucans. This component

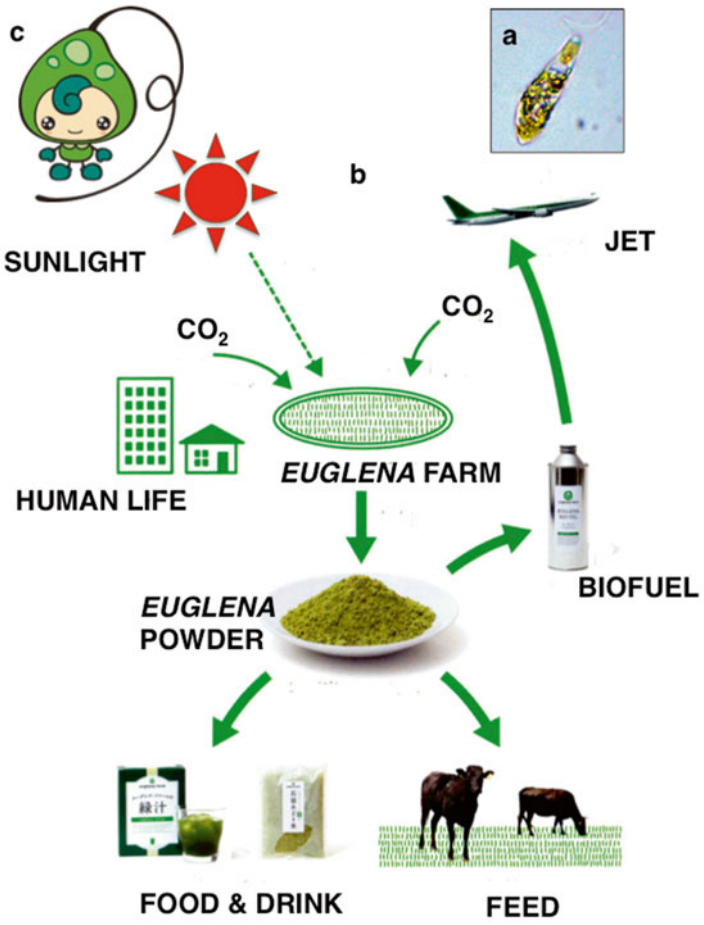

Fig. 26.1 Challenging utilization of aquatic protists for humans by a Japanese venture business, euglena Co., Ltd, Japan. (**a**) *Euglena gracilis* (about 0.05 mm in length), the main mass-cultured flagellate for industrialization. (**b**) Possible utilization of *Euglena* for industries for humans. (**c**) Mascot of euglena Co., Ltd., called "eugleen"

has been reported to function in improvement of metabolism of lipids, easing of atopic dermatitis, reinforcement of liver function, and possible restraint of absorption of purine body.

One of the efficient ways to utilize newly produced biomass is based on five "F's", i.e., food, fiber, feed, fertilizer, and fuel. Since the added value is higher in this order, the priority of more highly valued "F's" simultaneously guarantees both reduction of burdens on natural environments and great success in business. *Euglena* can be utilized in all "F's" (Fig. 26.1b). In addition to food supplements, it can be used for cosmetics, decomposable plastics, protein concentrates for livestock and cultured fish, soil enrichments, and jet fuel. euglena Co., Ltd. has been intensively searching for possibilities that jet fuel made from wax ester produced by *Euglena* could be substituted for fossil fuel, and that *Euglena* powder could dispel starvation in the world.

Generally mass culture of protists has long been challenged by many researchers. Its early application to welfare for human societies is truly desired, because serious issues concerning the human future have been coming out. However, cost performance and methods for protist culture are still in hot debate. Novel technologies and new social systems are absolutely needed for sustainable management of carbon-based energy flow. For example, recycling of livestock and food wastes for culturing protists can be combined with efficient utilization of lands such as fallow fields and industrial vacant lots for mass culture. CO_2 gas emitted from factories can be directly utilized for photosynthesis of cultured autotrophs. Protist products can be substituted for petrochemicals. Newly innovated technologies in applied protistology can be put to practical use in modern society.

The lovely mascot "Eugleen" of euglena Co., Ltd. (Fig. 26.1c) seems to symbolize an optimistic future for humans.

26.4 Negative Impacts on Human Societies

There are many protist groups that, directly or indirectly, have negative impacts on human society or life. We are not going to enumerate these examples; rather, we would like to focus on the cases where new data or techniques are being contributed toward understanding and toward control of these negative impacts.

26.4.1 Recent Success in Culturing Dinophysis*: Toward Understanding of Productive Mechanisms of Toxins*

The dinoflagellates are a biologically interesting group and thus have been a focus of various fields of biology, including cytology and molecular biology (see Chap. 2). At the same time, the group is apparently one of the notorious groups of protists that display a wide range of negative impacts on human society, i.e., harmful algal

blooms (HABs). Some dinoflagellates are known to produce potent toxins to cause PSP (see Chap. 24), DSP, NSP, and ciguatera fish poisoning (CFP). To predict HAB events, comprehensive research is needed, including taxonomical, ecophysiological, chemical and toxicological studies. Also, to study the detailed physiological features of possible causative organisms, establishment of culture strains is essential. However, this is not always an easy task. In the case of DSP, particularly, the establishment of culture strains of causative organisms was regarded to be very difficult, but this difficulty has been overcome since their rather peculiar nutritional strategy was unraveled.

DSP is a gastrointestinal disease caused by uptake of toxin-contaminated shellfish, and DSP outbreaks have been reported from Japan, southeastern Asia, North America, South America, Europe, South Africa, Australia and New Zealand (Reguera et al. 2012). The causative organism of DSP was first identified as a planktonic dinoflagellate, *Dinophysis fortii*, by Yasumoto et al. (1980), and currently 12 congeners are known to produce toxins such as ocadaic acid (OA) and its derivatives, named dinophysiotoxins (DTXs) and pectenotoxins (PTXs) (Yasumoto et al. 1985; Reguera et al. 2012). After the pioneering attempt by Barker (1935), despite a number of attempts, culturing *Dinophysis* spp. remained difficult for a long time (see references in Reguera et al. 2012). Toxigenic *Dinophysis* spp. were thought to be photosynthetic, because they possess chloroplasts. However, the chloroplasts of *Dinophysis* were different from those of typical dinoflagellate plastids and their possible affinities with cryptophyte chloroplasts were suggested based on the ultrastructure (Schnepf and Elbrächter 1988; Lucas and Vesk 1990). A molecular phylogenetic study of plastid-encoded genes indicated that the plastids of *Dinophysis* spp. are of cryptophyte origin and that they are not permanent chloroplasts but temporally acquired chloroplasts, termed kleptochloroplasts (= stolen chloroplasts; see Schnepf and Elbrächter 1992; Takishita et al. 2002; Hackett et al. 2003; Janson and Granéli 2003; Janson 2004). Janson (2004) demonstrated that the sources of cryptophytes are cryptomonad *Teleaulax* spp., suggesting the possibility that *Dinophysis* spp. do not ingest cryptomonad cells directly but obtain plastids as a secondary kleptochloroplast via a ciliate *Mesodinium rubrum*, which also utilizes cryptomonad prey as a kleptochloroplast (Taylor et al. 1969; Hibberd 1977). Janson's hypothesis (2004) was later proven to be true when Park et al. (2006) successfully cultured *Dinophysis acuminata* by feeding it with *M. rubrum*. The nutritional strategy of *Dinophysis* as revealed by Park et al. (2006) was extraordinary. The *Dinophysis* species myzocytotically ingests ciliates' kleptochloroplasts, which were originally obtained from cryptophytes, thus the serial kleptochloroplastidic system (cryptophyte –*Mesodinium*–*Dinophysis*) is required to maintain *Dinophysis* cultures. This method was confirmed to be applicable for other species of toxin-producing *Dinophysis* spp. (Nagai et al. 2008; Nishitani et al. 2008a,b; Park et al. 2008). Thanks to successful culture of *Dinophysis* spp., our understanding of physiological aspects of the organisms, especially regarding toxin production, has been rapidly increased. The studies using culture strains confirmed production of toxins by further *Dinophysis* spp. (Hackett et al. 2009; Kamiyama and

Suzuki 2009; Fux et al. 2011). Moreover, the effects of light, temperature, food availability and the growth phase on toxin production, toxin retention and toxin excretion have been investigated using culture strains (Nagai et al. 2011; Tong et al. 2011; Smith et al. 2012; Nielsen et al. 2013). These laboratory data, coupled with investigations in nature, are indispensable for better prediction of initiation, maintenance and termination of *Dinophysis* blooms. Currently, the ecological role and significance of DSP toxins are also largely unknown (Reguera et al. 2012; Nielsen et al. 2013). Experimental studies using cultures might answer these questions. For more details about harmful *Dinophysis* species, readers are referred to an excellent review by Reguera et al. (2012).

26.4.2 Recent Advance in Perkinsus *Studies*

Due to overfishing and destruction of the habitats of commercially harvested fish and other aquatic organisms, alternative aquaculture has been becoming more globally intensive, with an estimation of over 73 million MT in 2009 (FAO 2013). However diseases caused by many kinds of microorganisms and parasites have been prevalent in farms, frequently resulting in mass mortalities and economic losses. The economic and environmental importance of protistan pathogens in aquaculture has been well documented by Rhode (2005).

The alveolate *Perkinsus* has been well known as a pathogenic endoparasite of commercially important molluskans including oysters, clams, scallops and abalone, and can be regarded as a model organism (cf. Joseph et al. 2010). Oysters have been one of the most important aquaculture products in the world, with an annual catch of approximately 4.4 million MT in 2009 (FAO 2013), but has often suffered from lethal diseases caused by such parasitic protists. In farms of flat and eastern oysters, rapid declines in production or mass mortalities were recorded after the discoveries of pathogenic protists such as haplosporidians and perkinsids (Carnegie 2005). Transplantation of infected seed oysters has secondarily caused wide spreading of these diseases. The prevalence of *Perkinsus* in Manila clams and eastern oysters was remarkably high, as much as 60–100% in East Asian waters (Choi and Park 2010) and also up to 100% along the eastern coast of USA (Oliver et al. 1998), respectively. Cultured bivalves are also suggested to play an important role in controlling eutrophication by their high filter feeding activities to clear suspended particles at rates of 1–4 L/h (e.g., Rice 2000). It is likely that mass mortalities of cultured bivalves by these diseases alter the ecosystem around cultured areas. Considering the economic and environmental importance of *Perkinsus*, its infective mechanism at the molecular level was intensively investigated by Tasumi and Vasta (2007). According to their sophisticated experiment, the trophozoite or the infective and vegetative multiplication stage in the host tissue mimics food microalgae, leading to phygocytosis or successful passive entry into the host cells. Some kinds of immunological therapies should be innovated to avoid or reduce the damage in consideration of the molecular infective mechanism.

In contrast, attention is paid to another endoparasitic perkinsid *Parvilucifera*, since it kills bloom-forming dinoflagellates (Noren et al. 1999) as well as endoparasitic viruses, as is documented in detail in Chap. 20. These endoparasites are potential candidates to regulate red tide blooming artificially.

26.5 Response to Anthropogenic Effects

As is well known, the human impact on nature has been significant since the Industrial Revolution, which started around AD 1760. Anthropogenic perturbation has supplied carbon to inland waters by as much as 1.0 $PgCyr^{-1}$ since pre-industrial times, mainly owing to enhanced carbon export from soil, while 0.1 $PgCyr^{-1}$ of the perturbation carbon inputs into the open oceans (Regnier et al. 2013). Although scientific observations on marine protozoans were being made as far back as the nineteenth century (e.g., Meyen 1834), human activities had already been very active worldwide, thus the records did not reflect natural conditions. The major longer environment changes closely related to anthropogenic effects are enrichment of carbon dioxide by consumption of coal, oil and methane hydrates; the discharge of nitrogen and phosphorus matter from agricultural activity and industrial products; and the supply of iron from industrial products and iron ships (e.g., Tyrrell 2011). However, the important point with regard to these anthropogenic effects is that chemical elements are essential to maintain marine life. Thus, some anthropogenic effects contribute to enhancement of marine life, while others cause damage to marine life. This section briefly introduces both negative and positive impacts of anthropogenic effects on protists. The reader of this book may ask which is correct, but we place emphasis on the difficulty—and existence of strong contradictions between studies—of obtaining a clear answer.

Global warming has long been pointed out as relying largely on anthropogenic carbon dioxide emissions, but this hypothesis is still in dispute. Aside from the "true" cause, global warming is considered to create an ocean heat sink, sea-level rise, reduced ocean overturning, stratification of oceans and de-iced polar oceans (Tyrrell 2011). It is, however, very difficult to prove a cause-and-effect relation between marine protists and global warming, because the heat capacity of the total ocean is about 1000 times that of the total atmosphere. The warming phenomena were detected in the temperature of the Arctic Intermediate Water (AIW) in the Arctic Ocean as being at least 0.2°C between the 1950s–1960s and 1994, and as a new introduction of a polycystine *Ceratocyrtis histricosa* into the warming AIW after the 1950s–1960s based on the complete lack of this species in plankton samples in the 1950s and 1960s and the subsequently common occurrence of the representative species from AIW plankton samples in 2000 (Itaki et al. 2003). The existence of warm-water species in cold-water regions is not always due to global warming. In the Norwegian Sea, typical subtropical and tropical surface water polycystines like *Didymocyrtis tetrathalamus* were collected in 2010 (Bjørklund and Kruglikova 2003). These species are in general dominant at a temperature of about

25°C, but the water temperature recorded in 2010 was lower than 10°C. Similar "warm-water species pulses" were detected in the Norwegian Sea when the sea surface water was below 10°C for a century; thus these pulses were not caused by global warming and other abrupt intrusion events of warm waters.

The value of atmospheric CO_2 at the present day is approximately 380 ppm, the highest over the past 0.8 Myr, and its atmospheric CO_2 pressure (pCO_2) ranges from 0.03 to 0.04 kPa (Jarrold 2012). According to Sabine et al. (2004), one third of CO_2 released into the atmosphere is absorbed by the oceans, and the elevated pCO_2 causes ocean acidification of seawater. It is noted that increasing CO_2 had already started as far back as 18 Ka from 180 ppmv (pCO_2) to 280 ppmv in the Holocene (Petit et al. 1999). The age 18 Ka is the period when the glacial period ended. Carbon dioxide dissolves from the surface water, and subsequently alkalinity in seawater rises. The anthropogenic activity after the Industrial Revolution saw pH in seawater reduced on the order of 0.1 (Doney et al. 2009). It is likely that the elevated CO_2 contributes to increasing the concentrations of marine chlorophyll-*a*, but it substantially decreases not only in coastal areas but also in the oligotrophic subtropical gyres for decades (Gregg et al. 2003, 2005; Polovina et al. 2008; Irwin and Oliver 2009). The global primary productivity estimated from chlorophyll-*a* shows a strong inverse correlation with sea surface temperature (Behrenfeld et al. 2006; Martinez et al. 2009). This was caused by reduction of mixed layer depth and vertical mixing. The numerical simulations of marine chlorophyll-*a* concentrations in response to anthropogenic CO_2 emissions result in a 50 % decline of them between AD 2000 and AD 2200, although this model was estimated only on physical conditions and was not concerned with biological effects, due to uncertainty (Hofmann et al. 2011). The decline in marine chlorophyll-*a* concentrations is caused by weakened vertical mixing and shortage of nutrient supply from intermediate waters (probably below the thermocline) by increased sea surface temperatures, and by diminishment of nutrient supply via fresh flow from the land.

A 40-year monitoring of calcifying plankton and pH in the North Sea showed that the abundance of foraminifers and coccolithophores has risen over the last few decades, suggesting enhanced proliferation rather than damage to their survival (Beare et al. 2013). Instead, bivalve larvae and pteropods have decreased in abundance, and thus the tolerance ability with respect to ocean acidification differs among plankton groups. The sensitivity of foraminifers and coccolithophores to ocean acidification seems very complex. A culture experiment on coccolithophorid *Emiliania huxleyi* and *Gephyrocapsa oceanica* from 300 ppmv (pCO_2) or pH 8.2 to 750 ppmv (pCO_2) or pH 7.8 resulted in reduced calcite production at increased CO_2 concentrations. This was also accompanied by increased malformed coccoliths and incomplete coccospheres (Riebesell et al. 2000). However, the weight and size of coccoliths does not significantly change in acidification laboratory experiments, and coccoliths gain weight under the highest coccolithophore production, without an acidification effect (Beaufort et al. 2007). Furthermore, some clone types of *Emiliania huxleyi* survived or adapted themselves to ocean acidification in 500-generation selection culture experiments (Lohbeck et al. 2012). Increasing knowledge on the effects of ocean acidification on coccolithophores has led to the

opinion that coccolithophores have adapted to increased alkalinity in oceans. The effect of ocean acidification has also been examined with planktonic foraminifers. The shell weight of *Globigerina bulloides* in the Southern Ocean shows a strong negative correlation with the pCO_2 record during the past 50 Kyr: 30.3 μg at 18 Ka and 24.4 μg in the late Holocene (Moy et al. 2009). The pCO_2-related reduction in the shell weight of *Neogloboquadrina pachyderma*, a cold-water planktonic foraminifer, has also been observed in a culture experiment, but the weight reduction liberalizes if decreased pH is associated with increased temperature (Manno et al. 2012). This may explain why planktonic foraminifers have retained their standing stock for 40 years in the Norwegian Sea.

The acidification of seawater will also affect marine benthic protistans such as foraminifers. Benthic foraminifers may be differently influenced in relation to their habitat, as they live on sea grass, requiring sunlight for photosynthesis of algal symbionts, just on the seafloor surface, or in the sediments. According to Uthicke et al. (2013), any benthic foraminifers were almost absent with decreased pH (<7.9). By contrast, the field experiment with elevated pCO_2 resulted in no serious effect on *Ammonia aomoriensis*, a living benthic foraminifer (Haynert et al. 2013).

Recent studies on the impact of anthropogenic change on marine protists have occasionally led to different conclusions. However, we can infer what has happened in marine protists with increasing atmospheric warming, increasing pCO_2, and other changes in consideration of the fossil records. For instance, the atmospheric CO_2 has ranged between 300 and 1000 ppmv in the past 300 Myr, and the mean ocean surface pH is estimated to have ranged from 7.5 to 8.1 in this geologic time interval (Hönisch et al. 2012). Human begins may be frightened to lose ice from both the North Pole and the Antarctica, but the geological records reveal that the ice has brought ice-rafted debris since 45 Ma in the Northern Hemisphere, whereas it has covered the East Antarctic since 14 Ma in the Southern Hemisphere (Zachos et al. 2001; Moran et al. 2006). This means that the presence of ice in both hemispheres has a very short history in the Cenozoic. In addition to that, episodic fresh surface water relevant to the present temperate climate was widely distributed in the Arctic Ocean around 48 Ma (Brinkhuis et al. 2006). Thus, the geological record in the Cenozoic shows "abnormally" cold conditions in the past 2.88 Myr. Focused on the micropaleontological records, calcifying marine protists like calcareous nannofossils (roughly relevant to haptophytes), planktonic and benthic foraminifers have survived in association with evolutionary turnovers through severe threatened events like the end-Permian double extinction (approximately 265 Ma), the Triassic–Jurassic mass extinction (201 Ma), the Toarcian ocean anoxic event (approximately 180 Ma), PETM (approximately 55 Ma) and strong cooling trends since 2.6 Ma (Hönisch et al. 2012). In consideration of no extinction of the main protist groups in the past 300 Myr, the modern marine protists may survive through the anthropogenic effects. The abrupt environmental changes impacting on marine protists took thousands of years (Hönisch et al. 2012), but the impact by anthropogenic effects are too rapid, which has never been experienced in geologic time.

The fate of marine protists under the continuous anthropogenic effects should be considered along with two other points. One is the inflow of essential elements to

the oceans and the other is the long-term effect on the deep oceans. A total of 30 Gt phosphate, 0.003 % of the total marine phosphate, is considered to be used by marine organisms. Mineral phosphates are mined at a rate of 140–150 Mt y^{-1} and a huge volume of phosphates are supplied to the oceans. The inflow to the oceans is estimated as 0.02 Gt y^{-1}, twice as large as the pre-industrial period. This leads to a nitrogen–phosphorous imbalance in the seawater (Peñuelas et al. 2013). The inflow of dissolved iron by human activity may also perturb the productivity of HNLC (high nutrient–low chlorophyll) areas.

The anthropogenic effects will inevitably bring changes in protistan life regardless of positive or negative feedback. Further evaluations from various points of view are needed.

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