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DINOFLAGELLATES: A REMARKABLE EVOLUTIONARY EXPERIMENT[1]

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In this paper, we focus on dinoflagellate ecology, toxin production, fossil record, and a molecular phylogenetic analysis of hosts and plastids. Of ecological interest are the swimming and feeding behavior, bioluminescence, and symbioses of dinoflagellates with corals. The many varieties of dinoflagellate toxins, their biological effects, and current knowledge of their origin are discussed. Knowledge of dinoflagellate evolution is aided by a rich fossil record that can be used to document their emergence and diversification. However, recent biogeochemical studies indicate that dinoflagellates may be much older than previously believed. A remarkable feature of dinoflagellates is their unique genome structure and gene regulation. The nuclear genomes of these algae are of enormous size, lack nucleosomes, and have permanently condensed chromosomes. This chapter reviews the current knowledge of gene regulation and transcription in dinoflagellates with regard to the unique aspects of the nuclear genome. Previous work shows the plastid genome of typical dinoflagellates to have been reduced to single-gene minicircles that encode only a small number of proteins. Recent studies have demonstrated that the majority of the plastid genome has been transferred to the nucleus, which makes the dinoflagellates the only eukaryotes to encode the majority of typical plastid genes in the nucleus. The evolution of the dinoflagellate plastid and the implications of these results for understanding organellar genome evolution are discussed.

Key words: dinoflagellate; endosymbiosis; evolution; harmful algal blooms.

The dinoflagellates (division Pyrrhophyta, class Dinophyceae) are an important group of phytoplankton in marine and fresh waters. Their adaptation to a wide variety of environments is reflected by a tremendous diversity in form and nutrition and an extensive fossil record dating back several hundred million years (Graham and Wilcox, 2000). As swimming cells, they can flourish under conditions that are unsuitable for many nonmotile phytoplankton, a success due in part to unique behavior patterns, including diel vertical migration (migration through the water column on a 24-h cycle). Some dinoflagellates produce toxins that are dangerous to man, marine mammals, fish, seabirds, and other components of the marine food chain (Van Dolah, 2000). Others are bioluminescent and emit light; some function as parasites or symbionts that rely on host organisms for part of their nutrition. Many dinoflagellates are photosynthetic and, through endosymbiosis, have acquired a wide diversity of plastids from distant evolutionary lineages. The most common plastid in dinoflagellates has been subject to drastic evolutionary changes that we are only beginning to understand. An equal number of dinoflagellates obtain their carbon by ingesting other phytoplankton. Many are now being shown to have both of these traits—i.e., to be mixotrophic. It is thus no surprise that these organisms have been extensively studied and classified as plants by some workers and as animals by others.

General characteristics—Whether living as a swimming, solitary cell or a nonmotile symbiont within an invertebrate host, all living dinoflagellates have certain common characteristics (Steidinger, 1983). Most photosynthetic species contain chlorophylls *a* and c_2, the carotenoid beta-carotene, and a group of xanthophylls that appears to be unique to dinoflagellates, typically peridinin, dinoxanthin, and diadinoxanthin. These pigments give many dinoflagellates their typical golden-brown color. However, some dinoflagellates have acquired other pigments through endosymbiosis, including fucoxanthin (see the following plastid discussion). Two different cell types can be distinguished on the basis of the cell-wall covering or theca. The ''naked'' or unarmored forms have an outer plasmalemma surrounding a single layer of flattened vesicles. These cells are fragile and distort easily. Armored dinoflagellates have cellulose or other polysaccharides within each vesicle, giving the cells a more rigid, inflexible wall. These cellulose plates are arranged in distinct patterns (called ''tabulation''), which are extensively used as taxonomic ''fingerprints.'' For a detailed discussion of dinoflagellate taxonomy, see Fensome et al. (1993). The dinoflagellate nucleus is unique in several ways, as elaborated in more detail later. The chromosomes, for example, are easily visible at all stages of growth because they do not go through coiling and uncoiling, as is common in other phytoplankton, but instead remain permanently condensed. Dinoflagellates also have few or no nucleosomes associated with their DNA and a unique pattern of mitosis (Spector, 1984). Because these characteristics are so different from both eukaryotic and prokaryotic cells, a new intermediate kingdom, Mesokaryota, was once proposed for them (Dodge, 1965). Yet another distinguishing characteristic of dinoflagellates is that their motile cells have two unequal flagella. One is a flattened, ribbon-like flagellum, which encircles the cell in a transverse groove, providing propulsive and spinning force for the cell. The other flagellum is directed posteriorly along a longitudinal groove and presumably acts like a rudder for steering. Although all dinoflagellates share certain physiological and structural characteristics, they exhibit a tremendous diversity in external morphology. Some cells are small and smoothly spherical, whereas others have elaborate structures that resemble horns, wings, collars, or even arms and hands with fingers.

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Ecology—Several aspects of the behavior, physiology, and ecology of dinoflagellates are notable and will be highlighted next. These include swimming behavior, bioluminescence, heterotrophy, symbiosis, and toxicity.

Swimming behavior—As motile cells, dinoflagellates are capable of directed swimming behavior in response to a variety of parameters. These include chemotaxis, phototaxis, and geotaxis, for which movement is controlled by chemical stimuli, light, or gravity, respectively. It has long been observed that many dinoflagellates do not move randomly through the water column but instead aggregate at specific depths that can vary with the time of day. This vertical migration has proven to be a highly complex process that varies between species and with environmental or nutritional conditions (Cullen and MacIntyre, 1998). Velocities on the order of 1 m/h are common. Although light may not be the major factor that determines the directionality of vertical migration, it certainly affects the extent of that motion. Past observations that cells tend to aggregate closer to the water surface on cloudy or overcast days have been complemented by detailed laboratory studies that document the active selection of certain light levels by some dinoflagellates (Anderson and Stolzenbach, 1985). Whereas other nonmotile phytoplankton may sink or are unable to consistently obtain nutrients, dinoflagellates can position themselves in the water column to take full advantage of available light and nutrients.

Heterotrophy—About one-half of extant dinoflagellates lack a plastid or pigments to carry out photosynthesis (Gaines and Elbrachter, 1987). These heterotrophic species have both naked and armored cell walls and occur in every type of aquatic environment. Most naked heterotrophic dinoflagellates have flexible cell walls that allow them to engulf living cells and particles (termed phagotrophy), which can then be seen inside the colorless dinoflagellate. Some naked species deploy a thin, tubelike extension called a peduncle to penetrate prey and withdraw the contents. The feeding behavior of the armored or thecate heterotrophic dinoflagellates was completely unknown until recently. Some of these species have developed a remarkable pseudopod-like structure, that is extruded from the cell and flows around the prey, enveloping it so the contents can then be digested. Termed a ''feeding veil'' or ''pallium'' (Jacobson and Anderson, 1992), the retractile organelle easily spreads over long spines on diatoms and sometimes envelops as many as 70 diatoms in a chain (Fig. 1). Other types of phytoplankton, including dinoflagellates, are also used as food.

Bioluminescence—The spectacular display of blue sparkling light seen as waves break on beaches or as a boat passes through the water in the night is called bioluminescence. Many organisms in the ocean emit such light, although dinoflagellates are the only photosynthetic organisms capable of this behavior (Sweeney, 1987). It is widely accepted that dinoflagellates account for much of the planktonic bioluminescence in the ocean (Kelly and Tett, 1978). The biochemistry, physiology, and molecular biology of dinoflagellate luminescence are relatively well understood. The bioluminescence system consists of the enzyme luciferase, its substrate luciferin, and a protein that binds luciferin. Bioluminescence appears to be compartmentalized in discrete particles called ''scintillons'' within the cell. Nearly all luminescent organisms in the ocean emit light with a peak wavelength near 490 nm, and dinoflagellates are no exception. It is no coincidence that this blue–green color is the wavelength that is least attenuated in water and most visible to marine animals. Because attenuation of other wavelengths would be negligible over the short distances at which luminescence is thought to be effective in organism–organism interactions, it is commonly believed that it is blue–green not because it travels further in water but simply because it matches the photoreceptors of most marine organisms (i.e., it can be seen). The ecological advantage of bioluminescent flashes has been the subject of considerable speculation. One of the suggested functions, supported by experimental data, is that it decreases the grazing behavior of copepods (Buskey and Swift, 1983). Not all dinoflagellates are bioluminescent, however, and luminescent and nonluminescent strains of the same species are common. Bioluminescence may thus be considered a useful but nonessential survival strategy.

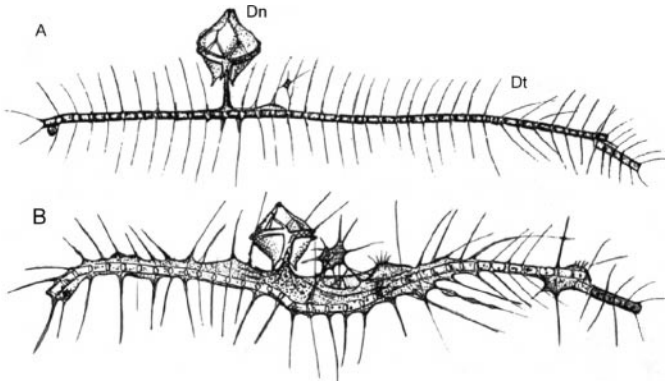

Fig. 1. An illustration of the dinoflagellate (Dn) *Protoperidinium depressum* feeding on a chain of diatoms (Dt) using a pallium, a retractile organelle that spreads over the long spines of diatoms so that the contents can be digested. Illustration by D. M. Jacobson (reproduced from Jacobson, 1987).

Symbiosis—Some dinoflagellates (called zooxanthellae) are capable of forming symbioses with a phylogenetically wide range of marine protists and invertebrate animals (for a review, see Trench, 1993, 1997). Within the dinoflagellate lineage, at least seven genera from four orders are found in symbiotic associations (Banaszak et al., 1993). The polyphyletic origin of symbiotic dinoflagellates supports the idea that this trait arose independently several times in evolutionary history (McNally et al., 1994). As with dinoflagellates in general, however, the molecular phylogenetic relationships of symbiotic dinoflagellates remain to be clarified. Interestingly, small subunit ribosomal RNA analyses show the diversity within the genus *Symbiodinium* to be comparable to that found between different genera or orders of free-living species (Rowan and Powers, 1992).

The hosts in dinoflagellate associations with other organisms include foraminifera, radiolarians, flatworms, anemones, jellyfish, and even bivalve mollusks. The best-studied relationship, however, is between zooxanthellae of *Symbiodinium* and hermatypic, or reef-forming corals. The relationship between corals and the dinoflagellate is a mutualistic symbiosis (i.e., both organisms benefit). Corals with a dinoflagellate symbiont calcify much faster than those without, an effect linked to photosynthetic fixation of CO_2 by the dinoflagellates (Marshall, 1996). A significant amount of photosynthetic product is excreted by the symbiotic dinoflagellates, primarily as glycerol. Up to 50% of the fixed carbon may be transferred to the

host (Paracer and Ahmadjian, 2000), in which it is converted mainly to lipids and proteins. A number of other small metabolites, such as glucose, alanine, and organic acids, are also translocated to the host. On the dinoflagellate side, many of these symbioses occur in oligotrophic waters in which nutrients are scarce in the water column. Movement of metabolites from the host to the algae is less well studied, but it is likely that the host can provide a variety of organic nutrients (e.g., urea, glycerophosphate, amino acids) as well as other compounds such as growth factors. This close reciprocal relationship between dinoflagellates and invertebrates, as typified by the *Symbiodinium*–coral association, is thought to contribute significantly to the ecological success of their respective hosts (Trench, 1987; Stanley and Swart, 1995).

Toxicity—A number of dinoflagellate species are known to produce potent neurotoxins, which are often associated with the phenomena commonly called ''red tides.'' This term can be quite misleading, because many toxic blooms occur when waters are not discolored, but other blooms, in which the high biomass and pigments of the dinoflagellates turn the water red are not toxic (Smayda, 1997). These outbreaks are now called harmful algal blooms or HABs. Documentation of HABs has expanded greatly over the last few decades, and presently, nearly every country with marine waters is known to be affected by these blooms (Hallegraeff, 1993). HAB toxins can affect humans, other mammals, seabirds, fish, and many other animals and organisms. One major category of impact occurs when toxic species are filtered from the water as food by shellfish, which then accumulate the algal toxins to levels that can be lethal to humans or other consumers (Shumway, 1989). The poisoning syndromes linked to dinoflagellates have been given the names paralytic (PSP), diarrhetic (DSP), neurotoxic (NSP), and azaspiracid shellfish poisoning (AZP). A fifth human illness, ciguatera fish poisoning (CFP) is caused by ciguatoxins produced by dinoflagellates that attach to surfaces in many coral reef communities (Lehane and Lewis, 2000). The final human illness linked to toxic algae is called possible estuary-associated syndrome (PEAS). This vague term reflects the poor state of knowledge of the human health effects of the dinoflagellate *Pfiesteria piscicida* and related organisms that have been linked to symptoms such as deficiencies in learning and memory, skin lesions, and acute respiratory and eye irritation, all after exposure to estuarine waters in which *Pfiesteria*-like organisms have been present (Burkholder et al., 1998).

''Blooms'' of neurotoxic dinoflagellates from several genera result in outbreaks of PSP, probably the most widespread of the poisoning syndromes. The economic, public health, and ecosystem impacts of PSP outbreaks take a variety of forms and include human intoxications and death from contaminated shellfish or fish, loss of natural and cultured seafood resources, impairment of tourism and recreational activities, alterations of marine trophic structure, and death of marine mammals, fish, and seabirds. PSP is caused by the saxitoxins, a family of heterocyclic guanidines that bind to sodium channels responsible for the flux of sodium in nerve and muscle cells. Saxitoxin, by mass, is 1000 times more potent than cyanide and 50 times stronger than curare. It is, like most of the other dinoflagellate toxins, just one member of a toxin family of related compounds. The origin of saxitoxins has been controversial as toxic species are paraphyletic within the genus *Alexandrium*, and there are toxic and nontoxic strains of the same species, which may relate to the hypothesis that the ability to produce the toxins actually lies in symbiotic bacteria and not the dinoflagellate (Silva, 1978; Kodama et al., 1988; Vasquez et al., 2001). Some researchers have suggested that bacteria associated with *Alexandrium* are capable of producing saxitoxins (Gallacher et al., 1997; Vasquez et al., 2001), whereas others argued that toxin production ability remains when all symbiotic bacteria have been removed (Hold et al., 2001). In this context, it is of note that the ability to produce saxitoxins has also been acquired by other organisms not closely related to *Alexandrium*. These include the dinoflagellates *Gymnodinium catenatum* (Oshima et al., 1993; Sako et al., 2001) and *Pyrodinium bahamense* var *compressum* (Usup et al., 1994), the cyanobacteria *Aphanizomenon flos-aquae* (Pereira et al., 2000) and *Planktothrix* sp. (Pomati et al., 2000), and other bacteria (Kodama et al., 1988; Levasseur et al., 1996). The explanation for the acquisition of toxin producing ability by such disparate organisms may be related to the apparent ease with which this trait has been acquired and lost within *Alexandrium* (Lilly, 2003).

Another important dinoflagellate toxin family is the brevetoxins, a suite of polycyclic ether compounds produced by *Karenia brevis* (Van Dolah, 2000). Brevetoxins bind with high affinity to the sodium channel, resulting in persistent activation and prolonged channel opening. As with saxitoxins, the brevetoxins are a family of compounds that exhibit different potencies. Brevetoxins can accumulate in filter-feeding shellfish, causing NSP, but other impacts occur because *K. brevis* is an unarmored dinoflagellate, and thus the cells are easily lysed. Released toxin can quickly be lethal to fish and other marine animals that are not filter feeders. Fish mortalities from *K. brevis* blooms (often true red tides) can be massive, involving tens of millions of wild fish of all types. Another impact from brevetoxins is a result of inhalation of aerosolized toxin in sea spray, which causes irritation and burning of the throat and upper respiratory tract of exposed humans. Marine mammals, especially the endangered Florida manatee, have recently been shown to be susceptible to brevetoxin ingestion or even inhalation (O'Shea et al., 1991).

Another family of polyether toxins is called the ciguatoxins (reviewed by Lehane and Lewis, 2000). These originate in the dinoflagellate *Gambierdiscus toxicus*, which has an epiphytic existence, living attached to seaweeds and other surfaces. Herbivorous fish accumulate the lipid-soluble toxin, which is passed up the food chain to higher predators, and ultimately to human consumers. It is estimated that over 50 000 people are affected annually (Ragelis, 1984). The ciguatoxins are structurally related to the brevetoxins and compete with brevetoxin for a site on the voltage-dependent sodium channel. The definition of ciguatera is complicated by the fact that *G. toxicus* is only one member of a diverse assemblage of benthic or epiphytic dinoflagellates, many of which produce toxins. Unlike the planktonic dinoflagellates, toxicity in the benthic coral reef dinoflagellates is common (Anderson and Lobel, 1987).

The diarrhetic shellfish toxins responsible for DSP are another class of polyether compounds produced by some species in the genera *Dinophysis* and *Prorocentrum*. This toxin class consists of at least eight congeners, including okadaic acid (van Dolah, 2000). These compounds are inhibitors of Ser/Thr protein phosphatases, which are critical components of signaling cascades in eukaryotic cells that regulate an array of cellular processes. Diarrhea associated with DSP is most likely

due to the hyperphosphorylation of proteins, including ion channels, in the intestinal epithelia, resulting in impaired water balance and loss of fluids.

A final group of dinoflagellate toxins is called the azaspiracids (AZAs), recently discovered to be associated with the heterotrophic species *Protoperidinium crassipes* (James et al., 2003). AZAs are potent, lipid-soluble neurotoxins, the pharmacology of which is generally unknown. Because consumption of contaminated shellfish by humans can result in symptoms of severe gastroenteritis, the syndrome may be confused with DSP. The full human etiology of AZA is unknown, but tests on laboratory mice have shown that chronic doses of AZA too low to cause acute illness result in damage to the liver, small intestine, and lymphoid tissues including the thymus and spleen. Low, chronic doses of AZA are also observed to be carcinogenic in laboratory mice, causing lung tumors (Ito et al., 2002). Cytological assays have indicated that AZAs are neither sodium channel blockers, like the PSP toxins, nor protease inhibitors, like the DSP toxins. AZAs cause apoptosis and inhibition of protein synthesis when applied in cell culture assays (Flanagan, 2001). AZA is the only known neurotoxin produced by a heterotrophic dinoflagellate, which raises obvious questions about the link between different food items and the toxicity of *P. crassipes* and also about the potential of other *Protoperidinium* species to produce this and similar toxins.

Evolutionary history of the dinoflagellates—There is a rich fossil and biogeochemical record for the dinoflagellates. Premesozoic fossils of dinoflagellates, however, have been controversial, and evidence for dinoflagellates comes primarily from fossilized cysts, first found from the early Triassic period (245–208 million years ago [mya], Fensome et al., 1999). There was clearly a dramatic increase in both numbers and diversity of dinoflagellates in the Jurassic (208–144 mya) and Cretaceous (144–66 mya), although they are declining today. The presence of dinosteranes, a sterol almost exclusively associated with dinoflagellates (related compounds are found in haptophytes; Withers, 1987; Volkman et al., 1990), also supports a mesozoic radiation of the dinoflagellates, showing a dramatic increase beginning in the Permian through the Cretaceous (Moldowan et al., 1996; Moldowan and Talyzina, 1998). Importantly, these compounds were also detected in rocks as far back as the Proterozoic, correlating with the presence of some acritarchs (fossilized cysts of unknown taxonomy), suggesting that these organisms may be among the ancestors of dinoflagellates (Moldowan et al., 1996; Moldowan and Talyzina, 1998; Fensome et al., 1999).

Molecular phylogenetic analyses place dinoflagellates in the kingdom Alveolata (Cavalier-Smith, 1991) with the ciliates and apicomplexans. This relationship is well supported in molecular trees (e.g., Gajadhar et al., 1991; Fast et al., 2002). Current data indicate that the ciliates are sister to the rest of the group, with the apicomplexans and dinoflagellates as sisters. The alveolates are often united with another group of protists, the Chromista (cryptophytes, haptophytes, and stramenopiles) that also contain chlorophyll *c* and tubular mitochondrial cristae (except for the cryptophytes that have flat cristae). Together these organisms were termed the ''chromalveolates'' (Cavalier-Smith, 1999). Recent analyses using nuclear genes have supported a sister relationship between the chromalveolate groups, the stramenopiles and alveolates (Van de Peer and De Wachter, 1997; Baldauf et al., 2000; Nozaki et al., 2003). Shared characteristics of the photosynthetic organelle (plastid) among chromalveolates and evidence from plastid gene analyses have led to the hypothesis that this organelle originated through secondary endosymbiosis of a red alga in their common ancestor (see the dinoflagellate plastids discussion).

Deciphering the internal relationships among dinoflagellates has been much more difficult. Most studies have focused on photosynthetic taxa, although recently, several important studies involving heterotrophic species have been published. Analyses of multiple proteins have shown the heterotrophic *Oxyrrhis marina* and the parasitic *Perkinsus marinus* are sister to the rest of the dinoflagellate lineage (Saldarriaga et al., 2003). Environmental PCR studies have revealed an amazing diversity of unidentified organisms that branch at the base of the dinoflagellates in phylogenetic trees. Using 18S rDNA amplified from seawater samples of picoplankton, López-García et al. (2001) and Moon-van der Staay et al. (2001) revealed diverse lineages that branch between *Perkinsus* and the dinoflagellates. López-García et al. (2001) discovered two well-supported clades of unidentified alveolates (one of which might be Syndiniales; Saldarriaga et al., 2001) at the base of the dinoflagellates, and they hypothesize this could reconcile the discrepancy between the dinoflagellate fossil record and the biogeochemical evidence (i.e., dinosteranes) for pre-mesozoic dinoflagellates. These small alveolates may have been responsible for the pre-mesozoic production of dinosteranes and are either not well preserved in the fossil record or have been misidentified as prokaryotes (López-García et al., 2001). These unidentified alveolates were discovered in aphotic regions of the water column, indicating they are heterotrophic.

Molecular analyses using small subunit (SSU) rDNA have been unable to resolve many relationships within the dinoflagellates, even though they have included a broad taxon sampling (Saunders et al., 1997; Gunderson et al., 1999; Saldarriaga et al., 2001). Analyses of the large subunit (LSU) rDNA have included fewer taxa but show greater phylogenetic support and resolve several major dinoflagellate clades (Daugbjerg et al., 2000). However, the relationships among these clades remain unclear, including relationships within the gymnodinoid, peridinoid, and prorocentroid groups (GPP complex, Saunders et al., 1997). Figure 2 is a schematic tree representing the current knowledge of dinoflagellate relationships using molecular data. Molecular analyses have generally supported the relationships determined using morphological characters (Fensome et al., 1999; Daugbjerg et al., 2000).

Dinoflagellate genetic structure and gene regulation—Dinoflagellates possess a number of remarkable genetic characteristics that distinguish them from other eukaryotes (reviewed in Rizzo, 1991). One of the most striking features is the large amount of cellular DNA that they contain. Most eukaryotic algae contain on average about 0.54 pg DNA/cell^{1}, whereas estimates of dinoflagellate DNA content range from 3–250 pg/cell^{1} (Spector, 1984), corresponding to approximately 3000–215 000 Mb (in comparison, the haploid human genome is 3180 Mb and hexaploid *Triticum* wheat is 16 000 Mb). It has been suggested that polyploidy or polyteny may account for this large cellular DNA content (Beam and Himes, 1984), but studies of DNA reassociation kinetics do not support this hypothesis. In the heterotrophic dinoflagellate *Crypthecodinium cohnii*, about one-half of the genome is comprised of unique sequences (1–3 copies) interspersed with repeats of approxi-

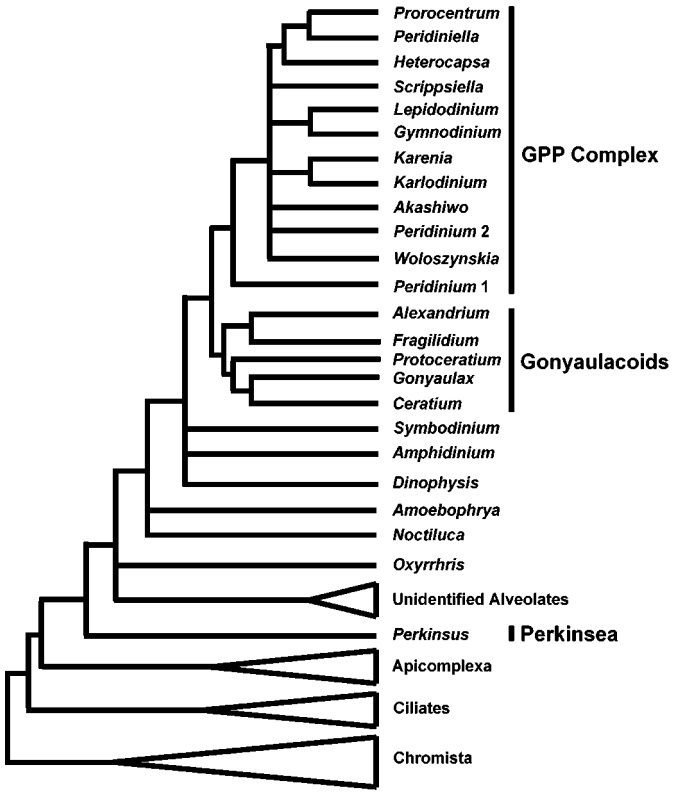

Fig. 2. A schematic phylogenetic tree of the dinoflagellates that illustrates currently supported relationships within the group using molecular data (see text for details).

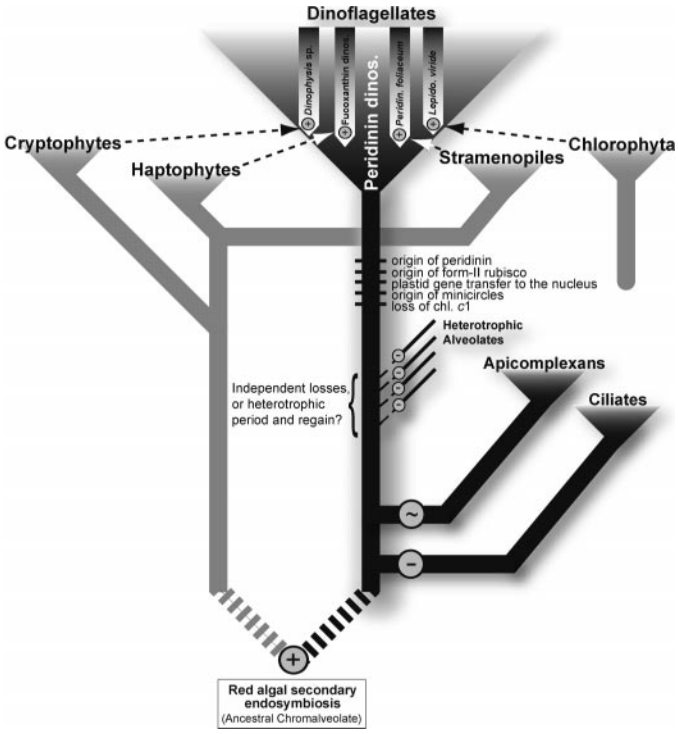

Fig. 3. An illustration showing the major events in plastid evolution in the alveolate lineage, with an emphasis on the dinoflagellates. The (+) indicates plastid gain, the (−) indicates plastid loss, and the (~) indicates the origin of the apicoplast in the apicomplexans. The putative multiple independent losses of the peridinin plastid in the dinoflagellate lineage are not shown. *Peridin*. is *Peridinium*, *Lepido*. is *Lepidodinium*, and chl. is chloropyll.

mately 600 nt (Allen et al., 1975; Hinnebusch et al., 1980). Reassociation kinetics indicated that the complexity of slowly renaturing unique DNA is about 1.5×10^9 base pairs, an amount typical of ''higher'' eukaryotes. In contrast, the complexity of the unique DNA in the autotrophic dinoflagellate *Wolosynskia bosteniensis* was calculated to be 1.32×10^{10} base pairs, about one order of magnitude larger than mammalian single copy DNA (Davies et al., 1988).

In addition to their disproportionately large genomes, dinoflagellate nuclei are unique in their morphology, regulation, and composition. Dinoflagellate nuclei vary in shape, including round, tetragonal, triangular, and kidney and horseshoe shapes, and they contain a large number of chromosomes (ca. 143 in *Alexandrium fundyense*) that remain attached to the nuclear envelope during cell division (Oakley and Dodge, 1974). The chromosomes are morphologically similar to one another (Loeblich, 1976) and remain permanently condensed throughout the cell cycle (Dodge, 1966). Dinoflagellates are the only eukaryotes with DNA that contains 5-hydroxymethylmuracil, which replaces 12–70% of the thymidine (Rae, 1976). In addition, dinoflagellate DNA contains 5-methylcytosine and the rare N^6-methyladenine (Rae and Steele, 1978). A well-characterized difference is the absence of typical histones in dinoflagellates and the presence of basic proteins that are involved in the organization of the genome (Rizzo, 1981). In addition, the upstream regions of genes lack typical eukaryotic transcriptional elements (e.g., TATA boxes) and downstream polyadenylation sites, implying potentially novel regulatory mechanisms (Lee et al., 1993; Le et al., 1997; Li and Hastings, 1998).

Studies of dinoflagellate gene expression have indicated that these organisms use both transcriptional and post-transcriptional regulation in roughly equal measure, with the iron superoxide dismutase of *Lingulodinium polyedrum* exhibiting both modes, depending upon the stimulus (Okamoto et al., 2001). Transcriptional regulation has been shown for peridinin-chlorophyll *a* binding protein (Triplett et al., 1993), S-adenosyl-homocysteine-hydrolase-like protein, methionine-aminopeptidase-like protein, and histone-like protein (Taroncher-Oldenburg and Anderson, 2000). Post-transcriptional regulation has been shown for luciferin-binding protein (Morse et al., 1989) and glyceraldehyde-3-phosphate dehydrogenase (Fagan et al., 1999). A recent study by Lin et al. (2002) revealed the presence of a novel type of substitutional RNA editing in several mitochondrial mRNAs in the dinoflagellates *Pfiesteria piscicida*, *Prorocentrum minimum*, and *Crypthecodinium cohnii*. RNA editing results in post-transcriptional retailoring of mRNA, which is manifest as changes in the RNA sequence when compared to that of the encoding DNA. Known substitutional mRNA editing mechanisms involve either U to C or C to U transitions. Dinoflagellate mitochondrial mRNA editing shows these changes, in addition to A to G transitions and a small number of transversions, which indicates that the dinoflagellates have multiple editing mechanisms or a single novel mechanism that can perform both types of changes (Lin et al., 2002; Gray, 2003). RNA editing systems are well known from a number of eukaryotes, but these are so far notably absent from ciliates and apicomplexans, both close relatives of the dinoflagellate lineage (e.g., Gajadhar et al., 1991; Edqvist et al., 2000; Rehkopf et al., 2000). This may

indicate that the RNA editing mechanism observed in dinoflagellates arose independently, early in the dinoflagellate lineage (Lin et al., 2002; Gray, 2003).

The unique physical features of dinoflagellate chromosomes are likely to affect both gene transcription and regulation. Dinoflagellate DNA is packaged at a protein : DNA ratio of 1 : 10, unlike the equimolar ratios found in other eukaryotes. Experimental evidence has indicated that the DNA is organized into two chromosomal regions: a main body composed of genetically inactive or ''silent'' DNA and a peripheral, diffuse region containing transcriptionally active DNA. This has been demonstrated through incorporation of tritiated adenine (Sigee, 1984), immunological detection of Z-DNA (which assists in unraveling chromosomal material) in extrachromosomal loops (Soyer-Gobillard et al., 1990), and mild restriction endonuclease digestion of isolated intact nuclei (Anderson et al., 1992). Dinoflagellate basic nuclear proteins have a much lower affinity for DNA than do common core histones (Vernet et al., 1990), and psoralen cross-linking reveals that only 20% of the genome is in protected regions that are organized in 10–15-kbp units separated by unprotected longer regions (Yen et al., 1978). Taken together, these findings have confirmed an earlier hypothesis (Soyer and Haapala, 1974) that transcription of active DNA occurs extrachromosomally where DNA processing enzymes may access the sequences outside of the condensed chromosomes. Furthermore, these findings have illustrated the unusual higher-order DNA structure present in the dinoflagellate nucleus. The timely expression of genes is directly related to such higher-order structures (for review, see Getzenberg et al., 1991).

All of these data indicate that the organization and regulation of dinoflagellate genes is very different from that of most other eukaryotes. Given the vast quantities of DNA in their cells, our basic knowledge of eukaryotic genetics and gene expression could be significantly increased by understanding dinoflagellates gene structure and transcriptional regulation. Unfortunately, it is the quantity of chromosomal DNA that has hampered genetic studies of dinoflagellates. DNA content makes it difficult to perform simple genomic hybridizations like Southern blots and impractical to construct genomic libraries or to consider sequencing the genome. To date, all of the data regarding gene regulation mechanisms in dinoflagellates has emerged sporadically, from studies of specific genes that are of interest for a particular function. The application of genomic technologies, such as expressed sequence tag (EST) sequencing and global gene expression profiling methods, would enable us to learn about many genes or transcripts simultaneously, even in uncharacterized systems like dinoflagellates. Global gene expression analyses have already been used to identify redox-regulated genes in the dinoflagellate, *Pyrocystis lunula* (Okamoto and Hastings, 2003).

The plastids of dinoflagellates—Among eukaryotes, acquisition of a photosynthetic organelle appears to be a rare event. The first plastid was probably acquired once from a cyanobacterium in the common ancestor of glaucophytes, red algae, and green algae (including land plants; Bhattacharya and Medlin, 1995). Reduction of the endosymbiont genome, gene transfer to the host nucleus, and evolution of a protein import system ensued to establish the primary plastid that is found in these lineages (McFadden, 1999). Secondary endosymbiosis has probably occurred three times, contributing plastids to chlorarachniophytes and euglenids (likely through independent green algal endosymbioses) and chromists and alveolates (from a red algal endosymbiosis; Bhattacharya et al., 2004; Hagopian et al., in press; Yoon et al., 2004). In contrast, plastid acquisition and loss is relatively common in the dinoflagellates (Saldarriaga et al., 2001). Plastid-containing dinoflagellates make up approximately one-half of the known taxa and are among the most environmentally and economically important of these protists. The majority of plastid-containing dinoflagellates contain the photopigment peridinin, however, the dinoflagellates also contain an amazing diversity of plastid types (Schnepf and Elbrächter, 1999). Currently, there are five plastids known in this group, each with its own evolutionary history, making this group the champions of plastid endosymbiosis among eukaryotes.

Peridinin-containing dinoflagellates—The most common type of plastid in dinoflagellates is surrounded by three membranes and contains peridinin as the major carotenoid. This pigment, although similar in structure to fucoxanthin, is unique to this group. These dinoflagellates, like *Euglena*, have independently evolved a tripartite N-terminal extension containing two hydrophobic domains for targeting nuclear-coded plastid proteins to the organelle (Nassoury et al., 2003). The plastid genome in peridinin plastids is also remarkably different from that of other photosynthetic eukaryotes. Normally, plastids contain a circular genome that, although varying in complexity and genetic content, is about 150 kilobases (kb) in size and encodes approximately 100 genes. Even the plastid genomes of nonphotosynthetic eukaryotes (e.g., *Plasmodium falciparum*, *Epifagus virginiana*, *Euglena longa*) are a single circular molecule with reduced gene content; i.e., lacking the genes involved in photosynthesis. In contrast, the plastid genome of peridinin-containing dinoflagellates is reduced and broken up into minicircles. Currently, only 16 proteins encoded on these minicircles have been found, in addition to the LSU and a putative SSU of the plastid ribosomal RNA and ''empty'' minicircles and those encoding pseudogenes (Zhang et al., 1999; Barbrook and Howe, 2000; Hiller, 2001; Zhang et al., 2002; Howe et al., 2003; Ellen et al., 2004; Laatsch et al., 2004). These sequences code for the core subunits of the photosystem, cytochrome b_6f, ATP synthase complex (*atpA*, *atpB*, *petB*, *petD*, *psaA*, *psaB*, *psbA–E*, *psbI*) and four other proteins (*ycf16*, *ycf24*, *rpl28*, and *rpl23*). The remaining genes required for photosynthesis have been lost from the plastid and moved to the nucleus. Remarkably, a recent paper from Laatsch et al. (2004) provided evidence (based on partial sequences) that the minicircles in the peridinin dinoflagellate *Ceratium horridum* are present in the nucleus rather than in the plastid of this species. This raises the possibility that minicircle genes in different dinoflagellates may be found in either, or potentially both, plastids and nuclei. Clearly, the extent and type of plastid gene transfer in different dinoflagellates needs to be carefully examined to understand fully plastid evolution in this lineage.

The localization of the majority of the plastid genome in the nucleus has been recently documented for three dinoflagellates (*Alexandrium tamarense*, *Amphidinium carterae*, and *Lingulodinium polyedrum*) through EST sequencing (Hackett et al., 2004; Bachvaroff et al., 2004). Hackett et al. (2004) analyzed a set of 6480 unique cDNAs from *A. tamarense*, focusing on genes that are normally plastid coded in other organisms. They showed that 15 genes (among others) that are found in the plastid in every other photosynthetic eukaryote have been moved to the nucleus in this species. The dinofla-

gellates are the only eukaryotes to have these plastid proteins in the nucleus. These genes have also acquired the tripartite N-terminal transit peptides to target them to the plastid (Nassoury et al., 2003). Bachvaroff et al. (2004) found similar results regarding the migration of the plastid genome from EST sequencing of two other dinoflagellates. They also identified many other plastid-associated genes that are typically in the nucleus in photosynthetic eukaryotes. These genes were likely transferred from the nucleus of the red algal secondary endosymbiont in the common ancestor of the alveolates. The forces behind this massive gene transfer are not yet understood; however, it is clear that the peridinin dinoflagellates have been able to overcome the barriers of gene transfer that restrict these genes to the plastid genome in other eukaryotes.

Several hypotheses have been proposed to explain why some organellar genes are transferred to the nucleus and others remain. Mutation by oxygen-free radicals and Muller's ratchet effect of nonrecombining genomes seem in general to favor the transfer of organellar genes to the nucleus (Allen and Raven, 1996; Martin and Herrmann, 1998). The few genes that remain in the plastid are primarily the core subunits of the photosystem, cytochrome b_6f, and ATP synthase complexes (*atpA, atpB, petB, petD, psaA, psaB, psbA–E, psbI*), which supports the idea of co-localization of genes and gene products for the redox regulation of gene expression (CORR hypothesis, see Allen, 2003). Under this scenario, the core subunits of the photosystem remain encoded in the plastid, close to the functional site of the proteins, which allows the organism to maintain tight control of the redox potential in the plastid and respond quickly to changes, maximize efficiency and minimize the creation of harmful free radicals. Maintaining transcription and translation of these genes in plastids may be especially important in organisms with multiple plastids, in which one plastid may require more of a particular protein than others. Unlike other eukaryotes that have drastically reduced plastid genomes because of a loss of photosynthesis due to a parasitic lifestyle, the peridinin dinoflagellates have drastically reduced their plastid genome while retaining this ability. This makes these organisms a model for understanding organelle-to-nucleus gene transfer and for evaluating gene transfer hypotheses.

The dinoflagellates have also lost chlorophyll c_1, which is present in the chromists and have traded form I ribulose 1,5-bisphosphosphate carboxylase/oxygenase (rubisco), which is a multisubunit complex formed by eight large and eight small subunits, for the anaerobic proteobacterial form II rubisco, which forms homodimers and higher order multimers (Whitney et al., 1995; Morse et al., 1995). Form II rubisco has a much lower specificity for CO_2 over O_2 than the form I enzyme, raising the question of how dinoflagellates carry out carbon fixation with this enzyme in the presence of oxygen (for a review, see Palmer, 1995). However, recent studies have indicated that dinoflagellates may have a carbon-concentrating mechanism that might overcome this problem (Leggat et al., 1999).

Evolution of the peridinin plastid—Because of the presence of chlorophyll *c*, it has been proposed that the peridinin plastid of dinoflagellates and the plastids of the chromists (cryptophytes, haptophytes, and stramenopiles) share a common ancestor through secondary endosymbiosis of a red alga (and the subsequent evolution of chlorophyll *c*; Cavalier-Smith, 1999). According to the chromalveolate hypothesis (see Evolutionary History section), a red algal plastid was acquired in the common ancestor of the chromists and alveolates, which includes the dinoflagellates. This plastid was maintained in the chromist lineage and went through significant changes in the alveolates. The plastid was lost in the ciliates and reduced to the nonphotosynthetic apicoplast in the apicomplexans. The dinoflagellates evolved a tripartite targeting signal to shuttle proteins to the plastid, which was no longer inside the endoplasmic reticulum (ER). The minicircle genes may have provided the best answer to the question of the origin of the peridinin plastid. Zhang et al. (1999) did phylogenetic analyses using a concatenated set of seven minicircle genes and found that the peridinin plastid was sister to the chromists and red algae. A red algal origin remains the most parsimonious solution to the provenance of minicircle genes, but this hypothesis awaits evaluation through a multigene analysis that incorporates a broader taxon sampling for these highly divergent sequences.

Analyses of nuclear plastid-targeted genes have supported a specific relationship between chromist and alveolate plastid genes (Fast et al., 2001; Harper and Keeling, 2003). However, the internal relationships among the chromists and dinoflagellates are poorly resolved and do not clearly show chromists and alveolates as sisters. In contrast, analyses of light-harvesting proteins, plastid SSU rDNA, and plastid *atpI* showed a specific relationship between the stramenopiles and peridinin dinoflagellates to the exclusion of the cryptophytes and/or haptophytes (Durnford et al., 1999; Tengs et al., 2000; Hackett et al., 2004). This may potentially indicate that the plastids of the alveolates are more closely related to stramenopiles. It is still unclear, however, if these results stem from phylogenetic artifacts, lateral transfers of stramenopile genes, or a tertiary endosymbiosis of a stramenopile that gave rise to the peridinin plastid. It also appears that, like the chlorarachniophyte *Bigelowiella natans* (Archibald et al., 2003), *A. tamarense* has genes transferred from distantly related algal lineages. Phylogenetic analyses indicate that delta-aminolevulinic acid dehydratase and *cox2b* in *A. tamarense* have a green algal origin (Hackett et al., 2004), which indicates that lateral gene transfer may be common among mixotrophic protists.

Fucoxanthin-containing dinoflagellates—Karenia brevis, *K. mikimotoi*, and *Karlodinium micrum* contain a plastid bound by three membranes that contain 19′-hexanoyloxy-fucoxanthin and/or 19′-butanoyloxy-fucoxanthin as accessory pigments but do not contain peridinin. Because these pigments are also found in haptophyte algae, this plastid is believed to have originated from a haptophyte alga through tertiary endosymbiosis (Tengs et al., 2000). A haptophyte nucleus (i.e., nucleomorph) has not been detected in these species, indicating that all genes on this genome necessary for plastid function have presumably been transferred to the nucleus of the dinoflagellate. Based on analyses of the plastid genes *psaA* and *psbA*, Yoon et al. (2002) suggested that these unarmored, fucoxanthin-containing dinoflagellates may be an early diverging lineage, and they proposed a model of dinoflagellate evolution (see Morden and Sherwood, 2002) in which peridinin taxa were a derived group. However, this suggestion has been controversial, and the analyses were most likely misled by codon usage heterogeneity in the minicircle DNA sequences used to erect the relationships (Inagaki et al., 2004). Ribosomal DNA trees have thus far not unambiguously positioned the fucoxanthin-containing taxa in the dinoflagellate tree (Saunders et al., 1997; Daugbjerg et al., 2000; Saldarriaga et al., 2001). Mitochondrial *cob*, however, appears to be a better marker of dinoflagellate

phylogeny and robustly supports a derived position of *K. brevis* and *K. micrum* among peridinin-containing taxa (H. Zhang, D. Bhattacharya, S. Lin, unpublished data). If, as it now seems substantiated, the fucoxanthin-containing dinoflagellates are derived from a peridinin-containing ancestor, it will be important to determine the fate of the nuclear-encoded plastid genes in this and other tertiary plastid-containing lineages. Have these organisms retained their nuclear-encoded plastid genes, replaced them by transfers from the haptophyte endosymbiont, or eliminated them in favor of plastid-encoded homologs? This question has been answered for one gene, *psbO*, which is in the nucleus and appears to originate through lateral transfer from the haptophyte endosymbiont in *K. brevis* (Ishida and Green, 2002).

Other plastids in dinoflagellates—There are three additional plastid types in dinoflagellates that are particularly significant because they may illustrate intermediate stages of endosymbiosis. Several dinoflagellates contain ''kleptoplasts,'' temporary plastids stolen from prey through myzocytosis (Schnepf and Elbrächter, 1999). A heterotrophic dinoflagellate consuming photosynthetic eukaryotic prey may be the first stage in plastid endosymbiosis (Schnepf, 1993). Members of the genus *Dinophysis* are perhaps in the earliest stages of plastid acquisition through endosymbiosis. Photosynthetic members of this genus contain a plastid of cryptophyte origin, which was originally determined by analyses of ultrastructural and pigment characteristics (Schnepf and Elbrächter, 1988; Vesk et al., 1996). Recently, several studies have confirmed the cryptophyte origin of the plastid with molecular data (Takashita et al., 2002; Hackett et al., 2003; Jansen and Granéli, 2003). However, there are some significant differences between the cryptophyte and *Dinophysis* plastids. The cryptophyte plastid is surrounded by four membranes and contains a nucleomorph, a remnant of the red algal endosymbiont nucleus. In contrast, only two membranes surround the plastid of *Dinophysis* sp. and the nucleomorph is absent. Importantly, many genes that are necessary to maintain the plastid are coded in the nucleomorph of cryptophytes (Douglas et al., 2001). This apparent lack of a nucleomorph and the fact that *Dinophysis* species do not survive for long in cell culture have raised the possibility that the plastid of *Dinophysis* is a kleptoplast. Unfortunately, molecular studies have been unable to resolve this issue due to low levels of polymorphism in both plastid and nuclear genes, and an unresolved tree of the host cells (Takishita et al., 2002; Guillou et al., 2002; Hackett et al., 2003; Janson and Granéli, 2003). Current data indicated that, either the plastid of *Dinophysis* is a kleptoplast that is acquired from the same species of cryptophyte present around the world, or it is a permanent plastid and the genus shows little sequence divergence. Analyses of more variable plastid loci and comparison to a resolved host tree will be required to conclusively answer this question.

The second plastid is that of *Peridinium foliaceum* and *P. balticum*, for which the plastid originated from a diatom and contains fucoxanthin as the main carotenoid (Chesnick et al., 1996, 1997). The diatom endosymbiont is clearly a permanent plastid, as this species grows autotrophically in culture. These dinoflagellates contain a three-membrane-bound structure called the stigma, or eyespot, that may be the remnant of the original peridinin plastid, although it contains no photopigments (Withers et al., 1977). The endosymbiont is separated from the dinoflagellate host by a single membrane. Amazingly, it still maintains a nucleus, mitochondria, ribosomes, and plastids within the ER lumen (Schnepf and Elbrächter, 1999). These species appear to represent an intermediate stage of endosymbiosis between engulfment and reduction of the endosymbiont to a small nucleus (the nucleomorph) and the plastid, as in cryptophytes and chloroarachniophytes. If there has been gene transfer from the diatom nucleus to the dinoflagellate nucleus in these species, this would indicate that a protein import system has evolved, which is a critical step in converting an endosymbiont into an organelle.

The final known plastid type is the prasinophyte plastid of *Lepidodinium viride* (Watanabe et al., 1987). This is the only plastid in the dinoflagellates that comes from outside the red plastid lineage, contains the photopigment prasinoxanthin, and lacks peridinin and fucoxanthin (Watanabe et al., 1991). As in *Dinophysis*, only two membranes surround this plastid, and other endosymbiont components are absent with the exception of ribosomes. In this species, the endosymbiont nucleus is absent, indicating that all genes necessary for maintenance of this plastid have been transferred to the nucleus of the dinoflagellate and reduction of the endosymbiont is complete. The two membrane-bound tertiary plastids in *Lepidodinium* and *Dinophysis* raise important questions about protein movement to the plastid. Have these organisms evolved a new set of protein import signals or have they possibly reverted to using the two-membrane import signal of primary plastid lineages?

It is clear that the dinoflagellates possess the most diverse array of plastids of any eukaryotic lineage. Whereas some data indicate that the most common peridinin plastid arose through secondary endosymbiosis from a red alga, it is interesting to note that no plastids have yet been found in lineages at the base of the dinoflagellates (*Perkinsus* and *Oxyrrhis*). In addition, a large group of unidentified alveolates, which are likely to be heterotrophic, has been discovered at aphotic depths in the ocean (López-García et al., 2001; Moon-van der Staay et al., 2001). These organisms also group near the base of the dinoflagellates in phylogenetic analyses. Photosynthetic dinoflagellates are not monophyletic, so the peridinin plastid was probably present early in dinoflagellate evolution and was lost as many as eight times in the radiation of the group (Saldarriaga et al., 2001). However, it is still unclear whether the presence of aplastidial lineages at the base of the dinoflagellates indicates that these taxa lost plastids independently or dinoflagellates experienced an early aplastidial phase. This would mean that the peridinin plastid arose through tertiary endosymbiosis, rather than being directly descended from a red algal secondary endosymbiosis. It is now clear that the peridinin plastid is related to the red algal/chromist plastids; however, current data cannot distinguish between these two possibilities. Recent studies have begun to clarify plastid evolution in the dinoflagellates, but many aspects of plastid evolution in this group remain to be resolved.

Conclusions—Scientific interest in the dinoflagellates has risen dramatically because of the increased frequency (or our enhanced ability of detection) and severity of toxic blooms and because of the important role these organisms play in the health of coral reefs. In the near future, application of genomic techniques will help the scientific community investigate many important aspects of these organisms and provide insights into ecology, cell biology, gene expression, and toxicity. Genomics has already shed light on the complex evolution of their plastid genomes and revealed the migration of the plastid genome to

the nucleus. At this point, the scale of the effort required to sequence one of the large dinoflagellate nuclear genomes makes this unfeasible. However, application of genomic techniques such as EST sequencing, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), and microarrays are already underway, which are likely to provide many fascinating insights into these unique organisms.

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