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Evolution

Cladistic analyses of combined traditional and molecular data sets reveal an algal lineage

(18S rRNA/chromophyte/chrysophyte/diatom/phylogeny)

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ABSTRACT **The chromophyte algae are a large and biologically diverse assemblage of brown seaweeds, diatoms, and other golden algae classified in 13 taxonomic classes. One subgroup (diatoms, pedinellids, pelagophytes, silicoflagellates, and certain enigmatic genera) is characterized by a highly reduced flagellar apparatus. The flagellar apparatus lacks microtubular and fibrous roots, and the flagellum basal body is attached directly to the nucleus. We hypothesize that the flagellar reduction is the result of a single evolutionary series of events. Cladistic analysis of ultrastructural and biochemical data reveals a monophyletic group that unites all taxa with a reduced flagellar apparatus, supporting our hypothesis. Phylogenetic analyses of 18S rRNA gene sequence data provide strong resolution within most of the major groups of chromophytes but only weakly resolve relationships among those groups. Some of the molecularly based most parsimonious trees, however, also unite the taxa with a reduced flagellar apparatus, although the diatoms are not included in this lineage. This grouping is further supported by *a posteriori* character weighting of the molecular data, suggesting that flagellar reduction occurred at least twice in parallel evolutionary series of events. To further test our hypothesis of a single evolutionary reduction in the flagellar apparatus, we combine the two data sets and subject the hybrid data matrix to parsimony analysis. The resulting trees unite the diatoms with the other reduced flagellar apparatus algae in a monophyletic group. This result supports our hypothesis of a single evolutionary reduction and indicates the existence of a previously unrecognized lineage of algae characterized by a highly reduced flagellar apparatus. Further, this study suggests that the traditional classification of the diatoms with the chrysophytes and xanthophytes in the division (= phylum) Chrysophyta, as presented in most textbooks, is unsatisfactory and that a significantly different classification should be employed.**

The chromophyte algae are a biologically diverse group consisting of an estimated one million living species representing 13 taxonomic classes (1, 2) that have phylogenetic affinities to some aquatic fungi and zooflagellates (3–5). Ecologically, the marine planktonic chromophytes (e.g., diatoms, haptophytes, and pelagophytes) account for ≈50% of oceanic primary production (6, 7) and marine phytoplankton *in toto* account for up to 40% of the global primary production (8, 9). Yet, despite the size and significance of the group, evolutionary relationships within the chromophytes went unstudied for decades. During the past 8 years this topic has received attention, based either upon traditional data (i.e., morphology, ultrastructure, and photosynthetic pigments) (3, 4, 10–13) or upon gene sequence data (2, 14–16). Traditional data sets emphasize ultrastructural features, especially those of the flagellar apparatus. The eukaryotic flagellum (including cilium) probably evolved only once, and regardless of life stage, flagella are considered homologous; i.e., a flagellum of a sperm cell is considered homologous to that of a flagellate phytoplankter or an asexual zoospore (10). Microtubular roots often anchor the flagellum or flagella, and they are the major component of the cell's cytoskeleton (17), often being active in specific cell activities [e.g., phagocytosis (18–20) and scale formation (21–23)]. The flagellar apparatus in many chromophyte classes has four microtubular roots, and in some cases a system II fiber or rhizoplast is also present (Fig. 1 *Left*) (10–12). The absolute configuration of the microtubular roots is remarkably consistent within an algal class and therefore is used as a modern basis for defining taxonomic classes (2, 10, 24).

Some chromophytes (diatoms, pedinellophytes, pelagophytes, silicoflagellates, and *Rhizochromulina*), however, have a highly reduced flagellar apparatus that completely lacks microtubular and fibrous roots (Fig. 1 *Right*) (25–30). As further reductions, diatom sperm lack the central pair of microtubules in their flagellum (i.e., 9 + 0, not 9 + 2) (25, 26), and the single flagellum of *Pelagomonas* is not even associated with a second barren basal body, a condition that is apparently unique among all vegetatively flagellate organisms (2). The simple flagellar apparatus of these algae is almost certainly the result of evolutionary reduction: the earliest lineages of protists have elaborate flagellar apparatuses (31) and most flagellates and ciliates have microtubular roots (17).

We hypothesized that these algae having a reduced flagellar apparatus form a monophyletic lineage within the chromophyte assemblage; i.e., their reductions are the product of a single evolutionary series of events and not of multiple independent parallel losses. To test the hypothesis, we assembled a traditional data set, and we determined 18S rRNA gene sequences for five relevant chromophyte species‖ and added these to published sequences for other organisms to form a molecular data set. In this paper, we describe the results of phylogenetic analyses of traditional and molecular data sets, separately and in combination, and we report a major lineage of chromophyte algae characterized by a highly reduced flagellar apparatus.

MATERIALS AND METHODS

Ultrastructural and biochemical features were selected from published data and coded as cladistic characters. The terminal taxa for this analysis were taxonomic classes, except for the divisions Bacillariophyta and Öomycota and the genera *Rhizochromulina* and *Pelagococcus* (nomenclatural modification is required before the two genera can be formally classified). Characters were selected whose states are fixed within termi-

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‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U14384–U14388).

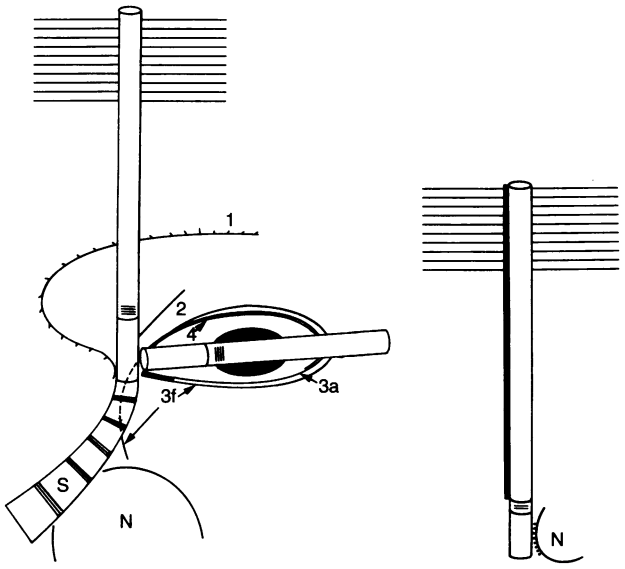

FIG. 1. (*Left*) Two-dimensional flagellar apparatus diagram for an ochromonad-type flagellate (Chrysophyceae). 1–4, roots 1–4; 3a and 3f, the "a" and "f" microtubules of root 3; S, the system II fiber (= rhizoplast); N, nucleus (see ref. 12). (*Right*) Two-dimensional flagellar apparatus diagram for *Pelagomonas calceolata* (Pelagophyceae) showing the absence of microtubular and striated roots. N, nucleus.

nal taxa for all examined species, and missing values were assigned when information was not available or when a character was not applicable (e.g., flagellate cells are unknown for *Pelagococcus subviridis*). One exception was the use of diatom sperm flagellar features for all diatoms even though only the centric diatoms produce flagellate sperm cells. The single multistate character was treated as unordered and the data matrix was analyzed using the "ie*" option of HENNIG86, which is guaranteed to find all the most parsimonious trees (32).

Total genomic DNA was isolated by phenol/chloroform extraction (33) from *Apedinella radians* (strain MUCC204), *Dictyocha speculum* (strain CCMP1381), *Pelagococcus subviridis* (strain CCMP1429), *Pseudopedinella elastica* (strain CCMP716), and *Rhizochromulina* cf. *marina* (strain CCMP237). The MUCC strain was obtained from the Melbourne University Culture Collection (Melbourne, Australia) and the CCMP strains were obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME). The nuclear encoded 18S rRNA genes were amplified (2) as two fragments by PCR using primers G01 (5′-CACCTGGTTGATCCTGCCAG-3′), G14 (5′-CCTTGGCAGACGCTTTCGCAG-3′), G04 (5′-CAGAGGTGAAATTCTTGGAT-3′), and G07 (5′-GCTTGATCCTTCTGCAGGTTCACCTAC-3′) which correspond to nt 1–20, 950–970, 913–932, and 1790–1816, respectively, in *Pelagomonas calceolata* (2). Sequencing primers included seven previously described (2) plus two new primers, G15 (5′-GATTACGTCCCTGCCCTTTGT-3′) and G16 (5′-ACAAAGGGCAGGGACGTAATC-3′), which correspond to nt 1628–1648 in *P. calceolata*. PCR products were directly sequenced with the Applied Biosystems Taq DyeDeoxy Terminator Cycle Sequencing kit in an Applied Biosystems model 373A automated DNA sequencer.

The full 18S rRNA gene sequences were added to a previous alignment of 21 organisms (2), plus *Emiliania huxleyii* (16) and *Chlamydomonas reinhardtii* (14). Sequences were aligned by the CLUSTALV (34) computer software program and refined by eye. Fifteen hundred ninety-seven unambiguously aligned nucleotides were converted to arrays of unordered, character state data by the "DNAtype" option of PAUP (35). Nucleotides were given equal weights and alignment gaps were treated as a fifth character state. With the "heuristic search" option and the tree bisection–reconnection branch-swapping algorithm of PAUP (35), most parsimonious cladograms were sought by random (50 replicates) sequential addition of taxa. Based upon strict consensus, trees obtained by relaxing parsimony one step at a time (up to five steps) were used to determine decay indices (36). Bootstrap sampling (100 replicates) was also completed (37). The molecular data were subjected to *a posteriori* character weighting by the successive-approximations method (38) by applying the "reweight characters" command in PAUP, where the truncated rescaled consistency index was used as the weighting factor in one successive iteration. Trees were rooted by the location at which the outgroup taxon (*Chlamydomonas reinhardtii*) joined the tree (39).

The traditional and molecular data sets were combined directly and analyzed with PAUP (35). Because some of the taxa for which sequence data were available have not been studied ultrastructurally and/or biochemically, it was necessary to assume, for the combined analysis, that they have the same character states as the members of their respective classes for which published data on ultrastructure and biochemistry are available.

RESULTS

The traditional characters and character states are shown in Table 1, and the data matrix for the taxa is shown in Table 2; the original sources for this information are summarized elsewhere (2, 11, 12). Of the 14 characters, 10 are flagellar characters, 2 are pigment characters, 1 is a mitotic character, and 1 is a Golgi body character. Only one multistate character (transitional helix) was included; this was treated as "unordered" because of uncertainty as to homology of the transitional helix above the major plate and that below it. Cladistic

Table 1. Characters and character states of the traditional data set

Character	Character states
R_1 and R_3 flagellar roots	0 = present
	1 = absent
R_2 and R_4 flagellar roots	0 = present
	1 = absent
System II fiber	0 = present
	1 = absent
Flagellar hairs	0 = present
	1 = absent
Flagellar hair structure	0 = smooth shafts
	1 = with lateral filaments
Paraxonemal rod	0 = present
	1 = absent
Transitional helix	0 = absent
	1 = present, above major plate
	2 = present, below major plate
Flagellum number	0 = one
	1 = two
Basal body number	0 = one
	1 = two
19′-Butanoyloxyfucoxanthin	0 = present
	1 = absent
Diatoxanthin	0 = present
	1 = absent
Basal body on nucleus	0 = present
	1 = absent
Sinking mitotic spindle	0 = present
	1 = absent
Golgi body located on posterior nuclear surface	0 = present
	1 = absent

Table 2. Ultrastructural and biochemical database used in the cladistic analyses

Chlorophyceae	0001?101111111
Bacillariophyta	1110000001000?
Chrysophyceae	00001111111111
Dictyochophyceae	1110002010007?0
Eustigmatophyceae	0000011111111??
Haptophyceae	0011?1?1100111
Öomycota	00?001111??111
Pedinellophyceae	1110002017?00?0
Pelagococcus	??????????00?01
Pelagophyceae	1110002000007?1
Phaeophyceae	00100101111111
Rhizochromulina	11100?201?00?0
Synurophyceae	01001111111111
Xanthophyceae	00000111110111

The Chlorophyceae are designated as the outgroup. Missing values are represented by question marks.

analysis of these data produced 10 most parsimonious trees (length = 20, consistency index = 0.75, retention index = 0.88); the strict consensus tree is shown in Fig. 2. All algal groups with a reduced flagellar apparatus (Bacillariophyta, Dictyochophyceae, Pedinellophyceae, Pelagophyceae including the nonflagellate *Pelagococcus*, and *Rhizochromulina*) formed a monophyletic group; relationships within this group were not completely resolved in the strict consensus tree.

Complete 18S rRNA gene sequences were obtained and deposited in GenBank under the following accession numbers: *Apedinella radians*, U14384; *Dictyocha speculum*, U14385; *Pelagococcus subviridis*, U14386; *Pseudopedinella elastica*, U14387; *Rhizochromulina* cf. *marina*, U14388. We will provide exact alignments on request. A cladistic analysis resulted in four most parsimonious solutions (length = 1649, consistency index = 0.56, retention index = 0.64). One of these was chosen for illustration (Fig. 3); a second tree was identical except for relationships within the pennate diatoms. In the other two most parsimonious trees, the branch uniting the Pelagophyceae and Dictyochophyceae/*Rhizochromulina*/Pedinellophyceae clades collapsed, as indicated by the decay index value of 0 on that branch in Fig. 3. In all four trees, the diatoms were a sister taxon to a clade including all chromophytes except the Haptophyceae, but the sister relationship was only weakly supported, having a low bootstrap value (<50) and a decay index of only 1. The Haptophyceae, represented by *E. huxleyii*,

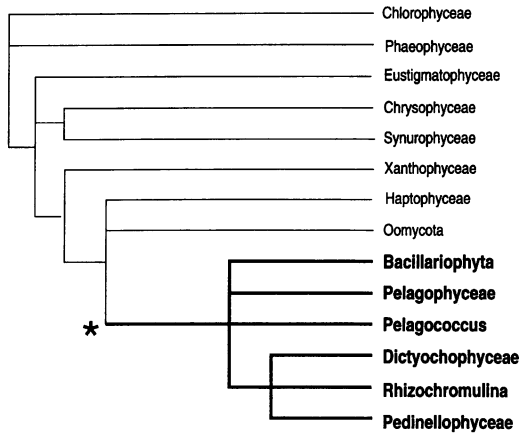

FIG. 2. Strict consensus tree of 10 most parsimonious cladograms (length = 20; consistency index = 0.75; retention index = 0.88) based upon 14 traditional (ultrastructural and biochemical) characters (Table 1). The clade of taxa with reduced flagellar apparatus is shown in boldface type; the single evolutionary reduction of the flagellar apparatus implied by this tree is indicated by an asterisk next to the branch uniting those taxa.

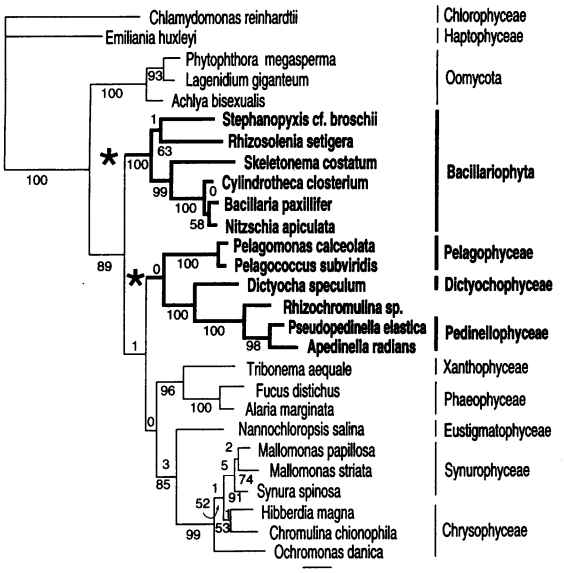

FIG. 3. One of four most parsimonious trees based upon 18S rRNA gene sequence data. Numbers above branches represent decay index values (internal nodes lacking values have decay indices that are >5); numbers below branches represent bootstrap values (internal nodes lacking values appeared in <50% of trees in the bootstrap analysis). Scale bar = 30 steps. The clades of reduced-flagellar-apparatus taxa are shown in boldface type; the two independent evolutionary reductions of the flagellar apparatus implied by this tree are indicated by asterisks next to the branches leading to those clades.

diverged deep within the tree and were separated from the other chromophytes by the Oomycota.

Using iterative *a posteriori* character weighting (38) for the molecular data, a single most parsimonious solution (length = 675,742, consistency index = 0.79, retention index = 0.80) was obtained after only one iteration. The structure of this tree was identical to that in Fig. 3. Bootstrap support for the clade of taxa with a reduced flagellar apparatus (excluding the diatoms) was 53%.

Cladistic analysis of the hybrid matrix resulting from direct combination of raw data sets resulted in two most parsimonious trees (length = 1676, consistency index = 0.56, retention index = 0.65). The two trees differed only in the relationships among the pennate diatoms (decay index = 0), and one of the trees is presented (Fig. 4). With the exception of the Haptophyceae, the trees placed the chromophyte algae in essentially three major lineages. These were the Eustigmatophyceae/Chrysophyceae/Synurophyceae clade (bootstrap value = 87, decay index > 5), the Phaeophyceae/Xanthophyceae clade (bootstrap value = 94, decay index > 5), and a clade which included all the taxa with reduced flagellar apparatuses (Bacillariophyta, Pelagophyceae including *Pelagococcus*, Dictyochophyceae, *Rhizochromulina*, and Pedinellophyceae) (bootstrap value = 54, decay index = 3).

DISCUSSION

Phylogenetic analysis of traditional data reveals a previously unrecognized monophyletic lineage of chromophyte algae characterized by having a reduced flagellar apparatus. Two of the four most parsimonious trees produced from phylogenetic analysis of unweighted 18S rRNA gene sequence data, as well as the single tree produced by *a posteriori* weighting of the molecular data, also support the recognition of this lineage, with the exclusion of the diatoms. Thus, the traditional data suggest a single evolutionary reduction of the flagellar apparatus, and the molecular data imply that at least two parallel evolutionary reductions occurred. The position of the diatoms

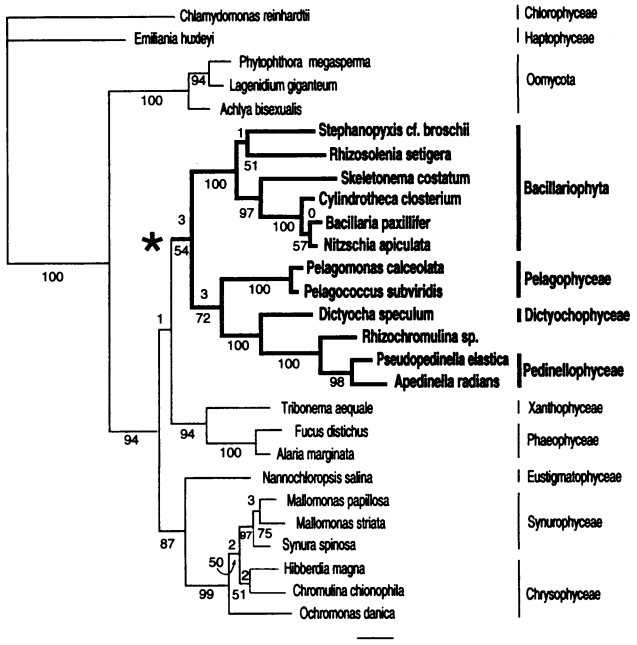

FIG. 4. One of two most parsimonious trees based upon combined traditional and molecular data. Numbers above branches represent decay index values (internal nodes lacking values have decay indices that are >5); numbers below branches represent bootstrap values (internal nodes lacking values appeared in <50% of trees in the bootstrap analysis). Scale bar = 30 steps. The clade of reduced-flagellar-apparatus taxa is shown in boldface type; the single evolutionary reduction of the flagellar apparatus implied by this tree is indicated by an asterisk next to the branch uniting those taxa.

in the molecular tree is only weakly supported, however, as evidenced by the low bootstrap value (<50) and a decay index of 1. The weak support for an independent flagellar reduction in diatoms becomes more evident when the two data sets are combined, because only two most parsimonious trees are found and both trees unite the diatoms with the other algae having a reduced flagellar apparatus. This supports the hypothesis that there was a single evolutionary reduction in the flagellar apparatus. We conclude that the molecular data do not provide strong support for the hypothesis that flagellar reduction occurred independently more than once, and we favor the hypothesis that the reduced-flagellar-apparatus algae form a single monophyletic lineage.

The strongest argument in favor of our conclusion is that it is supported by a combination of data from different sources. We agree with other workers (e.g., refs. 40 and 41) that combining of data sets strengthens an analysis by providing hypotheses that best explain all of the available evidence simultaneously. It has been argued that traditional and molecular data should not be combined if the two data sets strongly support conflicting hypotheses of phylogenetic relationship (42, 43), or the sheer size difference between the two data sets causes the molecular characters to swamp out the traditional ones when data are combined (44). In both of these cases, combining of data could select a tree with the wrong topology for the taxa under study because of differences between the phylogenetic histories of genes and those of the organisms that bear those genes (see ref. 45). Neither of these arguments applies to this study, however. The general concordance between the two data sets does not suggest different underlying phylogenies and, in spite of considerable size disparity between molecular (489 characters) and traditional (14 characters) data sets, direct combination of data does not generate the same trees as molecular data alone. The resulting trees (Fig. 4) instead combine strongly supported elements from each of the individual data-set analyses. With respect to the reduced flagellar apparatus group, it is in fact the traditional characters that dominate in the combined data analysis. Thus, the combination of data demonstrates the weak support in the molecular data for the resolution among the major chromophyte lineages in general and for the exclusion of the diatoms from the lineage of chromophytes with a reduced flagellar apparatus in particular.

It could be further argued that a change in one of our traditional characters, which represent complex structures each probably controlled by one to many genes, is much less likely than a change in an individual nucleotide of the 18S rRNA molecule. Differential weighting of characters has been advocated for cases in which rates of change are different for different types of characters (42, 46). This argument would justify increasing the relative weight of the traditional characters in the combined data, further strengthening the support for the reduced-flagellar-apparatus clade. For example, applying a weight of 2.0 to the traditional characters (data not shown) results in an increase of the decay index to >5 and of the bootstrap value to 92, in support of the reduced-flagellar-apparatus lineage. The choice of this particular weighting value is arbitrary, but, if our assumptions that the traditional characters are independent and that each represents a major genetic change are correct, it is probably conservative. In addition, the possibility of nonindependence of at least some of our molecular characters must be considered (47, 48), which would further support giving the molecular data a lower relative weight.

Our traditional data set emphasizes flagellar ultrastructural features for two reasons: flagellar apparatuses have been used extensively for over two decades as evolutionary markers in algae (2, 10–12, 17, 24, 49) and other reliable characters are rare. In choosing flagellar characters, we selected those which we believed were not functionally correlated and varied independently of each other; i.e., there was no *a priori* evidence that they would support one another. The analysis would have been strengthened by additional ultrastructural and molecular data, such as details for cell division or nucleotide sequences for other genes; however, these data were lacking at the time of our analysis.

Several significant findings emerge from this study regarding the phylogeny and classification of the chromophyte algae. First, the phylogenetic position of the newly described class Pelagophyceae was unresolved in a previous study (2). In this study, the Pelagophyceae are clearly related to the Dictyochophyceae, Pedinellophyceae and Bacillariophyta. Second, previous studies suggested an isolated phylogenetic position for the diatoms (15), whereas our study suggests that they are related to the other reduced-flagellar-apparatus algae. Third, although a relationship between the Dictyochophyceae and Pedinellophyceae was suggested on the basis of ultrastructural features (29), our molecular data and combined data-set analyses provide strong support (bootstrap value = 100) for this relationship. In addition to showing a close relationship between these two algal groups, it is now obvious that these algae are not members of the Chrysophyceae and that earlier taxonomic revisions establishing them as separate groups were well founded. Finally, it is apparent that the often used division Chrysophyta (chrysophytes, diatoms, xanthophytes), which appears in general biology, general botany, and phycology textbooks, is not a natural group and a new classification is required. In addition, *Pelagococcus*, *Rhizochromulina*, and probably several other marine "chrysophytes" can no longer be included in the Chrysophyceae. Therefore, we suggest abandoning Pascher's (50) definition for the division Chrysophyta and restricting "chrysophyte" to those algae belonging in the class Chrysophyceae *sensu stricto*. A number of nomenclatural changes are suggested by this work, and we plan to address these in a separate paper.

The evolutionary selection that led to the reduced flagellar apparatus is not known. There are an estimated one million species of diatoms (1, 51), and in terms of species diversity diatoms rank with insects, ascomycete fungi, and flowering plants. Without question, diatoms are successful single-celled aquatic organisms even though they do not rely upon flagella. In fact, a flagellum is present in only certain centric diatoms that produce flagellate sperm (51); pennate diatoms have amoeboid sperm and do not even have centrioles at the poles during mitosis (52). A number of nonflagellate species occur in the Pelagophyceae also (D.P. and R.A.A., unpublished data), and *Rhizochromulina* is amoeboid in its vegetative state, occasionally producing zoospores (27, 30). Among the reduced flagellar apparatus algae, only the silicoflagellates and pedinellids are typically flagellate organisms (28, 29). It is unclear whether these flagellates returned to the swimming state secondarily or whether they descended directly from an ancestral flagellate that first expressed the reduced flagellar apparatus.

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