Evolution and Cytological Diversification of the Green Seaweeds (Ulvophyceae)

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Abstract

The Ulvophyceae, one of the four classes of the Chlorophyta, is of particular evolutionary interest because it features an unrivaled morphological and cytological diversity. Morphological types range from unicells and simple multicellular filaments to sheet-like and complex corticated thalli. Cytological layouts range from typical small cells containing a single nucleus and chloroplast to giant cells containing millions of nuclei and chloroplasts. In order to understand the evolution of these morphological and cytological types, the present paper aims to assess whether the Ulvophyceae are monophyletic and elucidate the ancient relationships among its orders. Our approach consists of phylogenetic analyses (maximum likelihood and Bayesian inference) of seven nuclear genes, small subunit nuclear ribosomal DNA and two plastid markers with carefully chosen partitioning strategies, and models of sequence evolution. We introduce a procedure for fast site removal (site stripping) targeted at improving phylogenetic signal in a particular epoch of interest and evaluate the specificity of fast site removal to retain signal about ancient relationships. From our phylogenetic analyses, we conclude that the ancestral ulvophyte likely was a unicellular uninucleate organism and that macroscopic growth was achieved independently in various lineages involving radically different mechanisms: either by evolving multicellularity with coupled mitosis and cytokinesis (Ulvales—Ulotrichales and Trentepohliales), by obtaining a multinucleate siphonocladous organization where every nucleus provides for its own cytoplasmic domain (Cladophorales and *Blastophysa*), or by developing a siphonous organization characterized by either one macronucleus or millions of small nuclei and cytoplasmic streaming (Bryopsidales and Dasycladales). We compare different evolutionary scenarios giving rise to siphonous and siphonocladous cytologies and argue that these did not necessarily evolve from a multicellular or even multinucleate state but instead could have evolved independently from a unicellular ancestor.

Key words: multigene phylogeny, fast site removal, evolution, green algae, Chlorophyta, Ulvophyceae.

Research article

Introduction

The green plants or Viridiplantae evolved in two major lineages, the Chlorophyta and the Streptophyta (Lewis and McCourt 2004; O'Kelly 2007). The Streptophyta consists of several lineages of freshwater green algae from which the land plants evolved (McCourt et al. 2004). The Chlorophyta includes all other green algae and exhibits a high morphological, cytological, and reproductive diversity. Whereas considerable progress has been made in clarifying the relationships among the Streptophyta (Lemieux et al. 2007; Rodríguez-Ezpeleta et al. 2007), the evolutionary history of the Chlorophyta has been more difficult to elucidate. Four classes are recognized within the Chlorophyta: the predominantly marine planktonic Prasinophyceae, the freshwater or terrestrial Trebouxiophyceae and Chlorophyceae, and the Ulvophyceae, which are best known as marine macroalgae (green seaweeds) in coastal ecosystems. The Prasinophyceae form a paraphyletic assemblage of unicellular flagellates from which the Ulvophyceae, Trebouxiophyceae, and Chlorophyceae (UTC) are derived (Guillou et al. 2004). The latter three classes form a well-supported clade, but the relationships among them remain largely unresolved. Furthermore, the monophyly of the Trebouxiophyceae and Ulvophyceae has not been unequivocally demonstrated (Pröschold and Leliaert 2007; Turmel, Gagnon, et al. 2009).

In contrast to the Prasinophyceae, Trebouxiophyceae, and Chlorophyceae, which are unicells, colonies, or in a few cases simple filaments or sheet-like thalli, an unrivalled diversity of morphological and cytological designs has evolved within the Ulvophyceae, ranging from microscopic unicells to macroscopic multicellular plants, and giant-celled organisms with unique morphological, cellular, and physiological characteristics (Mine et al. 2008). Four main cytomorphological types can be distinguished in the Ulvophyceae.

The first type comprises unicellular algae with a single nucleus and chloroplast. This is present in some genera of uncertain affinity (e.g., *Oltmannsiellopsis*, *Ignatius*) and some early-branching Ulvales–Ulotrichales (Nakayama et al. 1996; Friedl and O'Kelly 2002; Watanabe and Nakayama 2007).

The second type has multicellular bodies composed of uninucleate cells. In external morphology, these algae are filamentous or sheet like. This cytomorphological type is present in most of the Ulvales–Ulotrichales and in the Trentepohliales.

The third type is known as the siphonocladous type and has multicellular bodies composed of multinucleate

cells as a consequence of uncoupled cell division and mitosis. The synchronously dividing nuclei are organized in nonmotile regularly spaced nucleocytoplasmic domains that are maintained by perinuclear microtubule arrays (McNaughton and Goff 1990; Motomura 1996). Despite lacking clear physical borders, such as a plasma membrane, these cytoplasmic domains behave like independent structural entities or pseudocells (Baluška et al. 2004). This cytomorphology is typical of the Cladophorales (including Siphonocladales) and *Blastophysa*, a genus of uncertain affinity.

The fourth type is better known as siphonous type and is characterized by plants consisting of a single giant tubular cell (Vroom and Smith 2003). It is present in the orders Bryopsidales and Dasycladales (Verbruggen et al. 2009). Siphonous cells generally contain thousands to millions of nuclei dividing by asynchronous mitosis. Most Dasycladales, however, remain uninucleate throughout much of their life cycle with a giant diploid nucleus that only divides at the onset of reproduction (Berger and Kaever 1992). In contrast to the siphonocladous type, the cytoplasm of siphonous algae exhibits vigorous streaming, enabling transportation of nutrients and organelles across the plant (Menzel 1987, 1994; Mine et al. 2001). Although some siphonous algae are tiny microscopic siphons, many form large and complex seaweeds that develop in the absence of cell division (Kaplan and Hagemann 1991; Mandoli 1998). Several species exhibit morphological differentiation into structures that resemble the roots, stems, and leaves of land plants and even have similar functions (Chisholm et al. 1996).

In order to understand the diversification of cytological types and morphological complexity that took place in the Ulvophyceae, the phylogenetic relationships among its orders need to be resolved. Several problems surround the classification of the Ulvophyceae. First, the monophyly of the Ulvophyceae has been questioned because it lacks unique ultrastructural synapomorphies (Mattox and Stewart 1984; O'Kelly and Floyd 1984; Gile et al. 2009; Zuccarello et al. 2009). Second, molecular phylogenetic studies have not fully resolved the relationships among the orders and the positions of some enigmatic genera (Chappell et al. 1991; Watanabe and Nakayama 2007). The fact that previous molecular phylogenetic analyses have not yielded a satisfactory resolution is due at least in part to the difficulty in obtaining a good combination of taxon and gene sampling. Single-gene analyses with good taxon sampling have suffered from poor resolving power due to the low number of characters (López-Bautista and Chapman 2003; Watanabe and Nakayama 2007). Conversely, studies based on longer alignments from complete organelle genomes have suffered from sparse and uneven taxon sampling (Pombert et al. 2004; Turmel, Otis, et al. 2009; Zuccarello et al. 2009), which can lead to systematic error in phylogenetic analysis (Brinkmann et al. 2005; Philippe et al. 2005). Moreover, the Bryopsidales have only been included in an early phylogenetic analysis based on partial small subunit (SSU) and large subunit ribosomal DNA sequence data (Zechman et al. 1990). An additional difficulty in resolving the relationships among the orders is their ancient (late Neoproterozoic) age (Butterfield et al. 1994; Verbruggen et al. 2009).

Resolving ancient relationships can be an arduous task that requires a sufficient amount of sequence data. Additional problems can arise because of substitution saturation, which introduces noise in the data set that can mask the signal in more slowly evolving sites and enhance nonphylogenetic signals, increasing chances of systematic error in phylogenetic reconstruction (Ho and Jermiin 2004). Various approaches have been proposed to avoid problems with saturation and systematic error, including fast site removal (site stripping). The rationale of this method is to remove noise from the data by eliminating sites that are most likely to contain homoplasy and focusing on the more informative slow-evolving positions for reconstruction of ancient relationships (Waddell et al. 1999; Delsuc et al. 2005; Rodríguez-Ezpeleta et al. 2007). Fast site removal has been applied in several studies aimed at resolving deep nodes (e.g., Ruiz-Trillo et al. 1999; Morgan-Richards et al. 2008). Other commonly used approaches to reduce inference bias regarding ancient relationships are the exclusion of the third codon position, analyses based on amino acid sequences, or the use of a covarion model.

In this study, we aim to evaluate the monophyly of the Ulvophyceae and gain understanding in the diversification of cytological designs by resolving the phylogenetic relationships among ulvophycean orders. Our approach consists of phylogenetic analysis of a ten-gene alignment composed of nuclear and plastid genes of 43 taxa representing the major lineages of the Viridiplantae with model-based techniques and carefully considering the selection of suitable partitioning strategies and models of sequence evolution. We introduce a procedure for fast site removal that is targeted at improving phylogenetic signal in a particular epoch of interest and evaluate the specificity of the removal of fast sites to retain signal about ancient relationships.

Material and Methods

Laboratory Work

Algal strain information, culture conditions and the procedures used for RNA isolation, cDNA library construction, primer design, reverse transcriptase polymerase chain reaction (PCR), PCR, cloning, sequencing, and gene orthology verification are described in detail in the Supplementary Material online.

Alignment and Model Selection

Protein-coding genes were aligned by eye based on their amino acid sequences. SSU nuclear DNA (nrDNA) was aligned based on RNA secondary structure (Cocquyt et al. 2009). There were only a few ambiguously aligned regions, which were not excluded a priori. Instead, we opted for a site-stripping approach (described below) that progressively removes fast evolving sites from the analysis. The ten loci were concatenated, yielding an alignment of 10,209 bases. In a few cases, sequences from different species were concatenated if their monophyly with respect

to other taxa in our alignment could be clearly demonstrated (supplementary table S1, Supplementary Material online; Campbell and Lapointe 2009). A suitable partitioning strategy and models of sequence evolution were selected with a three-step procedure based on the Bayesian information criterion. This method is described by Verbruggen et al. (2010) and illustrated in detail for the present data set in supplementary figure S1, Supplementary Material online.

Phylogenetic Analysis

Maximum likelihood (ML) analysis was performed with TreeFinder, which allows likelihood searches under partitioned models (Jobb et al. 2004). Due to the relatively low tree space coverage in TreeFinder compared with other ML programs, an analysis pipeline was created to increase tree space coverage by running analyses from many starting trees. A first set of starting trees was created by randomly modifying the PhyML guide tree by 100 and 200 nearest neighbor interchange (NNI) steps (50 replicates each). ML searches were run from these 100 starting trees and the three trees yielding the highest likelihood were used as the starting point for another set of NNI modifications of 20 and 50 steps (50 replicates each). A second set of ML searches was run from each of the resulting 300 starting trees. The tree with the highest likelihood resulting from this set of analyses was retained as the global ML solution. The bootstrap resampling method was used to assess statistical support. We used full searches on 1,000 bootstrapped alignments, starting each search from 50 randomized maximum parsimony start trees.

Bayesian phylogenetic inference was carried out with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Two parallel runs, each consisting of four incrementally heated chains, were run for 25 million generations, sampling every 1,000 generations. Convergence of log-likelihoods and parameter values was assessed in Tracer v1.4 (Rambaut and Drummond 2007). A burn-in sample, conservatively determined at 2.5 million generations, was removed before constructing a majority rule tree including all compatible bipartitions. We ran additional Bayesian phylogenetic analyses 1) with the covariotide option activated to address heterotachy using the same optimal partitioning strategy and model of sequence evolution; 2) with third codon positions excluded from the analysis using the same optimal partitioning strategy and model of sequence evolution, except that there are no partitions for third codon positions; and 3) with protein-coding genes recoded as amino acids, using the same optimal partitioning strategy and model of sequence evolution for the SSU nrDNA and a single partition with a WAG $+ \Gamma_8$ model for protein-coding genes (Abascal et al. 2005), to avoid potential effects of compositional heterogeneity at the DNA level.

Topological Hypothesis Testing

Approximately unbiased tests (AU test; Shimodaira 2002) were used to test the alternative relationships between some ulvophycean orders and between the UTC classes. Site likelihoods were calculated by ML optimization in TreeFinder using the complete data set and the same model specifications as for phylogenetic inference. AU tests were performed with CONSEL V0.1i (Shimodaira and Hasegawa 2001).

Targeted Removal of Fast Sites

We applied a modified site-stripping approach (Waddell et al. 1999) to improve the signal to noise ratio in the epoch of interest. Site-specific rates were calculated with the "substitution rates" standard analysis implemented in HyPhy (Pond et al. 2005). Site-specific rates were inferred under a global JC69 model using the inferred ML phylogeny as a guide tree. Progressive removal of the fastest sites, 5% each time, resulted in a set of alignments that were subjected to ML analysis as described above. We used a procedure that reveals the trend of signal change with increasing amounts of fast site removal to select an optimal site-stripping condition. First, branch lengths were made roughly proportional to evolutionary time by nonparametric rate smoothing (Sanderson 1997). Second, the epoch of interest (containing the UTC and ulvophycean diversification) was located in the tree. Third, the strength of phylogenetic signal, measured as average bootstrap values (BVs) in a sliding window across the rate-smoothed tree, was plotted for all site-stripping conditions. We will refer to such plots as bootstrap support profiles. Fourth, we calculated signal gain in the epoch of interest (G_E) by integrating the bootstrap support profile across the epoch of interest with numerical integration (rectangle rule). Finally, these gain values were compared between site-stripping conditions, leading to the conclusion that moderate site stripping (25% of characters removed) yielded maximum signal in the epoch of interest.

In order to assess the specificity of fast site removal to retain signal about ancient regions, we compared fast site removal with random site removal. To this goal, the bootstrap support profile of the tree inferred from the alignment from which the 25% fastest sites were removed was compared with a distribution of bootstrap support profiles resulting from the trees inferred from 100 alignments from which 25% randomly picked sites were removed.

Results

The concatenated matrix contained 43 taxa representing all major lineages of the green algae and ten genes (10,209 bases; supplementary fig. S2 and tables S1 and S2, Supplementary Material online). It is 63% filled at the gene level and 61% at the nucleotide level. The model evaluation procedure led us to partition the data into eight categories and apply completely unlinked GTR $+ \Gamma_8$ models to each (supplementary fig. S1, Supplementary Material online). The eight partitions were SSU nrDNA loops and stems (two partitions) and first, second, and third codon positions of the nuclear and plastid genes (three $\times$ two partitions).

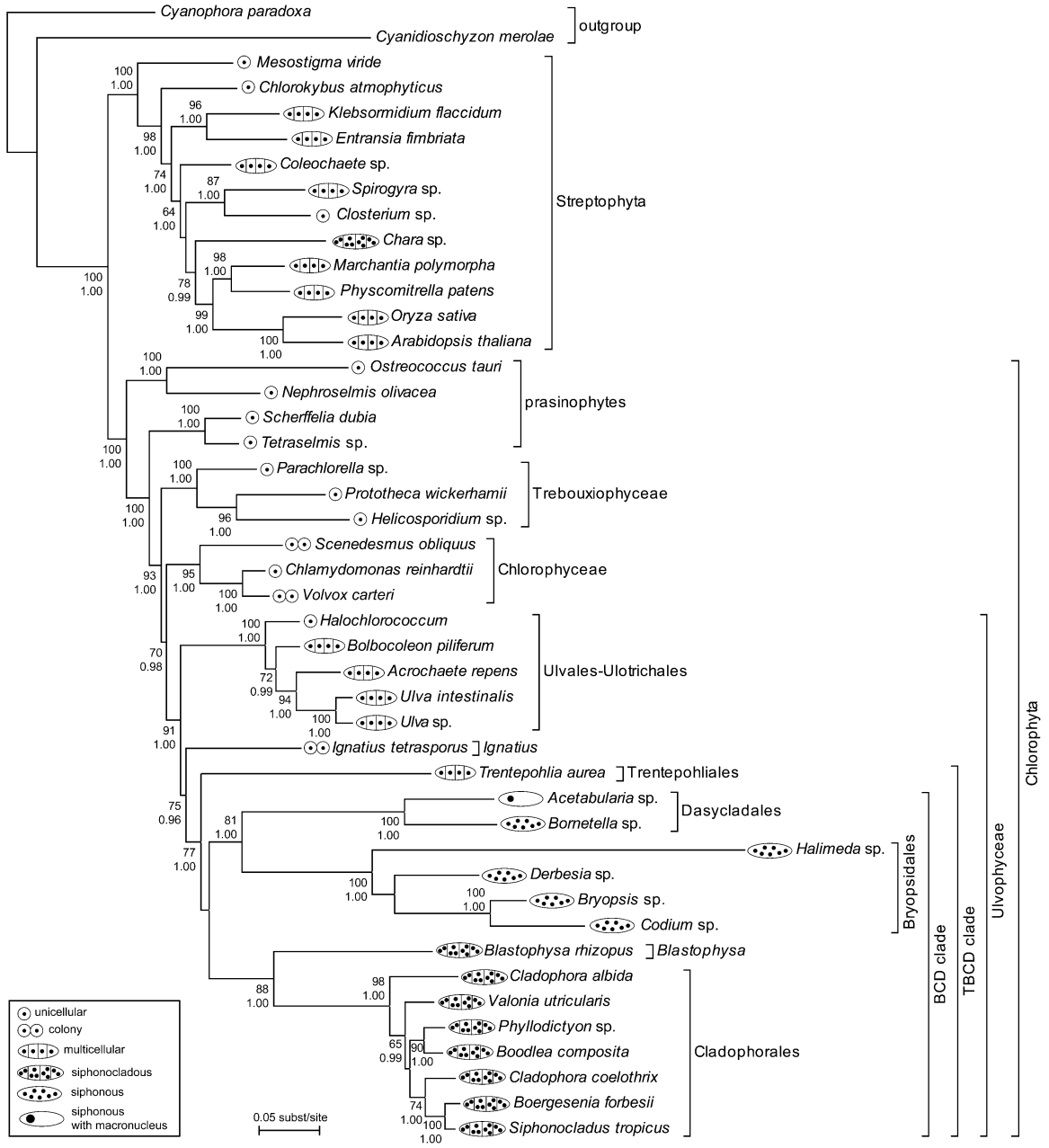

Fig. 1. Phylogeny of the green plant lineage obtained by ML inference of the 25% site-stripped data set containing seven nuclear genes, SSU nrDNA, and plastid genes *rbc*L and *atp*B. Numbers at nodes indicate ML BVs (top) and BI PP (bottom); values below, respectively, 50 and 0.9 are not shown. BCD clade stands for the orders Bryopsidales, Cladophorales, and Dasycladales, and TBCD clade stands for the orders Trentepohliales, Bryopsidales, Cladophorales, and Dasycladales.

The phylogenetic tree inferred from our data set shows high resolution of the backbone of the green algal tree of life, including most ulvophycean orders (fig. 1). The Ulvophyceae are recovered as a monophyletic group with strong support (BV = 91; posterior probabilities [PP] = 1.00). Congruent with earlier studies, the Ulvales–Ulotrichales clade, which predominantly consists of multicellular seaweeds, branches first. The genus *Ignatius* and the Trentepohliales, two relatively inconspicuous and somewhat neglected taxa both confined to subaerial habitats, form separate ancient lineages. The Trentepohliales are recovered as the sister lineage of the Bryopsidales, Cladophorales, and Dasycladales (BCD), together termed the Trentepohliales Bryopsidales, Cladophorales and Dasycladales (TBCD) clade, which contain the bulk of the green seaweeds. The Bryopsidales and Dasycladales are confidently recovered as sister lineages and the genus *Blastophysa* is sister to the Cladophorales. The branching order of ulvophycean orders is well resolved; only the short branch leading to the BCD clade did not receive acceptable levels of support.

Our phylogeny also provides information about the branching order of the UTC classes: the Trebouxiophyceae (fig. 1) is the first class to branch off, leaving the Chlorophyceae and Ulvophyceae as sister clades. The branch

joining Ulvophyceae and Chlorophyceae is very short, indicative of a rapid diversification. The AU test suggested that a sister relationship between Trebouxiophyceae and Ulvophyceae ($\ln L = -123831.41$; $P = 0.114$) was not significantly worse than the ML solution ($\ln L = -123822.64$; $P = 0.910$).

Incremental removal of fast evolving sites did not change the topology but yielded a positive trend in phylogenetic signal in the epoch corresponding to the radiation of UTC classes and ulvophycean orders (fig. 2*D*, G_E = gain in relevant epoch), with an optimum at moderate amounts of site removal (25%). Figure 2*C* clearly illustrates that statistical support for phylogenetic relationships within the relevant epoch is substantially improved by removing the 25% fastest sites at the expense of signal in more recent epochs that are outside the scope of our study. The tree in figure 1 was inferred from the data set with optimal signal in the relevant epoch (25% sites removed); the result of the phylogenetic analysis on the full data matrix is given in supplementary figure S3, Supplementary Material online. Other commonly used approaches that reduce inference bias for ancient relationships, such as exclusion of third codon positions, analysis with a covariotide model, and analyses at the amino acid level yielded virtually identical topologies (supplementary figs. S4–S6, Supplementary Material online).

The capacity of fast site removal to preferentially retain signal about ancient relationships follows from our experiments. First, the signal gain graph (fig. 2*C*) clearly shows that fast site removal increases the signal about ancient relationships (the function exceeds zero in the center part of the graph) and reduces signal about more recent relationships (the function is below zero on the right-hand side of the graph). Second, it follows from the bootstrap support profiles in figure 2*E* that the strength of the phylogenetic signal concerning ancient relationships is significantly higher in the alignment from which the 25% fastest sites were removed than in alignments from which 25% randomly picked sites are removed (black line above the gray ribbon). When it comes to signal about recent relationships, fast site removal does not yield significantly different results than random removal of sites (black line within the gray ribbon). Similarly, the removal of third codon positions performs considerably worse than fast site removal (fig. 2*F*) to the extent that removal of third codon positions has a negative gain in the relevant epoch ($G_E = -6.05$).

Discussion

Phylogeny of the Ulvophyceae

For the first time, the monophyly of the Ulvophyceae is inferred with extensive taxon sampling and high statistical support (BV = 91; PP = 1.00). In addition, we provide a solid hypothesis about the phylogenetic relationships among the main ulvophycean lineages. The only known early-diverging lineage absent from our analysis is the genus *Oltmannsiellopsis*, which is most commonly recovered near the base of the Ulvales–Ulotrichales clade (Watanabe and Nakayama 2007; Cocquyt et al. 2009). The most surprising result may be the grouping of the Dasycladales and Bryopsidales because ultrastructural features of the flagellar apparatus and the shared presence of a noncanonical genetic code suggest that Dasycladales are more closely related to Cladophorales (O'Kelly and Floyd 1984; Gile et al. 2009). Our molecular data confidently recover the Dasycladales as a sister to the Bryopsidales and the AU test shows that a sister relationship between Dasycladales and Cladophorales–*Blastophysa* is significantly less likely (AU test: $\Delta\ln L = 20.12$, $P = 0.001$). The phylogenetic position of *Ignatius* had never been fully resolved. It was either embedded in the Ulvales–Ulotrichales clade or clustered with the TBCD clade depending on the inference method used (Watanabe and Nakayama 2007). The relationship of *Ignatius* with the TBCD clade revealed in this study is corroborated by features of the flagellar apparatus (Watanabe and Nakayama 2007) and the shared presence of the elongation factor-1 alpha gene instead of the elongation factor-like gene, which is present in all earlier diverging chlorophytan lineages (Cocquyt et al. 2009). The enigmatic endophytic green alga *Blastophysa* is revealed to be sister to the Cladophorales (BV = 88; PP = 1.00), a relationship that is corroborated by morphological, ultrastructural, cytological, and biochemical features (O'Kelly and Floyd 1984; Chappell et al. 1991).

Cytomorphological Diversification

Based on the phylogeny and the distribution of cytological designs along its terminal taxa, we will discuss three alternative hypotheses about the cytomorphological diversification of green seaweeds (fig. 3). A common aspect between the hypotheses is that the ancestral ulvophyte was a unicellular organism with a single nucleus. This follows directly from the fact that several early-branching lineages are of this type. Another shared feature between the hypotheses is that multicellularity evolved independently in the Ulvales–Ulotrichales and the TBCD clades.

The three hypotheses differ in how multicellularity and siphonous cells evolved in the TBCD clade. The first hypothesis (fig. 3) suggests a single origin of multicellularity at the base of the TBCD clade. Although this hypothesis is parsimonious in requiring only a single gain of multicellularity, the phragmoplast-like cell division in Trentepohliales differs strongly from cytokinesis in the BCD taxa, which takes place by ingrowth of a diaphragm-like cross wall (McDonald and Pickett-Heaps 1976; Chapman et al. 2001), and for that reason, it may be more reasonable to propose that multicellularity evolved independently in the Trentepohliales and the BCD clade (hypothesis 2; fig. 3).

The first and second hypotheses differ from the third in how the siphonous cell structure has evolved. In hypotheses 1 and 2, a siphonocladous layout evolved from a multicellular ancestor of the BCD clade by uncoupling nuclear and cellular division. From this multicellular multinucleate design, the siphonous thallus structure evolved in the common ancestor of the Bryopsidales and Dasycladales. The

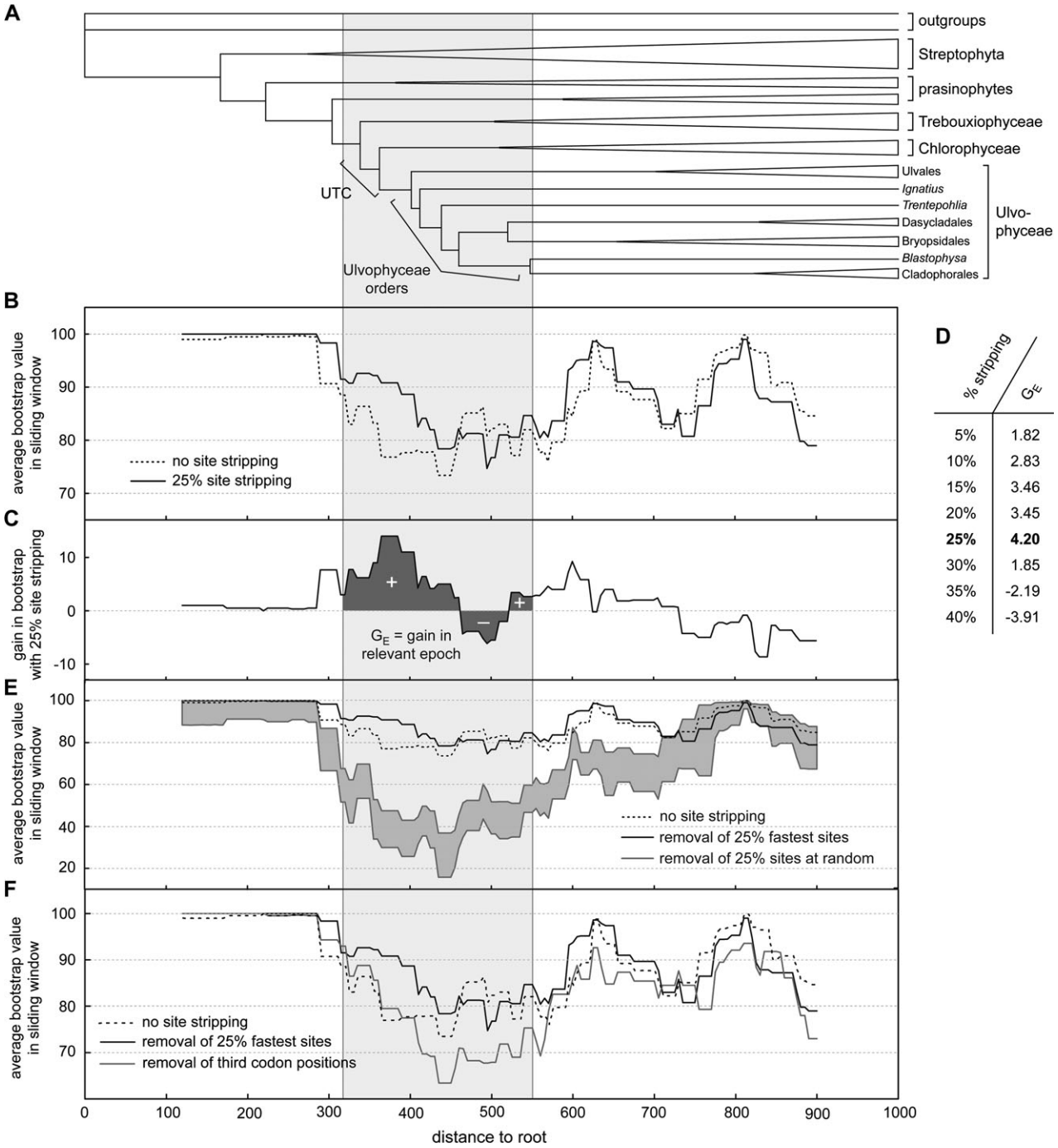

FIG. 2. Illustration of the site-stripping approach. (*A*) Based on a rate-smoothed tree, the epoch in which the relationships of interest are situated is selected (gray band across figure). (*B*) The strength of the phylogenetic signal in the data (measured as average BVs in a sliding window across the tree) is plotted for an analysis from which the 25% fastest evolving characters had been stripped and compared with the condition without site stripping. (*C*) The gain in BVs, calculated as the BV of site-stripped condition minus the BV of the condition without site stripping, is plotted. This graph shows net gain in older epochs and net loss in younger epochs. In this example, there is net gain in the epoch of interest (dark gray). (*D*) The gain in the epoch of interest is calculated for all site-stripping conditions. This table shows that moderate site stripping yields optimal phylogenetic signal in the relevant epoch. (*E*) Difference between removal of the 25% fastest sites (site stripping) and random removal of 25% of the sites. The strength of the phylogenetic signal in the site-stripped alignment (black line) is much higher than that of the randomly stripped alignments (gray ribbon) in the epoch of interest. The gray ribbon represents the 95 percentile of signal strength (measured as average BVs in a sliding window across the tree) obtained from the 100 alignments from which 25% of the sites were removed at random. (*F*) Difference between removal of the 25% fastest sites (site stripping) and removal of third codon positions. The strength of the phylogenetic signal in the site-stripped alignment (black line) is higher than that in the alignment from which the third codon positions are excluded (gray line) in the epoch of interest.

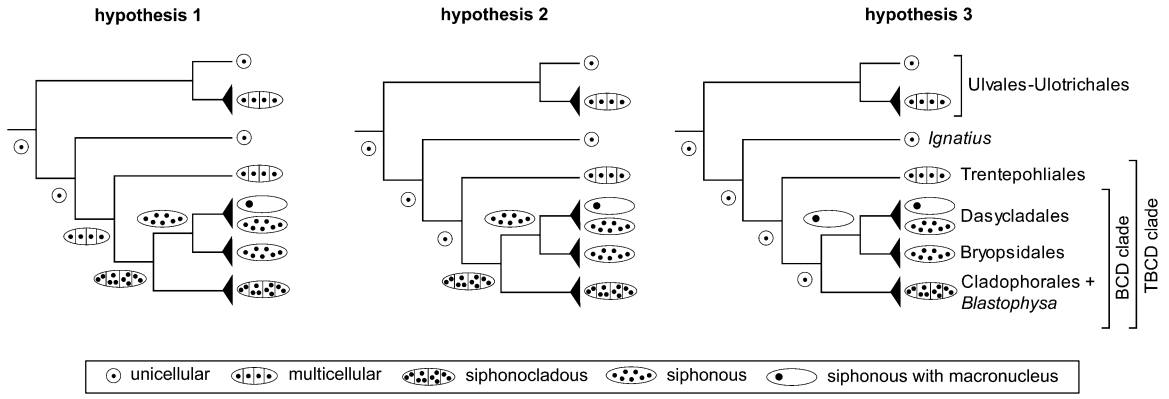

Fig. 3. Three hypotheses about cytomorphological diversification in the Ulvophyceae differing in the origin of multicellularity, siphonocladous, and siphonous design. See text for further explanation. BCD clade stands for the orders Bryopsidales, Cladophorales, and Dasycladales, and TBCD clade stands for the orders Trentepohliales, Bryopsidales, Cladophorales, and Dasycladales.

interpretation that a siphonous structure evolved through loss of multicellularity is in accordance with the occasional formation of cross walls in the vegetative tissue of *Ostreobium*, an early-diverging lineage of Bryopsidales (Hillis-Colinvaux 1984). Similarly, cross walls are still present at the base of reproductive structures in the Bryopsidineae, one of the two major bryopsidalean lineages, and at the base of reproductive structures and old hairs in some Dasycladales (Ngo et al. 2005).

In contrast, the third hypothesis does not require loss of multicellularity to form siphonous thalli. Instead, it assumes that the ancestor of the BCD clade was a uninucleate cell subjected to selective pressures for macroscopic growth in marine benthic environments. Two evolutionary pathways toward enlarged cells and macroscopic growth emerged. The first involved the evolution of multinucleate cells where every nucleus provides for its cytoplasmic domain. This pathway led to the siphonocladous cytomorphology present in the Cladophorales and *Blastophysa*. The second pathway, which led to the siphonous orders Bryopsidales and Dasycladales, involves two steps. Initially, the enlarged ancestral cell developed a macronucleus and cytoplasmic streaming, allowing increased transcription from a single nucleus and distribution of transcripts across the cell. The second step involves the evolution of multinucleate siphons, which occurred independently in both orders.

Various arguments support the third hypothesis. First, cytoplasmic streaming, a compulsory feature for this evolutionary scenario, is present in all representatives of the Bryopsidales and Dasycladales, providing strong evidence that this was the ancestral condition for the siphonous clade. Cytoplasmic streaming is not known to occur in any other Ulvophyceae. Second, the fact that a macronucleus is found in the Dasycladales (Liddle et al. 1976) and in the zygote of certain Bryopsidales (Burr and West 1971) suggests a shared origin of this feature. Third, true multinucleate siphons evolved independently in larger taxa of both orders, although in Dasycladales a macronucleus is always present in young life stages. The correlation of macronuclei with relatively small taxa and/or life stages (e.g., *Acetabularia*, *Bryopsis*) versus the presence of true multinucleate siphons in larger taxa and life stages (e.g., *Cymopolia*, most Bryopsidales) suggests that true multinucleate siphons evolved in response to the need for very big cells to form elaborate anatomies and macroscopic structures. An argument against the third hypothesis is that it requires independent gains of multicellularity in the siphonocladous and siphonous clades (only at the base of reproductive structures and old hairs in the latter). Despite the fact that in this respect, the third hypothesis is not as parsimonious as the previous two, the arguments listed above lead us to propose this hypothesis as a likely scenario for the cytomorphological diversification of the Ulvophyceae.

The evolution of siphonocladous and siphonous architectures coincides with several cytological and cytoskeletal specializations. These giant-celled green algae have evolved a unique mechanism of wounding response in which injured cells form a wound plug or contract their protoplasm into numerous spheres that regenerate into new cells and thalli (O'Neil and La Claire 1984; Menzel 1988; Kim et al. 2001). Giant-celled Cladophorales have evolved several specialized modes of cytokinesis, which are mediated by unique cytoplasmic and cytoskeletal reorganizations (Okuda, Mine, Morinaga, et al. 1997; Okuda, Mine, and Ueno 1997; Leliaert et al. 2007). In addition to facilitating transport of transcripts as mentioned above, the evolution of cytoplasmic streaming also allowed transport of nutrients, organelles, and so on throughout the siphonous algal body. In combination with morphological and anatomical changes, this allowed nutrient uptake from marine sediments (Littler et al. 1988; Chisholm et al. 1996) and chloroplast migration for optimal photosynthesis and avoidance of herbivory (Drew and Abel 1992). Such innovations most likely had selective advantages and are at least in part responsible for the ecological dominance of siphonous algae in tropical and warm-temperate coastal ecosystems (Vroom and Smith 2003).

Last, it warrants mentioning that some Ulotrichales (e.g., *Urospora* and *Acrosiphonia*) have independently evolved multinucleate cells. These genera are not present in our phylogeny, but it is known from previous studies that they

are nested in the Ulvales–Ulotrichales clade (Leliaert et al. 2009). In contrast to the situation in the Cladophorales, these organisms synchronize mitosis and cytokinesis in vegetative cells of mature filaments (Lokhorst and Star 1983).

Targeted Removal of Fast Sites

Ancient rapid radiations can be difficult to resolve even with large data sets for a number of reasons, including systematic error (Delsuc et al. 2005; Wiens et al. 2008). We have taken multiple precautions to avoid systematic error: the use of multiple genes, broad taxon sampling (Zwickl and Hillis 2002; Geuten et al. 2007), and the incremental removal of fast evolving characters. Fast sites can erode the phylogenetic signal of a data set by masking the more reliable signal of slow sites and removing them may reveal a more accurate signal (Delsuc et al. 2005).

We have introduced a procedure to determine a suitable level of site stripping. This method, based on the strength of the signal concerning relationships in a predefined historical epoch, can be useful in a number of situations. First, stripping an alignment of a certain proportion of fast sites has different effects on the signal at different depths in the phylogeny (e.g., fig. 2*C*), and it is convenient to be able to identify some optimal amount of site stripping to address a specific phylogenetic question. Second, some genome-scale studies have shown that competing signals can swap in and out of dominance along a gradient of incremental fast site removal (e.g., Lemieux et al. 2007), and in such situations, it would be very convenient to have an indication of which amount of site stripping is likely to yield the most reliable signal.

Our method measures the signal strength by averaging the BVs of the nodes inside the epoch of interest and evaluates how much signal is gained compared with an analysis of the complete data set (the G_E measure). The rationale of using BVs as a proxy of signal strength is that they reflect the clarity of the signal in the data, that is, are inversely related to the amount of conflict in the data (Felsenstein 1985; Soltis and Soltis 2003). A potential weakness of using BV is that it is not insensitive to node density and thus to taxon sampling. However, this effect is constant across site-stripping conditions and is not expected to have a strong influence on the determination of the optimal condition. Furthermore, it may seem as circular reasoning to use BV to decide on the most suitable site-stripping condition and subsequently use BV to come to conclusions about the strength of relationships. However, this is not entirely true because G_E is calculated over a wide temporal range and across all lineages in the relevant epoch, so it does not rely exclusively on the BV of the branches we wish to resolve. Furthermore, the approach can easily be adapted to use only nodes that are not of immediate interest to calculate G_E. The proper functioning of the method and the impact of the nodes of interest on the optimal condition thus relies on broad sampling of taxa that are not of immediate interest, which is the principal reason that our analyses span the entire green lineage instead of just the Ulvophyceae.

Although fast site removal is commonly employed to help resolve difficult phylogenetic questions, the validity of the method has not been studied in great detail. Our experiments yield insight in a few aspects of its behavior. First, the observation that fast site removal increases BV in ancient epochs at the expense of BV in more recent epochs indicates that it restores the signal contained in slow sites that was masked by fast sites in the full data set. Second, our comparison of signal strength in an alignment with 25% fast sites removed and a set of alignments with 25% random sites removed can be used to address the specificity of fast site removal to retain signal about old relationships. Although for recent epochs, the clarity of the signal in the alignment with the 25% fastest sites removed did not differ significantly from that of alignments from which random sites were removed, this was clearly the case for older epochs. In other words, removal of fast sites preferentially retains information about ancient relationships compared with random site removal, and this preferential retention of signal yields improved bootstrap support for old nodes compared with an analysis of the complete data set (fig. 2*E*). Similarly, comparison of signal strength between an alignment with 25% fast sites removed and an alignment with exclusion of third codon positions indicates that our site-stripping approach retains more information about ancient relationships in the alignment compared with the exclusion of third codon positions (fig. 2*F*).

Progress and Perspectives

The slow progress in green algal phylogenetics can in part be attributed to the limited availability of genomic data and the difficulties in consistently amplifying single-copy nuclear markers over the entire spectrum of a group of organisms that dates back well into the Proterozoic. Our results indicate that phylogenetic analyses of multiple markers from different genomic compartments show great promise in resolving ancient divergences within the green algae. The present phylogeny has provided us with a framework to advance our understanding of the nature of the morphological and cytological diversification of the Ulvophyceae. However, it provides only the first step toward understanding the origin of these features. The discovery of other early-branching Ulvophyceae (e.g., unicells, small filaments) or analyses including additional early-branching lineages (e.g., *Oltmannsiellopsis*) may be necessary to come to a definite conclusion about cytomorphological evolution in the Ulvophyceae. More profound insights into the genetic background of the transitions may benefit from genomics approaches. Further insights may be gained by inferring the timescale of green algal evolution, as this would permit an assessment of the timing of the crucial changes and the global ecological circumstances under which they took place. From a phylogenetic perspective, additional taxon sampling is required to assess the monophyly of the Trebouxiophyceae and Chlorophyceae and consolidate the relationships among UTC classes.

Supplementary Material

Supplementary text S1 and supplementary figures S1–S6 and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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