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Phylogeny of Oedogoniales, Chaetophorales and Chaetopeltidales (Chlorophyceae): inferences from sequence-structure analysis of ITS2

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• *Background and Aims* The green algal class Chlorophyceae comprises five orders (Chlamydomonadales, Sphaeropleales, Chaetophorales, Chaetopeltidales and Oedogoniales). Attempts to resolve the relationships among these groups have met with limited success. Studies of single genes (18S rRNA, 26S rRNA, *rbc*L or *atp*B) have largely failed to unambiguously resolve the relative positions of Oedogoniales, Chaetophorales and Chaetopeltidales (the OCC taxa). In contrast, recent genomics analyses of plastid data from OCC exemplars provided a robust phylogenetic analysis that supports a monophyletic OCC alliance.
• *Methods* An ITS2 data set was assembled to independently test the OCC hypothesis and to evaluate the performance of these data in assessing green algal phylogeny at the ordinal or class level. Sequence-structure analysis designed for use with ITS2 data was employed for phylogenetic reconstruction.
• *Key Results* Results of this study yielded trees that were, in general, topologically congruent with the results from the genomic analyses, including support for the monophyly of the OCC alliance.
• *Conclusions* Not all nodes from the ITS2 analyses exhibited robust support, but our investigation demonstrates that sequence-structure analyses of ITS2 provide a taxon-rich means of testing phylogenetic hypotheses at high taxonomic levels. Thus, the ITS2 data, in the context of sequence-structure analysis, provide an economical supplement or alternative to the single-marker approaches used in green algal phylogeny.

Key words: ITS2, sequence structure, secondary structure, phylogeny, Chlorophyceae, Oedogoniales, Chaetopeltidales, Chaetophorales.

INTRODUCTION

Chlorophyceae are one of three green algal classes currently allied in a UTC (Ulvophyceae + Trebouxiophyceae + Chlorophyceae) clade of core chlorophytes (Fig. 1). The UTC, Prasinophyceae and the new Palmophyllalean group (Zechman *et al.*, 2010) comprise Chlorophyta. Chlorophyta and Streptophyta (the latter including both algae and embryophytes) form Viridiplantae. Current assessments suggest that Viridiplantae, Glaucocystophyta and Rhodophyta form a monophyletic group, Archaeplastida.

Oedogoniales, Chaetophorales and Chaetopeltidales (OCC), which comprise three orders within the green algal class Chlorophyceae, each possess a suite of morphological and anatomical characters that bears witness to their independent status within the class (Lewis and McCourt, 2004). Oedogoniales are branched or unbranched filaments characterized by the production of stephanokont zoospores and sperm. The complexity of the multiple flagellar bases has blocked any assessment of the rotational symmetry of flagellar components (Lewis and McCourt, 2004). Ostensibly linked to the phycoplast type of cytokinetic apparatus, cell division in Oedogoniales is unique in that new cell wall material arises from a ring of polysaccharide that expands following the development of a circumferential dissolution of the parental cell wall immediately adjacent to the ring of nascent wall material (Pickett-Heaps, 1975). As the wall material expands and matures, the remnants of the circumferential wall dissolution, termed caps, mark each end of the new wall segment and, thus, remain etched on the cell surface as testimony to this unusual form of cell division (Pickett-Heaps, 1975). Oedogoniales also bear pyrenoids with cytoplasmic invaginations of the matrix (Hoffman, 1968; Buchheim *et al.*, 2001).

Like Oedogoniales, Chaetophorales largely comprise branched or unbranched filaments. In most cases, the ends of filaments or branches show demonstrable tapering (John *et al.*, 2003). In addition, a number of chaetophoralean genera exhibit heterotrichy (Fritsch, 1935; Smith, 1950; John *et al.*, 2003). Analysis of rotational symmetry in flagellar apparatus components of zoospores indicates that one pair of basal bodies exhibits clockwise (CW) rotation whereas the second pair is directly opposed (DO) or only slightly offset with CW rotation (Bakker and Lokhorst, 1984; Watanabe and Floyd, 1989). Cell division occurs by the formation of a cell plate that is preceded by the formation of a phycoplast system of microtubules (Lokhorst *et al.*, 1984). Pyrenoids of chaetophoralean taxa exhibit thylakoids that traverse the matrix (Stewart *et al.*, 1973; Buchheim *et al.*, 2001).

Chaetopeltidales comprise the most recent ordinal addition to the class Chlorophyceae (O'Kelly *et al.*, 1994). Chaetopeltidales are a diverse group of unicellular, thalloid and pseudofilamentous taxa. Members of the group are allied

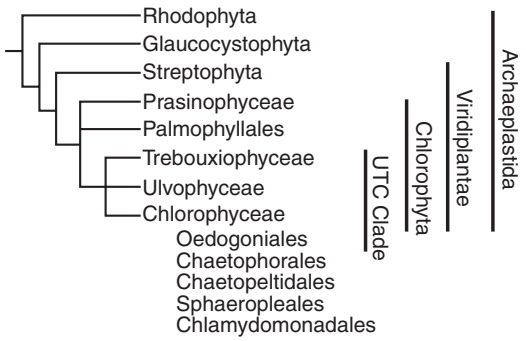

FIG. 1. Phylogenetic overview of Archaeplastida (Rhodophyta + Glaucocystophyta + Viridiplantae), Viridiplantae (Streptophyta + Chlorophyta) and UTC (Ulvophyceae + Trebouxiophyceae + Chlorophyceae) clade. Chlorophyceae comprises at least five orders, Oedogoniales, Chaetophorales, Chaetopeltidales, Sphaeropleales and Chlamydomonadales.

largely on the basis of shared features of the motile cell and flagellar apparatus. The zoospores of chaetopeltidalean taxa are quadriflagellate with both pairs of basal bodies in a strict (no offset) DO orientation (O'Kelly *et al.*, 1994). The zoospore cell body is covered with scales, a feature that appears to be unique among chlorophycean taxa (O'Kelly *et al.*, 1994).

In summary, there is little morphological or anatomical evidence to suggest that OCC form an alliance. Moreover, none of the earliest investigations to apply molecular approaches to the study of green algal phylogeny provided compelling evidence of an OCC clade. One of the first investigations of a subset of these taxa reported that 18S rRNA gene sequence data placed Oedogoniales as an early branching alliance within Chlorophyceae and identified Chaetophorales as the sister group to Chlamydomonadales, but the relative positions of these groups had only weak bootstrap support (Booton *et al.*, 1998*a*). Furthermore, no chaetopeltidalean taxa were included in this analysis. A subsequent study of 18S rRNA data for an expanded set of Chaetophorales and Chaetopeltidales demonstrated strong support for an alliance of these two groups at the base of Chlorophyceae (Sanchez-Puerta *et al.*, 2006). Unfortunately, no oedogonialean taxa were included in this investigation. In a study of 18S and 26S rRNA data, Buchheim *et al.* (2001) conducted a broad sampling of chlorophycean taxa, including representatives of Oedogoniales, Chaetophorales and Chaetopeltidales. An alliance of Chaetophorales and Chaetopeltidales was strongly supported by most analyses of these data (Buchheim *et al.*, 2001). Furthermore, the data also placed Oedogoniales as the earliest or an early branching clade within Chlorophyceae (Buchheim *et al.*, 2001). However, the data did not resolve the issue of the relative positions of Oedogoniales to Chaetophorales and Chaetopeltidales (Buchheim *et al.*, 2001). A Bayesian analysis of ribosomal data by Shoup and Lewis (2003) suggested that Oedogoniales, Chaetophorales and Chaetopeltidales formed a basal grade within Chlorophyceae, but, again, the relative positions of these groups were only poorly supported by the data. Analyses of plastid-encoded genes (*atp*B and *rbc*L) from major chlorophycean lineages have yet to provide a robust alternative to either 18S or 26S rRNA data (Verghese, 2007).

Caisova *et al.* (2011) presented one of the most comprehensive assessments of chaetophoralean diversity, based on 18S rRNA data. Results from their work demonstrated non-monophyly for a number of chaetophoralean genera. Of particular note, however, was the observation that a Bayesian analysis of 18S rRNA data supported a monophyletic OCC clade ($P = 0{\cdot}98$). However, the monophyly of OCC was not robust to method of analysis, and bootstrap values from RaxML, ML, MP and NJ approaches never exceeded 52 % (Caisova *et al.*, 2011).

The most recent and the most compelling investigations to tackle the question of the relative positions of Oedogoniales, Chaetophorales and Chaetopeltidales employed comparisons of plastid genomic data from exemplars for each of the recognized orders of chlorophycean green algae. The results of these investigations identified a deep divergence within Chlorophyceae, uniting Chlamydomonadales and Sphaeropleales into one group and allying Chaetophorales and Chaetopeltidales with Oedogoniales in a second fundamental group (Turmel *et al.*, 2008; Brouard *et al.*, 2010, 2011).

In addition to promoting a novel but well-supported alliance of green algal orders, the results from the genomic study of the plastid provide an excellent opportunity to evaluate an alternative to what are the *de facto* standards for single-marker phylogenetics of green algae, i.e. comparisons of 18S rRNA, 26S rRNA, *atp*B or *rbc*L. An emerging alternative is the internal transcribed spacer two (ITS2) from the rDNA array. DNA sequences from the ITS2 of green algae have most frequently been used to assess diversity within a species, within a genus or among closely related members of a family (An *et al.*, 1999; van Hannen *et al.*, 2000; Hegewald and Hanagata, 2000; Kang and Lee, 2002; Hegewald and Wolf, 2003; Krienitz *et al.*, 2004; Buchheim *et al.*, 2005; Vanormelingen *et al.*, 2007; Hegewald *et al.*, 2010). The ITS2 ranges from a mere 128 bp to 483 bp across the spectrum of chlorophytan diversity, far fewer than the >1000 bp that are typically included in comparisons using 18S rRNA, 26S rRNA, *rbc*L or *atp*B. Thus, one might reasonably challenge the notion that the ITS2, alone, could provide the necessary level of conservation plus variation to provide insight into the question of phylogenetic relationships among major lineages of the class Chlorophyceae. However, results from some investigations led researchers to propose that the ITS2 rRNA can be applied to comparisons of a broad taxonomic spectrum of green algae (Buchheim *et al.*, 2011) or even eukaryotes (Hershkovitz and Lewis, 1996; Mai and Coleman, 1997; Coleman, 2003, 2007; Schultz *et al.*, 2005). In light of these observations, some are promoting ITS2 as a DNA barcode (Chen *et al.*, 2010; Yao *et al.*, 2010). Furthermore, the use of a sequence-structure approach in analyses of ITS2 diversity has provided another layer of interpretation that improves, in theory and in practice, the efficiency of the ITS2 for reconstructing phylogeny (Wolf *et al.*, 2005, 2008; Seibel *et al.*, 2006, 2008; Selig *et al.*, 2008; Keller *et al.*, 2009, 2010; Hegewald *et al.*, 2010; Koetschan *et al.*, 2010).

The goals of this investigation are twofold. First, we intend to provide an independent test, utilizing a sequence-structure analysis of ITS2 from multiple exemplars, of the genomics hypothesis that unites OCC taxa within the class Chlorophyceae (Turmel *et al.*, 2008; Brouard *et al.*, 2010). As a consequence of the use of ITS2 in a test of the OCC hypothesis, our second goal is to provide an additional evaluation

of ITS2 as a tool for recovering phylogenetic signal at high taxonomic levels. If ITS2 continues to demonstrate utility for deep phylogeny assessments, it has the potential to offer a rapid, inexpensive and taxon-rich supplement or alternative to a comparative genomics approach that is powerful, but also time-intensive and expensive.

MATERIALS AND METHODS

Taxon selection

The broad spectrum of ingroup taxa was selected for the investigation because (1) parallel data from other markers already exist for these taxa and (2) this set of taxa allows us to expand previous work with ITS2 (Keller *et al.*, 2008) data that permit an investigator to compare data at multiple Linnean ranks. The OCC taxa included in the investigation (Table 1) include five oedogonialean taxa comprising two genera, six chaetophoralean taxa comprising five genera and three chaetopeltidalean taxa comprising two genera.

DNA extraction, amplification and sequencing

Extraction of genomic DNA from cultured cells of *Uronema belkae* SAG 34·86 was done using Dynabeads (DNA DIRECT Universal, Dynal Biotech, Oslo, Norway) according to the manufacturer's protocol. In addition to the *Uronema* extract, genomic DNA from cells of *Aphanochaete magna* (UTEX B 1909), *Chaetopeltis orbicularis* (UTEX LB 422), *Draparnaldia plumosa* (UTEX LB 423), *Hormotilopsis gelatinosa* (UTEX 104), *Hormotilopsis tetravacuolaris* (UTEX 946), *Schizomeris leibleinii* (UTEX LB 1228) and *Stigeoclonium helveticum* (UTEX 441) that had been collected as part of a previous study of 18S and 26S rRNA data (Buchheim *et al.*, 2001) was used for standard PCR amplification of the ITS array, including ITS2. Flanking primer sets of ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAG G-3′) or ITS3 (5′-GCAT CGATGAAGAACGCAGC-3′) and ITS4 (White *et al.*, 1990) were used for all amplifications. Purified and concentrated PCR product served as the template for cycle-sequencing using the flanking primers described above and ITS1 [5′-TC CGTAGGTGAACCTGCGG-3′ (White *et al.*, 1990)], ITS2 [5′-GCTGCGTTCTTCATCGATGC-3′ (White *et al.*, 1990)] and ITS7 (5′-CAAGAGCATGTCTGCCTCA-3′). Base calls from each fragment were edited and assembled into contigs using Sequencher (v. 4·9, Genecodes Corp., Ann Arbor, MI, USA). All sequences generated by this investigation were deposited in the nucleotide database at the National Center for Biotechnology Information [NCBI (Benson *et al.*, 2008), see Table 1].

Secondary structure prediction, alignment and phylogenetic analysis

ITS2 sequences were annotated according to Keller *et al.* (2009). ITS2 secondary structures of newly obtained sequences and of one sequence available at GenBank (Benson *et al.*, 2008) (Table 1) were folded with the help of RNAstructure (Mathews *et al.*, 2004) and then manually corrected. The new sequence-structure data and an outgroup (see below) were added to the sequence-structure alignment from Keller *et al.* (2008). The phylogenetic analysis followed the procedure outlined in Schultz and Wolf (2009) in accordance with Keller *et al.* (2010). A global, multiple sequence-structure alignment was generated in 4SALE v1·5 (Seibel *et al.*, 2006, 2008). Sequences and secondary structures were synchronously aligned, making use of an ITS2 sequence-structure-specific scoring matrix (Seibel *et al.*, 2006). Based on primary and secondary structure information, phylogenetic relationships were reconstructed by the neighbour-joining (NJ) method (Müller *et al.*, 2004), through the use of an ITS2 sequence-structure-specific, general time

TABLE 1. *List of OCC taxa added to the ITS2 sequence-structure alignment (Keller* et al.*, 2008) as a consequence of this investigation*

OCC taxa with ordinal affiliation	Provenance	NCBI accession number
Aphanochaete magna Godward (Chaetophorales)*	UTEX[a] B 1909*	HQ646383*
Bulbochaete rectangularis Wittrock (Oedogoniales)	UTEX LB 954	AY962677
Chaetopeltis orbicularis Berthold (Chaetopeltidales)*	UTEX LB 422*	HQ646378*
Draparnaldia plumosa Vaucher (C.A.Agardh) (Chaetophorales)*	UTEX LB 423*	HQ646379*
Hormotilopsis gelatinosa Trainor & Bold (Chaetopeltidales)*	UTEX 104*	HQ646380*
Hormotilopsis tetravacuolaris Arce & Bold (Chaetopeltidales)*	UTEX 946*	HQ646384*
Oedogonium cardiacum (Hassall) Wittrock (Oedogoniales)	UTEX LB 40	AY962675
Oedogonium nodulosum Wittrock (Oedogoniales)	FACHB[b] 996	DQ078301
Oedogonium oblongum Wittrock (Oedogoniales)	ACOI[c] 1118	AY962681
Oedogonium undulatum (Brébisson) A.Braun (Oedogoniales)	See Mei *et al.* (2007)	DQ178025
Schizomeris leibleinii Kützing (Chaetophorales)*	UTEX LB 1228*	HQ646381*
Stigeoclonium helveticum Vischer (Chaetophorales)*	UTEX 441*	HQ646382*
Uronema belkae G.M.Lokhorst (Chaetophorales)*	SAG[d] 34·86*	HQ646377*
Uronema sp. (Chaetophorales)	CCAP[e] 335/1B	FR717536

Ordinal affiliation, provenance of cultures and accession numbers for each set of sequence data are noted. Taxa for which new sequence data were generated for this investigation are noted with an asterisk*. Abbreviations: [a]UTEX, Culture Collection of Algae at the University of Texas at Austin; [b]FACHB, Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Science; [c]ACOI, Coimbra Collection of Algae; [d]SAG, Culture Collection of Algae (Sammlung von Algenkulturen der Universität Göttingen); [e]CCAP, Culture Collection of Algae and Protozoa (Plymouth, UK).

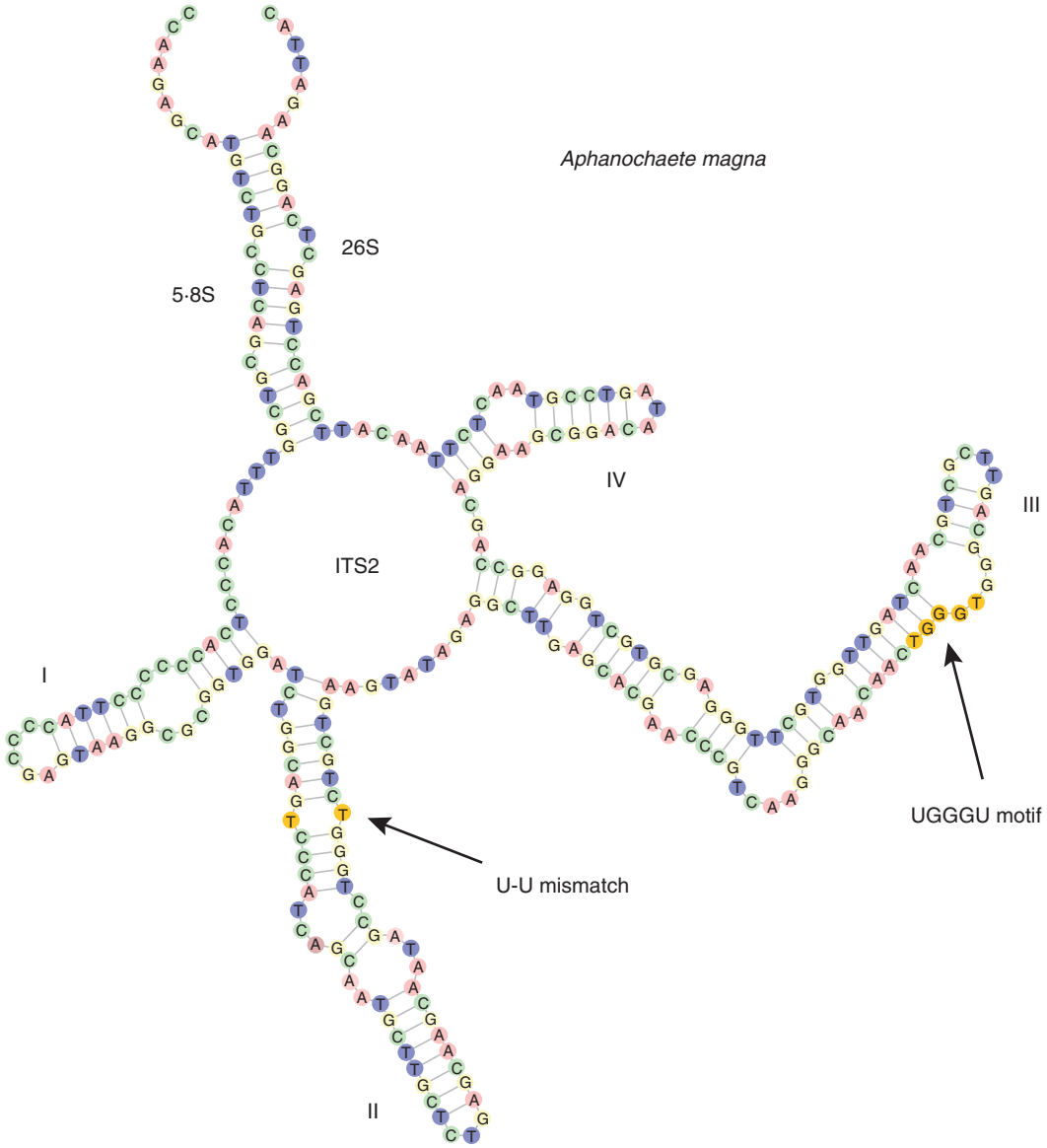

FIG. 2. 5·8S–26S rRNA hybridization and ITS2 secondary structure of *Aphanochaete magna* visualized with 4SALE (Seibel *et al.*, 2008). ITS2 helices are numbered I–IV. Typical ITS2 motifs are highlighted.

reversible (GTR) substitution model as implemented in ProfDistS v0.9.8 (Friedrich *et al.*, 2005; Rahmann *et al.*, 2006; Wolf *et al.*, 2008). Bootstrap support (Felsenstein, 1985) was estimated based on 100 pseudo-replicates. An appropriate outgroup was identified using the ITS2 sequence-structure BLAST available at the ITS2 Database (Koetschan *et al.*, 2010). The tree was rooted with two taxa classified as Ulvophyceae [*Acrochaete* sp. (gi:157695730) and *Ulva laetevirens* (gi:219525733)] and visualized using Treeview (Page, 1996). ITS2 sequence-structure data for ulvophycean taxa (serving as outgroup) and trebouxiophycean taxa [serving as supplemental taxonomic context; *Chlorella sorokiniana* (gi:207366656) and *Parachlorella beijerinckii* (gi:207366649)] were obtained from the ITS2 Database Version 3·0.3 (Schultz *et al.*, 2006; Selig *et al.*, 2008; Koetschan *et al.*, 2010).

RESULTS

Secondary structure

Folding of the chaetopeltidalean, chaetophoralean and oedogonialean ITS2 sequences revealed the typical features for secondary structure for eukaryotes, including the presence of helices I–IV (Mai and Coleman, 1997; Joseph *et al.*, 1999; Schultz *et al.*, 2005; Koetschan *et al.*, 2010). For illustration purposes, only the sequence for *Aphanochaete magna* is presented (Fig. 2).

Phylogenetic analysis

Results from analyses (Fig. 3) reveal strong bootstrap support (93 %) for a monophyletic Chlorophyceae. In addition, in the OCC, the taxa with clockwise absolute orientation

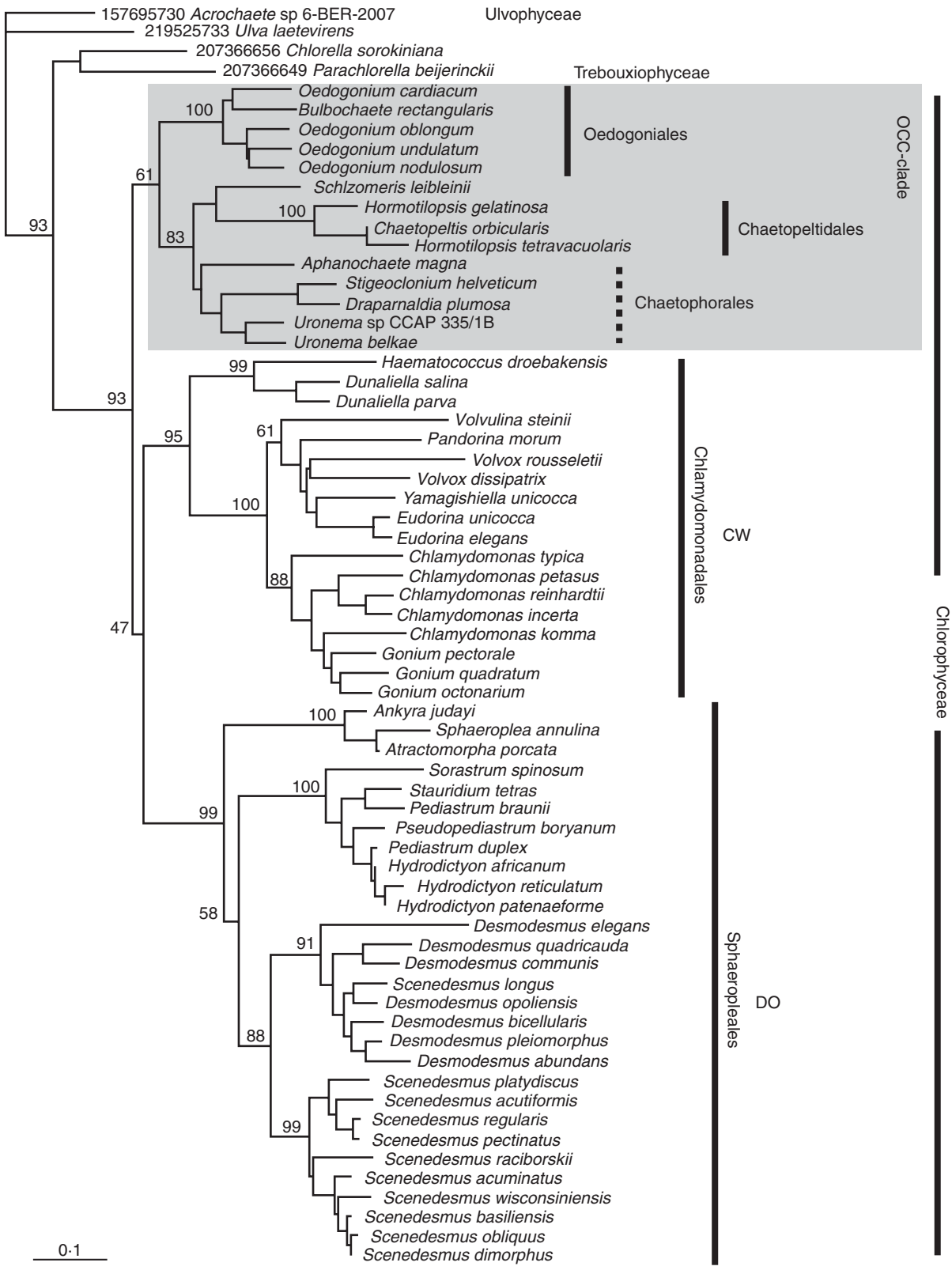

FIG. 3. ITS2 sequence-structure tree of Chlorophyta (rooted with Ulvophyceae). Bootstrap values based on 100 replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The OCC clade is shaded in grey.

of flagellar components (CW; Chlamydomonadales) and the taxa with directly opposed flagellar components (DO; Sphaeropleales) form independent, monophyletic groups (Fig. 3). Bootstrap values for monophyletic CW and DO clades are robust (95 and 99 %, respectively), but support for the OCC clade is not strong (<65 %). Multiple experiments (including sequence-only data; Supplementary Data Fig. S1, available online) that tested for topological stability indicated that the OCC clade is not robust to taxon deletion trials or to alternative substitution models (data not shown). The CW and DO clades form a monophyletic group, but bootstrap support for this alliance is weak (<50 %). Analyses of the data support a monophyletic Oedogoniales (100 %). An alliance of Chaetopeltidales and Chaetophorales is supported with modest bootstrap support (83 %). Chaetopeltidales are robustly resolved as monophyletic (100 % bootstrap support). *Aphanochaete magna* is resolved as the earliest branching lineage within a monophyletic alliance of chaetophoralean taxa, but with only weak bootstrap support (<50 %). The chaetophoralean taxon *Schizomeris leibleinii* is placed as sister to the rest of the chaetopeltidalean clade, albeit with only weak support (<50 %).

DISCUSSION

ITS2 and the OCC clade

Although bootstrap support is not strong, our sequence-structure analysis of ITS2 data corroborates the hypothesis, based on plastid genomic analysis, of a monophyletic OCC clade (Turmel *et al.*, 2008; Brouard *et al.*, 2010, 2011). Within the OCC clade, analyses of the ITS2 data strongly support Oedogoniales and Chaetopeltidales (*sensu stricto*) as independent groups. Robust support for Chaetophorales is lacking, but the ITS2 data provide modest support (>80 %) for a Chaetopeltidales + Chaetophorales alliance. Our results also corroborate the broader aspects of the plastid hypothesis (Turmel *et al.*, 2008; Brouard *et al.*, 2010, 2011). Results from analyses of the ITS2 data identify Chlamydomonadales (CW) and Sphaeropleales (DO) as sister taxa, albeit with weak support. Nonetheless, the ITS2 data recover the same split between the OCC and the CW/DO groups as observed in the plastid genomic data. As noted in the Introduction, all but one (Caisova *et al.*, 2011) of the previous investigations of 18S rRNA, 26S rRNA, *rbc*L or *atp*B failed to support a monophyletic OCC group. Moreover, only the Bayesian analysis of 18S data supported a monophyletic OCC clade (Caisova *et al.*, 2011). Thus, our hypothesis testing demonstrates that a sequence-structure analysis of ITS2 is a reasonable and economical alternative for single-marker assessments of chlorophycean taxa. This observation is particularly remarkable as the ITS2 comprises, at best, half the total of nucleotides available for comparisons using a standard, single-marker gene.

Aphanochaete and *Schizomeris*

Bootstrap values indicate that the relative positions of *Aphanochaete* and *Schizomeris*, both regarded as chaetophoralean taxa (Bourrelly, 1990; John *et al.*, 2003; Wehr and Sheath, 2003), are likely to be the most labile among the OCC taxa. Of particular note is the observation that *Schizomeris* is placed as sister to the rest of Chaetopeltidales. This finding challenges the monophyly of a traditional circumscription of Chaetophorales. O'Kelly *et al.* (1994) suggested that Chaetophorales were likely to be derived from a *Chaetopeltis*-like ancestor. Whereas neither our data nor the plastid inferences (Turmel *et al.*, 2008; Brouard *et al.*, 2010, 2011) support the specifics of this assertion, it is generally consistent with a relatively close alliance of the two orders.

Uronema and *Hormotilopsis*

The ITS2 data provide support for a monophyletic *Uronema* but offer a challenge to the monophyly of *Hormotilopsis*. In the case of *Hormotilopsis*, 18S rRNA data are available for both *H. gelatinosa* and *H. tetravacuolaris*. Analyses of these ribosomal data also fail to support a monophyletic *Hormotilopsis* (Booton *et al.*, 1998*b*). Unfortunately, our understanding of the spectrum of taxonomic diversity in Chaetopeltidales remains sufficiently impoverished as to preclude any alternative explanation for the non-monophyly of *Hormotilopsis*. Although it is possible that one or the other of the *Hormotilopsis* taxa may ultimately be relegated to another extant chaetopeltidalean taxon (e.g. *Phyllogloea* or *Dicranochaete*), there is currently no evidence to support such a conclusion. It also remains possible that the monophyly issue for *Hormotilopsis* might be resolved through additional taxon sampling. However, it seems likely that a new chaetopeltidalean taxon will need to be erected to accommodate the considerable nucleotide distance between the two species of *Hormotilopsis*.

Future work and new approaches

Our current understanding of molecular diversity in Chlorophyceae has focused on five orders: Chlamydomonadales, Sphaeropleales, Oedogoniales, Chaetophorales and Chaetopeltidales. However, there is evidence to suggest that at least two additional lineages of ordinal status might exist within Chlorophyceae. Studies of ribosomal data made compelling but incomplete cases for the existence of a *Cylindrocapsa* clade [*Cylindrocapsa*, *Treubaria*, *Trochiscia* and *Elakatothrix* (Buchheim *et al.*, 2001; Lewis and McCourt, 2004)] and a *Mychonastes* clade [*Mychonastes* and *Pseudodictyosphaerium* (Krienitz *et al.*, 2003; Lewis and McCourt, 2004)]. Sequence-structure analysis of ITS2 offers an excellent opportunity to explore further the phylogenetic position of these enigmatic, green algal taxa.

We also contend that another advantage that sequence-structure studies of ITS2 exert over the traditional, single marker investigations is the ability to quickly and inexpensively identify targets for the powerful, but decidedly more selective genomics approaches. For example, the results from this investigation suggest that *Aphanochaete* might be a good candidate for a plastid genomics analysis. Beyond the OCC clade, Sphaeropleales are a well-supported alliance, albeit comprising highly diverse taxa. Genomics data are available for *Scenedesmus*, but not for any other sphaeroplealean taxon.

Analyses of ITS2 (Keller *et al.*, 2008) and more traditional ribosomal data (Buchheim *et al.*, 2001; Wolf *et al.*, 2002; Shoup and Lewis, 2003; Lewis and McCourt, 2004) show that Sphaeropleaceae comprise a highly divergent lineage in the order. As a consequence, results from analyses of the ITS2 data indicate that additional members of Sphaeropleaceae (e.g. *Sphaeroplea* or *Atractomorpha*) are logical targets of a genomics investigation.

The results of this investigation provide more evidence of the potential utility of the ITS2, coupled with secondary structure analysis, for reconstructing the phylogeny of major chlorophytan lineages. However, one avenue of future work that has the capacity to further enhance the use of ITS2 is the development of character-based approaches to sequence-structure phylogenetics. Specifically, we envisage developing parsimony, likelihood and Bayesian approaches to sequence-structure analyses of ITS2. By employing character-based approaches, one can avoid criticisms associated with the use of an algorithmic method such as NJ (i.e. algorithmic methods, by definition, do not find optimal trees). Adapting character-based methods for sequence-structure analysis will probably lead to more computational complexity (as the character-based methods are exercises in hill-climbing); however, one will gain a powerful repertoire of statistical tests (e.g. tree comparison metrics) that are generally unavailable with algorithmic methods.

CONCLUSIONS

This two-fold test of sequence-structure analyses using ITS2 data provided corroborating evidence for the existence of an OCC clade in the class Chlorophyceae. In addition, the results presented here provide further support for the assertion that the ITS2 data (both primary sequence and secondary structure) possess phylogenetic signal of sufficient conservation and variability to permit its use in reconstructing the phylogeny of major green algal lineages. Not all nodes within our ITS2 analyses have robust support, but the results of this investigation, at the very least, show equivalent levels of topological congruence when genomics data are compared with the more traditional, single-marker genes. Moreover, generating ITS2 data can be accomplished with a considerably smaller investment in time and money than is needed for preparing complete sequences for the standard ribosomal or plastid genes. Given that studies of ITS2 can be completed in a taxon-rich framework with relative ease, this sequence-structure approach can serve as a guide for more focused, genomics investigations. These observations offer compelling evidence that the ITS2 should not be dismissed as a single marker of choice for reconstructing green algal phylogeny.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Figure S1: phylogenetic tree using only the ITS2 primary sequence information for alignment (CLUSTALX) and tree reconstruction (NJ).

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