Are Combined Analyses Better Than Single Gene Phylogenies? A Case Study Using SSU rDNA and rbcL Sequence Comparisons in the Zygnematophyceae (Streptophyta)

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Although the combination of different genes in phylogenetic analyses is a promising approach, the methodology is not well established and analyses often suffer from inadequate, noncongruent taxon sampling, long-branch attraction, or conflicting evolution models of the genes analyzed. Conflicts or congruence between multigene and single-gene phylogenies, as well as the assumed superiority of the multigene approach, are often difficult to assess solely because of incongruent taxon sampling. In the present study, a data set of 43 nuclear-encoded SSU rDNA and plastid-encoded rbcL gene sequences was generated from the same strains of conjugating green algae (Zygnematophyceae, Streptophyta). Phylogenetic analyses used the genes individually and in combination, either as concatenated sequences or with the log-likelihood summation method. Single-gene analyses, although mostly congruent, revealed some conflicting nodes and showed different patterns of statistical support. Combined analyses confidently resolved the conflicts between the single-gene analyses, enhanced phylogenetic resolution, and were better supported by morphological information. Long-branch taxa were not the same for the two genes analyzed, and, thus, their effect on phylogenetic resolution was minimized in the combined analyses.

Introduction
Recent years have seen combined analyses using two or more genes or even complete genomes become increasingly popular in molecular phylogenetic studies. Multigene phylogenies have been used to address the evolution of embryophyte land plants (Nickrent et al. 2000; Shaw and Allen 2000; Karol et al. 2001; Bowe, Coat, and dePamphilis 2002), animals (Mallat and Winchell 2002), various groups of algae (Nozaki et al. 2000; Hoef-Emden, Marin, and Melkonian 2002), and the radiation of eukaryotes (Baldauf et al. 2000; Bapteste et al. 2000). In most of these groups of organisms, single-gene analyses did not provide sufficient resolution or sometimes gave conflicting results, which is often ascribed to the limited number of alignable nucleotides or to differing rates of sequence evolution (Capesius and Bopp 1997; Nei, Kumar, and Takahashi 1998; Poe and Swofford 1999; Nickrent et al. 2000; Philippe 2000; Hoef-Emden, Marin, and Melkonian 2002). Thus, combined approaches are driven by the assumption that a larger number of characters improves phylogenetic accuracy and resolution (Hillis 1996). However, it is known that a strong bias in evolutionary rates (leading to long-branch attraction [LBA]) may persist and even increase when more and more characters are added (Sanderson and Shaffer 2002).

In practice, some multigene analyses are still leading to conflicting results, are sensitive to LBA, and do not significantly resolve all internal branches (e.g., Karol et al. 2001; Murphy et al. 2001; Bapteste et al. 2002). Insufficient taxon sampling because of limiting sequencing and computation capacities can be a major problem in multigene approaches, whereas single-gene phylogenies may recover the correct topology because of a better taxon representation (Graybeal 1998; Bapteste et al. 2002). Moreover, multigene analyses often deal with data sets originating from incongruent taxon sampling (i.e., different genes representing the same taxon did not originate from the same clonal source) and are still affected by unresolved methodological problems, especially model misspecifications (Cao et al. 1998; Bapteste et al. 2002; Hoef-Emden, Marin, and Melkonian 2002; Pupko et al. 2002). Because the characteristics of sequence evolution are rarely identical in different genes, an “average” model for a multigene data set may sufficiently deviate from single-gene models to favor spurious relationships in the analysis. Ideally, the combined analysis should allow for different sets of model parameters to be used for the different genes (Yang 1996; Bapteste et al. 2002; Pupko et al. 2002).

These general questions of relationship between single-gene and multigene analyses have been rarely studied using real sequence data. An ideal two-gene data set to resolve such questions should have the following characteristics: congruent taxon sampling, comparable phylogenetic resolution in single-gene trees, and presence of some conflict between single-gene trees that could be tested with the multigene approach and compared with independent evidence derived from morphological information. For the present study, we have generated such a data set comprising nuclear-encoded SSU rDNA and plastid-encoded rbcL sequences of 43 taxa (clonal strains) of zygnematophycean green algae.

Our model taxon, the class Zygnematophyceae, is characterized by a unique mode of sexual reproduction (conjugation) and occupies a still unresolved position within the streptophyte green algae (Chapman et al. 1998; Karol et al. 2001; Turmel et al. 2002a). Absence of flagellate reproductive stages and any trace of centriolar centrosomes in the Zygnematophyceae are presumably unique among the streptophyte green algae. Previous single-gene phylogenies using SSU rDNA (Bhattacharya et al. 1994; Surek et al. 1994; Chapman et al. 1998;
Materials and Methods

Cultures

The 43 strains of conjugating green algae used for this study were obtained from different sources (table 1) and grown in modified WARIS-H culture medium (McFadden and Melkonian 1986) at 20°C with a photon fluency rate of 40 µmol m⁻²/s in a 14/10 h light/dark cycle.

DNA Extraction, Amplification, and Sequencing

After mild ultrasonication to remove mucilage (Surek and Sengbusch 1981), total genomic DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). SSU rDNA and rbcL were amplified by polymerase chain reactions (PCR) using published protocols and 5′-biotinylated PCR primers (Marin, Klingberg, and Melkonian 1998). PCR and sequencing primers for SSU rDNA were described elsewhere (Marin, Klingberg, and Melkonian 1998; Gontcharov, Marin, and Melkonian 2003) for newly designed rbcL primers, see table 2). PCR products were purified with the Dynabeads M-280 system (Dynal Biotech, Oslo, Norway) and used for bidirectional sequencing reactions (for protocols, see Hoef-Emden, Marin and Melkonian [2002]). Gels were run on a Li-Cor IR² DNA sequencer.

Sequence Alignments and Tree Reconstructions

Sequences were manually aligned using the Olson Multiple Sequence Alignment Editing Program (Olsen 1990). For coding regions of the SSU rDNA of the Zygnematophyceae, the alignment was guided by primary and secondary structure conservation (Wuyts et al. 2000, 2001 [http://oberon.rug.ac.be:8080/rRNA/]). The alignments are available from the authors upon request. Phylogenetic trees were inferred with maximum-likelihood (ML), neighbor-joining (NJ), and maximum-parsimony (MP) criteria using PAUP version 4.0b10 (Swofford 1998) and Bayesian inference (BI) using MrBayes version 3.0b3 (Huelsenbeck and Ronquist 2001). SSU rDNA (1,722 unambiguously aligned nt) and rbcL data sets (1,353 nt) were analyzed separately and in combination (3,075 nt). Evolutionary models (for ML and NJ analyses) for the different data sets were selected via Modeltest version 3.04 (Posada and Crandall 1998). Distances used for NJ analyses were calculated by ML. ML and MP analyses used heuristic searches with a branch-swapping algorithm (tree bisection-reconnection). In BI, the Markov chains were run for one million generations, sampling every 100 generations for a total of 10,000 samples. The first 500 (rbcL set) or 1,000 (SSU rDNA and combined sets) samples were discarded as “burn-in.” The remaining samples were combined into a single file and analyzed using the sumt command in MrBayes. The robustness of the trees was estimated by bootstrap percentages (BP [Felsenstein 1985]) using 1,000 (NJ and MP) or 100 (ML) replications and by posterior probabilities (PP) in BI. Nonsignificant BP less than 50% and PP less than 0.90 were not included in figures. In MP, the stepwise addition option (10 heuristic searches with random taxon input order) was used for each bootstrap replicate. ML-bootstrap used a single heuristic search (starting tree via stepwise addition) per replicate.

Combined Analyses

For concatenated analyses, SSU rDNA and rbcL sequence data were fused as a “supergene” in one alignment and analyzed using a single “concatenated model” with averaged parameters. In addition, we performed a combined analysis via “log-likelihood summation” (LS [Yang 1996]) following the method described by Bapteste et al. (2002). The 1,000 “best” ML topologies of the SSU rDNA, rbcL and concatenated data sets (3,000 trees) were combined in a treefile and log-likelihood values were calculated separately for the SSU rDNA data set (with the SSU rDNA model) and the rbcL alignment (with the rbcL model). For each topology, the sum of both values was calculated and provided the tree optimality criterion. Similarly, the “LS” method was applied to the rbcL gene alone, and the data set was subdivided corresponding to the three codons. For each codon, as well as for the complete gene, the 1,000 best ML trees were determined (4,000 trees) with the appropriate model (table 3), and for each tree topology, the log-likelihood values were calculated separately for each codon (using the appropriate model). The sum of all three values again provided the tree optimality criterion.

Topology Tests

User-defined trees were generated by manually modifying the treefile of the “best tree” using TreeView version 1.6.2 (Page 1996). To compare user-defined
Table 1
Origin and Taxonomic Designation of Strains and EMBL/GenBank Accession Numbers of SSU and rbcL rDNA

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain</th>
<th>SSU rDNA</th>
<th>rbcL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bambusina borreri (brebissonii) (Raf.) Cl.</td>
<td>CCAC 0045</td>
<td>AJ428118</td>
<td>AJ553935</td>
</tr>
<tr>
<td>Closterium lunula (Müll.) Nitzsch ex Rafs</td>
<td>M 2156</td>
<td>AJ553916</td>
<td>AJ553936</td>
</tr>
<tr>
<td>Cosmarium contractum Kirchn.</td>
<td>SVCK 396</td>
<td>AJ428112</td>
<td>AJ553937</td>
</tr>
<tr>
<td>Cylindrocystis brebissonii Mensegh. ex De Bary</td>
<td>ACOI 55</td>
<td>AJ549228</td>
<td>AJ553938</td>
</tr>
<tr>
<td>C. brebissonii var. deserti Flechtnr et Johansen</td>
<td>UTEX B 2684</td>
<td>AJ553920</td>
<td>AJ553939</td>
</tr>
<tr>
<td>C. crassa De Bary</td>
<td>ACOI 788</td>
<td>AJ553918</td>
<td>AJ553940</td>
</tr>
<tr>
<td>C. cf. cashleakhæ Brook</td>
<td>M 2158</td>
<td>AJ553919</td>
<td>AJ553941</td>
</tr>
<tr>
<td>Cylindrocystis sp.</td>
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<td>AJ553917</td>
<td>AJ553942</td>
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<tr>
<td>Gonatozygon brebissonii De Bary</td>
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<td>AJ428083</td>
<td>AJ553944</td>
</tr>
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<td>C. brebissonii De Bary</td>
<td>SVCK 250</td>
<td>AJ553921</td>
<td>AJ553945</td>
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<td>G. kinalhinii (Arch.) Rabenh.</td>
<td>ACOI 350</td>
<td>AJ553939</td>
<td>AJ553946</td>
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<td>AJ553947</td>
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<td>AJ553943</td>
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<tr>
<td>Haplotaenium (Pleurotaenium) minutum</td>
<td>SVCK 302</td>
<td>AJ428125</td>
<td>AJ553948</td>
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<td>(Raf.) Bando</td>
<td>SVCK 428</td>
<td>AJ553929</td>
<td>AJ553949</td>
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<td>Heimania (Cosmoschlamydia) pusilla (Hilse) Coes.</td>
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<td>Mesotaenium caldariorum (Lagerch.) Hatn.</td>
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<td>AJ553952</td>
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<td>Mongeotia sp.</td>
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<td>Mongeotia sp.</td>
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<td>AJ553954</td>
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<td>AJ553955</td>
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<td>N. interruptum (Breb.) Lütkem.</td>
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<td>AJ428071</td>
<td>AJ553956</td>
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<tr>
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<td>AJ553925</td>
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<tr>
<td>N. oblongum</td>
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<td>AJ553958</td>
</tr>
<tr>
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<td>AJ553930</td>
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<td>P. exiguum W. West</td>
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<td>AJ553960</td>
</tr>
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<td>P. spirostreptoides Barker.</td>
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<td>AJ553961</td>
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<td>AJ553962</td>
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<td>AJ553963</td>
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<td>R. obtusa (Breb.) W. et G.S. West</td>
<td>SVCK 45</td>
<td>AJ428082</td>
<td>AJ553964</td>
</tr>
<tr>
<td>Spinoclosterium cuspitatum (Bayley)</td>
<td>NIES 325</td>
<td>AJ553931</td>
<td>AJ553965</td>
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<tr>
<td>Hirano</td>
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<tr>
<td>Spirogyra (Sirogonium) sticina (J.E. Smith) Wille</td>
<td>UTEX 1985</td>
<td>AJ428076</td>
<td>AJ553966</td>
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<tr>
<td>Spirogyra sp.</td>
<td>M 2157</td>
<td>AJ549231</td>
<td>AJ549239</td>
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<td>Spirogyra sp.</td>
<td>M 1810</td>
<td>AJ428074</td>
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<td>Spondylosium panduriforme (Heimerl)</td>
<td>SAG 52.88</td>
<td>AJ428124</td>
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<td>S. pulchrum (Bail.) Arch.</td>
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<td>Staurostrum lunatum Rafs</td>
<td>SVCK 15</td>
<td>AJ428106</td>
<td>AJ553971</td>
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<td>Staurastrum viride ex Rafs</td>
<td>SVCK 85</td>
<td>AJ428108</td>
<td>AJ553972</td>
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<td>Zygogonium deserti</td>
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<td>AJ553973</td>
</tr>
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<td>AJ553976</td>
</tr>
<tr>
<td>Zygogonium tunetanum Gauthier-Lievre</td>
<td>UTCC 136</td>
<td>AJ549230</td>
<td>AJ549235</td>
</tr>
</tbody>
</table>

Note.—Sequences determined for this study are indicated by accession numbers in bold face. ACOI = Coimbra Collection of Algae, University of Coimbra, Portugal (www.uc.pt/botanica/ACOI.htm); ASW = Culture Collection of Algae at the University of Vienna (www.univie.ac.at/IECB/hydrobotanik/); CCAC = Culture Collection of Algae at the University of Cologne, Germany (www.ccac.uni-koeln.de); M = Culture Collection Mellonian, Botanical Institute, University of Cologne, Germany; NIES = National Institute for Environmental Studies, Tsukuba, Japan (www.nies.go.jp/Biology/mec/home.htm); SAG = Sammlung von Algenkulturen, University of Göttingen, Germany (www.gwdg.de/~epsag/phylkologia/epsag.html); SVCC = Sammlung von Conjugaten-Kulturen, University of Hamburg, Germany (www.biologie.uni-hamburg.de/b-online/d44_1/44_1.htm); UTCC = University of Toronto Culture Collection of Algae and Cyanobacteria (www.botany.utoronto.ca/utex/); UTEX = Culture Collection of Algae at the University of Texas at Austin, U.S.A. (www.bio.utexas.edu/research/utex/); Taxon names in (parentheses) correspond to those used in the culture collection catalogue. [Mesotaenium endlicherianum] presumably represents a wrong determination of strain SAG 12.97.

topologies with the “best tree,” site-wise log-likelihoods were calculated for each topology in PAUP and used as input for CONSEL (Shimodaira and Hasegawa 2001), which calculates the probability values according to the Kishino-Hasegawa test (KH [Kishino and Hasegawa 1989]), the Shimodaira-Hasegawa test (SH [Shimodaira and Hasegawa 1999]), both weighted [w] and unweighted, and the approximately unbiased test (AU) using the multiscale bootstrap technique (Shimodaira 2002). CONSEL was also used to test incongruence between the three “best” ML trees (figs. 1–3) using the SSU rDNA, the rbcL, and the combined data set.
Table 2
Newly Designed Oligonucleotides Used for PCR and Sequencing (Seq) of rbcL

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MaGo1F</td>
<td>ATGTCACACAAAACNGAAAC</td>
</tr>
<tr>
<td>MaGo2F</td>
<td>ATGTCACACAAAACNGAAACTAAAGCWGG</td>
</tr>
<tr>
<td>MaGo3R</td>
<td>(5' - biotinylated) GTATCRATHGTWTCAAATTC</td>
</tr>
<tr>
<td>Seq-1F-rbcL</td>
<td>CACCACAAAACNGAAACTAAAGCWGG</td>
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<tr>
<td>Seq-2F-rbcL</td>
<td>TRTYACYTCTYTTTGG</td>
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<tr>
<td>Seq-4F-rbcL</td>
<td>ATCRATHGTWTCAAATTC</td>
</tr>
<tr>
<td>Seq-5F-rbcL</td>
<td>TTWGYYTATDGTCACAWCC</td>
</tr>
</tbody>
</table>

Results
Taxon Sampling

For this study, 18 new SSU rDNA and 41 new rbcL sequences were obtained from 43 strains of the Zygnematophyceae; the new sequences are available under GenBank accession numbers AJ553916 to AJ553976. For four nonmonophyletic genera (Mesotaenium, Cylindrocytis, Netrium, and Penium; [Gontcharov, Marin, and Melkonian 2003, and unpublished results]) three to five strains/species were included, whereas for the monophyla Spirogyra, Mougeotia, Zygnema, and Gonatozygon, two or three representative members (the most distantly related in SSU rDNA phylogenies) were selected. To cover the derived family Desmidiaceae (containing about 35 genera and approximately 2,000 species), 10 genera (one species/strain each) were analyzed, including its basal divergence in SSU rDNA phylogenies, Phymato-

docius nordstedtiana (Gontcharov, Marin, and Melkonian 2003).

Although the monophyly of the Zygnematophyceae is usually recovered (McCourt et al. 2000; Gontcharov, Marin, and Melkonian 2003), the position of this class within the streptophytes remains unsettled. Therefore, only unrooted phylogenies are chosen in this study.

SSU rDNA Phylogeny

The selected model for the SSU rDNA data set was TrN with gamma shape (Γ) and proportion of invariable sites (I). The value of one substitution rate category, C→T, was considerably elevated (6.9) compared to those of other categories (table 3).

In the ML phylogeny, the 43 sequences were arranged in two clusters corresponding to the orders Zygnematales and Desmidiaceae (fig. 1). This split was strongly supported by all methods except MP, and the longest internal branch (20 steps) separated these clusters. In the Desmidiaceae, two robust families/clades with long individual branches, Gonatozygaceae (GON) and Closteriaceae (CL), preceded a crown assemblage (designated DESM), which comprised the Desmidiaceae and two of three species of the Peniaceae analyzed (Penium spiriformis was a weakly supported sister taxon of DESM). The significance of DESM was only moderate in ML, NJ, and MP analyses (65% to 71%), but PP in BI analysis was 1.0 (fig. 1).

Within the Zygnematales, several genera obtained high support, namely Mougeotia, Zygnema, and Gonatozygon, and the long-branched Spirogyra (SPI). Zygnema and Zygogonion formed the moderately supported ZYG clade. Other genera were not mono-

Table 3
Evolutionary Models, Log-Likelihood Values (−ln L), and Settings Identified by Modeltest for Different Data Sets Used for Figure 1 to Figure 3 and for Special Analyses

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>SSU rDNA (Fig. 1)</th>
<th>rbcL (Fig. 2)</th>
<th>SSU rDNA + rbcL (Fig. 3)</th>
<th>SSU rDNA – SPI-clade</th>
<th>rbcL – 3a</th>
<th>rbcL (1 + 2)b</th>
<th>rbcL (1)c</th>
<th>rbcL (2)d</th>
<th>rbcL (3)e</th>
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</thead>
<tbody>
<tr>
<td>−ln L</td>
<td>9734.959</td>
<td>16535.103</td>
<td>27120.692</td>
<td>7874.399</td>
<td>1406.352</td>
<td>4072.608</td>
<td>2727.526</td>
<td>1170.204</td>
<td>11379.570</td>
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<td>I</td>
<td>0.5097</td>
<td>0.5558</td>
<td>0.6069</td>
<td>0.6163</td>
<td>0.6393</td>
<td>0.8283</td>
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<td>Γ</td>
<td>0.6565</td>
<td>1.4549</td>
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<td>0.7270</td>
<td>0.4056</td>
<td>1.9752</td>
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</table>

Base frequencies:
- A: 0.2647 0.2869 0.2661 0.2669 0.2844 0.2450 0.2071 0.2880 0.3312
- C: 0.2157 0.1529 0.1753 0.2057 0.1525 0.2489 0.2284 0.2560 0.1006
- G: 0.2684 0.1790 0.2331 0.2659 0.1784 0.2819 0.3687 0.2052 0.0563
- T: 0.2512 0.3812 0.3314 0.2615 0.3847 0.2242 0.1958 0.2507 0.5119
- GC: 0.4841 0.3319 0.4084 0.4716 0.3309 0.5308 0.5971 0.4612 0.1569

Rate matrix (I[G→T=1.00])
- [A→C]: 1.0000 1.5321 1.6666 1.0000 1.5572 2.6826 3.7679 1.0948 0.1674
- [A→T]: 1.0000 3.1598 2.8498 1.0000 3.0591 0.6810 0.4504 1.1309 0.6730
- [C→G]: 1.0000 1.6363 1.4417 1.0000 1.6368 1.2484 0.6887 5.4494 0.4214

Aligned nt: 1722 1353 3075 1722 1353 902 451 451 451
Constant nt: 1194 785 1979 1331 805 762 349 413 23
MP-inform.5 392 507 899 295 494 108 83 25 399
MP-uninf.6 136 61 197 96 54 32 19 13 29

a rbcL minus three long branches (LB), that is, Mesotaenium endlicherianum, M. caldariorum, and Netrium oblongum SVCK 255.
b rbcL with first and second codon positions only.
c rbcL with first (1), second (2), or third (3) codon position only.
d Informative and uninformative (but not constant) characters in MP.

Comparing SSU rDNA and rbcL Phylogenies
phyletic, specifically *Netrium*, *Mesotaenium*, and *Cylindrocystis*. A stable clade (MZC) combined four strains with almost identical sequences (1 to 4 nt difference only) belonging to three traditional genera: *Mesotaenium kramstai*, *Cylindrocystis* sp. strain UTEX 1926, *Zygmenopsis* sp. strain CCAP 699/1, and *Zygnemopsis minutum*. Within the Zygnematales, the branching order remained unresolved. *Roya* spp. and *Netrium interruptum* branched closer to the Desmidiales, followed by a polytomy comprising five branches (SPI, N-clade, *Netrium oblongum* SVCK 255, *Mesotaenium endlicherianum* and the “crown Zygnematales” [fig. 1]).

Because lineages within the Zygnematophyceae differed profoundly in evolutionary rates of the SSU rRNA gene, long-branch attraction (LBA) may have affected the phylogenetic analyses. *Spirogyra* was the longest SSU rDNA branch, and an analysis without this taxon produced an almost identical tree topology with similar significance in ML (not shown). However, deletion of *Spirogyra* had a noticeable effect on NJ and MP analyses in which the significance of many internal branches increased (not shown).

**rbcL Phylogeny**

The *rbcL* data set included the same strains as used for the SSU rDNA phylogeny and contained 1,353 aligned nt; the appropriate model was GTR+I+C255+I (table 3). Whereas the proportion of invariable sites (I) estimated for the *rbcL* alignment was comparable to that of the SSU rRNA data set, \( \Gamma \) was significantly higher (1.46 compared with 0.57), reflecting the more even distribution of substitutions in *rbcL*. In the substitution rate matrix, two categories ([A→G], [C→T]) attained much higher values in *rbcL* (7.5, 14.3) compared with SSU rDNA (2.1, 6.9 [table 3]).

In contrast to SSU rDNA analyses, the two zygnematophycean orders were not resolved as monophyla because in *rbcL* trees, two members of the Zygnematales (*Roya* and *Netrium oblongum* SVCK 255) diverged within the Desmidiales (fig. 2) (see below).

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**Fig. 1**—Unrooted ML (TrN+I+\( \Gamma \); for parameters, see table 3) phylogeny of the Zygnematophyceae based on 43 SSU rDNA sequences (1,722 aligned positions); very long branches are graphically (\( k \)) reduced to 50%. Nodes are characterized by BP and PP values: ML/ NJ/ MP/ BI. *[Mesotaenium endlicherianum]* presumably represents a wrong determination. For clade abbreviations, see Results. The longest internal branch reflects the split between both zygnematophycean orders, Zygnematales and Desmidiales.
Except for the orders Desmidiales and Zygnematales, rbcL analyses generally recovered the same clades as SSU rDNA phylogenies (figs 1 and 2). The lineages DESM, DESM/Penium spirostriolatum, ZYG, MCZ, and MOUG, which gained no (MOUG) or only moderate support in the SSU rDNA phylogeny, were robustly resolved by rbcL analyses (>90% BP in ML [fig. 2]). Similarly, the "crown-Zygnematales" (only topological support in SSU rDNA sequence comparisons) were significantly supported in the rbcL phylogeny (in ML and BI [fig. 2]). Among those four branches, which in SSU rDNA analyses represented the closest relatives of the "crown-Zygnematales" without significance (see fig. 1), rbcL placed SPI as sister to it, separated from all other Zygnematophyceae with high PP (1.00) but no or low BP (branch separating N and SPI [fig. 2]). The position of Roya and the long-branch taxon Netrium oblongum SVCK 255 within the Desmidiales, conflicting with the SSU rDNA phylogeny, was supported by PP only (branch separating CL and Roya/GON [fig. 2]). The four taxa constituting the MCZ-clade showed considerably divergent rbcL sequences, in contrast to their almost identical SSU rRNA genes (see fig. 1). Their branching pattern, with Mesotaenium kramstai as basal branch, was now significantly resolved (fig. 2). Moreover, rbcL revealed three Cylindrocystis species diverging as a paraphyletic assemblage grouped with MCZ (see fig. 2). The same Cylindrocystis species formed an unresolved polytomy in the SSU rDNA tree (see fig. 1).

In our rbcL analysis, Roya was only nonsignificantly associated with GON (fig. 2), in contrast to a previous rbcL study (McCourt et al. 2000), where Roya anglica (UTEX 934; accession number U38694) was sister to the Gonatozygon/Genicularia clade with 100% BP. Therefore, we resequenced the same strain (UTEX 934) and found 155 differences to the published sequence. When U38694 was included in our analysis (tree not shown), it was significantly positioned between Gonatozygon kinahani and the remaining Gonatozygon species, without affinity to Roya. We conclude that U38694 was derived from a Gonatozygon sp., presumably resulting from culture or DNA misplacement.

In rbcL phylogenies, Mesotaenium endlicherianum, M. caldariorum, and Netrium oblongum SVCK 255 were characterized by fast evolutionary rates and had long individual branches (>125 apomorphies), in contrast to the SSU rDNA analysis. Reversely, the SPI clade
(extremely long branched in SSU rDNA) revealed an average evolutionary rate in \(rbcL\) (figs. 1 and 2). Whereas in \textit{Mesotaenium caldariorum}, the first and second codon positions contributed only 10 of 128 autapomorphic characters, this number was higher (34 of 166 and 24 of 154) in \textit{Mesotaenium endlicherianum} and \textit{Netrium oblongum}, respectively, reflecting their deviating amino acid composition. Exclusion of the long-branch species from the \(rbcL\) data set led to very similar tree topologies (not shown) and model parameters except for a higher gamma-shaped parameter (table 3).

To investigate the possible impact of homoplasy and saturation in third codon positions (Nickrent et al. 2000; Nozaki et al. 2000), the first and second codon positions contributed only 10 of 128 autapomorphic characters, this number was higher (34 of 166 and 24 of 154) in \textit{Mesotaenium endlicherianum} and \textit{Netrium oblongum}, respectively, reflecting their deviating amino acid composition. Exclusion of the long-branch species from the \(rbcL\) data set led to very similar tree topologies (not shown) and model parameters except for a higher gamma-shaped parameter (table 3).

To investigate the possible impact of homoplasy and saturation in third codon positions (Nickrent et al. 2000; Nozaki et al. 2000), the first two (902 nt) and the third position (451 nt) were analyzed separately. Analyses using first and second codon positions (trees not shown) revealed the same basic topology as for the complete \(rbcL\) alignment (fig. 2). However, resolution and significance decreased considerably and previously supported clades were either not recovered at all (GON, SPI, and ZYG) or received no statistical support (DESM and the DESM/Penium spirostriolatum clade). In contrast, analyses using third codon positions recovered the same clades as the complete data set with similar statistical confidence (trees not shown; for model parameters see table 3). The log-likelihood summation (LS) method using separate models for each codon (table 3) identified a tree with the lowest sum of log-likelihoods (not shown), which was identical to the “concatenated” tree shown in figure 3 with two exceptions: (1) the branches of \textit{Phymatodocis} and \textit{Penium exiguum}/\textit{P. cylindricus} were separate, and (2) \textit{Netrium oblongum} SVCK 255 was sister to \textit{Roya spp}. (still positioned as sister to GON within the Desmidiales). Neither of these differences refers to significantly supported clades in figure 2.

Combined Analysis (SSU + \(rbcL\))

The analysis of combined SSU rDNA and \(rbcL\) data sets (3,075 nt [table 3]) with a concatenated model resolved almost all internal branches separating zygnematothecan lineages (fig. 3). The tree topology was similar to the SSU rDNA tree (fig. 1), but internal branches were

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**Fig. 3.**—Combined (concatenated) analysis of 43 Zygnematophyceae inferred from SSU rDNA and \(rbcL\) sequences (3,075 aligned nt) using ML (GTR+\(+\Gamma\); for parameters, see table 3). Details as in figure 1. As in the SSU rDNA phylogeny (fig. 1), the Zygnematales and Desmidiales are monophyletic, revealing the placement of \textit{Roya} and \textit{Netrium oblongum} SVCK 255 in the \(rbcL\) tree (fig. 2) as an artifact. Compared with both single-gene analyses (figs. 1 and 2), the combined tree reveals much better topological resolution, higher statistical support for internal branches, and a more regular significance distribution.
Table 4
Comparison Between the Best ML Trees (Figure 1 to Figure 3) Using the SSU rDNA, \( rbc \)L, and Combined Data Sets and User-Defined Trees by Kishino-Hasegawa and Shimodaira-Hasegawa Tests

| Tree compared            | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L | SSUr DNA | \( rbc \)L | SSU + \( rbc \)L |
|-------------------------|----------|------------|-----------------|----------|------------|-----------------|----------|------------|----------------|----------|------------|----------------|----------|------------|----------------|----------|------------|----------------|----------|------------|----------------|----------|------------|----------------|----------|------------|
| Best ML trees           |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| SSUr DNA                |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| \( rbc \)L              | Best     | 152.2      | 118.8           |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| SSU + \( rbc \)L        | 83.8     | Best       | 39.4            |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| 2) UD trees             |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| 1                       |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| 2                       |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| 3                       |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |
| 4                       |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |                 |          |            |

Note.—AU = \( P \)-value of the approximately unbiased test calculated from the multiscale bootstrap. KH = Kishino-Hasegawa test. SH = Shimodaira-Hasegawa test. wSH = weighted Shimodaira-Hasegawa test. The tree topologies tested: 1. The orders Desmidiales and Zygnematales are monophyletic (\( Netrium \) oblongum SVCK 255 and \( Roya \) are sisters to the Desmidiales); 2. Mesotaenium endlicherianum is a sister to the “crown Zygnematales”; 3. \( Penium \) cinctulum exiguum and \( P. \) spirostolatum are sisters; 4. The genus \( Netrium \) is monophyletic. ** 0 \( < P < 0.001 \).

* Probability of obtaining a more extreme \( T \)-value under the null hypothesis of no difference between the two trees.

* Difference in \(-\log\)-likelihood.
and KH tests (0.03 < P < 0.05). The combined ML tree was the least rejected (table 4). In the SH and wSH tests, this topology was not significantly rejected (P > 0.05).

Discussion

To exclude incongruent taxon sampling and likely artifacts associated with it, the two genes (rbcL and SSU rDNA) were sequenced exclusively from the same strain. The importance of this strict approach is shown by two examples: (1) two strains designated *Netrium oblongum* (M 1367 and SVCK 255; in morphology both corresponding to the species description) are in fact unrelated to each other, and (2) a database sequence (U38694) of strain UTEX 934 (*Roya anglica*) apparently was not derived from this strain, but refers to another genus, as shown in this study. Combining SSU rDNA and rbcL sequences of different strains may thus introduce taxon sampling artifacts, which could result in chimeric sequences and in single-gene trees in conflicting topologies. Our approach ensures that conflicts between single-gene topologies are derived from different patterns of sequence evolution between the genes.

SSU rDNA and rbcL sequence comparisons of 43 strains of the Zygnematophyceae were used to analyze the relation between single-gene versus combined analyses, gene concatenation versus log-likelihood summation, and bootstrap percentages (ML, NJ, and MP) versus posterior probabilities (BI), as an evolutionary case study based on empirical data. Previously published phylogenetic analyses using SSU rDNA sequence comparisons in the Zygnematophyceae suffered mostly from lack of resolution (Besendahl and Bhattacharya 1999; Denbho, Hendrayanti, and Ichimura 2001; Gontcharov, Marin, and Melkonian 2003), whereas in rbcL studies, taxon sampling was limited, with only one species per genus included (McCourt et al. 2000). In phylogenetic analyses of streptophyte green algae, different molecular markers favored conflicting tree topologies; for example, concerning the position of the genera *Mesostigma, Klebsormidium*, or the group studied here, the Zygnematophyceae (Marin and Melkonian 1999; Lemieux, Otis, and Turmel 2000; McCourt et al. 2000; Karol et al. 2001; Cimino and Delwiche 2002; Delwiche et al. 2002; Martin et al. 2002; Turmel et al. 2002a).

Single-Gene Data and Analyses

In the Zygnematophyceae, ribosomal (SSU rDNA) and chloroplast (rbcL) genes studied revealed considerable differences in their evolutionary dynamics as reflected by model parameters (patterns of nucleotide substitutions, Γ, base composition). In both genes, the C→T substitution category is conspicuously high in comparison with the remaining frequencies (table 3), a situation, which for SSU rDNA may be related to pairing constraints at the transcript (rRNA) level (G-C→G-U) and for rbcL (an even higher C→T value) is caused by asymmetrical codon usage (Morton 1994). The gamma-shaped parameter estimated for the SSU rDNA data set is nearly three-fold lower than that for rbcL, in which the variability distribution reflects the regular codon structure. Moreover, the length of the Rubisco large subunit is conserved in the Viridiplantae (476 amino acids), and the variability at the amino acid level is rather moderate (reviewed by Kellogg and Juliano [1997]). In the Zygnematophyceae, less than 80% of the sequence variability of rbcL refers to third codon positions. Although third codon positions are sometimes down-weighted or excluded from the phylogenetic analyses because of codon degeneration and homoplasy (Nickrent et al. 2000; Nozaki et al. 2000), restriction of our analyses to first and second codon positions resulted in greatly diminished resolution (see also McCourt et al. [2000]). However, using only the third codon position, the topology and resolution reflected that obtained for all positions, thus, demonstrating that most of the phylogenetic signal resides in the third codon.

Not surprisingly, SSU rDNA and rbcL data led to selection of different models of evolution and model parameters (table 3), but these models were not in conflict (TrN is nested within GTR) and do not prevent a combined analysis using a concatenated (averaged) model (here: GTR).

In general, both single-gene trees show a high degree of congruence and largely recover the same taxa and clades with comparable BP and PP support. However, the analyses also reveal some conflicts that are not caused by incongruence in taxon sampling. The most obvious discrepancy between the two single-gene trees relates to the split between the orders Zygnematales and Desmidiales, which is resolved in the SSU rDNA analysis but not in rbcL phylogenies (McCourt et al. 2000; this study); in our rbcL study, the Desmidiales are mixed with two zygnematalan branches (*Roya* and *Netrium oblongum* SVCK 255). However, even this incongruence refers to internal branches without significant bootstrap support in the rbcL analysis (in contrast to PP [see below]), and moving *Roya* and *N. oblongum* SVCK 255 to the Zyg nematales is not rejected in user-defined topology tests (table 4). As an example for congruence, the polyphyly of *Mesotaenium, Cylindrocystis*, and *Netrium* is clearly revealed in both single-gene phylogenies.

Combined Analyses

When both data sets were combined as a concatenated “supergene” and analyzed with a single average model, the resulting phylogeny was superior to both single-gene analyses when the statistical support of internal branches is considered. Specifically, concatenated data significantly (BP and PP) resolved the major conflict between rbcL and SSU rDNA trees (Desmidiales-Zygnematales divergence) in favor of the SSU rDNA analysis (i.e., monophyly of both groups in the unrooted phylogeny). Several other internal branches (especially basal branches of the Zygnematales), which in single-gene phylogenies were not or weakly supported, obtained higher significance in the combined analysis (see Results). In general, the combined analysis was dominated by the phylogenetic signal of the SSU rDNA data set, whereas rbcL contributed sufficient sequence diversity to improve resolution within clades (see MZC clade where SSU rDNAs are almost
identical) but also to increase the overall significance of the branches.

It is somewhat illegitimate (a logical circle) to use higher statistical support for branches as the only criterion to regard combined analysis as superior (i.e., closer to the “true tree” than the rbcL analysis). Of course, there is no a priori knowledge of the “true tree.” However, cell-wall characters provide some independent evidence for comparing evolutionary hypotheses in the Zygnematales. The SSU rDNA tree reveals a single evolutionary transition from simple cell walls (Zygnematales) towards complex cell walls in the monophyletic Desmidiales, without homoplasious character changes. The conflicting rbcL scenario, if correct, would imply two additional changes (i.e., reversals: complex—simple wall) for those Zygnematales that are rooted within the Desmidiales (Roya and Netrium oblongum SVCK 255). Thus, the parsimony criterion applied to cell-wall structures (one versus three character changes), as well as the combined SSU rDNA+rbcL analyses, both favor the SSU rDNA topology and emphasize the value of complex cell walls as a phylogenetic marker.

In a concatenated analysis, it is possible that the longer or the more variable data set dominates the “average” model of sequence evolution and, thus, the resulting topology. However, the LS method applied here (which avoids the use of an average model) agreed with the results obtained by the concatenated analysis. There are, of course, conditions under which concatenated phylogenetic analyses will fail. Whenever different genes evolve under deviating rules (i.e., models), a concatenated analysis may be significantly worse than phylogenies that allow the application of separate models, as shown by Pupko et al. (2002). In the Zygnematales, the two single-gene models were not in conflict (see above), but some model parameters, especially the gamma-shaped and the C→T substitution category, differed considerably between SSU rDNA and rbcL (see Results). The concatenated model (averaged parameters) apparently did not violate the evolutionary characteristics of both genes.

Combined Analyses and Long Branches

It has been proposed that fast-evolving taxa with long undivided branches should be better analyzed by using another, slow-evolving, gene to avoid LBA (Philippe 2000). Fortunately, all long-branch taxa in our analyses are either fast-evolving in rbcL (M. endlicherianum and N. oblongum SVCK 255) or in the SSU rRNA gene (S. pyriformis) but not in both data sets. Our results show that the combination of slow-evolving and fast-evolving genes can also resolve the phylogenetic position of a taxon with a fast-evolving gene, in particular when improved taxon sampling does not help to subdivide the long branch (e.g., SSU rDNA in S. pyriformis). It appears that the combined approach can successfully extract the phylogenetic signal from the fast-evolving gene and nevertheless reduce LBA (see also Hoef-Emden, Marin, and Melkonian 2002). The latter is illustrated by comparison of the positions of Mesotaenium endlicherianum and Netrium oblongum SVCK 255 in single-gene and combined analyses. Both taxa have long branches in the rbcL phylogeny but not in the SSU rDNA phylogeny. Their position in the rbcL tree (fig. 2) most likely reflects a LBA artifact because in combined analyses, they comprise shorter (albeit still relatively long) branches (no LBA), and their placement confirms the SSU rDNA topology with better significance. The most conspicuous long branch in our analyses refers to the genus Spirogyra in SSU rDNA trees, in which Spirogyra was previously positioned as a sister to all other Zygnematophyceae (Besendahl and Bhattacharya 1999) or as one of the basal divergences of the class (Gontcharov, Marin, and Melkonian 2003). In the rbcL phylogeny, Spirogyra is not a long-branch taxon, but interestingly, the position of Spirogyra does not contradict rooted (Gontcharov, Marin, and Melkonian 2003) or unrooted (this study) SSU rDNA trees. We conclude that the position of Spirogyra in the SSU rDNA trees was likely not the result of an LBA artifact.

The examples discussed above may provide some confidence that the deep-level phylogeny of the Zygnematophyceae based on the conservative SSU rRNA gene agrees better with organismal data and is less sensitive to various artifacts than analyses using the homoplasious and largely saturated gene rbcL. However, the latter gene resolves shallow evolutionary relationships much better where the conserved SSU rDNA lacks variability.

Phylogeny of the Zygnematophyceae

Although the Zygnematales, characterized by a simple cell wall (consisting of only one piece, no pores [Mix 1972]; plesiomorphic character state), form a clade in the unrooted trees, this taxon is not monophyletic because the root of the Zygnematophyceae falls within the Zygnematales, and, thus, reveals this order as paraphyletic (McCourt et al. 2000; Gontcharov, Marin, and Melkonian 2003). Chloroplast shape (three types) and level of organization (unicellular or filamentous) vary in the Zygnematales and have previously been used for classification (e.g., Palla 1894; Randhawa 1959; Yamagishi 1963). Generally, none of these proposals gains support by our molecular phylogenetic analyses. We have tentatively identified seven lineages within the traditional Zygnematales, namely Roya, N, S, the “crown-Zygnematales,” and three individual taxa (Mesotaenium endlicherianum, Netrium interruptum, and N. oblongum SVCK 255). Three filamentous genera (Spirogyra, Mougeotia, Zygnema) are resolved as monophyletic, whereas other genera are not monophyletic. Obviously, in the Zygnematales, the genetic diversity at the genus level was severely underestimated by traditional taxonomists because the importance of the organizational level (unicellular versus filamentous) and chloroplast shape (axial plateglike, stellate, or helical) has been overestimated. These characters may have originated or have been lost several times independently in the group. Our phylogenies place unicellular organisms as basal diversifications of some zygnematalean clades (“crown-Zygnematales,” MZC, and MOUG) tentatively suggesting that unicells could perhaps be ancestral to these lineages.

Probably the most interesting genus resolved here as nonmonophyletic is Netrium—species analyzed form three independent branches, each characterized by a different number of chloroplasts per cell (1, 2, or 4), differing
positions of the nucleus in the cell, and varying nuclear behavior during cytokinesis (Pickett-Heaps 1975; Jarman and Pickett-Heaps 1990; unpublished observations). The three Netrium branches occupy a key position between the other Zygnematales and the Desmidiales, supporting previous rooted analyses containing only one Netrium species (as sister of the Desmidiales [McCourt et al. 2000; Gontcharov, Marin, and Melkonian 2003]). In the latter publication, we erroneously reported two species of Netrium as having identical SSU rDNA sequences; however, these sequences actually originated from the same culture, Netrium interruptum strain M 1021. Our expanded taxon sampling in this study reveals the Desmidiales as originating from a paraphyletic stock of derived unicellular Zygnematales (i.e., Netrium and Roya branches).

The Desmidiales, a clade defined by derived cell-wall characters, is well supported (but not in the rbcL phylogeny [see above]). The molecular phylogeny within the Desmidiales as revealed here and by previous studies (McCourt et al. 2000; Gontcharov, Marin, and Melkonian 2003) reflects the increasing complexity of the cell-wall ultrastructure. Among the four families described, the Gonatozygaceae and Closteriaceae are confirmed, whereas the concept of the Peniaceae (cell wall consisting of several segments separated by shallow groove(s); simple pores perforating only the outer cell wall layer) and the Desmidaceae (constricted cells composed of two semicells with complex [simple in Phymatocodiocis {Engels and Lorch 1981}] cell wall pores) are in need of revision. Two of three species of Penium analyzed form a robust clade with the Desmidaceae (DESM). The third Penium species (P. spirostriolatum) forms a clade with DESM, although no morphological synapomorphy is presently known. Because of its simple cell-wall structure, Penium is usually not regarded as closely related to the Desmidiales.

Bayesian Phylogenetics

Bayesian inference, a recently introduced method for inferring molecular phylogenies (Huseldenbeck and Ronquist 2001; Rannala and Yang 1996), provides a statistical confidence measure (PP) for branches and is much faster than ML bootstrap analysis. However, PP values are often much higher than ML BP and thus, the reliability of this method has recently been controversially discussed (Huseldenbeck et al. 2002; Suzuki, Glazko, and Nei 2002; Alfaro, Zoller, and Lutzoni 2003; Douady et al. 2003). Based on simulation studies or using real sequence data, some authors considered ML BP as too conservative (Hillis and Bull 1993; Murphy et al. 2001; Wilcox et al. 2002; Alfaro, Zoller, and Lutzoni 2003), whereas others concluded that PP is too optimistic (Suzuki, Glazko, and Nei 2002). In our empirical study, the level of support for branches by PP or BP is roughly similar, although we also found several branches with significant PP support (≥0.95), which were not substantiated by significant BP values. One branch in the rbcL phylogeny, namely the branch separating CL and Royal/GON (fig. 2), defines an artificial divergence that does not exist in the SSU rDNA topology and in the combined analysis (see Results). This artificial branch receives no BP values in the rbcL analysis, but considerable support by Bayesian inference (PP = 0.99 [fig. 2]). It is known that Bayesian analysis is sensitive to small-model misspecifications (Waddell, Kishino, and Ota 2001; Buckley 2002, Buckley et al. 2002), here probably related to the individual long-branch taxa Mesotaenium endlicherianum and Netrium oblongum SVCK255 and the high level of homoplasy in the rbcL gene. We conclude that Bayesian inference can be positively misleading as exemplified in our case study and suggest that PP support should always be confirmed by traditional bootstrap analyses.

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