Molecular phylogenetics, morphological variation and colony-form evolution in the family Hydrodictyaceae (Sphaeropleales, Chlorophyta)

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A phylogenetic analysis of 26S and internal transcribed spacer–2 ribosomal DNA (rDNA) data from members of the freshwater green algal family Hydrodictyaceae, including multiple taxa from both culture collections and new isolates, sets the stage to explore morphological variation and patterns of colony-form evolution within the family. These analyses particularly focus on Pediasstrum boryanum, P. tetras, P. duplex, Hydrodictyon and Sorastrum. The genera Hydrodictyon and Sorastrum are derived from within Pediasstrum in the individual and combined analyses, indicating a pattern of colony-form evolution within the family from two-dimensionality to three-dimensionality. In some cases the gene topologies reveal discrepancies in the morphological characters used to delimit species, varieties and forms within Pediasstrum. Some isolates, such as those of H. reticulatum, exhibited little or no genetic variation between different geographic localities. Additional data and taxa are needed to better resolve and support these relationships, but the present results illustrate that some taxonomic revisions will be necessary.

INTRODUCTION

The freshwater green algal family Hydrodictyaceae (Sphaeropleales, Chlorophyceae) comprises four morphologically distinct genera that display an extreme dichotomy in growth form with two- and three-dimensional colonies expressed. Pediasstrum Meyen 1829 and Euastropsis Lagerheim 1894 form flat, two-dimensional colonies, whereas Sorastrum Kützing 1845 and Hydrodictyon Roth 1800 form three-dimensional colonies. Despite the extreme differences in colony morphology, the genera share characteristics of reproduction and gamete flagellar apparatus ultrastructure (Bold & Wynne 1985; Wilcox & Floyd 1988). The Hydrodictyaceae reproduce asexually via biflagellated zoospores formed by the cleavage of cell contents in the adult colonies. The zoospores undergo a swarming period within the parental cell wall, or in a vesicle derived from the inner layer of the parental wall (Pocock 1960; Davis 1967; Marchant & Pickett-Heaps 1972). During swarming, the swimming cells aggregate, withdraw their flagella and form miniature daughter colonies that are released from the parental cell wall or vesicle; cell expansion results in full-sized coenobia.

Recent studies of the class Chlorophyceae using morphological and ultrastructural data in a noncladistic approach, and phylogenetic analyses based on 18S ribosomal DNA (rDNA) sequence data, have supported the close relationship of Pediasstrum and Hydrodictyon (Deason et al. 1991; Wilcox et al. 1992; Lewis 1997; Buchheim et al. 2001). However, those studies did not include Sorastrum or Euastropsis. In 2001. Nevertheless, the relationships among the four genera were not thoroughly explored. A preliminary analysis of the nuclear small subunit that included two species of Pediasstrum [P. duplex Meyen and P. boryanum (Turpin) Meneghini] and one species of Hydrodictyon [H. reticulatum (Linnaeus) Lagerheim] provided intriguing evidence that the morphologically distinct Hydrodictyon may be derived from Pediasstrum (L.A. Lewis, personal observation). An expanded phylogenetic analysis of 18S rDNA data including nine sequences of Pediasstrum representing six species, one Hydrodictyon sp., and one Sorastrum strain, indicated that Pediasstrum is not monophyletic (Carlson 2002). However, because of the limited taxon sampling in these previous studies, an analysis including more species of each genus is necessary for a definitive understanding of the phylogenetic relationships within the family.

The genus Pediasstrum has a relatively extensive fossil record and the numerous species, varieties and forms recognized by Bigeard (1933), Sulek (1969) and Parra (1979) are used as ecological indicators in paleolimnological reconstruction (Komařek & Jankovská 2001). Though designated morphospecies have been widely used in recent literature, the taxonomy of both fossils and extant taxa has been described as somewhat dubious (Nielsen 2000; Komárek & Jankovská 2001). The difficulties in assigning names to particular phenotypes and defining species limits is most likely due to the polymorphic nature of the genus (Nielsen 2000; Komárek & Jankovská 2001). Though Komárek & Jankovská (2001) compiled an identification key for use with extant and fossil taxa, the morphological characteristics used to define species of Pediasstrum have not been tested for plasticity, so their taxonomic significance is not known.

This paper focuses on resolving phylogenetic relationships within the Hydrodictyaceae using an expanded set of taxa and characters from the nuclear large subunit ribosomal RNA gene (26S rDNA), 5.8S rDNA and the internal transcribed spacer–2 regions (ITS-2). Inclusion of isolates representing the three known species of Hydrodictyon (H. reticulatum, H. africanaum Yamanouchi and H. patenaeforcome Pocock) allows us to test...
Table 1. List of taxa included in present study, their strain number and GenBank accession numbers. All sequences were obtained for this study, except 26S rRNA data for UTEX LB1364 and UTEX 138. Locality information is listed in Appendix 1.

| Species                        | Strain    | 26S rDNA   | ITS       |
|--------------------------------|-----------|------------|-----------|
| Hydrodictyon africana         | UTEX LB782| AY534693   | AY577739  |
| Hydrodictyon patenaiforme     | CCAP 236/3| AY534698   | AY577736  |
| Hydrodictyon reticulatum      | CBS       | AY534695   | AY577735  |
| Hydrodictyon reticulatum      | UTEX LB515| AY534694   | AY577738  |
| Hydrodictyon reticulatum      | SF0201NY  | AY534696   | AY577737  |
| Hydrodictyon reticulatum      | ML0301CT  | AY534697   | AY577746  |
| Hydrodictyon reticulatum      | NZ0301    | AY534710   | AY577747  |
| Pediasstrum angulosum         | UTEX LB1366| AY534724  | AY577729  |
| Pediasstrum biradiatum        | UTEX 37   | AY536059   | AY577732  |
| Pediasstrum boryanum var. longicorne | UTEX 1372| AY536060   | AY577727  |
| Pediasstrum boryanum var. cornutum | UTEX LB470| AY534692   | AY577743  |
| Pediasstrum boryanum var. forcipatum | SAG 87.81| AY534707   | AY577752  |
| Pediasstrum boryanum var. brevicorne Braun | SAG 261-7| AY534708   | AY577731  |
| Pediasstrum boryanum          | OL0301MN  | AY534721   | AY577759  |
| Pediasstrum boryanum          | EL0203CT  | AY534715   | AY577741  |
| Pediasstrum braunii           | SAG 43.85 | AY534705   | AY577756  |
| Pediasstrum duplex var. asperum | UTEX LB1364| AY534725  | AY577734  |
| Pediasstrum duplex            | EL0201CT  | AY534725   | AY577742  |
| Pediasstrum duplex            | SAG 84.80 | AY534706   | AY577748  |
| Pediasstrum duplex            | SR0201NJ  | AY534710   | AY577755  |
| Pediasstrum duplex            | SF0202NY  | AY534718   | AY577757  |
| Pediasstrum duplex            | SB0201VA  | AY534717   | AY577753  |
| Pediasstrum duplex            | LN0201NC  | AY534726   | AY577744  |
| Pediasstrum duplex            | LW0201NC  | AY534727   | AY577745  |
| Pediasstrum integrum          | SAG 29.81 | AY534703   | AY577750  |
| Pediasstrum kawraisky         | SAG 35.81 | AY534704   | AY577751  |
| Pediasstrum privum            | SAG 36.81 | AY534709   | AY577763  |
| Pediasstrum simplex           | UTEX LB1601| AY534724  | AY577726  |
| Pediasstrum tetras            | UTEX 38   | AY534699   | AY577755  |
| Pediasstrum tetras            | HL0202W1  | AY534712   | AY577760  |
| Pediasstrum tetras            | SF0203NY  | AY534719   | AY577761  |
| Pediasstrum tetras            | EL0207CT  | AY534701   | AY577762  |
| Pediasstrum sp.               | UTEX LB144| AY534723   | AY577728  |
| Pediasstrum sp.               | CL0201VA  | AY534711   | AY577740  |
| Pediasstrum sp.               | HL0201W1  | AY534725   | AY577730  |
| Pediasstrum sp.               | RL0201FR  | AY534713   | AY577749  |
| Sorastrum americanum (Bohlin) Schmidle | SAG 13.94| AY534702   | AY577758  |
| Sorastrum spinulosum          | UTEX LB2452| AY534700  | AY577725  |
| Sorastrum spinulosum          | IL0201MN  | AY534714   | AY577733  |
| Neochloris aquatica           | UTEX 138  | AY277563   | AY577764  |

the monophyly of Hydrodictyon. The phylogeny is also used in conjunction with scanning electron microscope images as a framework to explore the evolution of two- and three-dimensional colonies and morphological variation in the Hydrodictyaceae. Incorporating new isolates increases the representation of genetic and morphological variation not present in culture collection isolates and allows us to test the boundaries of recognized morphospecies within Pediasstrum.

MATERIAL AND METHODS

Taxa

Isolates of multiple species of Pediasstrum, three species of Hydrodictyon, two species of Sorastrum and Neochloris aquatica Starr (Neochloridaceae, Sphaeropleales) (outgroup) were obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX), the Culture Collection of Algae and Protozoa in the United Kingdom (CCAP), the Sammlung von Algenkulturen in Göttingen, Germany (SAG) or Carolina Biological Supply (CBS). In addition, 18 new isolates were collected from the United States, France, and New Zealand. These new taxa were obtained by micropipette isolation under a light microscope from plankton tows with a 48 µM mesh plankton net. Information regarding strain number and species designation is included in Table 1; locality information can be found in Appendix 1. The new isolates are housed in the authors’ culture collection at the University of Connecticut.

The cultures were maintained at 20°C with a 16:8 h light:dark regime. The liquid growth medium consisted of a 50:50 mixture of Bold’s Basal Medium (BBM) (Bold 1949; Bischoff & Bold 1963) and a modified version of soilwater liquid medium (Pringsheim 1946). The BBM was made without the trace element solutions and instead with micronutrients (S.F. McManus & Lewis: Hydrodictyaceae phylogeny 583). The cultures were housed in the authors’ culture collection at the University of Connecticut. Following Komárek & Jankovská (2001), taxonomic designations have been tentatively assigned to the new isolates based on examination using light microscopy and scanning electron microscopy. Representatives of 10 species, 5 varieties and 4 undetermined species of Pediasstrum, 3 species of Hydrodictyon, and 2 species of Sorastrum were included in the phylogenetic analyses. Euastrupsis was not included in these analyses due to lack of available material.

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DNA extraction, polymerase chain reaction amplification and sequencing

DNA extraction from living cells was performed using the Qiagen DNEasy Plant Extraction kit (Qiagen Inc, Valencia, CA, USA). The purity and concentrations of the DNA were analysed and compared with known standards by running 4 μl of extracted DNA on 40 ml, 0.9% agarose gels stained with 2 μl ethidium bromide solution. 26S rDNA amplification and sequencing primers are described in Buchheim et al. (2001), and primers for amplification and sequencing of the ITS region are described in White et al. (1990). Two microlitres dimethyl sulfoxide (DMSO) was incorporated into the 25 μl PCR reactions to assist with primer binding. Due to difficulties amplifying the ITS region for some taxa, a combination of the small subunit (18S) primer N18J (Shoup & Lewis 2003) and large subunit 26B primer was used. The PCR conditions were 26 cycles of 94°C for 1 min to denature, annealing at 54°C for 45 s and an extension step at 72°C for 1 min 45 s. These cycles were followed with a final extension step at 72°C for 8 min 15 s. Quantification of the PCR products was done by running 4 μl of the double-stranded PCR product on 0.9% agarose gels stained with ethidium bromide. The QiaQuick Nucleotide Purification kit (Qiagen Inc, Valencia, CA, USA) was used to clean the PCR product, and cycle sequencing of the purified PCR product was done with the ABI PRISM Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA), but with 10 μl reactions. Once the ITS region was amplified, sequences were obtained using the ITS region specific primers (White et al. 1990). The cycle sequencing conditions were 27 cycles of denaturing at 96°C for 30 s, annealing at 50°C for 15 s, and 4 min extension at 60°C. Unincorporated dye terminators were removed from the final sequenced product using ethanol precipitation; automated sequencing was done on an ABI PRISM 3100 Genetic Analyzer.

Sequence editing and alignment

Sequences were assembled into contigs and edited using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Forward and reverse primers were used for sequencing reactions to insure double-stranded coverage and assist with ambiguous base calls. Each sequence was compared to the National Center for Biotechnology Information (NCBI) database using BLAST (Altschul et al. 1990) to check for contaminants. An initial alignment was done using Clustal X (Thompson et al. 1997) and the final alignment was done by eye using MacClade 4.0 (W.P. Maddison & D.R. Maddison 1992) and PAUP* 4.0b10 (Swofford 2002). Because of difficulties aligning the ITS-1 region due to its hyper-variable nature, this region was excluded from the analyses and only the 5.8S and ITS-2 regions were included. The ITS-2 sequences were submitted to the Mfold (Zuker 2003) web server to estimate secondary structure. These structures were used to assist with alignment of the variable ITS-2 region. Completed sequences were deposited in GenBank (NCBI) and the corresponding accession numbers are listed in Table 1. The alignments are available from TreeBase (http://www.treebase.org/treebase/).

Phylogenetic analyses

Estimation of phylogenetic relationships was done using maximum likelihood (ML) and Bayesian methods with PAUP* 4.0b10 (Swofford 2002) and MrBayes v3.0b4 (Huelsenbeck & Ronquist 2001). Neochloris aquatica was selected as the representative outgroup taxon for the 26S data analyses based on the findings of Buchheim et al. (2001) and BLAST searches. A preliminary analysis with the additional taxa Characium hindakii Lee & Bold (GenBank accession AF183466), Neochloris vigensis Archibald (AF277654), Sphaeroplea soleirii var. crassisspeta (Duby) Montag, ex Kützing (AF183485), Schroederia setigera (Schröder) Lemmermann (AF277657), Chaetophora incrassata (Hudson) Hazen (AF183471), Ouracoccus multisporus Bischoff & Bold (AF277655) and Scedesmus obliquus (Turnip) Kützing (AF183482), supported the monophyly of the family Hydrodictyaceae (tree not shown); these taxa were excluded from the final analyses to reduce the computer time required. To maintain consistency, N. aquatica was selected as outgroup for the ITS-2 data set, and the ITS-2 region was amplified and sequenced following the protocol outlined above. The 26S and ITS-2 data sets were analysed separately, then combined for analysis after the Partition Homogeneity test (Incongruence Length Difference or ILD test) (Farris et al. 1994) in PAUP* illustrated there was no significant signal conflict (P = 0.13). The 26S data set contained 1937 aligned characters for 40 taxa (39 ingroup, 1 outgroup). Of these, 1786 characters were constant and 78 were parsimony informative. The ITS-2 data set (5.8S + ITS-2 regions) contained 505 characters; 359 characters were unambiguously aligned and the remaining characters were excluded from analyses. Of the 359 included characters, 255 were constant and 49 were parsimony informative. Modeltest version 3.06 (Posada & Crandall 1998) evaluated the appropriate model of evolution to be used for the ML analyses. The General Time Reversible plus gamma plus invariant sites (GTR+Γ+I) model of evolution for the 26S data set was selected based on the Akaike Information Criterion (AIC). The ML estimates of the parameter values are as follows: freqA = 0.2585, freqC = 0.2120, freqG = 0.2991, freqT = 0.2304, R<sub>A</sub>C = 0.9609, R<sub>A</sub>G = 6.8254, R<sub>A</sub>T = 1.8868, R<sub>G</sub>G = 0.4374, R<sub>G</sub>T = 14.2051, R<sub>T</sub>T = 1.0, gamma distribution shape parameter (α) = 0.7079, proportion of invariable sites (I) = 0.8194. The SYM+Γ+I model was selected by AIC in Modeltest for the ITS-2 data set with the following parameters estimated: equal base frequencies, R<sub>A</sub>C = 2.5273, R<sub>A</sub>G = 3.3378, R<sub>A</sub>T = 2.5709, R<sub>C</sub>G = 0.5643, R<sub>C</sub>T = 6.8580, R<sub>C</sub>C = 1.0, α = 0.5748, I = 0.3766. Modeltest selected GTR+Γ+I model for the combined data set (freqA = 0.2586, freqC = 0.2181, freqG = 0.2915, freqT = 0.2317, R<sub>A</sub>C = 2.1562, R<sub>A</sub>G = 4.9975, R<sub>A</sub>T = 2.2580, R<sub>C</sub>G = 0.6612, R<sub>C</sub>T = 1.0, R<sub>C</sub>C = 11.4711, R<sub>T</sub>T = 1.00, α = 0.6565, I = 0.7642). The ML analyses were done using a heuristic search algorithm with 10 random addition sequence replicates and tree-bisection–reconnection branch swapping, setting the values estimated by Modeltest. Bootstrap support values were obtained from 100 bootstrap replicates under a heuristic search of one random addition sequence replicate. Nodes with bootstrap values ≥ 80 will be regarded as being well-supported, representing > 95% accuracy (Hillis & Bull 1993).

Bayesian analyses were done initially using the GTR+Γ+I
model. However, we found that separate Markov chain Monte Carlo (MCMC) runs failed to converge when both rate heterogeneity parameters (I+Γ) were simultaneously estimated. Individual runs plateaued, but the resulting trees had different topologies and the parameters different optima. To circumvent this problem, we instead modelled rate heterogeneity by removing the invariant sites parameter and increasing the number of gamma rate categories from 4 to 15, the number of rate categories determined by running a likelihood ratio test for various numbers of categories. The combined data set was therefore analysed under the GTR+Γ model with 15 rate categories. We performed two MCMC runs, each for three million generations and consisting of one cold chain and three hot chains. A tree was saved every 100 generations yielding 30,000 trees per run. After confirming the two runs converged, the trees were combined (discarding the first 1500 trees as burn-in), and a 50% majority rule consensus of the remaining 57,000 trees was obtained. Nodes with Bayesian posterior probabilities ≥95% will be considered well-supported, representing relationships that are approximately 95% accurate (Alfaro et al. 2003).

Specimen preparation for Scanning Electron Microscopy

Specimens were fixed overnight at room temperature in 2% glutaraldehyde–BBM solution, rinsed three times with dH2O and postfixed with 2% osmium tetroxide for 3 h at room temperature. After three rinses with dH2O, fixed colonies were collected on 0.4 μM HTTP Isopore® Membrane (Millipore, Billerica, MA, USA) filters using Millipore Swinnex (Millipore) re-usable filter holders connected to 10 ml syringes, and rinsed thoroughly with dH2O. The specimens were then dehydrated following a graded ethanol series, and the algae collected on the filters were re-suspended in 100% ethanol. One drop of this suspension from each isolate was mounted on silicon chips and allowed to air dry. This air-dry method was used following Parra (1979) to better observe wall ultrastructure. Dehydrated samples of Hydrodictyon were critical point dried with CO2 for 3 h and mounted on stubs with double-sided tape. The silicon chips were glued onto SEM stubs with double-sided tape. The silicon chips were glued onto SEM stubs with silver paint. All specimens were sputter coated with gold for 1 min 15 s and observed using a DSM982 Gemini Zeiss Field Electron Microscope (Zeiss, Oberkochen, Germany).

RESULTS

In preliminary analyses of 26S data, the published Hydrodictyon reticulatum sequence (GenBank accession AF183477) was found to have a much longer branch than the other isolates of that genus, so we re-extracted, amplified and sequenced the strain from Carolina Biological Supply. Upon comparison, it was found that the published sequence differed by 40 bases from our sequence, the majority of these differences near the 3’ end and potentially due to electrophoresis errors or inaccurate editing. The analyses discussed in this paper include the new 26S sequence.

Two taxa, P. biradiatum Meyen (UTEX 37) and P. boryanum var. longicorne Reinsch (UTEX LB1372), contain putative introns in the 26S gene. These regions are regarded as putative group I introns based on BLAST comparisons. The Pediastrum biradiatum sequence contains one intron 414 bases long and located 856 bases from the 5’ end of the gene. The Pediastrum boryanum var. longicorne sequence contains either two or three introns. One intron begins 853 bases from the 5’ end, which corresponds to three bases before the insertion point of the P. biradiatum intron. A potential second intron is directly adjacent to the first intron; the combined length of these regions is 870 bases. Determination of whether one or two introns are present and the precise length of the intron(s) was not possible due to difficulties sequencing completely through this region. The third intron begins 965 bases from the 3’ end of the putative second intron and is 405 bases in length. The intron sequences were excluded from the phylogenetic analyses.

Genetic variation within species

Multiple isolates of five species, H. reticulatum, P. duplex, P. boryanum, P. tetras (Ehrenberg) Ralfs, and S. spinulosum Nägeli were included in these analyses, along with single isolates of additional species. No base pair differences were found among multiple geographically distinct and morphologically similar representatives within the species H. reticulatum and S. spinulosum in either the 26S or the ITS-2 data. All but one strain of P. boryanum grouped together; P. boryanum var. longicorne (UTEX LB1372) is extremely different genetically, exhibiting between 68 and 70 bp differences out of 2442 nucleotides from the other P. boryanum sequences, and morphologically resembles the genus Tetraëdron Kützing rather than Pediastrum. Two geographically separated strains of P. duplex, EL0201CT from Connecticut and UTEX LB144 from Tennessee, exhibit no base pair differences. Likewise, two isolates collected from separate lakes in North Carolina with P. duplex var. gracillimum morphology, LN0201NC and LW0201NC, exhibit no base pair differences. Conversely, a great deal of molecular variation was apparent between taxa in the P. tetras + P. privum clade (HL0202WI, SF0203NY, EL0207CT, SAG 36.81). The absolute number of pairwise differences in the P. tetras + P. privum clade ranged from 0 to 24 in the 26S data set (0–0.01286 HKY85+G corrected distances), and 0–18 (0–0.05842 HKY85+G corrected distances) in the ITS-2 data set.

Maximum likelihood analyses of 26S and ITS-2 data

ML analyses were done separately on the 26S and ITS-2 data, and then in combination under the assumption that the data were combinable (based on the ILD test; see Material and Methods). The resulting trees are shown in Figs 1, 2 and 3, respectively. The details of the 26S and ITS-2 trees will be explored in combination and the results of the combined analyses (Fig. 3) are described in the next section.

Overall, the 26S data offered more resolution of the ingroup when compared with the ITS-2 data, indicating more phylogenetic signal in the 26S rDNA data. Analyses of these data recovered Sorastrum as a morphological group, however this taxon is paraphyletic in the ITS-2 gene topology. Pediastrum tetras was recovered as paraphyletic in regards to P. privum in both analyses, and P. boryanum is monophyletic in both analyses with the exception of P. boryanum var. longicorne (UTEX LB1372). The isolate P. boryanum var. longicorne (UTEX LB1372) is most likely Tetraëdron based on mor-
Fig. 1. ML tree estimated from nuclear 26S rDNA gene sequence data of 39 isolates from the family Hydrodictyaceae (Sphaeropleales) plus one outgroup taxon, Neochloris aquatica (Neochloridaceae, Sphaeropleales). Names in bold represent wild isolates, other taxa were obtained from culture collections. Taxonomic groups are bracketed and labelled with appropriate genus or species (or both). Bootstrap values ≥ 50 indicated above the branches. Branch lengths are proportional to the expected number of substitutions per site indicated by scale bar. *Pediastrum boryanum* var. longicorne (UTEX LB1372) is placed in quotes due to its questionable taxonomic designation.

Phylogeny and its divergent 26S and ITS-2 sequences (see discussion). Although *Hydrodictyon* is not supported as monophyletic in the ITS-2 tree (because of insufficient variation), there is strong evidence for this taxon using the 26S and combined data. A fifth major morphological taxon, *P. duplex*, was not resolved as monophyletic in any of the analyses.

The placement of three additional taxa, RL0201FR (*Pediastrum* sp.; Figs 4, 5), *P. simplex* (Meyen) Lemmermann...
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Fig. 2. ML tree estimated from ITS rDNA data (5.8S + ITS-2 regions) of the family Hydrodictyaceae. Names in bold represent wild isolates, the remaining were obtained from culture collections. Taxonomic groups are bracketed and labelled. Bootstrap support values ≥ 50 are shown above the branches. *Pediastrum boryanum var. longicorne* (UTEX LB1372) is placed in quotes due to its questionable taxonomic designation.

(UTEX LB1601; Figs 6, 7), *P. biradiatum* (UTEX 37; Fig. 8) and CL0201VA (*Pediastrum* sp.; Figs 10, 11), also differed substantially in the analyses of individual data sets.

The 26S data group *Sorastrum* with *P. boryanum* var. longicorne (UTEX LB1372), and this clade is sister to the *P. duplex* plus *Hydrodictyon* complex, whereas the ITS-2 data group *Sorastrum* with *P. biradiatum* and place this clade as sister to the *P. tetrass + P. privum + P. boryanum* var. longicorne + *P.
Fig. 3. ML tree estimated from a combined 26S rDNA + ITS rDNA data set. Taxonomic groups bracketed and labelled. The SEM images included represent the general morphology of each group. *Sorastrum americanum* (SAG 13.94), scale bar = 10 μM; *P. duplex* (EL0201CT), scale bar = 20 μM; *H. reticulatum* (CBS), scale bar = 100 μM; *P. boryanum* (SAG 87.81), scale bar = 20 μM; *P. tetras* (HL0202WI), scale bar = 5 μM. Bootstrap values ≥ 50 noted above nodes. Italised values below branches represent Bayesian posterior probabilities ≥ 50%. *Pediastrum boryanum* var. *longicorne* (UTEX LB1372), in quotes, is most likely *Tetraedron. braunii* clade; the *P. tetras + P. privum* clade occurs as sister to *P. braunii* in the 26S tree. The *P. duplex* group is poorly resolved in the ITS-2 tree and is closely related to *Hydrodictyon* in the 26S tree. The placement of the *P. boryanum* clade, excluding *P. boryanum* var. *longicorne* (UTEX LB1372), reveals a close relationship to *P. kawraiskyi* and *P. integrum* in both the ITS-2 and 26S trees. RL0201FR (Figs 4, 5) was poorly supported as sister taxon to *P. angulosum* (Ehrenberg) Meneghini (UTEX LB1366 and HL0201WI) in the 26S rDNA tree (ML bootstrap support, BS, < 50) (Fig. 1), but occurred at the base of the tree outside of the *Pediastrum* spp. clades in the ITS-2 tree (Fig. 2). *Pediastrum simplex* (UTEX LB1601; Figs 6, 7) occurred on its own in the ITS-2 tree (Fig. 2), but grouped with isolates LN0201NC (*P. duplex*) and LW0201NC (*P. duplex*) in the 26S tree. The ITS-2 data placed *P. biradiatum* (UTEX 37; Fig. 8) with the *Sorastrum* spp. with a low BS support (Fig. 2), and the 26S rDNA data did not place it with any particular clade (Fig. 1). *Pediastrum boryanum* var. *longicorne* (UTEX LB1372; Fig. 9) was sister taxon to the *Sorastrum* clade in the 26S tree (Fig. 1), and the ITS-2 data placed it as sister to *P. braunii* Wartmann (SAG 43.85; Fig. 2), though poorly supported. CL0201VA (*Pediastrum*...
Figs 4–9. *Pediastrum* spp., SEM.

Fig. 4. Colony of RL0201FR illustrating a lack of intercellular spaces. Scale bar = 20 μM.

Fig. 5. View of a marginal cell of RL0201FR showing detail of net-like wall pattern with granules. Scale bar = 5 μM.

Fig. 6. *Pediastrum simplex* (UTEX LB1601). The morphology of this isolate is characteristic of *P. simplex* and differs from other isolates included in this study by possessing only one process on each marginal cell. Scale bar = 10 μM.

Fig. 7. Portion of marginal cell of *P. simplex* (UTEX LB1601). Note extensive cell wall sculpturing. Scale bar = 2 μM.

Fig. 8. *Pediastrum biradiatum* (UTEX 37). Colony with intercellular spaces and cells that divide into two lobes, with each lobe further divided into two processes. Scale bar = 10 μM.

Fig. 9. *Pediastrum boryanum* var. *longicorne* (UTEX LB1372). This isolate was not observed to produce *Pediastrum*-type colonies and it exhibits wall ultrastructure that is not typical of *P. boryanum*. The taxonomic designation is questionable and is most likely *Tetraëdron*. Scale bar = 5 μM.
trum sp.; Figs 10, 11) consistently failed to group with any particular clade, but was nested within the genus Pediastrum.

Phylogenetic analyses of the two-gene data set
The ML analysis of the combined data (Fig. 3) was most similar to the 26S topology (Fig. 1). The combined analysis failed to resolve *P. duplex* as monophyletic as in the separate gene analyses. RL0201FR (Figs 4, 5) was poorly supported (BS support < 50) as a sister taxon to *P. angulosum* (UTEX LB1366 and HL0201W1), and *P. simplex* (UTEX LB1601; Figs 6, 7) grouped with two new isolates, LN0201NC (*P. duplex*) and LW0201NC (*P. duplex*). The placement of *P. biradiatum* (UTEX 37; Fig. 8) differed from both the 26S and ITS-2 topologies; in the combined data, it appears as a poorly supported sister taxon to the *Sorastrum* clade (Fig. 3). The combined data did not group *Pediastrum boryanum* var. longicorne (UTEX LB1372; Fig. 9) with any clade (Fig. 3).

The Bayesian analysis (not shown) of the combined data recovered the same major groupings as in the ML topology; however, their placement varied. The *P. tetras + P. privum* clade occurred on its own at the base of the tree, and the remaining taxa grouped together. The *P. boryanum, P. duplex* (excluding LN0201NC and LW0201NC) and *Hydrodictyon* groups formed a polytomy containing CL0201VA, the *Sorastrum* clade occurred outside of this polytomy and *P. boryanum* var. *longicorne* was placed on its own outside of the polytomy and *Sorastrum* clade. Removing *P. boryanum* var. *longicorne* (UTEX LB1372) from the analysis resulted in better resolution of the polytomy (not shown). The *Hydrodictyon* and *P. simplex* (UTEX LB1601) were placed outside the *P. duplex* and *Hydrodictyon* group. One major difference was the placement of *P. braunii* (SAG 43.85), which no longer grouped basal to *P. tetras*, but was placed at the base of the tree. There was more support (posterior probability = 0.96) for the node uniting all taxa (excluding *P. braunii* SAG 43.85 and the *P. tetras + P. privum* clade) in the analysis excluding *P. boryanum* var. *longicorne* (UTEX LB1372) (not shown).

Phylogenetic groupings and phenotypic variation
A description of the morphological characteristics of the four morphological groups recovered in the combined analysis, *P. boryanum*, *P. tetras* (including *P. privum*), *Hydrodictyon*, and *Sorastrum*, as well as the *P. duplex* taxa, is used to address species boundaries and patterns of colony-form evolution. The characteristics highlighted for each *Pediastrum* species are summarized using Komárek & Jankovska (2001), and the information for *Hydrodictyon* and *Sorastrum* was extracted from Prescott (1951). Additional information regarding the morphology of individual taxa was extracted from scanning electron micrographs obtained for this study.

Five isolates form the *P. boryanum* clade and share characteristics typical of the species (Fig. 3). The coenobia of *P. boryanum* lack intercellular spaces, or if spaces are present, they are small and irregular, the marginal cells have a V- or U-shaped incision, and the wall ornamentation is usually granular. *Pediastrum kawraiskyi* Schmidle (SAG 35.81) is a sister taxon to the *P. boryanum* clade and *P. integrum* NaÈgeli (SAG 29.81) is highly supported as a sister taxon to *P. boryanum* and *P. kawraiskyi*. The coenobia of *P. kawraiskyi* (SAG 35.81) are typified by lacking intercellular spaces and the marginal cells are divided into two lobes perpendicular to the plane of the coenobium that are terminated by short processes; the wall structure is typically granular. The marginal cells of *P. integrum* lack an incision in the marginal cells, the processes are smaller than those in *P. boryanum*, and the wall structure consists of regularly arranged granules. Overall, the group containing *P. boryanum*, *P. kawraiskyi* and *P. integrum* can be described as lacking intercellular spaces and exhibiting varying degrees of granular wall ultrastructure.
Figs 12–15. Pediastrum spp., SEM.

Fig. 12. Pediastrum tetras, UTEX 38. Colony exhibiting the typical P. tetras morphology from Czechoslovakia. The cells are trapezoidal in shape and each has a deep incision/sinus. Note overall similarities to Fig. 13. Scale bar = 10 μM.

Fig. 13. Pediastrum tetras, HL0202WI. Isolate obtained from Wisconsin, USA, with the typical P. tetras morphology. Note the morphological similarities to UTEX 38 in Fig. 12, however there are 39 bp differences out of 2442 bases. Scale bar = 10 μM.

Fig. 14. Pediastrum tetras, EL0207CT. Four-celled colony of P. tetras isolated in Connecticut, USA. Scale bar = 5 μM.

Fig. 15. Pediastrum privum, SAG 36.81. Colony with four cells, each with a slight concave depression rather than a deep incision/sinus as seen in P. tetras. Though morphologically distinct, this isolate differs from EL0207CT (Fig. 14) by only 7/2442 bases. Scale bar = 5 μM.

The P. duplex group, excluding LN0201NC and LW0201NC, is made up of seven isolates that are characterized by coenobia with intercellular spaces and marginal cells with V-like incisions. The cell wall ultrastructure varies from smooth and lacking ornamentation to coarsely wrinkled or irregular net-like sculpture (Fig. 3) (Komárek & Jankovská 2001). Two new isolates (LN0201NC and LW0201NC) with P. duplex var. gracillimum West & West morphology were found to be more closely related to P. simplex (UTEX LB1601; Figs 6, 7) in the combined molecular phylogenetic tree. Pediastrum simplex differs from P. duplex by possessing only one process per cell. Considering the differing conclusions drawn from phenotypic and genetic data, it is clear that additional information is necessary before a determination about the validity of the characteristics of P. duplex can be made. Inclusion of data from additional strains of P. simplex might also help to clarify these relationships.

In P. tetras, the coenobia lack intercellular spaces, the marginal cells have narrow incisions and are trapezoidal in shape, and the wall ultrastructure varies from irregularly net-like to warty. The clade includes one isolate of P. tetras (UTEX 38, Fig. 12), and three new isolates tentatively designated P. tetras (Figs 13, 14). Also included is P. privum (Printz) Hegewald (SAG 36.81), which is characterized as lacking intercellular spaces and having marginal cells with only a slight concavity (Fig. 15). Pediastrum privum and one new isolate assigned to P. tetras (EL0207CT, Fig. 14) group as highly supported sister taxa (BS = 96, Bayes = 100) and differ only at seven of 2442 sites (HKY+G distance = 0.00309). The morphology of EL0207CT is four to eight cells with deep incisions in the marginal cells (Fig. 14). The isolate from Czechoslovakia (UTEX 38, Fig. 12) is morphologically similar to isolates HL0202WI and SF0203NY; however, there are 39 bp distinguishing them (HKY+G distance = 0.01784). These intriguing differences between molecular relatedness and morphological similarities highlight the need for more detailed investigations of the characters defining this species.

The three-dimensional, macroscopic coenobia of Hydro-
dictyon are composed of multinucleate cells that join at their ends to form a cylindrical net with five- or six-sided meshes (Fig. 3) (Prescott 1951). The three *Sorastrum* isolates are well supported as a clade in the combined ML topology and are characterized by microscopic, spherical colonies made up of reniform, pyriform, cuneate or pyramidal cells with stalks that connect with stalks of other cells at the center of the colony (Fig. 3) (Prescott 1951; Fritsch 1965). Both *Hydrodictyon* and *Sorastrum* are shown to be most closely related to forms of *Pediastrum* with intercellular spaces in the 26S rDNA and combined trees.

**DISCUSSION**

Data from the expanded taxon sampling of culture collections and new isolates included in this study, as well as other analyses including multiple outgroup taxa (not shown), support the monophyly of the family Hydrodictyaceae and permitted a more thorough investigation of the relationships among the genera. Our results, which are also concordant with Carlson’s (2002) 18S rDNA study, indicate that the genus *Pediastrum* is paraphyletic. In addition, the 26S and ITS-2 data provide a complex picture of colony-form evolution within the Hydrodictyaceae. In all the analyses, the genera exhibiting three-dimensional colonies, *Hydrodictyon* and *Sorastrum*, are each nested within *Pediastrum* indicating independent cases of three-dimensional colony evolution (Figs 1–3). The combined and 26S trees place *P. duplex* and *P. angulosum* as the closest relatives of *Hydrodictyon*, although the ITS-2 data provide no information regarding this relationship. An intermediate step in the evolution of *Hydrodictyon* colonies may be an increase in size of intercellular spaces in two-dimensional colonies, possibly represented within the closely related *P. duplex* group, which exhibits intercellular spaces. The transition from the *Pediastrum* form to the *Hydrodictyon* form would likely begin at the zoospore level, with an increase in the number of zoospores joining to five or six, resulting in the formation of meshes. The newly formed colony would then undergo extreme expansion creating larger intercellular spaces and lengthened individual cells. This trend of increased size of intercellular spaces is a hypothesis that requires ancestral state reconstruction. Because support is weak and additional taxa are necessary to determine the exact species relationships within this family, ancestral state reconstruction is not possible but will be explored in future studies of the Hydrodictyaceae. The relationship of *Sorastrum* to *Pediastrum* is not clear in these analyses, precluding exploration of possible colony-form evolution scenarios.

The inclusion of data from new isolates provided greater detail regarding the amount of genetic diversity within and among species than that from culture collections alone, and acted as a test of the boundaries of recognized morphospecies. Monographic works on *Pediastrum* have described species, varieties and forms based on such characteristics as the size and shape of the marginal cells, presence or absence of intercellular spaces and pattern and extent of cell wall sculpturing. Within *Pediastrum*, the *P. boryanum* clade was consistently recovered as a clade in the phylogenetic analyses, with the exception of *P. boryanum* var. *longicorne*. The *P. boryanum* clade, indicated in Fig. 3, is typified by lacking intercellular spaces [except variety *cornutum* (Racicborski) Sulek that has small spaces], and exhibiting granular wall ultrastructure. The isolates making up this well-supported clade exhibit the *P. boryanum*-morphology; however, some isolates in the analyses that have this morphology did not group within the *P. boryanum* clade. Using light and electron microscopy, the morphology of the marginal cells, lack of intercellular spaces, and type of cell wall ornamentation indicates the isolate RL0201FR best fits the morphology described for *P. boryanum* var. *boryanum* (Figs 4, 5) (following Komárek & Jankovská 2001). However, this isolate does not group with the well-supported *P. boryanum* clade as expected, and instead groups with the *P. duplex* clade in trees from both the 26S and combined analyses, though this placement is poorly supported (BS < 50). The characteristics of CL0201VA most closely ally with those of *P. boryanum* var. *forcipatum* (Corda) Chodat, but this isolate occurs on its own in all three trees, rather than within the *P. boryanum* clade (Figs 1–3). These two examples illustrate the morphological characteristics used to delimit *Pediastrum* species can be misleading and that these characteristics are convergent, ancestral, or plastic and vary depending on ecological factors.

The number of taxa recognized in three monographs, Bigeard (1933), Sulek (1969) and Parra (1979), differs with respect to the number of varieties and forms. One of the main distinctions made between the varieties and forms has been based on the density of cell wall sculpturing. Qualitative statements describing the extent of the cell wall sculpturing were used by Bigeard (1933) and Sulek (1969), whereas Parra (1979) used quantitative measurements of granule/wart density, particularly among varieties of *P. boryanum*. However, due to the lack of overlap in the way that ornamentation was scored among the various authors (Bigeard 1933; Sulek 1969; Parra 1979), it has been difficult for other researchers to assign appropriate taxonomy to new isolates (Nielsen 2000). Bigeard (1933), Parra (1979) and Nielsen (2000) concluded from general observations that a small number of the morphological characteristics within the genus *Pediastrum* may exhibit variation depending on the environment or age of the coenobium, but the potential for environmental influence on these characters has not been explored experimentally. Some preliminary data indicate that varying nutrient levels in the growth medium affect the phenotypic expression of isolate CL0201VA, with the length of the processes and density of the granules on the cell walls varying (H.A. McManus and L.A. Lewis, unpublished observations). Further studies are being conducted to explore the full range of variation within this strain as well as RL0201FR and other cultures with *P. boryanum*-morphology.

*Pediastrum tetras* has marginal cells that are trapezoidal in shape with a deep, narrow incision. Within the *P. tetras + P. privum* clade there is disagreement between the morphological and molecular data that raises questions regarding the taxonomy within *P. tetras*. Two morphologically similar isolates, UTEX 38 and HL0202WI, exhibit 39 bp differences mostly found in the ITS-2 region, whereas two dissimilar isolates, *P. privum* and EL0207CT, exhibit only 7 bp differences. Komárek & Jankovská (2001) note that several varieties have been assigned to *P. tetras* and they may represent separate taxonomic units. The results of the molecular phylogenetic analysis support this statement and highlight the need for fur-
ther sampling and morphological studies to clarify both taxonomy and characters used to delimit boundaries between taxa with *P. tetras*-like morphology.

Relationships among most of the isolates with the *P. duplex* morphology characterized by intercellular spaces, marginal cells with V-like incisions and wall sculpturing smooth or waved with irregular net-like pattern would indicate that the general *P. duplex* morphology is relatively stable. However, both LN0201NC and LW0201NC would be assigned to *P. duplex* var. *gracilimum* using morphology, whereas the molecular data place them outside the *P. duplex* group. This indicates the *duplex* morphology is of questionable taxonomic value because it may span across several clades. Again, no definite conclusions can be drawn from these results because the support for the grouping of the *P. duplex* taxa is poor, and the placement of the two *P. duplex* var. *gracilimum* isolates with *P. simplex* is only supported in the Bayesian analysis, with a posterior probability of 0.91. However, zero out of 57,000 Bayesian topologies support the monophyly of *P. duplex*, making it appear unlikely that additional data will unite the *duplex* taxa.

Three of the four taxa that vary in their placement between the individual data set analyses, *P. biradiatum* (UTEX 37; Fig. 8), *P. boryanum* var. *longicorne* (UTEX LB1372; Fig. 9), and *P. simplex* (UTEX LB1601; Figs 6, 7), differ morphologically from the other taxa included. The lack of closely related isolates may be causing the sequences of *P. biradiatum* and *P. simplex* to vary in their position, and including morphologically similar isolates may resolve this discrepancy. *Pediastrum boryanum* var. *longicorne* (UTEX LB1372), in particular, was expected to group with the other *P. boryanum* sequences. Instead, it consistently occurs independently at the base of the tree. Upon further examination of its morphology, this culture does not form colonies typical of *P. boryanum*, but grows as unicells that resemble the genus *Tetraedron* (Fig. 9) and suggests this culture strain was misidentified. The genus *Tetraedron* consists of cells that are always solitary and free-floating and are typically triangular, quadrangular or polygonal (Smith 1950). The young cells are uninucleate and the mature cells may contain two, four or eight nuclei (Smith 1950). *Tetraedron* has been suggested to be grouped within Hydrodictyaceae based on the cell morphology and development [Probst 1988], however, Watanabe et al. (1988) have proposed its placement elsewhere and basal to the coenobial taxa (Probst 1926); Starr (1954)), however, Watanabe et al. (1988) have proposed its placement elsewhere and basal to the coenobial genera based on ultrastructure. Other authors have grouped *Tetraedron* within families such as Oocystaceae (Smith 1950), Chlorococaceae (Bourrelly 1966), and Ettl and Gärtnert (1995) synonymize *Tetraedron* with *Chlorotetraedron*. At this time there are too many contradictions within the literature to be confident in the current phylogenetic placement of *Tetraedron*. In all of these cases an increased taxon sampling is needed to better test the placement of these isolates.

**Future directions**

A well-resolved phylogeny is necessary to fully explore colony-form evolution in the Hydrodictyaceae, and to determine the taxonomic importance of morphological characters within the morphologically diverse genus *Pediastrum*. The results presented here indicate that some of the characters used to define species, varieties, and forms of *Pediastrum*, such as wall morphology and length of processes, span across taxa and taxonomic revisions will be needed. With hopes of constructing a more robust estimate of the phylogeny of the Hydrodictyaceae, additional taxa are being added, and molecular data from the plastid-encoded *rbcL* gene are currently being collected. Our understanding of the morphological variation of *Pediastrum* will become increasingly clear with an expanded taxon sampling and insights gained from the study of phenotypic plasticity.

In addition, there is need for wider geographical sampling. With the exception of three isolates of *Hydrodictyon*, despite the reported worldwide distribution for some species, all of the isolates included in the present study are from the Northern Hemisphere. With this bias, the proposed cosmopolitan distribution of these species cannot be evaluated. Inclusion of additional strains of *Pediastrum* spp. from various localities, especially from the Southern Hemisphere, will provide important information on geographic variation and species distributions.

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### Appendix 1. Locality information of isolates included in present study.

| Species                        | Strain  | Locality                                |
|--------------------------------|---------|-----------------------------------------|
| Hydrodictyon africanaum        | UTEX LB782 | Cape Flats, South Africa               |
| Hydrodictyon patenaeforme      | CCAP 236/3 | unknown                                |
| Hydrodictyon reticulatum       | CBS      | Indiana, USA                            |
| Hydrodictyon reticulatum       | UTEX LB515 | Nashville, Tennessee, USA              |
| Hydrodictyon reticulatum       | SF0201NY | Stockport Flats, New York, USA         |
| Hydrodictyon reticulatum       | ML0301CT | Mirror Lake, Storrs, Connecticut, USA  |
| Hydrodictyon reticulatum       | NZ0301   | pond near Arapuni, North Island, New Zealand |
| Pediastrum angulosum           | UTEX LB1366 | Podbansko pool, High Tatra Mountains, Czechoslovakia |
| Pediastrum biradiatum          | UTEX 37  | Sweden                                  |
| Pediastrum boryanum var. longicorne | UTEX 1372 | Pond Brehyne, Doksy, Czechoslovakia    |
| Pediastrum boryanum var. cornutum | UTEX LB470 | Malham Tarn, Yorkshire, UK              |
| Pediastrum boryanum var. forcipatum | SAG 87.81 | Schöhssee/Pöln, Germany               |
| Pediastrum boryanum var. brevicorne | SAG 261-7 | unknown                                |
| Pediastrum boryanum            | OL0301MN | Otter Lake, Minnesota, USA             |
| Pediastrum braunii             | EL0203CT | Eagleville Lake, Storrs, Connecticut, USA |
| Pediastrum duplex var. asperum  | UTEX LB1364 | Machovo jezero pond, Doksy, Czechoslovakia |
| Pediastrum duplex              | EL0201CT | Eagleville Lake, Storrs, Connecticut, USA |
| Pediastrum duplex              | SAG 84.80 | Schöhssee/Pöln, Germany               |
| Pediastrum duplex              | SR0201NJ | Spruce Run Lake, New Jersey, USA       |
| Pediastrum simplex             | SAG 29.81 | unknown                                |
| Pediastrum simplex             | SAG 35.81 | Steinhuder Meer, Germany               |
| Pediastrum simplex             | SAG 36.81 | Kuusjärvi, Saukkoalhti near Jyväskylä, Finland |
| Pediastrum tetras              | UTEX LB1601 | unknown                                |
| Pediastrum tetras              | UTEX 38   | Czechoslovakia                          |
| Pediastrum tetras              | HL0202WI | Hook Lake, Wisconsin, USA              |
| Pediastrum tetras              | SF0203NY | Stockport Flats, New York, USA         |
| Pediastrum tetras              | EL0207CT | Eagleville Lake, Storrs, Connecticut, USA |
| Pediastrum sp.                 | UTEX LB144 | Nashville, Tennessee, USA             |
| Pediastrum sp.                 | CL0201VA | Claytor Lake, Virginia, USA            |
| Pediastrum sp.                 | HL0201WI | Hook Lake, Wisconsin, USA              |
| Pediastrum sp.                 | RL0201FR | Reilhac Lake, France                   |
| Sorastrum americanum           | SAG 13.94 | Lake Itasca State Park, North Deming Pond, Minnesota, USA |
| Sorastrum spinulosum           | UTEX LB2452 | Finland                                |
| Sorastrum spinulosum           | IL0201MN | Lake Itasca State Park, Itasca Lake, Minnesota, USA |
| Neochloris aquatica            | UTEX 138  | Aquarium in Bloomington, Indiana, USA  |