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PHYLOGENETIC PLACEMENT OF *BOTRYOCOCCUS BRAUNII* (TREBOUXIOPHYCEAE) AND *BOTRYOCOCCUS SUDETICUS* ISOLATE UTEX 2629 (CHLOROPHYCEAE)[1]

Hoda H. Senousy, Gordon W. Beakes, and Ethan Hack[2]

School of Biology, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, UK

The phylogenetic placement of four isolates of *Botryococcus braunii* Kützing and of *Botryococcus sudeticus* Lemmermann isolate UTEX 2629 was investigated using sequences of the nuclear small subunit (18S) rRNA gene. The *B. braunii* isolates represent the A (two isolates), B, and L chemical races. One isolate of *B. braunii* (CCAP 807/1; A race) has a group I intron at *Escherichia coli* position 1046 and isolate UTEX 2629 has group I introns at *E. coli* positions 516 and 1512. The rRNA sequences were aligned with 53 previously reported rRNA sequences from members of the Chlorophyta, including one reported for *B. braunii* (Berkeley strain). Phylogenetic trees were constructed using distance, weighted maximum parsimony, and maximum likelihood, and their reliability was estimated using bootstrap analysis for distance and parsimony and Bayesian inference for likelihood. All methods showed, with high bootstrap or credibility support, that the four isolates of *B. braunii* form a monophyletic group whose closest relatives are in the genus *Choricystis* in the Trebouxiophyceae, whereas the previously reported *B. braunii* sequence is from a member of the Chlamydomonadales in the Chlorophyceae and isolate UTEX 2629 is a member of the Sphaeropleales in the Chlorophyceae. Polyphyly of these sequences was confirmed by Kishino-Hasegawa tests on artificial trees in which sequences were moved to a single lineage.

Key index words: **18S rRNA sequences; *Botryococcus braunii*; *Botryococcus sudeticus*; *Botryosphaera*; *Botryosphaerella*; Chlorophyta; hydrocarbons; lipids**

Algae of the genus *Botryococcus* Kützing are noted for their ability to accumulate large amounts of lipids, especially hydrocarbons. They have a distinctive appearance, growing as colonies of cells held together by a matrix that often contains droplets of hydrocarbons; each cell is typically embedded in a cup-like sheath (Metzger et al. 1991, Metzger and Largeau 1999). Reproduction is autosporic. The genus has a long fossil history, being known from the Carboniferous Period, and is the major contributor to torbanite oil shales ("boghead coals") (Temperley 1936, Tyson 1995). *Botryococcus* is of contemporary significance as a potential source of renewable energy in the form of hydrocarbon fuels (Metzger et al. 1991, Metzger and Largeau 1999, Banerjee et al. 2002). The best known species is *Botryococcus braunii* Kützing. This organism has a worldwide distribution in fresh and brackish water and is occasionally found in salt water. Although it grows relatively slowly, it sometimes forms massive blooms (Metzger et al. 1991, Tyson 1995). *Botryococcus braunii* strains differ in the hydrocarbons that they accumulate, and they have been classified into three chemical races, called A, B, and L. Strains in the A race accumulate alkadienes; strains in the B race accumulate botryococcenes, which are triterpenoids; and strains in the L race accumulate lycopadiene, a tetraterpenoid (Metzger et al. 1985, 1990, 1991, Metzger and Largeau 1999). Whether the chemical races correspond to different evolutionary lineages has not been reported.

How *Botryococcus* should be classified into species has not been resolved. The most elaborate classification is that of Komárek and Marvan (1992), who proposed 13 species on morphological grounds. The morphological differences they used are relatively subtle, and Plain et al. (1993) found that a single isolate could resemble more than one species, depending on growth conditions. Therefore, the species descriptions of Komárek and Marvan (1992) have not been widely used, and most publications continue to refer to a single species, *B. braunii*. However, one clearly distinctive species is sometimes included in the genus *Botryococcus*, *B. sudeticus* Lemmermann. This is different enough from *B. braunii* that in 1922 Chodat (cited in Vazquez-Duhalt and Greppin 1987b) placed it in a new genus, *Botryosphaera* (also called *Botryosphaerella*; Silva 1970), but this distinction has not been universally adopted (Fritsch 1935). Vazquez-Duhalt and Greppin (1987b) and Vazquez-Duhalt (1991) described an alga that they isolated as a contaminant of a culture of *B. braunii* strain CCAP 807/1. This alga, which they identified as *Botryococcus sudeticus*, is deposited in the UTEX Culture Collection of Algae as isolate UTEX 2629. Vazquez-Duhalt and Greppin (1987b) found this alga to accumulate large amounts of neutral lipids, principally triacylglycerols, that contain a high proportion of oleic acid. The composition of its oil resembles that of olive oil, and so its utilization as a food oil has been proposed (Vazquez-Duhalt and Greppin 1987a). Thus, the type of lipid that isolate UTEX 2629 accumulates is quite different from the hydrocarbon lipids found in the three chemical races of *B. braunii*.

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[2]Author for correspondence: e-mail ethan.hack@ncl.ac.uk.

TABLE 1. *Botryococcus* isolates studied in this investigation.

Isolate	Site where isolated	Country of origin	Chemical race	Reference
Botryococcus braunii				
CCAP 807/1	Madingley Brick Pits, Cambridge	UK	A	Largeau et al. (1980) (isolated by Droop in 1950)
Titicaca[a]	Lake Titicaca	Bolivia	A	Metzger et al. (1989)
Songkla Nakarin[a]	Reservoir, Songkla Nakarin University, Khao Kho Hong	Thailand	L	Metzger and Casadevall (1987)
Ayamé[a]	Lake Ayamé	Ivory Coast	B	Metzger et al. (1988)
Botryococcus sudeticus				
UTEX 2629	Subisolate from isolate CCAP 807/1 of *B. braunii*	UK?		Vazquez-Duhalt (1991)

CCAP, Culture Collection of Algae and Protozoa, Windermere, Cumbria, UK; UTEX, Culture Collection of Algae, University of Texas at Austin, Texas, USA.

[a]From the collection of Dr. P. Metzger, Ecole Nationale Supérieure de Chimie de Paris.

As has been the case for many autosporic algae, the taxonomic position of *Botryococcus* has been difficult to establish, but it has become generally accepted that this genus belongs to the green algae (Hirose and Ogasawara 1977, Schnepf and Koch 1978). On the basis of molecular and ultrastructural evidence, the green algae are classified in two divisions, Chlorophyta and Streptophyta; the latter also includes higher plants (Friedl 1997, Chapman et al. 1998). The division Chlorophyta includes three principal classes, the Chlorophyceae, the Trebouxiophyceae, and the Ulvophyceae, that are believed to be monophyletic and to form a monophyletic group. A basal class, the Prasinophyceae, is paraphyletic (Friedl 1997, Chapman et al. 1998). The classes are defined morphologically by the properties of motile cells, which *Botryococcus* lacks, but also by DNA sequence relationships. Sawayama et al. (1995) sequenced the 18S rRNA of a single strain (the Berkeley strain) of *B. braunii*. They found that it was most closely related to sequences from two members of the Chlorophyceae, *Chlamydopodium vacuolatum* (Lee & Bold) Floyd et al. and *Dunaliella parva* Lerche, and so concluded that *B. braunii* is a member of the Chlorophyceae.

Our objective was to define the relationships among members of the different chemical races of *B. braunii* and to determine whether *B. braunii* and *B. sudeticus* form a monophyletic group. We sequenced the nuclear 18S rDNA of four isolates of *B. braunii* (*sensu lato*), two belonging to the A chemical race and one each to the B and L races, and of *B. sudeticus* isolate UTEX 2629. Unexpectedly, we found that the sequences from the new isolates are unrelated to the sequence reported by Sawayama et al. (1995). Moreover, the sequence from isolate UTEX 2629 is not closely related to any of the *B. braunii* sequences.

MATERIALS AND METHODS

Culture of algae. The *Botryococcus* isolates used in this investigation are listed in Table 1. The algae were grown as unialgal cultures in sterilized Jaworski's medium (Beakes et al. 1988), as recommended by the Culture Collection of Algae and Protozoa (CCAP), with constant irradiance (approximately 15 μmol photons $\cdot m^{-2} \cdot s^{-1}$) at 22° C in aerated Erlenmeyer flasks (typically 250 mL medium in a 1-L flask). The medium was renewed every 3 to 4 weeks until sufficient biomass (>0.5 g fresh mass) was obtained for DNA extraction.

Isolation of algal DNA. Algae were collected and centrifuged for 20 min at room temperature at 8000 rpm in a JA-13 rotor in a Beckman model J2-21 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Depending on the amount of oil they contained, the algae accumulated as a pellet at the bottom of the tube or floated at the top. In either case the cells were transferred into 1.5-mL microcentrifuge tubes and centrifuged again for 10–15 min at full speed (13,000 rpm) at room temperature in a Hettich Mikroliter bench-top microcentrifuge (Andreas Hettich GmbH & Co., Tuttlingen, Germany). Again, cells were collected from the top or the bottom of the tube. Centrifugation was repeated several times until approximately 0.5 g fresh mass of algal cells was collected in one tube. Harvested cells were stored at −20° C. For DNA extraction, cells were ground to a powder in liquid nitrogen in a precooled mortar and pestle. DNA was isolated using a hexadecyltrimethylammonium bromide method as described by Hayakawa (1997) and stored at −20° C.

rDNA amplification and sequencing. Primers used are shown in Table 2. The nuclear 18S rRNA gene of each isolate was amplified in three overlapping sections, using primer pairs CV1 and CV2, CV3 and CV4, and CV3 and ITS2B (White et al. 1990, Sawayama et al. 1995). PCR was performed essentially as described by Kidd and Ruano (1995) with 2 units of Taq DNA polymerase (Bioline, London, UK) in the manufacturer's buffer and 2.5 mM $MgCl_2$, 0.3 mM dNTPs, and 0.10 μM primers in a final volume of 100 μL. An equal volume of mineral oil (Sigma, Sigma-Aldrich Company, Ltd., Poole, Dorset, UK) was added to each tube, and the tubes were incubated in a Techne PHC-3 thermal cycler (Techne, Duxford, Cambridge, UK) with an initial incubation at 95° C for 5 min followed by 35 to 40 cycles of 95° C for 30 s, 45° C for 30 s, and 72° C for 2 min, and a final incubation at 72° C for 10 min.

Direct sequencing of PCR products was carried out by a commercial service (Qiagen, Hilden, Germany). Each PCR product was sequenced from the ends with the forward and reverse primers used for amplification. The intermediate primers BB1 and BB2 were used to complete the forward and reverse sequences, respectively, of the PCR product CV1–CV2 for all *B. braunii* isolates. For *B. sudeticus*, the intermediate primer 5IF was used to complete the forward sequence of the PCR product CV1–CV2 and the intermediate primer 5IR was used to complete the reverse sequence. The intermediate

TABLE 2. Primers used for PCR and DNA sequencing.

Primer name	Primer sequence	Orientation
CV1	5′-TACCTGGTTGATCCTGCCAGTAG-3′	Forward
CV2	5′-CCAATCCCTAGTCGGCATCGT-3′	Reverse
CV3	5′-AGATACCGTCGTAGTCTCAACCATAA-3′	Forward
CV4	5′-ACCTTGTTACGACTTCTCCTTCCTC-3′	Reverse
ITS2B[a]	5′-GCTGCGTTCTTCATCGGTGC-3′	Reverse
BB1	5′-CGGTAATTCCAGCTCCAATAG-3′	Forward
BB2	5′-ACGAGCTTTTTAACTGCAAC-3′	Reverse
BB3	5′-ATGGCCGTTCTTAGTTGGTG-3′	Forward
IIR	5′-CACCACCTAGTCTCTGAACC-3′	Reverse
5IF	5′-CTAATCCCGTGGTGAGCTTG-3′	Forward
5IR	5′-ACCACGGGATTAGCATATTG-3′	Reverse

Primers CV1, CV2, CV3, and CV4 (our designations) are from Sawayama et al. (1995). All other primers were designed for this study.

[a]A at position 17 in the original ITS2 primer (White et al. 1990) was changed to G in ITS2B.

primer IIR was used to complete the reverse sequence of the PCR product CV3–CV4 for CCAP 807/1 only. The intermediate primer BB3 was used to complete the forward sequence of the PCR product CV3–ITS2B. Sequence assembly was carried out using the UK Human Genome Mapping Project Resource Centre (Hinxton, UK) computing facilities using programs in the EMBOSS package (Rice et al. 2000) and the DIALIGN program (Morgenstern 1999). The sequences determined in this study have been deposited in the EMBL databank. The DDBJ/EMBL/GenBank accession numbers are AJ581910 (*B. braunii* Ayamé isolate), AJ581911 (*B. braunii* Songkla Nakarin isolate), AJ581912 (*B. braunii* Titicaca isolate), AJ581913 (*B. braunii* CCAP 807/1 isolate), and AJ581914 (*B. sudeticus* UTEX 2629 isolate).

Phylogenetic analysis. A set of approximately 300 aligned algal 18S rRNA sequences with predicted secondary structures was obtained from the rRNA Web Server (now at http://oberon.fvms.ugent.be:8080/rRNA/; Wuyts et al. 2002). The sequences determined in this study were added to the alignment using the DCSE program (De Rijk and De Wachter 1993) after the introns had been removed and the ends of the rRNA sequences identified by comparison with other algal 18S rRNA sequences. A neighbor-joining tree was constructed from this alignment using CLUSTAL with correction for multiple substitutions (Thompson et al. 1994). For further analysis, a set of 53 sequences was chosen from the full set. Table 3 shows the species selected and the DDBJ/EMBL/GenBank accession numbers for the sequences. These sequences were checked against the EMBL Nucleotide Sequence Database and changed to the EMBL version where the rRNA Web Server version differed. The alignment of the sequences was manually improved with DCSE with the assistance of predicted rRNA secondary structure. The final alignment used for phylogenetic analyses contained 1859 positions. This alignment has been submitted to TreeBASE (http://www.treebase.org/treebase/). The trees were constructed using two subsets of these positions. First, all positions in the alignment except the ends that were of varying lengths in different organisms (i.e. alignment positions 1–39, 1823–1859) were included. Second, ambiguous positions in the alignment (287–289, 513–516, 690–692, 1400–1410, 1757–1769) were excluded as well.

Trees were constructed using PAUP* version 4.0 beta 10 (Swofford 2001) and MrBayes (Hall 2001, Huelsenbeck and Ronquist 2001) and drawn with the programs NJplot (Perrière and Gouy 1996) and TreeView (Page 1996). Three independent types of data analysis were used: distance (neighbor joining, for preliminary analysis, and minimum evolution), weighted maximum parsimony, and likelihood (maximum likelihood and Bayesian inference). Except for Bayesian inference, the methods were based on Krienitz et al. (2001). Gaps were treated as missing data. Heuristic search conditions were with starting trees built stepwise with 10 random additions of taxa, using the tree bisection-reconnection branch-swapping algorithm to find the best tree. The best scoring tree was held at each step. The rRNA sequences of the prasinophytes *Nephroselmis olivacea* and *Pseudoscourfieldia marina* were used as the outgroup. In the maximum parsimony analyses, unweighted bootstrap parsimony analysis was carried out and the results were used to weight sites (rescaled consistency index over an interval of 1–1000; Bhattacharya 1996). The weights were used as input for weighted bootstrap analysis (2000 replications). For distance and maximum likelihood analyses, a suitable model for the process of DNA substitution was chosen using the program MODELTEST 3.06 (Posada and Crandall 1998). The best model was found to be the TrN + I + G model (unequal transition rates, equal transversion rates, variation of rates among sites follows the gamma distribution) with some sites invariant. With inclusion of all positions except the ends, the relative rates were 4.7845 for C ↔ T, 2.2841 for A ↔ G, and 1.0000 for transversions; the proportion of invariant sites was 0.5042; and the value of the shape parameter (α) of the gamma distribution was 0.6036. Bootstrap analyses with 1000 replications were done with the minimum evolution method.

The program MrBayes was used to derive a phylogenetic tree by Bayesian inference. The program was run with the general time reversible model with six relative substitution rates. Some sites were allowed to be invariant, whereas the remaining sites had a gamma distribution of rates. The MrBayes program estimates the relative base substitution rates, the proportion of invariant sites, and the value of the shape parameter (α) for the gamma distribution. Four simultaneous Monte Carlo chains were run for 1,000,000 generations. The current tree was saved to a file every 100 generations, giving 10,000 trees. A consensus tree was created with a burnin value equal to 2000; that is, the first 2000 trees were ignored when the consensus tree was created.

To show the extent of agreement between the different methods, consensus trees were constructed from two sets of trees, using the strict method (100% agreement) in both cases. The first set was the 12 trees produced by neighbor joining, minimum evolution, unweighted and weighted maximum parsimony, maximum likelihood, and MrBayes, with inclusion and exclusion of ambiguous positions in each case. The second set was six trees representing the three different approaches to analysis: minimum evolution (as a distance method), weighted parsimony, and maximum likelihood, including and excluding

TABLE 3. Algal 18S rRNA sequences used in this study and their DDBJ/EMBL/GenBank accession numbers.

Taxon	Accession number
Trebouxiophyceae	
Chlorella ellipsoidea Gerneck	X63520
Chlorella kessleri Fott & Nováková	X56105
Chlorella luteoviridis Chodat	X73997
Chlorella minutissima Fott & Nováková	X56102
Chlorella mirabilis Andreyeva	X74000
Chlorella saccharophila (Krüger) Migula	X63505
Chlorella Beijerinck sp. symbiont of *Hydra viridissima* strain HvT	X72707
Chlorella vulgaris Beijerinck	X13688
Choricystis minor (Skuja) Fott	X89012
Choricystis (Skuja) Fott sp. SAG 251–2	X81965
Dictyochloropsis reticulata (Tschermak-Woess) Reisigl	Z47207
Fusochloris perforata (Lee & Bold) Floyd et al. (*Characium perforatum* Lee & Bold)	M62999
Microthamnion kuetzingianum Nägeli	Z28974
Myrmecia biatorellae (Tschermak-Woes & Plessl) Petersen	Z28971
Nanochlorum eucaryotum Wilhelm et al.	X06425
Parietochloris pseudoalveolaris (Deason & Bold) Watanabe & Floyd	M63002
Prototheca wickerhamii Tubaki & Soneda	X74003
Trebouxia impressa Ahmadjian	Z21551
Trebouxia magna Archibald	Z21552
Trebouxia usneae (Hildreth & Ahmadjian) Gärtner	Z68702
Watanabea reniformis Hanagata et al.	X73991
Chlorophyceae	
Chaetopeltidales	
Floydiella terrestris (Groover & Hofstetter) Friedl & O'Kelly (*Planophila terrestris* Groover & Hofstetter)	U83127
Chaetophorales	
Chaetophora incrassata (Hudson) Hazen	U83130
Chlamydomonadales (CW group)	
Asteromonas gracilis Artari	M95614
Botryococcus braunii Kützing Berkeley strain	X78276
Chlamydomonas moewusii Gerloff	U41174
Chlamydomonas reinhardtii Dangeard	M32703
Chlamydopodium vacuolatum (Lee & Bold) Floyd et al.	M63001
Chlorococcum hypnosporum Starr	U41173
Dunaliella salina (Dunal) Teodoresco	M84320
Ettlia minuta (Arce & Bold) Komárek (*Chlorococcopsis minuta* (Arce & Bold) Watanabe & Floyd))	M62996
Pleurastrum insigne Chodat	Z28972
Polytoma anomale Pringsheim	U22931
Protosiphon botryoides Klebs	U41177
Spermatozopsis similis Preisig & Melkonian	X65557
Stephanosphaera Cohn sp. UTEX LB 2409	U70798
Sphaeropleales	
Ankistrodesmus stipitatus (Chodat) Komárková-Legnerová	X56100
Bracteacoccus minor (Chodat) Petrova	U63097
Characiopodium hindakii (Lee & Bold) Floyd et al.	M63000
Chlorella zofingiensis Dönz	X74004
Hydrodictyon reticulatum (Linnaeus) Lagerheim	M74497
Neochloris aquatica Starr	M62861
Pediastrum duplex Meyen	M62997
Scenedesmus obliquus (Turpin) Kützing	X56103
Scenedesmus vacuolatus (Shihara & Kraus) Kessler et al.	X56104
Ulvophyceae	
Acrosiphonia Agardh sp. SAG–127.80	U03757
Gloeotilopsis planctonica Iyengar & Philipose	Z28970
Ulothrix zonata (Weber & Mohr) Kützing	Z47999
Ulva rigida Agardh	AJ005414
Prasinophyceae	
Nephroselmis olivacea Stein	X74754
Pseudoscourfieldia marina (Throndsen) Manton	X75565
Scherffelia dubia (Perty) Pascher	X68484
Tetraselmis striata Butcher	X70802

Synonyms given are names used in the NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) at the time of writing.

positions in each case. The resulting consensus trees were identical to one another. Artificially rearranged trees were constructed with the aid of the TreeView program. Likelihoods of trees constructed by different methods and of rearranged trees were compared by means of the Kishino-Hasegawa test (Kishino and Hasegawa 1989) with test distributions generated by the resampling estimated log-likelihood method (Kishino et al. 1990).

RESULTS

Four different isolates of *B. braunii* (*sensu lato*) and *B. sudeticus* isolate UTEX 2629 were examined. The *B. braunii* isolates represent the different phytochemical groups within this organism (A, B, and L) and originated from four widely separated geographic locations (Table 1). Figure 1 shows substantial differences in the colony morphology of these isolates. The A race isolates (CCAP 807/1 and Titicaca) were broadly similar in appearance, characteristically producing distinct botryoid clusters of bright green cells interconnected by fine barely discernible colorless threads, which were barely visible even with differential interference contrast phase optics (Fig. 1a). The Titicaca isolate also produced some more diffuse colonies with well-separated cells connected by fine bifurcating threads (Fig. 1b). Both of these A race isolates were bright green throughout most of their growth but became slightly more yellowish in older static cultures. The L race isolate (Songkla Nakarin) produced dense colonies of dark olive-green cells embedded in a dark brown pigmented matrix that made cell detail difficult to discern (Fig. 1c). The cluster illustrated in Figure 1c was just a small fragment that had detached from a much larger colony. The B race isolate (Ayamé) produced large ovoid cells located in discrete cups at the tips of distinctive broad banded strands (Fig. 1d),

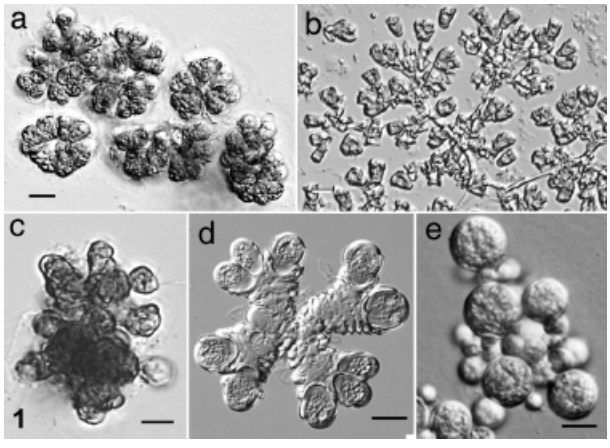

FIG. 1. Light micrographs illustrating the morphology of the isolates analyzed in this study (see Table 1 for details). Scale bars, 10 μm. All images except c (bright field optics) were taken under differential interference phase contrast (DIC) optics. a, CCAP 807/1 isolate; b, Tititicaca isolate; c, Songkla Nakarin isolate; d, Ayamé isolate; e, UTEX 2629 (*Botryococcus sudeticus*) isolate.

which were orange pigmented, giving the whole culture a reddish green appearance. Finally, *B. sudeticus* isolate UTEX 2629 produced dense rather indeterminate clusters of spherical cells ranging from 4 to 20 μm in size (Fig. 1e); the cultures remained bright to dark green throughout their growth phases. The clusters were not linked by visible strands.

Comparison with other algal rDNA sequences showed that *B. braunii* isolate CCAP 807/1 (chemical race A) contains an intron at *Escherichia coli* position 1046 and *B. sudeticus* UTEX 2629 contains introns at *E. coli* positions 516 and 1512 (not shown). The 18S rRNA genes of the other *B. braunii* isolates contain no introns. Intron sequences were removed for phylogenetic analysis. As a preliminary step, a neighbor-joining tree was constructed from the new sequences and approximately 300 aligned sequences of algae in the division Chlorophyta. This tree (not shown) suggested that the four studied *B. braunii* isolates formed a monophyletic group within the Trebouxiophyceae and were not closely related to the previously characterized Berkeley strain of *B. braunii* (Sawayama et al. 1995) or to *B. sudeticus* isolate UTEX 2629, both of which were in the Chlorophyceae. For further analysis, a representative subset of chlorophyte sequences was chosen, with a bias toward algae that from the preliminary analysis were likely to be related to the isolates examined in this study.

The Kishino-Hasegawa statistical test (Kishino and Hasegawa 1989) was carried out to compare the likelihoods of the trees constructed by different methods of analysis. Table 4 shows no significant difference in likelihood between the maximum likelihood tree produced by including all positions except the ends (the best tree, as expected) and the trees produced by the other methods of analysis, either including or excluding ambiguous positions, except that the minimum evolution trees produced by including ($P = 0.004$) and excluding ($P = 0.009$) ambiguous positions had significantly lower likelihoods. The maximum likelihood tree also had significantly higher likelihood than the consensus tree ($P < 0.0005$).

According to maximum likelihood analysis (Fig. 2), all sequences except the four from prasinophytes formed a single monophyletic group. The same result was obtained with the maximum parsimony and distance methods (Fig. 3). This group was highly supported (89%) in credibility tests using MrBayes and in bootstrap tests by the maximum parsimony (98%) and distance (99%) criteria. This large group comprises three lineages, corresponding to the classes Chlorophyceae, Trebouxiophyceae, and Ulvophyceae. Maximum likelihood analysis (Fig. 2) indicated a sister-group relationship between the Trebouxiophyceae and the Chlorophyceae. This relationship received high credibility support in the MrBayes method (99%) and moderate bootstrap support (68%) in the minimum evolution method, but it is not represented in the consensus tree (Fig. 3) because in maximum parsimony and neighbor-joining trees the Chlorophyceae shared a sister-group relationship with the Ulvophyceae rather than the Trebouxiophyceae (not shown).

A monophyletic origin of the Trebouxiophyceae received high bootstrap and credibility support using both minimum evolution (85%) and MrBayes (97%) methods but relatively low bootstrap support using weighted parsimony (51%). All the methods of analysis showed that the four *B. braunii* isolates examined in this study form a monophyletic group within the Trebouxiophyceae (Figs. 2 and 3). This group received 100%

TABLE 4. Comparisons of the maximum likelihood tree, including ambiguous positions, with trees constructed by different methods of analysis.

Method of analysis	Positions[a]	$-\ln(L)$[b]	Diff $-\ln(L)$[c]	KH test P
Maximum likelihood	Including	16186.80	(Best)	
Maximum likelihood	Excluding	16188.26	1.46	0.920
MrBayes	Including	16188.31	1.51	0.801
MrBayes	Excluding	16189.16	2.35	0.869
Weighted parsimony	Including	16198.23	11.43	0.405
Unweighted parsimony	Excluding	16201.07	14.27	0.448
Weighted parsimony	Excluding	16202.48	15.68	0.381
Neighbor joining	Excluding	16204.02	17.22	0.362
Unweighted parsimony	Including	16204.50	17.70	0.198
Neighbor joining	Including	16225.36	38.56	0.060
Minimum evolution	Excluding	16257.50	70.70	0.009*
Minimum evolution	Including	16259.34	72.54	0.004*
Consensus		16326.61	139.81	<0.0005*

Likelihoods of trees were calculated under the model in which ambiguous positions are included in the analysis. The Kishino-Hasegawa statistical test (KH test) was carried out to evaluate significance of differences in likelihood. Trees are arranged in descending order of likelihood.

*$P < 0.05$.

[a]Including, trees constructed with all positions except the ends included; Excluding, trees constructed with ends and ambiguous positions in the alignment excluded.

[b] $-\ln(L)$, negative logarithm of the likelihood of the tree.

[c]Diff $-\ln(L)$, difference between the negative logarithms of the likelihoods of the tested tree and the maximum likelihood tree.

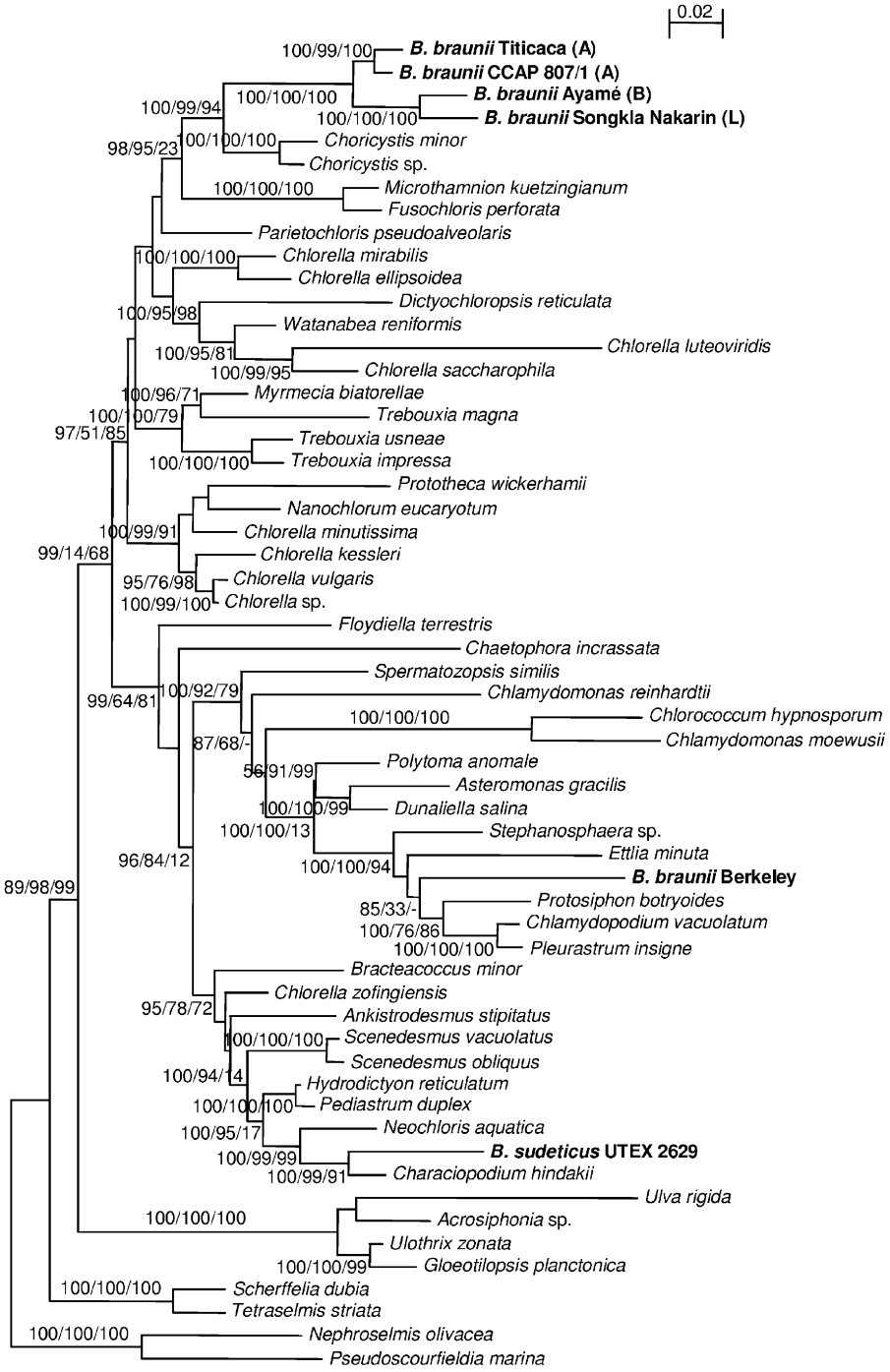

FIG. 2. Phylogenetic tree of 18S rRNA sequences inferred with the maximum likelihood method. Numbers shown at the branches are the credibility percentages using Bayesian inference (left), and bootstrap percentages using weighted parsimony (middle), and minimum evolution (right) methods. Percentages are shown only for branches that have credibility values above 50%. The horizontal lengths are proportional to the estimated number of substitutions per site. The scale bar is for 0.02 substitutions per site. Taxa shown in bold letters are the isolates whose 18S rRNA sequences were determined in this study.

bootstrap and credibility support. The closest relative of the four *B. braunii* isolates is the genus *Choricystis*. This relationship was well supported in bootstrap and credibility tests using the minimum evolution (94%), weighted parsimony (99%), and MrBayes (100%) methods. According to the maximum likelihood tree (Fig. 2), the *B. braunii/Choricystis* lineage has a sister-group relationship with *Microthamnion kuetzingianum* and *Fusochloris perforata*. This relationship received high bootstrap support using weighted parsimony (95%) and high credibility support using MrBayes (98%) but is not represented in the strict consensus tree

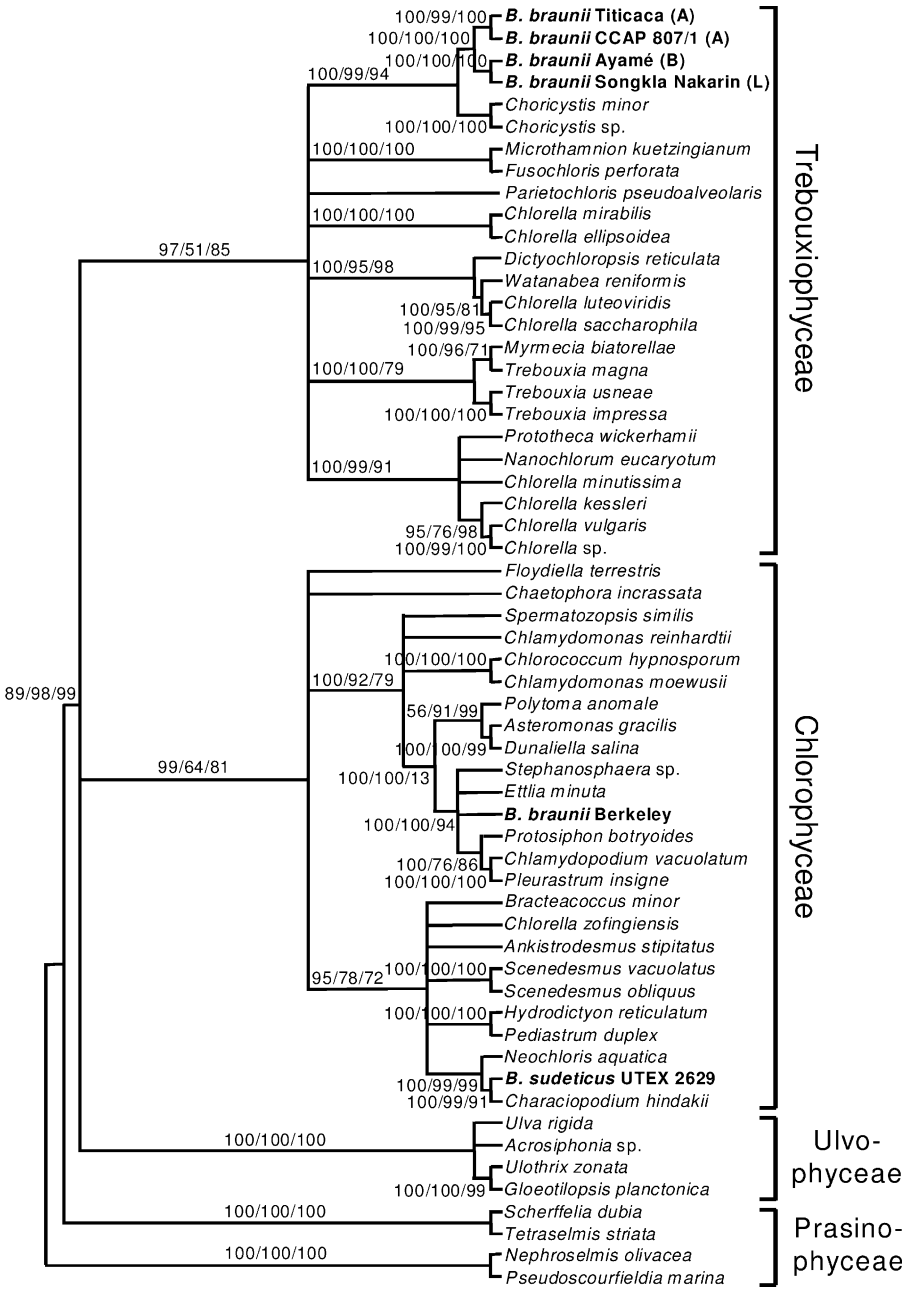

FIG. 3. Consensus tree from trees inferred with different methods of analysis. The numbers shown at the branches are credibility percentages determined using Bayesian inference (left) and bootstrap values determined using weighted parsimony (middle) and minimum evolution (right). Taxa shown in bold letters are the isolates whose 18S rRNA sequences were determined in this study.

(Fig. 3) because it was not supported by distance analysis. The distance methods suggested that *Microthamnion kuetzingianum*, *Fusochloris perforata*, and *Parietochloris pseudoalveolaris* share a common ancestor and collectively have a sister-group relationship with the *B. braunii/Choricystis* lineage (not shown). There were no clear relationships between the *B. braunii/Choricystis*, *Microthamnion/Fusochloris*, and *Parietochloris* lineages and other groups in the Trebouxiophyceae.

Monophyly of the Chlorophyceae was well supported in bootstrap and credibility tests using the minimum evolution (81%) and MrBayes (99%) methods but received only moderate bootstrap support with the weighted parsimony method (64%) (Figs. 2 and 3). The consensus tree (Fig. 3) shows four identifiable lineages in the Chlorophyceae (Nakayama et al. 1996). These represent the Chaetopeltidales (*Floydiella terrestris*), the Chaetophorales (*Chaetophora incrassata*), the Sphaeropleales (Deason et al. 1991), and the "CW group" or Chlamydomonadales (Buchheim et al. 1996). The CW group was well resolved by bootstrap analyses using minimum evolution (79%) and weighted

TABLE 5. Comparisons between the maximum likelihood tree, including ambiguous positions and artificial trees using the Kishino-Hasegawa statistical test (KH test).

Tree	$-\ln(L)$[a]	Diff $-\ln(L)$[b]	KH test P
Maximum likelihood tree	16186.80	(Best)	
Botryococcus braunii (Berkeley strain) with other *B. braunii* isolates in Trebouxiophyceae	16259.15	72.35	0.007*
All *B. braunii* isolates with *B. braunii* (Berkeley strain) in Chlorophyceae	16327.08	140.28	<0.0005*
Botryococcus braunii isolates except Berkeley strain with *B. sudeticus* UTEX 2629 in Chlorophyceae	16338.96	152.16	<0.0005*
Botryococcus sudeticus UTEX 2629 with *B. braunii* in Trebouxiophyceae	16351.69	164.89	<0.0005*

Likelihoods of trees were calculated under the model in which ambiguous positions are included in the analysis.
*$P < 0.05$.
[a] $-\ln(L)$, negative logarithm of the likelihood of the tree.
[b] Diff $-\ln(L)$, difference between the negative logarithms of the likelihoods of the tested tree and the maximum likelihood tree.

parsimony (92%) and by MrBayes (100%). Within this group four lineages are identifiable. The Berkeley strain of *B. braunii* (Sawayama et al. 1995) is a member of one of these lineages, the "*Dunaliella* clade" (Nakayama et al. 1996). In the maximum likelihood tree (Fig. 2), *B. braunii* (Berkeley strain) clusters with *Protosiphon botryoides*, *Chlamydopodium vacuolatum*, and *Pleurastrum insigne* within the *Dunaliella* clade. This clustering was supported in the credibility test using MrBayes (85%) but not in the bootstrap tests using minimum evolution (<5%) and weighted parsimony (33%). The clustering of the *Protosiphon botryoides/Chlamydopodium vacuolatum/Pleurastrum insigne* lineage with *B. braunii* (Berkeley strain), *Ettlia minuta*, and *Stephanosphaera* sp. UTEX LB 2409 was, however, highly supported in the bootstrap and credibility tests using the minimum evolution (94%), weighted parsimony (100%), and MrBayes (100%) methods. Bootstrap support for monophyly of the *Dunaliella* clade was very low (13%) with the minimum evolution method, but it was well supported (100%) using weighted parsimony and MrBayes.

All the methods of analysis showed that *Botryococcus sudeticus* UTEX 2629 is a member of the Sphaeropleales in the Chlorophyceae and is most closely related to *Characiopodium hindakii* (Figs. 2 and 3). A sister-group relationship between these species was highly supported in the bootstrap tests using minimum evolution (91%) and weighted parsimony (99%) and also in the credibility test using MrBayes (100%). *Neochloris aquatica* groups with the *B. sudeticus/Characiopodium hindakii* lineage. This relationship was highly supported in the bootstrap tests using minimum evolution (99%) and weighted parsimony (99%) and in the credibility test using MrBayes (100%). Monophyly of the Sphaeropleales, including *B. sudeticus*, within the Chlorophyceae was highly supported in the credibility test using MrBayes (95%) and moderately supported in the bootstrap tests using minimum evolution (72%) and weighted parsimony (78%).

The analysis of the trees led to three principal conclusions: 1) that the new *B. braunii* isolates are in the Trebouxiophyceae, 2) that *B. braunii* (Berkeley strain) (Sawayama et al. 1995) is in the Chlamydomonadales in the Chlorophyceae, and 3) that *B. sudeticus* UTEX 2629 is in the Sphaeropleales in the Chlorophyceae. To test the reliability of these conclusions, the branches of the maximum likelihood tree were rearranged manually and the Kishino-Hasegawa statistical test was used to compare the resulting artificial trees with the maximum likelihood tree. Table 5 shows highly significant differences between the maximum likelihood tree and all the rearranged trees. The tree in which the branches were rearranged to put *B. braunii* (Berkeley strain) in the Trebouxiophyceae with the new *B. braunii* sequences was the least unlikely ($P = 0.007$). P values for the differences between the maximum likelihood tree and all the other artificial trees were less than 0.0005.

DISCUSSION

Conclusions from molecular studies of phylogeny depend on the quality of the sequence alignment and the method of tree construction. It is common practice to exclude highly variable regions from the analysis because they cannot be aligned unambiguously (Friedl 1997, Chapman et al. 1998). In this study we compared the effect of including all positions and excluding highly variable regions to assess the sensitivity of the analysis to uncertainty in the alignment. The trees produced with exclusion and inclusion of ambiguous positions were almost identical to one another and their likelihoods were indistinguishable by the Kishino-Hasegawa test. Most importantly, the tree topologies are identical for the tested *Botryococcus* isolates whether or not ambiguous positions are included.

All the phylogenetic trees inferred from 18S rRNA gene sequences show that four diverse isolates of *B. braunii* form a monophyletic group within the class Trebouxiophyceae. This group includes two well-separated lineages. The first comprises two isolates belonging to chemical race A, which synthesizes alkadienes and trienes from fatty acids (Metzger and

Largeau 1999). The second comprises isolates belonging to races B and L. Members of both these chemical races accumulate isoprenoid hydrocarbons, but race B synthesizes triterpenoid botryococcenes, whereas race L synthesizes lycopadiene, a tetraterpenoid. Although triterpenoids and tetraterpenoids are synthesized by different pathways in higher plants, in members of the division Chlorophyta they probably share a common biosynthetic origin in the plastids (Schwender et al. 2001). Thus, the hydrocarbons of the B and L races would be biosynthetically related, and there seems to be a correspondence between phylogeny and chemistry within *B. braunii*. Analysis of internal transcribed spacer (ITS) sequences of rDNA from a larger set of isolates supports this view (unpublished data).

The closest known relative of the four isolates of *B. braunii* is the genus *Choricystis*. This relationship is strongly supported in all methods of analysis. Other than 18S rRNA sequences, no characteristics that would specifically support the relationship are known. Like *B. braunii*, *Choricystis* species are autosporic coccoids (Friedl 1997), but they have a much smaller cell size ($<3\,\mu m$) and so are considered to be picoplankton. Reductions in cell size to picoplanktonic dimensions have occurred several times in the division Chlorophyta (Krienitz et al. 1999), so that a relationship between genera with very different cell sizes is not unusual. The relationship of the *Botryococcus/Choricystis* lineage to other members of the Trebouxiophyceae is less clear than the relationship between *Botryococcus* and *Choricystis*. Maximum likelihood and parsimony analyses indicate a sister-group relationship of the *Botryococcus/Choricystis* lineage with a lineage containing zoosporic organisms, the filamentous *Microthamnion kuetzingianum* and the coccoid *Fusochloris perforata* (Friedl and Zeltner 1994). This relationship is not supported by the distance methods, in which the zoosporic coccoid *Parietochloris pseudoalveolaris* (Watanabe and Floyd 1989, Deason et al. 1991) is the closest relative of *Microthamnion kuetzingianum* and *Fusochloris perforata* (not shown). Because of the inconsistent position of *Parietochloris pseudoalveolaris*, these algae appear as three separate lineages (*Botryococcus/Choricystis*, *Microthamnion/Fusochloris*, and *Parietochloris*) in the consensus tree. Nevertheless, the evidence as a whole indicates that all three lineages form a monophyletic group. Presumably, the ancestor of this group was a flagellate, and flagella were lost in the ancestor of the *B. braunii/Choricystis* lineage—an example of the loss of flagella that has evidently occurred many times in the green algae (Friedl 1997, Chapman et al. 1998).

The 18S rRNA sequence reported by Sawayama et al. (1995) for *B. braunii* (Berkeley strain) is from a member of the Chlorophyceae, in contrast to the isolates characterized in this study. This organism falls into the *Dunaliella* clade in the Chlamydomonadales or CW group, whose other members have motile cells with clockwise-oriented basal bodies (Mattox and Stewart 1984, Buchheim et al. 1996, Nakayama et al. 1996). This conclusion is consistent with previous findings. Sawayama et al. (1995) found *B. braunii* to be related to *Chlamydopodium vacuolatum* and *Dunaliella parva*. Friedl (1997) found that *B. braunii* was in the CW group but the *Dunaliella* clade was not well supported in his analysis, whereas Chapman et al. (1998), with a similar but not identical set of organisms, found a monophyletic *Dunaliella* clade that includes *B. braunii* (Berkeley strain). The difference may be because of the uncertain position of *Spermatozopsis similis*, which was included by Friedl (1997) but not by Chapman et al. (1998). Within the *Dunaliella* clade, the relationship of *B. braunii* (Berkeley strain) with *Stephanosphaera* sp. UTEX LB 2409, *Ettlia minuta*, and a lineage including *Protosiphon botryoides*, *Chlamydopodium vacuolatum*, and *Pleurastrum insigne* is highly supported by all the different methods of analysis. This group of organisms is remarkably diverse. It was first described as containing the uninucleate coccoids *E. minuta* and *C. vacuolatum* (Lewis et al. 1992), but subsequent analysis has added filamentous organisms (*Pleurastrum insigne*; Friedl and Zeltner 1994), coccoids with multinucleate vegetative cells (*Protosiphon botryoides*; Nakayama et al. 1996), and colonial flagellates (*Stephanosphaera* sp. UTEX LB 2409; Buchheim et al. 1997). Given this diversity, it would not seem surprising to find an organism such as *B. braunii* in the group, except that *B. braunii* would be the only known member with no motile stage.

The conclusion that the reported sequence for *B. braunii* (Berkeley strain) is not closely related to the sequences from our tested *B. braunii* isolates is substantiated by the Kishino-Hasegawa statistical test, which shows that the maximum likelihood tree is significantly better than artificial trees in which *B. braunii* (Berkeley strain) is moved to the Trebouxiophyceae with our *B. braunii* isolates or our isolates are moved to the Chlorophyceae with the Berkeley strain. From this evidence, either *B. braunii* is polyphyletic or the sequences reported by us or Sawayama et al. (1995) are not from *B. braunii*. Many algal genera defined by morphological criteria have been shown to be polyphyletic by ultrastructural or molecular analysis, but the morphology and chemical characteristics of *B. braunii* are highly distinctive. The Berkeley strain was isolated as a typical *B. braunii* synthesizing botryococcenes (Wolf et al. 1985). We found that four diverse isolates of *B. braunii*, including one that synthesizes botryococcenes like the Berkeley strain, form a monophyletic group. Thus, polyphyly of *B. braunii* is unlikely to explain the results. The alternative is that either our cultures or those of Sawayama et al. (1995) contained a green alga other than *B. braunii*. Association of other algae with *B. braunii* has been reported. Metzger et al. (1985) found that *B. braunii* cells in a sample from Australia had a closely associated alga that supplanted the *B. braunii* in liquid culture. Similarly, Vazquez-Duhalt (1991) isolated *B. sudeticus* isolate UTEX 2629 from a culture of *B. braunii* CCAP 807/1 in which it seems to have at least partially replaced *B. braunii*. Extensive observations of our cultures have not,

however, revealed any evidence of algal contamination, and we obtained unique sequences without cloning the PCR products. It is improbable that all four strains would contain an undetected set of contaminants whose diversity parallels the chemical diversity in *B. braunii* and whose rDNA was consistently amplified in preference to that of *B. braunii*. Taken as a whole, therefore, the evidence indicates that *B. braunii* is monophyletic and is in the Trebouxiophyceae. The sequence reported by Sawayama et al. (1995) is almost certainly from a different organism.

Our results show that in contrast to *B. braunii*, *B. sudeticus* UTEX 2629 is a member of the Sphaeropleales in the Chlorophyceae. The conclusion that isolate UTEX 2629 is unrelated to *B. braunii* is substantiated by the Kishino-Hasegawa statistical test, which shows highly significant differences between the maximum likelihood tree and trees in which the branches were rearranged to put our tested *B. braunii* isolates with UTEX 2629 in the Chlorophyceae or to put UTEX 2629 with our tested *B. braunii* isolates in the Trebouxiophyceae. That *B. sudeticus* UTEX 2629 is unrelated to *B. braunii* is consistent with the observation that it accumulates large amounts of triacylglycerols rather than hydrocarbons (Vazquez-Duhalt and Greppin 1987b). All methods of analysis indicate that this organism is most closely related to *Characiopodium hindakii*, which is a zoosporic, multinucleate, unicellular coccoid (Floyd et al. 1993). UTEX 2629 and *C. hindakii* cluster with *Neochloris aquatica*, which is also a zoosporic, multinucleate, unicellular coccoid (Watanabe and Floyd 1989, Deason et al. 1991). The apparent relationship of UTEX 2629 to zoosporic taxa despite its apparent lack of a flagellated stage is consistent with the loss of flagellated stages in other members of the Sphaeropleales (Wilcox et al. 1992, Buchheim et al. 2001).

Many green algae contain group I introns in their rRNA genes. These introns form distinct lineages defined by their insertion sites, which are identified by the equivalent position in the rRNA of *E. coli*. They can undergo lateral transfer or can be lost (Bhattacharya et al. 1996). Thus, they can be helpful in interpreting relationships, but it is not always clear whether they are synapomorphic or symplesiomorphic characters. *Botryococcus braunii* isolate CCAP 807/1 contains an intron at *E. coli* position 1046 of the 18S rDNA, which is not present in the other three *B. braunii* isolates. Comparison with other intron sequences and consultation of the Comparative RNA Web Site (http://www.rna.icmb.utexas.edu/; Cannone et al. 2002) indicates that it is a typical 1046 intron. The 18S rRNA genes of *Choricystis* species contain from one to three introns (Hepperle and Schlegel 2002), but all have a 1046 intron. Thus, a 1046 intron could have been present in the common ancestor of *Choricystis* and *B. braunii* and been lost in the other *B. braunii* isolates. This would involve two separate losses, in the Titicaca (race A) lineage and in the Songkla Nakarin (race L)/Ayamé (race B) lineage. Alternatively, the intron may have been transferred laterally to an ancestor of *B. braunii* CCAP 807/1. The 18S rRNA gene of *B. sudeticus* isolate UTEX 2629 differs from the *B. braunii* genes in that it contains two introns, one of them at *E. coli* position 516 and the other near the 3′ end of the 18S sequence at *E. coli* position 1512. The closest known relative of UTEX 2629, *Characiopodium hindakii*, has no 18S rDNA introns, but *Neochloris aquatica*, which is closely related to UTEX 2629 and *Characiopodium hindakii*, has a 516 intron (Lewis et al. 1992). The sequence of this intron has not to our knowledge been reported. There are, however, introns similar to both the UTEX 2629 introns in some other members of the Sphaeropleales, so that these introns may be ancestral.

The results of this investigation show that *B. braunii* forms a well-resolved monophyletic group, but the branches connecting the various isolates are comparable in length with those connecting separate species (e.g. *Choricystis* species) or even genera (e.g. *Microthamnion* and *Fusochloris*). Thus, the molecular data are consistent with the view that *B. braunii* should be regarded as more than one species. "*Botryococcus sudeticus*" UTEX 2629 is unrelated to *B. braunii* and so should be transferred to a separate genus. The distinctive morphology of this isolate also indicates that it is a different organism from *B. sudeticus* as described by Lemmermann (1896). We discuss the morphology and ultrastructure of UTEX 2629 in more detail in a later article.

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