Symbiosis, 23 (1997) 125–148
Balaban, Philadelphia/Rehovot

Species Relationships in the Lichen Alga *Trebouxia* (Chlorophyta, Trebouxiophyceae): Molecular Phylogenetic Analyses of Nuclear-Encoded Large Subunit rRNA Gene Sequences

THOMAS FRIEDL* and CLAUDIA ROKITTA
Fachbereich Biologie, Allgemeine Botanik, Universität Kaiserslautern, POB 3049, 67653 Kaiserslautern, Germany, Tel. +49-631-2052360, Fax. +49-631-2052998, E-mail. friedl@rhrk.uni-kl.de

Received December 11, 1996; Accepted May 22, 1997

Abstract

Sequences of the 5' region of the nuclear-encoded large subunit (26S) rRNA genes were determined for seven species of *Trebouxia* to investigate the evolutionary relationships among these coccoid green algae that form the most frequently occurring photobiont in lichen symbiosis. Phylogenies inferred from these data substantiate the importance of certain chloroplast characters for tracing species relationships within *Trebouxia*. The monophyletic origin of the "*Trebouxia* cluster" which comprises only those species that have centrally located chloroplasts and distinct pyrenoid matrices interdispersed by a thylakoid tubule network is clearly resolved. However, those species of *Trebouxia* with a chloroplast closely appressed to the cell wall at certain stages and an indistinct pyrenoid containing regular thylakoids are distantly related to the *Trebouxia* cluster; these species may represent an independent genus. These findings are corroborated by analyses of available complete 18S rDNA sequences from *Trebouxia* spp. There are about 1.5 times more variable positions in the partial 26S rDNA sequences than in the full 18S sequences, and most of these positions are

*The author to whom correspondence should be sent.

Presented at the Third International Lichenological Symposium (IAL3), September 1–7, 1996, Salzburg, Austria

0334-5114/97/$05.50

clustered in two neighbouring variable domains of the 26S rRNA. Hence, in future studies short partial sequences that encompass both domains of the 26S rRNA may be sufficient for resolving close relationships and allow unambiguous species identifications.

Keywords: *Trebouxia*, Trebouxiophyceae; green algae, lichen algae, lichens; molecular phylogeny, taxonomy; large subunit (26S) rRNA, small subunit (18S) rRNA

1. Introduction

The unicellular coccoid green alga *Trebouxia* De Puymaly is the most frequently occurring photobiont in lichen fungi, being present in approximately 20% of all lichens and mainly associated within the ascomycetes order Lecanorales (Rambold and Triebel, 1992; Friedl and Büdel, 1996). Lichens are the symbiotic phenotype of nutritionally specialised fungi (mycobionts) that derive carbon nutrition from algal and/or cyanobacterial photobionts which are located extracellulary within a matrix of fungal hyphae (Honegger, 1991; Palmqvist et al., 1997). It has been a major concern in many studies on the taxonomy, biogeography and population dynamics in lichens that the phenotypic and genotypic variety within the unicellular green algal photobionts is largely not understood. Within *Trebouxia*, the scarcity of morphological characters makes it particularly difficult to achieve a reliable species identification. The conserved morphological features of these species may be correlated with their high degree of adaptation to the lichenized life style and this phenotypic conservation may belie a significant genotypic diversity. Recent morphological studies of available cultured *Trebouxia* species have particularly emphasised certain characters of the chloroplast as being important taxonomic markers, i.e. the overall morphology of the plastid as seen in the light microscope (Ettl and Gärtner 1984; Gärtner 1985) and the ultrastructure of pyrenoids (Friedl, 1989; Ascaso et al., 1995). Other studies have brought attention to the different modes of autospore formation which distinguish species of *Trebouxia* and which are correlated with two different patterns of the development of zoospores into vegetative cells ("cell cycles"; Friedl, 1993). These features have been used to separate another genus, *Pseudotrebouxia* Archibald, from *Trebouxia* (Archibald, 1975; Hildreth and Ahmadjian, 1981; Melkonian and Peveling, 1988), and to distinguish two subgenera within *Trebouxia* (Tschermak-Woess, 1989). Nevertheless, despite being valuable for species identification, further characters are necessary to assess the phylogenetic relationships among *Trebouxia* spp.

Sequence comparisons of ribosomal RNA genes have been found particularly useful for inferring phylogenetic relationships among and within genera of

coccoid green algae (e.g. Huss and Sogin, 1990; Lewis et al., 1992; Steinkötter et al., 1994; Friedl, 1996; Nakayama et al., 1996). Recent analyses of the small subunit (18S) rRNA genes have revealed the relationships of some *Trebouxia* species (*T. asymmetrica* Friedl and Gärtner, *T. impressa* Ahmadjian and *T. magna* Archibald) to other lichenized and non-symbiotic green algae (Friedl and Zeltner 1994; Friedl, 1995). The 18S rDNA phylogenies suggest that *Trebouxia* forms a paraphyletic assemblage since *T. magna* is more closely related to Myrmecia spp. than to the other *Trebouxia* spp. Phylogenetic analyses of 18S rDNA sequences have substantiated the importance of motile cell features for tracing evolutionary relationships among coccoid green algae (e.g. Lewis et al., 1992; Nakayama et al., 1996). Since motile cell features are almost identical among *Trebouxia* spp. (Melkonian and Peveling, 1988), the correlation between morphological features and phylogenetic structures within *Trebouxia* is unclear. Although structures of vegetative cells have been found misleading for the assessment of evolutionary relationships among genera of coccoid green algae, since they are often homoplastic (the result of parallel evolution, e.g. in *Characium, Neochloris,* and *Myrmecia;* Lewis et al., 1992; Friedl, 1995), they may, however, be important for tracing relationships at the species level.

In an effort to further explore the phylogenetic relationships among species of *Trebouxia* we have extended our studies to the 5' portion of the nuclear-encoded large subunit (26S) rRNA. This part of the molecule contains hypervariable regions which are known to be among of the most rapidly evolving portions of rRNA-encoding eukaryotic DNA (Hassounoa et al., 1984; Michot and Bachellerie, 1987; Scholin et al., 1994a,b). These so-called "D1" - "D6" domains (Hassouna et al., 1984; Michot and Bachellerie, 1987; Lenaers et al., 1991; "expansion segments", Kolosha and Fodor, 1990) have successfully been used to investigate evolutionary relationships among species and strains, e.g. in dinoflagellates (Lenaers et al., 1989, 1991; Scholin et al., 1994a), and foraminifera (Pawlowski et al., 1994a,b). These sequences have also been applied to allow species identification of unialgal isolates in diatoms (Miller and Scholin, 1996; Scholin et al., 1996). Phylogenetic studies in green algae that included sequence information of the 26S rRNA molecule have used only short RNA fragments (about 100–200 nucleotides long) that are dispersed over the molecule, and these sequences have been analysed only in connection with short fragments from the 18S rRNA (e.g. Buchheim and Chapman, 1991; Larson et al., 1992). In this study, 26S rDNA sequences have been determined for seven species of *Trebouxia* (Table 1) which exhibit various chloroplast morphologies, pyrenoid ultrastructures and both types of autospore formation found within the genus. Our aim was to evaluate the phylogenetic usefulness of these morphological characters used in the taxonomy of *Trebouxia*. In addition,

Table 1. Investigated algal taxa, their lichen fungal source (if symbiotic), data base accession, and references for the publication of the rRNA sequence data. Only those algae are listed from which 26S rDNA sequences have been determined in this study. SAG, algal culture collection at Göttingen (Schlösser, 1994); UTEX, algal culture collection at Austin, Texas (Starr and Zeikus, 1993); UKL, University of Kaiserslautern.

Organism (strain)	Source	Sequence accession nos.	Reference
Trebouxia arboricola De Puymaly (SAG 219-1a)	Unknown	Z95381 (26S) Z68705 (18S)	this paper Bhattacharya et al. (1996)
Trebouxia asymmetrica (SAG 48.88)	*Diploschistes diacapsis* (Ach.) Lumbsch	Z95380 (26S) Z21553 (18S)	this paper Friedl and Zeltner (1994)
Trebouxia gelatinosa Ahmadjian (UTEX 905)	*Parmelia caperata* (L.) Ach.	Z95382 (26S)	this paper
Trebouxia erici Ahmadjian (UTEX 911)	*Cladonia cristatella* Tuck.	Z95379 (26S)	this paper
Trebouxia impressa (UTEX 892)	*Physcia stellaris* (L.) Nyl.	Z95383 (26S) Z21552 (18S)	this paper Friedl and Zeltner (1994)
Trebouxia jamesii (Hildreth and Ahmadjian) Gärtner (UKL-86.132E1)	*Hypogymnia physodes* (L.) Nyl.	Z95384 (26S) Z68700 (18S)	this paper Bhattacharya et al. (1996)
Trebouxia usneae (Hildreth and Ahmadjian) Gärtner (UKL-87.019A1)	*Parmelia tinctorum* Nyl.	Z95385 (26S) Z68702 (18S)	this paper Bhattacharya et al. (1996)
Leptosira terrestris (SAG 463-3)	Soil	Z95378 (26S) Z28973 (18S)	this paper Friedl and Zeltner (1994)

complete 18S rDNA sequences already available for *Trebouxia* spp. (Friedl and Zeltner, 1994; Bhattacharya et al., 1996) have been analyzed to test the congruence of relationships derived from the two rRNA coding regions.

2. Materials and Methods

Cultures of *Trebouxia* spp. and *Leptosira terrestris* (Fritsch and John) Printz (Table 1) were grown as described in Friedl (1989). DNA was extracted from log phase cultures of these algae. rRNA coding regions were amplified using the polymerase chain reaction protocols (PCR, Saiki et al., 1988) and directly sequenced over both strands using the dideoxy sequencing method (Sanger et al., 1977; as described in Friedl and Zeltner, 1994; Friedl, 1996). Oligonucleotide primers for the PCR amplification and sequencing of the 5' portion of 26S rRNA coding regions are listed in Table 2. Since most sequencing primers used for the 26S rDNA have initially been designed for analyses of fungal rDNAs (e.g., Vilgalys and Hesters, 1990; Armaleo and Clerc, 1991; Rehner and Samuels, 1994), some primer sequences were slightly modified for the proper use with green algae (see Table 2).

Table 2. Sequences of oligonucleotide primers used for PCR and 26S rDNA sequencing in this study. The sequences were deduced from conserved regions of the 26S rRNA, were taken from Vilgalys and Hester (1990) (a), Armaleo and Clerc (1991) (b), donated from R. Vilgalys (c) (Vilgalys, unpubl.) or were taken from Vilgalys and Hester (1990) and slightly modified for the proper use with green algae (d). Annealing positions of primers are compared to the 26S rRNA sequence of *Chlorella ellipsoidea* (D17810).

Primer name	5'–3' sequence	Annealing position	Comment
LR0R	ACCCGCTGAACTTAAGC	26–42	5' PCR; b
LR7	TACTACCACCAAGATCT	1438–1422	3' PCR; a
LRF1	GCATATCAATAAGCGGA	41–57	sequencing, forward
LRF2	GAACAAGTACCGTGAGG	345–361	sequencing, forward
LRF3	TCTAACATGTATGCGAG	658–674	sequencing, forward
LRF4	TGCTGACGTGCAAATCG	863–879	sequencing, forward
LR17R	TAACCTATTCTCAAACTT	1025–1042	sequencing, forward; c
LR1850	CCTCACGGTACTTGTTC	361–345	sequencing, reverse
LR3	CCGTGTTTCAAGACGGG	648–632	sequencing, reverse; d
LR5	TCCTGAGGGAAACTTCG	957–941	sequencing, reverse; d
LR6	CGCCAGTTCTGCTTACC	1131–1115	sequencing, reverse; a

The 26S rDNA sequences determined from *Trebouxia* spp. and *L. terrestris* in this study were manually aligned with the rRNA coding region from *Chlorella ellipsoidea* Gerneck (database accession number D17810; Aimi et al. 1994) using the multiple sequence alignment editor SeqEdit (Olsen et al., 1992). Determination of the *L. terrestris* sequence provided an additional outgroup for testing root placement. Choice of outgroup has been shown to be critical for reliable determination of the polarity of character evolution (Adachi and Hasegawa, 1995). *Trebouxia* spp., *L. terrestris* and *Chl. ellipsoidea* are related within the class Trebouxiophyceae (Friedl, 1995). Other 18S rDNA sequences were obtained from the Genbank/EBI data base and manually aligned with approximately 100 rRNA coding regions from trebouxiophytes as well as other green algae, charophytes, and land plants using the SeqEdit program. The secondary structure of the 5' portion of the 26S rRNA from *Trebouxia usneae* (Fig. 1) was constructed according to the rRNA model of the complete 26S rRNA of *Chlorella ellipsoidea* available from the URL http://pundit.colorado.edu:8080/RNA/23S/eucarya.html. This secondary structure model and that of the 18S rRNA from *Trebouxia usneae* [based on the rRNA model of *Gloeotilopsis paucicellulare* (Vischer) Friedl; Friedl, 1996] were used to refine the alignments of 26S and 18S rRNA coding regions, respectively. The alignments used are available upon request from Thomas Friedl; the secondary structure model of the 5' portion of the 26S rRNA from *T. usneae* (Fig. 1) is also available in PICT format.

Three independent types of data analyses were used to assess the evolutionary relationships resolved in the rDNA phylogenies. Only the relationships congruent among all three methods were regarded as significantly resolved. For phylogenetic analyses with the 26S rDNA sequences, *Chlorella ellipsoidea* was used as an outgroup taxon. For analyses with 18S rDNA sequences *Nephroselmis olivacea* Stein and *Pseudoscourfieldia*

Figure 1. Secondary structure model of the 5' portion of the large-subunit (26S) ribosomal RNA from *Trebouxia usneae* (strain UKL-87.019A1) and adjacent portion of the 5.8S rRNA (sequence accession numbers Z68702 and Z95384). The model is based on the 26S rRNA secondary structure model of *Chlorella ellipsoidea* (see text). Black boxes indicate the nucleotide substitutions that are variable among the 26S rRNA coding regions from species of the *Trebouxia* cluster in Fig. 3. Circled nucleotides are identical among members of the *Trebouxia* cluster, but distinguish *T. erici* from other *Trebouxia* spp. Shaded areas mark the binding sites of both PCR primers. The portion of the determined sequences that have been used for phylogenetic analyses is indicated by arrows. The sequence track that could not be sequenced for *T. jamesii* is indicated by grey bars. D1-D6 correspond to the variable domains of 26S rRNAs as determined in Hassouna et al. (1984).

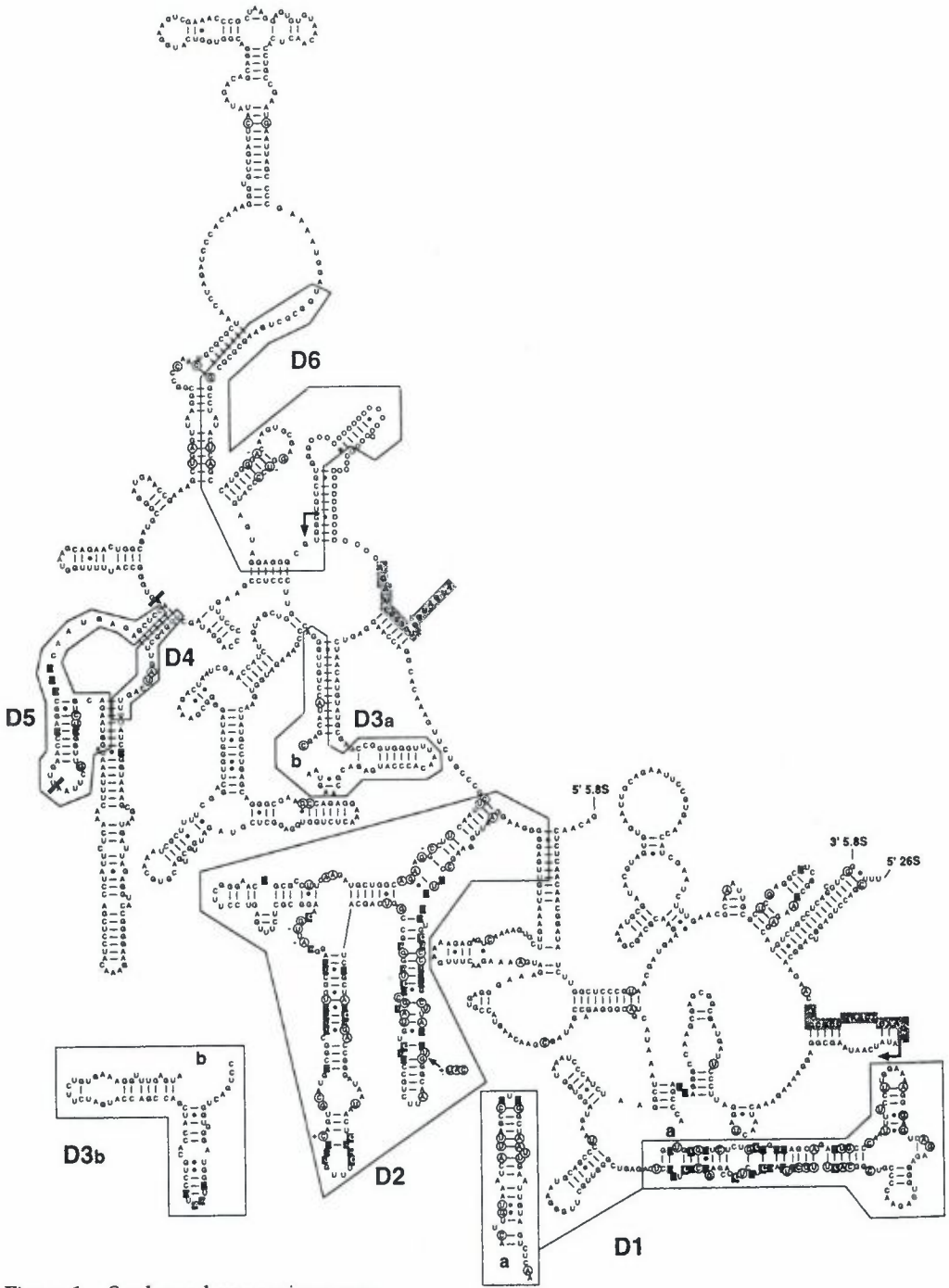

Figure 1. See legend on previous page.

marina (Throndsen) Manton were used to root the phylogenies (Steinkötter et al., 1994). For the distance method analysis, pairwise similarities were calculated using the two parameter correction by Kimura (1980). The neighbor-joining reconstruction (Saitou and Nei, 1987) with jumbled taxon addition (PHYLIP 3.5c; Felsenstein, 1993) was then used to select a phylogenetic tree from the distance matrix. Maximum parsimony analyses were performed using PAUP 3.1.1 (Swofford, 1993). For analyses of the 26S rDNA sequences the nucleotide positions were unweighted and the branch and bound algorithm was used. For analyses of the 18S rDNA sequences the nucleotide positions were weighted (rescaled consisteny index over an interval of 1-1000) using PAUP (Bhattacharya and Medlin, 1995; Friedl, 1996). A random addition of sequences with 10 replicates and a branch swapping algorithm (TBR, or tree bisection-reconnection) was used. The 26S rDNA gene tree topology (Fig. 3) was identical when the sequences were analyzed with the same options in PAUP as the 18S rDNAs. Support for internal branches in the parsimony and neighbor-joining trees were estimated using the bootstrap method (Felsenstein, 1985). For maximum-likelihood analyses, the program fastDNAml (Olsen et al., 1994) was used with the global search option. This involved rearrangements of partial trees crossing the maximum number of branches, i.e. 6 branches with the 26S data set (9 taxa), and 31 branches with the 18S data set (34 taxa), respectively. Rearrangements of the full trees involved the same (maximum) number of branches to cross.

Small-subunit rRNA sequences used for comparisons in this study (except those listed in Table 1) are as follows (with Genbank/EBI accession numbers where available): *Acrosiphonia* sp. (U03757), *Ankistrodesmus stipitatus* (Chodat) Komárková-Legnerová (X56100), *Asteromonas gracilis* Artari (M95614), *Chlamydomonas reinhardtii* Dangeard (M32703), *Chlorella ellipsoidea* SAG 211-1a (X63520), *Chl. saccharophila* var. *saccharophila* SAG 211-9b (Krüger) Migula (X63505), *Chl.* sp. (Hydra symbiont, strain HvT) (X72707), *Chl. vulgaris* Beijerinck SAG 211-11b (X13688), *Choricystis minor* (Skuja) Fott (X89012), *Dunaliella salina* (Dunal) Teodoresco (M84320), *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess (Z47207), *Fusochloris perforata* (Lee and Bold) Floyd, Watanabe et Deason (M62999), *Gloeotilopsis paucicellulare* (Z47997), *Hydrodictyon reticulatum* (L.) Lagerheim (M74497), *Nannochloris* sp. SAG 251-2 (Huss and Hümmer, unpubl.), *Microthamnion kuetzingianum* Nägeli (Z28974), *Myrmecia biatorellae* Boye-Petersen (Z28971), *M. israeliensis* (Chantanachat and Bold) Friedl (M62995), *Nephroselmis olivacea* (X74754), *Pleurastrum insigne* Chodat (Z28972), *Pseudendoclonium basiliense* Vischer (Z47996), *Pseudoscourfieldia marina* (X75565), *Scenedesmus obliquus* (Turp.) Kützing (X56103), *Scherffelia dubia* (Perty) Pascher (X68484), *Tetracystis aeria* Brown and Bold (U41175),

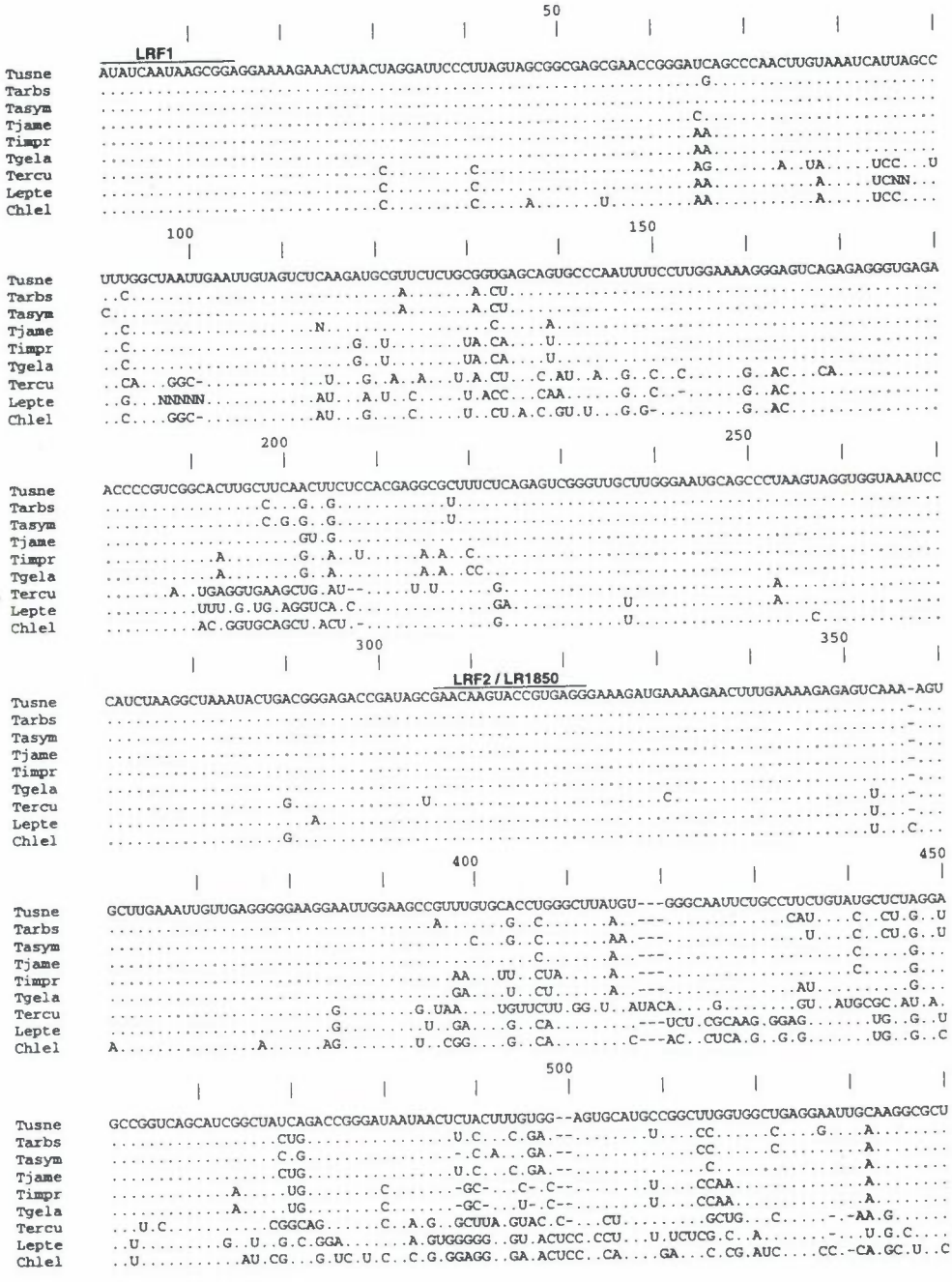

Figure 2. See legend on next page.

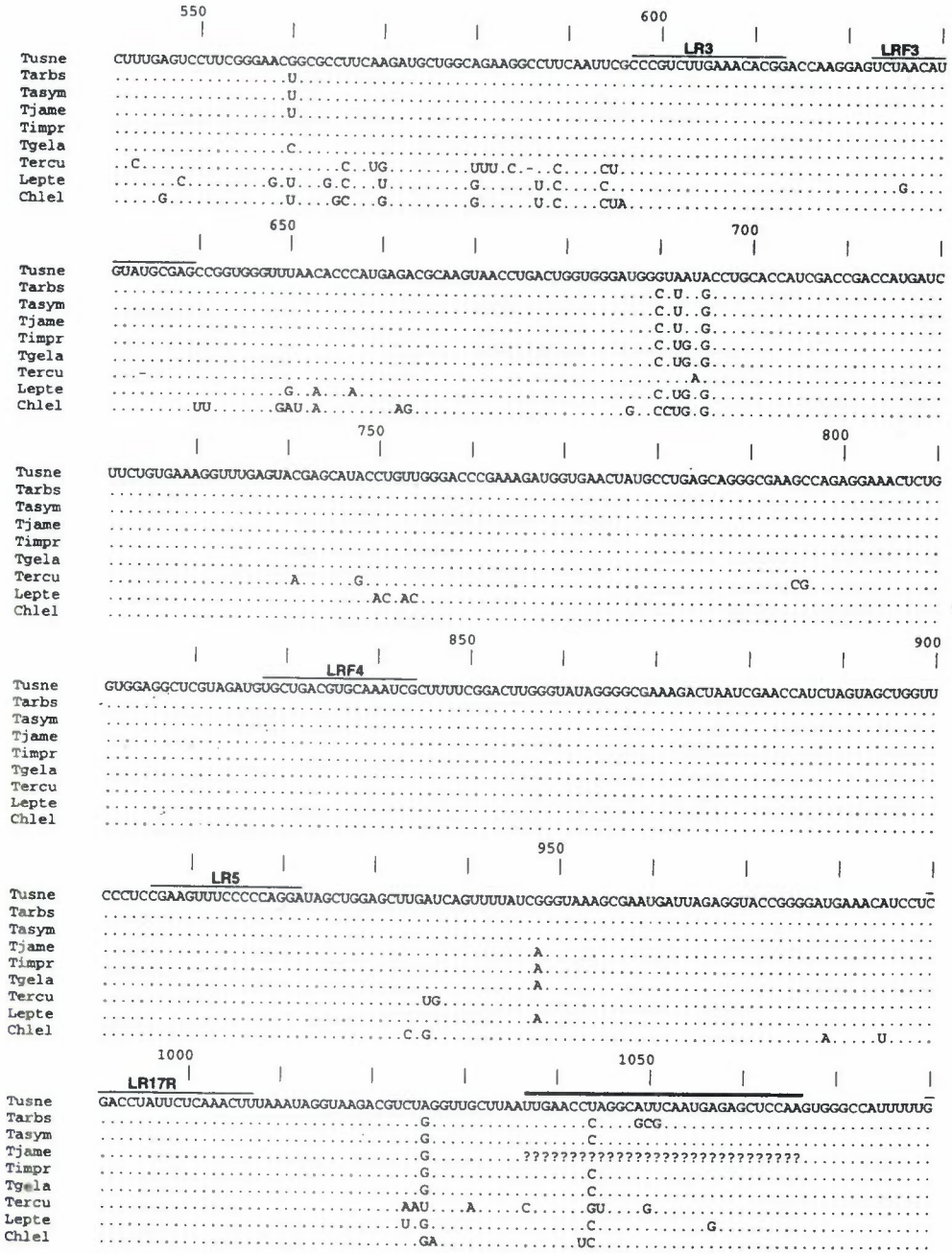

Figure 2. See legend on next page.

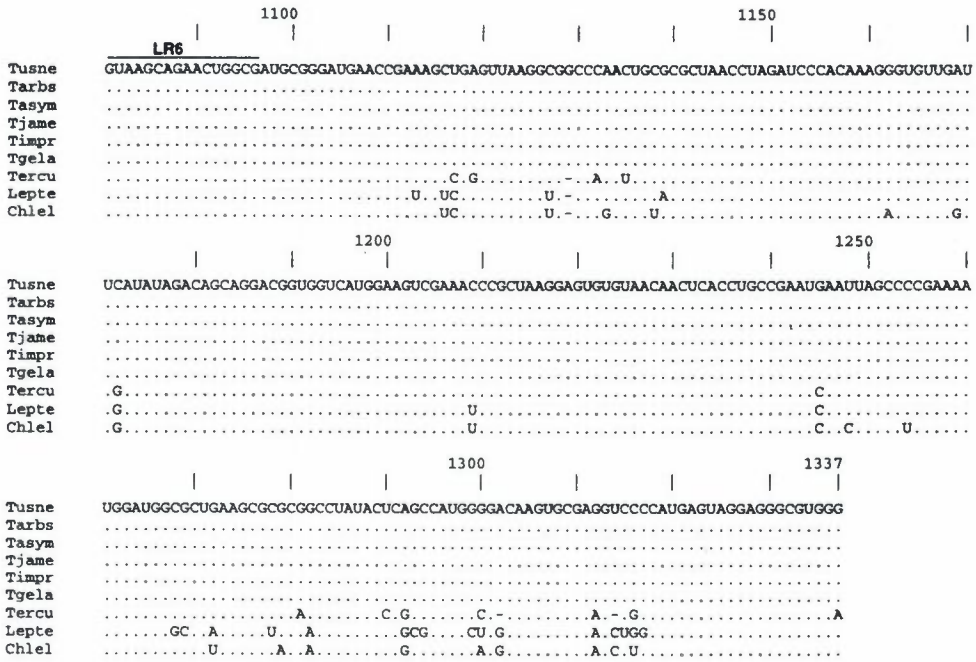

Figure 2. Proposed 26S rDNA alignment for species of *Trebouxia, Leptosira terrestris* and *Chlorella ellipsoidea* that has been used for the phylogenetic analysis in Fig. 3. Species abbreviations are as follows: Tusne = *Trebouxia usneae;* Tarbs = *T. arboricola;* Tasym = *T. asymmetrica;* Tjame = *T. jamesii;* Timpr = *T. impressa;* Tgela = *T. gelatinosa;* Tercu = *T. erici;* Lepte = *Leptosira terrestris;* Chlel = *Chlorella ellipsoidea. T. usneae* is used as the reference sequence. All equivalent positions are indicated by a period, and alignment gaps are shown as a dash. Alignment positions 1 to 1337 correspond to *Chlorella ellipsoidea* 26S rRNA positions 43 and 1373, respectively (D17810; Aimi et al., 1994). Thin lines above the sequences mark portions from which sequencing primers have been deduced (Table 2). The thick line shows the sequence track that could not be sequenced for *T. jamesii* and, therefore, was omitted from the phylogenetic analysis.

Tetraselmis striata Butcher (X70802), *Trebouxia magna* (Z21552), and *Ulothrix zonata* (Weber and Mohr) Kützing (Z47999).

The partial large-subunit ribosomal RNA sequences determined in this study are available from the Genbank/EBI database under the accession numbers listed in Table 1.

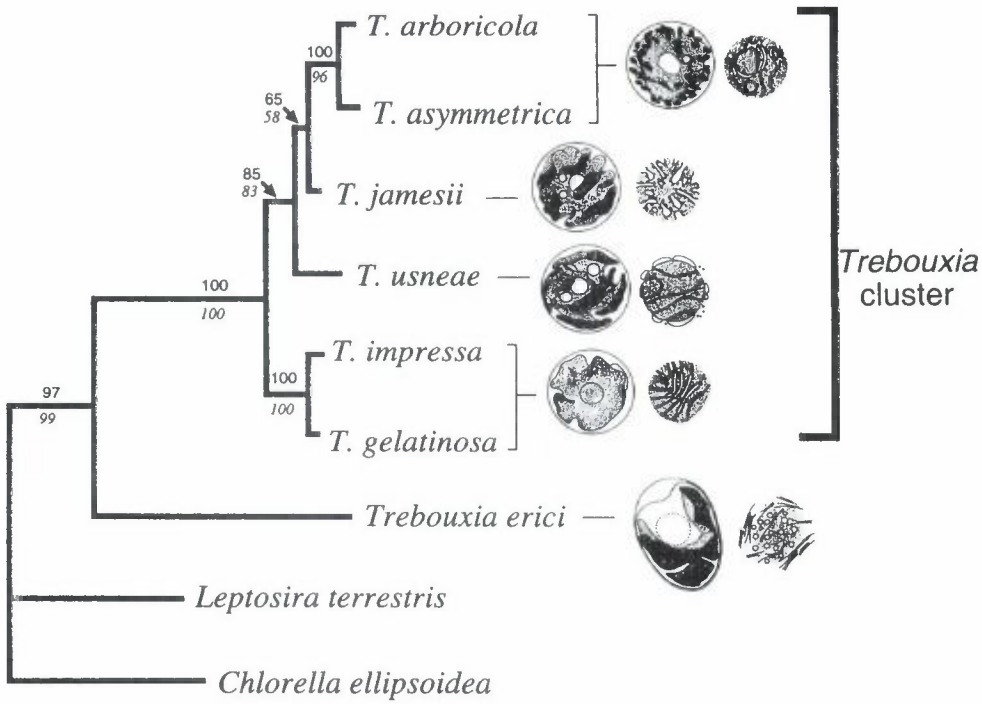

Figure 3. Phylogenetic analysis of partial 26S rDNA sequences from *Trebouxia* spp., *Leptosira terrestris* and *Chlorella ellipsoidea*. The phylogeny shown has been inferred with the maximum-likelihood method (fastDNAml). Bootstrap values were computed independently for 500 resamplings using the neighbor-joining (above lines) and the maximum parsimony methods (below lines), respectively. The unweighted maximum parsimony analysis (PAUP) resulted in a single most parsimonious tree which was 420 steps long and had a consisteny index (CI) of 0.87. Arrows are used to indicate bootstrap values on nodes where these numbers do not fit on the branches. Schematic drawings of chloroplast morphologies (left) and pyrenoid ultrastructures (right) that are characteristic for the *Trebouxia* species investigated are presented besides the species names. These chloroplast characters are very similar within the *T. arboricola*/*T. asymmetrica* and *T. impressa*/*T. gelatinosa* clades, respectively (see text). Therefore, for the *T. arboricola*/*T. asymmetrica* clade, schematic drawings of chloroplast morphology and pyrenoid ultrastructure from *T. arboricola* are shown as an example, whereas for the *T. impressa*/*T. gelatinosa* clade schematic drawings of the *T. impressa* chloroplast morphology and the *T. gelatinosa* pyrenoid ultrastructure are presented.

3. Results and Discussion

The nine 26S rDNA sequences used in this study are homologous for a continuous stretch of about 1.3 kb of the 5' portion of the large-subunit rRNA molecule (Fig. 1). This region corresponds to about 41% of the complete 26S rRNA sequence from *Chlorella ellipsoidea* (Aimi et al., 1994) and encompasses the variable domains D1-D6 (Hassouna et al., 1984). A total of 1307 nucleotide positions were aligned unambiguously (Fig. 2). The final data set contained 291 variable sites of which 136 were parsimony-informative. The tree topology that was inferred from the 26S rDNA sequences (Fig. 3) is very robust, since it was identical for all the three methods used.

The evolutionary relationships inferred from 26S rDNA sequence comparisons are congruent with a grouping of *Trebouxia* spp. based on chloroplast characters, i.e., chloroplast morphology and pyrenoid ultrastructure (Fig. 3). This finding substantiates the importance of these morphological characters for assessing species relationships within *Trebouxia*. The monophyletic origin of those *Trebouxia* spp. that have a centrally located chloroplast with clearly defined pyrenoid matrices ("protein bodies"; Ascaso et al., 1995) is well supported; they form the "*Trebouxia* cluster" (Fig. 3). However, *T. erici* which contains a chloroplast with a position closely appressed to the cell wall at certain stages (Ahmadjian, 1960) and without a distinct pyrenoid (Friedl, 1989) is rather distant in the 26S rDNA phylogeny from the other *Trebouxia* spp. In species within the *Trebouxia* cluster, chloroplast thylakoids invaginate the pyrenoid matrix (Ascaso et al., 1995) forming tubules of different shapes which are arranged in various patterns (Fisher and Lang, 1971; Friedl, 1989). However, in *T. erici* (and also in a few other *Trebouxia* spp., see below), the thylakoids within the pyrenoids are not distinct from those of photosynthetically active parts of the chloroplast (Friedl, 1989).

Species relationships within the *Trebouxia* cluster as inferred from the 26S rDNA sequences are consistent with the observation of various types of chloroplast morphologies (Ettl and Gärtner, 1984; Gärtner, 1985) and pyrenoid ultrastructures (Friedl, 1989) in these species. Resolution within the *Trebouxia* cluster is provided by short internal internodes. However, they are supported by high bootstrap values except for the clustering of *T. jamesii* with *T. arboricola / T. asymmetrica*, and this indicates that the support for these internodes is largely uncontradicted (Bandelt et al., 1995). In the rDNA data, *T. impressa* and *T. gelatinosa* form a monophyletic lineage (99.3% sequence identity) that is clearly separated from the grouping of other *Trebouxia* spp. within the cluster. *T. impressa* and *T. gelatinosa* have rather similar chloroplast morphologies with thick and short lobes giving rise to a "massive"

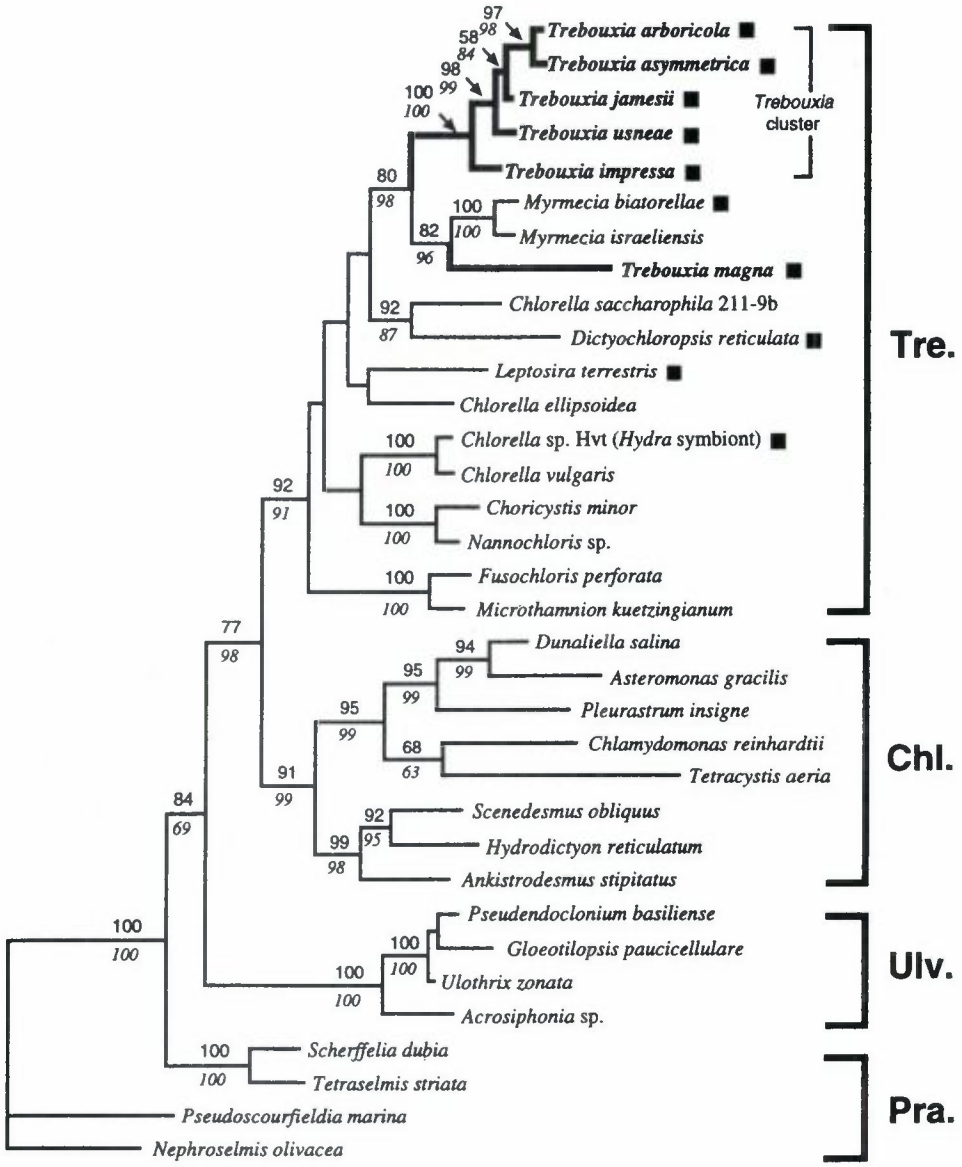

Figure 4. Phylogenetic analysis of complete 18S rDNA sequences from *Trebouxia* spp., members of the classes Trebouxiophyceae (Tre), Chlorophyceae (Chl.), Ulvophyceae (Ulv.), and Prasionophyceae (Pra.). The paraphyly of *Trebouxia* is marked by thick lines. Shaded squares besides species names indicate symbiotic taxa. The phylogeny is based on 1714 unambiguously aligned sequence positions for 34 green algal taxa with 551 variable sites of which 375 were parsimony-informative. The phylogeny shown has been inferred with the

appearence of the plastid in the light microscope (Gärtner, 1985). Both species also share a parallel arrangement of thylakoid channels in the pyrenoid ultrastructure (compare Figs. 4 and 12 in Friedl, 1989). In all other species within the *Trebouxia* cluster, various chloroplast morphologies with thin and long lobes are present with different patterns of pyrenoid thylakoids. Chloroplast morphologies are most similar between *T. arboricola* and *T. asymmetrica* as these have the "crenulate" pattern in common (i.e. thin chloroplast lobes in surface view; Gärtner, 1985; Friedl and Gärtner, 1988). There is also a 26S rDNA sequence identity of 98.6% among these two species. *T. jamesii* and *T. usneae* form independent lineages within the *Trebouxia* cluster. These species exhibit chloroplast morphologies and pyrenoid ultrastructures rather different from each other and from *T. arboricola*/*T. asymmetrica*.

Chloroplast morphology in *Trebouxia* spp. is most clearly expressed in culture, and not within lichen thalli. It also varies during developmental stages of *Trebouxia* from small autospores into fully grown vegetative cells (Ettl and Gärtner, 1984). This feature makes it more difficult to interpret than pyrenoid ultrastructure. In contrast to chloroplast morphology, pyrenoid ultrastructure is a stable character for delineating *Trebouxia* spp. as it does not change in cultures on different media and within lichen thalli (Friedl, 1989). Pyrenoids appear to be functionally important organelles for both lichenized and free-living green algae as they are thought to constitute an important part of a photosynthetic CO_2 concentrating mechanism (CCM; Badger et al., 1993; Palmqvist, 1993; Palmqvist et al., 1994), which allows the cell to accumulate and maintain a higher concentration of inorganic carbon inside the chloroplast in relation to the environment (Badger et al., 1993). However, there are also green algal photobionts lacking a pyrenoid (e.g. *Coccomyxa* and *Myrmecia*) and these have developed a different CO_2 acquisition strategy (Palmqvist et al., 1994; Smith and Griffiths, 1996). The phylogenetic significance of these differences has, however, not yet been thoroughly investigated (Palmqvist, 1997). Interestingly, the presence of ribulose-bisphophatase carboxylase (Rubisco) has been detected in pyrenoids of *Trebouxia* (Ascaso et al., 1995).

Figure 4. Continuation: maximum-likelihood method (fastDNAml). Bootstrap values were computed independently for 500 resamplings using the neighbor-joining (above lines) and the maximum parsimony methods (below lines), respectively. Only bootstrap values above 50% that define nodes shared by the maximum likelihood, neighbor-joining, and weigthed parsimony trees are recorded. The weighted maximum parsimony analysis (PAUP) resulted in a single most parsimonious tree which was 525730 steps long and had a consisteny index (CI) of 0.74. Arrows are used to indicate bootstrap values on nodes where these numbers do not fit on the branches.

In contrast to chloroplast characters, differences in autospore formation ("cell cycles"; Friedl, 1993) appear unreliable as a phylogenetic marker within *Trebouxia* spp. Species that share cell cycle A (*T. asymmetrica, T. arboricola, T. impressa* and *T. jamesii*) and cell cycle B (*T. gelatinosa, T. usneae* and *T. erici*), respectively, do not group together in the 26S rDNA phylogeny, rather they are separated from each other in different lineages (Fig. 3). Therefore, the separation of the genus "*Pseudotrebouxia*" from *Trebouxia* (Archibald, 1975; Hildreth and Ahmadjian, 1981) or the distinction of two subgenera within *Trebouxia* (Tschermak-Woess, 1989) which were mainly based on differences in autospore formation appears not justified.

In 18S rDNA phylogenies, *Trebouxia* is clearly paraphyletic as *T. magna* is more closely related to *Myrmecia* spp. than to other *Trebouxia* spp. (Friedl and Zeltner, 1994; Fig. 4). Since *T. arboricola* (strain SAG 219-1a), the type species of the genus *Trebouxia* (Gärtner, 1985), is within a cluster of closely related species in both 18S and 26S phylogenies (*Trebouxia* cluster; Figs. 3 and 4), a monophyletic genus *Trebouxia* is represented only by these species, and would exclude *T. magna*. The latter species has to be attributed to another genus (see below). The isolated position of *T. erici* in the 26S phylogeny (Fig. 3) may indicate that this species also lies outside of *Trebouxia*. If so, this result would be similar to that found with *T. magna* in the 18S phylogeny. This conclusion is supported from evolutionary distances of 26S rDNA sequences. The objective 26S rRNA distance estimates between *T. erici* and members of the *Trebouxia* cluster (average 0.136) are slightly more than between these and *L. terrestris* (average 0.126). The genus *Leptosira* is phylogenetically distinct from *Trebouxia* within the Trebouxiophyceae (Friedl, 1996). These observations highlight the genetic difference between *T. erici* and species within the *Trebouxia* cluster. *T. erici* and *T. magna* lack a distinct pyrenoid matrix (Friedl, 1989) and both taxa share a chloroplast that assumes a parietal position at certain stages (Ahmadjian, 1960; Gärtner, 1985). These features are also characteristic for a few other *Trebouxia* spp., e.g. *T. irregularis, T. excentrica* (Friedl and Gärtner, 1988; Friedl, 1989), and *Trebouxia phycobiontica* (Tschermak-Woess) Tschermak-Woess (= *Asterochloris phycobiontica*; Tschermak-Woess, 1980; 1989, Ettl and Gärtner, 1995). Therefore, the position of these taxa within *Trebouxia* appears doubtful and needs to be further investigated. We speculate that additional rDNA sequence comparisons will show the position of these taxa in a single genus that is independent of the *Trebouxia* cluster shown in Figs. 3 and 4.

In the 18S rDNA phylogeny the topology for species within the *Trebouxia* cluster (Fig. 4) is identical with that inferred from the 26S data. The three different phylogeny reconstruction methods were congruent for the 18S rDNA data, except for the relationships among lineages of the Trebouxiophyceae (see

discussion in Friedl, 1995). With the exception of *T. gelatinosa*, the 18S sequences have determined for the same *Trebouxia* spp. as for the 26S rDNA analyses. The congruence in both topologies for the *Trebouxia* cluster (Figs. 3 and 4) suggests the presence of a strong phylogenetic signal in the rRNA operon of *Trebouxia* spp., although the species relationships are resolved by only short internal internodes in both data sets. Another congruent result is that *Leptosira terrestris* is rather distant from *Trebouxia* spp. in both phylogenies which is in contrast to previous assumptions based on vegetative cell morphology (for discussion see Friedl and Zeltner, 1994). In the 26S rDNA phylogeny, *L. terrestris* is grouped together with *Chlorella ellipsoidea* which was chosen as an outgroup taxon. Also, *L. terrestris* and *Chl. ellipsoidea* are separated from *Trebouxia* spp. in the 18S phylogeny (Fig. 4). However, the *Chl. ellipsoidea*/*L. terrestris* clade and its position relative to other lineages of the Trebouxiophyceae is not supported in bootstrap analyses. The available data are insufficient to reject the hypothesis that *L. terrestris* is a sister species to the *Trebouxia arboricola-Dictyochloropsis reticulata* clade.

Phylogenetic analyses of the 18S data set (Fig. 4) reveals *Trebouxia* spp. and other lichen symbionts, *Dictyochloropsis reticulata*, *Leptosira terrestris*, and *Myrmecia biatorellae* to be related within a monophyletic evolutionary lineage, the Trebouxiophyceae (Friedl, 1995, 1996). This class also includes many *Chlorella* spp. and other coccoid green algae that lack motile stages. Most members of the Trebouxiophyceae known so far are found in terrestrial habitats or in lichen symbiosis. Symbiotic coccoid green algae are dispersed on several independent lineages within that class (Fig. 4). The Trebouxiophyceae shares a sister group relationship with the Chlorophyceae; both lineages are clearly separated from the Ulvophyceae (Friedl, 1996). The radiation of these three lineages represents the major diversification of green algae which forms a sister group with the charophyte/land plant lineage; it is preceded by the divergence of a heterogenous assemblage of scaly flagellates, the Prasinophyceae (Steinkötter et al., 1994; Fig. 4).

The partial 26S rDNA sequences among members of the *Trebouxia* cluster (Fig. 3) used in this study contain about 1.5 times more variable positions and more than double as many parsimony-informative sites than the corresponding data set of complete 18S rRNA coding regions (Table 3). The evolutionary distances among these species are about two to three times greater within the 26S data set than among the corresponding 18S rDNAs (Table 4). The 26S rDNA distance values between the *Trebouxia* cluster and *Leptosira terrestris*, and those between *Trebouxia* cluster/*Chlorella ellipsoidea* are even greater than between the corresponding 18S distance values from *Trebouxia* spp. and the prasinophyte *Nephroselmis olivacea* which has been used as an outgroup taxon to root the 18S rDNA phylogeny (Table 4). These findings are consistent

with the higher resolving power of 26S rDNA analyses for elucidating close species relationships among green algae.

Table 3. Comparisons of variable and parsimony-informative sequence positions found among members of the *Trebouxia* cluster (see Fig. 3) in coding regions of their 18S and 26S rRNAs. *Partial sequences encompass only regions V4 and V9 of the 18S rRNA secondary structure model (see text); **partial sequences encompass only variable domains D1 and D2 of the 26S rRNA secondary structure model (see Fig. 1).

	18S rDNA	26S rDNA
Variable/parsimony-informative sites	52/18	73/42
% variable/parsimony-informative sites in the total number of considered sites	3.0/1.1	5.6/2.4
Variable/parsimony-informative sites in partial sequences	32/14*	66/39**
% of the total number of variable/parsimony-informative sites in partial sequences	61.6/77.8*	90.4/92.9**

Table 4. Comparisons of evolutionary distances among members of the *Trebouxia* cluster (Fig. 3, except for *T. gelatinosa* where no 18S rDNA sequence is available) with *Leptosira terrestris*, and their corresponding outgroup taxa calculated from their 18S/26S rDNA sequences (after Kimura 2-parameter correction in DNADIST, PHYLIP 3.5.c; see text). Abbreviations are: Tas = *Trebouxia asymmetrica*; Tja = *T. jamesii*; Tus = *T. usneae*; Tim = *T. impressa*; Lep = *Leptosira terrestris*; Pra = Prasinophyceae (*Nephroselmis olivacea*); Chl = *Chlorella ellipsoidea*.

Tas	Tja	Tus	Tim	Lep	Pra/Chl
T. arboricola					
0.004/0.011	0.010/0.018	0.015/0.032	0.020/0.035	0.060/0.129	0.111/0.145
T. asymmetrica					
	0.010/0.019	0.014/0.028	0.017/0.037	0.058/0.130	0.109/0.144
T. jamesii					
		0.008/0.021	0.014/0.028	0.055/0.122	0.109/0.142
T. usneae					
			0.016/0.037	0.053/0.131	0.107/0.147
T. impressa					
				0.051/0.124	0.105/0.148

Among the 26S rDNA sequences from members of the *Trebouxia* cluster almost all variable sites are located in two neighbouring regions of the secondary structure model; these regions correspond to the highly variable domains D1 and D2 (Hassouna et al., 1984; Michot and Bachellerie, 1987; Scholin et al., 1994a; Fig. 1) and encompass about 90% of the variable and parsimony-informative sites (Table 3). Similarly, the majority of variable/informative sites among the 18S rDNA sequences from *Trebouxia* spp. (except *T. magna*) is found in two regions of the molecule, described as V4 (stems E23-1 and E23-2) and V9 (end of stem 49) in the 18S rRNA secondary structure model of Van de Peer et al. (1994). In the 26S rDNA sequences, there are 112 additional variable sites that distinguish *Trebouxia erici* from the other *Trebouxia* spp., most of these sites are also clustered in domains D1 and D2 (Fig. 1). Outside of these domains (e.g. in domains D3–D6 of Hassouna et al., 1984), there is very little sequence variation, even among the other trebouxiophycean green algae, *Leptosira terrestris* and *Chlorella ellipsoidea* (Fig. 2). Therefore, short partial sequences that encompass D1 and D2 should be sufficient for tracing species relationships (Pawlowski et al., 1994b; Scholin et al., 1994a). The analysis of a reduced data set where only these two domains were retained from the 26S sequences of the *Trebouxia* cluster (380 nucleotides) resulted in an identical topology as inferred from the sequences of lengths 1.3 kb (not shown). Sequences of domains D1 and D2 of the large subunit rRNA have been used as species-specific rRNA targets to discriminate between toxic and non-toxic species of the dinoflagellate genus *Alexandrinum* (Scholin et al., 1994a) and the diatom genus *Pseudo-nitzschia* (Scholin et al., 1994a,b, 1996; Miller and Scholin, 1996). Fluorescently labeled oligonucleotides, complementary to species-specific rRNA target locations, have been successfully applied as probes to unambiguously identify unialgal isolates (Miller and Scholin, 1996; Scholin et al., 1996). Based on the alignment presented in Fig. 2 we believe that it is possible to identify species-specific "signature sequences" also for *Trebouxia*. It is encouraging to develop and to test specific oligonucleotide probes that allow the unambiguous and relatively simple identification of species of *Trebouxia* within lichen thalli without the time-consuming efforts of culturing.

The scarcity of variable sites in regions outside of the two highly variable domains makes it doubtful whether 26S rDNA sequences are useful for tracing more distant phylogenetic relationships among the green algae, e.g. among genera that are related at the order or class levels. Domains D1 and D2 may be too variable for taxonomic studies above the species level because of multiple substitutions at sites. The conserved areas in the 5' part of the 26S rRNA have been found to be very important for investigating distant relationships among different lineages of the eukaryote kingdoms (e.g. Lenaers et al., 1988; Perasso

et al., 1989), but mostly only partial sequences (about 500 nucleotides) are yet available (Pawlowski et al., 1994a).

Other regions of the rRNA operon that are very useful for resolving close relationships at or below the species level in green algae are the internal transcribed spacer (ITS) regions between the 18S and 26S rRNA genes (e.g. Kooistra et al., 1992; van Oppen et al., 1993; Friedl, 1996). For *Trebouxia*, a small number of ITS sequences is already available (Bhattacharya et al., 1996) and these data are very promising for elucidating close evolutionary relationships among the species. However, ITS regions may sometimes be too variable and/or show considerable length variation which renders impossible a reliable alignment of these sequences (e.g. Bakker et al., 1995). In such cases, sequence analyses of the two highly variable regions from the 26S rDNA may be particulary advantageous as they are almost constant in length (at least among closely related species) and their alignment is facilitated by conserved rRNA secondary structure models.

Acknowledgements

This research was supported by grants from the Deutsche Forschungsgemeinschaft to T.F. (DFG Fr 905/1-2, 1-4, 1-5, and 1-6). The authors express their sincere thanks to P. Lockhart, D. Bhattacharya, and K. Palmqvist for valuable comments on the manuscript; A. Täuber and S. Wallisch for their excellent technical assistance; U. Jensen and U.G. Maier for encouragement and provision of laboratory facilities.

REFERENCES

Adachi, J. and Hasegawa, M. 1995. Phylogeny of whales: dependence of the inference on species sampling. *Molecular Biology and Evolution* **12**: 177–179.

Ahmadjian, V. 1960. Some new and interesting species of *Trebouxia*, a genus of lichenized algae. *American Journal of Botany* **47**: 677–683.

Aimi, T., Yamada, T., Yamashita, M., and Murooka, Y. 1994. Characterization of the nuclear large-subunit rRNA-encoding gene and the group-I self-splicing intron from *Chlorella ellipsoidea* C-87. *Gene* **145**: 139–144.

Archibald, P.A. 1975. *Trebouxia* de Puymaly (Chlorophyceae, Chlorococcales) and *Pseudotrebouxia* gen. nov. (Chlorophyceae, Chlorosarcinales). *Phycologia* **14**: 125–137.

Armaleo, D. and Clerc, P. 1991. Lichen chimeras: DNA analysis suggests that one fungus forms two morphotypes. *Experimental Mycology* **15**: 1–10

Ascaso, C., Valladares, F., and de los Rios, A. 1995. New ultrastructural aspects of the lichen photobiont *Trebouxia* (Microthamniales, Chlorophyta). *Journal of Phycology* **31**: 114–119.

Badger, M.R., Pfanz, H., Büdel B., Heber, U., and Lange, O.L. 1993. Evidence for the functioning of photosynthetic CO_2-concentrating mechanisms in lichens containing green algal and cyanobacterial photobionts. *Planta* **191**: 57–70.

Bandelt, H.J., Forster, P., Sykes, B.C., and Richards, M.B. 1995. Mitochondrial portraits of human populations using median networks. *Genetics* **141**: 743–753.

Bakker, F.T., Olsen, J.L., and Stam, W.T. 1995. Evolution of nuclear rDNA ITS sequences in the *Cladophora albida/sericea* clade (Chlorophyta). *Journal of Molecular Evolution* **40**: 640–651.

Bhattacharya, D. and Medlin, L. 1995. The phylogeny of plastids: a review based on comparisons of small-subunit ribosomal RNA coding regions. *Journal of Phycology* **31**: 489–498.

Bhattacharya, D., Friedl, T., and Damberger, S. 1996. Nuclear-encoded rDNA group I introns: origin and phylogenetic relationships of insertion site lineages in the green algae. *Molecular Biology and Evolution* **13**: 978–989.

Buchheim, M.A. and Chapman, R.L. 1991. Phylogeny of the colonial green flagellates: a study of 18S and 26S rRNA sequence data. *BioSystems* **25:** 85–100.

Ettl, H. and Gärtner, G. 1984. Über die Bedeutung der Cytologie für die Algentaxonomie, dargestellt an *Trebouxia* (Chlorellales, Chlorophyceae). *Plant Systematics and Evolution* **148**: 135–147.

Ettl, H. and Gärtner, G. 1995. *Syllabus der Boden-, Luft- und Flechtenalgen*. Gustav Fischer, Stuttgart, Jena, New York.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 66–70.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inferences Package), Version 3.5c. Distributed by the author, Department of Genetics, University of Washington, Seattle.

Fisher, K.A. and Lang, N.J. 1971. Comparative ultrastructure of cultured species of *Trebouxia*. *Journal of Phycology* **7**: 155–165.

Friedl, T. 1989. Comparative ultrastructure of pyrenoids in *Trebouxia* (Microthamniales, Chlorophyta). *Plant Systematics and Evolution* **164**: 145–159.

Friedl, T. 1993. New aspects of the reproduction by autospores in the lichen alga *Trebouxia* (Microthamniales, Chlorophyta). *Archiv für Protistenkunde* **143**: 153–161.

Friedl, T. 1995. Inferring taxonomic positions and testing genus level assignments in coccoid green lichen algae: a phylogenetic analysis of 18S ribosomal RNA sequences from *Dictyochloropsis reticulata* and from members of the genus *Myrmecia* (Chlorophyta, Trebouxiophyceae cl. nov.). *Journal of Phycology* **31**: 632–639.

Friedl, T. 1996. Evolution of the polyphyletic genus *Pleurastrum* (Chlorophyta): inferences from nuclear-encoded ribosomal DNA sequences and motile cell ultrastructure. *Phycologia* **35**: 456–469.

Friedl, T. and Büdel, B. 1996. Photobionts. In: *Lichen Biology*. T. Nash, ed. Cambridge University Press, Cambridge, pp. 8–23.

Friedl, T. and Gärtner, G. 1988. *Trebouxia* (Pleurastrales, Chlorophyta) as a phycobiont in the lichen genus *Diploschistes*. *Archiv für Protistenkunde* **135**: 147–158.

Friedl, T. and Zeltner, C. 1994. Assessing the relationships of some coccoid green lichen algae and the Microthamniales (Chlorophyta) with 18S ribosomal RNA gene sequence comparisons. *Journal of Phycology* **30**: 500–506.

Gärtner, G. 1985. Die Gattung *Trebouxia* PUYMALY (Chlorellales, Chlorophyceae). *Archiv Hydrobiologie,* Supplement **74**(4) [*Algological Studies* **41**]: 495–548.

Hassouna, N., Michot, B., and Bachellerie, J.P. 1984. The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increases of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Research* **12**: 3563–3583.

Hildreth, K.C. and Ahmadjian, V. 1981. A study of *Trebouxia* and *Pseudotrebouxia* isolates from different lichens. *Lichenologist* **13**: 65–86.

Honegger, R. 1991. Functional aspects of the lichen symbiosis. *Annual Reviews in Plant Physiology* **42**: 553–578.

Huss, V.A.R. and Sogin, M.L. 1990. Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. *Journal of Molecular Evolution* **31**: 432–442.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of sequence evolution. *Journal of Molecular Evolution* **16**: 111–120.

Kolosha, V.O. and Fodor, I. 1990. Nucleotide sequence of *Citrus limon* 26S rRNA gene and secondary structure model of its RNA. *Plant Molecular Biology* **14**: 147–161.

Kooistra, W.H.C.F., Stam, W.T., Olsen, J.L., and van den Hoek, C. 1992. Biogeography of *Cladophoropsis membranacea* based on comparisons of nuclear rDNA ITS sequences. *Journal of Phycology* **28**: 660–668.

Larson, A., Kirk, M.M., and Kirk, D.L. Molecular phylogeny of the volvocine flagellates. *Molecular Biology and Evolution* **9**: 85–105.

Lenaers, G., Maroteaux, L., Michot, B., and Herzog, M. 1989. Dinoflagellates in evolution. A molecular phylogenetic analysis of large-subunit ribosomal RNA. *Journal of Molecular Evolution* **29**: 40–51.

Lenaers, G., Nielsen, H., Engberg, J., and Herzog, M. 1988. The secondary structure of large subunit rRNA divergent domains, a marker for protist evolution. *BioSystems* **21**: 215–222.

Lenaers, G., Scholin, C.A., Bhaud, Y., Saint-Hilaire, D., and Herzog, M. 1991. A molecular phylogeny of dinoflagellate protists (Pyrrhophyta) inferred from the sequence of 24S rRNA divergent domains D1 and D8. *Journal of Molecular Evolution* **32**: 53–63.

Lewis, L.A., Wilcox, L.W., Fuerst, P.A., and Floyd, G.L. 1992. Concordance of molecular and ultrastructural data in the study of zoosporic chlorococcalean green algae. *Journal of Phycology* **28**: 375–380.

Melkonian, M. and Peveling, E. 1988. Zoospore ultrastructure in species of *Trebouxia* and *Pseudotrebouxia* (Chlorophyta). *Plant Systematics and Evolution* **158**: 183–210.

Michot, B. and Bachellerie, J.P. 1987. Comparisons of large subunit rRNAs reveal some eukaryote specific elements of secondary structure. *Biochemie* **69**: 11–23.

Miller, P.E. and Scholin, C.A. 1996. Identification of cultured *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes. *Journal of Phycology* **32**: 646–655.

Nakayama, T., Watanabe, S., Mitsui, K., Uchida, H., and Inouye, I. 1996. The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18SrDNA sequence data. *Phycological Research* **44**: 47–55.

Olsen, G.J., Matsuda, H., Hagstrom, R., and Overbeek, R. 1994. fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer Applications in the Biosciences (CABIOS)* **10**: 41–48.

Olsen, G.J., Overbeek, R., Larsen, N., Marsh, T.L., McCaughey, M.J., Maciukenas, M.A., Kuan, W-M., Macke, T.J., Xing, Y., and Woese, C.R. 1992. The ribosomal database project. *Nucleic Acids Research* **20**: 2199–2200.

Palmqvist, K. 1993. Photosynthetic CO_2 use efficiency in lichens and their isolated photobionts: the possible role of a CO_2 concentrating mechanism. *Planta* **191**: 48–56.

Palmqvist, K., de los Rios, A., Ascaso, C., and Samuelsson, G. 1997. Photosynthetic carbon acquisition in the lichen photobionts *Coccomyxa* and *Trebouxia* (Chlorophyta). *Physiologia Plantarum* (in press).

Palmqvist, K., Samuelsson, G., and Badger, M.R. 1994. Photobiont-related differences in carbon acquisition among green-algal lichens. *Planta* **195**: 70–79.

Pawlowski, J., Bolivar, I., Guiard-Maffia, J., and Gouy, M. 1994a. Phylogenetic position of foraminifera inferred from LSU rRNA gene sequences. *Molecular Biology and Evolution* **11**: 929–938.

Pawlowski, J., Bolivar, I., Fahrni, J., and Zaninetti, L. 1994b. Taxonomic identification of foraminifera using ribosomal DNA sequences. *Micropaleontology* **40**: 373–377.

Perasso, R., Baroin, A., Qu, L.H., Bachellerie, J.P., and Adoutte, A. 1989. Origin of the algae. *Nature* **339**: 142–144.

Rambold, G. and Triebel, D. 1992. The interlecanoralean associations. *Bibliotheca Lichenologica* **48**: 1–201.

Rehner, S.A. and Samuels, G.J. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycological Research* **98**: 625–634.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., and Horn, G. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487–491.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

Sanger, F., Nicklen, F., and Coulsen, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**: 5463–5467.

Schlösser, U.G. 1994. SAG – Sammlung von Algenkulturen at the University of Göttingen. Catalogue of strains 1994. *Botanica Acta* **107**: 111–186.

Scholin, C.A., Herzog, M., Sogin, M., and Anderson, D.M. 1994a. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrinum* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *Journal of Phycology* **30**: 999–1011.

Scholin, C.A., Villac, M.C., Buck, K.R., Krupp, J.M., Powers, D.A., Fryxell, G.A., and Chavez, F.P. 1994b. Ribosomal DNA sequence discriminate among toxic and non-toxic *Pseudo-nitzschia* species. *Natural Toxins* **2**: 152–165.

Scholin, C.A., Buck, K.R., Britschgi, T., Cangelosi, G., and Chavez, F.P. 1996. Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. *Phycologia* **35**: 190–197.

Smith, E.C. and Griffiths, H. 1996. The occurrence of the chloroplast pyrenoid is correlated with the activity of a CO_2-concentrating mechanism and carbon isotope discrimination in lichens and bryophytes. *Planta* **198**: 6–16.

Starr, R.C. and Zeikus, J.A. 1993. UTEX – the culture collection of algae at the University of Texas at Austin. *Journal of Phycology* **29** (supplement): 1–106.

Steinkötter, J., Bhattacharya, D., Semmelroth, I., Bibeau, C., and Melkonian, M. 1994. Prasinophytes form independent lineages within the Chlorophyta: evidence from ribosomal RNA sequence comparisons. *Journal of Phycology* **30**: 340–345.

Swofford, D.L. 1993. PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign.

Tschermak-Woess E. 1980. *Asterochloris phycobiontica*, gen. et spec. nov., der Phycobiont der Flechte *Varicellaria carneonivea*. *Plant Systematics and Evolution* **135**: 279–294.

Tschermak-Woess, E. 1989. Developmental studies in trebouxioid algae and taxonomical consequences. *Plant Systematics and Evolution* **164**: 161–195.

Van de Peer, Y., Van den Broeck, I., De Rijk, P., and De Wachter, R. 1994. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research* **22**: 3488–3494.

van Oppen, M.J.H, Olsen, J.L., Stam, W.T., van den Hoek, C., and Wiencke, C. 1993. Arctic-antarctic disjunctions in the benthic seaweeds *Acrosiphonia arcta* (Chlorophyta) and *Desmarestia viridis/willii* (Phaeophyta) are of recent origin. *Marine Biology* **115**: 381–386.

Vilgalys, R. and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.