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

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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

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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 Budel, 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 lifestyle 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 (Hassouna 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.

<table>
<thead>
<tr>
<th>Organism (strain)</th>
<th>Source</th>
<th>Sequence accession nos.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tre bouxia arboricola</em> De Puymaly (SAG 219-1a)</td>
<td>Unknown</td>
<td>Z95381 (26S)</td>
<td>this paper</td>
</tr>
<tr>
<td><em>Tre bouxia gelatinosa</em> Ahmadjian (UTEX 905)</td>
<td><em>Parmelia caperata</em> (L.) Ach.</td>
<td>Z95380 (26S)</td>
<td>this paper</td>
</tr>
<tr>
<td><em>Tre bouxia impressa</em> (UTEX 892)</td>
<td><em>Physcia stellaris</em> (L.) Nyl.</td>
<td>Z95379 (26S)</td>
<td>this paper</td>
</tr>
<tr>
<td><em>Tre bouxia jamesii</em> (Hildreth and Ahmadjian) Gärtner (UKL-86.132E1)</td>
<td><em>Hypogymnia physodes</em> (L.) Nyl.</td>
<td>Z95383 (26S)</td>
<td>this paper</td>
</tr>
<tr>
<td>Leptosira terrestris (SAG 463-3)</td>
<td>Soil</td>
<td>Z95385 (26S)</td>
<td>Bhattacharya et al. (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z68700 (18S)</td>
<td>this paper</td>
</tr>
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<td></td>
<td></td>
<td>Z95378 (26S)</td>
<td>Friedl and Zeltner (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z28973 (18S)</td>
<td>this paper</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Primer name</th>
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<th>Annealing position</th>
<th>Comment</th>
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<tr>
<td>LR0R</td>
<td>ACCCGCTGAACCTAAGC</td>
<td>26–42</td>
<td>5' PCR; b</td>
</tr>
<tr>
<td>LR7</td>
<td>TACTACCAACAAAGATCT</td>
<td>1438–1422</td>
<td>3' PCR; a</td>
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<tr>
<td>LRF1</td>
<td>GCATATCATAAAGCGGA</td>
<td>41–57</td>
<td>sequencing, forward</td>
</tr>
<tr>
<td>LRF2</td>
<td>GAACAGTGACCGCGAGG</td>
<td>345–361</td>
<td>sequencing, forward</td>
</tr>
<tr>
<td>LRF3</td>
<td>TCTACATGTATGCGAGG</td>
<td>658–674</td>
<td>sequencing, forward</td>
</tr>
<tr>
<td>LRF4</td>
<td>TGGCTAAGCGAAATCG</td>
<td>863–879</td>
<td>sequencing, forward</td>
</tr>
<tr>
<td>LR17R</td>
<td>TAACCTATTCCTCAAACTT</td>
<td>1025–1042</td>
<td>sequencing, forward; c</td>
</tr>
<tr>
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<td>CCTCAGCTGACTTGTCC</td>
<td>361–345</td>
<td>sequencing, reverse</td>
</tr>
<tr>
<td>LR3</td>
<td>CCGTGTTTTCAAGACGGG</td>
<td>648–632</td>
<td>sequencing, reverse; d</td>
</tr>
<tr>
<td>LR5</td>
<td>TCCGAGGGAAACTTCG</td>
<td>957–941</td>
<td>sequencing, reverse; d</td>
</tr>
<tr>
<td>LR6</td>
<td>CGCCAGTTCTGCTTACC</td>
<td>1131–1115</td>
<td>sequencing, reverse; a</td>
</tr>
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</table>
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 consistency 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 fastDNAmli (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 ellipsioida 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 paucicellularis (Z47997), Hydrodictyon reticulatum (L.) Lagerheim (M74497), Nannochloris sp. SAG 251-2 (Huss and Hümmer, unpubl.), Microthamnion kuetzingianum Nägeli (Z28974), Myrmechia biatorelliae Boye-Petersen (Z28971), M. israeliensis (Chantanachat and Bold) Friedl (M62995), Nephroselmis olivacea (X74754), Pleurastrum insignis Chodat (Z28972), Pseudendoclonium basiliense Vischer (Z47996), Pseudoscurfieldia marina (X75565), Scenedesmus obliquus (Turp.) Küting (X56103), Scherffelia dubia (Perty) Pascher (X68484), Tetracystis aerea 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 *Chiarella 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 = *Chiarella 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 *Chiarella 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 consistency 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 Prasinoaphyceae (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
appearance of the plastid in the light microscope (Gartner, 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; Gartner, 1985; Friedl and Gartner, 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 Gartner, 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 CO2 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 CO2 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-bisphosphate carboxylase (Rubisco) has been detected in pyrenoids of Trebouxia (Ascaso et al., 1995).

Figure 4. Continuation: maximum-likelihood method (fastDNAm). 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 weighted 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 consistency 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 "Pseu dotrebouxia" 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 Myrmezia 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
discussed 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).

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

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.

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

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SPECIES RELATIONSHIPS IN TREBOUXIA


