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The Distribution of Group I Introns in Lichen Algae Suggests That Lichenization Facilitates Intron Lateral Transfer

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The nuclear-encoded small subunit ribosomal DNA gene of many lichen-forming green algae in the genus *Trebouxia* contains a group I intron at *Escherichia coli* genic position 1512. We studied the evolutionary history of the 1512 intron in *Trebouxia* spp. (Trebouxiophyceae) by analyzing intron and "host" cell phylogenies. The host trees were constructed by comparing internal transcribed spacer regions of rDNA. Maximum-likelihood, maximum-parsimony, and distance analyses suggest that the 1512 intron was present in the common ancestor of the green algal classes Trebouxiophyceae, Chlorophyceae, and Ulvophyceae. The 1512 intron, however, was laterally transferred at least three times among later-diverging *Trebouxia* spp. that form lichen partnerships. Intron secondary structure analyses are consistent with this result. Our results support the hypothesis that lichenization may facilitate 1512 group I intron lateral transfer through the close cell-to-cell contact that occurs between the lichen algal and fungal symbionts in the developing lichen thallus. © 2000 Academic Press

INTRODUCTION

Group I introns are short sequences (200–500 bp) of a conserved primary and secondary structure that often have the ability to catalyze their own excision from preRNAs (i.e., are "self-splicing," Zaug *et al.,* 1983; Cech, 1988). The sporadic and wide distribution of group I introns in the nuclear rDNA and organellar genes of green algae, fungi, ciliates, red algae, and different amoebas suggests that these sequences are highly successful at invading and maintaining themselves in eukaryotic genomes (Gargas *et al.,* 1995; Turmel *et al.,* 1995; Nishida *et al.,* 1998; Bhattacharya 1998; Cho *et al.,* 1998; Schroeder-Diedrich *et al.,* 1998; Watanabe *et al.,* 1998).

The green algae (Chlorophyta) are particularly rich in nuclear-encoded rDNA introns and are an excellent model for understanding the origin and phylogeny of these sequences (for review, see Bhattacharya, 1998). Most green algal nuclear rDNA group I introns are members of subgroup IC1 on the basis of their conserved primary and secondary structure (Damberger and Gutell, 1994), and introns at specific rDNA genic sites (e.g., 943, 1056, 1506, and 1512; numbering based on the *Escherichia coli* gene) form distinct phylogenetic lineages (Van Oppen *et al.,* 1993; Wilcox *et al.,* 1994; Bhattacharya *et al.,* 1994, 1996). Analyses of intron primary and secondary structure are consistent with the view that frequent lateral transfers between the intron lineages during the evolution of the green algae may not have occurred. Support for this hypothesis comes from the detailed phylogenetic analysis of group I introns and rDNA genes from taxa that have presumptively maintained an intron(s) since the origin of the lineage (e.g., 942, 1046, and 1506 introns; Bhattacharya *et al.,* 1994, 1996). In such cases, the intron and rDNA phylogenies are generally congruent. On the basis of these findings, we have adopted the following working hypothesis regarding group I intron evolution in green algal nuclear genomes: the sporadic distribution of group I introns in the Chlorophyta is best explained by ancient intron origins, at least prior to the near simultaneous radiation of the three "advanced" green algal classes, the Trebouxiophyceae, Chlorophyceae, and Ulvophyceae (hereafter referred to as the TCU). Both rDNA (Friedl, 1997) and actin coding region (T. Friedl, A. Nickles, D. Bhattacharya, unpublished data) phylogenies clearly support a monophyletic origin of each of the TCU classes. Thereafter, lateral transfers appear to have been rare events in the Chlorophyta, whereas intron loss has been common. This model of intron evolution is restricted to the nuclear rDNA genes because mobile introns have been described in both the chloroplast and the mitochondrion of green algae and land plants (e.g., Lonergan and

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Gray, 1994; Turmel *et al.,* 1995; Adams *et al.,* 1998; Cho *et al.,* 1998; Watanabe *et al.,* 1998).

Here, we have studied the evolutionary history of the 1512 group I intron in members of the genus *Trebouxia,* the most frequent green algal partner (photobiont) in lichen symbioses. The lichen symbiosis is a useful tool for investigating the "movement" of homologous nuclear-encoded group I introns across species boundaries and for understanding the mechanisms of intron transfer. This is important because the mechanism of lateral transfer is one of the most important, yet least understood, aspects of intron evolution (see Woodson, 1996). Our phylogenetic analyses of the group I intron and the "host" algal cells show that after a prolonged period of stability within the free-living Trebouxiophyceae and other green algae, the 1512 intron has become mobile within lichenized *Trebouxia* spp. Results of splicing analyses suggest that 1512 intron mobility occurs at the RNA level through "reverse-splicing" (Woodson and Cech, 1989).

MATERIALS AND METHODS

Algal Cultures, DNA Extraction, and PCR Analyses

Axenic photobiont cultures were obtained from different culture collections (see Table 1) and were maintained as described in Friedl (1989). Total nucleic acids were isolated from exponentially growing algal cultures (Friedl, 1995), and nuclear-encoded ITS regions, including 1512 introns (if present) at the 3′-terminus of the SSU rDNA gene, were amplified with the PCR method (Saiki *et al.,* 1988). The PCR primers were NS7M and LR1850 (Bhattacharya *et al.,* 1996) and the reaction conditions were as in Friedl and Zeltner (1994). PCR products were directly sequenced using the dideoxy sequencing method (Sanger *et al.,* 1977) with the oligonucleotide primers described in Beck *et al.* (1998). Fluorescent-labeled primers were used in sequencing reactions with Licor L4000 and ALFexpress automated sequencers.

Construction of Group I Intron and "Host" Cell Phylogenies

The group I intron and ITS rDNA sequences determined in this study were compared with available photobiont and green algal group I intron sequences (see Table 1). The accession numbers (where available) of the group I introns used in this study (apart from those listed in Table 1) are as follows: Trebouxiophyceae—*Choricystis minor* (X89012); Chlorophyceae—*Dunaliella salina* (M24320), *Characium saccatum* (M84319); Ulvophyceae—*Gloeotilopsis paucicellulare* (Z47997), *G. sarcinoideum* (Z47998), *Urospora penicilliformis* (Van Oppen *et al.,* 1993). The 1512 intron sequences were manually aligned with the aid of secondary-structure models (see Fig. 4; Cech *et al.,* 1994; Bhattacharya *et al.,* 1996), as were the ITS rDNAs (Mai and Coleman, 1997), using the multiple sequence alignment editor SeqEdit (RDP; Maidak *et al.,* 1997) and the program SeqApp (Gilbert, 1992). Ambiguous positions within the alignment were excluded prior to the phylogenetic analyses. The intron and ITS rDNA alignments are available from T.F.

Two different 1512 intron data sets were prepared to test the stability of clusters within the intron phylogenies. The first data set (331 sequence positions of which 214/163 were variable/parsimony-informative sites) contained *Trebouxia* spp., *Choricystis minor,* and chlorophycean and ulvophycean sequences (with the

TABLE 1

Strains/Species of *Trebouxia* Used in This Paper and the GenBank Accession Nos. of the ITS and 1512 Group I Intron Sequences

Trebouxia species	Strain	Accession No.	Source
T. arboricola 11C3	92.011C3[2]	Z68703	Bhattacharya *et al.* (1996)
T. arboricola 11A1[1]	92.011A1[2]	AJ249481	This study
T. arboricola[1] 11C5	92.011C5[2]	Z68705	Bhattacharya *et al.* (1996)
T. arboricola 45[1]	86.045E1[2]	AJ249482	This study
T. arboricola 60[1]	87.060E1[2]	AJ249564	This study
T. arboricola SAG[1]	SAG 219-1a	Z68705	Bhattacharya *et al.* (1996)
T. asymmetrica	SAG48.88	AJ249565	This study
T. corticola[1]	UTEX 909	AJ249566	This study
T. galapagensis[1]	UTEX 2230	AJ249567	This study
T. gelatinosa 72	87.072B1[2]	AJ249575	This study
T. gelatinosa 108	86.108B2[2]	Z68697	Bhattacharya *et al.* (1996)
T. gelatinosa 15[1]	87.015A1[2]	AJ249568	This study
T. gelatinosa UTEX[1]	UTEX 905	AJ249569	This study
T. gigantea	UTEX 2231	AJ249577	This study
T. higgensiae[1]	UTEX 2232	AJ249574	This study
T. impressa 26A	87.026A6[2]	AJ249576	This study
T. impressa M26	M-96.026D4[3]	AZ007386H	Beck *et al.* (1998)
T. impressa M27	M-96.027D1[3]	AZ007383H	Beck *et al.* (1998)
T. impressa 17[1]	87.017E1[2]	AJ249570	This study
T. jamesii 156	86.156C3[2]	Z68701	Bhattacharya *et al.* (1996)
T. jamesii 01[1]	86.001E1[2]	Z68699	Bhattacharya *et al.* (1996)
T. jamesii 132[1]	86.132E1[2]	Z68700	Bhattacharya *et al.* (1996)
T. jamesii 73[1]	85.073A1[2]	AJ249571	This study
T. spec.[1]	98.003B2[2]	AJ249572	This study
T. usneae 17[1]	87.019A1[2]	Z68702	Bhattacharya *et al.* (1996)
T. usneae UTEX[1]	UTEX 2235	AJ249573	This study

Note. SAG, culture collection of algae at the University of Göttingen, Germany (Schlösser, 1994); UTEX, culture collection at the University of Austin, Texas (Starr and Zeikus, 1993).

[1] 1512 Group I intron present in the SSU rDNA coding region.

[2] Private culture collection of T.F.

[3] Private culture collection of A. Beck and G. Rambold (Beck *et al.,* 1998).

ulvophytes as the outgroup taxa). The second data set (371 sequence positions, 178/127 variable/parsimony-informative sites) contained only the photobiont sequences (i.e., *Trebouxia* spp.). To infer putative lateral transfers of the 1512 group I introns, an ITS data set was constructed that contained the same photobiont taxa as in the second intron data set (except for *T.* spec., for which only a partial ITS sequence is available). This ITS data set contained 397 sequence positions with 194/137 variable/parsimony-informative sites. To understand the frequency of 1512 intron gain and loss among the different evolutionary lineages of *Trebouxia* spp., another larger ITS data set was prepared that included *Trebouxia* spp. without the 1512 intron. Analysis of these sequences allowed us to test whether the branching orders resolved in phylogenies inferred from the smaller ITS data set were stable with the addition of a broader sample of taxa. The latter data set (397 sequence positions) contained 198/162 variable/parsimony-informative sites.

Phylogenetic Analyses

All data sets were subjected to maximum-parsimony, distance, and maximum-likelihood analyses. Maximum-parsimony and distance analyses were done using PAUP* V4.0b1a (Swofford, 1999) and maximum-likelihood analyses were implemented with the program fastDNAml (Olsen *et al.,* 1994). In the maximum-parsimony analyses, the sites were weighted (rescaled consistency [RC] index over an interval of 1–1000; Bhattacharya, 1996) and then used as input for a bootstrap analysis (2000 replications). Starting trees were built stepwise with 10 random additions of taxa, using the tree bisection–reconnection branch-swapping algorithm to find the best tree. Best-scoring trees were held at each step. Distance analyses were done in two ways. In the first approach, the neighbor-joining method (Saitou and Nei, 1987) was used to build a tree from a distance matrix calculated using the Kimura two-parameter model (Kimura, 1980). In the second approach, the minimum evolution criterion (Rzhetsky and Nei, 1992) was used in conjunction with the HKY85 distance correction (Hasegawa *et al.,* 1985). For the minimum evolution method, a heuristic search procedure was used (as above) and site-to-site rate heterogeneity was modeled as a gamma distribution (Yang, 1994) with discrete rate categories approximating the gamma distribution having the shape parameter $\alpha = 0.5$. Bootstrap analyses with 2000 replications were done with both distance methods. For the maximum-likelihood analyses, the transition/transversion ratio was estimated from the minimum evolution tree as implemented in PAUP*. Maximum-likelihood searches were done with the global search option and rearrangements of the full tree until no better likelihood score was found for the topologies. We also used the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) to investigate the interrelationships of the group I introns in the inferred phylogenies. For these tests, alternative topologies were constructed with the program TreeView (V1.5.3; Page, 1996) from phylogenies inferred with the maximum-likelihood method. The constrained phylogenies were compared to the "best" maximum-likelihood tree using functions implemented in PAUP*. Finally, conserved and variable sites in the 1512 group I introns of *Trebouxia* spp. were identified with MacClade (Maddison and Maddison, 1992) for the secondary-structure analyses.

Splicing Assays

DNA fragments containing the different 1512 group I introns were isolated using the PCR method and the primers Treb-5′:$^{5'}$GGGGGATCCTAATACGACTCACTA TAGGGCGATCCCTGCCCTTTGTACACACCGCCCGT-CGCTCCTA$^{3'}$ and Treb-3′:$^{5'}$GGG GAATTCCGGGAGC-CAAGATATCCGTTGTTGAGAGTTG$^{3'}$ and the reaction conditions described above. The Treb-5′ primer encodes the promoter sequence for T7 RNA polymerase. The PCR primers recognize conserved sequences that are 123 bp upstream (in the SSU rDNA gene) and 323 bp downstream (in the 5.8S rDNA gene) from the position of the 1512 intron in the *Trebouxia* rDNA repeat. This ensures that the 1512 intron can be successfully amplified from all *Trebouxia* species and that the intron-containing transcripts contain the flanking exon sequences that are required for the P1 and P10 secondary-structure interactions required for intron splicing (Burke, 1988; Cech *et al.,* 1994). The PCR products were purified with a Qiaquick column (Qiagen) and made blunt ended with Klenow polymerase (Promega) prior to T7 RNA transcription in the presence of an RNase inhibitor (RNasin; Stratagene). We tested introns from the following taxa for self-splicing activity: *T. arboricola* SAG219.1a/92.011A1/86.045E1/87.060E1, *T. gelatinosa* UTEX905/87.014A3/87.015A1, *T. impressa* 87.017E1, and *T. jamesii* 85.073A1.

If splicing did not go to completion in the transcription buffer (40 mM Tris–HCl [pH 8.0], 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 30 mM DTT, 400 μM rNTPs), then the $MgCl_2$ concentration was increased to 158 mM and the RNAs incubated at 42°C for an additional 30 min. These conditions favor *in vitro* intron splicing (see Oliveira and Ragan, 1994 for details). The separation and detection of RNA splicing products was done as described in Besendahl and Bhattacharya (1999).

RESULTS AND DISCUSSION

Phylogeny of the ITS Region and the 1512 Group I Intron in Trebouxia and in Other Green Algae

Our phylogenetic analyses are consistent with a stable inheritance (or loss) of the 1512 group I intron throughout the evolutionary history of the TCU classes

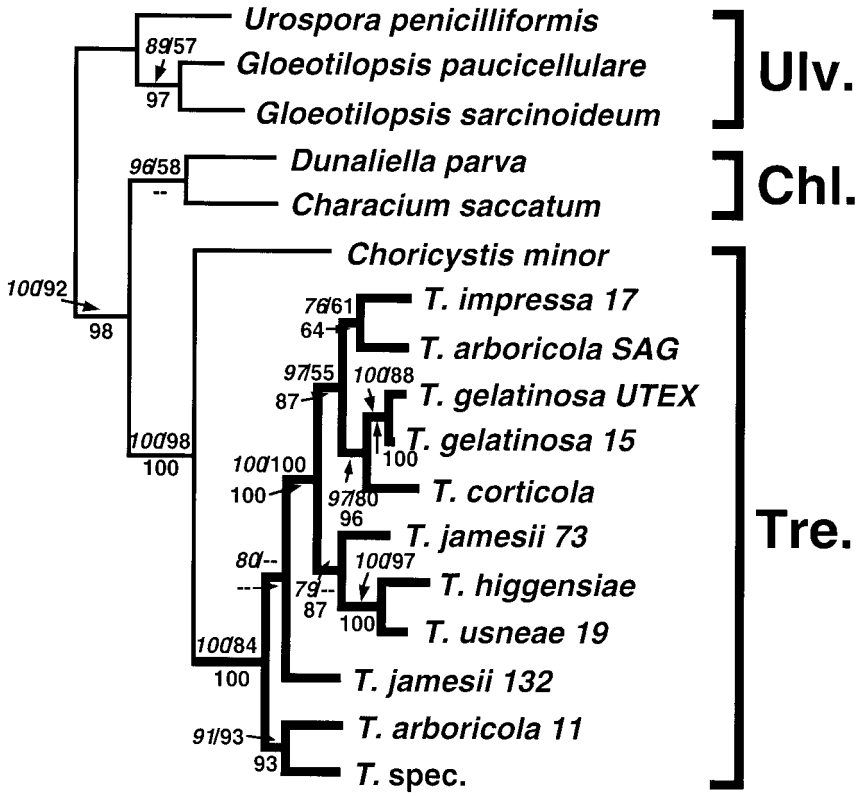


FIG. 1. Phylogeny of 1512 group I introns from *Trebouxia* spp., *Choricystis minor* (Trebouxiophyceae, Tre.), and members of the Chlorophyceae (Chl.) and the Ulvophyceae (Ulv.) based on the analyses of 331 aligned sequence positions (163 parsimony-informative sites). This tree is rooted on the branch leading to the ulvophyte group I intron sequences and was inferred with the maximum-likelihood method. Numbers shown at the branches result from bootstrap analysis (2000 replicates). Values that are left of slash-mark were inferred with a weighted maximum-parsimony analysis, those right of the slash-mark with the minimum evolution method, and those below the branches with a neighbor-joining analysis. Bootstrap values were recorded only for those lineages that are supported by >50% of all replicates. The arrows indicate bootstrap values that did not fit on the branches. Branches leading to members of the genus *Trebouxia* are shown as thick lines.

(Fig. 1). The maximum-likelihood, maximum-parsimony, and distance bootstrap analyses show that the 1512 intron in the TCU forms three distinct lineages, in agreement with rDNA trees (Melkonian and Surek, 1995; Friedl, 1997; Bhattacharya and Medlin, 1998). If there was a lateral transfer of the 1512 intron, then this must have occurred at or near the time of origin of the three lineages because the tree shown in Fig. 1 (though based on limited data) is not consistent with recent lateral transfers across the TCU classes. Comparison of the ITS and 1512 group I intron phylogenies of *Trebouxia* spp. provides, however, evidence against a stable ancestry of the intron within this genus (Fig. 2B, see below). Though vertically inherited in the common ancestor of *Trebouxia* spp., the 1512 intron has, thereafter, undergone multiple lateral transfers. This was suggested in Bhattacharya *et al.* (1996) regarding the 1512 intron in *T. arboricola* (strain SAG 219-1a) and is now firmly established with the addition of a larger number of *Trebouxia* sequences in the phylogenies.

ITS trees. Regarding host cell evolution, the ITS trees (Figs. 2A and 3) show that members of the genus *Trebouxia* form four distinct lineages: that defined by *T. arboricola* + *T. asymmetrica* + *T. gigantea* (see Fig. 3), that defined by *T. jamesii,* that defined by *T. impressa* + *T. gelatinosa,* and that defined by *T. corticola* + *T. usneae* (strains 19 and UTEX) + *T. galapagensis*/*higgensiae.* The monophyletic origin of each these morphologically distinct lineages is supported by high bootstrap values. The ITS sequences of *T. corticola*/*T. usneae* UTEX and *T. galapagensis*/*higgensiae,* respectively, are identical and these taxa may be regarded as synonymous species. *T. usneae* is a paraphyletic taxon in the ITS rDNA phylogenies. This and the other relationships are consistent with similarities seen in chloroplast morphology and pyrenoid ultrastructure and are, therefore, likely to reflect the evolutionary relationships of the species/strains (T. Friedl and H. Seeberger, unpublished). In cases in which the same taxa have been studied, the relationships seen in the ITS phylogenies (e.g., the close relationship of *T. gelatinosa* with *T. impressa*) are also found in comparisons of highly variable regions of the large subunit rDNA (Friedl and Rokitta, 1997). The relationship of *T. jamesii*

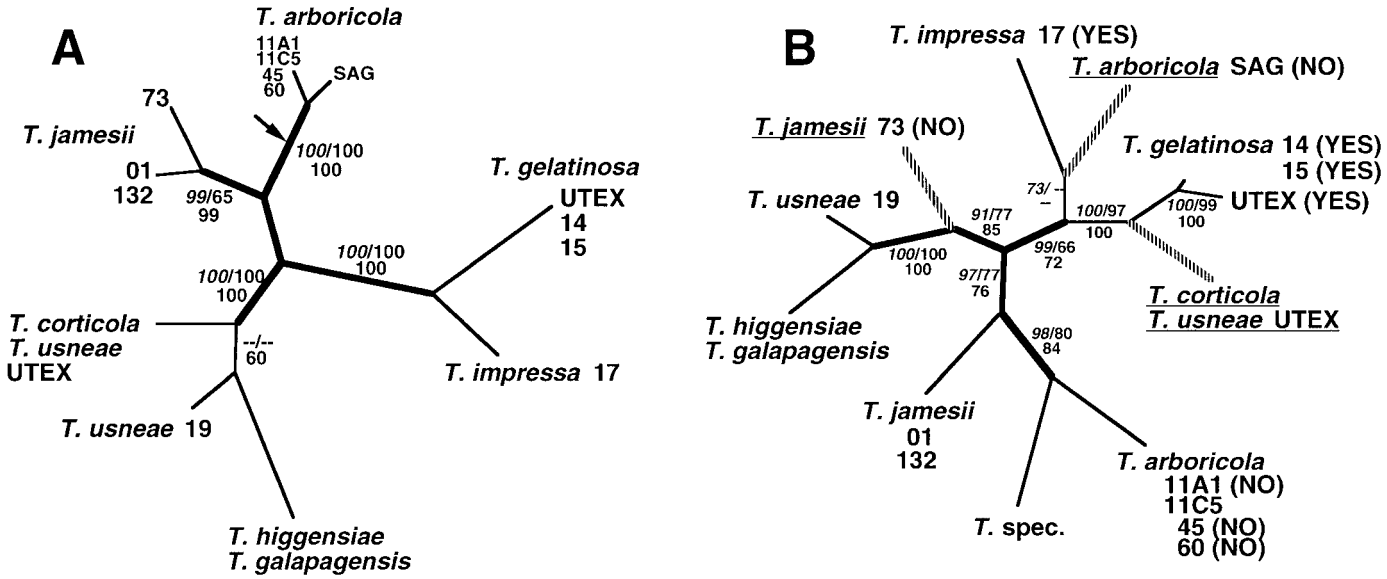


FIG. 2. Unrooted phylogenies of ITS-1,2 rDNA (A) and 1512 group I intron (B) sequences from *Trebouxia* spp. The ITS analysis included 397 aligned sequence positions (137 parsimony-informative sites), whereas the intron analysis included 371 sequence positions (127 parsimony-informative sites). Both trees were inferred with the maximum-likelihood method. The arrow in (A) indicates the position of *T.* spec. strain 98.003B2, as inferred from independent analyses using a partial ITS rDNA sequence. Numbers shown at the branches result from bootstrap analysis (2000 replicates). Values that are left of slash-mark were inferred with a weighted maximum-parsimony analysis, those right of the slash-mark with the minimum evolution method, and those below these numbers with a neighbor-joining analysis. Bootstrap values were recorded only for those lineages that are supported by >50% of all replicates. Where identical ITS sequences for several taxa or strains were determined, only one sequence was used for the analyses; the others were simply added to the figure. The branches shown in hatch marks in (B) with underlined taxon names are candidates for 1512 intron lateral transfers. Introns from the studied taxa that were tested for self-splicing capacity are indicated with a YES if they self-splice *in vitro* or with a NO if they do not.

with other *Trebouxia* spp. is, however, not unequivocally resolved because this taxon is in different positions in phylogenies inferred from the two ITS data sets (Figs. 2A and 3). This is reflected in the lack of bootstrap support (i.e., <50%) for the internal nodes that connect *T. jamesii* with other *Trebouxia* spp in Figs. 2A and 3. The different strains of *T. jamesii,* however, always group together with high bootstrap support in both ITS phylogenies. All taxa in these analyses are lichenized, except for *T. corticola,* which may possibly be free living (Ettl and Gärtner, 1995).

Intron trees. The four lineages of *Trebouxia* spp. described above are also resolved in the intron tree (Fig. 2B) with three clear exceptions. The presence of *T. arboricola* SAG and *T. corticola / T. usneae* UTEX within the lineage formed by *T. gelatinosa* and *T. impressa* (Fig. 2B) is not consistent with the ITS tree. In addition, *T. jamesii* has multiple origins within the intron phylogeny, with strain 73 intron being closely related to the lineage formed by the *T. usneae* 19 and *T. higgensia / T. galapagensis* introns, whereas the other *T. jamesii* strains (01 and 132) form a sister group to *T. arboricola* (except strain SAG) and *T.* spec. in the intron phylogeny (Fig. 2B). The polyphyletic origin of *T. jamesii* and *T. arboricola,* as well as the position of *T. corticola / T. usneae* UTEX, is well supported by bootstrap analyses in the intron phylogenies (Figs. 1 and 2B).

To test these results, the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) was used to assess alternate intron trees. An intron phylogeny was prepared in which the different strains/species of *T. jamesii* and *T. arboricola* were forced to share a monophyletic origin and *T. corticola / T. usneae* UTEX was positioned as a sister group of *T. usneae* 19 and *T. higgensia / T. galapagensis* (as in the ITS phylogenies, Fig. 2A). This alternate tree was found to be "significantly worse" than the best likelihood tree shown in Fig. 2B ($P < 0.05$). Trees in which only one of the above-mentioned "misplaced" intron sequences was forced into the relationship supported by the ITS tree also resulted in log likelihood scores that were "significantly worse" ($P < 0.05$) than the best intron tree. These results suggest that the 1512 intron has been laterally transferred on three separate occasions in the studied *Trebouxia* spp. (transfers denoted by underlined taxon names and stippled terminal branches [Fig. 2B] or indicated by gray circles [Fig. 3]). We postulate that a 1512 intron related to that found in *T. impressa* 17 was laterally transferred into the SSU rDNA gene of *T. arboricola* SAG, whereas a strain/species of *T. gelatinosa* likely gave rise to the intron in *T. corticola / usneae* UTEX. Finally, the *T. jamesii* 73 intron appears to share a close evolutionary relationship with 1512 introns found in *T. usneae* 19 and *T. higgensia / T. galapa-*

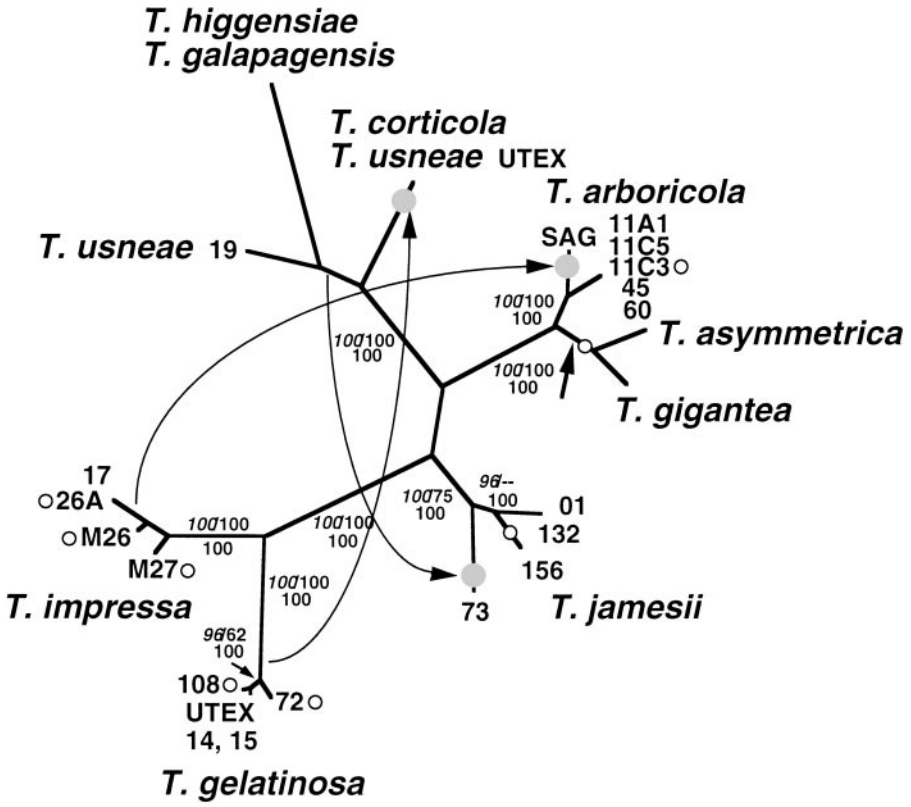


FIG. 3. Unrooted phylogeny of ITS-1,2 rDNA sequences from *Trebouxia* spp. This tree was inferred with the maximum-likelihood method using 397 aligned sequence positions (162 parsimony-informative sites). Numbers shown at the branches result from bootstrap analysis (2000 replicates). Values that are left of slash-mark were inferred with a weighted maximum-parsimony analysis, those right of the slash-mark with the minimum evolution method, and those below these numbers with a neighbor-joining analysis. Bootstrap values were recorded only for those lineages that are supported by >50% of all replicates. Where identical ITS sequences for several taxa or strains were determined, only one sequence was used for the analyses; the others were simply added to the figure. Putative lateral transfers (and the directions of these transfers) between lineages of *Trebouxia* spp. are shown with arrows. Lineages involved in the intron transfers are marked with a grey circle. Putative intron losses are shown with open circles beside the strain designations. A thick arrow indicates the position of *T.* spec. strain 98.003B2, as inferred from an independent analysis using a partial ITS rDNA sequence.

gensis. Lateral transfers of the 1512 intron have not yet been found in the free-living Trebouxiophyceae (i.e., *Choricystis minor*) or in other members of the TCU, though strains/species within genera containing the 1512 group I intron (e.g., *Gloeotilopsis*) have not yet been extensively sampled.

Intron loss. We mapped the presence/absence of 1512 group I introns in *Trebouxia* spp. in the phylogeny shown in Fig. 3. Given the existence of the 1512 intron in the common ancestor of *Trebouxia* species, this intron has been lost from taxa (marked with open circles beside the strain designations in Fig. 3) at least eight times during the evolutionary history of this group. In addition, the 1512 intron must also have been lost independently three times from the strains that presently contain laterally transfered introns; i.e., intron loss must precede intron gain. Detailed analyses of the distribution of the 1512 intron in different lichen algal strains provide additional evidence of the dynamic nature of intron gain and loss. For example, about 28% of 85 tested strains (as part of another study) contained the 1512 group I intron (Friedl, unpublished data). In this study, *Trebouxia* strains that encoded identical or nearly identical ITS rDNAs, but were associated with taxonomically different fungi, often differed by the presence/absence of 1512 group I introns (e.g., strains of *T. arboricola, T. gelatinosa,* and *T. impressa;* Fig. 3). In the case of *T. arboricola,* two strains (11A1 and 11C5) differed from a third strain (11C3) by the presence of an intron in the former taxa (Fig. 3). All three strains were taken, however, from the same lichen sample of *Pleurosticta acetabulum.* Two strains of *T. impressa,* 17 and M26, although isolated from two different lichen genera, were identical over their ITS rDNA regions, but only one of these strains, 17, contained a 1512 intron. The 1512 intron may also be transported within its host cell to taxonomically distantly related lichens. An isolate of *T. impressa* that was identical over its 1512 group I intron and ITS rDNA sequences with *T. impressa* strain 17 (associated with *Parmelina carporrhizans*) has been found in a distantly related lichen, *Physcia stellaris* (P. Pfeiffer,

unpublished data). A single algal strain may, therefore, frequently come in contact with different lichen fungi. This occurs when an algal partner from a symbiotic lichen propagule serves as a photobiont source for different lichen fungi (Beck *et al.,* 1998). These observations show that, given mobile introns, the lichen symbiosis may facilitate the spread of 1512 introns among a large number of algal strains that coexist in the same or different fungal thalli.

Intron Secondary-Structure Analysis

We tested the finding of 1512 lateral transfers in the Trebouxiophyceae by studying intron secondary structure. To do this, we mapped base substitutions on the secondary structures of different 1512 group I introns (see Fig. 4). In general, this analysis showed that the *Trebouxia* 1512 group I introns have secondary structures that are typical of subgroup IC1 introns (see Cech *et al.,* 1994). The core of the 1512 intron (e.g., P4, P7) is highly conserved in both primary sequence and secondary structure among all *Trebouxia* species, and the peripheral loops are the regions of both nucleotide substitutions and insertions/deletions (Fig. 4). We also found good evidence for the existence of an IGS interaction at the 5′ terminal region of the 1512 introns.

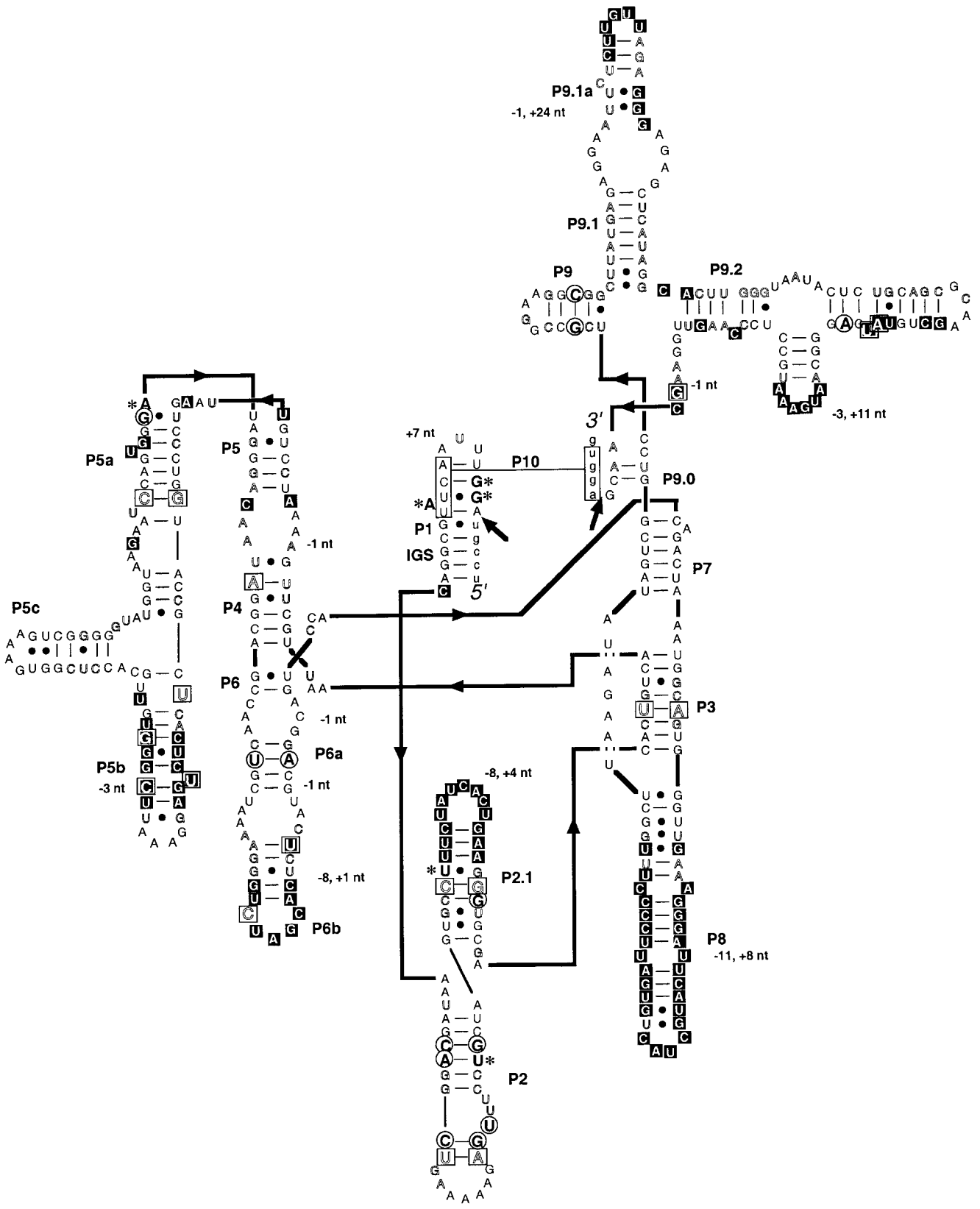
Regarding lateral transfers, the secondary-structure comparisons lead to two major conclusions. First, the high sequence conservation over much of the 1512 group I intron secondary structure in *Trebouxia* spp. supports convincingly the monophyletic origin of these introns. We do not believe that the 1512 introns in species of *Trebouxia,* including those that have been laterally transferred, originate from outside this genus. This hypothesis is supported by more exhaustive analyses that include group I introns in different rDNA sites from a variety of green algae and fungi (e.g., Bhattacharya *et al.,* 1996). Second, inspection of the intron secondary structures shows that there are a number of nucleotides (shown in the open boxes in Fig. 4) that are shared exclusively by the laterally transferred 1512 intron of *T. corticola*/*T. usneae* UTEX with other introns of *T. arboricola* SAG, *T. gelatinosa,* and *T. impressa* (see Fig. 2B). This is consistent with a close evolutionary relationship between these introns and an origin of the *T. corticola*/*usneae* UTEX intron from a sequence that is closely related to the introns in *T. gelatinosa.* In addition, positions of identity shared between the introns of *T. corticola*/*usneae* UTEX, *T. arboricola* SAG, *T. gelatinosa,* and *T. impressa* are found both within conserved core regions (e.g., P3, P4) and within less-conserved peripheral helices (e.g., P2, P6b). Together, these observations support the hypothesis that the phylogeny shown in Fig. 2B reflects accurately the evolutionary history of the 1512 introns.

Intron Mobility

Two major mechanisms are known to lead to group I intron lateral transfer. Mobility at the DNA level may be facilitated by an endonuclease (ENase) that recognizes a long sequence (15–35 nt) within a homologous intronless copy of the coding region (Lambowitz and Belfort, 1993). Initiation of a double-strand break in the DNA of the intronless coding region is followed by a unidirectional conversion event that results in intron insertion (Belfort and Perlman, 1995; Johansen *et al.,* 1997). This process, called intron "homing," is highly efficient at introducing introns into the alleles of a gene but is not expected to lead to the frequent lateral transfer of group I introns into heterologous sites (intron transposition) because of the high endonuclease sequence specificity (Dujon, 1989; but see Bryk *et al.,* 1993). Group I introns may also integrate into RNAs through a reversal of the splicing reaction (reverse-splicing). In reverse-splicing, a free intron inserts into a homologous or a heterologous RNA by recognizing a short (4- to 6-nt) sequence at the 5′ splice site, the internal guide sequence (IGS; Woodson and Cech, 1989; Cech *et al.,* 1994; Thompson and Herrin, 1994; Roman and Woodson, 1995, 1998). *In vitro* and bacterial expression analyses have shown that the *Tetrahymena thermophila* large subunit (LSU) rDNA group I intron can integrate, through reverse-splicing, into both homologous and heterologous sites in the LSU rRNA of *E. coli* (Woodson and Cech, 1989; Roman and Woodson, 1998).

Regarding the *Trebouxia* spp. 1512 introns, two lines of evidence lead us to favor the reverse-splicing model of intron lateral transfer in this genus. First, none of the 1512 introns encode an ENase open reading frame

FIG. 4. Consensus secondary structure of group I introns at position 1512 in the SSU rDNA of *Trebouxia* spp. superimposed on the secondary-structure model of the *T. corticola*/*T. usneae* UTEX intron (drawn according to the conventions of Cech *et al.,* 1994). The 5′ and 3′ splice junctions are shown with the large arrows as are the locations of the pairing segments P1–P10 (the P10 interaction is shown with the open boxes). Solid lines are used to position secondary-structure elements that are believed to interact in close proximity. The positions thatare conserved in all *Trebouxia* 1512 group I introns are shown in the normal text; positions shown in the contour text encoded one of two different nucleotides, whereas positions in the filled boxes encoded more than two different types of nucleotides or a gap was needed in the alignment to mark this position. The nucleotides in the open boxes mark positions that are shared between the putatively laterally transferred *T. corticola*/*T. usneae* UTEX intron and other intron sequences from *T. impressa* 17, *T. arboricola* SAG, and *T. gelatinosa* (see Fig. 2B) to the exclusion of introns from all other investigated species/strains. The circled nucleotides are invariant among all available trebouxiophyte intron sequences (i.e., from *Trebouxia* spp. and *Choricystis minor*) but variable among other 1512 intron sequences. Asterisks mark those sequence positions that are invariant among *Trebouxia* spp. 1512 introns but different from the corresponding sequence of *C. minor.* The length variations of particular distal pairing elements in the *Trebouxia* spp. 1512 introns are shown with the number ranges.


P9.1a
-1, +24 nt
P9.1
P9
P9.2
-1 nt
-3, +11 nt
3′
+7 nt
P10
P9.0
*A
P1
IGS
5′
P5a
P5
-1 nt
P5c
P4
P6
-1 nt
P6a
P5b
-3 nt
-1 nt
-8, +1 nt
P6b
P7
P3
-8, +4 nt
P2.1
P8
-11, +8 nt
P2

(ORF). Intron homing would require that these ORFs were once present in some or all of the 1512 introns and that the encoded ENases allowed homing into the homologous 1512 site in the SSU rDNA within and presumably between species. Spread of the intron within rDNA gene families could result either from homing or from the processes of concerted evolution. The complete lack of such ORFs is, therefore, hard to explain, given our broad sampling of *Trebouxia* spp. Alternatively, an ENase encoded in another gene or intron may have mediated 1512 mobility in trans. Although we cannot address the second scenario, we note that intron homing occurs through the double-strand-break-repair pathway (Szostak, 1983). This process results in coconversion of nucleotide markers around the intron insertion site, such that the donor flanking sequences would be found in the host (e.g., Moran *et al.,* 1992). To address this issue, we studied the exonic sequences flanking the insertion site of the 1512 intron in *Trebouxia* spp. that had been vertically inherited or laterally transferred. The 5′ flanking region of these introns was completely conserved but the 3′ flanking region showed polymorphisms within the ITS sequence. The ITS sequence starts 12 bp downstream from the 1512 intron insertion site. Analysis of the ITS polymorphisms showed, however, no sites uniquely shared by taxa containing laterally transferred introns and the donors of these introns (e.g., *T. corticola/usneae* and *T. gelatinosa,* respectively). Sites were exclusively shared by the host cell lineages resolved in Fig. 2A (e.g., *T. corticola/usneae* and *T. higgensiae/galapagensis;* results not shown).

A second line of evidence that favors reverse-splicing-mediated 1512 intron transfer comes from our analyses of intron splicing. Our experiments show that several strains of *T. arboricola* do not self-splice *in vitro* (see Fig. 2B). Interestingly, the only introns that did successfully complete the splicing reaction under the conditions used in our assay (*T. impressa,* all *T. gelatinosa* strains) were also the sources of the laterally transfered introns in *T. arboricola* SAG and *T. corticola/usneae.* This suggests that self-splicing ability may be an important component of 1512 intron mobility, an hypothesis that is supported by bacterial expression analyses of the self-splicing *T. thermophila* intron. Analyses of the *T. thermophila* intron show that reverse-splicing, which is relatively inefficient in comparison to the forward reaction (Woodson, 1996), occurs in *E. coli* only when large amounts of the catalytically active intron RNA are produced in the cell (Roman and Woodson, 1995, 1998). Mutations introduced into the *T. thermophila* intron that shut down or limit (e.g., G264 to A in the intron core) self-splicing also inhibit reverse-splicing in bacteria (Roman and Woodson, 1998). This suggests that an intron that splices poorly or is dependent on host cell splicing mediation is unlikely to be a mobile element. Self-splicing ability may, therefore, be a requirement for reverse-splicing-mediated group I intron mobility.

We realize, however, that the results of the splicing analysis are preliminary in nature and need to be tested with the remainder of the *Trebouxia* spp. 1512 introns. In addition, a variety of splicing conditions need to be used (see Besendahl and Bhattacharya, 1999) to ensure that the present results do not reflect primarily the failure to provide the correct *in vitro* conditions to facilitate splicing (work in progress). If we accept, however, that self-splicing ability is an important component of mobility, then it is not clear why the laterally transferred introns in *T. jamesii* 73 and *T. arboricola* SAG no longer self-splice. One possible explanation is that regulation of splicing by the host cell (resulting in loss of self-splicing ability) occurs soon after lateral transfer. Lineages that are characterized by regulated splicing, such as *T. arboricola* spp., may restrict autocatalysis of laterally transferred introns. In such cases, novel introns would lose self-splicing ability soon after their origin (e.g., *T. arboricola* SAG).

To conclude, the results of our study underline the dynamic evolutionary history, not only of the 1512 group I intron in lichenized *Trebouxia* spp. but also of the algal cells themselves. The appearance of identical, intron-containing *Trebouxia* strains in different lichen fungi suggests that the process of lichenization may facilitate the movement of introns and their host cells among different lichen partnerships. We have previously proposed (Bhattacharya *et al.,* 1996) that the lateral transfer may be mediated by a viral vector during the early development of lichens. During this time, different strains/species of *Trebouxia* may inhabit a single lichen thallus and come in direct cell-to-cell contact (Friedl, 1987; Ott, 1987). Lichen maturation leads to the elimination of all but one strain of photobiont that then exclusively inhabits the lichen thallus. That 1512 group I intron mobility occurs only among *Trebouxia* photobionts (and not with the mycobiont) is supported by the finding that the various mycobiont rDNA group I introns that are now available (DePriest and Been, 1992; Gargas *et al.,* 1995) show no primary or secondary structure similarity to the algal 1512 introns. We speculate that virus(es) that specifically infect *Trebouxia* may mediate the lateral transfer of 1512 introns into related strains, a scenario that is consistent with previous observations of viral particles during transmission electron microscopic studies of lichen thalli (L. Goff, pers. comm.). Furthermore, viral-mediated lateral transfer of group I introns in algal cells has been previously documented (see Yamada *et al.,* 1995; Bhattacharya *et al.,* 1996; Nishida *et al.,* 1998). A process such as reverse-splicing could then lead to the integration of self-splicing introns into homologous sites of the nuclear-encoded rRNA genes in different lichen algae.

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