Nuclear-Encoded rDNA Group I Introns: Origin and Phylogenetic Relationships of Insertion Site Lineages in the Green Algae

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Group I introns are widespread in eukaryotic organelles and nuclear-encoded ribosomal DNAs (rDNAs). The green algae are particularly rich in rDNA group I introns. To better understand the origins and phylogenetic relationships of green algal nuclear-encoded small subunit rDNA group I introns, a secondary structure-based alignment was constructed with available intron sequences and 11 new subgroup IC1 and three new subgroup IB3 intron sequences determined from members of the Trebouxiophyceae (common phycobiont components of lichen) and the Ulvophyceae. Phylogenetic analyses using a weighted maximum-parsimony method showed that most group I introns form distinct lineages defined by insertion sites within the SSU rDNA. The comparison of topologies defining the phylogenetic relationships of 12 members of the 1512 group I intron insertion site lineage (position relative to the *E. coli* SSU rDNA coding region) with that of the host cells (i.e., SSU rDNAs) that contain these introns provided insights into the possible origin, stability, loss, and lateral transfer of IC1 group I introns. The phylogenetic data were consistent with a viral origin of the 1512 group I intron in the green algae. This intron apppears to have originated, minimally, within the SSU rDNA of the common ancestor of the trebouxiophytes and has subsequently been vertically inherited within this algal lineage with loss of the intron in some taxa. The phylogenetic analyses also suggested that the 1512 intron was laterally transferred among later-diverging trebouxiophytes; these algal taxa may have coexisted in a developing lichen thallus, thus facilitating cell-to-cell contact and the lateral transfer. Comparison of available group I intron sequences from the nuclear-encoded SSU rDNA of phycobiont and mycobiont components of lichens demonstrated that these sequences have independent origins and are not the result of lateral transfer from one component to the other.

Introduction

Group I introns are characterized by a distinct RNA secondary structure and a common splicing pathway (Burke et al. 1987). Many group I introns have the capacity to catalyze (via the ribozyme) their own excision from coding regions (Cech 1990; Cech, Damberger, and Gutell 1994). The widespread and often sporadic distribution of group I introns in nuclear-encoded ribosomal DNA (rDNA, Saldanha et al. 1993; Bhattacharya et al. 1994; Vader et al. 1994), in chloroplast and mitochondrial coding regions, in phages, in eubacteria (Lambowitz and Belfort 1993), and in viruses of eukaryotes (Yamada et al. 1994) strongly suggests that these sequences are capable of lateral transfer between evolutionarily distinct lineages (Vader et al. 1994; Yamada et al. 1994; Belfort and Perlman 1995). Several lines of evidence support this hypothesis. Group I introns in the genomes of plastids, mitochondria, phages, and eubacteria may have open reading frames (ORFs) that encode endonucleases (ENases) to mediate their sequence-specific "homing," at the DNA level into homologous, intronless coding regions (Dujon 1989). Homing may also result in the lateral transfer of group I introns between mitochondria and chloroplasts (Turmel et al. 1995).

Group I intron mobility may also occur at the RNA level from the reversal of the splicing reaction in which an excised intron recognizes a short 5' flanking sequence in the coding region and inserts itself into the RNA (Lambowitz and Belfort 1993). Reverse transcription of the intron-containing coding region followed by general recombination with the intronless genomic copy would result in the lateral transfer of the group I intron. Reverse splicing has been demonstrated in vitro for the *Tetrahymena thermophila* subgroup IC 1 (Cech, Damberger, and Gutell 1994) LSU group I intron; reverse splicing results in the reinsertion of the *T. thermophila* intron into its natural target site in the LSU rRNA and its lateral transfer into the heterologous β-globin mRNA (Woodson and Cech 1989). Successful reverse splicing of the *T. thermophila* group I intron requires only a four base (CUCU) recognition sequence at the 5' flanking region to form the PI helix (Kim and Cech 1987) that is required for both forward and reverse splicing reactions.

The loss of group I introns, a phenomenon that must happen frequently in nature due to the "optional" distribution of group I introns in closely related taxa, may also occur at the RNA level (Lambowitz and Belfort 1993; Van Oppen, Olsen, and Stam 1993; Vader et al. 1994). Intron loss would result from the reverse transcription of an intronless RNA followed by general recombination with the intron-containing genomic copy of the coding region (Dujon 1989). Given, therefore, that there are existing mechanisms to explain the mobility and loss of group I introns, it is worthwhile to study the distribution of group I introns among eukaryotes to determine the possible role of such mechanisms in explaining the observed distribution.

The subgroup IC1 (Michel and Westhof 1990) nuclear-encoded rDNA group I introns offer one possibility to model the evolution of these highly divergent sequences. The IC1 introns are of a relatively great length

Key words: green algae, group I introns, lateral transfer, lichens, molecular evolution, phylogeny, secondary structure, small subunit ribosomal DNA.

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(350-450 nucleotides [nt]) are, in general, readily alignable along most of the sequence and, remarkably, of the more than 50 subgroup IC1 group I introns known from taxa that form the "crown" (Knoll 1992; Wainright et al. 1993) of the eukaryotic tree of life (e.g., green algae, charophytes, ciliates, fungi, red algae), none encodes a mobility-conferring ORE This final observation excludes the extrachromosomal ENase-containing, mobile rDNA group I introns of the primitive myxomycetes, *Physarum polycephalum* and *Didymium iridis* (Johansen, Johansen, and Haugli 1992; Ruoff, Johansen, and Vogt 1992), and *Naegleria* spp. (De Jonckheere 1993). The myxomycetes and *Naegleria* spp. are the earliest diverging eukaryotic taxa known to contain group I introns (De Jonckheere 1993) on the basis of SSU rDNA sequence comparisons (Hinkle and Sogin 1993; McFadden, Gilson, and Hill 1994). That all known nuclear-encoded rDNA group I introns in crown group taxa lack ENases suggests that aspects of their distribution may be explained by RNA-mediated processes such as reverse splicing and loss. This hypothesis does not, however, exclude the possibility that ENases were once found within these rDNA introns, mediated their lateral transfer, and then were lost over time. ENases themselves may be the mobile components of group I introns with their acquisition leading to intron mobility and their loss to intron stability or loss over time (Belfort 1991; Loizos, Tiller, and Belfort 1994). If ENases have played a dominant role in group I intron mobility within nuclear-encoded rDNAs in crown group taxa, then independent and complete loss, so far as is known, of these sequences has to be invoked to explain the present distribution.

In this paper, we report 11 new subgroup IC1 and two new subgroup IB3 group I introns that interrupt the SSU rDNA of trebouxiophyte and ulvophyte green algae and present phylogenetic analyses of group I introns and the SSU rDNAs that contain them. The green algae are surprisingly rich in group I introns and are, therefore, a model group to probe the evolution of these sequences (Bhattacharya et al. 1994; Vader et al. 1994). Our phylogenetic analyses provide putative examples of the origin, stability, lateral transfer, and loss of subgroup IC1 group I introns within the 15 12 intron lineage (position relative to the *E. coli* coding region). Further, results of phylogenetic analyses of the numerous group I introns in the SSU rDNA of the lichen fungi, *Cladonia* spp. and *Lecanora dispera,* are consistent with a reverse-splicing-mediated origin of some of these intron lineages. Taken together, these data suggest that RNA-mediated processes such as reverse splicing and loss are important components of the evolution of nuclear-encoded rDNA group I introns. Our results also suggest that most green algal nuclear-encoded rDNA group I introns originated relatively "late" in evolution (Palmer and Logsdon 1991).

Materials and Methods

Ribosomal DNA Sequence Determination

Total nucleic acids were isolated from exponentially growing unialgal cultures of *Pleurastrum paucicellulare* and *Protoderma sarcinoidea* (Ulvophyceae, Chlorophyta) and members of the genus *Trebouxia* (Trebouxiophyceae, Chlorophyta; see table 1) as described in Fried1 (1995). Nuclear-encoded SSU rDNA coding regions were amplified using the polymerase chain reaction protocols (PCR, Saiki et al. 1988) and the following 5' and 3' primers, respectively, that are complementary to the termini of rDNA sequences (Hamby et al. 1988): NS 1,5′-GTAGTCATATGCTTGTCTC-3′;18L, 5′-CACCTACGGAAACCTTGTTACGACTT-3′. The internal-transcribed-spacer (ITS) region of the rDNA operon from *Trebouxia* spp., *P. paucicellulare,* and *P. sarcinoidea* was isolated using the PCR-method and the following 5' and 3' primers, respectively: NS7m, 5′-GGCAATAACAGGTCTGT-3'; LR1850, 5′-CCTCA CGGTACTTGTTC-3'. The PCR reaction conditions were as in Fried1 and Zeltner (1994).

Single-stranded template DNA was prepared for sequencing using either biotinylated amplification primers and the Dynabeads M-280 system (Dynal AS, Hultman et al. 1991) or by treating the PCR products with the enzymes Exonuclease I and Shrimp Alkaline Phosphatase according to the manufacturer's instructions (United States Biochemical, Cleveland, Ohio), after which the PCR products were denatured by heating to 100°C for 3 min and chilled on wet ice. SSU rDNA and ITS PCR products were sequenced directly using the dideoxy sequencing method (Sanger, Nicklen, and Coulson 1977) and a collection of oligonucleotide primers complementary to conserved regions of eukaryotic SSU rDNAs (El-

Table 1
New SSU rDNA and Group I Intron Sequences Reported in this Manuscript with Strain Identifications, Culture Sources and Database Accession Numbers Shown

Group I Introns	Accession Numbers	Intron Positions
Trebouxia usnea		
UBT-87.019A1	268702	516, 1512
Trebouxia arboricola		
SAG 291-1a* . .	268705	516, 1512
Trebouxia arboricola		
UBT-92.011C3* . .	268703	—
Trebouxia arboricola		
UBT-92.01 1C5* .	268704	1512
Trebouxia gelatinosa		
UTEX 905*	268698	1512
Trebouxia gelatinosa		
UBT-86.108B2*	268697	
Trebouxia jamesii		
UBT-86.132E1	268700	1512
Trebouxia jamesii		
UBT-86.001E1* . .	268699	1512
Trebouxia jamesii		
UBT-86.156C3* .	268701	
Pleurastrum paucicellulare		
SAG 463-1	247997	***943, 1046, 1512***
Protoderma sarcinoidea		
UTEX ***1 7 1 0***	247998	***943, 1512***

NOTE.-UBT = University of Bayreuth. The positions of the group I introns in the SSU rDNAs are also shown. Species marked with an asterisk (*) have been sequenced only over the ITS region and the group I intron(s) in the SSU rDNA (if these exist in the coding region).

wood, Olsen, and Sogin1985), trebouxiophyte ITS regions, and group I introns (unpublished data).

Host Cell Phylogeny

The SSU rDNA coding regions of ***P. paucicellulure, P.*** *sarcinoidea, T. jamesii* (UBT-86.132E1), and ***T.*** *usneae* (UBT-87.019A1) were manually aligned (Olsen 1990) with rDNA sequences from 55 eukaryotes (i.e., host cells) representing 1 red alga (Rhodophyta), 2 fungi (Eumycota), and 52 green algae, charophytes, and land plants. Representatives of each major lineage of green algae (i.e., Chlorophyceae, Prasinophyceae, Trebouxiophyceae [*sensu* Fried1 1995], Ulvophyceae) were included in the phylogenetic analysis; these sequences are available from Gutell (1993) or the RDP (Maidak et al. 1994) and GenBank/EBI-EMBL/DDBJ databases. Unambiguously aligned sequence positions within the green algae/glaucocystophytes (1,700 nt, alignment available from TF) with the red algal/fungal rDNA sequences as outgroups were analyzed with a distance method using a Kimura (1980) matrix as input for a neighbor-joining phylogenetic reconstruction (Saitou and Nei 1987) with jumbled taxon addition and the transition/transversion ratio = 2 (PHYLIP V.3.5, Felsenstein 1993). Bootstrap analyses (500 replications, Felsenstein 1985) were done to assess the stability of topological elements in the host cell phylogeny.

To determine the phylogenetic relationships within the genus ***Trebouxiu,*** the ITS sequences determined in this study were aligned and a total of 629 nt used as input for maximum-parsimony and neighbor-joining analyses (alignment available from TF). In the maximum-parsimony analysis, the sites were weighted (rescaled consistency [RC] index over an interval of 1–1,000) and then used as input for a bootstrap analysis (500 replications) using the branch-and-bound search method (PAUP V3.1.1, Swofford 1993). Bootstrap analysis (500 replications) using the neighbor-joining method was implemented with the ITS data set as described above.

Group I Intron Phylogeny

The newly determined subgroup IC1 group I intron sequences were included in an alignment (Olsen 1990) of 35 introns from this subgroup, including those from 6 distinct nuclear-encoded SSU rDNA insertion site subfamilies, 2 introns interrupting different protein-coding regions in the genomes of ***Chlorellu*** spp. viruses (Yamada et al. 1994), and 7 introns from subgroup IB3 (position 5 16) as outgroup for the IC1 lineage phylogenetic analyses. The unambiguous alignment of these highly divergent sequences was aided by the juxtaposition of homologous secondary structure elements. This alignment is nearly identical to that presented in Bhattacharya et al. (1994) and is available upon request from D.B. Two hundred forty-two sequence positions from the group I intron alignment could be used as input for the phylogenetic analyses. A second data set of subgroup IC1 intron sequences was prepared that included only members of the 1512 intron lineage with the *Chlorella* spp. viral group I introns (see below) as outgroup. The relatively close phylogenetic relationship between the 1512 intron sequences allowed the inclusion of 325 nt in the phylogenetic analyses. An analysis was also done using only 1512 introns from members of the Trebouxiophyceae and the latter alignment. To investigate the phylogenetic relationships of the distinctive lichen mycobiont SSU rDNA group I introns (subfamily classification unknown, Damberger and Gutell 1994), members of six different intron site lineages from ***Cludoniu*** spp. and ***L. dispersu*** were aligned and 207 nt used for the midpoint rooted phylogenetic analyses (alignment available from D.B.).

Phylogenetic analyses of the large data set of subgroup IC1 and IB3 group I intron insertion site lineages were implemented with a weighted maximum-parsimony method as described above except that a heuristic procedure (TBR, tree bisection reconnection) was used to search for the best trees. Bootstrap analyses (500 replications) were done with this data set both with and without the highly divergent subgroup IB3 introns as outgroup. Phylogenetic analyses of the 15 12 intron lineage was done with the maximum-parsimony (branch-and-bound search) and neighbor-joining methods as described above with all the 15 12 introns with and without the *Chlorella* spp. viral intron sequences as outgroup; the same analyses were also done with only the 1512 group I introns from ***Trebouxiu*** spp. The mycobiont intron sequences from C. ***chlorophueu*** and ***L. dispersu*** were analyzed with the maximum-parsimony (branch-and-bound search), neighbor-joining (as described above), and maximum-likelihood methods (fastDNAml V1.0, Olsen et al. 1994). For the maximum-likelihood method, the global search option was used with rearrangements of partial trees initially crossing one branch and rearrangements of the full tree crossing eight branches. A transition/transversion ratio of 2 was used for the fastDNAml analysis.

Group I introns used in the phylogenetic analyses (excluding those determined in this study) and their GenBank/EBI-EMBL/DDBJ database accession numbers (where available) are as follows: Subgroup IC1—*Ankistrodesmus* ***stipitutus (X56100), Churucium succuturu*** (M84319), ***Chlorellu ellipsoideu (X63520), C. mirubilis (X74000), C. luteoviridis (X73998), C. sorokiniunu (X73993), Chlorellu*** virus CVB 11 (D2963 1), ***Chlorellu*** virus CVU1(17367), ***Choricystis minor (X89012),*** *Dunaliella parva* (M62998), *Dunaliella salina* (M84320), ***Gloeotilopsis plunctonicu*** (Z28970), ***Mesotuenium*** *calduriorum* ***(X75763), Pneumocystis curinii (X13687), Pneumocystis curinii*** LSU (M86760), ***Protomyces inouye*** (D11377), ***Urosporu*** *penicilliformis* (Van Oppen, Olsen, and Stam 1993), *Ustilago* ***muydis (X62396), Zygnemopsis circumcurinutu (X79495);*** Subgroup IB3—*Chlorella* ***luteoviridis (X73997), C. succhurophilu (X73991),*** *Klebsormidium* ***flaccidum (X735520),*** *Muriella aurantiaca* ***(X91268), Rhodosporidium ducryoidum*** (D13459); Mycobiont introns-Cludoniu *grayi* (Z14025), C. *merochlorophaea* (Z14026), *Lecanora* ***dispersu (L37734).***

Results and Discussion

SSU rDNA Host Cell Phylogeny

Phylogenetic relationships between all 59 SSU rDNAs included in this study are shown in figure 1. The green plants form a distinct lineage defined by the Charophyceae, the Embryophyta, and the Chlorophyta (Steinkbtter et al. 1994). Within the Charophyceae, the Zygnematales ("desmids") share a unique common origin (Surek et al. 1994). The monophyletic Chlorophyta is subdivided into at least four distinct lineages, the Chlorophyceae, the Trebouxiophyceae, the Ulvophyceae, and a group of scaly flagellates, the Prasinophyceae, that diverge at the base of the Chlorophyta (Steinkötter et al. 1994). *Nephroselmis olivacea/Pseudoscourfieldia marina* represent the prasinophytes in this analysis. The polyphyletic genus *Chlorella* spp. (Huss and Sogin 1990) is distributed within the Chlorophyceae/Trebouxiophyceae. The Ulvophyceae, a large assemblage of morphologically diverse green algae, is represented in figure 1 by members of the Ulotrichales (G. *planctonica, P. paucicellulare, P. sarcinoidea)* and the Acrosiphoniales *(Acrosiphonia* spp.). The Trebouxiophyceae defines a green algal class that was recently erected on the basis of SSU rDNA sequence comparisons (Fried1 1995) and includes many principally lichen-forming algae (phycobionts). There is good bootstrap support for most of these groupings. Base composition was relatively conserved within the SSU rDNA sequences shown in figure 1, with the A content ranging from 24% to 28% and the G+C content ranging from 45% to 51%; within the Chlorophyta, these values were 24%-26% and 46%-5 1%, respectively. Base composition is not expected to bias the neighbor-joining analysis of the SSU rDNA data set.

The SSU rDNA coding regions of the ulvophytes, *P. paucicellulare* and G. *planctonica,* were found to differ at only one sequence position though these taxa are morphologically distinct. This U → G substitution is located in a bulged region of element 8 in the SSU rRNA secondary structure model of Neefs et al. (1993). Sequence analyses of PCR products from independent template preparations from *P. paucicellulare* and G. *planctonica* verify this base substitution.

Group I introns are sporadically distributed throughout the green algae. Two exceptions are the numerous 15 12 group I introns in the genus *Trebouxia* (Trebouxiophyceae, see below) and the 1506 intron in the Zygnematales (Charophyceae); all studied members of the Zygnematales (except *Spirogyra* spp.) contain a putative homologous group I intron at position 1506 of their SSU rDNA (Bhattacharya et al. 1994). In contrast, no embryophyte has yet been found that contains a nuclear-encoded SSU rDNA group I intron. The Eumycota and the Rhodophyta also contain taxa with SSU rDNA group I introns. These lineages are phylogenetically removed from each other and from the green plants though they all arise from within the crown group radiation.

Phylogenetic Relationships of Group I Intron Sequences

The phylogenetic relationships between the subgroup IC1 group I introns analyzed in this study show that, in agreement with previous reports (Bhattacharya et al. 1994; Vader et al. 1994; Van Oppen, Olsen, and Stam 1993), insertion site subfamilies form distinct lineages (fig. 2). Within the Chlorophyta, the most significant result is the close phylogenetic relationship between the 15 12 intron lineage and the group I introns interrupting protein coding regions in the *Chlorella* spp. viruses. The close phylogenetic relationship of the viral introns to the 15 12 rDNA introns suggests that eukaryotic viruses may have facilitated the spread of these group I introns. Both maximum-parsimony analyses (i.e., with and without the IB3 introns) position the viral intron sequences as a sister group to the 1512 group I intron lineage with bootstrap support for this relationship (85% and 94%, respectively).

The *Chlorella* spp. viral group I introns interrupt a protein with significant sequence similarity to eukaryotic transcription elongation factor SII (CVU1) in one case and to a protein of unknown function in the other case (CVB 11, Yamada et al. 1994). Southern blot analyses show that both these protein coding regions are widespread and highly conserved among viral species that infect *Chlorella* spp.; the viral group I introns are, however, optional (Yamada et al. 1994). The close phylogenetic relationship between the two *Chlorella* viral group I introns both to each other (78% sequence similarity, Yamada et al. 1994) and to the rDNA 15 12 introns supports the hypothesis of Yamada et al. (1994) that the *Chlorella* spp. viral group I introns are/were mobile genetic elements that may have been one source of eukaryotic group I introns. The lack of an ORF in the viral group I introns and limited flanking sequence similarity (there is, however, a 5/6 match in the 5' flanking sequence [GA(A/U)CCU]) suggest a mechanism such as reverse splicing (Woodson and Cech 1989) may have mediated the transposition(s) of this intron into the heterologous viral protein coding regions and into the green algal rDNA (and see below). Another putative example of reverse-splicing-mediated group I intron transposition has recently been described between a plant-parasitic fungus and its host. Nishida and Sugiyama (1995) identified an identical group I intron in the rDNA of the fungus *Protomyces inouye,* intron A (position 943, see fig. 2), and in the host plant, *Youngia japonica.* The 943 intron in Y. *japonica* was not located in the rDNA, suggesting that its origin was the result of a lateral transfer; these introns do not encode a mobility-conferring ORE

The maximum-parsimony analyses of the group I intron lineages are consistent with a single origin of the 943 group I intron in the common ancestor of the chlorophytes/ulvophytes. It is not yet clear, however (due to the limited data), whether these introns were found in the common ancestor of these green algal classes and then lost multiple times or whether the introns had multiple origins within the rDNAs. That the chlorophyte and

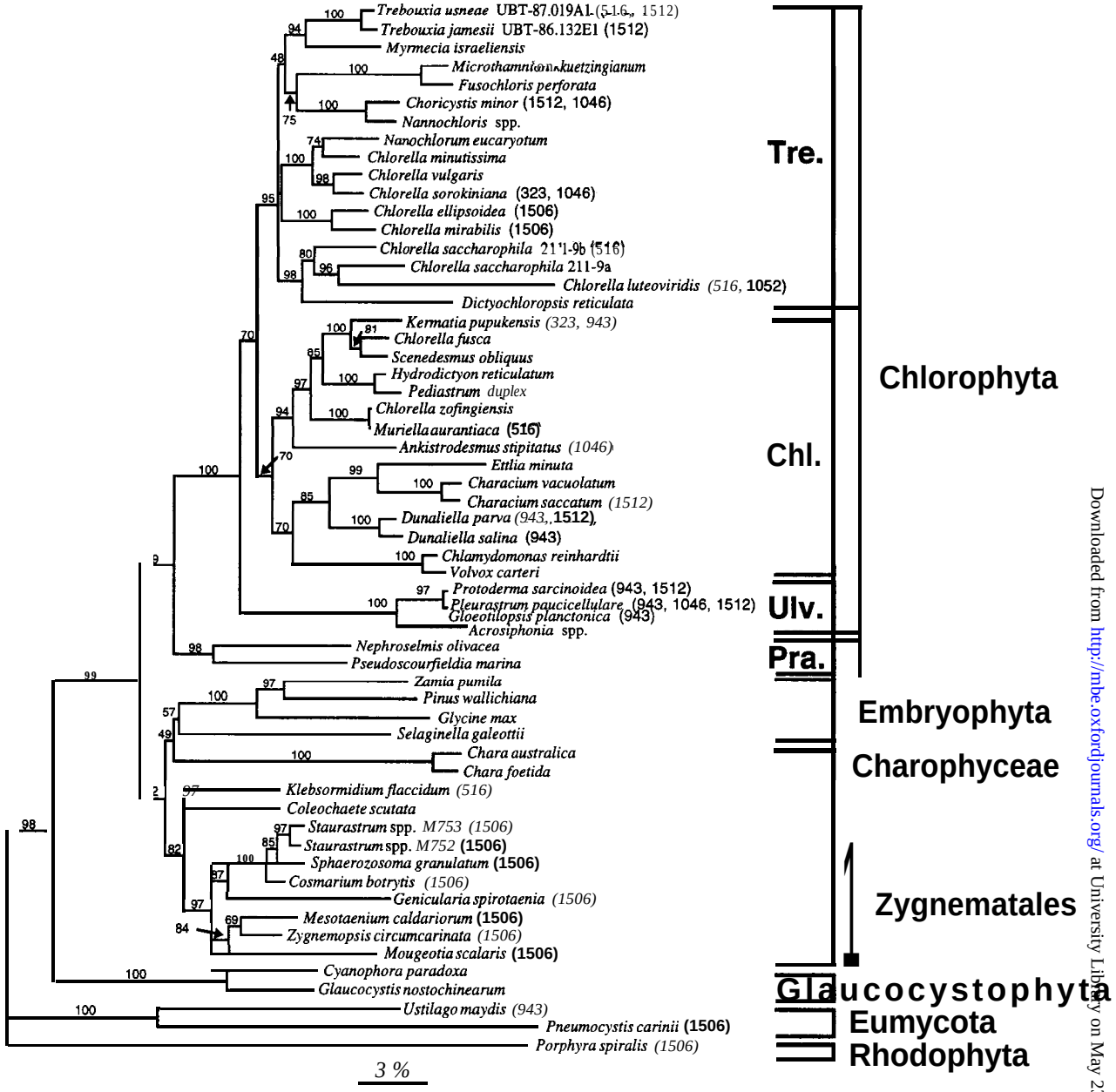

FIG. 1 .-Phylogenetic analyses of SSU rDNA coding regions. Unrooted tree constructed with the neighbor-joining method (Saitou and Nei 1987) based on structural distances (Kimura 1980) between SSU rDNA coding regions. A total of 1,700 nt was considered. Evolutionary distances are represented in the horizontal axis by the sum of branch lengths separating taxa. The distance that corresponds to 3% sequence divergence is indicated by the scale. Bootstrap percentage values based on 500 resamplings of these data are shown at the internal nodes. Arrows are used to indicate bootstrap values at nodes where these numbers do not tit on the branches. Taxa that contain a group I intron(s) are indicated. The *Pleurastrum paucicellulare* rDNA sequence, which differs at only one position from this coding region in *Gloeotilopsis planctonica* (see text), was not analyzed but simply added to the figure. The following shortened taxon names are used: Tre.-Trebouxiophyceae, Chl.—Chlorophyceae, Ulv.—Ulvophyceae, Pra.-Prasinophyceae,

ulvophyte 943 group I introns form distinct groups within a monophyletic green algal cluster (bootstrap values = 82% and 90%) independent of the fungal 943 introns suggests that if these introns were frequently laterally transferred, then closely related intron(s) that homed to the 943 site invaded the rDNA of these taxa.

The 943 insertion site group I intron sequence of *P. paucicellulare* differed with respect to one base substitution from the homologous intron in G. *planctonica*, as is the case with their SSU rDNAs. This verified C → U change is located in domain P5b (see Cech, Damberger, and Gutell 1994 for secondary structure model). The near identity of both host cell and group I intron sequences is consistent with a close evolutionary relationship between *P. paucicellulare* and G. *planctonica* rather than with a recent lateral transfer of the same 943 intron into these taxa.

Base compositions were more variable within the group I intron sequences (see fig. 2). That base composition may vary widely within an insertion site lineage

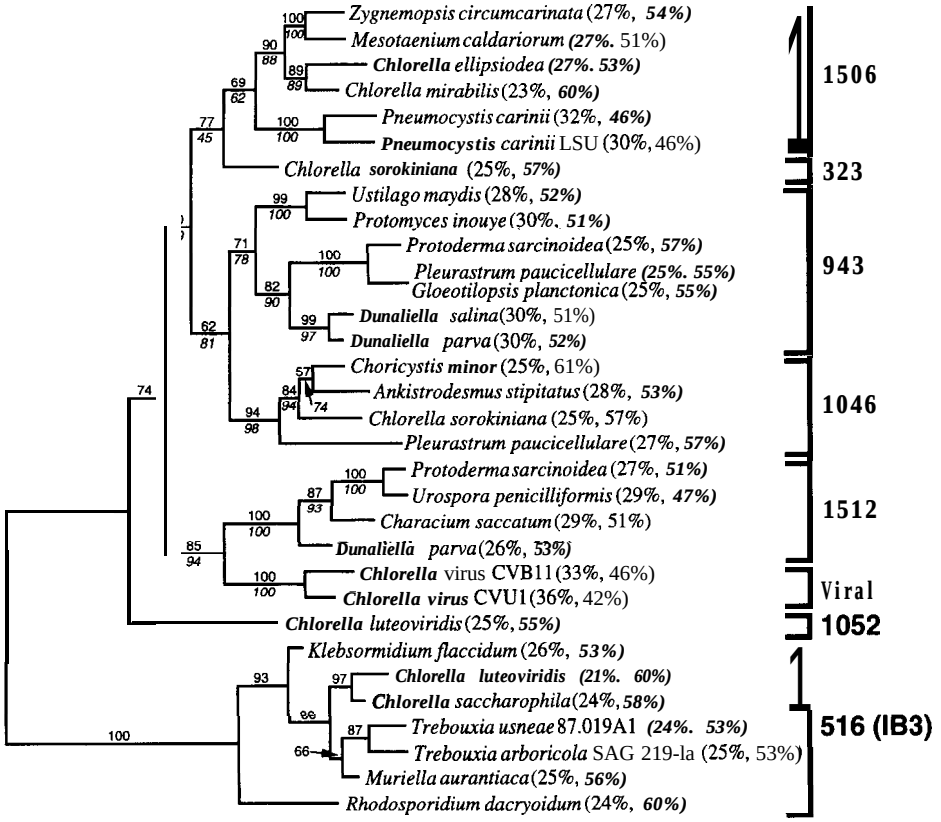

FIG. 2.—Phylogenetic analyses of subgroup IC1 and subgroup IB3 group I introns. Bootstrap consensus phylogram inferred with a weighted (RC index over the interval 1–1,000) maximum-parsimony method (PAUP V3.1 .1, Swofford 1993) using 242 aligned nucleotides. A heuristic search (TBR, tree bisection reconnection) procedure was used and the MULPARS option. The tree shown is rooted within the branch leading to the 516 group I introns and has a consistency index (CI) of 0.59. The bootstrap values (500 replications; Felsenstein 1985) above the internal nodes are inferred from an analysis that includes the IB3 intron sequences whereas the bootstrap values below the internal nodes in italic script were inferred from a maximum-parsimony analysis that excluded the IB3 introns. The *Pleurastrum paucicellulare* 943 intron sequence, which differs at only one position from this intron in *Gloeotilopsis planctonica (see* text), was not analyzed but simply added to the figure. Arrows are used to indicate bootstrap values at nodes where these numbers do not fit on the branches.

independent of the nucleotide composition of the SSU rDNAs containing these sequences has been shown for the Zygnematales 1506 introns (Bhattacharya et al. 1996). There is, however, no clear evidence from figure 2 that base composition is solely responsible for the resolved insertion site groupings. Within the 1046 insertion site lineage, for example, which is strongly supported in the bootstrap analyses (94%, 98%), the G+C content varies from 53% to 61%.

Analyses of the 15 12 Intron Lineage in the Green Algae

The phylogenetic analyses show a close evolutionary relationship between the green algal group I introns inserted at position 1512 (figs. 2 and 3A). The maximum-parsimony analyses are consistent with a monophyletic origin of the 1512 group I intron in the Chlorophyceae/Trebouxiophyceae/Ulvophyceae with a sister group relationship between chlorophytes and trebouxiophytes as in the rDNA phylogeny (fig. 1). Exclusion of the *Chlorella* spp. viral intron sequences resulted in higher bootstrap support for these results. Within the Trebouxiophyceae, both coding region and intron phylogenies supported a sister group relationship between *C. minor* and a monophyletic *Trebouxia* spp. grouping. An alternative explanation for these data is that the 15 12 intron originated simultaneously, multiple times, either (1) in the common ancestors of the three algal classes (i.e., the same intron has been laterally transferred into these diverse taxa in the order that simulates the monophyletic origins of the three algal classes) or (2) thereafter within the rDNA of these taxa. The latter hypothesis, though clearly possible regarding the origin of the 15 12 intron in the chlorophytes and ulvophytes (where the data are limited), is considered less likely regarding the trebouxiophyte 1512 introns. It is unlikely that multiple, independent group I intron lateral transfers explain the origin of the seven trebouxiophyte 1512 intron sequences since chance alone would dictate against the grouping of the trebouxiophyte introns to the exclusion of the chlorophyte and ulvophyte sequences. To test this hypothesis further, a comparison was made of the best trees inferred from the maximum-parsimony analysis of the 15 12 intron data set with the lengths of 10,000 random trees derived from these data (PAUP V3.1.1, Swofford 1993) to see if the phylogeny shown in figure *3A* is significantly better than a random grouping of these sequences that would be expected if lateral transfers of

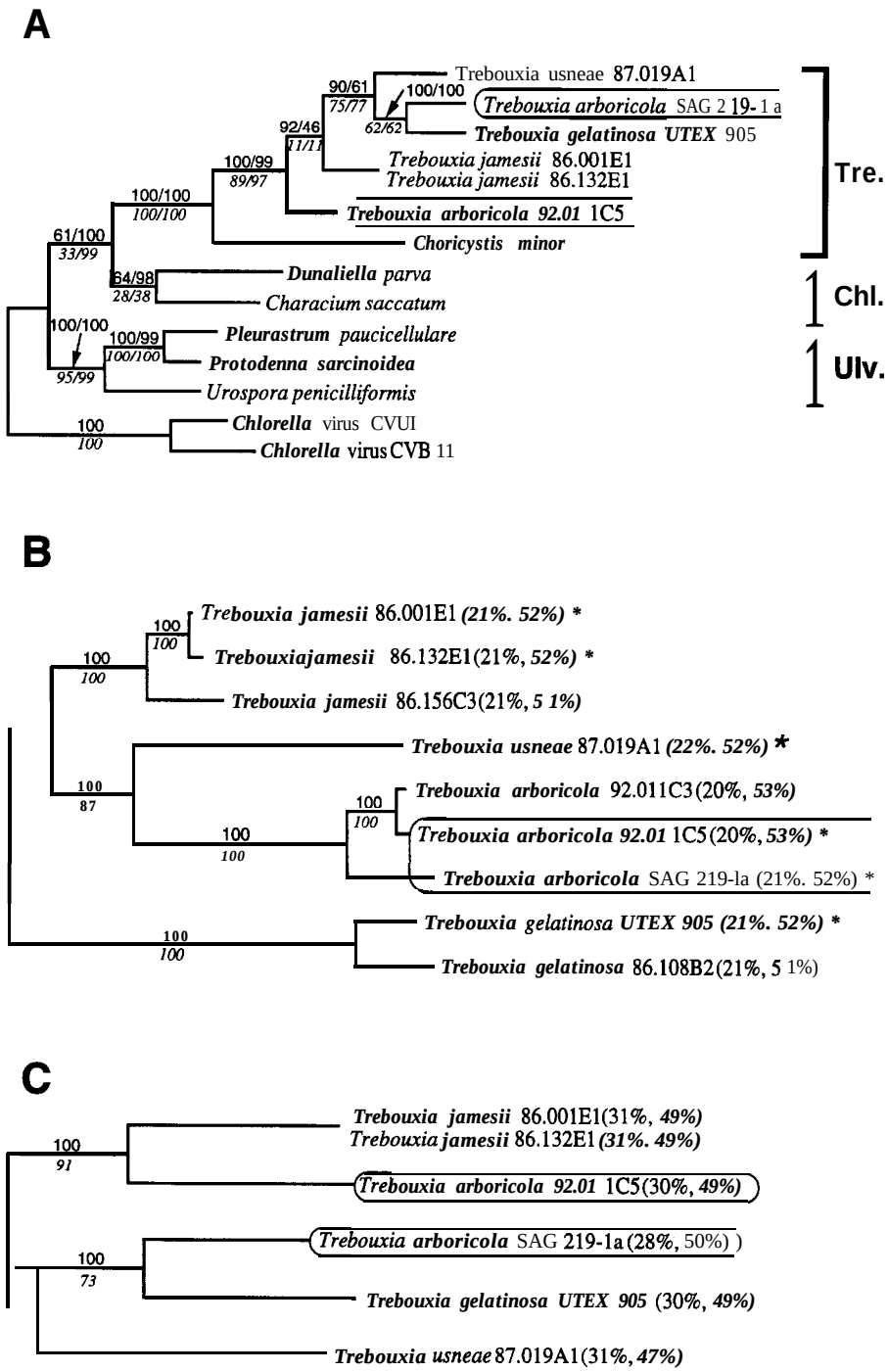

FIG. 3.-Evolutionary analyses of trebouxiophyte 15 12 group I introns and ITS regions. ***(A)*** Bootstrap consensus phylogram of 15 12 group I introns inferred with the weighted maximum-parsimony method using a branch-and-bound search and 325 aligned nucleotides; this phylogeny has a CI of 0.86 and is rooted within the branch leading to the ***Chlorella*** spp. viral group I introns. Bootstrap values (500 replications) shown above the internal nodes were inferred from the parsimony analysis with and without the viral introns as outgroup whereas the values shown below the internal nodes were inferred from a neighbor-joining (Saitou and Nei 1987) bootstrap analysis using structural distances calculated according to Kimura (1980) with and without the viral intron sequences. The following shortened taxon names are used: Tre.-Trebouxiophyceae, Chl.-Chlorophyceae, Ulv.-Ulvophyceae. (*B*) Bootstrap consensus phylogram of ITS regions from ***Trebouxia*** spp. using a weighted maximum-parsimony method and a branch-and-bound search and 629 aligned nucleotides. This tree has been midpoint rooted and has a CI of 0.96. Bootstrap values (500 replications) shown above the internal nodes were inferred from the parsimony analysis, whereas the values shown below the internal nodes were inferred from a neighbor-joining bootstrap analysis. Taxa containing the 1512 group I intron are marked with an asterisk (*).(*C*) Bootstrap consensus phylogram of 1512 group I intron sequences from ***Trebouxia*** spp. using a weighted maximum-parsimony method and a branch-and-bound search and 325 aligned nucleotides. This tree has been midpoint rooted and has a CI of 1.00. Bootstrap values (500 replications) shown above the internal nodes were inferred from the parsimony analysis, whereas the values shown below the internal nodes were inferred from a neighbor-joining bootstrap analysis. The *T. arboricola* taxa that are believed to have polyphyletic origins of their 1512 group I introns are shown boxed. The percent adenine (A) and cytidine+guanidine (C+G) contents of the ITS and 1512 intron sequences are shown in *B* and *C,* respectively. Arrows are used to indicate bootstrap values at nodes where these numbers do not fit on the branches.

the 15 12 intron had occurred promiscuously during the evolution of these algae. The 10,000 random trees were uniformly sampled from the set of all possible trees to test the distribution of tree lengths that would result if a subsample of all possible trees was evaluated (Swofford 1993). This analysis shows that the 15 12 intron data set contains significant evolutionary signal (g1 = -0.75, $P < 0.01$; Hillis and Huelsenbeck 1992) with the best trees (three most parsimonious trees) inferred from a branch-and-bound search being removed by 130 parsimony steps from the mean (570 steps) of the distribution of the 10,000 random trees.

If it is accepted that the 15 12 group I intron had a unique origin in the common ancestor of the Chlorophyceae/Trebouxiophyceae/Ulvophyceae, then its evolution can be studied in terms of its subsequent vertical inheritence, loss, and lateral transfer. To address these issues within the Trebouxiophyceae, for which the greatest sequence data exist, a phylogeny was created with the ITS sequences from nine species/strains of the lichen algal genus *Trebouxia,* and this phylogeny was compared to that of the 15 12 introns in these taxa. The maximum-parsimony and neighbor-joining analyses of the ITS sequence data (fig. 3B) show that the *Trebouxia* spp. lichen phycobionts comprise four distinct lineages *(T. jamesii, T. usneae, T. arboricola,* and *T. gelatinosa)* with significant bootstrap support for these clades. The ITS phylogeny also shows that loss of the 15 12 intron has occurred frequently in *Trebouxia* spp. *(e.g., T. jamesii* [86.156C3], *T. arboricola* [92.001C3]). Phylogenetic analyses of the *Trebouxia* spp. 15 12 group I introns also resolve four clades with one important difference from the ITS tree: the *T. arboricola* clade is now polyphyletic (fig. 3C). This result strongly suggests that the group I intron(s) in *T. arboricola* spp. are of independent origins. Since the sequenced introns from this taxon group exclusively with 15 12 introns from other *Trebouxiu* spp., it can be anticipated that one or both of the *T. arboricola* group I introns have originated from other members of this genus. Analysis of the base compositions of the 1512 introns shows that this result is likely not due to base compositional bias since these sequences have relatively conserved nucleotide compositions in *Trebouxia* spp. (i.e., 1512 group I introns: A, 28%–31%; C+G, 47%-50%).

It is not surprising that group I intron lateral transfer may have occurred between *Trebouxia* spp., since different strains/species within this genus are known to occupy the same lichen thallus (as the phycobiont) during early development of the lichen and there may be direct cell-to-cell contact during this period (Fried1 1987; Ott 1987). Full development of the lichen results in the elimination of all but one strain/species that then exclusively inhabits the lichen thallus. Examination of morphological characters of *Trebouxia* spp. phycobionts isolated from different regions of a mature lichen thallus has shown identity (unpublished data). Further, that the group I introns within the phycobionts do not originate from the mycobiont component of the lichen (or vice versa, DePriest and Been 1992) is clearly demonstrated by our data. There is no significant primary sequence or secondary structure similarity between the 1512 introns from the Trebouxiophyceae and the group I introns from the mycobionts, *Cladonia* spp. and *L. dispersa.*

Group I Intron Origins

Woodson and Cech (1989) proposed three reasons why group I intron transposition to new sites may not be limited to ORF-encoded endonucleases. First, many group I introns lack ORFs (this was also the case for the *T. thermophila* LSU intron studied by Woodson and Cech [1989], which was shown to be laterally transferred among *Tetruhymenu* spp. [Sogin et al. 19861). Second, ENase-mediated intron conversion requires extensive flanking sequence identity (14-40 nt, Lambowitz and Belfort 1993) that is not found among the various rDNA group I intron lineage insertion sites. And, third, despite the high transposition frequency to homologous sites by many ENase-encoding group I introns, transposition to new sites is not favored (Lambowitz and Belfort 1993). These data suggest that transposition by reverse splicing or loss at the RNA level may explain elements of the distribution of nuclear-encoded rDNA group I introns. To further investigate the possible role of reverse splicing in the spread of rDNA group I introns, the numerous nuclear-encoded SSU rDNA group I introns of the mycobionts *Cladonia* spp. and *L. dispersa* (all lacking ORFs) were studied with phylogenetic methods. These mycobionts are remarkable in containing up to seven group I introns in their SSU rDNA (i.e., *L. dispersa)* and in variability in intron composition between populations (DePriest 1993; Gargas, DePriest, and Taylor 1995). It is likely that the lichen mycobionts are a useful model system for studying the gain/loss of group I introns.

Results of the phylogenetic analyses of the mycobiont introns are shown in figure 4. The maximum-parsimony and neighbor-joining analyses identified, with bootstrap support, four clades within this intron family, the 1516, 1199+287, 1046 and 1216+1389 clades. Group I introns at positions 1046 and 1516 are found in the closely related *Cladonia* spp. and *L. dispersa;* these introns likely existed in the common ancestor of these mycobionts and were vertically inherited. The group I introns at positions 1216 and 1389 are also phylogenetically related to each other but are located at different sites in the rDNA of the mycobionts. These introns may share a monophyletic origin and have been laterally transferred from one site in the rDNA to another via reverse splicing. Support for this scenario comes from the identical sixmer 5' flanking sequence (5′-UGCCCU-3′) of the 1216 and 1389 group I introns whereas their 3' flanking sequences share only 2/6 identity. Similarly, the 287 and 1199 group I introns in C. *merochlorophaea* and *L. dispersa* may originate from one intron that has independently invaded these heterologous positions in the SSU rDNA of these two taxa. The 287 and 1199 introns share 6/7 5' flanking sequence identity (5′-AAC[A/G]GGU-3′) and, again, no significant similarity in the 3' flanking sequence (see fig. 4A). Support for a monophyletic origin of the 287 and 1199 group I introns in the mycobionts comes also from high

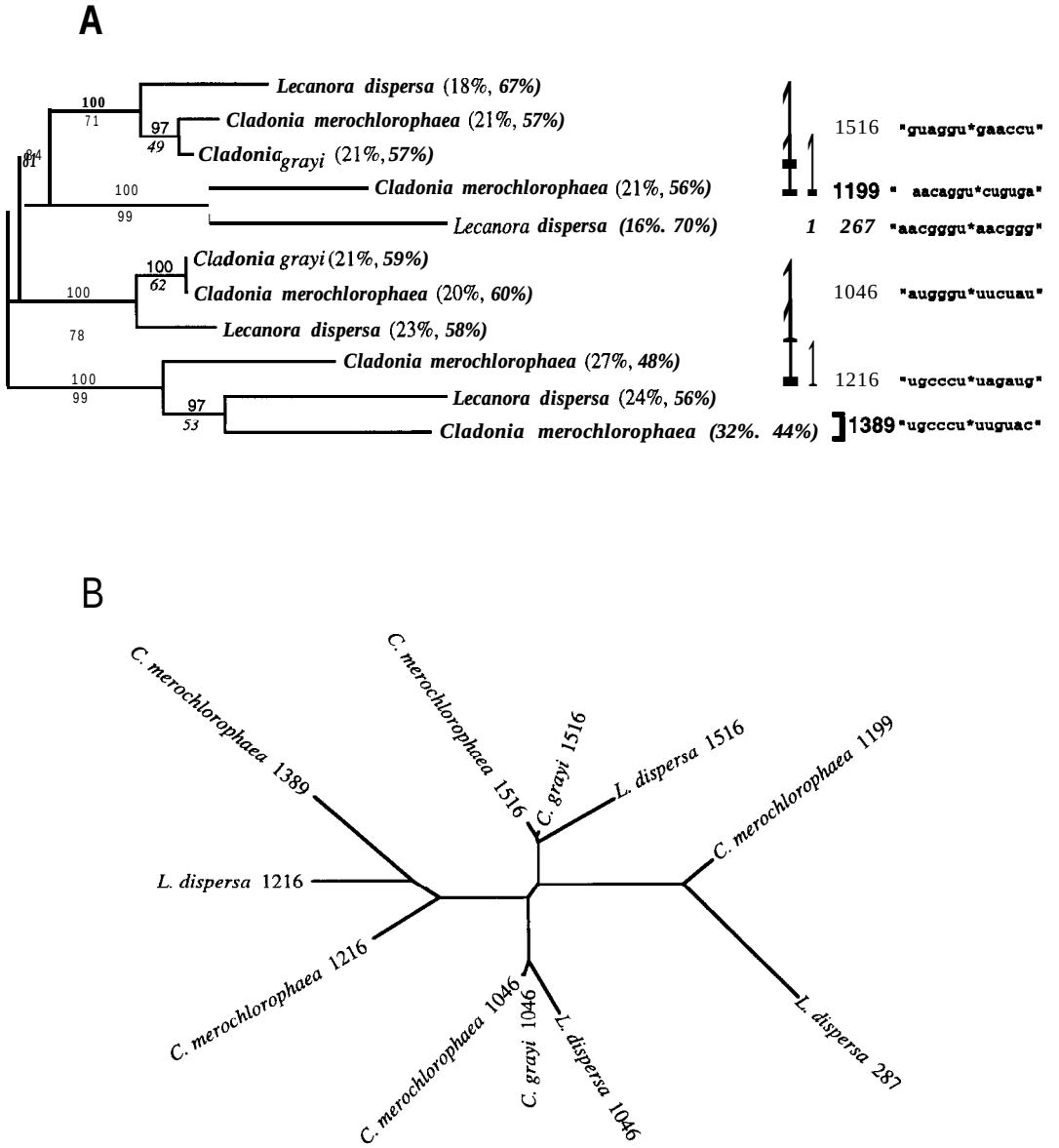

FIG. 4.-Phylogenetic analyses of group I introns from the mycobionts ***Cladonia*** spp. and *Lecanora* ***dispersa. (A)*** Bootstrap consensus phylogram of mycobiont introns using a weighted maximum-parsimony method and a branch-and-bound search and 207 aligned nucleotides. This tree has been midpoint rooted and has a CI of 0.89. Bootstrap values (500 replications) shown above the internal nodes were inferred from the parsimony analysis, whereas the values shown below the internal nodes were inferred from a neighbor-joining bootstrap analysis. The percent adenine (A) and cytidine+guanidine (C+G) contents of the mycobiont intron sequences are also shown. (B) Maximum-likelihood tree of mycobiont group I introns (fastDNAmlV1.0, Olsen et al. 1994) using the global search option with rearrangements of partial trees initially crossing one branch and rearrangements of the full tree crossing eight branches and a transition/transversion ratio of 2.

similarity (20/21 nt) that is centered around distinctive P7 and P3 sequences (i.e., 5′-AACGCAUGCAAAG[U/C]GGUGGGCC-3′; P7 and P3 sequences shown underlined, respectively) not shared by the other mycobiont introns. Since the mycobiont intron sequences have highly variable nucleotide compositions (see fig. 4*A*), a maximum-likelihood analysis was done to test the results of the maximum-parsimony and neighbor-joining analyses. The fastDNAml phylogeny (fig. 4*B*) is identical to that shown in figure 4A.

To further test the hypothesis of a reverse-splicing-mediated origin of the mycobiont 287 and 1199 introns and the 15 12 intron in the green algae, secondary structures were created of the conserved P1 and P10 domains that form the internal guide sequence (IGS) and contribute to 3′-splice site binding and facilitate the excision of the group I introns (Cech, Damberger, and Gutell 1994). The aim of this analysis was to compare the "native" P1 and P10 domains in these introns formed with the exon flanking sequences with those created when the intron is positioned in a foreign site of the SSU rDNA (i.e., after lateral transfer), the reasoning being that if reverse splicing mediated the lateral transfer of these rDNA group I introns, then it is to be expected that the introns would be able to recreate the conserved P1 and P10 helices in the putative foreign site to ensure proper

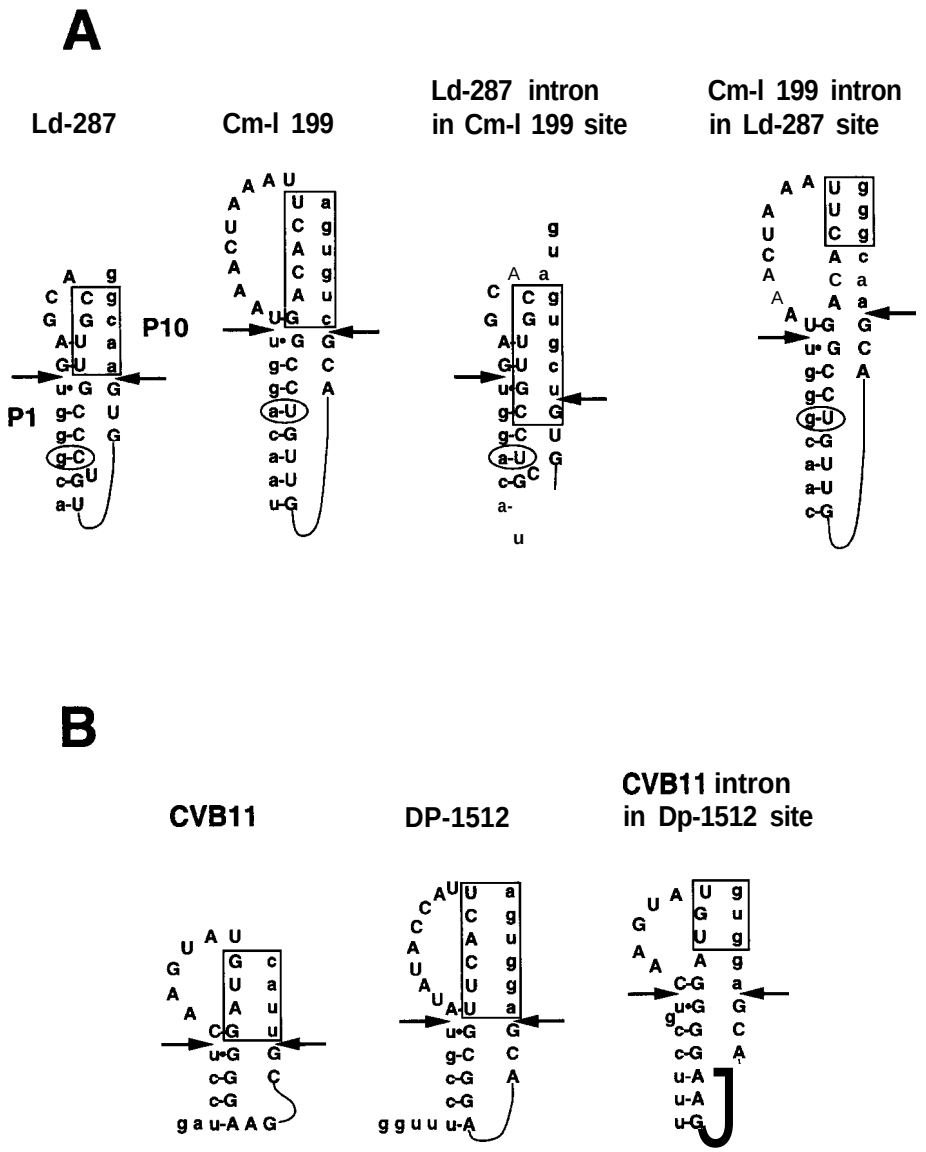

FIG. 5.—Putative secondary structures of helices P1 and P10 in group I introns. *(A)* The Pl and P10 (boxed) helices of the *Lecanora dispersa* 287 (Ld-287) and the *Cladoniamerochlorophaea* 1199 (Cm-l 199, DePriest and Been 1992) group I introns in both the "native" and heterologous positions in the SSU rRNA. The A/G base substitution in the 5' flanking sequence of the 287 and 1199 introns are circled (see text for details). (B) The Pl and P10 (boxed) helices of the *Chlorella* spp. viral (CVB11, Yamada et al. 1994) and the *Dunaliella parva* 1512 (Dp- 1512, Wilcox et al. 1992) group I introns in their "native" positions and when the viral intron is inserted into the 1512 site in the SSU rRNA. The arrows indicate the 5' and 3' splice sites in these introns with exon sequences shown in lower case and intron sequences in upper case. The intron sequences between PI and P10 are represented with a solid line.

excision and survival of the host organism. The P1 and P10 domains should remain conserved over time, although the remainder of the group I intron would be expected to accumulate mutations leading to overall intron sequence divergence.

The results of the secondary structure analyses of domains P1 and P10 are shown in figure 5 and indicate that the 287 and 1199 group I introns from the mycobionts would, theoretically, be able to form stable P1 helices in the foreign sites (fig. 5*A*); the direction of lateral transfer is not known in this case, although it is likely to have been mediated by a foreign vector (e.g., eukaryotic virus). The A/G exchange in the 5' flanking sequence of these introns is a conservative substitution that maintains the P1 base-pairing (see circled regions in fig. *5A*). Alignment of the 3' flanking sequence from the putative foreign sites of introns 287 and 1199 also shows regions that are complementary to P1 in these introns and could, potentially, form the P10 interaction. Similarly, the group I intron from the *Chlorella* spp. virus (CVB11) can form a stable P1 helix in the conserved 15 12 rDNA insertion site; there is also evidence for a P10 interaction in this case. The direction of lateral transfer is assumed to be from the virus to the alga in figure *5B*.

Conclusions

That group I introns lacking ENase coding regions may be stable genetic elements for millions of years was

first shown by the finding of a group I intron positioned in the homologous site of the $tDNA^{Leu}$ of cyanobacteria and diverse plastids (Kuhsel, Strickland, and Palmer 1990; Xu et al. 1990). These data suggest that the $tDNA^{Leu}$ intron was present in the progenitor(s) of plastids and therefore could have been stably inherited (or lost) for at least 500 million years (Kuhsel, Strickland, and Palmer 1990). Within the nuclear-encoded rDNA, phylogenetic analyses of the 1506 group I introns in the Zygnematales are consistent with the vertical inheritence of this intron for 450-500 million years (Bhattacharya et al. 1994). That ENase-lacking group I introns may also be mobile genetic elements is suggested by the close phylogenetic relationship between the ORF-lacking *Chlorella* spp. viral group I introns and the 15 12 rDNA introns, the identity of the 943 intron in *P. inouye* and the intron in the genome of Y. *japonica* (Nishida and Sugiyama 1995), the putative lateral transfer of the 15 12 intron in *T. arboricola* spp., and the phylogenetic analyses of the mycobiont introns. We interpret our data as supporting the multiple origins of nuclear-encoded rDNA introns in the green algae with subsequent loss in most taxa, stable inheritence in some, and lateral transfer in others (e.g., *T. arboricola* spp.). The complete lack of intron-encoded ENases in these taxa, the finding of a high 5' flanking sequence similarity among some introns from heterologous sites in the rDNA that share close sequence similarity, and the ability of these latter introns to form stable P1 and P10 helices in the foreign sites are consistent with the scenario that reverse splicing may have mediated the lateral transfer of these introns.

In conclusion, the availibility of the new intron sequences in the phylogenetic analyses have provided interesting insights into group I intron evolution that, taken together, suggest that most green algal nuclear-encoded rDNA group I introns originated relatively "late" (Palmer and Logsdon 1991) in evolution (i.e., after the crown group radiation). The clustering of most intron lineages on the basis of insertion sites suggests that promiscuous lateral transfers into heterologous sites are relatively rare in nuclear-encoded rDNA group I intron evolution (the exceptions are detailed above). Understanding further the role of homing of group I introns into homologous sites via a mechanism such as reverse splicing or via ENAse-mediated lateral transfer, followed by loss of the ORF, will require phylogenetic analyses of additional intron sequences.

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