

Complex Evolutionary Patterns of tRNA^{Leu}_{UAA} Group I Introns in Cyanobacterial Radiation

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Based on the findings that plastids and cyanobacteria have similar group I introns inserted into tRNA^{Leu}_{UAA} genes, these introns have been suggested to be immobile and of ancient origin. In contrast, recent evidence suggests lateral transfer of cyanobacterial group I introns located in tRNA^{Leu}_{UAA} genes. In light of these new findings, we have readdressed the evolution and lateral transfer of tRNA^{Leu}_{UAA} group I introns in cyanobacterial radiation. We determined the presence of introns in 38 different strains, representing the major cyanobacterial lineages, and characterized the introns in 22 of the strains. Notably, two of these strains have two tRNA^{Leu}_{UAA} genes, with each of these genes interrupted by introns, while three of the strains have both interrupted and uninterrupted genes. Two evolutionary distinct clusters of tRNA genes, with the genes interrupted by introns belonging to two distinct intron clusters, were identified. We also compared 16S rDNA and intron evolution for both closely and distantly related strains. The distribution of the introns in the clustered groups, as defined from 16S rDNA analysis, indicates relatively recent gain and/or loss of the introns in some of these lineages. The comparative analysis also suggests differences in the phylogenetic trees for 16S rDNA and the tRNA^{Leu}_{UAA} group I introns. Taken together, our results show that the evolution of the intron is considerably more complex than previous studies found to be the case. We discuss, based on our results, evolutionary models involving lateral intron transfer and models involving differential loss of the intron.

Group I introns share conserved sequence motifs and secondary structure (3, 20). They occur in a variety of locations: in mitochondrial, chloroplast, cyanellar, and nuclear genetic systems in eukaryotes, as well as in bacteria and bacteriophages. Many of these introns are mobile, either by the action of a homing endonuclease or possibly through reverse splicing and reverse transcription (19, 40). There has been a controversy as to whether group I introns are of early or late evolutionary origin (25, 35). In this regard, the group I introns in tRNA^{Leu}_{UAA} genes, widespread both in chloroplasts and cyanobacteria, have attracted substantial attention because of the presumed immobility and ancient origin (1, 26). These introns are anticipated to be older than the divergence of cyanobacteria and chloroplasts more than 1 billion years ago (6, 17, 41).

There is evidence for lateral transfer in the cyanobacterial radiation of group I introns located in tRNA^{fMet}_{CAU} genes (1). Recently, it has also been found that group I introns located in tRNA^{Leu}_{UAA} anticodon loops may have polyphyletic origin (30). On the basis of these findings, we have readdressed tRNA^{Leu}_{UAA} group I intron origin and evolution, both by analyzing the distribution of this intron in the cyanobacterial radiation and by comparative analysis of the 16S rDNA and tRNA^{Leu}_{UAA} intron/exon evolution. Our study is based on 16S rDNA and tRNA^{Leu}_{UAA} intron and exon sequences from a total of 38 different strains, representing the major cyanobacterial lineages. We have included several evolutionarily tightly clustered groups—based on 16S rDNA analysis—in our study, because the existence of closely related strains with and without introns may imply intron mobility (19).

MATERIALS AND METHODS

Sample and sample preparation. The organisms used in this work (Table 1) were classified according to the criteria given in *Bergey's Manual of Systematic Bacteriology* (4). Most of the strains investigated are maintained at the Norwegian Institute for Water Research (NIVA). The cultures are maintained in a constant temperature room 17 ± 2°C in growth medium Z8 (23). The strains were originally isolated from their natural habitat as single cells or filaments. The cultures at NIVA are routinely examined for contaminations by microscopy. Furthermore, all cultures used in this work have been characterized by direct sequencing of 16S rDNA without cloning. No mixed sequences were observed in these experiments. The cultures are confirmed to be unialgal (containing a single cyanobacterial strain) based on these criteria. However, the existence of uncharacterized heterotrophic bacteria in the cultures cannot be ruled out because these bacteria may be closely associated with or attached to the cyanobacteria (24). The cultivation of strains and DNA extraction for PCR amplification were done as described by Rudi et al. (32). For Southern hybridization analysis, DNA was isolated from approximately 50-mg (wet weight) cell pellets by a standard phenol-chloroform method (31).

PCR amplification and sequencing. Primer pair 5'GCGGAATGGTAGA CGCTACGGA3' (CA)-5'TGGGGGTGGGGGACTTGAC3' (CB) was constructed to selectively amplify cyanobacterial tRNA^{Leu}_{UAA} genes (30). Primer 5'CCGTCGAGTCTCTGCACCTTC3' (CR; complementary to the conserved intron element R) was constructed for amplification with primer CA. Primer 5'CAGTCTCAAATTCAGGGAAACC3' (CS; complementary to the conserved intron element P) was constructed for amplification with primer CB. Primer pairs CA-CR and CS-CB were used to amplify intron-containing genes, while primer pair CR-CS was used to generate intron-specific hybridization probes.

The PCR cycling parameters were as follows: for primer pair CA-CB, 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s; for primer pair CA-CR, 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; for primer pair CB-CS, 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and for primer pair CR-CS, 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. Between 30 and 40 cycles were used in the amplification reactions. All reactions were initiated with a 4-min denaturation at 94°C and ended with 7-min extension at 72°C. PCR amplification and direct DNA sequencing of the amplification products were done as described by Rudi et al. (32).

Southern hybridization. Approximately 1 µg of DNA was digested overnight at 37°C with 1 U of *Hind*III (Promega, Madison, Wis.). The restricted DNA was then heated to 65°C for 5 min, immediately loaded on a 1.5% agarose gel, and run at 4°C at 45 V for 5 h. The DNA was transferred and cross-linked to GeneScreen hybridization membranes as recommended by the manufacturer (NEN, Boston, Mass.). The membranes were hybridized at 50 to 55°C as described by Galau et al. (11).

Single-stranded ³²P-labeled probes were generated from PCR-amplified DNA by using a Random Primer DNA labeling kit (Boehringer Mannheim, GmbH, Mannheim, Germany) as described by Espelund et al. (7). Amplification prod-

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TABLE 1. Strains of cyanobacteria and prochlorophytes used in this study and EMBL accession numbers for tRNA^{Leu}_{UAA} intron and 16S rDNA sequences

Strain	EMBL accession no.	
	Intron	16S rDNA
Cyanobacteria		
<i>Chroococcales</i>		
<i>Microcystis</i> sp. strain NIVA-CYA 324/1	None	z82708
<i>Microcystis aeruginosa</i> NIVA-CYA 43	None	z82784
<i>Microcystis aeruginosa</i> NIVA-CYA 57	y13474	z82785
<i>Microcystis aeruginosa</i> NIVA-CYA 143	aj228694	z82786
<i>Microcystis aeruginosa</i> NIVA-CYA 228/1	aj228695	z82783
<i>Cyanothece aeruginosa</i> NIVA-CYA 258/2	None	z82775
<i>Synechococcus leopoliensis</i> NIVA-CYA 20	aj228715	z82780
<i>Synechococcus</i> sp. strain NIVA-CYA 328	None	z82779
<i>Oscillatoriales</i>		
<i>Planktothrix agardhii</i> NIVA-CYA 29	aj228702	z82796
<i>Planktothrix agardhii</i> NIVA-CYA 299	aj228701	z82799
<i>Planktothrix mougeotii</i> NIVA-CYA 11	aj228700	z82795
<i>Planktothrix prolifica</i> NIVA-CYA 320	aj228703	z82798
<i>Phormidium</i> sp. strain NIVA-CYA 203	None	z82792
<i>Phormidium</i> sp. strain NIVA-CYA 202	aj228704	z82794
<i>Phormidium</i> sp. strain NIVA-CYA 177	None	z82790
<i>Arthrospira fusiformis</i> NIVA-CYA 136/2	aj228714	z82793
<i>Tychonema bourellyi</i> NIVA-CYA 261/1	None	z82791
<i>Pseudanabaena limnetica</i> NIVA-CYA 276/6	None	z82778
<i>Spirulina subsalsa</i> NIVA-CYA 163	None	z82787
<i>Spirulina subsalsa</i> NIVA-CYA 164	None	z82788
<i>Nostocales</i>		
<i>Anabaena</i> sp. strain NIVA-CYA 267/4	None	z82802
<i>Anabaena lemmermannii</i> NIVA-CYA 281/1	None	z82797
<i>Anabaena lemmermannii</i> NIVA-CYA 83/1	None	z82801
<i>Anabaena lemmermannii</i> NIVA-CYA 266/1	None	z82800
<i>Aphanizomenon gracile</i> NIVA-CYA 103	aj228706	z82806
<i>Aphanizomenon flos-aquae</i> NIVA-CYA 142 ^a	aj228697	z82809
	aj228707	
<i>Nostoc</i> sp. strain NIVA-CYA 246	aj228705	z82803
<i>Nostoc</i> sp. strain NIVA-CYA 124	aj228711	z82776
<i>Nostoc</i> sp. strain NIVA-CYA 194	aj228708	z82805
<i>Nostoc</i> sp. strain NIVA-CYA 308 ^a	aj228696	z82804
	aj228712	
<i>Nostoc commune</i>	aj228709	y12687
<i>Nostoc flagelliforme</i>	aj228710	y12688
<i>Pleurocapsales</i>		
<i>Chroococciopsis thermalis</i> PCC 7203	None	z82789
<i>Dermocarpella incassata</i> PCC 7326	None	z82807
<i>Pleurocapsa minor</i> PCC 7327	aj228713	z82810
<i>Dermocarpa violacea</i> PCC 7301	None	z82777
Prochlorophytes		
<i>Prochlorales</i>		
<i>Prochlorothrix hollandica</i> NIVA-5/89	aj228698	z82782
<i>Prochlorothrix</i> sp. strain NIVA-8/90	aj228699	z82781

^a Strain with two introns.

ucts with primers CA-CB, not containing introns, from *Anabaena lemmermannii* NIVA-CYA 266/1 were used as a tRNA^{Leu}_{UAA} exon-specific probe. The intron-specific probe was generated by nested amplification with the primer pair CR-CS of a gel-purified intron-containing PCR product amplified with the primer pair CA-CB from *Nostoc* sp. strain NIVA-CYA 194.

Phylogenetic reconstruction. The sequences were aligned both manually and by the computer algorithm PILEUP from the Genetics Computer Group (Madison, Wis.) Wisconsin Package (13). Sites that appeared to be ambiguously aligned were not considered in the phylogenetic analysis. For the 16S rDNA data, 489 aligned positions (nucleotides [nt] 346 to 845 relative to the *Escherichia coli* rDNA sequence) were considered in the phylogenetic analysis; for the tRNA^{Leu}_{UAA} intron data, 346 positions were considered. For positions in the intron data set where only a subset of the data could be aligned, the missing characters were substituted by N's for the other taxa. All tRNA^{Leu}_{UAA} sequences used in the analysis were sequenced in this work, while the 16S rDNA sequences are from

Rudi et al. (32), with the addition of *Nostoc commune* and *Nostoc flagelliforme* (EMBL accession no. y12687 and y12688, respectively).

Separate phylogenetic trees were constructed with the neighbor-joining method (33) from the Trecon software package (39), the maximum-parsimony method (10) from Phylogenetic Analysis Using Parsimony (PAUP; version 3.1.1) and the minimal-evolution method from PAUP* 4.0 developed by D. L. Swofford (Illinois Natural History Survey, Champaign), and finally the maximum-likelihood method (8) using the Phylogeny Inference Package (PHYLIP; version 3.5) developed by J. Felsenstein (Department of Genetics, University of Washington, Seattle). The Kimura two-parameter model (16), with a transversion: transition weight of 2:1, was used to compute the distance matrices for the neighbor-joining analysis. The minimal evolution tree was constructed by using LogDet distances (19a). A heuristic tree search on the LogDet distances was conducted with parameters set to default values. For the maximum-parsimony analysis, the heuristic search algorithm implemented in the PAUP package was used to find the shortest trees. The rescaled consistency index was calculated for each position in the alignment, and this criterion was then used in the tree construction. To investigate the phyletic structure of the data, the tree length skewness was also determined for 100,000 randomly generated trees and compared to critical values given by Hillis and Huelsenbeck (14). In the maximum-likelihood analysis, we used a transition: transversion ratio of 2 and empirically determined base frequencies. To infer the confidence levels of the branch points in the constructed tree, bootstrap analysis (9) was used. Consensus trees were constructed from 500 bootstrap replicates for the neighbor-joining analysis and from 100 replicates for both the maximum-parsimony analysis and the tRNA^{Leu}_{UAA} intron maximum-likelihood and minimal-evolution analyses. Because of the extensive computational requirements, only 40 replicates were used for the 16S rDNA maximum-likelihood analysis. Unfortunately, we could not use the minimal-evolution analysis with LogDet distances for 16S rDNA because of the extensive computational requirements.

We also tested whether the tree topologies generated with the 16S rDNA alignment and the tRNA^{Leu}_{UAA} intron data for the same taxa were significantly different. This analysis was done with the Templeton-Felsenstein test (38) for user-defined trees, as implemented in the DNAPARS program of the PHYLIP package.

RESULTS

The characterization of tRNA^{Leu}_{UAA} group I introns was done both by screening for the presence or absence of introns by PCR and Southern hybridization analysis and by comparative sequence analysis of intron and 16S rDNA evolution.

Intron distribution determined by PCR amplification. The PCR primer pair (CA-CB) complementary to the tRNA^{Leu}_{UAA} exon generated PCR products with sizes corresponding to tRNA genes both with (270 to 365 nt) and without (approximately 60 nt) introns. In addition, double bands suggest the presence of two intron-containing genes in the strains *Aphanizomenon flos-aquae* NIVA-CYA 142 and *Nostoc* sp. strain NIVA-CYA 308 (Fig. 1). Strains belonging to the *Microcystis* category gave amplification products with the predicted lengths of genes both with and without introns, as previously reported by Rudi and Jakobsen (30). All strains generating bands cor-

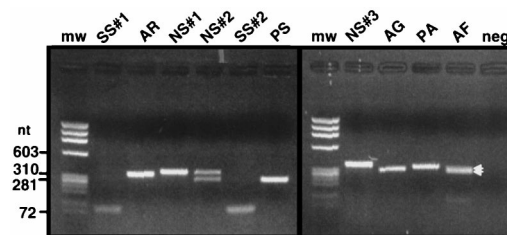


FIG. 1. Amplification products with the tRNA^{Leu}_{UAA} exon-specific primer pair CA-CB. The PCR products were electrophoresed in an ethidium bromide-stained 1.5% agarose gel for 30 min at 100 V. Twenty percent of the amplification product was loaded in each lane. Strain abbreviations: SS#1, *Spirulina subsalsa* NIVA-CYA 164; AR, *Arthrospira fusiformis* NIVA-CYA 136/2; NS#1 and NS#2, *Nostoc* sp. strains NIVA-CYA 194 and 308; SS#2, *Spirulina subsalsa* NIVA-CYA 163; PS, *Phormidium* sp. strain NIVA-CYA 202; NS#3, *Nostoc* sp. strain NIVA-CYA 124; AG, *Aphanizomenon gracile* NIVA-CYA 103; PA, *P. agardhii* NIVA-CYA 29; AF, *A. flos-aquae* NIVA-CYA 142. The two intron bands amplified for *A. flos-aquae* NIVA-CYA 142 (see arrows) are not properly separated in this gel. mw, molecular weight standard; neg, negative control.

		anticodon domain	variable region - extra loop	
		*****##*=====*****##*		
APH. FLOS. N-C 142 ^a	CYANOB	CTTAAAATCCGTCGGGG-CA----	ACCCGTGAGG	C Y A N O B
NOSTOC SP. N-C 308 ^b	CYANOB	YTTWAAATCCGTCGGG--CATNNNNNNNNNNNN		
MICR. AER. N-C 143	CYANOB	CTTAAAATCCGTCGGG--CTTT--	GCCTTTGGGG	
PLANKT. AG. N-C 299	CYANOB	CTTAAAATCCGTCGA--CCCTTATAAAGGTCGTGG		
PLEUR. MINOR. P 7327	CYANOB	CTTAAAATCCGTCGA--CCCT-AAAAGGTCGTGAGG		
SYNECH. LE. N-C 20	CYANOB	CTTAAAATCCGTCGA--CTGTAAAAGGTCGTGAGG		
ARTH. FUS. N-C 136/2	CYANOB	CTTAAAATCCGTCGA--CCTTCACCCGTCGTGG		
PHORM. SP. N-C 202	CYANOB	CTTAAAATCCGTCGG--CTGGAGACGCGTGG		
PROCHL. SP. N 8/90	CYANOB	YTTAAAATCCGTCGA--CC----GTAG-GTGAGG		
APH. FLOS. N-C 142 ^a	CYANOB	CTTAAAATCCGTCGA--CTTTAAAACGGTCGTGGG		
NOSTOC SP. N-C 308 ^b	CYANOB	CTTAAAATCCGTCGA--CGTAAAANNNNNNNNNN		
NOSTOC SP. N-C 246	CYANOB	CTTAAAATCCGTCGA--CCTTAAAACGGTCGTGG		
CYANOPHORA PARAD.	CYANEL	CTTAAAATCCGTCGA--TTGTATAGATCGTGAGG		
MARCHANTIA POLYM.	CHLORO	CTTAAAATCCGTCGG--CTTTAAAGACCGTGAGG		
ORYZA SATIVA	CHLORO	CTTAAAATCCGTCGA--CTTTTATAAGTCGTGAGG		
ZEA MAYS	CHLORO	CTTAAAATCCGTCGA--CTTTTATAAGTCGTGAGG		
SORGHUM BICOLOR	CHLORO	CTTAAAATCCGTCGA--CTTTTAAAATCGTGAGG		
NICOTIANA TABACUM	CHLORO	CTTAAAATCCGTCGA--CTTTAAAATCGTGAGG		
VICIA FABA	CHLORO	CTTAAAATCCGTCGA--CTTTAAAATCGTGAGG		
EUGLENA GRACILIS	CHLORO	CTTAAAATCCGTCG---CTT-ATPAAAGCGTACCG		
MYCOPLASMA CAPRIC.	EUBACT	CTTAAAATCCGTCGGG--CTTAAATPCCGTCGCG		
MYCOPLASMA PNEUMO.	EUBACT	CTTAAAATCCGTCGATAGCA---ATAGCGTGCCG		
ACHOLEPLASMA LAID.	EUBACT	CTTAAAATCCGTCGTCGATAA---AAGCCATGTCC		
STREPTOMYCES COEL.	EUBACT	CTTAAAATCCGTCGTCGCTT--CGAGGGCGTGCCG		
STAPHYLOCC. AURE.	EUBACT	CTTAAAATCCGTCGCGTGTAGT--GATCACCGTACCG		
BACILLUS SUBTILIS	EUBACT	CTTAAAATCCGTCGCGTGTAGT--GACTACCGTGCCG		
E. COLI	EUBACT	CTTAAAATCCGTCGCGTGTAGT--CGCTGTGCGG		
AZORARCUS SP. BH72	EUBACT	CTTAAAATCCGTCGCGTGTAGT--GACTACCGTGCCG		
HAEMOPHILUS INFLU.	EUBACT	CTTAAAATCCGTCGCG---CTTTCG-ACGCGTGCCA		
PHAGE T4	VIRUS	CTTAAAATGCTGCGGAATGATTT--CCTGTGGGT		

FIG. 2. Compilation of tRNA^{Leu}_{UAA} exon sequences. All the tRNA^{Leu}_{UAA} sequences in the tRNA database (36) were compiled and compared to the partial exon sequences identified in this work. The figure includes the two putative classes A and B of tRNA^{Leu}_{UAA} genes found for the cyanobacteria (CYANOB) in this work. *, conserved position (conserved in 90% or more of the species); #, position conserved only for cyanobacteria and chloroplasts (CHLORO), i.e., conserved in 90% or more of the cyanobacteria and chloroplasts and 40% or less conserved for the other species; =, variable position (position 80% or less conserved for all species); ^a, interrupted by cluster II intron; ^b, interrupted by cluster I intron. EUBACT, eubacteria. Species abbreviations: APH. FLOS., *Aphanizomenon flos-aquae*; MICR. AER., *Microcystis aeruginosa*; PLANKT. AG., *Planktothrix agarhii*; PLEUR. MINOR., *Pleurocapsa minor*; SYNECH. LE., *Synechococcus leopoliensis*; ARTH. FUS., *Arthrospira fusiformis*; PHORM., *Phormidium*; PROCHL., *Prochlorothrix*; CYANOPHORA PARAD., *Cyanophora paradoxa*; MARCHANTIA POLYM., *Marchantia polymorpha*; MYCOPLASMA CAPRIC., *Mycoplasma capricolum*; MYCOPLASMA PNEUMO., *Mycoplasma pneumoniae*; ACHOLEPLASMA LAID., *Acholeplasma laidlawii*; STREPTOMYCES COEL., *Streptomyces coelicolor*; STAPHYLOCC. AURE., *Staphylococcus aureus*; E.COLI., *Escherichia coli*; HAEMOPHILUS INFLU., *Haemophilus influenzae*.

responding to uninterrupted genes were also screened with the primer pairs CA-CR and CS-CB (primers CR and CS are complementary to the highly conserved intron regions R and P, respectively), enabling selective amplification of intron-containing genes. No amplification products were obtained with these primer pairs for the strains without introns, while the strains verified to contain introns (by amplification with the CA-CB primer pair) also gave amplification products with CA-CR and CS-CB primers (results not shown).

Identification of tRNA^{Leu}_{UAA} genes. For all sequenced PCR products, the exons flanking the introns contain both the anticodon and the conserved sequences 5'CTTGAAATCCGT3' in the anticodon loop and stem typical for cyanobacterial tRNA^{Leu}_{UAA} genes (Fig. 2) (36). The UAA anticodon was also identified by sequencing of the amplification products for the strains lacking introns (results not shown). We found two putative clusters of tRNA^{Leu}_{UAA} genes in the cyanobacterial radiation. In the following discussion, these clusters are designated types A and B, respectively (Fig. 2).

Identification of intron sequences. All of the inserted sequences have the regions corresponding to P, Q, R, and S (Fig. 3), which are generally conserved among group I introns (20). Our data set defines two clear intron clusters (clusters I and II), as judged from the conserved intron regions. The introns belonging to cluster I are inserted into the type A tRNA genes, while introns belonging to cluster II are inserted into type B genes. In each of the strains shown to have two intron-containing tRNA^{Leu}_{UAA} genes, *A. flos-aquae* NIVA-CYA 142 and *Nostoc* sp. strain NIVA-CYA 308, both cluster I and cluster II introns are present.

The intron belonging to cluster I for the strain *Planktothrix agardhii* NIVA-CYA 29 has a secondary structure resembling the characteristic structure of group I introns (Fig. 4). The secondary structure of the intron sequences belonging to cluster II has previously been reconstructed for *Microcystis aeruginosa* NIVA-CYA 57 (30). Both of these structures resemble what is expected for group I introns belonging to the IC3 subgroup (20).

		conserved intron elements				
		P	Q	R	S	
species	strains	intron (bp)				
Micr. aer.	N-C143	226	TCAAATTCGGGGAAACCT	GGCAATCCCAGCCAAGC	GAAGGTGTAGAGACTAGACGGG	GGTGAAGGGATAGTCCA
Micr. aer.	N-C228/1	226
Micr. aer.	N-C57	226
Nostoc sp.	N-C308i2	226G.....
Aph. flos-aquae	N-C142i2	209TC.A.....
Prochlor. holl.	N-C5/89	267A.....T.....C.....C.....A.....G.....
Prochlor. sp.	N-C8/90	269A.....T.....C.....C.....A.....G.....
Aph. gracile	N-C103	238C.A.....T.....C.....C.....A.....G.....
Aph. flos-aquae	N-C142i1	237C.A.....T.....C.....C.....A.....G.....
Nostoc sp.	N-C124	298C.A.....T.....C.....CT.....A.....G.....
Nostoc sp.	N-C308i1	296C.R.....C.....T.....C.....CC.....A.....G.....
Nostoc sp.	N-C194	~295C.A.....T.....C.....CC.....A.....G.....
Nostoc commune		302C.A.....T.....C.....CC.....A.....G.....
Nostoc flagelliforme		274C.A.....T.....C.....C.....A.....G.....
Plankt. moug.	N-C11	258A.....T.....C.....C.....A.....A.G.....
Plankt. ag.	N-C299	258A.....T.....C.....C.....A.....A.G.....
Plankt. ag.	N-C29	259A.....T.....C.....C.....A.....A.G.....
Plankt. prol.	N-C320	259A.....T.....C.....C.....A.....A.G.....
Phorm. sp.	N-C202	216A.....T.....C.....C.....A.....G.....
Nostoc sp.	N-C246	248C.A.....T.....C.....C.....A.....A.G.....
Arthrospira fus.	N-C136/2	269C.A.....T.....C.....C.....A.....G.....
Synech leop.	N-C20	240C.A.....T.....C.....A.....A.....G.....
Pleur. minor	PCC7327	250A.....T.....C.....CA.....A.....A.G.....

FIG. 3. Conserved intron elements. The intron elements P, Q, R, and S, generally conserved among group I introns (20), are shown for the cyanobacterial introns characterized in this work. The two introns present in *A. (Aph.) flos-aquae* NIVA-CYA 142 and *Nostoc* sp. strain NIVA-CYA 308 are annotated with i1 and i2, respectively. Dots indicate identity to *M. aeruginosa* (Micr. aer.) NIVA-CYA 143, while lines indicate gaps in the alignment. Prochlor. holl., *Prochlorothrix hollandica*; Plankt. moug., *Planktothrix mougeotii*; Plankt. ag., *P. agardhii*; Plank. prol., *P. prolifica*; Phorm., *Phormidium*; Arthrospira fus., *Arthrospira fusiformis*; Synech leop., *Synechococcus leopoliensis*; Pleur., *Pleurocapsa*.

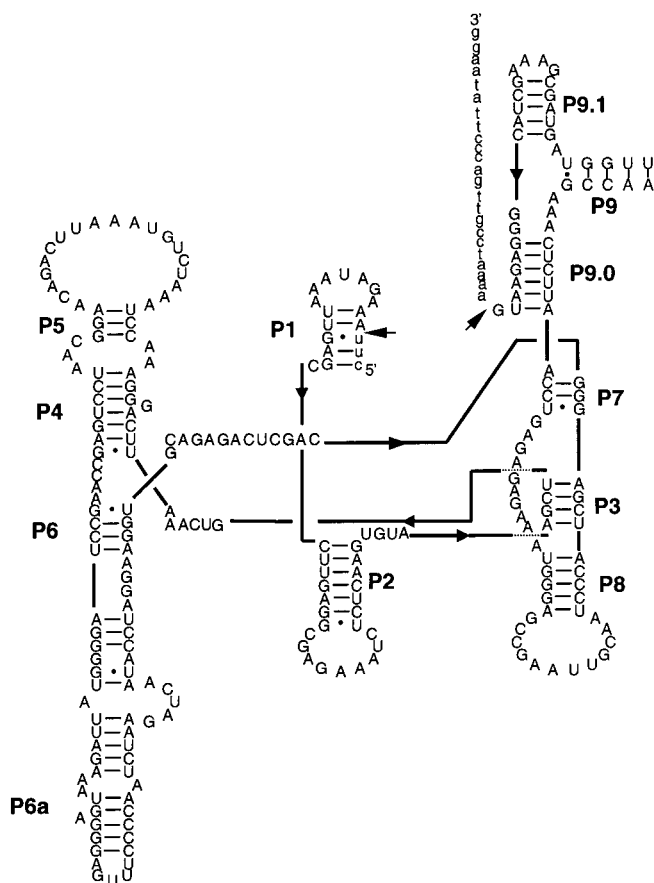


FIG. 4. Secondary structure of the *P. agardhii* NIVA-CYA 29 intron (cluster I). The structure is shown in the format described by Cech et al. (5), with marked secondary structure elements (P1 to P9). The sequences in lowercase letters represent exon sequence, while arrows indicate splice sites.

Distribution of introns in the *Nostoc* category determined by Southern hybridization. Selected strains belonging to the *Nostoc* category with both intron-containing genes and genes without introns (as determined by PCR) were used in Southern hybridization analysis. Only a single hybridizing band was identified for all strains with a tRNA^{Leu}_{UAA} exon probe generated from *Anabaena lemmermannii* NIVA-CYA 266/1 (Fig. 5A). This suggests that the two tRNA genes identified by PCR in *Nostoc* sp. strain NIVA-CYA 308 and in *A. flos-aquae* NIVA-CYA 142, respectively, are located on the same restriction fragments. All *Nostoc* strains containing introns gave hybridizing bands with a intron probe generated from *Nostoc* sp. strain NIVA-CYA 194 (Fig. 5B), while *Anabaena lemmermannii* NIVA-CYA 266/1 did not give a hybridizing band with this probe. This finding, together with the PCR results, shows that *Anabaena lemmermannii* NIVA-CYA 266/1 has an uninterrupted tRNA^{Leu}_{UAA} gene.

Comparison of 16S rDNA and tRNA^{Leu}_{UAA} intron evolution. The tree length distribution skewness ($g_1 = -0.557$) shows that the 16S rDNA evolutionary tree contains significant phylogenetic signals ($P < 0.01$) (11). We could identify eight statistically supported groups of strains (*Nostoc*, *Planktothrix*, *Phormidium/Tychonema*, *Spirulina*, *Dermocarpa/Dermocarpella*, *Microcystis*, *Synechococcus*, and *Prochlorothrix*) (Fig. 6A). None of the strains in the *Phormidium/Tychonema*, *Spirulina*, and *Dermocarpa/Dermocarpella* groups contain introns (as inferred from PCR). In the *Nostoc*, *Synechococcus*, and *Microcystis*

groups, on the other hand, there are both intron-containing strains and strains without introns. All strains in the *Planktothrix* and *Prochlorothrix* groups contain introns.

The tRNA^{Leu}_{UAA} intron data (346 aligned positions) provide significant phylogenetic signals ($g_1 = -0.90$) ($P < 0.01$) (11). In general, the minimal evolution analysis with LogDet distances gave the tree with the best statistical support. There is a large base composition bias for the different introns; the LogDet algorithm corrects for such biases (19a). We were able to identify statistically supported groups for the introns in *Aphanizomenon*, *Nostoc* sp. strain NIVA-CYA 308/124, and *N. commune/Nostoc* sp. strain NIVA-CYA 194 in the *Nostoc* category, in addition to the group formed by *Nostoc* sp. strain NIVA-CYA 246/*Phormidium* sp. strain NIVA-CYA 202. We also identified the introns found in *Planktothrix*, *Prochlorothrix* and *Microcystis* as phylogenetic groups (Fig. 6B). The phylogenetic reconstruction also confirm the clustering (cluster I and II) defined from the conserved intron regions (compare Fig. 3 and 6B).

The intron groups belonging to cluster I for the *Nostoc* strains are relatively distantly related to each other (Fig. 6B), while all *Nostoc* strains are clustered in the 16S rDNA evolutionary tree (Fig. 6A). Furthermore, the intron located in the strain *Nostoc* sp. strain NIVA-CYA 246 is more closely related to the intron in strain *Phormidium* sp. NIVA-CYA 202 (88% identity) compared to the other *Nostoc* introns belonging to cluster I (85 to 86% identity). In contrast, *Nostoc* sp. strain NIVA-CYA 246 is approximately 95% identical to the other *Nostoc* strains for 16S rDNA, while for this locus NIVA-CYA 246 shows only 88.5% identity to *Phormidium* sp. NIVA-CYA 202 (Fig. 6A). This discrepancy is also supported by a phylogenetic reconstruction involving 346 aligned intron positions (Fig. 6B) and by an analysis involving 204 unambiguously aligned positions where only the *Nostoc* introns, *Phormidium* sp. strain NIVA-CYA 202, and *Synechococcus leopoliensis* NIVA-CYA 20 (outgroup) were considered. In the last analysis, NIVA-CYA 246 and 202 formed a separate clade from the other *Nostoc* introns, with bootstrap values of 66, 75, and 79% for the maximum-likelihood, maximum-parsimony, and neighbor-joining analyses, respectively (data not shown). The introns in *Nostoc* sp. strains NIVA-CYA 308 and 124 also form a clade in the phylogenetic reconstruction displayed in Fig. 6B, while for 16S rDNA these organisms are located on separate branches. The introns in *Prochlorothrix* (NIVA-CYA

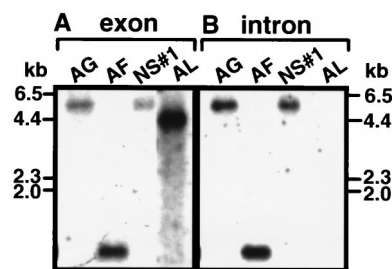


FIG. 5. Southern hybridization using a tRNA^{Leu}_{UAA} exon (A) or an intron (B) probe for selected *Nostoc* strains. The exon probe was generated from CA-CB primer amplification products for *Anabaena lemmermannii* NIVA-CYA 266/1 (AL; a strain confirmed to have an uninterrupted tRNA^{Leu}_{UAA} gene). The intron probe was obtained from an internal fragment of the intron from the strain *Nostoc* sp. strain NIVA-CYA 194 amplified with primer pair CR-CS. Genomic DNA digested with *Hind*III was separated, blotted, and hybridized as described in Materials and Methods. The same membrane was used for each of the hybridization experiments, providing exact assignment of the hybridizing bands from different experiments. The molecular weight standard is *Hind*III-digested λ DNA. Strain abbreviations are as for Fig. 1.

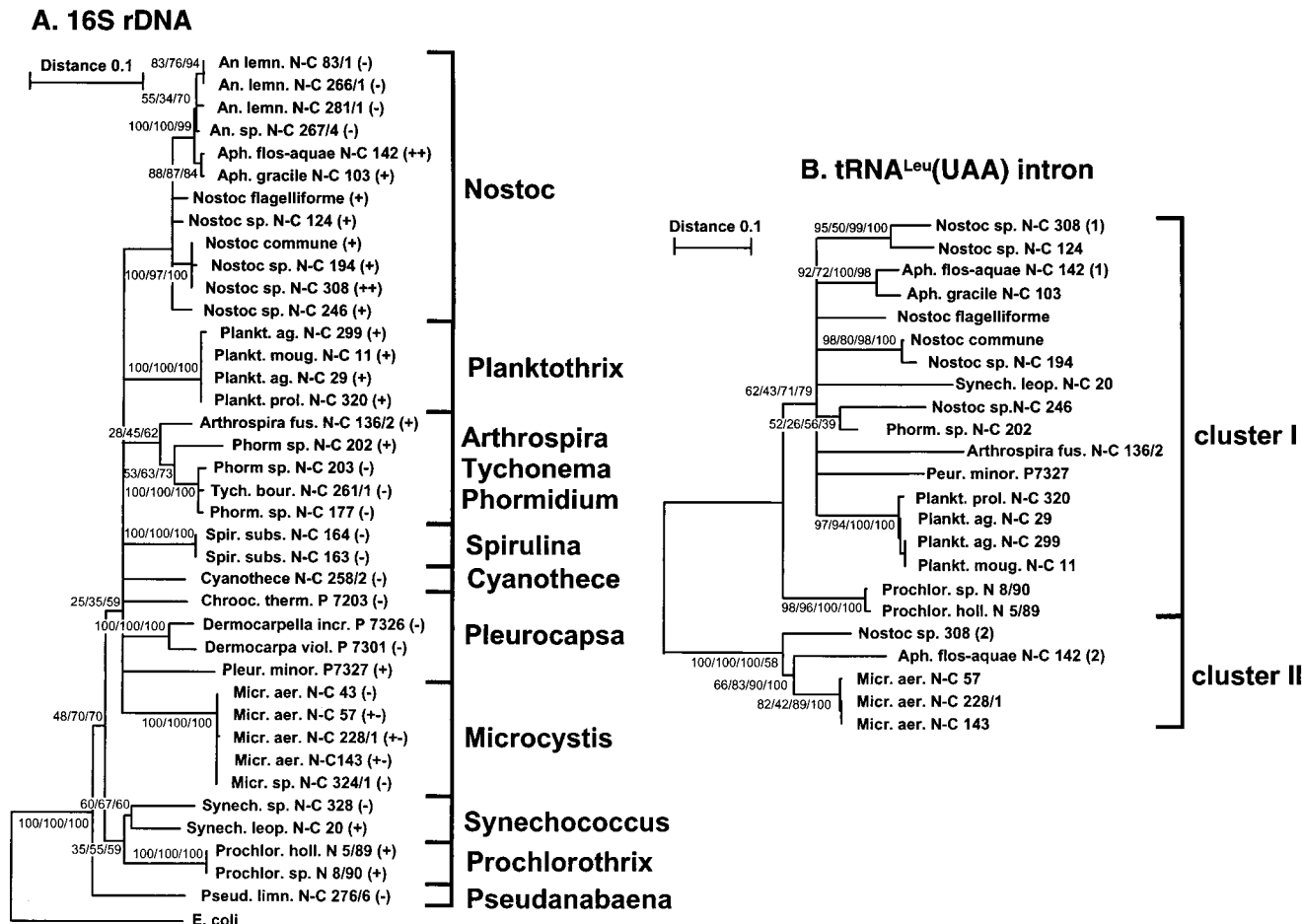


FIG. 6. Evolutionary trees for 16S rDNA (A) and tRNA^{Leu}_{UAA} introns sequences (B). The trees were built using the neighbor-joining, maximum-parsimony, and maximum-likelihood methods. The trees shown in panels A and B (based on 489 and 346 aligned positions, respectively) are consensus trees for the branches supported by $\geq 25\%$ of the bootstrap trees in all of the phylogenetic methods tested. The genetic distances between two strains are Kimura distances expressed in substitutions per nucleotide in the neighbor-joining tree. Numbers at the nodes (minimal evolution [for the intron tree]/maximum likelihood/maximum parsimony/neighbor joining) indicate the percentage of the bootstrap trees in which the cluster descending from the node was found. In panel A, the presence and absence of introns are indicated with (+) and (-), respectively. The respective clone numbers (N-C, NIVA-CYA; N, NIVA; P, Pasteur) are given for each branch. Species abbreviations: An. lemn., *Anabaena lemmermannii*; Tych. bour., *Tychonema bourleyi*; Spir. subs., *Spirulina subsalsa*; Cyanothecae, *Cyanothecae aeruginosa*; Chrooc. therm., *Chroococcidiopsis thermalis*; Dermocarpella incr., *Dermocarpella incrassata*; Dermocarpella viol., *Dermocarpella violacea*; Pseud. limn., *Pseudanabaena limnetica*. All other species are abbreviated as in Fig. 2 and 3.

8/90 and 5/89) and *S. leopoliensis* NIVA-CYA 20 are in separate clades, while for 16S rDNA these organisms are located in the same clade. Finally, the intron belonging to cluster II for *A. flos-aquae* NIVA-CYA 142 is closer to the *Microcystis* introns compared to the *Nostoc* sp. NIVA-CYA 308 intron (Fig. 6B).

The evolutionary patterns for the introns seem dissimilar in the distinct lineages as defined from 16S rDNA phylogenetic reconstruction (Fig. 6A). There are both strains without introns and strains with evolutionarily divergent introns in the clustered *Nostoc* lineage. In the tightly clustered *Planktothrix* group, on the other hand, all strains contain evolutionarily closely related introns. Finally, in the *Microcystis* group, closely related introns are present in some *Microcystis* strains, while other strains contain no introns (30).

We used the Templeton-Felsenstein test (38) implemented in the DNAPARS program in the PHYLIP software package to further test whether the 16S rDNA and tRNA^{Leu}_{UAA} intron topologies are significantly different for strains containing introns belonging to cluster I. In this comparison, we used unambiguous alignments of 479 positions for 16S rDNA

and 204 positions for introns belonging to cluster I. The DNAPARS program was used to generate the tree topologies for the 18 strains with introns belonging cluster I. The shortest trees generated with both data sets were used as user-defined trees for the tRNA^{Leu}_{UAA} data. The 16S rDNA tree required 246 steps to explain the data, while the tRNA^{Leu}_{UAA} tree required 221 steps. The variance in step differences as determined by the step differences at individual positions for the two trees is 7.37, which is a significant difference according to the Templeton-Felsenstein test.

DISCUSSION

Two tRNA^{Leu}_{UAA} genes. Since the strains used in our study are confirmed to be unialgal (see Materials and Methods), there still may be a low level of commensals in the cultures. Hypothetically, the two clusters of group I introns could reside in tRNA^{Leu}_{UAA} genes in different bacteria. However, this is unlikely because the PCR primers used in this work were designed for selective amplification of cyanobacterial and chloroplast tRNA^{Leu}_{UAA} genes, excluding other known eubacterial genes

(36). Both the type A and B tRNA^{Leu}_{UAA} genes identified in this work group with the chloroplast genes, confirming a cyanobacterial origin of these gene clusters (Fig. 2). Furthermore, no introns have yet been identified in tRNA^{Leu}_{UAA} genes from other eubacteria (26). Taken together, this is strong evidence that the intron-containing tRNA^{Leu}_{UAA} genes are of cyanobacterial origin. Two divergent elongator tRNA^{Met}_{CAU} genes (73% identity) have also been identified in *Methanococcus jannaschii*, a methanogenic archaeon (2). Furthermore, studies of a tRNA operon in gamma purple bacteria suggests that tRNA operons can be hot spots for rearrangements and that gene duplications and deletions are common evolutionary events (12).

Introns belonging to each of the intron clusters I and II are inserted in the A and B tRNA^{Leu}_{UAA} genes, respectively (Fig. 2). As shown by Rudi and Jakobsen (30), introns belonging to cluster II are closer related to introns located in tRNA^{Arg}_{CCU} and tRNA^{Leu}_{CAU} genes (21a, 27) than to the tRNA^{Leu}_{UAA} introns belonging to cluster I. This finding suggests ancient lateral transfer of the intron between different tRNA genes.

Possible mechanisms for intron evolution. There are strains with and without introns in both the evolutionarily tightly clustered (as defined from 16S rDNA) *Microcystis* group (introns belonging to cluster II) and the *Nostoc* group (introns belonging to cluster I). This finding can be interpreted as relatively recent gain and/or loss of the introns in these groups. Furthermore, there are four examples where topologies are different for the 16S rDNA and the intron trees (compare Fig. 6A and B). The tree topology generated for the introns belonging to cluster I is also significantly different from that of the 16S rDNA topology.

There are different evolutionary patterns for group I introns located in the distinct clustered groups defined from 16S rDNA (Fig. 6), which may indicate variance in the stability of introns located in the different lineages. Group I introns without open reading frames (ORF's) can possibly transpose through reverse transcription and splicing (19, 21, 29, 40, 42). The reverse transcriptase activity in prokaryotes can be provided from a retron element (37), for example. Retron elements are of special interest because they are mobile and not universally distributed among closely related strains (15, 28), which could explain a possible difference in evolutionary patterns for the different lineages. Experiments addressing whether lateral transfer for tRNA group I introns is correlated with reverse transcriptase activity are thus called for.

Homing endonucleases may be mobile elements, e.g., through invasion of group I introns (18, 34). Because only one of seven identified tRNA^{Met}_{CAU} introns contains a putative homing endonuclease ORF (1), this ORF might be mobile. Indeed, the current tRNA^{Leu}_{UAA} introns distribution can also be explained assuming a mobile homing endonuclease for this intron, although no ORFs have yet been detected. Hypothetically, homing endonucleases conferring mobility to introns might also be encoded in *trans*. Thus, further work is needed to address whether there exists a homing endonuclease for the tRNA^{Leu}_{UAA} intron.

Is lateral transfer a main force in the evolution of tRNA group I introns in cyanobacteria? It has been suggested that the evolution of tRNA^{Leu}_{UAA} group I introns is fundamentally different from that of tRNA^{Met}_{CAU} introns (1, 26). The tRNA^{Leu}_{UAA} introns were anticipated to be stable and of ancient origin (1, 17, 26, 41), while the tRNA^{Met}_{CAU} introns were suggested as recent invaders of the genomes (1, 26).

An intron stability scenario for tRNA^{Leu}_{UAA} group I introns is, according to Paquin et al. (26), more parsimonious than a mobility scenario. They explain the distribution of group I introns in the cyanobacterial radiation (26) by four intron

losses and suggest that the intron mobility theory will require at least 13 insertions into each intron-containing strain. On the other hand, they also noted that the intron distribution could be explained through four insertions at earlier evolutionary stages into the intron-containing lineages. However, Paquin et al. (26) preferred the stability scenario, arguing that that an intron loss is more likely than an intron gain.

Paquin et al. (26) assume in their evolutionary model, based on the parsimony principle, that the data contain no homoplasy, that is, several events of gain or loss in a single branch. Such an assumption cannot be justified statistically without knowing the actual likelihood of these events. To our knowledge, the likelihood of intron gain or loss in the cyanobacterial radiation has never been calculated.

Several putative events of tRNA^{Leu}_{UAA} intron loss and/or gain could be traced in the cyanobacterial radiation in our data. Notably, we identified intron-negative strains in both the *Nostoc* lineage (confirmed by Southern hybridization) and the *Synechococcus* lineage. According to Paquin et al. (26), these two lineages contain only intron-positive strains. We also identified intron-positive strains in the *Microcystis* lineage, which the same authors found to contain only intron-negative strains. Thus, our data do not support a model involving only a few events of loss of an ancient intron (26). Our data may in fact indicate homoplasy, with events of intron gain and/or loss in several of the cyanobacterial lineages. The intron also seems vertically inherited in some of the clustered groups, so that the balance between intron gain and/or loss is still unknown. Finally, our data suggested no fundamental differences in the tRNA^{Leu}_{UAA} evolutionary patterns compared to the structurally similar tRNA^{Met}_{CAU} group I introns.

An argument for the intron stability view for tRNA^{Leu}_{UAA} introns is that the cyanobacterial ancestor of chloroplasts, engulfed by a eukaryote more than 1 billion years ago, contained a tRNA^{Leu}_{UAA} and not a tRNA^{Met}_{CAU} intron (26). There is fairly good evidence for a monophyletic origin of chloroplasts (6, 22). Thus, the presence of tRNA^{Leu}_{UAA} introns and the absence of the intron in tRNA^{Met}_{CAU} genes in chloroplasts may simply be explained by that the chloroplast ancestor coincidentally contained a tRNA^{Leu}_{UAA} gene with an intron and a tRNA^{Met}_{CAU} gene without intron, as seen for many of the current cyanobacterial species. The reason for the apparent stability of the intron in chloroplasts may be that the introns require a bacterial environment for mobility, e.g., reverse transcriptase activity or homing endonucleases.

With the data presented here, the evolution of the intron located in the tRNA^{Leu}_{UAA} gene is considerably more complex than previous studies have found to be the case. Our data suggest that an evolutionary model involving both lateral transfer and differential loss of the intron should be considered. However, experimental data demonstrating the properties of the introns are probably needed to resolve the tRNA^{Leu}_{UAA} intron stability-versus-intron mobility controversy.

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