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Sporadic Distribution of tRNA$_{CCU}^{Arg}$ Introns among α-Purple Bacteria: Evidence for Horizontal Transmission and Transposition of a Group I Intron

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A group I intron interrupts the tRNA$_{CCU}^{Arg}$ gene of the α-purple bacterium *Agrobacterium tumefaciens* (B. Reinhold-Hurek and D. A. Shub, Nature [London] 357:173–176, 1992). In this study, we assess the distribution of the corresponding intron among 12 additional species of α-purple bacteria. Of 10 newly identified tRNA$_{CCU}^{Arg}$ genes, we found only two that contained an intron homologous to that of the *Agrobacterium* tRNA$_{CCU}^{Arg}$ intron. This restricted and scattered distribution of the tRNA$_{CCU}^{Arg}$ intron among α-purple bacteria is consistent with a recent origin and horizontal transmission. Primary and secondary structural similarities between tRNA$_{UAA}^{Leu}$ introns found in strains of the cyanobacterium *Microcystis aeruginosa* (K. Rudi and K. S. Jacobsen, FEMS Microbiol. Lett. 156:293–298, 1997) and α-purple tRNA$_{CCU}^{Arg}$ introns suggest that these introns share a more recent common ancestor than either does with other known cyanobacterial tRNA$_{UAA}^{Leu}$ introns.

Group I introns are present in cellular and viral genes in eukaryotes and eubacteria (6, 15, 16). Although these introns interrupt a number of different protein-coding and RNA genes of eukaryotes and bacteriophages, the only insertion site observed so far in eubacteria is the sequence of the anticodon loop of tRNA genes. Eubacterial group I introns were first identified in the tRNA$_{UAA}^{Leu}$ genes of five cyanobacterial species (13, 22), prompting speculation that these introns would be widely distributed among cyanobacteria. In conjunction with the observation that most plastid genomes also possessed a homologous intron, an ancient origin predating the endosymbiotic event that gave rise to plastids was proposed for the tRNA$_{UAA}^{Leu}$ intron (13, 22). The phylogenetic distribution of tRNA$_{UAA}^{Leu}$ introns among cyanobacteria was later determined, and it was shown that the distribution was not universal but, nevertheless, was consistent with an ancient origin (17). This conclusion was challenged by the discovery of tRNA$_{UAA}^{Leu}$ introns in some strains of the cyanobacterium *Microcystis aeruginosa* which, however, seem to have originated independently of the previously characterized cyanobacterial tRNA$_{UAA}^{Leu}$ introns through horizontal transfer (19). Conversely, tRNA fMet group I introns are sporadically distributed among cyanobacteria (17), a finding that corroborated the initial suggestion of a relatively recent origin of these introns during cyanobacterial evolution (3).

It has previously been demonstrated that two other eubacterial tRNA genes are interrupted by group I introns, the tRNA$_{CAU}^{Ile}$ gene of *Azoarcus* sp. BH72 (a β-purple bacterium) and the tRNA$_{CCU}^{Arg}$ gene of *Agrobacterium tumefaciens* A136 (an α-purple bacterium) (18). Initial data suggested that similar introns are likely to be widespread among proteobacteria,

as the *Azoarcus* and *Agrobacterium* intron probes cross-hybridized to genomic DNA from a number of purple bacteria (11, 18). Because determining their phylogenetic distribution is a valuable tool to assess the evolutionary history of group I introns (2, 17), we decided to survey the distribution of the tRNA$_{CCU}^{Arg}$ intron among α-purple bacteria.

A tRNA$_{CCU}^{Arg}$ intron in *Azospirillum halopraeferens*. It has been shown that a restriction fragment from the genomic DNA of the α-purple bacterium *A. halopraeferens* Au5 hybridized to an *Azoarcus* intron probe (18). The signal was also observed when the Southern hybridization was repeated with an *Agrobacterium* tRNA$_{CCU}^{Arg}$ intron probe (11). The hybridizing region was cloned in pBSM13 as a 4.3-kb *Pst*I-*Sst*I restriction fragment (pAGAU1.1) and subsequently subcloned as a 400-bp *Ava*II fragment (pAGAU1.2). The latter insert was completely sequenced on both strands, revealing a tRNA$_{CCU}^{Arg}$ gene interrupted by a potential group I intron inserted after the U of the CCU anticodon (the same position as the *Agrobacterium* intron). The 217-bp *Azospirillum* intron sequence folds into a bona fide group I secondary structure (Fig. 1) and shares 69% identity with the *Agrobacterium* intron (Table 1). In addition, like the *Agrobacterium* intron, it self-splices in vitro (11, 18).

PCR amplification of tRNA$_{CCU}^{Arg}$ genes from various α-purple bacteria. Based upon the exonic tRNA sequences of *Agrobacterium* and *Azospirillum*, we designed degenerate primers for amplifying tRNA$_{CCU}^{Arg}$ genes from various α-purple bacteria. We performed PCRs (17) on either the extracted DNAs or cell pellets from 11 species, with the primers ARG-5′ (5′-GTCC[G/A/T]CGATGCTCA[G/A][C/T]A[A/G]GATA-3′) and ARG-3′ (5′-TGGTGTCC[G/A/C]C[G/T][G/A][G/C][G/A/T]GGA[A/T]TCGAACC-3′). These primers were expected to amplify a sequence that includes the entire anticodon stem and loop of the tRNA. Although PCR products of ~75 bp, the expected size of an uninterrupted tRNA gene, were amplified from all the samples, a PCR product of approximately 300 bp, the expected size of an intron-containing gene was also amplified from the DNA of *Anaplasma marginale* (Fig. 2). Cloning and sequencing of three independent clones confirmed the identity of this product as a tRNA$_{CCU}^{Arg}$ gene interrupted by a group I intron inserted after the U of the CCU anticodon. The *Anaplasma* intron is similar both in

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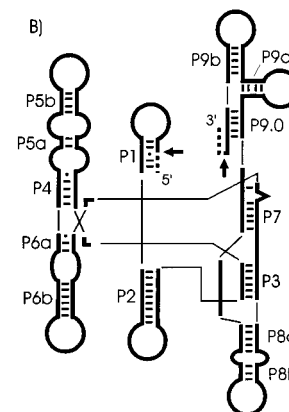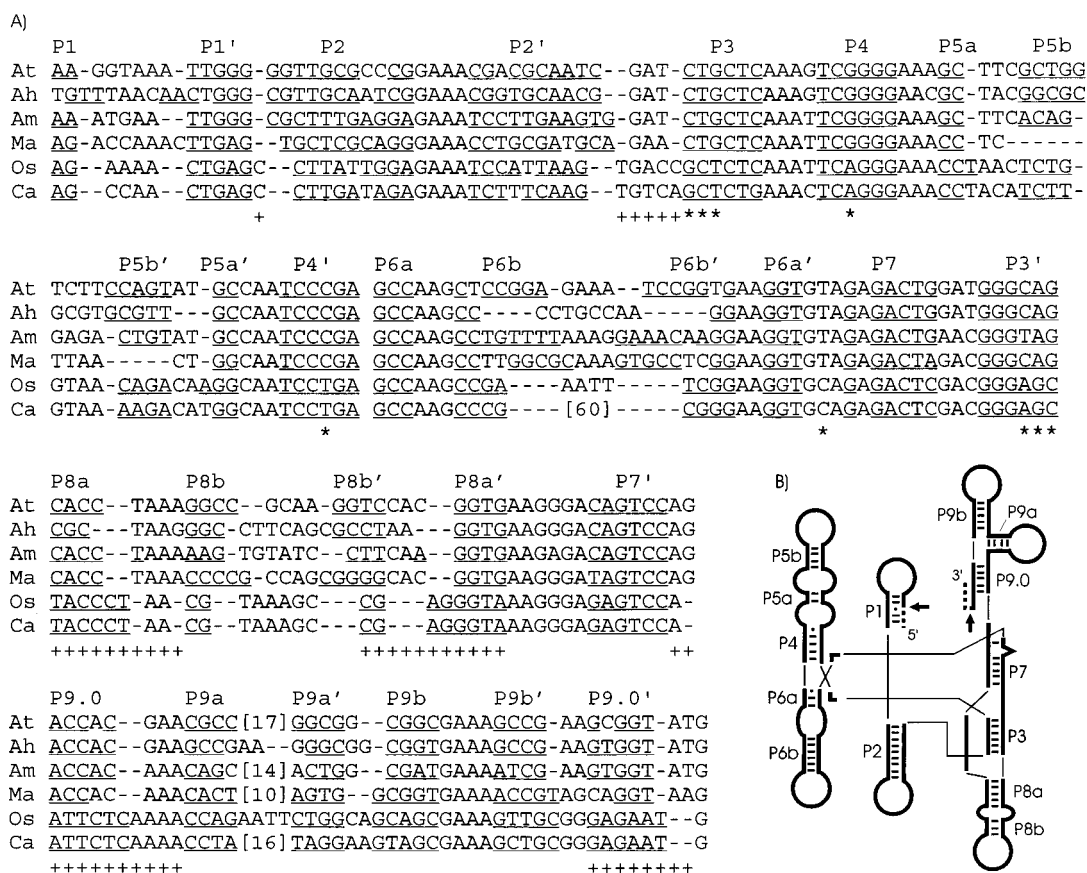
A)

```
     P1        P1'      P2              P2'             P3           P4    P5a    P5b
At  AA-GGTAAA--TTGGG-GGTTGCGCCCGGAAACGACGCAATC--GAT-CTGCTCAAAGTCGGGGAAAGC-TTCGCTGG
Ah  TGTTTAACAACTGGG-CGTTGCAATCGGAAACGGTGCAACG--GAT-CTGCTCAAAGTCGGGGAACGC-TACGGCGC
Am  AA-ATGAA--TTGGG-CGCTTTGAGGAGAAATCCTTGAAGTG-GAT-CTGCTCAAATTCGGGGAAAGC-TTCACAG-
Ma  AG-ACCAAACTTGAG--TGCTCGCAGGGAAACCTGCGATGCA-GAA-CTGCTCAAATTCGGGGAAACC-TC-----
Os  AG--AAAA--CTGAGC--CTTATTGGAGAAATCCATTAAG--TGACCGCTCTCAAATTCAGGGAAACCTAACTCTG-
Ca  AG--CCAA--CTGAGC--CTTGATAGAGAAATCTTTCAAG--TGTCAGCTCTGAAACTCAGGGAAACCTACATCTT-
                 +                             +++++***          *
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         P5b'   P5a'    P4'     P6a       P6b          P6b'    P6a'   P7           P3'
At  TCTTCCAGTAT-GCCAATCCCGA GCCAAGCTCCGGA-GAAA--TCCGGTGAAGGTGTAGAGACTGGATGGGCAG
Ah  GCGTGCGTT---GCCAATCCCGA GCCAAGCC----CCTGCCAA-----GGAAGGTGTAGAGACTGGATGGGCAG
Am  GAGA-CTGTAT-GCCAATCCCGA GCCAAGCCTGTTTTAAAGGAAACAAGGAAGGTGTAGAGACTGAACGGGTAG
Ma  TTAA-----CT-GGCAATCCCGA GCCAAGCCTTGGCGCAAAGTGCCTCGGAAGGTGTAGAGACTAGACGGGCAG
Os  GTAA-CAGACAAGGCAATCCTGA GCCAAGCCGA----AATT-----TCGGAAGGTGCAGAGACTCGACGGGAGC
Ca  GTAA-AAGACATGGCAATCCTGA GCCAAGCCCG----[60]-----CGGGAAGGTGCAGAGACTCGACGGGAGC
          *                                           *                ***
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```
     P8a      P8b       P8b'      P8a'      P7'
At  CACC--TAAAGGCC--GCAA-GGTCCAC--GGTGAAGGGACAGTCCAG
Ah  CGC---TAAGGGC-CTTCAGCGCCTAA---GGTGAAGGGACAGTCCAG
Am  CACC--TAAAAAG-TGTATC--CTTCAA--GGTGAAGAGACAGTCCAG
Ma  CACC--TAAACCCCG-CCAGCGGGGCAC--GGTGAAGGGATAGTCCAG
Os  TACCCT-AA-CG--TAAAGC---CG---AGGGTAAAGGGAGAGTCCA-
Ca  TACCCT-AA-CG--TAAAGC---CG---AGGGTAAAGGGAGAGTCCA-
    ++++++++++       +++++++++++             ++
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     P9.0        P9a      P9a'     P9b      P9b'     P9.0'
At  ACCAC--GAACGCC[17]GGCGG--CGGCGAAAGCCG-AAGCGGT-ATG
Ah  ACCAC--GAAGCCGAA--GGGCGG-CGGTGAAAGCCG-AAGTGGT-ATG
Am  ACCAC--AAACAGC[14]ACTGG--CGATGAAAATCG-AAGTGGT-ATG
Ma  ACCAC--AAACACT[10]AGTG--GCGGTGAAAACCGTAGCAGGT-AAG
Os  ATTCTCAAAACCAGAATTCTGGCAGCAGCGAAAGTTGCGGGAGAAT--G
Ca  ATTCTCAAAACCTA[16]TAGGAAGTAGCGAAAGCTGCGGGAGAAT--G
    ++++++++++               +++++++
```

B)

FIG. 1. Intron sequence alignment and secondary structures. (A) DNA sequences of tRNA$^{Arg}_{CCU}$ introns of the α-purple bacteria *A. tumefaciens* (At) (18), *A. halopraeferens* (Ah), and *A. marginale* (Am) are aligned with tRNA$^{Leu}_{UAA}$ introns from three distantly related cyanobacteria, *M. aeruginosa* (Ma) (19), *Oscillatoria* PCC 6304 (Os) (17), and *Calothrix* PCC 7101 (Ca) (17). Phylogenetically conserved base-paired regions (P1 to P9) (15) are indicated, with putative base-pairings underlined. Proposed base-pairing patterns of the *Microcystis* intron have been altered from the original report (19) to better conform to the conserved secondary structure of group I introns (15). Numbers in brackets indicate omitted sequence, and dashes represent gaps introduced to improve the alignment. Primary sequence (*) or secondary structural (+) similarities pointing to a closer relationship of the *Microcystis* tRNA$^{Leu}_{UAA}$ introns to the tRNA$^{Arg}_{CCU}$ introns, rather than to the other known cyanobacterial tRNA$^{Leu}_{UAA}$ introns, are shown below the alignment. Note that these emphasized primary and secondary structural motifs are conserved among the cyanobacterial tRNA$^{Leu}_{UAA}$ introns (17), excluding the *Microcystis* introns. (B) Schematic representation of the consensus secondary structure of eubacterial tRNA group I introns, drawn according to Cech et al. (5). Phylogenetically conserved stems (P1 to P9), Watson-Crick base pairs (bars), and G-U pairs (dots) are shown according to Michel and Westhoff (15). Exons (dashed lines), the intron (thick lines), and splice sites (arrows) are indicated. Thin lines are used to join helical domains.

primary sequence and secondary structure to the other known tRNA$^{Arg}_{CCU}$ group I introns (Fig. 1; Table 1).

To substantiate the apparent absence of introns in the tRNA$^{Arg}_{CCU}$ genes of the other 10 species (Fig. 2), we sequenced up to 10 different tRNA-sized clones for each of them. Interestingly, the primers amplified various tRNA^{Arg} genes, not exclusively tRNA$^{Arg}_{CCU}$ genes as intended (Table 2). For instance, the tRNA-sized PCR product from *Anaplasma* resulted from the amplification of a cognate tRNA$^{Arg}_{CCG}$ gene, not from a second, intronless copy of the tRNA$^{Arg}_{CCU}$ gene. In 4 of the 10 species we surveyed, the tRNA$^{Arg}_{CCU}$ gene was not amplified. Alignment of tRNA gene sequences revealed that the first four nucleotides of tRNA$^{Arg}_{YCU}$ as amplified by our initial set of primers were invariably 5'-GAGC-3' (Fig. 3), whereas most of the other tRNA^{Arg} sequences started with 5'-GAGT-3' (data not shown). We exploited this discrepancy at the fourth position by designing a primer, ARG-5'EXT (5'-CC[G/A/T]CGA TGCTCA[G/A][C/T]A[A/G]GATAGAGC-3'), to specifically amplify tRNA$^{Arg}_{YCU}$ from those four species for which we failed to detect the tRNA$^{Arg}_{CCU}$ gene. Using the ARG-5'EXT and ARG-3' primers, we were able to amplify the tRNA$^{Arg}_{CCU}$ genes from two additional species (Table 2). All PCR mixtures were subjected to Southern hybridization with a radiolabeled *Agrobacterium* intron-specific probe, and as expected, only the intron-containing product of *Anaplasma* hybridized to the probe (data not shown). In summary, we identified a tRNA$^{Arg}_{CCU}$ gene from 11 of 13 species, three of which contained an intron (Table 2). We could not amplify the corresponding gene from two species, *Rhizobium etli* and *Rickettsia prowazekii*. The ge-

TABLE 1. Identity of intron and small subunit rRNA sequences[a]

	% Identity					
	At	Ah	Am	Ma	Os	Ca
At		69	66	66	47	47
Ah	85		62	56	45	45
Am	83	80		63	53	51
Ma	77	77	76		50	54
Os	76	74	73	85		85
Ca	74	73	72	83	83	

[a] Values above the diagonal are from intron sequences, those below are from rRNA sequences. At, *A. tumefaciens*; Ah, *A. halopraeferens*; Am, *A. marginale*; Ma, *M. aeruginosa*; Os, *Oscillatoria* PCC 6304; Ca, *Calothrix* PCC 7101.

TABLE 2. PCR results and distribution of the tRNA$_{CCU}^{Arg}$ intron

Species	tRNA^{Arg} genes amplified[a]					Intron in tRNA$_{CCU}^{Arg}$
	CCU	UCU	ACG	CCG	Total	
Agrobacterium tumefaciens A136[b]						+[d]
Anaplasma marginale	3	0	0	3	6	+
Azospirillum halopraeferens Au5[b]						+
Bradyrhizobium japonicum	1	2	4	0	7	−
Ehrlichia risticii	1	2	1	0	4	−
Erythrobacter longus OCh 119	0 (1)	0 (1)	6 (1)	4	13	−
Methylobacterium extorquens	1	2	7	0	10	−
Paracoccus denitrificans	1	1	0	2	4	−
Rhizobium etli CE3	0	3 (4)	0	7 (2)	17[c]	?
Rhodobacter sphaeroides ATH 2.4.1	0 (2)	10	0	0	12	−
Rhodocista centenaria	1	0	0	0	1	−
Rhodospirillum rubrum S.1	2	0	0	0	2	−
Rickettsia prowazekii	0	3 (7)	7	0	18[c]	−[e]

[a] The identities of the amplified genes were determined by the anticodon sequence of their encoded tRNAs. PCR was performed with the pair of primers ARG-5′ and ARG-3′ or ARG-5′EXT and ARG-3′ (numbers in parentheses). After these data were obtained we discovered that the nucleotides complementary to positions 8 and 9 of the *Agrobacterium* tRNA of both 5′ PCR primers were transposed, resulting in potential mismatches. Both primers were resynthesized to be complementary at those positions, and PCR revealed no new products indicating the presence of an intron. Sequencing of 12 clones from these PCRs of *R. etli* DNA revealed the same anticodon species that had been determined previously. Two clones were determined from PCR of *R. prowazekii* DNA; neither had an anticodon for Arg.

[b] These species were not screened by PCR. The data were deduced from genomic clones.

[c] A tRNA$_{UUU}^{Lys}$ gene (*R. etli*) and a tRNA$_{GCU}^{Asn}$ gene (*R. prowazekii*) were also amplified by PCR with the ARG-5′EXT and ARG-3′ primers.

[d] Data from reference 18.

[e] The tRNA$_{CCU}^{Arg}$ gene is lacking from the *R. prowazekii* genome (1).

nome of *R. prowazekii* has been completely sequenced and, as in some other bacterial genomes (e.g., *Mycoplasma genitalium* [10] and *Haemophilus influenzae* [9]), the tRNA$_{CCU}^{Arg}$ gene is lacking (1). Assuming a G-U wobble, tRNA$_{UCU}^{Arg}$ can substitute for tRNA$_{CCU}^{Arg}$ in decoding AGG triplets. Therefore, it is possible that the tRNA$_{CCU}^{Arg}$ gene is also missing from the genome of *R. etli*.

Distribution and evolution of the tRNA$_{CCU}^{Arg}$ intron among α-purple bacteria. The 13 species we have selected for this study represent only a sampling of the diversity of α-purple bacteria. Nevertheless, the distribution of the tRNA$_{CCU}^{Arg}$ intron is informative because of the phylogenetic relationship among the species we surveyed. We believe the distribution of tRNA$_{CCU}^{Arg}$ introns is best explained by a recent origin and horizontal transmission, rather than by an ancient origin and differential loss in various α-purple lineages. Our reasoning is as follows. First, the three species possessing a tRNA$_{CCU}^{Arg}$ intron, *A. tumefaciens*, *A. halopraeferens*, and *A. marginale*, are widely divergent α-purple bacteria (Fig. 4; see also the phylogenetic tree compiled by the Ribosomal Database Project [RDP] in reference 14). Second, *A. marginale* and *Ehrlichia risticii* branch within a monophyletic clade of closely related species, but only the *Anaplasma* tRNA$_{CCU}^{Arg}$ gene contains an intron. The same is also true for *A. halopraeferens* (intron present) and *Rhodocista centenaria* (also known as *Rhodospirillum centenum*) (no intron).

Group I intron mobility is mediated by intron-encoded homing endonucleases or, alternatively, occurs via reverse splicing (for a review of group I intron mobility, see reference 21). Unlike reverse splicing, in which only the intron sequence is transferred, endonuclease-dependent mobility is accompanied by coconversion of flanking sequences. Interestingly, pairwise comparisons of the amplified regions of the tRNA genes shown in Fig. 3 reveal a striking similarity among the three intron-containing species (1, 2, or 3 differences, respectively, in 30 bp). On the other hand, with the exception of two identical pairs (*E. risticii*-*Bradyrhizobium japonicum* and *Methylobacterium extorquens*-*R. centenaria*) all of the other pairwise comparisons display 6 to 17 differences with a mean of 11 differ-

ences (note that, as expected, the 7-bp sequence defining the anticodon loop is virtually identical among all 11 species). Considering the fact that the three intron-containing species are not particularly closely related (Fig. 4), this observation is reminiscent of a recent endonuclease-dependent invasion. Although none of the introns described in this study contains an open reading frame (ORF), it would not be surprising if an ORF-containing intron is present in some other, unsurveyed species. For example, in a recent survey of cyanobacteria, tRNA-fMet genes of seven distantly related species were interrupted by highly similar introns. However, only one of these introns contained an endonuclease-encoding ORF (4, 17).

Recently, Rudi and Jacobsen showed that group I introns in tRNA$_{UAA}^{Leu}$ genes were sporadically distributed in strains of the cyanobacterium *M. aeruginosa*; six introns were found in 16 strains (19). Three of these introns were sequenced and shown to be almost identical (>99.5% identity) but only <61.5% identical to the previously described cyanobacterial tRNA$_{UAA}^{Leu}$ introns (19), whereas the latter are highly similar among themselves (Table 1; see also reference 17). Therefore, it was sug-
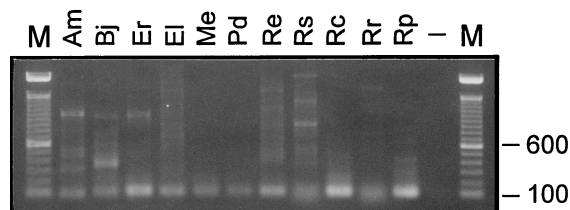

FIG. 2. PCR amplification of tRNA^{Arg} genes. The migration in a 1% agarose gel of the PCR amplification products from DNA or cell pellets of various α-purple bacteria is shown. Am, *A. marginale*; Bj, *B. japonicum*; Er, *E. risticii*; El, *Erythrobacter longus*; Me, *M. extorquens*; Pd, *Paracoccus denitrificans*; Re, *R. etli*; Rs, *Rhodobacter sphaeroides*; Rc, *R. centenaria*; Rr, *Rhodospirillum rubrum*; Rp, *R. prowazekii*; −, no DNA (negative control); M, 100-bp DNA marker (Gibco/ BRL) (sizes are indicated on the right side of the gel). Some of the samples shown here are the results of a reamplification (Am, Bj, Me, and Pd). *R. etli* and *R. prowazekii* are the two species from which the tRNA$_{CCU}^{Arg}$ genes could not be detected.

```
              A          D              D    Ac    ↓     Ac        T           T      A
      At  GTCCACGTAGCTCAGCAGGATAGAGCACAGGATTCCTAATCCTGGGGTCGGAGGTTCGAATCCTCTCGTGGACACCA
I+    Ah  GTCCGCGTAGCTCAGCAGGATAGAGCACAGGATTCCTAATCCTGGGGCCGTGGGTTCGAATCCCGCCGCGGACACCA
      Am              GAGCACAGGATTCCTAATCCTGGGGTCGTG
      Bj              GAGCAACAGATTCCTAATCTGTGTGTCGTG
      Er              GAGCAACAGATTCCTAATCTGTGTGTCGTG
      El              ACCAGATTCCTAATCTGGGGGCCGCA
I-    Me              GAGNAAGCGTTTCCTAAACGCTAGGTCGGC
      Pd              GAGCAGCCCCTTCCTAAGGGGCAGGTTACA
      Rs                GATCCCCTCCTAAGGGATAGGTTGCA
      Rc              GAGCAAGCGTTTCCTAAACGCTAGGTCGGC
      Rr              GAGCGTTGGATTCCTAATCCAAAGGCCACA
                                       ═        ▬▬▬
```
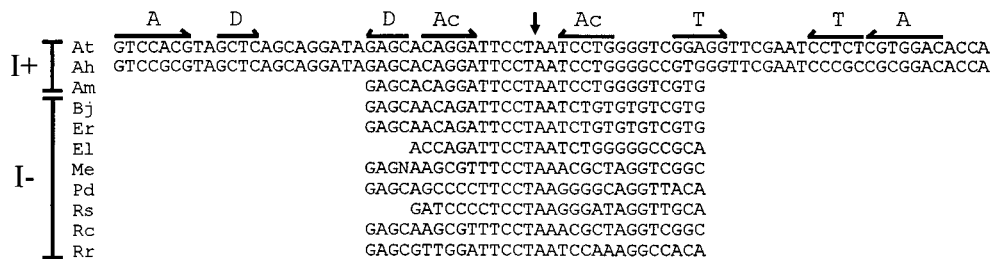
FIG. 3. Alignment of tRNA$^{Arg}_{CCU}$ gene sequences. Base-pairing in the acceptor stem (A), anticodon stem (Ac), D stem (D), and T stem (T), the intron insertion site (arrow), the anticodon sequence (underline), and the position used to restrict amplification to tRNA$^{Arg}_{YCU}$ genes (double underline) are indicated. N, any of the four possible nucleotides. I+ and I−, intron plus and minus, respectively. At, *A. tumefaciens*; Ah, *A. halopraeferens*; Am, *A. marginale*; Bj, *B. japonicum*; Er, *E. risticii*; El, *E. longus*; Me, *M. extorquens*; Pd, *P. denitrificans*; Rs, *R. sphaeroides*; Rc, *R. centenaria*; Rr, *R. rubrum*. Except for At and Ah, the tRNA$^{Arg}_{CCU}$ genes were amplified by PCR. Sequences of the introns (shown in Fig. 1) and primers are omitted. The shorter sequences for *E. longus* and *R. sphaeroides* denote the use of the longer primer, ARG-5′EXT.

gested that the tRNA$^{Leu}_{UAA}$ introns in cyanobacteria are polyphyletic and that the *Microcystis* introns originated independently through horizontal transfer (19). The *Microcystis* tRNA$^{Leu}_{UAA}$ introns most likely share a more recent common ancestor with the α-purple tRNA$^{Arg}_{CCU}$ introns than either does with the other cyanobacterial tRNA$^{Leu}_{UAA}$ introns. This is supported by comparisons of primary sequences (Table 1; see also reference 19) and secondary structures (Fig. 1), as well as phylogenetic analyses (19 and data not shown). However, the origin of the tRNA$^{Leu}_{UAA}$ introns, excluding the *Microcystis* introns, appears to be monophyletic (17). Taken together, these results suggest that the *Microcystis* introns originated from a tRNA$^{Arg}_{CCU}$-like intron by horizontal transfer. This represents an interesting case of intron transposition and horizontal transmission between widely divergent species (cyanobacteria and α-purple bacteria).

Nucleotide sequence accession numbers. The intron sequences reported here have been deposited in GenBank under accession numbers AF081791 (*A. marginale*) and AF081792 (*A. halopraeferens*).
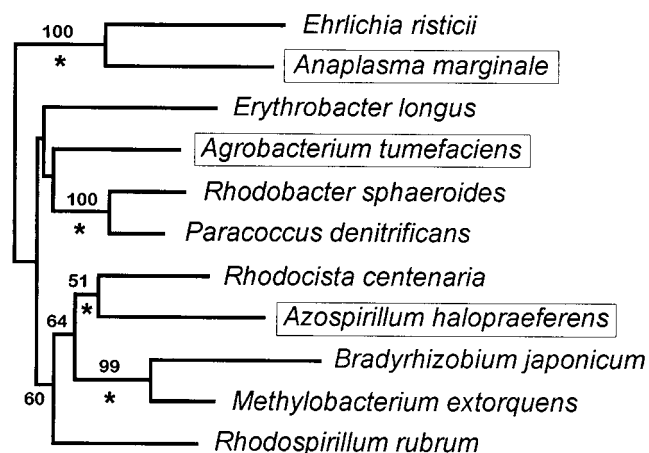
FIG. 4. rRNA phylogenetic tree. Prealigned small subunit rRNA sequences were extracted from the RDP web site (14) for the 11 species whose tRNA$^{Arg}_{CCU}$ genes were detected. The tree was inferred with DNADIST (Kimura two-parameter model [12], with a transition/transversion ratio of 2) and NEIGHBOR (20) as implemented in PHYLIP (8). Bootstrap (7) values were deduced from 100 replicates (only those values higher than 50 are shown). Branches conserved between this tree and that published on the RDP web site (14) are indicated (*), and species harboring a tRNA$^{Arg}_{CCU}$ intron are boxed. Note the scattered distribution of the three tRNA$^{Arg}_{CCU}$ intron-containing species; a monophyletic clade grouping these three species is supported by a bootstrap value of <1%. The topology shown has been suggested by the branching position of an outgroup (γ-purple bacterium *Ectothiorhodospira shaposhnikovii*).

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