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Group-I introns with unusual sequences occur at three sites in nuclear 18S rRNA genes of *Acanthamoeba lenticulata*

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Abstract Seven of eleven isolates of *Acanthamoeba lenticulata* were found to have group-I introns located at one of three positions within the 18S rRNA gene. The introns are 636–721-bp long and are absent from mature rRNA. They lack open reading frames that could encode any known endonucleases. Sequences of introns from the same site in different isolates are 86.0–98.9% identical, while from different sites they are 24.2–29.8% identical. The most closely related introns from other organisms are in the 18S rRNA genes of several green algae where the 17.0–23.6% identity is mostly limited to a highly conserved core of base pairs including P, Q, R and S. Because the *A. lenticulata* introns only occur in one *Acanthamoeba* lineage, they were probably acquired after the divergence of this species.

Key words Group-I introns · Ribosomal DNA · Acanthamoeba

Introduction

Acanthamoeba is a genus of small free-living amoebae that are widely distributed in the environment and occasionally are opportunistic pathogens of humans and other animals. Nuclear group-I introns in *Acanthamoeba* were first discovered in the small subunit rRNA genes (18S rDNA) from two isolates, *A. griffini* S7 and *A. lenticulata* PD_2S (Gast et al. 1994). Both isolates were obtained from aquatic environments. Neither isolate is known to be pathogenic, but several other isolates of these two species have been associated with disease. For example, *Acanthamoeba griffini* H37 was recently identified as the cause of *Acanthamoeba* keratitis in a human (Ledee et al. 1996). Also, several isolates of *Acanthamoeba lenticulata* from nasal passages of healthy humans were found to be pathogenic to mice (De Jonckheere and Michel 1988).

The introns of *A. griffini* S7 and *A. lenticulata* PD_2S are very different (Gast et al. 1994). The primary sequence of the *A. griffini* intron has a 59% nucleotide identity with the nuclear 5′ 18S rDNA intron of the green alga *Dunaliella parva*. The *A. lenticulata* intron appears to have little primary sequence identity with any other group-I intron except in the core P, Q, R and S regions. It was suggested that the *A. griffini* intron might have arisen from a common ancestor of algae and acanthamoebae, or from horizontal transfer between these organisms. Since acanthamoebae are known to phagocytize algae, it was suggested that the amoeba introns might have been acquired from ingested algae. The possibility that the acquisition occurred after the branching of the *A. griffini* lineage is supported by the observation that the primary sequence and site of insertion of the intron are unique among the 18S rRNA genes of more than 50 isolates that we have examined from 16 other *Acanthamoeba* species.

Stronger evidence for the possible transfer of rRNA gene introns from algae to acanthamoebae comes from studies of group-I introns in mitochondrial 23 S rRNA genes. Three such introns, designated AcLSU.m1, AcLSU.m2 and AcLSU.m3, are found in *Acanthamoeba castellanii* Neff (Lonergan and Gray 1994; Turmel et al. 1995). AcLSU.m1 is similar to a chloroplast 23 S rDNA intron in *Chlamydomonas pallidostigmatica* (CpLSU.2) (Turmel et al. 1995) and AcLSU.m3 is similar to CrLSU, a chloroplast 23S rRNA gene intron in the green alga *Chlamydomonas reinhardtii* (Lonergan and Gray 1994). The discovery that these amoebal and algal introns include open reading frames encoding homologous endoncleases provided additional evidence consistent with a transfer of

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Table 1 *A. lenticulata* strains employed in this study

Strain[a]	ATCC no.	Intron type (AlSSU.n)	GenBank 18S rDNA sequence accession no.[b]	Source of isolate
45	50703	–	U94730	Human nasal mucosa, Germany
72/2	50704	–	U94732	Human nasal mucosa, Germany
7327	50705	–	U94731	Swimming pool, France
68-2	50427	–	U94733	Scipio Creek, Florida, USA
118	50706	n1	U94736	Human nasal mucosa, Germany
PD_2S	30841	n1	U94741	Medicinal pool, France
E18-2	50690	n1	U94735	Marine sewage site, Philadelphia, USA
53-2	50691	n1	U94737	Marine sewage site, New York Bight, USA
NJSP-3-2	50429	n1	U94738	Sewage treatment plant, New Jersey, USA
25/1	50707	n2	U94740	Human nasal mucosa, Germany
Jc-1	50528	n2	U94739	Jones Creek, New York, USA
407-3a	50692	n3	U94734	Marine sewage site, Philadelphia, USA

[a] All cultures from Germany or France, except PD_2S, were received from Dr. Johan F. De Jonckheere, Institute of Hygiene and Epidemiology, Brussels, Belgium. The rest of the cultures were obtained from Dr. Thomas A. Nerad, American Type Culture Collection, Rockville, Maryland

[b] Sequences include introns where they occur, except for PD_2S where the intron sequence was submitted previously and has the accession number U02539 (Gast et al. 1994)

introns between algae and *Acanthamoeba* (Turmel et al. 1995).

The previously described 18S rDNA intron of *A. lenticulata* PD_2S is similar to the *A. griffini* intron in that it has been found in only one *Acanthamoeba* species, thus suggesting that it was acquired after branching of the *A. lenticulata* lineage. New data reported here for 18S rDNA from 11 additional isolates of *A. lenticulata* describe three distinct intron primary sequences. The data do not rule out the possibility of a common origin for the three types, but are more consistent with the possibility that each was acquired separately after the branching of the species. No substantial evidence was found for an algal origin of the introns in this species.

Materials and methods

Gene and intron nomenclature. The nuclear small subunit rRNA genes of *Acanthamoeba* are referred to as "18S rDNA". They have previously also been designated *"Rns"* (Gast et al. 1996). The intron designations used, e.g., AlSSU.n1, are consistent with prior designations of group-I introns from the mitochondrial large subunit rRNA genes of *Acanthamoeba* (Lonergan and Gray 1994) and reflect the taxon, gene, and genetic compartment where the introns were found; introns are numbered 1, 2 or 3 according to their 5′ to 3′ position within the small subunit rRNA genes from various isolates of this species.

Amoeba culture and nucleic acid extraction. The amoeba strains listed in Table 1 were cultured in 25-cm^2 culture flasks in optimal growth medium (OGM) as described previously (Byers et al. 1980). *A. lenticulata* tolerates temperatures of up to 40 °C (De Jonckheere and Michel 1988), but grew well at 27 °C in these studies. Amoebae were harvested by low-speed centrifugation for 10 min and the amoebal pellet was lysed using 500 µl of UNSET lysis buffer (8 M Urea, 2% sodium dodecyl sulfate, 0.15 M NaCl, 0.001 M EDTA, 0.1 M Tris pH 7.5) (Hugo et al. 1992). The lysate was phenol/chloroform-extracted until the protein interface was no longer present and the nucleic acid was ethanol-precipitated. DNA was dried under vacuum and re-suspended in 30–60 µl of sterile double-distilled water. One microliter was used for amplification of the 18S rRNA gene. For RNA isolation, amoebae were cultured axenically in 75-cm^2 tissue-culture flasks in 15 ml of OGM. They were harvested as above and RNA was extracted using the RNeasy Total RNA Kit (Qiagen, Chatworth). Following the protocol described in the kit, amoebae were lysed in 700 µl of lysis buffer, using syringe homogenization, and RNA was collected on a spin-column filter and eluted with DEPC-treated water as specified in the tissue-culture extraction procedure. RNA was quantified using a UV-spectrophotometer and diluted to less than 200 ng/reaction for the reverse transcription and PCR amplification which immediately followed extraction, to avoid RNA degradation by ribonuclease.

PCR and RT-PCR. 18S rDNA was amplified in overlapping fragments using primers specific for nuclear small ribosomal subunit genes (Table 2, Wilcox et al. 1992). Conditions used for amplification were hot soak at 95 °C for 4 min followed by 35–40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C using *Taq* DNA polymerase (Gibco/BRL, Gaithersburg). PCR products were band-isolated, glass-bead purified, and cloned into PCRII using the TA

Table 2 Primers used in PCR and RT-PCR[a]

Primer	Priming direction	Sequence	Location (bp)
SSU1	5′ → 3′	CCGCGGCCGCGTCGACTGGTTGATCCTGCCAGTAG	1–23
SSU2	3′ → 5′	CCGCGGCCGCGGATCCTGATCCCTCCGCAGGTTCAC	2281–2303
570C	5′ → 3′	GTAATTCCAGCTCCAATAGC	656–675
857C	5′ → 3′	CCCGTTCCTGCTATTGA	834–850
892C	5′ → 3′	GTCAGAGGTGAAATTCTTGG	1120–1140
892	3′ → 5′	CCAAGAATTTCACCTCTGAC	1120–1140
1262C	5′ → 3′	GTGGTGCATGGCCGTTCTTA	1584–1603
1262	3′ → 5′	GAACGGCCATGCACCAC	1584–1600
1200C	5′ → 3′	CAGGTCTGTGATGCCC	1826–1841
1200	3′ → 5′	GGGCATCACAGACCTG	1826–1841

[a] Primer sequences are written with the 5′ end at the left. Locations refer to bp positions in the Neff strain of *A. castellanii* (Gunderson and Sogin 1986)

cloning method (Invitrogen, San Diego). Cloned fragments and PCR products were sequenced using the dsCycle Sequencing Kit (Gibco/BRL, Gaithersburg). Reverse-transcriptase PCR (RT-PCR) was performed using the GeneAmp Thermostable *rTth* Reverse Transcriptase RNA PCR Kit (Perkin-Elmer/Cetus, Norwalk). An additional aliquot of reverse primer had to be added for the PCR step. RT-PCR products were band-isolated, glass bead-purified and sequenced directly.

Sequence alignment, secondary structure modeling and identity searches. The18S rDNA gene sequences and intron sequences have been deposited in GenBank (Bilofsky and Burks 1988) (Table 1). Sequences were aligned using ESEE v. 3.0 s (Cabot and Beckenbach 1989) and alignments are based on secondary structure. The intron secondary structure model of Cech et al. (1994) was used to determine the putative secondary structures of the introns in regions of conserved structure, and Zucker's PCFold (Zucker and Stiegler 1981) was used to predict the structures of less-conserved regions based on minimal energy folding. BLAST (Altschul et al. 1990) searches of GenBank and EMBL databases were performed to identify introns with similar primary sequences and to look for potential open reading frames in the introns. Genepro, v. 4.20 (Riverside Scientific) was used to search for open reading frames.

Results

The three group-I introns of *A. lenticulata*

Acanthamoeba 18S rDNA typically is about 2300 bp in length. These genes in *A. lenticulata* isolates were PCR-amplified in several overlapping fragments in order to determine the gene size and the putative location of introns. Four of the eleven isolates examined have a typical 18S rDNA of about 2300 bp in length. The remaining seven isolates have genes of atypical lengths, about 3000 bp, as previously observed for *A. lenticulata* PD_2S (Gast et al. 1994). PD_2S plus six of the seven new intron-containing isolates have the inserts in the same PCR product. The seventh new isolate, 407-3a, has an insert in a second PCR product further downstream and nearer the 3′ end of the gene. Sequencing of the fragments containing the inserts revealed three different intron insertion sites (Fig. 1).

Introns from E18-2, 53-2, NJSP-3-2 and 118 range in size from 636 to 656 bp and are located at the PD_2S intron insertion site adjacent to helix 33 between nucleotides 1485 and 1486 (Fig. 1). They are designated as AlSSU.n1. The equivalent nucleotide positions in the *Escherichia coli* 16S rRNA gene, typically used for reference (Gutell 1993), are 943 and 944. Introns from Jc-1 and 25/1, which are 698 and 679 bp in size, are located adjacent to helix 34 between nucleotides 1498 and 1499 and are designated as AlSSU.n2. The equivalent *E. coli* nucleotide positions are 956 and 957. The 721-bp intron from 407-3a is located in helix 32 between nucleotides 2111 and 2112 and is designated as AlSSU.n3. The equivalent *E. coli* positions are nucleotides 1389 and 1390.

The geographical distributions of both 18S rDNA intron-containing and intron-free strains are both widespread and overlapping. Strains without introns and strains with the same intron type (AlSSU.n1 and AlSSU.n2) were found on both sides of the Atlantic Ocean (Table 1). In addition,

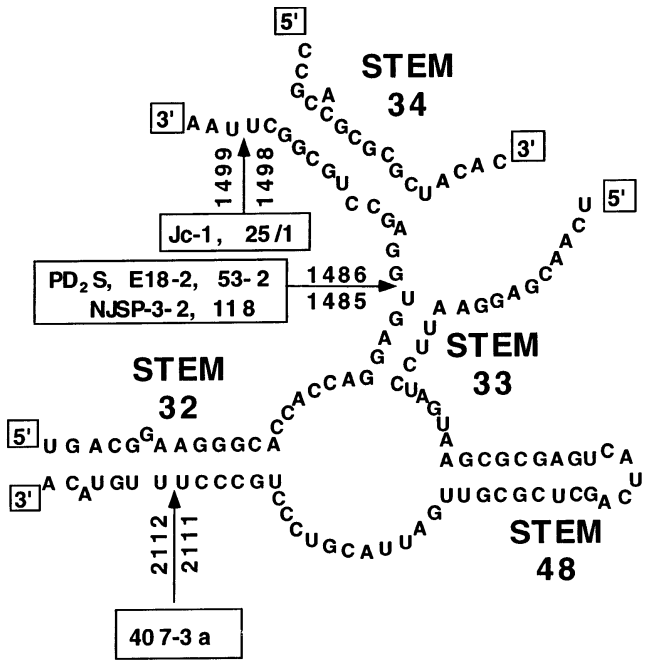

Fig. 1 The sites of intron insertion within the secondary structure of *Acanthamoeba* 18S rRNA. The sequence and base pair numbering are from the reference strain *A. castellanii* Neff (Gunderson and Sogin 1986; GenBank Accession #U07416). The numbering of stems is after Neefs et al. (1993)

two strains with different intron types, E18-2 (AlSSU.n1) and 407-3a (AlSSU.n3), were found at the same oceanic sewage-dump site. Although four isolates were obtained from human nasal mucosa, it is interesting that *A. lenticulata* has not yet been identified in human disease.

Relationships between the presence or absence of introns, the types of intron sequences, and phylogenetic relationships among strains of *A. lenticulata* are shown in Fig. 2. The *A. lenticulata* tree is based on alignments of complete coding sequences for mature 18S rRNA. The branching pattern is more detailed than that in a previous analysis of the entire genus because the sample is more homogeneous and, consequently, more sequence could be aligned reliably. As can be seen, all of the strains that contain AlSSU.n1 are very closely related to one another. This would be consistent with the acquisition of this intron by a common ancestor of these strains after the appearance of an ancestral form of *A. lenticulata*. Intron AlSSU.n2, however, is shared by two strains which have divergent 18S rRNA coding sequences. The occurrence of this intron must be explained by either an ancestral state within *A. lenticulata*, followed by loss from most strains, or the independent acquisition of the intron by strains 25/1 and Jc-1. In strain 25/1, this may have been associated also with the loss of intron AlSSU.n1. The occurrence of intron AlSSU.n3 in strain 407-3a suggests a unique acquisition of this intron by this strain. The lack of introns in four of the six *A. lenticulata* strains in the clade at the bottom of Fig. 2 suggests that the common ancestor of these six strains either lacked any intron, or lost intron AlSSU.n1

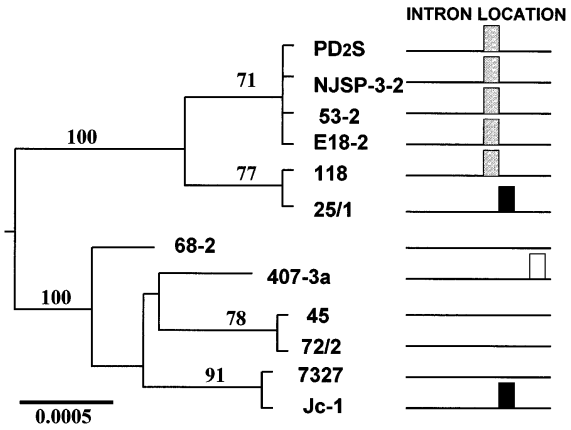

Fig. 2 Relationships between the evolutionary tree for the complete 18S rDNA coding sequences of the 12 *A. lenticulata* strains and the type and relative location of introns found within the 18S rDNA regions. The tree results from a neighbour-joining analysis using MEGA (Kumar et al. 1993) with the Kimura two-parameter correction. Sequence alignments used differ from those in Table 3 in that indels are excluded in this analysis. The *distance bar* represents 0.5 bp substitutions per 1000 bp. Values for 1000 bootstrap runs are indicated on the branches; bootstrap values <50% are not reported. Introns AlSSU.n1, AlSSU.n2 and AlSSU.n3 are represented by *shaded, filled and open boxes*, respectively. Relative positions of introns are not to an exact scale

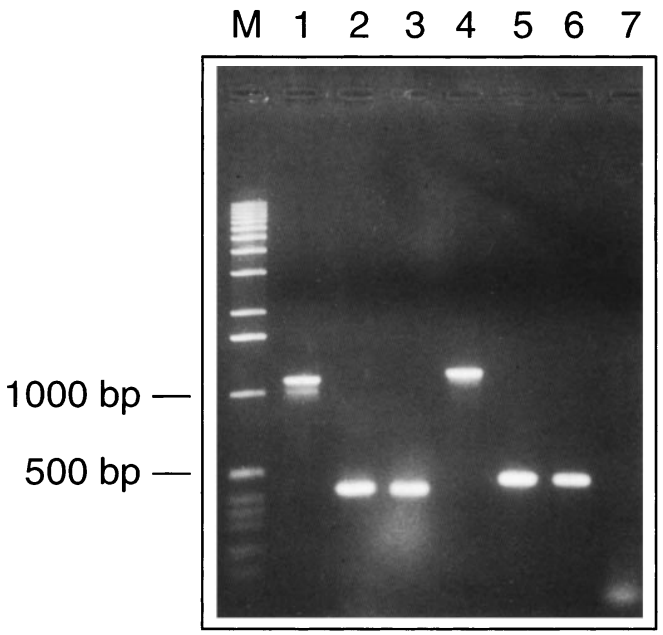

Fig. 3 PCR and RT-PCR results for representative introns Jc-1 and 407-3a showing that the introns are absent from the mature rRNA. *Lane M*, 1-kb DNA ladder (Gibco/BRL, Gaithersburg). *Lanes 1 and 4*, PCR products from Jc-1 and 407-3a. *Lanes 2 and 5*, positive control amplification products from 68-2. *Lanes 3 and 6*, RT-PCR products from Jc-1 and 407-3a. *Lane 7*, negative control for reaction contamination

after diverging from the ancestor of the upper six strains in the figure. Either interpretation suggests that at least two of the introns were acquired after the divergence of the ancestral *A. lenticulata* form from other members of the genus *Acanthamoeba*.

Introns are absent from mature 18S rRNA

Gast et al. (1994) used RT-PCR to demonstrate that the 18S rRNA intron of *A. lenticulata* PD_2S is removed during RNA maturation. PD_2S is representative of isolates containing AlSSU.n1 introns. Therefore, we used RT-PCR to examine the effects of RNA processing in strains Jc-1 and 407-3a which represented isolates containing introns AlSSU.n2 and AlSSU.n3, respectively. Figure 3 shows the results of PCR-amplification and RT-PCR for Jc-1, 407-3a and the intronless strain 68-2 which was used as a positive control. Lanes 1 and 4 are PCR-amplifications of intron-containing DNA fragments from Jc-1 and 407-3a, respectively. The bands are approximately 1150 bp in length. Lanes 2 and 5 are positive controls for PCR amplifications of the same DNA fragment from strain 68-2. These control bands are approximately 400 and 450 bp long, respectively, and are the expected sizes of the RT-PCR products for these regions if the introns are absent from the mature rRNA. Lanes 3 and 6 are RT-PCR products from Jc-1 and 407-3a, respectively. The RT-PCR band sizes correspond to the sizes of the fragments that lack introns. Lane 7 is a negative control reaction that does not contain a template. Only unused primer is present at the bottom of the gel.

The introns do not encode any known endonucleases

A Genepro search for potential open reading frames within the introns was performed. The largest ORFs identified could encode 80–122 amino acids and are present in the AlSSU.n1 introns of PD_2S, NJSP-3-2 and 118. Comparison of the amino-acid sequences inferred from the translation of these short ORFs with sequence databases produced no matches suggestive of possible function. No similarities to any known endonucleases were found. Comparison of the open reading frames in the five strains that have the AlSSU.n1 intron indicate that they would not encode a common protein because of multiple frameshifts due to insertions or deletions.

Intron secondary structure

The model of Cech et al. (1994) for the conserved secondary structure of group-I introns was used to determine the putative secondary structures of the various introns. The less-conserved regions, stems P5 and P9, were folded using minimal energy folding (Zucker and Stiegler 1981). Representative secondary structures for introns at the various insertion sites are shown in Fig. 4. Although the three structures differ from each other, especially at stems P5 and P9, all introns inserted at a single site can be folded to fit the representative structure for that site with only minor modifications to the lengths of stems or loops. In-

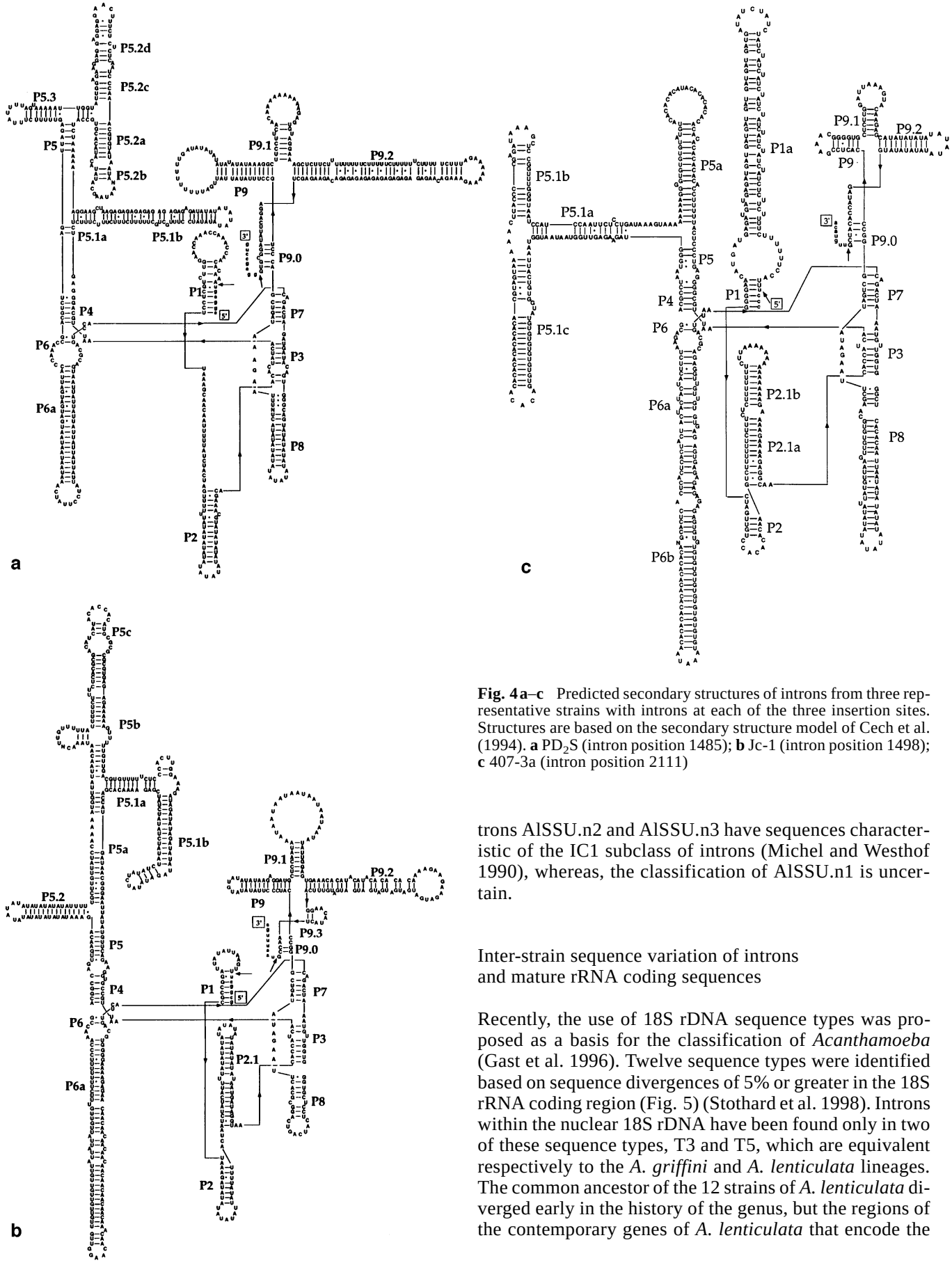

Fig. 4a–c Predicted secondary structures of introns from three representative strains with introns at each of the three insertion sites. Structures are based on the secondary structure model of Cech et al. (1994). **a** PD_2S (intron position 1485); **b** Jc-1 (intron position 1498); **c** 407-3a (intron position 2111)

trons AlSSU.n2 and AlSSU.n3 have sequences characteristic of the IC1 subclass of introns (Michel and Westhof 1990), whereas, the classification of AlSSU.n1 is uncertain.

Inter-strain sequence variation of introns and mature rRNA coding sequences

Recently, the use of 18S rDNA sequence types was proposed as a basis for the classification of *Acanthamoeba* (Gast et al. 1996). Twelve sequence types were identified based on sequence divergences of 5% or greater in the 18S rRNA coding region (Fig. 5) (Stothard et al. 1998). Introns within the nuclear 18S rDNA have been found only in two of these sequence types, T3 and T5, which are equivalent respectively to the *A. griffini* and *A. lenticulata* lineages. The common ancestor of the 12 strains of *A. lenticulata* diverged early in the history of the genus, but the regions of the contemporary genes of *A. lenticulata* that encode the

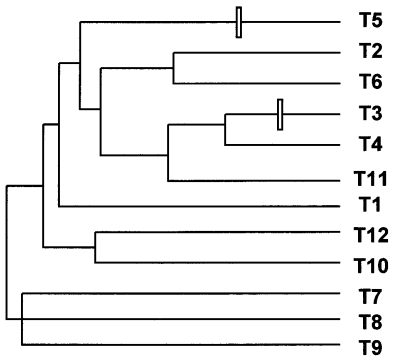

Fig. 5 Evolutionary tree relating the 18S rRNA gene-sequence types T1–T12 described by Stothard et al. (1998). Sequence type T5 represents all strains of *A. lenticulata* and sequence type T3 represents all strains of *A. griffini*. *Boxes* are placed on lineages in which group-I introns occur in the 18S rDNA

intron-free mature rRNA differ by no more than 13 bp and have nucleotide identities of 99.4–100% (Table 3).

The introns are more variable than the mature rRNA coding regions. Sequences of the AlSSU.n1 introns of PD_2S, E18-2, 53-2, NJSP-3-2 and 118 are 94.0–98.9% identical; the AlSSU.n2 introns of Jc-1 and 25/1 are 86% identical while the AlSSU.n3 intron of strain 407-3a is unique (Table 3). In contrast, differences among the introns at different sites are large. The sequence of AlSSU.n3 is only 26.2–27.5% identical to sequences of AlSSU.n1 and 24.2–24.5% identical to sequences of AlSSU.n2. Likewise, AlSSU.n1 is only 28.4–29.2% identical to AlSSU.n2. Intron identities are greatest in the highly conserved P, Q, R and S regions essential for intron excision (Table 4). Although percent identities between sequences of introns at the different locations are much lower than those between sequences of introns at the same location, they still are slightly higher than the percent identities between sequences of *A. lenticulata* introns and the introns of other organisms such as green algae (Table 3).

No close relatives of the *A. lenticulata* rDNA introns have been found

The amoeba introns are unusual in having highly repetitive sequences consisting of approximately 150 to 300 bp of di-, tri- and tetra-nucleotide repeats. BLAST searches of Genbank and EMBL databases using complete *A. lenticulata* intron sequences failed to find any close relatives. After removing the repetitive sequences and repeating the BLAST search, the truncated amoeba sequences are most similar to 18S rDNA introns of the green algae *Chlorella mirabilis*, *Chlorella ellipsoidea* and *D. parva* (the 3′ intron). When the complete amoebal introns are aligned with these algal introns, the sequences are 14.8–23.6% identical. The identities that were found, however, are predominantly in the highly conserved P, Q, R and S sequences.

Discussion

The group-I introns of *Acanthamoeba* 18S rDNA occur at four different positions in the two species in which they have been found. In *A. griffini*, a single intron, now designated AgSSU.n1, is located in strains S7 and TIO:H37 after base pair 643 of the *Acanthamoeba* reference sequence (Gast et al. 1994; Ledee et al. 1996). In *A. lenticulata*, AlSSU.n1, n2 and n3 are located after base pair 1485, 1498 and 2111, respectively. In these two species, introns at the four different sites have sequence identities of 14.8–23.6%, whereas introns at a single site in different strains have sequence identities of at least 86% (Table 2). Similar se-

Table 3 Percent identities. Comparisons of complete intron-free rRNA coding sequences above the diagonal, and of complete intron sequences below the diagonal [a]

Strain	72/2	68-2	7327	PD_2 S	E18	53-2	NJSP	118	25/1	Jc-1	407
	No intron	No intron	No intron	Intron n1	Intron n1	Intron n1	Intron n1	Intron n1	Intron n2	Intron n2	Intron n3
45	100	99.6	99.7	99.5	99.5	99.5	99.5	99.5	99.5	99.7	99.7
72/2	–	99.6	99.7	99.9	99.5	99.5	99.5	99.5	99.5	99.7	99.7
68-2		–	99.8	99.5	99.5	99.5	99.5	99.5	99.5	99.9	99.8
7327			–	99.6	99.6	99.6	99.6	99.6	99.6	99.9	99.7
PD_2S				–	100	100	100	99.9	99.9	99.4	99.5
E18-2				94.8	–	100	100	99.9	99.9	99.4	99.5
53-2				94.4	97.7	–	100	99.9	99.9	99.4	99.5
NJSP-3-2				95.2	98.9	98.0	–	99.9	99.9	99.4	99.5
118				94.0	98.4	98.4	98.8	–	100	99.4	99.5
25/1				29.2	28.4	28.6	28.4	28.5	–	99.4	99.5
Jc-1				29.8	28.8	28.9	29.2	28.9	86.0	–	99.7
407-3a				27.3	26.6	26.2	26.5	26.6	24.2	24.5	–
D. parva (3′)				22.1	22.3	21.3	21.6	21.4	22.3	23.6	19.8
C. mirabilis				14.8	16.8	18.7	14.9	14.8	16.8	17.0	20.8
C. ellipsoidea				17.0	16.7	16.9	17.2	17.1	16.7	16.6	19.8

[a] Alignments used for calculations of % identity are based on structure and each indel base pair counts as a separate site (Stothard et al. 1998)

Table 4 Comparison of P, Q, R and S sequences in *A. lenticulata* introns at different loci [a]

Item	P	Q	R	S
Consensus	AAUUNCNN- GAAN	AAUNNGNA GC	GUUCAGAGACUANA	AAGAUAUAGUCC
AlSSU.n1	AAUUGCAG- GGAG	GAUCUGCA GC	GUCCACAGACUAGA	AAGAAAUAGUCG
AlSSU.n2	AAUUGCCGUGGAA	GACCGGCA GC	GUUCACAGACUAAA	AAGAUAUAGUCG
AlSSU.n3	AAUUGCGG- GGAG	AAUCCGCA GC	GUUAACAGACUAAA	AAGAUAUAGUCG
	P4	P6	P7	

[a] Consensus sequences were derived by Cech (1988). Underlining indicates regions that base pair to form the catalytic core of group-I introns. Stems P4, P6 and P7 of the secondary structure models (Fig. 4) are formed by base pairing of P with Q, of Q with R, and of R with S, respectively, as indicated by the connecting lines below the underlined sequences

quence relationships for introns at the same site and at different sites have been reported for other organisms (Gargas et al. 1995).

The occurrence of *Acanthamoeba* 18S rDNA introns only in the T3 and T5 lineages, and the relatively low sequence identity among introns at the four different positions, suggests that intron acquisition occurred independently at each of the four locations after branching of the T3 and T5 lineages (Figs. 2 and 5). The high sequence identity among the AlSSU.n1 introns is consistent with their descent from an ancestral intron initially acquired at that position (Fig. 2). The absence of an AlSSU.n1 intron in strain 25/1 is most likely explained by intron loss. If the two AlSSU.n2 introns are descended from a common ancestor within the T5 lineage, then multiple losses would be required to account for the present-day occurrence of the intron only in strains 25/1 and Jc-1.

All group-I introns of 18S rDNA in the two *Acanthamoeba* species occur at sites where group-I introns also occur in other organisms. For example, group-I introns occur at the same site as AgSSU.n1 in green algae (Gargas et al. 1995) and in the amoeboflagellate *Naegleria* (De Jonckheere 1993, 1994). Group-I introns also are found in fungi and green algae at the same site as AlSSU.n1, in acellular slime molds at the same site as AlSSU.n2, and in fungi at the same site as AlSSU.n3 (Gargas et al. 1995). One explanation for the repeated appearance of introns at selected sites is that physical accessibility of the sites may be an important factor in intron acquisition (Gargas et al. 1995). Differences in the physical environment might also influence the likelihood of intron loss at different sites.

Present day *Acanthamoeba* 18S rDNA introns may have descended from introns acquired by ancestral acanthamoebae from other locations within the genome of this genus. Alternatively, they may have been acquired from other organisms. The case for acquisition of some *Acanthamoeba* mitochondrial large subunit rRNA genes from algae has been discussed in the Introduction. A high sequence identity between the *A. griffini* 18S rDNA intron and an 18S rDNA intron in the green alga *D. parva* has also been noted. Relationships between the introns of *A. lenticulata* and algae are more distant. BLAST searches failed to find any close relatives of the *A. lenticulata* introns when complete intron sequences were used for the searches. However, searches using truncated introns identified 18S rDNA introns from three species of green algae, including *D. parva*, as the closest relatives of the *A. lenticulata* introns. The identities were mostly restricted to the P, Q, R and S sequences that are highly conserved among introns of eukaryotes, whereas identities between nuclear 18S rDNA introns of green algae and *A. griffini* and between organelle 23S rDNA introns of green algae and *A. castellanii* extend beyond the P, Q, R and S sequences. Thus, relationships between the group-I introns of *A. lenticulata* and those of the green algae appear to be the more ancient.

The 18S rDNA introns in some species, such as in the amoeboflagellate *Naegleria*, encode a putative homing endonuclease (Johansen et al. 1993). However, most nuclear 18S rDNA group-I introns do not have open reading frames and there is no evidence in *Acanthamoeba* for any ORF encoding any known endonucleases that could promote intron mobility. The ORF for the putative homing endonuclease of *Naegleria* is missing from the 18S rDNA of some species and this has been attributed to loss from the intron (De Jonckheere 1994). It is possible that the 18S rDNA group-I introns of ancestral acanthamoebae also had, and then lost, genes for endonucleases, but there is no current evidence to support that possibility. It is more likely that homing endonucleases are encoded in other parts of the genome. We have shown here and previously (Gast et al. 1994) that intron sequences are absent from the mature 18S rRNA of *A. griffini* and *A. lenticulata*, presumably as a result of splicing activity. Thus, the less-likely possibility remains that mobility of the *Acanthamoeba* introns could be promoted by reverse splicing (Woodson and Cech 1989).

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