Small subunit ribosomal RNA evolution in the genus *Acanthamoeba*

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Reverse transcription of small subunit ribosomal RNA (srRNA) was used to determine the partial nucleotide sequences of the srRNA of seven isolates of *Acanthamoeba*. These seven sequences and the sequence of the corresponding region in an *A. castellanii* previously totally sequenced were compared in order to investigate evolution of the srRNA gene in *Acanthamoeba*. The results of the comparisons were consistent with the hypotheses that the genus is monophyletic, that the Pussard and Pons grouping system is valid, that the *Acanthamoeba* expansion segments in the srRNA gene evolve at a much faster rate than the rest of the gene, and that the extent of nucleotide sequence divergence in the genus *Acanthamoeba* is roughly similar to that differentiating vertebrates and invertebrates.

Introduction

The genus *Acanthamoeba* is composed of free-living amoebae which are common in soil and in marine and fresh waters. There are more than 20 nominal species, erected mainly on the criterion of cyst morphology, but the validity of most of these is uncertain. Opportunistic pathogens, responsible for amoebic encephalitis (Simon & Wilson, 1986) or keratitis (Stehr-Green *et al.*, 1989), have been assigned to several species.

Pussard & Pons (1977) divided *Acanthamoeba* species into three groups, based on cyst morphology. Group I consists of relatively large (cyst diameters greater than 18 µm) *Acanthamoeba* spp., with distinctly stellate endocysts. The ectocyst is smooth, more or less spherical, and coincident with the endocyst at the end of each ray, where there is an excystment pore. Group II and group III species are smaller (cyst diameters less than 19 µm) *Acanthamoeba* spp. Group II *Acanthamoeba* spp. have polygonal to stellate endocysts and irregular or wrinkled ectocysts. Pores occur in a depression in the ectocyst, at

the angles of the endocyst wall. Group III consists of *Acanthamoeba* spp. with rounded or slightly angular endocysts, with the ectocyst thinner and smooth or slightly wrinkled. The two walls are less widely separated than those in the other groups, so that pores occurring where the ends and ectocyst coincide are often obscure.

However, isoenzyme studies indicate that these morphological features are not reliable specific characters (Costas & Griffiths, 1980, 1984, 1985; Daggett *et al.*, 1982; De Jonckheere, 1983). In particular, the number of pores, which has been used to differentiate some species, can vary with culture conditions such as the ionic composition of the medium (Sawyer, 1971). Costas & Griffiths (1986) considered that the variation among some *Acanthamoeba* species overlaps so extensively that they should be regarded as species complexes for which the binomial nomenclature is 'inappropriate'.

Genetic comparisons at the nucleic acid level have become a powerful tool for solving phylogenetic problems. Sequencing of small subunit ribosomal RNA (srRNA) has already been used to study protists, demonstrating, for example, that the rhizopodan groups represented by *Naegleria*, *Acanthamoeba* and *Dictyostelium* are not monophyletic (Clark & Cross, 1988; Baverstock *et al.*, 1989). It has also been shown that the extent of divergence among *Naegleria* species is similar to that between mammals and frogs (Baverstock *et al.*,

Abbreviations: lrRNA, large subunit ribosomal RNA; srRNA, small subunit ribosomal RNA.

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1989). Restriction endonuclease patterns of ribosomal DNA have confirmed the genetic diversity of *Naegleria* species, and have shown that the type species of the genus, *N. gruberi*, is a polyphyletic complex of species (Clark *et al.*, 1989).

In the only *Acanthamoeba* species studied by such techniques, *A. castellanii*, the srRNA molecule is characterized by several large expansion segments, making it one of the largest known srRNAs (Gunderson & Sogin, 1986). These additional segments of nucleotides are the apparent reason for the increase in size of eukaryotic large subunit ribosomal RNA (lrRNA) and srRNA (Clark, 1987; Spencer *et al.*, 1984; Hassouna *et al.*, 1984; Gonzalez & Schmickel, 1986). They have been variously referred to as D (divergent) regions (Hassouna *et al.*, 1984), V (variable) regions (Spencer *et al.*, 1984), and expansion segments (Clark *et al.*, 1984). We believe that the latter terminology is the most appropriate, and it will consequently be used here.

Given the genetic heterogeneity among members of the genus *Naegleria* and the apparent morphological differences among *Acanthamoeba* species, this study was undertaken with four aims: (1) to determine the extent of srRNA gene variation in the genus *Acanthamoeba*; (2) to test the correlation of srRNA sequences with the three morphological groups of Pussard & Pons (1977); (3) to test the monophyly of the genus *Acanthamoeba*; and (4) to determine whether species of *Acanthamoeba* in general possess the expansion segments characteristic of *A. castellanii* and compare the level of variation in the expansion segments with that in more conserved regions.

Methods

Amoebal strains and growth conditions. Seven isolates, representing the three morphological groups and therefore the greatest presumed diversity, were chosen for study (Table 1). All came from the culture collection of the State Water Laboratory. Five isolates were the type strain or most widely used reference strain for separate species. A sixth isolate (AC-151) was an unidentified group I *Acanthamoeba* (nr. *A. astronyxis*) with a higher temperature tolerance than is usual for this

group. The seventh isolate (AC-102) was an undescribed group II *Acanthamoeba* (Pussard & Pons, 1977) with predominantly naviculate cysts. AC-013 was derived from the well-studied NEFF strain of *A. castellanii*, but has been in culture in Australia for at least 17 years.

Amoebae were grown at 30 °C in PYNFH medium modified as described in the ATCC Media Handbook (American Type Culture Collection, 1984). Most strains were harvested every 48 h from the tissue culture flasks and centrifuged at 1500 *g* for 10 min. The pellet was resuspended in 9 vols guanidine hydrochloride buffer and stored at -20 °C until the RNA was extracted. Strain AC-102 grew more slowly than the others in axenic culture, and the interval between successive harvests from the culture flasks was 4 to 5 d.

Extraction of RNA. Total cellular RNA was extracted from the amoebae by thawing the pellets in the presence of 6 M-guanidine hydrochloride, 0.2 M-sodium acetate pH 5.4, and 1 mM-2-mercaptoethanol and homogenization in a tissue grinder. The procedure has been described in detail elsewhere (Brooker *et al.*, 1980; Johnson *et al.*, 1986).

Sequencing. Reverse transcription of the total cellular RNA of the amoebae using three primers (A, GA/TATTACCGCGGCG/TGCTG; B, CCGTCAATTCA/CTTTA/GAGTT; C, ACGGGCG-GTGTGTA/GC) has been described elsewhere (Qu *et al.*, 1983; Lane *et al.*, 1985). These primers bind to sequences almost universally conserved in prokaryotes and eukaryotes. They cover nucleotide positions 1114–1131, 2298–2317 and 3389–3404, respectively, as listed by Dams *et al.* (1988). The DNA generated was sequenced by the deoxy/dideoxy chain-termination method, fractionated on 6% (w/v) polyacrylamide/8 M-urea nongradient gels, 0.4 mm thick, and visualized by autoradiography of the ³²S gels with X-ray film (Johnson & Baverstock, 1989). At least three sequencing reactions and determinations were carried out for each primer to ensure the accuracy of the nucleotide sequences obtained.

Extent of nucleotide sequence divergence. Sequences obtained here were aligned by eye with each other and with the published sequence of *A. castellanii*. Because our study of nucleotide sequence divergence focuses on nucleotide substitutions within and outside the expansion segments, we measured nucleotide sequence divergence as the number of nucleotide substitutions *per se*. Thus gaps did not score as a nucleotide substitution difference in this analysis.

Phylogenetic analyses. Phylogenetic relationships among the strains of *Acanthamoeba* were assessed using DNAPARS in the PHYLIP package (version 3.1) of J. Felsenstein. Gaps were treated as a fifth character. The robustness of the parsimony analysis was assessed by bootstrapping the data 20 times (Felsenstein, 1985), using DNABOOT, also in the PHYLIP package.

Table 1. Characteristics and origins of strains of amoebae examined

Strain	Species	Pussard & Pons group	Temperature tolerance (°C)	Axenic growth	Pathogenic to mice	Origin
AC-009 (ATCC 30867)	<i>tubiasii</i>	I	35	Good	NT	Fresh water, USA
AC-151		I	40	Good	NT	Fresh water, Madagascar
AC-013 (ATCC 30010)	<i>castellanii</i>	II	38	Luxuriant	No	Soil, USA
AC-010 (ATCC 30871)	<i>polyphaga</i>	II	38	Luxuriant	Yes	Fresh water, USA
AC-102		II	40	Poor	NT	Fresh water, S. Australia
AC-014 (ATCC 30810)	<i>palestinensis</i>	III	38	Good	No	Soil, Israel
AC-001 (ATCC 30111)	<i>culbertsoni</i>	III	40	Luxuriant	Yes	Contaminant of mammalian cell culture

NT, Not tested.

(a) A-primer sequence

AC	ACGGCGACGAUUCAUU-CAAAUUCUGCCCUAU-CAACUUCGAUGGUAGGAUAGAGGCCUAC-CAU-GG
AC-013	*****.....-X.....-.....
AC-010	*****.....X.....A.....X.....X.....-X.....
AC-102	A.....-.....X.....-.....
AC-014	**.....A.....-.....-.....
AC-001	*****XC.....-X.....-.....-X.....-X.....
AC-151	*****.....A.....C.....
AC-009	*****.....
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AC	UC-GUAACG-GGUAACGGAGAAUAGGGUU-CGAUU-CCGGA-GAGG---GAGC-C-UGAGAAU-GGCU
AC-013	..-...X.C...XX.....-X...G.X...XX.XX---...-G.....C....
AC-010	..A...X.C...X.XXX.....-...X..C..-..XXXXX...U.G.....C....
AC-102	..-...C.....-...X.....-XXAXX-...-G.....C....
AC-014	..-...X.C.....X.....-...-X.XXXA..XXXX-...-G.....C....
AC-001	..-...C..X.X.XXX.....X.X.....-...A..XX---...-.....C....
AC-151	..-...CG..XXC..XXX.....-.....-...XX---X.....-.....
AC-009	..-...XCG..XXX..XXX.....-.....-...XXXX-...-.....-.....
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AC	ACCA-CUU-CU--AA-GG-AAGGCAGCAGGCGCGCAAUUAACCCA-A-UCCCGACACGGGGAGGU-A-GU
AC-013	X...U...-X.--...-XX.....XXXX...X...X..G...-...XX.....XG-XUX..
AC-010	X...U...-...-...-XX.....XXX...X...G.G...-...XX.....G.G.U..
AC-102	X..XU...G.XUG..G..-XX.....XXX...X...-...-...XX.....G.G.U..
AC-014	X...U...X.--...-GXX.....XXX.....-G...A...XX.....G.G.U..
AC-001	...-...-...-...-XX.....XXX.....-...-...XX.....G.-.-..
AC-151	...-...-...-...-XX.....XXXX...--..XX...-...-XXX.....X.-.-..
AC-009	X...-...-...-...-XX.....XXXXX...X...-...-G.C.XXXX.....X.-.-..
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AC	GA-C-A-AUAA-A-U-A-A-CAAUACAG--GCGCUCGAUAAGAGUCUUGUAAUUGGAAUGAGU-ACAAUU
AC-013	..G.U.U...U.G.G.G.U.....GC.-X.....X.....-.....
AC-010	..G.U.U...U.G.G.G.U.....GC.GC.....G.....
AC-102	..G.U.U...U.G.G.G.U.....GC..C.....G.....
AC-014	..G.U.U...U.G.G.G.U.....GC.-X.....G.....
AC-001	..-.-.-.-.-.-.-.-.-GC.GC.....C.....-.....
AC-151	..-.-.-.-.-.-.-.-X.....GU--C.....XXX.....X.....X.....-.....
AC-009	..-.-.-.-.-.-.-.-X.....GU--C.....XXX.....X.....-.....
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AC	UAAACCCCUUACGAGUAA
AC-013	...X.....X.XXX.XX
AC-010	*****
AC-102	*****
AC-014	*****
AC-001	*****
AC-151	.XXX...*****
AC-009	.XX-...X.*****

Fig. 1 (continued overleaf). Partial sequences of the seven strains of *Acanthamoeba* studied aligned by eye with the homologous regions for AC. *, No data; ., same as AC; -, gap; X, base not determined. Regions underlined in AC indicate expansion segments. The blocks used for phylogenetic analysis are overlined.

Results

The sequences obtained from the A-, B- and C-primers of *Acanthamoeba* are shown in Fig. 1. They were aligned by

eye with each other and with the published sequence for *A. castellanii* of an unspecified strain (Gundersen & Sogin, 1986). This strain is referred to as AC in the analysis described here.

(b) B-primer sequence

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AC      AUUUA-UGAAAGAUUAAC-UUCUGCGAAAGCAUCUGCCAAG-GAUGUUUUA-UUAAUCAAGAACGAAAG
AC-013  *****
AC-010  *****CAC.CXX...A...X...XXXX.X..
AC-102  *****C...-.....XXGAC...
AC-014  .....G.....X.G.....A.....G.....GXX...GA
AC-001  *****-...XX...-.....XX-.....
AC-151  *****-...XX-.....
AC-009  .....-.....-.....-.....XX-.....

AC      --UUA--GGGGAUC-GAAGA-CGA-UCAGA-U-ACCGUCGUA-GUCUUAACCAUAA--AC-GAUGCCGA-
AC-013  --...-X.X...-...XX-.X.-.XX...-A.X.....-...XXX.....GA..U...U.--
AC-010  --...A-...X...-...A...-...-A.X.....G...XX.U.UCU.UU..A..C.AAUUG
AC-102  --.GAU.....X.....A..C....C...-X.....-...XXX.....--...-...X..-
AC-014  CG...--.....-.....-.....-...X.....-...XXX.....--...-.....-
AC-001  --...--.....-.....-.....-...X.....-...XX...-.....--...-.....-
AC-151  --...--.....-.....-...U...-...XXX.....-.....--...-C.....-
AC-009  --...--.....-.....-...U...-...X.....-.....--...-C.....-

AC      C-CAGCG-----AUUA-GGAG-A-CGU-UG-----AA-UACAAAA---CA-CCA
AC-013  .-X...CAGACUGACUUA..G.-...C.G...G..X--UAGUXU-CAU-...-U-----U-...
AC-010  .XX...XAX-C-G-----A...-...G..GCAUAGU---CAUC...U-----X-...
AC-102  .-X...-----...-...-...-...AGAUAUCGACA--C...-----...
AC-014  .-----...-...-...-...-...-...-...-...-...-...-...-...-...
AC-001  .-----...-...-...-...-...-...-...-...-...CAC..U...
AC-151  .-----...G-...-...-...U-----...-...X..GG
AC-009  .-----...G-...-...-...U-----...-...-...GG

AC      CCAUC----GGC--GCGGUC-GUCCUUGGC--GUCUGUCCCUUUC-AACGGGGGCAGGCGCG--A-GGG
AC-013  .X...-----X-.....-...XX...-...XXXX...XX--...X
AC-010  .X...-----X.X-.....-...G.C..GGC...-...CCXX-.X...CX.X..X
AC-102  .....-C..U--...X..U.....XX...GU.GG.C.CGCXXGXXXAUGGX--.U.CXGG..-
AC-014  ....A-----CAU--AAUUA-..A.A...CA...AAUUG-..GUGX---AUXXXUU-.U-----
AC-001  UUUAG-----CAUGGU..X.UUCAAA.AUU.C---U.U.UG---G-...UU---UUU-....A
AC-151  AAG.GGXGCC..-GG.G...-...XCUUUG---.C.GUX--XXX...XXCXGA..AGA-GCXXXX
AC-009  AGG.U-----XCG...-...G..C.X--X...G.--G-...-C.....-...XXXX

AC      CGG-UUUAGCCCGGUGGCACCGGUGAAUGACUCCCCUAGCAGCUUGUGAGAAAUCAU-AAGUCUUUGG
AC-013  ...-...-G.X...X.....X.....-...XXXX...
AC-010  ...-...CG.X...X.....X.....-...XX.*****
AC-102  ..A-...C-.U.A...X.UX.....X.....-...XX.*****
AC-014  -----...X.....X.....-...*****
AC-001  ACXX..CGU..U.A...AU.U..X.....-...*****
AC-151  XUCC...G.G.U.C.UC...UAAA.G.....C.....C...*****-
AC-009  -U-...G.G.X.C.UC...UXAA.G.....C.....C...U*****

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Presence of expansion segments

The srRNA of *A. castellanii* is characterized by a number of large expansion segments (Gunderson & Sogin, 1986). These regions are underlined in Fig. 1. One of these expansion segments is close to and upstream from the B-primer, and was therefore accessible by the reverse-

transcription method described here. All eight isolates studied here possess an expansion segment in precisely the same position (Fig. 1*b*). Although there is considerable variation among the species in both the size and base sequence of this expansion segment, sufficient similarity remains to be reasonably certain that the expansion segments are truly homologous, with the

(c) C-primer sequence

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AC.      AGAUGUUCUGGGCCGCGACGCGCGCUACACUGAUUAAUCCAACGAG-UCCGCUUCAUUCGAGGCGCGGAUGC
AC-013   *****
AC-010   *****
AC-102   *****
AC-014   *****
AC-001   ....X.X..C..GXX..X.....U.....U-CX.....G.....UAGAU
AC-151   *****...GXX-UXGXAA.GACUGG
AC-009   *****.....U.....U...A..G.XCXUXGA-ACGUG.GX

AC.      CGUUGGGGUCAAACCC-----AACUGUGUCGCGUGCCUGCAUC-GCGCCUGG
AC-013   *****
AC-010   *****..XCGGCUGA..XXX...XU...X...
AC-102   *****..UX...XXX...XU.....
AC-014   ****.....X..-----UAUCGU.....-.-.....XX...XU.....
AC-001   -XG.U-UUGGUUUGUGUGAGACAUAACAAXAC---UAUUUC---X.....XX...-.....
AC-151   -.CC-UUCGU-CAXGX-----..C.X.GA.GC.G.XG.XC.X--UC....
AC-009   -.GX.C.UCGUXCU.UGGAUCGACAAGGCUXGXXGUGCGG-.XUC.C.UGC.G.XG.XC.X--UC....

AC.      GCCGAUAGGUCCGGGUAAUCUUUG-CAAAUUAUUCGUGCU-GGGGAUAGAUC-AUUGUAAUUAUUGAU-
AC-013   *****..X.XXX.....-.....-..X....X.X..-XX.....X....G
AC-010   ..X.....U.XXX.....-.....U.....-.....G
AC-102   ..G.....A..XX.....-.....U.....X.....-
AC-014   .....XX.....-.....U.....-.....-
AC-001   .....U..XX.....-.....X.....U.....-
AC-151   .....U..XX.....UG.....X.....-.....-
AC-009   .....U..XX.....UG.....C.....-.....-

AC.      CUUC--AACGAGGAUUCUAGUAAGC-GCGAGUCAUCAGCUCGCGUU
AC-013   ..AUCG..G.X..X.....UAC.....AC..AU..AGU...C
AC-010   ....G-..GUX..X.....U.UAC.....*****
AC-102   ....--..XUX..X.....*****
AC-014   ....--..XUX..X.....-..A.U*****
AC-001   ....--..XUX.....U.-.....*****
AC-151   ....--..XUX.....X--.....*****
AC-009   ....--..XX..X.....X--.....*****

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possible exception of the expansion segment in AC-151. For example, all strains except AC-151 and AC-009 possess the sequence AAXUAC at the start of the expansion segment in AC and AC-009 possesses the sequence GGCAGGCGCG in common with AC in the middle of the expansion segment.

A second expansion segment occurs upstream from the C-primer, but its entire length was accessible to the reverse transcription technique only for AC-001 and AC-009 (Fig. 1c). In both strains the expansion segment occupied the same position as in AC. For AC-001, the expansion segment showed strong homology with that of AC at the 5' end. Homology of the expansion segment in AC-009 was less certain. Because we were unable to sequence sufficient of the C-region of the remaining strains to find homologous flanking regions, the presence

of the expansion segment in these strains is less certain. However, there is reasonable homology between the 3' end of the expansion segment of AC and the presumed homologous regions of AC-010, AC-102, and AC-014, suggesting that these strains also possess the expansion segment.

Nucleotide sequence divergence among strains

An assessment of the extent of nucleotide sequence divergence among strains is constrained somewhat by a small inherent inaccuracy of the reverse transcriptase method of about 1% (Lane *et al.*, 1985). Therefore only divergences exceeding 1% will be regarded as significant.

In order to assess variation among strains, only those sequences common to all eight strains were used. The analysis is presented in Tables 2 and 3.

For the A-region (Table 2a), the extent of nucleotide sequence divergence among the strains of *Acanthamoeba* is about equivalent to that between the toad (*Xenopus*) and the mammals shown, but less than that between vertebrates and the single invertebrate (*Artemia*) represented.

For the C-region common to all strains of *Acanthamoeba*, the maximum extent of nucleotide sequence divergence among the strains of amoebae far exceeds that between mammals and the toad, and is closer to that between vertebrates and the invertebrate (Table 2c).

Analysis of the B-region is confounded somewhat by the presence of the expansion segment in the *Acanthamoeba* strains. For comparison with the animal sequence, the expansion segment was removed from the

Table 2. Percentage nucleotide sequence divergence (excluding gaps) among eight strains of *Acanthamoeba* compared with that among six species of animals for the homologous regions of (a) A-region (213 nucleotides); (b) B-region excluding *Acanthamoeba* specific expansion segments (161 nucleotides); (c) C-region (88 nucleotides)

(a)		1	2	3	4	5	6	Animals*	
AC	1			0	0	2	1	7	1 <i>Homo</i> (human)
AC-013	2	0			0	2	1	7	2 <i>Mus</i> (mouse)
AC-010	3	1	1			2	1	7	3 <i>Rattus</i> (rat)
AC-102	4	1	0	1			3	8	4 <i>Oryctolagus</i> (rabbit)
AC-014	5	0	0	0	0			6	5 <i>Xenopus</i> (clawed toad)
AC-001	6	1	1	1	1	0			6 <i>Artemia</i> (brine shrimp)
AC-151	7	1	2	2	2	2	1		
AC-009	8	1	2	2	2	2	2	0	
<i>Acanthamoeba</i>		1	2	3	4	5	6	7	8
(b)		1	2	3	4	5	6	Animals	
AC	1			0	0	1	1	8	1 <i>Homo</i>
AC-013	2	1			0	1	1	8	2 <i>Mus</i>
AC-010	3	6	7			1	1	8	3 <i>Rattus</i>
AC-102	4	2	5	9			2	9	4 <i>Oryctolagus</i>
AC-014	5	1	2	7	3			7	5 <i>Xenopus</i>
AC-001	6	0	1	6	2	1			6 <i>Artemia</i>
AC-151	7	3	4	9	5	4	3		
AC-009	8	3	4	9	5	4	3	0	
<i>Acanthamoeba</i>		1	2	3	4	5	6	7	8
(c)		1	2	3	4	5	6	Animals	
AC	1			0	0	2	4	18	1 <i>Homo</i>
AC-013	2	19			0	2	4	18	2 <i>Mus</i>
AC-010	3	13	14			2	4	18	3 <i>Rattus</i>
AC-102	4	9	17	8			2	19	4 <i>Oryctolagus</i>
AC-014	5	7	16	7	2			18	5 <i>Xenopus</i>
AC-001	6	8	16	7	4	4			6 <i>Artemia</i>
AC-151	7	10	18	9	7	7	3		
AC-009	8	10	16	8	6	6	6	4	
<i>Acanthamoeba</i>		1	2	3	4	5	6	7	8

* Data from Dams *et al.* (1988).

Table 3. Percentage nucleotide sequence divergence (excluding gaps) among the eight strains of *Acanthamoeba* for the expansion segment in the B-region (116 nucleotides) compared with the B-region excluding the expansion segment (161 nucleotides)

		1	2	3	4	5	6	7	8		B-region expansion segment
AC	1		3	9	18	22	32	29	19	1	AC
AC-013	2	1		3	10	19	29	23	18	2	AC-013
AC-010	3	6	7		14	18	29	26	19	3	AC-010
AC-102	4	2	5	9		16	23	28	28	4	AC-102
AC-014	5	1	2	7	3		26	33	31	5	AC-014
AC-001	6	0	1	6	2	1		33	34	6	AC-001
AC-151	7	3	4	9	5	4	3		15	7	AC-151
AC-009	8	3	4	9	5	4	3	0		8	AC-009
B-region non-expansion segment		1	2	3	4	5	6	7	8		

analysis and the comparison was restricted to homologous regions. Here again, the maximum extent of nucleotide sequence divergence among the strains far exceeds that between the mammal and the toad, and is similar to that between the vertebrates and the invertebrate (Table 2b).

Variation in the B-region expansion segment

Table 3 shows the extent of nucleotide sequence divergence among strains of *Acanthamoeba* for the expansion segment, compared with that for the remainder of the B-sequence. The extent of nucleotide sequence divergence in the expansion segment far exceeds that of the remaining sequence by up to 10-fold, and among some strains is so high that alignments of this region are tenuous. Nevertheless, it appears that the expansion segment shows not only a high rate of base substitution, but also a high prevalence of insertion/deletion (Table 3).

Phylogenetic relationships

Phylogenetic relationships were assessed using the sequences common to all eight strains (Fig. 1). The parsimony analysis yielded two equally parsimonious trees, each of 385 steps (consistency index = 60%). Because no other organism sequenced so far has an srRNA similar to that of *Acanthamoeba*, there is no appropriate outgroup available to root the tree. The strict consensus tree for the two unrooted trees is shown in Fig. 2.

The results of the bootstrap analysis are shown in Fig. 3. This analysis reveals that the consensus tree is

rigorously supported by the data. Namely, all nodes but two were found in all 20 bootstraps. Of the remaining two, one was supported in 19 of the 20 bootstraps. The

Fig. 2. Strict consensus tree of the two most parsimonious trees found, showing inferred relationships among the eight strains of *Acanthamoeba*.

Fig. 3. Results of bootstrapping the data 20 times. The values at the nodes indicate the number of times out of 20 bootstraps that that node was supported by the data.

only node weakly supported by bootstrapping (10 out of 20 bootstraps) was the one that was ambiguous on the parsimony analysis.

Discussion

Monophyly of the genus Acanthamoeba

The genus *Acanthamoeba* is characterized by the possession of short, tapering sub-pseudopodia ('acanthopodia') in the amoeboid trophozoite stage and excystment pores sealed by opercula which persist in the vacant cyst wall after excystment. The cyst wall has two layers, with cellulose in the endocyst (inner wall). Morphological features may be convergent, however, especially in unicellular organisms. For example, the amoeba genus *Comandonia*, which has a similar cyst structure with pores, has recently been placed in a different family from *Acanthamoeba* (Page, 1988). Molecular data, and especially nucleotide sequence data, offer the advantage of providing phylogenetic data that are totally independent of morphological data.

At the molecular level, *Acanthamoeba castellanii* is characterized by the presence of large expansion segments in the srRNA. The present study has shown that all seven strains of *Acanthamoeba* studied possess at least one of these expansion segments, and may possess a second. In the sense of Hennig (1966), these inserts represent 'synapomorphies', i.e. unique derived character states in common. Their presence argues very strongly for monophyly of the genus, and hence that the morphological features used to diagnose the genus are truly homologous.

Nature of variation in the srRNA of Acanthamoeba

The srRNA of mammals is characterized by three expansion segments (Dams *et al.*, 1988). Virtually all of the nucleotide sequence variation among the species of mammals for which sequences are available are confined to these expansion segments. Within the vertebrates, the human and *Xenopus* srRNA genes have an overall divergence of only 2.5%, and humans and rodents have overall divergences of 0.45% (rat) and 0.37% (mouse). However, if we consider just the expansion segments, then the respective figures are *Xenopus* 6.4%, rat 1.4%, and mouse 1.3% (Gonzalez & Schmickel, 1986). A similar situation is also found with vertebrate lrRNA (Gonzalez *et al.*, 1985). Additionally, these expansion segments show a higher rate of insertion/deletion than the rest of the molecule. The hypotheses advanced to explain this evolutionary trend suggest that there are strong selective constraints within the evolutionarily-conserved regions, but that the expansion segments

evolve freely without strong functional or structural constraints (Larson & Wilson, 1989).

The present study has revealed a similar pattern among monophyletic members of the genus *Acanthamoeba*. Here, the expansion segments show up to 30-fold the rate of nucleotide sequence divergence of the remaining regions. It seems that the non-expansion segments are evolving under considerable selective constraint. Even if it is allowed that the expansion segments themselves are evolving in a non-selected (neutral) manner, there must be strong selective constraint on the remainder of the molecule.

Extent of nucleotide sequence divergence among strains of Acanthamoeba

While it is generally accepted that a genus of protists does not encompass the equivalent genetic diversity of (say) a genus of mammals, the actual extent of nucleotide sequence diversity within genera has been poorly documented in lower groups. In a study based on srRNA sequences of the amoeboid genus *Naegleria* Baverstock *et al.* (1989) showed that the extent of nucleotide sequence divergence among species was of approximately the same magnitude as that between mammals and frogs. The present study has shown that the extent of the nucleotide sequence divergence of the genus *Acanthamoeba* is even higher – roughly similar to that between vertebrates and invertebrates.

Woese (1987) has suggested that the rRNA gene is the 'ultimate molecular chronometer'. While it is unlikely that the srRNA molecule is exhibiting purely clock-like behaviour (Guoy & Li, 1989) it does seem to evolve at a sufficiently uniform rate to be used to provide an approximate time-frame for past cladogenic events.

The vertebrates arose at least 500 million years ago, so the vertebrate-invertebrate divergence is at least this old. Thus, even on a very rough molecular clock, the extant members of the genus *Acanthamoeba* date back to a common ancestor many millions of years ago. Presumably, rates of morphological evolution in this genus have been very slow indeed.

The extent of divergence of AC-013 from AC presents a problem. Nominally, both are the NEFF strain, although they have been separate for nearly 20 years. An investigation of the provenance of the NEFF strains in various laboratories, using other genetic techniques, would clearly be useful.

Phylogenetic relationships among Acanthamoeba

The validity of and relationships between many of the named *Acanthamoeba* species are unclear. Isoenzyme studies (Costas & Griffiths, 1980, 1984; Daggett *et al.*,

1982; De Jonckheere, 1983) suggest that morphological characters have been interpreted inconsistently and may be inherently unsuitable for species diagnosis. Several species names are certainly synonyms (De Jonckheere, 1983). In general, these studies used too few enzymes, without formal genetic interpretation, to clarify relationships further.

The three morphological groups of species proposed by Pussard & Pons (1977) seem to be a more practical use of morphological criteria, albeit at a more superficial level. Most clinical isolates from amoebic keratitis cases belong to group II, while most from encephalitis cases belong to group III. If these groups prove to be 'natural' sub-generic groups, they would be useful in sorting strains for more detailed examination in, for example, a rigorous isoenzyme study.

The groups of Pussard and Pons (1977) have been criticized by Costas & Griffiths (1985), who used a typing scheme based on the semi-quantitative API Zym system. Their study placed *A. palestinensis* with five group II *Acanthamoeba* species in a single API Zym class (common numerical profile). Other group II and group III strains and the single group I strain had unique numerical profiles. They mistakenly placed *A. hatchetti* in group III. Given that the numerical profiles are essentially phenetic groups, the only serious obstacle to the Pussard and Pons system is the position of *A. palestinensis*.

The phylogenetic analysis of srRNA sequence in the various strains of *Acanthamoeba* described here has provided a relatively rigorous unrooted tree of their relationships. Unfortunately, the analysis is dogged by the absence of a suitable outgroup. Nevertheless, the unrooted consensus tree has 12 branches, so there are only 12 possible rooting positions.

The group I strains, *A. tubiashi* (AC-009) and AC-151, are clearly sister taxa. By contrast, *A. palestinensis* (AC-014) is only distantly related to *A. culbertsoni* (AC-001), the other group III strain, confirming the biochemical evidence of Costas & Griffith (1985). Furthermore, *A. palestinensis* has a temperature tolerance in the range of group II *Acanthamoeba* (generally <40 °C), whereas group III isolates grow at (at least) 40 °C. A case can be made for modifying the definitions of groups II and III to include physiological criteria (specifically, temperature tolerance) and to permit morphological convergence of some species. By assigning *A. palestinensis* to an amended group II, the tree becomes consistent with the three sub-generic groups. A further qualification is that monophyly of the amended group III could not be inferred in any case, since only one strain (AC-001) was included. This analysis does, however, give some confidence in the usefulness of the subgeneric groups of Pussard & Pons (1977) for preliminary identification.

References

- AMERICAN TYPE CULTURE COLLECTION (1984). *American Type Culture Collection Media Formulations*. Rockville, MD: ATCC.
- BAVERSTOCK, P. R., ILLANA, S., CHRISTY, P. E., ROBINSON, B. S. & JOHNSON, A. M. (1989). srRNA evolution and phylogenetic relationships of the genus *Naegleria* (Protista: Rhizopoda). *Molecular Biology and Evolution* **6**, 243–257.
- BROOKER, J. D., MAY, B. K. & ELLIOTT, W. H. (1980). Synthesis of δ -amino laevulinic acid synthase in vitro using hepatic mRNA from chick embryos with induced porphyria. *European Journal of Biochemistry* **106**, 17–24.
- CLARK, C. G. (1987). On the evolution of ribosomal RNA. *Journal of Molecular Evolution* **25**, 343–350.
- CLARK, C. G. & CROSS, G. A. M. (1988). Small-subunit ribosomal RNA sequence from *Naegleria gruberi* supports the polyphyletic origin of amoebas. *Molecular Biology and Evolution* **5**, 512–518.
- CLARK, C. G., TAGUE, B. W., WARE, V. C. & GERBI, S. A. (1984). *Xenopus laevis* 28S ribosomal RNA: a secondary structure model and its evolutionary and functional implications. *Nucleic Acids Research* **12**, 6197–6220.
- CLARK, C. G., CROSS, G. A. M. & DE JONCKHEERE, J. F. (1989). Evaluation of evolutionary divergence in the genus *Naegleria* by analysis of ribosomal DNA plasmid restriction. *Molecular and Biochemical Parasitology* **34**, 281–296.
- COSTAS, M. & GRIFFITHS, A. J. (1980). The suitability of starch-gel electrophoresis of esterases and acid-phosphatases for the study of *Acanthamoeba* taxonomy. *Archiv für Protistenkunde* **123**, 272–279.
- COSTAS, M. & GRIFFITHS, A. J. (1984). The esterases and acid-phosphatases of *Acanthamoeba* (Amoebida, Acanthamoebidae). *Protistologica* **20**, 27–31.
- COSTAS, M. & GRIFFITHS, A. J. (1985). Enzyme composition and the taxonomy of *Acanthamoeba*. *Journal of Protozoology* **32**, 604–607.
- COSTAS, M. & GRIFFITHS, A. J. (1986). Physiological characterization of *Acanthamoeba* strains. *Journal of Protozoology* **33**, 304–309.
- DAGGETT, P.-M., SAWYER, T. K. & NEVAD, T. A. (1982). Distribution and possible interrelationships of pathogenic and nonpathogenic *Acanthamoeba* from aquatic environments. *Microbial Ecology* **8**, 371–386.
- DAMS, E., HENDRIKS, L., VAN DE PEER, Y., NEEFS, J. M., SMITS, G., VANDENBEMPT, I. & DE WACHTER, R. (1988). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research* **16**, r87–r173.
- DE JONCKHEERE, J. F. (1983). Isoenzyme and total protein analysis by agarose isoelectric focusing and taxonomy of the genus *Acanthamoeba*. *Journal of Protozoology* **30**, 701–706.
- FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- GONZALEZ, I. L. & SCHMIKEL, R. D. (1986). The human 18S ribosomal RNA gene: evolution and stability. *American Journal of Human Genetics* **38**, 419–427.
- GONZALEZ, I. L., GORSKI, J. L., CAMPEN, T. J., DORNEY, D. J., ERICKSON, J. M., SYLVESTER, J. E. & SCHMIKEL, R. D. (1985). Variation among human 28S ribosomal RNA genes. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 7666–7670.
- GUNDERSON, J. H. & SOGIN, M. L. (1986). Length variation in eukaryotic rRNAs: small subunit rRNAs from the protists *Acanthamoeba castellanii* and *Euglena gracilis*. *Gene* **44**, 63–70.
- GUOY, M. & LI, W. (1989). Phylogenetic analysis based on rRNA sequences supports the archaeobacterial rather than the eocyte tree. *Nature, London* **339**, 145–147.
- HASSOUNA, N., MICHOT, B. & BACHELLERIE, J. P. (1984). The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Research* **12**, 3563–3583.
- HENNIG, W. (1966). *Phylogenetic Systematics*. Urbana: University of Illinois Press.
- JOHNSON, A. M. & BAVERSTOCK, P. R. (1989). Rapid ribosomal RNA sequencing and the phylogenetic analysis of protists. *Parasitology Today* **5**, 102–105.

- JOHNSON, A. M., McDONALD, P. J. & ILLANA, S. (1986). Characterization and in vitro translation of *Toxoplasma gondii* ribonucleic acid. *Molecular and Biochemical Parasitology* **18**, 313–320.
- LANE, D. J., PACE, B., OLSEN, G. J., STAHL, D. A., SOGIN, M. L. & PACE, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences of phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6955–6959.
- LARSON, A. & WILSON, A. C. (1989). Patterns of ribosomal RNA evolution in salamanders. *Molecular Biology and Evolution* **6**, 131–154.
- PAGE, F. C. (1988). *A New Key to Freshwater and Soil Gymnamoebae*. Ambleside: Freshwater Biological Association.
- PUSSARD, M. & PONS, R. (1977). Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). *Protistologica* **13**, 557–598.
- QU, L. H., MICHOT, B. & BACHELLERIE, J. P. (1983). Improved methods for structure probing in large RNAs: A rapid 'heterologous' sequencing approach is coupled to the direct mapping of nuclease accessible sites. *Nucleic Acids Research* **11**, 5903–5920.
- SAWYER, T. K. (1971). *Acanthamoeba graffini*, a new species of marine amoebae. *Journal of Protozoology* **18**, 650–654.
- SIMON, M. W. & WILSON, H. D. (1986). The amebic meningoencephalitis. *Pediatric Infectious Diseases Journal* **5**, 562–569.
- SPENCER, D. F., SCHNARE, M. N. & GRAY, M. W. (1984). Pronounced structural similarities between the small subunit ribosomal RNA genes of wheat mitochondria and *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 493–497.
- STEHR-GREEN, J. K., BAILEY, T. M. & VISVESVARA, G. S. (1989). The epidemiology of *Acanthamoeba* keratitis in the United States. *American Journal of Ophthalmology* **107**, 331–336.
- WOESE, C. R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221–271.