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Subgenus Systematics of *Acanthamoeba*: Four Nuclear 18S rDNA Sequence Types

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ABSTRACT. Classification of *Acanthamoeba* at the subgenus level has been problematic, but increasing reports of *Acanthamoeba* as an opportunistic human pathogen have generated an interest in finding a more consistent basis for classification. Thus, we are developing a classification scheme based on RNA gene sequences. This first report is based on analysis of complete sequences of nuclear small ribosomal subunit RNA genes (*Rns*) from 18 strains. Sequence variation was localized in 12 highly variable regions. Four distinct sequence types were identified based on parsimony and distance analyses. Three were obtained from single strains: Type T1 from *Acanthamoeba castellanii* V006, T2 from *Acanthamoeba palestinensis* Reich, and T3 from *Acanthamoeba griffini* S-7. T4, the fourth sequence type, included 15 isolates classified as *A. castellanii, Acanthamoeba polyphaga, Acanthamoeba rhysodes,* or *Acanthamoeba* sp., and included all 10 *Acanthamoeba* keratitis isolates. Interstrain sequence differences within T4 were 0%–4.3%, whereas differences among sequence types were 6%–12%. Branching orders obtained by parsimony and distance analyses were inconsistent with the current classification of T4 strains and provided further evidence of a need to reevaluate criteria for classification in this genus. Based on this report and others in preparation, we propose that *Rns* sequence types provide the consistent quantititive basis for classification that is needed.

Supplementary key words. Keratitis, pathogenic amoeba, ribosomal RNA genes, ssu rDNA.

ACANTHAMOEBA is a genus of ubiquitous free-living amoebae that are opportunistic pathogens of humans and other animal species [22, 30]. In humans, the most common disease is *Acanthamoeba* keratitis, a painful sight-threatening eye infection, but acanthamoebae also cause granulomatous amebic encephalitis, a fatal brain infection, and are known to infect other organs in AIDS patients [18, 37, 38]. It is unknown whether the different types of infection are caused by different subgroups of the genus or whether all acanthamoebae are capable of causing disease in the eyes, in the central nervous system, and in other organ tissues. An answer to this question depends on a reliable method of classifying isolates.

Classification of *Acanthamoeba* at the subgenus level has been particularly problematic, but molecular diversity within the genus is large and offers hope for development of a more consistent taxonomy. Daggett et al. [11], in a previous cladistic study, identified 15 different isozyme electrophoretic zymograms (phenotypes) in the genus. These authors found numerous inconsistencies between their data and the existing isolate identifications. These inconsistencies could be due partly to problems with interpretation of zymograms, but most likely are due primarily to the absence of consistent criteria for classification of isolates. Johnson et al. [23] concluded that rRNA sequence divergence within the genus *Acanthamoeba* is roughly similar to that differentiating vertebrates and invertebrates. A large rDNA sequence diversity also has been found in the data reported here.

In addition to detection, diagnosis and classification, interest in the function and evolution of processes in *Acanthamoeba* will undoubtedly be stimulated by recent discoveries, which include the complete sequence of the mitochondrial genome of *Acanthamoeba castellanii* Neff [4], the identification of the first cases of mitochondrial tRNA editing [27, 28] and evidence for the possible horizontal transfer of introns between algal chloroplasts and *Acanthamoeba* mitochondria [29, 35]. Selection of the most appropriate *Acanthamoeba* species for study would be important. Our ribosomal DNA data and *Rns* types would be useful for distinguishing between closely or distantly related isolates.

Cyst size and structure have been used to divide species of *Acanthamoeba* into morphological Groups I, II and III [36]. Further study of intrageneric relationships has been based on criteria such as isoenzyme patterns [11, 12, 21], physiological characteristics [9], immunological reactivities [13], mitochondrial DNA restriction fragment length polymorphisms (mtRFLP) [2, 10, 17, 41], and partial sequences of nuclear small subunit rRNA [23]. In spite of these various approaches, inconsistencies in classification persist. Of those approaches that have been tried, the use of rRNA gene sequences seems most likely to succeed. In this report, we describe the sites and extent of interstrain sequence variation in nuclear small ribosomal subunit RNA genes (*Rns*). We identify four distinct sequence types and propose using them as units of subgenus classification. Most *Acanthamoeba* keratitis isolates cluster in a single sequence type. Subsequent reports will describe additional *Rns* types and present analyses of those nuclear or mitochondrial rDNA sequences.

MATERIAL AND METHODS

Strains and culture conditions. The terms "strain" and "isolate" are used interchangeably throughout this report. *Acanthamoeba* cultures were grown axenically as monolayers in Corning 25 cm^2 culture flasks at 27° C–30° C in OGM [6]. Table 1 lists the strains used and their sources. The Ma isolate, obtained from Dr. E. Willaert, was identified as *Acanthamoeba polyphaga* based upon morphology, but later was identified as *A. castellanii* by reactivity with hyperimmune sera [30]. A soil isolate from Japan with an mtRFLP pattern identical to Ma was identified as *A. polyphaga* JAC/S2 [41]. Reactivity with hyperimmune sera was used to identify *Acanthamoeba* sp. 88-2-27 as *A. castellanii* or *A. polyphaga* or *Acanthamoeba rhysodes* and *Acanthamoeba* sp. 88-2-37 as *A. castellanii* or *A. polyphaga* (Osato, M., pers. commun.).

Gene nomenclature. Lonergan and Gray [29] have adopted names for *Acanthamoeba* mitochondrial rRNA genes that are based on nomenclature for *Schizosaccharomyces pombe* [25]. We propose the same mnemonics for the nuclear genes, but with the recommendation of the Commission on Plant Gene Nomenclature that nuclear genes can be designated by using upper case for the first letter of the mnemonic and organelle genes can be designated with lower case for the first letter [8]. The mitochondrial large and small subunit RNA genes have been named *rnl* and *rns*. Therefore, we propose that the corresponding large and small subunit nuclear genes should be

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Table 1. *Acanthamoeba* isolates analyzed.

Strains	ATCC no.	GenBank no.[a]	Source
Group II			
A. castellanii			
Castellani	50374	U07413	Yeast culture (London, UK)
Ma	50370	U07414	Keratitis (New York, NY)
Neff	50373	U07416	Soil (Pacific Grove, CA)
CDC:0981:V006[b]	50494	U07400	Brain (Atlanta, GA)
CDC:0184:V014[b]	50492	U07401	Keratitis (India)
CDC:0786:V042[b]	50493	U07403	Keratitis (IL)
CDC:0180:1[b]	50491	U07405	Lung (Pittsburg, PA)
A. griffini S-7	30731	U07412	Shallow beach (New London, CT)
A. polyphaga			
JAC/S2[c]	50372	U07415	Soil (Japan)
73-1-16[d]	50371	U07407	Keratitis (Houston, TX)
CDC:0885:V029[b]	50495	U07402	Keratitis (Boston, MA)
A. rhysodes			
85-6-116[d]	50368	U07406	Keratitis (Houston, TX)
Singh	—	U07417	—
Group III			
A. palestinensis			
Reich	30870	U07411	Soil (Israel)
Group Not Identified			
Acanthamoeba sp.			
82-12-324[d]	—	U07408	Keratitis (Houston, TX)
88-2-27[d]	50369	U07409	Keratitis (Houston, TX)
88-2-37[d]	50497	U07410	Keratitis (Houston, TX)
CDC:0688:V125[b]	—	U07404	Keratitis (Los Angeles, CA)

[a] GenBank accession numbers for *Rns* sequences.
[b] Gift from Dr. Govinda Visvesvara, Atlanta, GA.
[c] Gift from Dr. Takuro Endo, Tokyo, Japan.
[d] Gift from Dr. Michael Osato, Houston, TX.

named *Rnl* and *Rns*. The nuclear large and small subunit rRNA genes in *Acanthamoeba* also have been referred to as the 26S and 18S rRNA genes, respectively.

DNA and RNA isolation, PCR amplification and DNA sequencing. Amoebae were harvested from confluent cultures (~1 × 10^6 amoebae) by low speed centrifugation (~1000 *g*, 5–10 min). The cell pellet was either resuspended in 500 μl of lysis buffer (200 μg Proteinase K, 0.2% sodium dodecyl sulfate, 10 mM Tris·HCl pH 7.4, 10 mM NaCl, 10 mM EDTA [3]) and incubated at 60° C for 2 h, or resuspended in 500 μl of UNSET (8 M urea, 2% sodium dodecyl sulfate, 0.15 M NaCl, 0.001 M EDTA, 0.1 M Tris·HCl pH 7.5 [20]). The lysate was phenol–chloroform extracted twice and the nucleic acid precipitated by ethanol. The dried nucleic acid pellet was resuspended in 40 μl sterile, double distilled water. PCR amplification [32] of the complete *Rns* gene used 1 μl of whole cell DNA, oligonucleotide primers complementary to the 5′ and 3′ ends of the gene (SSU1, SSU2) [16, 40], and a standard amplification program (30 cycles; 1 min, 94° C; 2 min, 45° C; 3 min, 72° C). PCR amplification of shorter segments of *Rns* used internal primers to conserved *Rns* sequences [40].

PCR products representing complete *Rns* genes were cloned into pBluescriptSK(−) (Stratagene, La Jolla, CA) or M13 (Boehringer Mannheim, Indianapolis, IN) for double stranded or single stranded dideoxy sequencing and to preserve the product for future reference. Direct double stranded dideoxy sequencing (dsCycle Sequencing Kit; Gibco/BRL, Gaithersburg, MD) of band isolated PCR products was also used to obtain sequence rapidly. At least 85% of each molecule was sequenced on both strands. The remainder, which was in conserved regions at the ends, was sequenced on a single strand at least twice.

Alignment and phylogenetic analysis. *Acanthamoeba* sequences were deposited in GenBank [1] (Table 1) and aligned sequences are available upon request from TJB or RJG. Amoeba strains were deposited at the American Type Culture Collection (Rockville, MD) (Table 1). All sequences were aligned using the Eyeball Sequence Editor (ESEE v. 1.09e [7]) and alignments were based on primary and secondary structure conservation [31]. The 5′ and 3′ terminal 23 bases are primer specified for all of the *Acanthamoeba* sequences and were not included in any of the analyses.

When sequences for the 18 *Acanthamoeba* strains were aligned to each other, 2050 unambiguously aligned sites were identified; 46 of these sites were considered informative for the phylogenetic analysis because they were ditypic (at least two isolates shared a base that differed from the rest at that position). The number of alignable sites increased to 2163 if sequences for V006, Reich and S-7 were removed, but only 33 were considered informative. All sites that could be aligned unambiguously were used to analyze branch order within the genus *Acanthamoeba*. Bootstrapped parsimony using heuristic search algorithms with rearrangement by tree bisection-reconnection (PAUP 3.0 [34]) and neighbor-joining distance analysis with Kimura 2-parameter correction (DNADIST and NEIGHBOR, PHYLIP [14]) were used for phylogenetic reconstructions. Dissimilarity values were calculated as a percentage of mismatched bases in pairwise comparisons of sequences without the removal of unique gaps or ambiguous positions. Hence, they are not evolutionary distances. Evolutionary distances were calculated based upon the dataset where all ambiguous and gap positions were removed. Dissimilarity values are used to emphasize the amount of sequence variability between iso-

Table 2. Dissimilarity values[a] (above diagonal) and evolutionary distances[b] (below the diagonal) in pairwise comparisons of *Rns* sequences from 18 *Acanthamoeba* strains.

Species	Sequence type: Strain	T1 V006	T2 Reich	T3 S-7	T4 Cast.	T4 Ma	T4 Neff
A. castellanii	V006	—	11.9	10.8	9.3	9.4	9.9
A. palestinensis	Reich	0.0545	—	10	10.1	10	10.5
A. griffini	S-7	0.0384	0.0408	—	6.7	6	6.6
A. castellanii	Castellani	0.0354	0.0403	0.0246	—	2	2.9
A. castellanii	Ma	0.0354	0.0393	0.0226	0.0039	—	3
A. castellanii	Neff	0.0349	0.0388	0.0241	0.0034	0.0034	—
A. castellanii	V014	0.0359	0.0383	0.0246	0.0078	0.0078	0.0044
A. castellanii	V042	0.0349	0.0408	0.0241	0.0024	0.0034	0.0019
A. castellanii	180:1	0.0354	0.0398	0.0231	0.0044	0.0024	0.0039
A. polyphaga	JAC/S2	0.0354	0.0393	0.0226	0.0039	0.0000	0.0034
A. polyphaga	73-1-16	0.0339	0.0398	0.0231	0.0034	0.0044	0.0029
A. polyphaga	V029	0.0354	0.0393	0.0226	0.0039	0.0000	0.0034
A. rhysodes	Singh	0.0344	0.0368	0.0251	0.0063	0.0073	0.0049
A. rhysodes	85-6-116	0.0339	0.0398	0.0221	0.0034	0.0044	0.0039
Acanthamoeba sp.	82-12-324	0.0333	0.0403	0.0226	0.0029	0.0039	0.0034
Acanthamoeba sp.	88-2-27	0.0354	0.0378	0.0241	0.0053	0.0053	0.0019
Acanthamoeba sp.	88-2-37	0.0354	0.0414	0.0246	0.0029	0.0039	0.0024
Acanthamoeba sp.	V125	0.0354	0.0398	0.0231	0.0044	0.0024	0.0039

[a] Dissimilarity values (% differences) were percentages of mismatched nucleotides in pairwise comparisons of aligned sequences after removal of common gaps, the 23 bases at each end and the intron of *A. griffini*.

[b] Distances were calculated from the aligned dataset with all gaps and ambiguous/unalignable bases removed. There were 2050 bases in the dataset. DNADIST with the Kimura 2-parameter correction was used. Evolutionary distances are represented as nucleotide changes per base.

lates, and tend to be almost twice the calculated evolutionary distance.

RESULTS

DNA sequence heterogeneity among *Acanthamoeba* isolates. Sequences were obtained for *Rns* from 18 isolates. The sizes of all genes were ~2300 bp except for that of *A. griffini*, which had an additional 518-bp group I intron [16]. Sequences were highly conserved over most of the molecule. We sequenced *Rns* from the Ohio State University line of *A. castellanii* Neff, which has been cultured in our lab for ~25 yr, for comparison with a sequence previously reported for the Neff strain [19]. We found only a single base difference, the absence of C at position 2197. This nucleotide is absent from all 18 of our sequences. An *A. palestinensis* Reich *Rns* sequence available in GenBank (#L09599) (Patterson, D. J., Sogin, M. L. & Medlin, L., unpubl. data) matched our Reich sequence except at position 763, where we report an extra T, and at position 2182, where we report an A instead of a G. These differences occurred in highly conserved regions at positions that were not phylogenetically informative within the genus. These results, which may be due to PCR, sequencing or reading errors, are consistent with our perception that errors from all sources occur at a frequency of less than 5 bp per 1,000.

Sequence variation within a single species was as great as between species. For example, dissimilarity values between strains of *A. castellanii* ranged from 1.1% (Castellani vs. V042) to 9.9% (V006 vs. Neff), whereas they ranged from 0.18% (*A. castellanii* Ma vs. *A. polyphaga* V029) to 11.9% (*A. castellanii* V006 vs. *A. palestinensis* Reich) between strains from different species (Table 2). All sequence variation among the 18 isolates was in 12 stem or loop regions previously identified as expansion segments (Fig. 1) [19, 23]. These are regions of size and sequence heterogeneity among small subunit rRNA genes from different eukaryotes. Although some isolates shared the same sequence in several variable regions, only CDC:0688:V125 and CDC:180:1 had identical sequences throughout the gene. These two strains also had identical sequences for their mitochondrial *Rns* genes and for the highly variable spacer region that lies between the large subunit and small subunit mitochondrial rDNAs (D. Ledee, in preparation). There were two other very closely related sets of strains: *A. castellanii* V042 and *Acanthamoeba* sp. 88-2-37 differed at four positions located in regions E23-1 and 29 (Fig. 1; Table 2); and *A. castellanii* Ma, *A. polyphaga* JAC/S2 and *A. polyphaga* V029 differed from each other at four positions located in variable regions E10-1, E23-1 and E45-1. Differences between sequences from all other isolates ranged from 18 to 272 bases.

Phylogenetic analyses. The position of the genus *Acanthamoeba* near the burst of eukaryotic divergence in a global phylogeny was originally proposed by Sogin et al. [33]. Phylogenetic relationships within the genus were examined with parsimony and neighbor-joining analyses (Fig. 2A, B). When all 18 sequences were aligned and informative sites were identified, parsimony analysis gave trees with very low bootstrap values (Fig.2A). Isolates S-7, Reich and V006 appear to form a clade, but their *Rns* sequence differences were greater than 10% different from each other and at least 6% different from all other isolates (Table 2). Distance-based phylogenetic reconstruction illustrated the small evolutionary distances within T4 and the larger ones between S-7, Reich and V006 (Fig. 2B). It is apparent with both analyses that the branch order within T4 is not significant.

Differences between *Acanthamoeba Rns* genes fell into four classes that we call sequence types. The types are defined as sequences or groups of sequences that differ from all other sequences by at least 6%, have a minimum of 134 base differences, or an evolutionary distance greater than 0.8% in the current dataset (Table 2 and Fig. 2B). Three of the types are currently represented by a single strain: Type T1, *A. castellanii* V006; T2, *A. palestinensis* Reich; and T3, *A. griffini* S-7. T4, the fourth type, includes the 15 remaining isolates. Eleven of these T4 strains were previously referred to as Ribocluster A [15], a term we now discard. Overall sequence variation within T4 (Table 2; strains 4–18) ranged from 0%–4.3% (0–98 base differences) and averaged 2.5%. Dissimilarities between sequence types ranged from 6%–11.9% (134–272 base differences) and averaged 8.9%. Evolutionary distances, which ex-

Table 2. Extended.

T4 V014	T4 V042	T4 180	T4 JAC	T4 16	T4 V029	T4 Singh	T4 116	T4 324	T4 27	T4 37	T4 V125
9.6	9.3	9.8	9.4	9.6	9.5	9.8	8.9	9.2	9.6	9.3	9.8
10.7	10.4	10.5	10	10.3	10.1	10	10.3	10.3	10.8	10.4	10.5
7	6.7	6.7	6.1	6.7	6	7.2	6.6	6.7	6.8	6.9	6.7
3.8	1.1	2.7	1.9	1.1	1.9	3.2	1.5	0.89	3.6	1.3	2.7
3.8	1.8	1.8	0.2	2.4	0.2	3.9	2.3	2	3.3	1.8	1.8
3.6	2.6	3.4	3.1	2.9	3.1	3.7	3.1	3.1	3.2	2.8	3.4
—	3.3	4.3	3.8	3.4	3.8	3.4	3.2	3.4	0.8	3.2	4.3
0.0063	—	2.4	1.8	1.3	1.7	3.1	1.1	1.5	3	0.2	2.4
0.0063	0.0039	—	1.9	2.5	2	4	2.4	2.8	3.9	2.4	0
0.0078	0.0034	0.0024	—	2.4	0.2	3.9	2.3	2	3.4	1.8	1.9
0.0044	0.0019	0.0049	0.0044	—	2.3	3.5	1.5	1.9	3.4	1.2	2.5
0.0078	0.0034	0.0024	0.0000	0.0044	—	3.9	2.3	1.9	3.4	1.7	2
0.0063	0.0049	0.0068	0.0073	0.0058	0.0073	—	3.1	3.4	3.1	3.2	4
0.0044	0.0029	0.0039	0.0044	0.0010	0.0044	0.0058	—	1.2	3.3	1.1	2.4
0.0049	0.0024	0.0044	0.0039	0.0005	0.0039	0.0063	0.0005	—	3.5	1.4	2.8
0.0024	0.0039	0.0039	0.0053	0.0049	0.0053	0.0039	0.0049	0.0053	—	3.1	3.9
0.0058	0.0005	0.0044	0.0039	0.0015	0.0039	0.0053	0.0024	0.0019	0.0044	—	2.4
0.0063	0.0039	0.0000	0.0024	0.0049	0.0024	0.0068	0.0039	0.0044	0.0039	0.0044	—

cluded differences in the highly variable and unalignable regions, ranged from 0.0–0.8 within T4 (0–16 unambiguous base differences) and from 2.2–5.4 between isolates from different sequence types (45–111 unambiguous base differences).

DISCUSSION

Phylogeny and taxonomy based on *Rns*. In this study we have examined *Rns* gene sequences from 13 *Acanthamoeba* Group II strains (four species), one Group III strain (one species) and four unclassified isolates. Seventeen of the sequences determined from the 18 isolates differed from each other by at least four nucleotides. The *Rns* gene sequences were identical for *Acanthamoeba* sp. V125, isolated from an eye infection in California in 1988, and *A. castellanii* 180:1, isolated from a lung infection in Pennsylvania in 1980. Their identity was confirmed by sequencing the same genes from two separate shipments of the strains.

T4 strains have a worldwide distiribution with isolates from Asia, Europe and North America. Recent discovery of additional strains of *A. griffini* [26] indicates that T3 also has a worldwide distribution. Data presented here indicate that T4 includes representatives of three different species, *A. castellanii, A. polyphaga* and *A. rhysodes* (Fig. 2). It includes all 10 of the keratitis isolates included in our study, as well as *A. castellanii* Neff, the single strain that has been used for the great majority of all previous cellular and molecular studies.

Parsimony and distance analyses failed to resolve details of branching in T4, confirming inconsistencies in prior species classifications. *A. castellanii* Ma differs from five other isolates of this species (excluding V006) by 41–85 nucleotides, but differs from *A. polyphaga* V029 and *A. polyphaga* JAC/S2 by only four nucleotides each (Table 2). Likewise, *A. castellanii* Castellani differs from the five other isolates of *A. castellanii* by 62–89 nucleotides, but differs from *A. rhysodes* 85-6-116 by only 33 nucleotides. The two *A. rhysodes* isolates also differed from each other by 69 nucleotides, well within the range of differences seen for isolates of the other T4 species. The ambiguous identification of *Acanthamoeba* sp. 88-2-27 (*A. castellanii, A. polyphaga,* or *A. rhysodes*) and *Acanthamoeba* sp. 88-2-37 (*A. castellanii* or *A. polyphaga*) based on reactivity with hyperimmune sera (Osato, M., pers. commun.) actually is conistent with the *Rns* data in that they both are clearly members of T4.

Although the ambiguity in assigning species names to T4 strains suggests a need for reclassification of some isolates, no reliable way to subdivide all members of T4 has yet been found

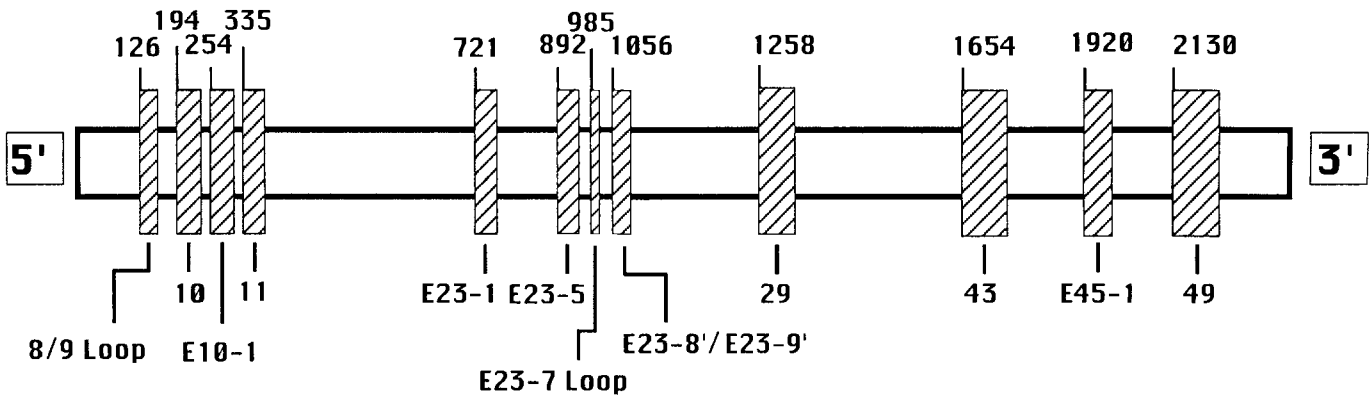

Fig. 1. Distribution of the 12 variable regions along the *Rns* gene of *Acanthamoeba*. The location of variable regions is indicated by numbers below the figure that identify small subunit rRNA stems or loops predicted by Neefs et al. [31]. Numbers above the figure indicate the base pair where the stems and loops start in our sequence for *A. castellanii* Neff (GenBank #U07416).

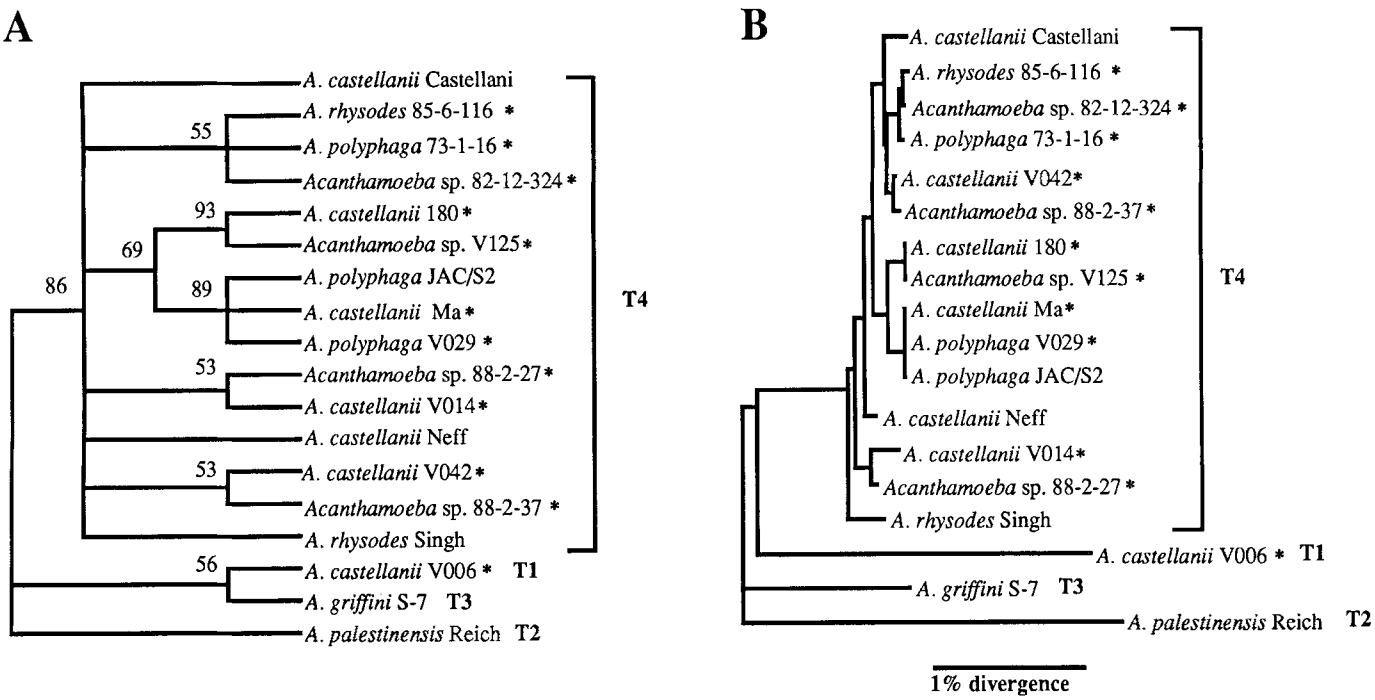

Fig. 2. **A**. Parsimony tree for *Rns* sequences from 18 *Acanthamoeba* isolates. The tree was constructed using 500 bootstrap replications of heuristic searches with tree bisection-reconnection rearrangements. This tree is unrooted. Bootstrap values below 50% were not shown. **B**. Distance tree for *Rns* sequences from 18 *Acanthamoeba* isolates. This tree is unrooted. The evolutionary distance matrix (Table 2, lower triangle) was calculated using Kimura 2-parameter correction and the tree was constructed using neighbor-joining. The scale bar represents the evolutionary distance equivalent to 1%. (*), Human disease isolate. (T1-T4), Strains with differing *Rns* sequence types as described in the text.

and no quantitative boundaries for species have been determined. A reasonable possibility would be to assign all T4 strains to a single species, but preliminary evidence suggests that sequence variation in this group of isolates may be several times higher than the interstrain variation of named species in other sequence types (Shroeder–Diedrich, J., DRL, Stothard, D., & TJB, unpubl. observ.). Although our *Rns* sequence data do not provide a reliable subdivision of T4, other molecular approaches might be more successful. In 1996, an international cooperative project to coordinate various molecular approaches to the systematics of *Acanthamoeba* was initiated at the Sixth International Conference on Amoebae in Adelaide, Australia. Thus, it would be premature to rename T4 strains at this time. Whenever possible, however, new isolates should be typed on the basis of *Rns* sequences for comparison with other typing methods. Identification of the sequence type could be achieved by sequencing *Rns* genes, as described here, by use of *Rns*-specific PCR (Lehmann, O. J., Keys, M. J.,Green, S. M., Kilvington, S., McGill, A. R., Elkington, P. J. & Watt, P. J., 1995. Early diagnosis of *Acanthamoeba* keratitis with the polymerase chain reaction. Abstract. *Invest. Ophthalmol. Vis. Sci.*, **36**: S182), or in situ probes [15].

***Rns* sequence types and isozyme patterns**. The most extensive phylogenetic analysis of *Acanthamoeba* was based on isoenzyme electrophoretic patterns for 71 isolates including 15 identified species [11]. This study identified 15 *Acanthamoeba* lineages, but noted inconsistencies between the various lineages and the classification of strains. Eight of the 15 lineages contained more than one species and several species occurred in multiple lineages. In some cases, the isozyme patterns linked strains that were clearly morphologically distinct. The authors assumed this result most likely was due to problems of strains classified based primarily on morphological criteria. It is not clear, however, whether the problem is with the other criteria or with their application by different taxonomists.

Isoenzyme patterns can be very helpful for classification purposes, but problems in their use with *Acanthamoeba* have been noted. Jacobson and Band [21] discovered a large heterogeneity in patterns obtained from environmental isolates of *A. polyphaga*. Although this might be attributed to difficulty in identifying *Acanthamoeba* species, they also reported that patterns changed when environmental isolates were grown axenically under laboratory selection. If this type of change was due to changes in gene expression or post-translational processing of the enzymes, rather than to selective replication of strains in a mixed culture, it could be problematic for isoenzyme-based classification. The use of stable DNA sequences as a basis for classification should eliminate this type of problem.

***Rns* sequence types and mitochondrial restriction fragment length polymorphisms**. Analyses in our lab of genomic mitochondrial DNA RFLP (mtRFLP) [2, 5] indicated a high degree of variation among *Acanthamoeba* isolates. However, more recently, several labs have been able to cluster strains based on identical mtRFLP [17, 24, 41]. Unfortunately, the published literature indicates few strains that have been examined both with mtRFLP and *Rns* sequences. When we compared *Rns* sequences from the Japanese isolate *A. polyphaga* JAC/S2 and the North American isolate *A. castellanii* Ma, which have identical mtRFLP [41], we found they differed by only four nucleotide pairs (Table 2). More extensive studies suggest similar close relationships between mtRFLP and *Rrn* sequence types in other strains (RJG, DRL, Yagita, K. & Endo, T., unpubl. observ.). This relationship should be examined further since RFLPs are generally easier to obtain than sequences and might be an alternative approach to recognize the clusters of strains identified by sequence types. One disadvantage of using mt-

RFLP to identify isolates, however, is that relatively large numbers of amoebae are required for an analysis.

18S rRNA sequences. Johnson et al. [23] were the first to use ribosomal nucleic acid sequences to study the phylogeny of the genus *Acanthamoeba*. They used reverse transcriptase to directly sequence three segments of 18S rRNA transcribed from seven isolates representing all three morphological groups. Their analysis suggested that the sequence divergence in *Acanthamoeba* was comparable to that between vertebrates and invertebrates. Although our data are inconsistent with some of their reported sequences, the authors are correct in reporting a relatively high degree of 18S rRNA sequence diversity within the genus. For general use, however, DNA sequencing is much easier than direct RNA sequencing.

Pathogenicity. The pathogenicity of an *Acanthamoeba* isolate cannot be determined from DNA sequence information alone. However, as we and others have noted [2, 17, 41], close phylogenetic relatedness can be one useful characteristic in the evaluation of an isolate's pathogenic potential. We suggest, for example, that the environmental isolate *A. polyphaga* JAC/S2 should be considered a potential human pathogen. The very close relationship of this isolate to the human eye isolates *A. castellanii* Ma and *A. polyphaga* V029 is indicated by the *Rns* data presented here (Table 2 and Fig. 2) and by sequences determined in our lab for a number of additional genes (DRL, unpubl. data). The most closely related strains in this study are V125, an eye isolate, and 180:1, a lung isolate. So far, we have been unable to find any genetic differences between them. Although these are different isolates, their genetic similarity is the best evidence available that any isolate might be capable of infecting more than one target tissue.

Vodkin et al. [39] designed PCR primers that appeared to distinguish pathogenic and nonpathogenic *Acanthamoeba* isolates. Their primers appeared to support amplification of a DNA fragment from nonpathogenic, but not from pathogenic acanthamoebae. The sequencing that we have done for this study reveals, however, that one of their primers is complementary to a highly variable region in the genomic DNA and that its ability to promote amplification is unrelated to pathogenicity. For example, the nonpathogen-specific PCR product could be obtained from the human disease isolate *A. rhysodes* 85-6-116. We cannot rule out the possibility that all isolates of *Acanthamoeba* are potential human pathogens. However, in this study and others to be reported later, in which we have examined *Rns* sequences from more than 50 strains, the large majority of the human disease isolates have T4 sequences. Thus, it seems reasonable that all T4 strains should be considered potential pathogens.

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LITERATURE CITED

1. Bilofsky, H. S. & Burks, C. 1988. The GenBank genetic sequence data bank. *Nucl. Acids Res.*, **16**:1861–1864.

2. Bogler, S. A., Zarley, C. D., Burianek, L. L., Fuerst, P. A. & Byers, T. J. 1983. Interstrain mitochondrial DNA polymorphism detected in *Acanthamoeba* by restriction endonuclease analysis. *Mol. Biochem. Parasitol.*, **8**:145–163.

3. Burg, J. L., Grover, C. M., Pouletty, P. & Boothroyd, J. C. 1989. Direct and sensitive detection of a pathogenic protozoan *Toxoplasma gondii* by polymerase chain reaction. *J. Clin. Microbiol.*, **27**:1787–1792.

4. Burger, G., Plante, I., Lonergan, K. M. & Gray, M. W. 1995. The mitochondrial DNA of the amoeboid protozoan, *Acanthamoeba castellanii*: complete sequence, gene content and genome organization. *J. Mol. Biol.*, **245**:522–537.

5. Byers, T. J., Hugo, E. R., Stewart, V. J. 1990. Genes of *Acanthamoeba*: DNA, RNA and protein sequences (a review). *J. Protozool.*, **37**:17S–25S.

6. Byers, T. J., Akins, R. A., Maynard, B. J., Lefken, R. A. & Martin, S. M. 1980. Rapid growth of *Acanthamoeba* in defined media; induction of encystment by glucose-acetate starvation. *J. Protozool.*, **27**:216–219.

7. Cabot, E. L. & Beckenbach, A. T. 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. *Comput. Appl. Biosci.*, **5**:233–234.

8. Commision on Plant Gene Nomenclature. 1994. Nomenclature of sequenced plant genes. *Plant. Mol. Biol. Reptr.*, **12**:S81.

9. Costas, M. & Griffiths, A. J. 1986. Physiological characterization of *Acanthamoeba* strains. *J. Protozool.*, **33**:304–309.

10. Costas, M., Edwards, S. W., Lloyd, D., Griffiths, A. J. & Turner, G. 1983. Restriction enzyme analysis of mitochondrial DNA of members of the genus *Acanthamoeba* as an aid in taxonomy. *FEMS Microbiol. Lett.*, **17**:231–234.

11. Daggett, P.-M., Lipscomb, D. S., Thomas, K. & Nerad, T. A. 1985. A molecular approach to the phylogeny of *Acanthamoeba*. *Biosystems*, **18**:399–405.

12. De Jonckheere, J. F. 1983. Isoenzyme and total protein analysis by agarose isoelectric focusing, and taxonomy of the genus *Acanthamoeba*. *J. Protozool.*, **30**:701–706.

13. Epstein, R. J., Wilson, L. A., Visvesvara, G. S., & Plounde, E. G. 1986. Rapid diagnosis of *Acanthamoeba* keratitis from corneal scraping using indirect fluorescent antibody staining. *Arch. Opthalmol.*, **104**:1318–321.

14. Felsenstein, J. 1989. PHYLIP—phylogeny inference package, vers. 3.2. *Cladistics*, **5**:164–166.

15. Gast, R. J. & Byers, T. J. 1995. Genus- and subgenus-specific oligonucleotide probes for *Acanthamoeba*. *Mol. Biochem. Parasitol.*, **71**:255-260.

16. Gast, R. J., Fuerst, P. A. & Byers, T. J. 1994. Discovery of group I introns in the nuclear small subunit ribosomal RNA genes of *Acanthamoeba*. *Nucl. Acids Res.*, **22**:592–596.

17. Gautom, R. K., Lory, S., Seyedirashti, S., Bergeron, D. L. & Fritsche, T. R. 1994. Mitochondrial DNA fingerprinting of *Acanthamoeba* spp. isolated from clinical and environmental sources. *J. Clin. Microbiol.*, **32**:1070–073.

18. Gullet, J., Mills, J., Hadley, K., Podemski, B., Pitts, L. & Gelber, R. 1979. Disseminated granulomatous *Acanthamoeba* infection presenting as an unusual skin lesion. *Am. J. Med.*, **67**:891–896.

19. 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.

20. Hugo, E. R., Stewart, V. J., Gast, R. J. & Byers, T. J. 1992. Purification of amoeba mtDNA using the UNSET procedure. *In*: Soldo, A. T. & Lee, J. J. (ed.), Protocols in Protozoology. Allen Press, Lawrence, Kansas. P. D-7.1.

21. Jacobson, L. M. & Band, R. N. 1987. Genetic heterogeneity in a natural population of *Acanthamoeba* polyphaga from soil, an isoenzyme analysis. *J. Protozool.*, **34**:83–86.

22. John, D. T. 1993. Opportunistically pathogenic free-living amebae. *In*: Kreier, J. (ed.), Parasitic Protozoa, 2d ed. Academic Press, San Diego. **1**:143–246.

23. Johnson, A. M., Fielke, R., Christy, P. E., Robinson, B. & Baverstock, P. R. 1990. Small subunit ribosomal RNA evolution in the genus *Acanthamoeba*. *J. Gen. Microbiol.*, **136**:1689–1698.

24. Kilvington, S., Beeching, J. R., & White, D. G. 1991. Differentiation of *Acanthamoeba* strains from infected corneas and the environment by using restriction endonuclease digestion of whole-cell DNA. *J. Clin. Microbiol.*, **29**:310–314.

25. Kohli, J. 1987. Genetic nomenclature and gene list of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.*, **11**:575–589.

26. Ledee, D. R., Hay, J., Byers, T. J., Seal, D. V. & Kirkness, C. M. 1996. *Acanthamoeba griffini*: molecular characterization of a new corneal pathogen. *Invest. Ophthalmol. Vis. Sci.*, **37**:544–550.

27. Lonergan, K. M. & Gray, M. W. 1993a. Editing of transfer RNAs in *Acanthamoeba castellanii* mitochondria. *Science*, **259**:812–816.

28. Lonergan, K. M. & Gray, M. W. 1993b. Predicted editing of additional transfer RNAs in *Acanthamoeba castellanii* mitochondria. *Nucl. Acids Res.*, **21**:4402.

29. Lonergan, K. M. & Gray, M. W. 1994. The ribosomal gene region in *Acanthamoeba castellanii* mitochondrial DNA. A case of evolutionary transfer of introns between mitochondria and plastids? *J. Mol. Biol.*, **239**:476–499.

30. Ma, P., Willaert, E., Juechter, K. B. & Stevens, A. R. 1981. A case of keratitis due to *Acanthamoeba* in New York, New York, and features of 10 cases. *J. Infect. Dis.*, **143**:662–667.

31. Neefs, J.-M., Van de Peer, Y. D., De Rijk, P., Chapelle, S. & De Wachter, R. 1993. Compilation of small ribosomal subunit RNA structures. *Nucl. Acids Res.*, **21**:3025–3049.

32. Saiki, R., Walsh, P. S., Levenson, C. & Erlich, H. 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc. Nat. Acad. Sci.* USA, **86**:6230–6234.

33. Sogin, M. L., Elwood, H. J. & Gunderson, J. H. 1986. Evolutionary diversity of eukaryotic small-subunit rRNA genes. *Proc. Nat. Acad. Sci.* USA, **83**:1383-1387.

34. Swofford, D. L. 1990. PAUP: Phylogenetic Analysis Using Parsimony, Vers. 3.0. Illinois Natural History Survey, Champaign, Illinois.

35. Turmel, M., Côté, V., Otis, C., Mercier, J. P., Gray, M. W., Lonergan, K. M. & Lemieux, C. 1995. Evolutionary transfer of ORF-containing group I introns between different cellular compartments (chloroplast and mitochondrion). *Mol. Biol. Evol.*, **12**:533–545.

36. Visvesvara, G. S. 1991. Classification of *Acanthamoeba*. *Rev. Infect. Dis.*, **13**(S5):S369–S372.

37. Visvesvara, G. S. 1993. Epidemiology of infections with free-living amebas and laboratory diagnosis of Microsporidiosis. *Mt. Sinai J. Med.*, **60**:283–288.

38. Visvesvara, G. S. & Stehr-Green, J.K. 1990. Epidemiology of free-living ameba infections. *J. Protozool.*, **37**:25S–33S.

39. Vodkin, M. H., Howe, D. K., Visvesvara, G. S. & McLaughlin, G. L. 1992. Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. *J. Protozool.*, **39**:378–385.

40. Weekers, P. H. H., Gast, R. J., Fuerst, P. A. & Byers, T. J. 1994. Sequence variations in small-subunit ribosomal RNAs of *Hartmannella vermiformis* and their phylogenetic implications. *Mol. Biol. Evol.*, **11**: 684–690

41. Yagita, K. & Endo, T. 1990. Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains in Japan. *J. Protozool.*, **37**:570–575.

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