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The Evolutionary History of the Genus *Acanthamoeba* and the Identification of Eight New 18S rRNA Gene Sequence Types

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ABSTRACT. The 18S rRNA gene (*Rns*) phylogeny of *Acanthamoeba* is being investigated as a basis for improvements in the nomenclature and taxonomy of the genus. We previously analyzed *Rns* sequences from 18 isolates from morphological groups 2 and 3 and found that they fell into four distinct evolutionary lineages we called sequence types T1–T4. Here, we analyzed sequences from 53 isolates representing 16 species and including 35 new strains. Eight additional lineages (sequence types T5–T12) were identified. Four of the 12 sequence types included strains from more than one nominal species. Thus, sequence types could be equated with species in some cases or with complexes of closely related species in others. The largest complex, sequence type T4, which contained six closely related nominal species, included 24 of 25 keratitis isolates. *Rns* sequence variation was insufficient for full phylogenetic resolution of branching orders within this complex, but the mixing of species observed at terminal nodes confirmed that traditional classification of isolates has been inconsistent. One solution to this problem would be to equate sequence types and single species. Alternatively, additional molecular information will be required to reliably differentiate species within the complexes. Three sequence types of morphological group 1 species represented the earliest divergence in the history of the genus and, based on their genetic distinctiveness, are candidates for reclassification as one or more novel genera.

Supplementary key words. Classification, granulomatous amoebic encephalitis, keratitis, multiple rDNA alleles, opportunistic pathogen, phylogeny.

ACANTHAMOEBA has been isolated from air, soil, freshwater, saltwater, wild animals and humans. In healthy humans, it can cause the sight-threatening eye disease *Acanthamoeba* keratitis. In immunocompromised individuals, it can cause the fatal disease granulomatous amebic encephalitis or disseminated infections of other tissues [16, 19, 20, 23, 31]. Identification of amoebae from the genus *Acanthamoeba* is relatively easy after they have been grown in culture. Classification at the subgenus level is a problem, however, even for expert taxonomists [30].

The nomenclature and taxonomy of *Acanthamoeba* have been revised many times [30], most recently ~20 years ago [26, 28]. Pussard and Pons [26] divided the genus into three morphological groups based on cyst size and shape (Table 1). Group 1 species have large cysts compared to the other groups, with a smooth ectocyst and a stellate endocyst. Group 2 species have a wrinkled ectocyst and the endocyst can be stellate, polygonal, triangular, or oval. Group 3 species typically have a thin, smooth ectocyst with a round endocyst. However, Sawyer first observed that ionic strength of the growth medium can alter the shape of cyst walls [27], thus, substantially reducing the reliability of cyst morphology as a taxonomic characteristic. Pussard and Pons [26] used the number of ostioles in the cysts as defining characters, but this character has been mentioned infrequently in subsequent descriptions of species.

In the last decade, several groups have used analysis of isoenzyme electrophoretic patterns to address intrageneric relationships and to test the morphological classification scheme. These studies discovered extensive diversity among isolates of *Acanthamoeba* as exhibited by unique isoenzyme patterns, especially for group 1 species. Moura, Wallace and Visvesvara [22] found good agreement between isoenzyme patterns and morphological groups, but their study was limited and included only one group 2 isolate, *A. castellanii* Castellani. De Jonckheere [8], Daggett et al. [7], and Costas and Griffiths [6], studied larger groups of isolates including 30, 71 and 37 strains, respectively. Each study divided isolates of *Acanthamoeba* into several different groups that often were inconsistent with species and/or morphological group designations. Each study suggested a need for revision of the classification.

Analyses of *Acanthamoeba* mitochondrial DNA restriction fragment length polymorphisms (mtRFLP), although limited in number of isolates used, also revealed a large degree of interstrain genetic diversity [2, 33]. Species belonging to groups 2 and 3, especially *A. castellanii* and *A. polyphaga,* were shown to be polyphyletic.

Isoenzyme patterns and mtRFLP analyses have highlighted ambiguities in the morphology-based classification scheme, but neither approach directly addressed the phylogeny of *Acanthamoeba.* Johnson et al. [17] analyzed partial nuclear 18S rRNA sequences from seven isolates of *Acanthamoeba* and obtained results that were concordant with the classification of Pussard and Pons, but only five species were included. Our lab recently began an investigation of 18S rRNA gene (*Rns*) phylogeny using 18 isolates of *Acanthamoeba* from morphological groups 2 and 3 [12]. These groups were chosen because they contain species most frequently isolated from human infections. The amount of sequence dissimilarity among the 18 isolates ranged from 0–11.9% and the various isolates subdivided into four rDNA sequence types. Fifteen of the isolates formed a tight phylogenetic cluster designated sequence type T4. The remaining three isolates each had sequences that were very distinct from the major group and, thus, were placed in sequence types T1–T3.

Here we examine 18S rDNA sequence variation in a group of 53 strains that includes the original 18 isolates plus 35 new isolates. Sixteen species and all three morphological groups are represented. We have identified eight more sequence types and have discovered that morphological group 1 isolates are so highly divergent that they probably could represent one or more unique genera.

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MATERIALS AND METHODS

Acanthamoeba isolates. All isolates were grown axenically as monolayers in Corning 25 cm^2 culture flasks at 27–30° C in optimal growth media (OGM) [4]. Table 1 lists all isolates used, their sources and GenBank accession numbers for the 18S rDNA sequences. Cultures were provided by the following individuals who were at the institutions indicated at the time cultures were received: Dr. T. A. Nerad, American Type Culture Collection (ATCC), Rockville, MD; Dr. X-y. Jin, Beijing Institute of Ophthalmology (BIO), Beijing, PRC; Dr. Govinda S. Visvesvara, Centers for Disease Control (CDC), Atlanta, GA; Dr. M. S. Osata and Dr. K. R. Wilhelmus, Cullen Eye Institute (CEI), Baylor University School of Medicine, Houston, TX; Dr. J. Liu, East China Normal University (ECNU), Shanghai, PRC; Dr. A. Stevens; Gainesville Veterans Administration Hospital (GVA), FL; Dr. J. De Jonckheere, Institute of Hygiene and Epidemiology (IHE), Brussels, Belgium; Dr. T. Endo and Dr. K. Yagita, Japanese National Institute of Health (JNIH), Tokyo; Dr. T. K. Sawyer, National Marine Fisheries Service (NMFS), Oxford, MD; Dr. J. Hay and Dr. D. V. Seal, Tennent Institute of Ophthalmology (TIO), Glasgow, Scotland; SRZ, Universidad Nacional Autónoma de México(UNAM); Dr. P. H. H. Weekers, University of Nijmegen (UNI), The Netherlands; Dr. J. Neff, Vanderbilt University (VUN), Nashville, TN.

DNA isolation. Cells were harvested from confluent cultures by low speed centrifugation (5–10 min at 500–1,000 *g*) and lysed with UNSET [11, 15]. Nucleic acids were isolated from the lysate by phenol-chloroform extraction and ethanol precipitation. The DNA was sedimented by centrifugation and the pellet was resuspended in double distilled water or Tris-EDTA (TE) buffer and quantified.

PCR primer design and amplification. Primers for use in the polymerase chain reaction (PCR) amplification of the entire 18S rRNA gene were designed based on known conserved sequences of the 18S rRNA gene (SSU1 and SSU2) [32]. The primers contained *Sal*I and *Bam*HI sites at the 5′ ends to facilitate cloning of PCR products. Typical 100 µl PCR reaction conditions included 40 pmol of each primer, 500 ng–1µg of genomic DNA, 1.25 mM dNTP, 2–4 mM $MgCl_2$, and 2.5 units of Taq polymerase in a 1× reaction buffer (Perkin-Elmer Cetus, Norwalk). PCR was performed for 30–40 cycles, using the following parameters: 95° C (1 min), 48–53° C (2 min), 72° C (3 min). PCR amplifications of shorter segments of the 18S rRNA gene were done using internal primers [11, 32].

DNA sequencing. Direct sequencing of PCR products and of cloned products used the BRL dsCycle Sequencing Kit (Gibco/BRL, Gaithersburg) as described by the manufacturer. To assure sequence fidelity, all amplified genes were sequenced from pooled, multiple PCR products. Seven strains appeared to produce multiple sequencing ladders (i.e. multiple alleles). For these strains, pooled PCR products suspected of containing multiple alleles were restricted and cloned into *Sal*I/*Bam*HI restricted pBluescriptSK- (Stratagene, LaJolla), or the PCR products were cloned directly into the TA cloning vector (Invitrogen, LaJolla). Cloned products then were sequenced by double stranded dideoxy sequencing. In order to assure sequence fidelity and maintain allele differences, multiple clones were sequenced and sequence was obtained from both strands.

Alignment of sequences. Nucleotide sequences were aligned with the assistance of the program ESEE (Eyeball Sequence Editor) [5] after identifying homologous positions on the basis of secondary structure [14, 24]. One master alignment was made and used for all further comparisons and phylogenetic reconstructions. The 18S rRNA genes of *Acanthamoeba* are approximately 2,300–2,700 bp in length. However, ten isolates from two different species contained group 1 introns [11; JMS-D et al., unpubl. data]. Intron sequences were not included in our analyses. Percent nucleotide similarities were calculated using all sites of the 18S rRNA gene. Large insertion or deletion events were treated as multiple sites rather than as single sites in the calculation. The percent similarity corresponds to the number of sites that are identical between two isolates, aligned to each other as in the master alignment, divided by the total number of sites compared after common gaps have been removed. Alignments are available at our Web site, www.bio-sci.ohio-state.edu/~pfuerst/!acanth.htm, or from DRS, RJG, TJB or PAF.

Phylogenetic analysis. Introns, regions of ambiguous alignment and regions specified by PCR primers SSU1 and SSU2 were removed from the master alignment so that only homologous sites were compared. As a result, the phylogenetic analysis included 1,863 sites. Once aligned, nucleotide sequences were compared and a distance matrix, which is available at our Web site or upon request, was generated using DNADIST in the PHYLIP package (Ver. 3.5) [9]. Phylogenetic inferences were made using neighbor-joining (NEIGHBOR) with the Kimura two-parameter correction for multiple substitutions as implemented in PHYLIP. The data also were examined using the maximum parsimony program DNAPARS and the maximum likelihood program DNAML in PHYLIP. Bootstrap analyses were done by generating 1,000 datasets at random using SEQBOOT. Consensus neighbor-joining and consensus parsimony trees were generated from bootstrapped datasets and bootstrap values were obtained. All analyses were performed on an IRIX Indigo (Silicon Graphics, Inc.) workstation.

Subcluster analysis. The phylogenetic analysis that included all 53 strains required deletion of regions that were ambiguous when all 12 sequence types were included, but contained phylogenetically useful sites for the more closely related taxa T3, T4 and T11. Therefore, the master alignment was modified in two ways and a subcluster analysis was performed: 1) only taxa within sequence types T2, T3, T4, T6 and T11 were included; and 2) ambiguous regions were re-defined for the subcluster dataset. T2 and T6 taxa were included in order to place the root joining T3, T4 and T11. This subcluster dataset included 35 taxa. Due to smaller regions of ambiguity between these taxa, the phylogenetic analysis of these groups was based on 2,090 *Rns* sites. Analyses of the subcluster dataset were performed as described above for the full dataset.

RESULTS

***Rns* sequence heterogeneity**. The average eukaryotic *Rns* gene is 1,800–1,900 bp in length [13], but the homologous *Acanthamoeba* genes contain additional base pairs in 11 regions of size heterogeneity indicated by red bases (Fig. 1). These regions, first recognized in *Acanthamoeba* by Gunderson and Sogin [13] in comparisons of the 18S rRNA sequence of *A. castellanii* Neff with comparable sequences of other eukaryotes, are referred to as expansion segments. They occur in the eukaryotic *Rns* variable regions V1–V7 [29]. The black bases in the 18S rRNA sequence of *A. castellanii* Castellani represent the regions that are conserved in the genus although a number of isolated variable sites also occur within these regions.

Due to extra nucleotides found in expansion segments, the *Rns* genes from morphological group 2 and group 3 isolates of *Acanthamoeba* were ~2,300 bp. The major exceptions were the genes from two isolates of *A. griffini* and eight isolates of *A. lenticulata* that contained group 1 introns and, thus, were larger [11; JMS-D, unpubl. data]. The *Rns* genes from morphological group 1 isolates *A. astronyxis, A. comandoni* and *A. tubiashi* were 2,600–2,700 bp. Using the small subunit rRNA structure

proposed by Neefs et al. [24] as a reference, most of the additional base pairs found in these isolates occurred as insertions in four regions: i) stems 10 and 11; ii) stem E23-1; iii) the loop between stems E23-8 and E23-9; and iv) stem 49 (Fig. 1). Due to these additional insertions, *Rns* sequences from these group 1 isolates were only ~65% similar to sequences from all other isolates and evolutionary distances between group 1 and other isolates were 9.9–11.2%.

Of the 53 *Rns* sequences obtained here and in our previous study [12], 41 had unique rRNA coding sequences. Eight of the 12 strains with rRNA coding sequences that were not unique belonged to a single species, *A. lenticulata,* one of the two *Acanthamoeba* species in which *Rns* introns have been found [11]. If the introns were included in sequence comparisons [JMS-D, unpubl. data], then six of the eight *A. lenticulata* sequences and 47 (89%) of the 53 *Rns* sequences were unique. Three pairs of strains that shared identical alleles remained; 1) *A. lenticulata* isolates 45 (sequence #36) and 72/2 (#37), 2)lung isolate *A. castellanii* 180:1 (#14) and keratitis isolate *A.* species V125 (#28), as previously reported [12], and 3) keratitis isolate *A.* species 88-2-37 (#27) and one allele of keratitis isolate *A.* species H30 (#31)(see below).

Multiple alleles. It has been estimated that amoebae of *A. castellanii* Neff have several hundred copies of the *Rns* gene [3], but only one allele has been detected. Evidence suggesting, however, that amoebae from other strains can have more than one *Rns* allele was found for seven of the 53 isolates studied here: #3, GE3a; #30, Jin-E5; #31, H30; #33, M95:7:45; #50, Lilly A1; #51, RF-B-1; and #53, V013. Only one allele has been detected so far in all other isolates.

Initial evidence for the presence of multiple alleles most frequently came from observing overlapping (i.e. ambiguous) regions in sequence ladders obtained from whole or partial *Rns* PCR products (Fig. 2B). The individual ladders could be resolved by sequencing cloned PCR products as demonstrated for isolate GE3a (Fig. 2A, C). The presence of several alleles in a PCR product could have three explanations: 1) Taq polymerase errors; 2) the presence of genetically different strains in the amoeba culture, or 3) the presence of several different alleles in individual amoebae. The multiple alleles are unlikely to be due to Taq polymerase errors. The numbers of differences between alleles in H30, the most carefully studied strain, were 1–4 bp per 1,000, whereas, our error rate for sequences from strains with only one *Rns* allele consistently was 0.5–1 bp per 1,000 [12]. The allele-specific differences within a strain were observed with several different primer sets and multiple PCR reactions. Strains 88-2-37 and H30 share one identical allele, but, whereas, only one allele was found in 88-2-37, two other alleles were found in H30. It is unlikely in this case that Taq repeatedly would make the same allele-specific mistakes in the DNA of one strain and not the other. Also, allele-specific insertion/deletion events were found in some strains with multiple alleles in addition to the point mutations that are characteristic of Taq errors. Thus, we conclude that the allelic differences are not due to Taq errors. The possibility that the presence of multiple alleles in H30 was due to PCR amplification from cultures that were mixtures of strains was ruled out by PCR amplifying from cloned amoeba cultures. The same three alleles were found in each of the cloned cultures. Thus, we conclude that individual amoebae can have more than one *Rns* allele.

Neighbor-joining analysis. The phylogeny inferred from the distance matrix for all 53 sequences is shown in Fig. 3. To use as much of the data as possible, this analysis has no outgroup. The *Rns* sequences from other genera that are closest to those of *Acanthamoeba* are from *Hartmannella vermiformis* (GenBank No. M95168) and *Balamuthia mandrillaris* (No. AF019071) Evolutionary distances determined from alignments that included all three genera were 0.00–7.19 substitutions per 100 bases between *Acanthamoeba* strains (0.00–3.27 if group 1 strains were excluded), whereas, they were 7.42–9.43 between *Balamuthia* and *Acanthamoeba* and 13.92–17.04 between *Hartmannella* and *Acanthamoeba.* When *Balamuthia,* the closest relative to *Acanthamoeba,* was used as the outgroup, we obtained regions of ambiguous alignment that were not ambiguous when only *Acanthamoeba* sequences were compared. The *Rns* sequence of *B. mandrillaris* is only 1,974 bp because it does not contain the expansion segments found in *Acanthamoeba.* Thus, it was necessary to delete most of the expansion segments found in *Acanthamoeba* and, consequently, most of the phylogenetic information in order to align *Rns* sequences of *B. mandrillaris* with *Acanthamoeba.*

Assuming approximately equal rates of substitution in all taxa examined, this analysis showed an early divergence of the three group 1 isolates. Consequently, these three isolates were used to root the phylogenetic tree examining the remainder of the taxa. A bootstrap analysis of the data was performed. Twelve lineages characterized by 12 distinct sequence types (T1–T12) (see below) were observed. Four of these types have been described previously [12]. With the exception of T5, which includes 12 isolates identified as *A. lenticulata,* the sequence types that include more than one strain (T2, T3, T4 and T11) all have isolates from more than one species and, thus, are species complexes.

Parsimony analysis. A total of 569 equally parsimonious trees were generated from a maximum parsimony analysis of all 53 taxa. Therefore, a bootstrap analysis was done to generate a consensus parsimony tree. The tree was nearly identical to the neighbor-joining tree in Fig. 3 and, thus, is not shown. The main difference was that T3, T4 and T11 formed a single clade for the following reason. From the 1,863 homologous sites examined, 216 were informative for parsimony analysis. However, the group 1 species *A. astronyxis, A. comandoni* and *A. tubiashi* were uniquely characterized by 93 (43.1%) of the informative sites and isolates of *A. lenticulata* (T5) were uniquely characterized by 22 (10.2%) of the informative sites. Therefore, 115 (53.2%) of the sites specifically identified group 1 species or *A. lenticulata.* The remaining 101 informative sites were not enough to significantly separate T3, T4 and T11.

Subcluster analysis. In an attempt to resolve the ambiguous branching patterns obtained in the T3, T4, and T11 region using all 53 taxa, a subcluster analysis or compartmentalization [21] was performed using neighbor-joining (Fig. 4). The phylogeny from the 53-taxon dataset revealed that T2 and T6 taxa diverge immediately prior to the divergence of T3, T4, and T11 taxa. Therefore, T2 and T6 were used to root the subcluster tree. This subcluster analysis included an additional 227 sites for a total of 2,090, of which 55 were variable and 49 informative. Compartmentalization showed distinction between T3, T4 and T11 clades. However, it did not significantly define relationships among all taxa within T4. The distribution of named species within T4 remains polyphyletic in some cases and unresolved in others. It appears that T4 strains are too closely related to be resolved fully by use of *Rns* sequences.

***Rns* sequence types.** Nucleotide sequence similarities/dissimilarities based on our alignment were examined in the 12 evolutionary lineages. The maximum dissimilarities between isolates within sequence types were the 4.9% in T2 and 4.4% in T4, whereas, the minimum dissimilarities between these sequence types and others were 5.2% between T2 and T6 and 5% between T4 and T11 (Table 2). Differences between all other sequence types were greater. Thus, in this study, differences between sequence types were at least 5% and were always

Table 1. Strains used in this study.

#	Seq. Type	Current species classification	Proposed revised classification[a]	Strain name (isolate source)	Morphological group	GenBank Ref. No.	Source
1	T1	*A. castellanii*	*A.* species	*(CDC:0981:V006)[b]	2	U07400	GAE[e], brain, Georgia, USA
2	T2	*A. palestinensis*		*Reich (ATCC:30970)	3	U07411	Soil, Israel
3	T2	*A. pustulosa*	*A. palestinensis*	GE 3a (ATCC:50252)	3	AF019050	Water, France
4	T2	*A. polyphaga*	*A. palestinensis*	CCAP:1501/3c (Weekers:UNI)	2	AF019051	Old distilled water, USA
5	T3	*A. griffini*		*S-7 (Stevens:GVA)	2	U07412	Beach bottom, Connecticut, USA
6	T3	*A. griffini*		Hay & Seal (TIO:H37)	2	S81337	Keratitis, Scotland, UK
7	T3	*A. polyphaga*	*A. griffini*	Panola Mt. (ATCC:30487)	2	AF019052	Soil, Georgia, USA
8	T3	*A. pearcei*	*A. griffini*	Sawyer (ATCC:50435)	1	AF019053	Sewage dump, Atlantic Ocean, USA
9	T4	*A. castellanii*		*Castellani (ATCC:3001/ATCC:50374)[c]	2	U07413	Yeast culture, London, UK
10	T4	*A. castellanii*		*Ma (ATCC:50370)	2	U07414	Keratitis, New York, USA
11	T4	*A. castellanii*		*Neff (Neff:VUN/ATCC:50373)	2	U07416	Soil, California, USA
12	T4	*A. castellanii*		*(CDC:0184:V014/ATCC:50492)	2	U07401	Keratitis, India
13	T4	*A. castellanii*		*(CDC:0786:V042/ATCC:50493)	2	U07403	Keratitis, Illinois, USA
14	T4	*A. castellanii*		*(CDC:180:1)	2	U07405	Lung infection, Pennsylvania, USA
15	T4	*A. lugdunensis/A. polyphaga*		*Garcia (CEI:73-01-16/ATCC:50371)	2	U07407	Keratitis, Texas, USA
16	T4	*A. hatchetti*		2AX1	2	AF019060	Marine sewage dump, USA
17	T4	*A. polyphaga*		*Jac/S2 (ATCC:50372)	2	U07415	Soil, Japan
18	T4	*A. polyphaga*		Page 23 (ATCC:30871)	2	AF019061	Freshwater pond, WI, USA
19	T4	*A. polyphaga*		*(CDC:0884:V029)	2	U07402	Keratitis, Massachusetts, USA
20	T4	*A. polyphaga*		HC-2 (Rodriguez-Zaragoza:UNAM)	2	AF019056	Mexico
21	T4	*A. polyphaga*		Nagington (ATCC:30873)	2	AF019062	Keratitis, England, UK
22	T4	*A. culbertsoni*	*A.* species	Diamond (CDC)	3	AF019057	Keratitis, OH, USA
23	T4	*A. rhysodes*		*Singh (ATCC:30973)	2	U07417	Soil, England, UK
24	T4	*A. rhysodes*		*Haas (CEI:85-6-116/ATCC:50368)	2	U07406	Keratitis, Texas, USA
25	T4	*A.* species		*Galka (CEI:85-12-324/ATCC:50496)	nd[d]	U07408	Keratitis, Texas, USA
26	T4	*A.* species		*Fernandez (CEI:88-2-27/ATCC:50369)	nd	U07409	Keratitis, Texas, USA
27	T4	*A.* species		*Rawdon (CEI:88-2-37/ATCC:50497)	nd	U07410	Keratitis, TX, USA
28	T4	*A.* species		*(CDC:0688:V125/ATCC:50498)	nd	U07404	Keratitis, California, USA
29	T4	*A.* species		Liu-E1 (ATCC:50709)	nd	AF019055	Keratitis, China
30	T4	*A.* species		Jin-E5 (ATCC:50710)	nd	AF019054	Keratitis, Beijing, China
31	T4	*A.* species		Hay & Seal (TIO:H30)	nd		Keratitis, England, UK
32	T4	*A.* species		Vazaldua (CEI:M95:5:27)	nd	AF019058	Keratitis, Texas, USA
33	T4	*A.* species		Rodriguez (ATCC:50711)	nd	AF019059	Keratitis, Texas, USA
34	T5	*A. lenticulata*		PD2S (ATCC:30841)	3	U94741	Swimming pool, France
35	T5	*A. lenticulata*		NJSP-3-2 (ATCC:50429)	3	U94738	Sewage plant, New Jersey, USA
36	T5	*A. lenticulata*		45 (ATCC:50703)	3	U94730	Nasal mucosa, Germany
37	T5	*A. lenticulata*		72/2 (ATCC:50704)	3	U94732	Nasal mucosa, Germany
38	T5	*A. lenticulata*		68-2 (ATCC:50427)	3	U94733	Freshwater stream, Florida, USA
39	T5	*A. lenticulata*		7327 (ATCC:50705)	3	U94731	Water, France
40	T5	*A. lenticulata*		E18-2 (ATCC:50690)	3	U94735	Sewage dump site, Atlantic Ocean, USA
41	T5	*A. lenticulata*		53-2 (ATCC:50691)	3	U94737	Sewage sump site, New York Bight, USA
42	T5	*A. lenticulata*		118 (ATCC:50706)	3	U94736	Nasal mucosa, Germany
43	T5	*A. lenticulata*		25-1 (ATCC:50707)	3	U94740	Nasal mucosa, Germany
44	T5	*A. lenticulata*		Jc-1 (ATCC:50428)	3	U94739	Freshwater stream, New York, USA
45	T5	*A. lenticulata*		407-3a (ATCC:50692)	3	U94734	Acid waste dump site, Atlantic Ocean, USA
46	T6	*A. palestinensis*		2802 (ATCC:50708)	3	AF019063	Swimming pool, France
47	T7	*A. astronyxis*		Ray & Hayes (ATCC:30137)	1	AF019064	Lab water, Washington, USA
48	T8	*A. tubiashi*		OC-15C (ATCC:30867)	1	AF019065	Freshwater, Maryland, USA
49	T9	*A. comandoni*		Comandon & de Fonbrune (ATCC:30135)	1	AF019066	Soil, France
50	T10	*A. culbertsoni*		Lilly A1 (ATCC:30171)	3	AF019067	Human cell culture, Indiana, USA

Table 1. Cont.

#	Seq. Type	Current species classification	Proposed revised classification[a]	Strain name (isolate source)	Morphological group	GenBank Ref. No.	Source
51	T11	*A. stevensoni*		RB:F:1 (ATCC:50388)	2	AF019069	Marine sediment, New York, USA
52	T11	*A. hatchetti*		BH-2 (Sawyer:NMFS)	2	AF019068	Brackish water, Maryland, USA
53	T12	*A. healyi*		(CDC1283:V013)	3	AF019070	GAE, brain, Barbados, BWI

[a] Revised species designation suggested by the *Rns* phylogeny.
[b] The designation in parentheses identifies the source of the culture we originally received (See Materials and Methods). Cultures received from CDC, CEI and IHE were provided by G. S. Visvesvara, M. S. Osata or K. R. Wilhelmus, and J. de Jonckheere, respectively. (*), strains used in our previous analysis [12].
[c] An ATCC number following (/) within parentheses was assigned after sequencing.
[d] nd, not determined.
[e] GAE, Granulomatous Amoebic Encephalitis.

greater than differences within sequence types. Seven isolates, *A. castellanii* V006 (T1), *A. palestinensis* 2802 (T6), *A. astronyxis* Ray (T7), *A. tubiashi* OC-15C (T8), *A. comandoni* Comandon and deFonbrune (T9), *A. culbertsoni* Lilly A-1 (T10) and *A. healyi* V013 (T12), which were identified as separate lineages, were 5.2–19.2% different from all other isolates. Five species, *A castellanii, A. culbertsoni, A. hatchetti, A. palestinensis,* and *A. polyphaga* each included strains with more than one sequence type and, thus, appear to be polyphyletic.

DISCUSSION

Phylogeny and taxonomy. The current classification of *Acanthamoeba* relies heavily on morphological characters plus immunological assays or isozyme electrophoresis. Three morphological groups have been described and subdivided into a number of species (Table 1) [26, 30]. Our *Rns* sequence data also indicate that each of the morphological groups consists of several different lineages identified here as different sequence types, but the sequence types and species are not uniformly equivalent. For example, isolates with the same sequence type, such as *A. rhysodes* Singh (#23) and *A. polyphaga* 1501/3d (#4), or *A. griffini* S-7 (#5) and *A. polyphaga* Panola Mt. (#7) (Fig. 4), currently are classified as different species. Conversely, several species clearly are polyphyletic; for example, several isolates of *A. castellanii* and *A. polyphaga* have T4 sequence types, whereas, other isolates of these species have T1, T2 or T3 types. The isolates of *A. culbertsoni, A. palestinensis* and *A. hatchetti* also are polyphyletic (Fig. 3).

Correlations between morphological groups, species and *Rns* sequence types for the strains studied here indicate that group 1 includes sequence types T7, T8 and T9; group 2 includes T3, T4 and T11; and group 3 includes T1, T2, T5, T6, T10, and T12. Only four isolates, *A. polyphaga* 1501/3c (T2), *A. culbertsoni* Diamond (T4), *A. castellanii* V006 (T1) and *A. pearcei* 50435(T3), appear to be in conflict with this arrangement (Fig. 3).

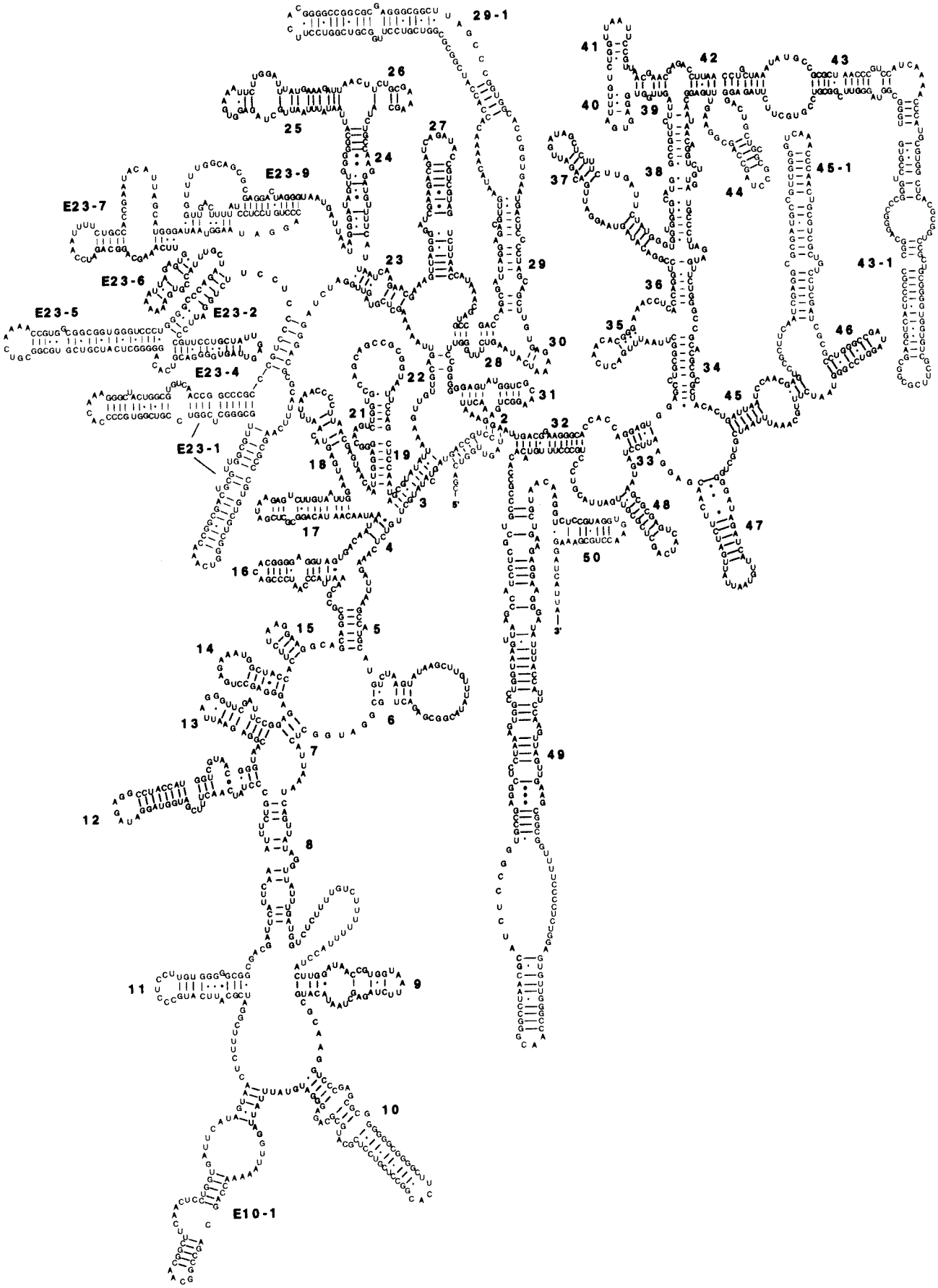
Strain 1501/3c is classified as *A. polyphaga* and, thus, is presumed to be a group 2 isolate [30]. Its *Rns* sequence indicates, however, that it belongs in T2 with group 3 isolates (Fig. 3). We propose below that the closely related T2 isolates we have examined all should be classified as *A. palestinensis* and, therefore, propose that *A. palestinensis* is a more appropriate species name for strain 1501/3c.

A. culbertsoni Diamond probably also is misclassified. Its *Rns* sequence type places it in T4 with group 2 isolates, whereas, strain A-1, the type strain for *A. culbertsoni,* has a T10 *Rns* sequence and is a group 3 strain. The Diamond strain is not likely to be *A. culbertsoni,* but our results do not enable us to suggest which of the six T4 species it might belong.

A. pearcei has been classified as a group 1 species [25], but the *Rns* sequence of the strain we examined is typical of T3 which consists of group 2 strains. The *A. pearcei* sequence is 98.3% similar to that of *A. griffini,* but only ~64% similar to sequences of the three authentic group 1 species. Although the large size and cyst morphology of *A. pearcei* have been used to characterize this species as a group 1 isolate, the cyst size range overlapped that of *A. griffini* in the same study. Furthermore, the published photographs of the cyst morphology [25] look very similar to a photograph of cysts from *A. griffini* H37 [18]. Thus, it is likely that *A. pearcei* is an invalid synonym for *A. griffini.*

A. castellanii V006 has a very different sequence type (T1) from *A. castellanii* Castellani (T4), the type strain for the species. Based on its sequence type, V006 branches with group 3 isolates (Fig. 3), whereas, *A. castellanii* is a group 2 species [30]. Thus, V006 is a candidate for a new species name and the possibility that it is a group 3 strain should be considered.

Reclassification of five strains, *A. castellanii* V006, *A. polyphaga* 1501/3c, *A. polyphaga* Panola Mt., *A. culbertsoni* Diamond and *A. palestinensis* 2802 would result in limiting each species to a single sequence type although some sequence types would continue to include more than one species. The rationale for reclassification of strains 1501/3c, Diamond and V006 has been discussed above. The Panola Mt. strain should be reclassified because it has a T3 sequence type, whereas, the majority of *A. polyphaga* strains are T4. The Panola Mt. sequence is only 0.9% dissimilar from S7, the type strain for *A. griffini,* and, thus, should be reclassified as this species. De Jonckheere [8] described a close relationship between *A. pustulosa* and *A. palestinensis* based on isozyme comparisons and suggested the possibility that *A. pustulosa* is an invalid synonym for *A. palestinensis.* Our results support that conclusion. His study also led to the reclassification of *A. lenticulata* 2802 to *A. palestinensis* 2802. The *Rns* phylogeny agrees that 2802 (T6) is not likely to be *A. lenticulata* (T5); its *Rns* sequence is 19.1–19.3% dissimilar from those of that species (Table 2). The *Rns* phylogeny also agrees that 2802 is most closely related to the T2 strains including *A. palestinensis* Reich (5.2–5.9% dissimilar). We have placed it in a separate sequence type because its dissimilarity from T2 sequences exceeds that found so far for dif-


E23-1
E23-2
E23-4
E23-5
E23-6
E23-7
E23-9
E10-1
5'
3'

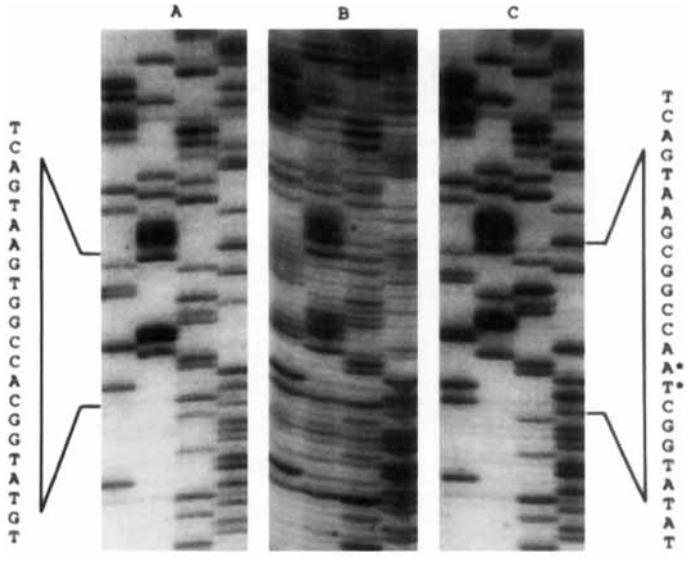


Fig. 2. Evidence for multiple alleles in the 18S rDNA sequence(s) obtained from *A pustulosa* GE3a. **B**. Overlapping sequence obtained directly from the PCR products using primer 892c [11, 32]. **A**, **C**. Two different sequences obtained after cloning the PCR products. The sequences are located in stem 29.1 approximately at base 1321 in GE3a. The sequence reads in the 5′ to 3′ direction from the bottom to the top. The two sequences indicate the presence of two different alleles.

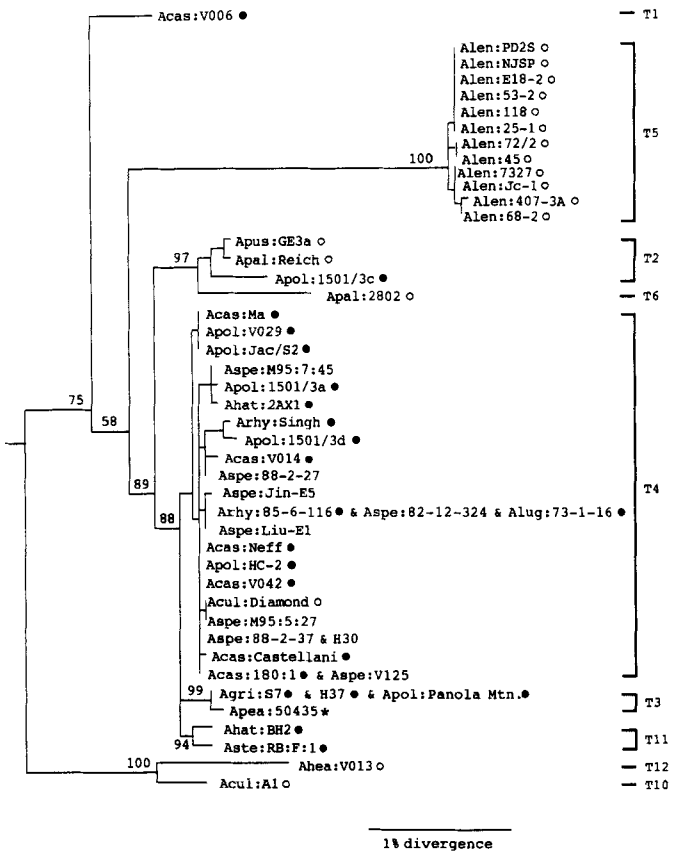


Fig. 3. Neighbor-joining tree based on the 18S rRNA genes of 53 isolates. The tree was rooted with the three group 1 isolates *A. astronyxis* (T7), *A. tubiashi* (T8) *and A. comandoni* (T9). Abbreviations for species are Acas, *A. castellanii*; Acul, *A. culbertsoni*; Agri, *A. griffini*; Ahea, *A. healyi*; Ahat, *A. hatchetti*; Alen, *A. lenticulata*; Alug, *A. lugdunensis*; Apal, *A. palestinensis*; Apea, *A. pearcei*; Apol, *A. polyphaga*; Apus, *A. pustulosa*; Arhy, *A. rhysodes*; Aspe, *Acanthamoeba* sp.; and Aste, *A. stevensoni.*

Bootstrap values are based on 1,000 bootstrap replicates and are placed at the nodes to which they apply. Values below 50 and for the closely related strains are omitted. (★), (●), and (○), identify strains of morphological groups 1, 2 and 3, respectively. Groups are not indicated where the species has not been identified.

ferences within other sequence types. Nevertheless, the two sequence types are relatively closely related and we see no compelling reason to rename this isolate at this time.

As indicated above, it generally is agreed that identification of *Acanthamoeba* isolates at the species level is very problematical [30]. Prior molecular studies as well as the studies we have done here indicate that there are numerous inconsistencies between the classification of strains and the evolutionary lineages. Our analyses have helped to more accurately identify lineages within the three morphological groups, but they have not succeeded in clarifying evolutionary relationships among the closely related strains that are included within a single sequence type. We attempted to identify branching patterns of lineages within sequence type T4 and failed. Twenty five isolates from six species are clustered in T4. The isolates are closely related with maximum nucleotide dissimilarities of 4.4% and evolutionary distances less than 0.44%. A subcluster analysis including T4 examined an additional 227 sites that were in regions of ambiguity in the full alignment. However, this analysis still did not further resolve relationships among the several species and they remained intermixed(Fig. 4). One clade of seven strains that has a neighbor-joining bootstrap value of 79% (Fig. 4) might be used as the basis for one species. At present, however, this clade has isolates from three different species. It remains to be seen whether other molecular methods such as isozyme electrophoresis, mitochondrial DNA RFLPs, or sequences of other genes will be helpful in reliably identifying other significant clades within T4. Unfortunately, at present we are unable to evaluate which method has the most promise for further resolving *Acanthamoeba* phylogeny because there is too little overlap between the strains used in our work and those used by others. Alternatively, it may be impossible with available methods to resolve the branching patterns within sequence types. In that case, we propose that each sequence type should be equated with a single species. For example, the various species in T4 all might be reclassified as *A. castellanii,* because the sequence type includes the type strain for that species [12].

The *Rns* sequences suggest an early divergence of group 1 species *A. astronyxis, A. comandoni* and *A. tubiashi* from the remaining taxa after the contemporary lines diverged from a common ancestor (Table 2). The large degree of genetic divergence between these group 1 taxa and the rest of the genus

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Fig. 1. The distribution of variable sites in the 18S rRNA of *Acanthamoeba.* The proposed secondary structure of 18S rRNA from *A. castellanii* Castellani is based on the work of Gunderson and Sogin [13], Gutell [14] and Neefs et al. [24]. Large boldface numbers in black identify stems. We folded E23-1 using the lowest-energy configurations determined with the program PC FOLD [32]. Black and red bases identify conserved and highly variable regions respectively. Green bases were primer determined.

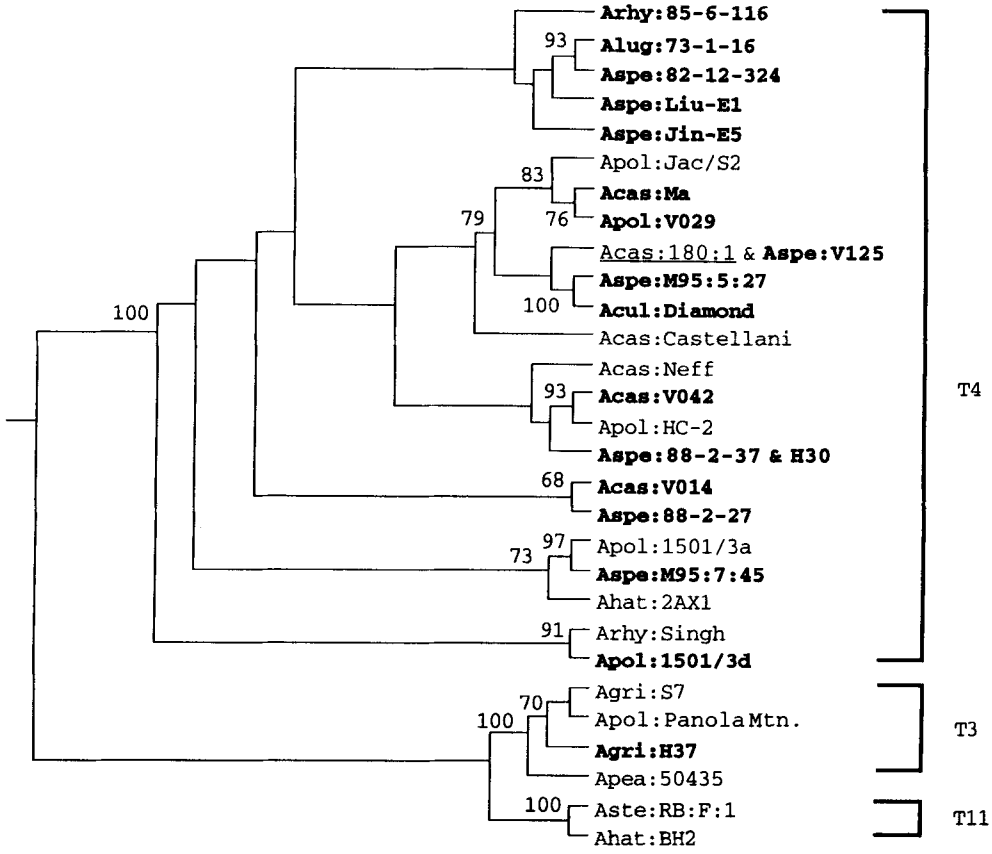

Fig. 4. Compartmentalization consensus neighbor-joining tree based on 35 strains. Bootstrap values are based on 1,000 replicates. The tree is rooted by sequence types T2 and T6. Isolates from *Acanthamoeba* keratitis and from a lung infection are identified by bold font and underlining, respectively.

suggests that they could be considered members of one or more novel genera. The divergence of group 1 taxa is followed by diversification of isolates that belong to group 3 and then the divergence of a monophyletic cluster of group 2 isolates arising from within group 3 (Fig. 3).

A. lenticulata isolates are the most divergent of the group 3 strains. In addition to a distinctive sequence type (T5), the presence of group 1 introns (no relationship to morphological group 1) in some of these isolates [11] further segregates them from the rest of the genus. Although *A. griffini* isolates also have group 1 introns [11], they are very distinct from those found in *A. lenticulata* (JMS-D, unpubl. data). The line leading to *A. lenticulata* is very long, suggesting an ancient divergence event. However, the strains of this species have a maximum nucleotide dissimilarity of 0.6% and, thus, form a tight clade. This suggests that the isolates have diverged recently from a common ancestor. However, eight of the 12 strains examined contain group 1 introns of three very different types (JMS-D, unpubl. data). Thus, this might be an interesting species for the study of intron inheritance.

Rns sequence variation provides useful quantitative information for identifying *Acanthamoeba* taxa. The boundaries of the taxa are arbitrary, however, especially if reproduction in *Acanthamoeba* is asexual as generally believed. In this study, we found that *Rns* sequences from 12 different evolutionary lineages identified by phylogenetic analyses were at least 5% dissimilar and that sequence variation among sequence types was greater than that within types where more than one se-

Table 2. Percent dissimilarities between and within sequence types.[a]

No.	ST	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
1	T1	—										
3	T2	12.0–12.9	**2.2–4.9**									
4	T3	10.6–11.0	11.3–12.3	**0.8–1.9**								
25	T4	9.7–10.8	11.2–13.8	6.0–7.5	**0.0–4.4**							
12	T5	18.7–18.9	17.8–18.8	18.0–20.8	16.4–17.6	**0.0–0.6**						
1	T6	13.5	5.2–5.9	12.6–13.2	12.9–13.5	19.1–19.3	—					
1	T7	35.3	35.6–35.8	35.2–35.3	34.2–34.9	36.5–36.6	36.0	—				
1	T8	34.5	35.0–35.3	34.9–35.1	34.0–34.7	36.5–36.6	35.1	19.2	—			
1	T9	37.6	36.3–36.8	36.1–36.5	35.7–36.3	37.5–37.6	37.1	29.2	19.2	—		
1	T10	16.2	17.6–17.7	17.1–17.2	17.3–18.2	21.6–22.0	17.6	35.6	35.1	36.7	—	
2	T11	10.9–11.1	11.4–12.5	5.6–6.6	5.0–6.3	17.0–17.4	13.0–13.4	34.6–34.8	34.3–34.4	35.8–36.2	17.4–18.2	**2.5**
1	T12	18.0	18.9–19.5	19.4–20.8	19.8–21.2	23.8–24.1	18.5	36.4	35	35.8–36.2	12.1	19.0–21.5

[a] The first column is the number of strains of each sequence type. Values in columns T1–T12 are ranges of percent dissimilarities in pairwise comparisons of sequences from the same or different sequence types. Boldface numbers are dissimilarity ranges within sequence types.

quence was available. Although the levels of dissimilarity between sequence types observed here are consistent with the overall phylogeny, we don't mean to imply that the same levels would apply to any other organism.

The amoeba sequence types do not overlap at present, but might in the future as more sequences are examined. In addition, it must be recognized that the sequence comparisons are a function of the sequence alignments and will differ somewhat with the addition of new strains and new alignments. Thus, the distances calculated for this paper differ slightly from those we have published previously for a smaller set of sequences [12].

There are at least seven named species whose rDNA sequences remain to be determined: *A. divionensis, A. echinulata, A. jacobsi, A. mauritaniensis, A. quina, A. royreba* and *A. terricola.* Given the large molecular diversity within the genus, the discovery of new sequence types would not be a surprise.

Multiple alleles and classification. Our evidence indicates that more than one *Rns* allele can be found in amoebae cloned from an isolate. This is a potential problem for construction of phylogenetic trees because there is no way to determine which alleles from different isolates are homologous. It is not clear how many strains have more than one allele because rare alleles might not show up during PCR. Our experience suggests that most isolates have one predominant allele that results in a single sequence ladder. Based on the limited data that we have obtained, the variation among sequences of alleles is less than among sequences of different isolates within a sequence type. Allele selection should not have a major impact on classification because the various alleles from a single isolate all appear to belong to the same sequence type.

Pathogenicity. It is noteworthy that 17 of 18 human keratitis isolates for which complete *Rns* sequences were available had T4 sequence types. *A. griffini* H37, which has a T3 type, is the only exception (Fig. 3) [18]. Another seven keratitis isolates, which were not included in this analysis because only partial sequences were determined, also have been identified as T4 strains. The divergence of T4 and T3 from a common ancestor (Fig. 3) suggests that the ability to cause keratitis might have evolved only once in the evolution of *Acanthamoeba.* However, T4 isolates are found worldwide and simply may be more common in eye isolates because they are more prevalent in the environment. It is possible that eye infections involving other sequence types will surface in a larger sample of keratitis isolates.

Mannis et al. [16] reported two cases of keratitis caused by *A. culbertsoni.* Identification was based on an immunofluorescence assay. As we have shown, the *A. culbertsoni* type strain, Lilly A-1, had a T10 sequence, but the Diamond strain had a T4 sequence. Therefore, we cannot be certain whether the strains isolated by Mannis et al. were likely to represent a new sequence type associated with keratitis, or whether they actually were T4 isolates, possibly misclassified as *A. culbertsoni.* A similar problem is found with a previous report of keratitis associated with *A. hatchetti* [16]. In our study, the type strain for this species, BH2 (#52), had a distinctive T11 sequence type, but a second strain, 2AX1 (#16), had a T4 sequence type. Thus, we cannot be certain whether the *A. hatchetti* strain isolated by Cohen et al. is likely to represent a new keratitis sequence type, or whether it also had a T4 type. Nevertheless, the possibility that eye infections could be caused by strains from sequence types other than T3 and T4 is suggested by evidence discussed below of an association of T1 and T12 isolates with other human infections and of the pathogenicity of *Acanthamoeba* to animal models.

The observation that T3 strain H37 (#6) manifested disease with an atypical, punctate appearance, rather than the ring infiltrate characteristic of T4 keratitis isolates [18], is consistent with the genetic distinction between T3 and T4. Further study is necessary, however, to determine whether there really is any sequence type specificity in the appearance of the disease.

Our study includes two isolates from granulomatous amoebic encephalitis, *A. healyi* V013 (T12) and *A. castellanii* V006 (T1) plus one isolate from a lung infection, *A. castellanii* 180:1 (T4). Thus, with the inclusion of T3 mentioned above, at least four of the 12 sequence types are associated with human infection. Others have reported CNS infections caused by *A. palestinensis, A. castellanii, A. rhysodes, A. astronyxis* and *A. culbertsoni* [16]. Thus, although we cannot be certain without testing the sequence types directly, it is possible that types T2 or T6 and T7 also may be opportunistic human pathogens.

Experimental infection of animal models has been demonstrated. De Jonckheere [8] infected mice with several isolates whose sequence types have been identified here. These include *A. lenticulata* PD_2S (T5), *A. culbertsoni* Lilly A-1 (T10) and *A. hatchetti* BH-2 (T11). As discussed above, the latter two of these species have been identified in human disease, but the sequence types of the strains found in the infections are uncertain.

Laboratory identification of sequence types. In order for *Rns* sequences to be widely useful in the classification of *Acanthamoeba,* practical methods for determining sequence types are needed. A number of laboratories will have the capability of sequencing *Rns* from new isolates. The new sequences can be compared with those that we have donated to GenBank® [1] and our master alignments which are available at our Web site or upon request. The alignments also can be used for development of alternative methods for identifying sequence types. For example, we previously described a slot blot method for identifying *Acanthamoeba* by hybridizing genus- or subgenus-specific oligonucleotide probes with genomic DNA [10]. We now have improved methods using PCR with slot blotting, sequencing of a short diagnostic fragment, or in situ hybridization using oligonucleotides with nonradioactive labels [DRS, JMS-D, unpubl. data]. We have developed methods that are genus-specific and T4-specific. Detection of T4 isolates was emphasized because they appear to be very abundant and the dominant sequence type in association with disease, but the techniques could be used to develop methods specific for other sequence types for clinical or biodiversity studies.

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