Advantages of Using Mitochondrial 16S rDNA Sequences to Classify Clinical Isolates of *Acanthamoeba*

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Purpose. This work was intended to test the classification of *Acanthamoeba* into genotypes based on nuclear ribosomal RNA gene (18S rDNA, *Rns*) sequences. Nearly all *Acanthamoeba* keratitis (AK) isolates are genotype *Rns*T4. This marked phylogenetic localization is presumably either due to an innate potential for pathogenicity or to a peculiarity of the gene sequences used. To differentiate between these possibilities, relationships among isolates have been reexamined, using a second gene.

Methods. Phylogenetic relationships among isolates of *Acanthamoeba* were studied, using sequences of the mitochondrial small subunit ribosomal RNA gene (16S rDNA; *rns*). Genotypes based on complete sequences of approximately 1540 bp were determined for 68 strains, by using multiple phylogenetic analyses.

Results. Each strain's mitochondria contained a single intron-free *rns* sequence (allele). The 68 strains had 35 different sequences. Twenty-eight strains had unique sequences, and 40 strains each shared one of the seven remaining sequences. Eleven mitochondrial *rns* genotypes corresponding to 11 of 12 previously described nuclear *Rns* genotypes were identified. Genotype *rns*T4 was subdivided into eight distinct clades, with seven including *Acanthamoeba* keratitis (AK) isolates.

Conclusions. The phylogenetic clustering of AK isolates was confirmed and thus is not specific to the nuclear gene. *Rns* and *rns* sequences are both suitable for genotyping of *Acanthamoeba.* However, the mitochondrial sequences are shorter and more consistent in length, have a higher percentage of alignable bases for sequence comparisons, and have none of the complications caused by multiple alleles or introns, which are occasionally found in *Rns*. In addition, the more common occurrence of strains with identical *rns* sequences simplifies identification and clustering of isolates. (*Invest Ophthalmol Vis Sci.* 2003;44:1142–1149) DOI:10.1167/iovs.02-0485

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Amoebae belonging to the genus *Acanthamoeba* have a worldwide distribution and inhabit a wide variety of environmental niches. They have been isolated from soil, fresh- and saltwater, air, humans, and various domestic and feral animals.[1,2] Until relatively recently, identification and classification of acanthamoebae involved in human diseases, including the sight-threatening eye infection *Acanthamoeba* keratitis (AK) and other often fatal infections, depended on morphologic or molecular characters that have been difficult to interpret.[2,3,4] However, the introduction of DNA typing has made it possible to characterize isolates on the basis of more consistent and readily interpreted characters. Classification of specimens into genotypic clades based on phylogenetic analysis of the nuclear 18S ribosomal RNA gene (*Rns* or 18S rDNA) has been particularly useful in taxonomic and epidemiologic studies of this genus. That approach has identified a genotype clade, *Rns*T4, which contains most of the AK isolates.[5,6,7] It has been assumed that phylogenetic trees based on the *Rns* sequences represent the evolutionary history of the genus. If this is correct, then it is likely that strains with the *Rns*T4 genotype especially, but also to a lesser extent the *Rns* T3 and T11 genotypes, share an evolutionary adaptation that enhances their ability to infect the eye. Alternatively, if the genotype clusters identified using *Rns* are anomalies that unite more distantly related strains, multiple explanations for the pathogenicity are more likely. The former correlation also is important if the *Rns* phylogeny is to have any value in revisions of the confused *Acanthamoeba* taxonomy in a way that will be epidemiologically useful. The possibility that the *Rns* phylogeny also represents the history of the genus is best tested by comparison of *Rns* trees with those obtained using sequences of other genes. Thus, in this study we asked how trees based on the mitochondrial small subunit rRNA gene (*rns* or 16S rDNA) compared with the *Rns* trees. The *rns* gene was selected because it has a function comparable to that of *Rns* but is likely to be under different evolutionary constraints because it is located within a cell organelle other than the nucleus. We also have examined the relative advantages of using either the mitochondrial or the nuclear rDNA genes for classification, tracking strains, or differentiating between closely related isolates.

Materials and Methods

Cultures

Table 1 summarizes the *Acanthamoeba* isolates/strains used in this study, including those from AK cases. All strains were grown axenically in 25 cm^2 canted-neck tissue culture flasks containing 5 mL of Neff's optimal growth medium (OGM) at 30°C, as described previously.[23] Approximately 1×10^6 cells of *Acanthamoeba* were harvested from each flask as soon as a confluent monolayer was observed. However, much smaller populations also were used and produced sufficient DNA for PCR applications.

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Isolation, Amplification, and Sequencing of DNA

DNA was extracted as previously described, using a scaled-down version of the UNSET method or by using an extraction kit (DNeasy; Qiagen, Inc., Valencia, CA).[6,24] The final volume of extract was 30 μL in distilled water. Mitochondrial *rns* sequences were amplified by PCR using forward primers mt1 or tALA and reverse primer mt1541 (Table 2).[25] The primers were based on sequences of *A. castellanii* Neff.[26] Primer mt1, designed for the 5′ end of the gene, did not work with some strains due to sequence variability sufficient to preclude amplification. In those cases, primer tALA (based on a conserved region in an alanine tRNA gene located upstream from *rns*) replaced mt1. One μL of whole-cell DNA extract was used for amplification of either complete or partial gene sequences. The PCR amplification program included 35 cycles of 1 minute at 94°C, 2 minutes at 45°C, and 3 minutes at 72°C. PCR products were cloned into PBSK+ (Stratagene, La Jolla, CA) or PCR II vector (Invitrogen, La Jolla, CA) to preserve the product for future reference. Internal primers were designed to sequence across the gene (Fig. 1, Table 2). Initially, sequencing of either direct or cloned PCR products used direct double-stranded manual sequencing methods (ds Cycle Sequencing Kit; Gibco, Gaithersburg, MD). In direct sequencing of PCR products, multiple products were sequenced. The entire gene was sequenced, including more than 60% of the gene covered on both strands. In the cases in which the gene was cloned, the entire gene was sequenced in both directions. In later stages of the study, sequencing was performed with an automated fluorescent sequencing system (ABI 310; Applied Biosystems, Inc., Foster City, CA), using the same primers and a kit (ABI Prism BigDye Terminator Cycle Sequencing Kit; Applied Biosystems) according to the manufacturer's protocols.

Sequence Alignment and Phylogenetic Analysis

Sequences were aligned using ESEE and/or ClustalX.[27,28] Alignments were based on both primary sequence and secondary structure[29] and are available from one of the authors (GCB; booton.1@osu.edu). Twenty-two bases at the 5′ end and 19 bases at the 3′ end of the gene, which were determined by the primers, were excluded from the analysis. In the 68 sequences examined, 1312 sites (~85% of the total number of sites) could be aligned unambiguously. Variation was at least ditypic at 236 sites, which therefore were considered phylogenetically informative. Distances were calculated from the aligned sequences in MEGA2.1 using the Kimura 2 parameter model.[30] Phylogenetic gene trees were reconstructed in MEGA2.1 using maximum parsimony, neighbor-joining, and minimum evolution methods. Bootstrap analysis as a test of the reliability of the tree reconstruction was also performed in MEGA2.1. The trees were rooted with *Balamuthia mandrillaris* as an outgroup, because previous work in our laboratory on nuclear 18S rDNA showed that *B. mandrillaris* is closely related to the *Acanthamoeba* species. We have also obtained the mitochondrial 16S rDNA from a number of *B. mandrillaris* strains.[31] Analyses using the *Balamuthia*, *Acanthamoeba*, and mitochondrial 16S gene sequences from additional genera also support use of *Balamuthia* as an outgroup to *Acanthamoeba* species. The *rns* sequences obtained in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), and the accession numbers for each isolate are listed in Table 1.

Results

DNA Sequence Heterogeneity of *rns*

Sequences were obtained for the *rns* coding region from all 68 isolates of *Acanthamoeba*. The gene ranged from 1514 to 1578 bp in length and averaged approximately 1540 bp. There were 35 different *rns* sequences among the 68 isolates. This is a significantly lower level of variation than found in the *Rns* genes. Although many copies of *rns* are expected in each amoeba, presumably in proportion to the number of mitochondria, there was no evidence for more than one kind of *rns* allele in any isolate. Similarly, no introns were found in the *rns* from any of the isolates. The most variable regions of the *rns* were observed in seven of nine regions identified by Lonergan and Gray[32] as being variable among different organisms. These variable regions constituted approximately 27% of the gene. Less concentrated sequence variation also occurred throughout the remainder of the gene. Sequence dissimilarities of 16S *rns* between previously identified genotypes (based on 18S *Rns* analysis) are presented in Table 3. These calculated dissimilarities are based on the aligned sequences (see Fig. 1) and represent 1312 nucleotides of the 1692-bp alignment. Dissimilarities based on the entire sequence alignment (1668 bp, which excludes the 5′ and 3′ ends determined by the amplification primers) are also shown, in parentheses, in Table 3. Dissimilarities within genotypes (where multiple strains were available) are also presented in Table 3 in bold font. Dissimilarities (expressed as percentages) between genotypes based on the aligned region ranged from 2.2% (T7 versus T9) to 14.0% (T3 versus T8). In general, as found in our previous studies of nuclear 18S *Rns,* the species *A. astronyxis* (T7), *A. tubiashi* (T8), and *A. comandoni* (T9) were the most distant from the remainder of the *Acanthamoeba* genotypes, with *rns* dissimilarities ranging from 12% to 14% versus the remaining genotypes. However, the dissimilarities between these three morphologic group I genotypes (T7, -8, and -9) ranged from 2.2% to 2.8% and, thus, were much lower than observed for the nuclear gene.[5] Even when the whole alignment is used to calculate dissimilarities, the range between these three genotypes is only 4.3% to 5.0%.

Phylogeny and Correlations between Genotypes and Species

Phylogenetic relationships among isolates were examined using maximum parsimony (M), neighbor joining (N), and minimum evolution (E) analyses. M analysis identified a major clade, designated genotype *rns*T4, which included 53 different strains having 22 different *rns* sequences. Six of the 22 sequences found within T4a, b, d, f, and h, occurred in more than one strain (Fig. 2, Table 4). The *rns*T4 clade was supported with an M bootstrap value of 100% of 100 replicates. Sequence dissimilarities among T4 strains ranged from 0% to 3.2% for the alignment data set used for phylogenetic reconstruction (0%–7.9% across entire alignment). The *rns*T4 clade had eight identifiable branches that we designate *rns*T4a to *rns*T4h. One branch included a single strain (*rns*T4c). The rest of the branches included clades of 2 to 10 sequences and each was supported by M bootstrap values of 100. Five of these six clades were also supported in N and E analyses, with bootstrap values ranging from 84% to 100%. Only clade *rns*T4f, which contained the two strains *A. castellanii* strain Neff (ATCC 50373) and *A. castellanii* strain Pussard 425 (ATCC 30134) and was supported with a 100% M, was not supported by bootstrapping using the other two methods. Two of these branches each contained a species type-strain. These were *rns*T4a (*A. castellanii*), and *rns*T4e (*A. royreba*). In addition, although the type-strain was not tested here, *rns*T4h included nine strains, all identified as *A. mauritaniensis* based on sequence identity with the nuclear *Rns* sequence of the species type-strain. Ten additional branches corresponded to the previously described nuclear *Rns* genotypes T1 to T3, T5, and T7 to T12. Thus, the corresponding mitochondrial genotypes were designated *rns*T1 to -T5 and *rns*T7 to -T12. No strain from the genotype designated *Rns*T6 was available for the present study and, therefore, a T6 mitochondrial *rns* could not be determined.

Table 1. *Acanthamoeba* Strains Used for Sequencing of *rns* and *Rns*

Acanthamoeba Species Isolates	rDNA Genotype Clades*	Source†	GenBank Accession Number
Morphological group I species			
A. astronyxis Ray and Hayes, 1954[8]			
1. Type-strain,‡ Ray and Hayes, ATCC 30137	T7	Lab water (Washington state, USA)	AF479546
A. tubiashi Lewis and Sawyer, 1979[9]			
2. Type-strain, NMFS OC-15C, ATCC 30867	T8	Freshwater (Maryland, USA)	AF479545
A. comandoni Pussard, 1964a[10]			
3. Type-strain, A1P, ATCC 30135	T9	Soil (France)	AF479544
Morphological group II species			
A. castellanii Douglas, 1930[11]			
4. Type-strain, Castellani, ATCC 50374	T4	Yeast culture (UK)	AF479528
5. Ma, ATCC 50370	T4	Keratitis (New York, USA)	AF479533
6. Neff, ATCC 50373	T4	Soil (California, USA)	AF479560
7. CDC V014	T4	Keratitis (India)	AF479550
8. CDC V042, ATCC 50493	T4	Keratitis (USA)	AF479529
9. CDC 0180:1	T4	Lung infection (Pennsylvania, USA)	AF479520
10. Pussard 425§, ATCC 30134 (formerly *A. terricola* Pussard, 1964b[12])	T4	Soil (France)	AF479561
11. JAC E2§‖	T4	Keratitis (Japan)	AF479497
12. JAC E3§‖	T4	Keratitis (Japan)	AF479498
13. JAC E4	T4	Keratitis (Japan)	AF479555
A. griffini Sawyer, 1971[13]			
14. Type-strain, S7, ATCC 30731	T3	Beach bottom (Connecticut, USA)	AF479562
A. mauritaniensis Pussard and Pons, 1977[14]			
15. SAWE 90/1‖, ATCC 50676	T4	Keratitis (South Africa)	AF479510
16. SAWE 92/2‖, ATCC 50677	T4	Keratitis (South Africa)	AF479511
17. SAWE 95/6‖, ATCC 50684	T4	Keratitis (South Africa)	AF479512
18. SAWE 93/3‖, ATCC 50678	T4	Keratitis (South Africa)	AF479513
19. SAWE 94/4‖, ATCC 50679	T4	Keratitis (South Africa)	AF479514
20. SAWE 94/5‖, ATCC 50680	T4	Keratitis (South Africa)	AF479515
21. SAWL 93/1‖, ATCC 50681	T4	Keratitis (South Africa)	AF479516
22. SAWL 91/3‖, ATCC 50682	T4	Keratitis (South Africa)	AF479517
23. SAWL 91/4‖, ATCC 50683	T4	Keratitis (South Africa)	AF479518
A. polyphaga (Puschkarew), Page, 1967[15]			
24. JAC/S2, ATCC 50372	T4	Soil (Japan)	AF479527
25. CEI 73-01-16, ATCC 50371 (also identified as *A. lugdunensis*[16])	T4	Keratitis (Texas, USA)	AF479557
26. CDC V029, ATCC 50495	T4	Keratitis (Massachusetts, USA)	AF479526
27. Sawyer, CCAP 1501/3C	T2	Freshwater (USA)	AF479543
28. TV8, ATCC 30921	T4	Shore (Antarctica)	AF479522
29. UNAM HC-2	T4	Keratitis (Mexico)	AF479496
30. CCAP, 1501-3D, ATCC 30873	T4	Keratitis (UK)	AF479537
31. Panola Mtn., ATCC 30487	T3	Soil (Georgia, USA)	AF479535
A. rhysodes Singh, 1952[17]			
32. CEI:85-6-116, ATCC 50368	T4	Keratitis (Texas, USA)	AF479553
Morphological Group III Species			
A. culbertsoni Singh and Das, 1970[18]			
33. Diamond	T4	Keratitis, (Ohio, USA)	AF479521
34. CDC 409§.	T10	Horse brain (USA)	AF479542
A. healyi Moura, Wallace and Visvesvara, 1992[19]			
35. Type-strain, CDC V013, ATCC 30866	T12	GAE, brain (British West Indies)	AF479548
A. lenticulata Molet and Ermolieff-Braun, 1976[20]			
36. Type-strain, PD_2S, ATCC 30841.	T5	Swimming pool, France	AF479541
37. SAWS 87/1, ATCC 50685	T5	Sewage sludge (South Africa)	AF479538
38. SAWS 87/2‖, ATCC 50686	T5	Sewage sludge (South Africa)	AF479539
39. SAWS 87/3‖, ATCC 50687	T5	Sewage sludge (South Africa)	AF479540
A. palestinensis Reich, 1935[21]			
40. Type-strain Reich, ATCC 30870	T2	Soil (Israel)	AF479563
A. royreba Willaert, Stevens and Tyndall, 1978[22]			
41. Type-strain, Oak Ridge.	T4	Human tissue culture	AF479559
Strains with no species identification			
Acanthamoeba species			
42. CEI 82-12-324, ATCC 50496	T4	Keratitis (Texas, USA)	AF479499
43. CEI 88-2-27, ATCC 50369	T4	Keratitis (Texas, USA)	AF479558
44. CEI 88-2-37, ATCC 50497	T4	Keratitis (Texas, USA)	AF479554
45. CDC V125, ATCC 50498	T4	Keratitis, (California, USA)	AF479524
46. Liu-E1, ATCC 50709	T4	Keratitis (China)	AF479500
47. JAC 324, Galka	T4	Keratitis, (Texas, USA)	AF479505
48. LVPEI 402/97	T4	Keratitis (India)	AF479506
49. LVPEI 773/96	T4	Keratitis (India)	AF479507
50. LVPEI 1060/96	T4	Keratitis (India)	AF479549

(*continues*)

Table 1. (*continued*). *Acanthamoeba* Strains Used for Sequencing of *rns* and *Rns*

Acanthamoeba Species Isolates	rDNA Genotype Clades*	Source†	GenBank Accession Number
51. LVPEI 749/98	T4	Keratitis (India)	AF479552
52. LVPEI 1002/99	T4	Keratitis (India)	AF479551
53. LVPEI 1035/99	T4	Keratitis (India)	AF479508
54. LVPEI 98/00	T4	Keratitis (India)	AF479509
55. CDC V504	T4	Keratitis (Italy)	AF479519
56. CDC V017	T4	Nasal sinus infection (USA)	AF479523
57. OHSU M002§	T4	Keratitis (Oregon, USA)	AF479504
58. CDC V328	T4	GAE	AF479501
59. CDC V382	T4	Skin infection (USA)	AF479502
60. CDC V390	T4	Skin infection (USA)	AF479503
61. CDC V383	T4	Keratitis (Argentina)	AF479534
62. CDC V168	T4	Skin infection (USA)	AF479525
63. CDC V006	T1	GAE, brain (Georgia, USA)	AF479547
64. JAC 9E′	T4	AK (Japan)	AF479556
65. JAC Kamph‖	T4	AK (Japan)	AF479532
66. JAC 473U‖	T4	AK (Japan)	AF479530
67. JAC E7‖	T4	AK (Japan)	AF479531
68. OHSU M001	T11	Keratitis (Oregon, USA)	AF479536

NMFS, National Marine Fisheries Service; CDC, Centers for Disease Control; JAC, Japanese National Institutes of Health; SAWE, South African Witwatersrand University: Eye isolate; SAWL, South African Witwatersrand University: Contact Lens isolate; CEI, Cullen Eye Institute, Baylor University, Houston, Texas; UNAM, National Autonomous University of Mexico; CCAP, Culture Collection of Algae and Protozoa; SAWS, South African Witwatersrand University: Sewage isolate; LVPEI, L. V. Prasad Eye Institute; OHSU, Oregon Health Sciences University.

* Both *Rns* and *rns* have been sequenced, except where noted otherwise. All *rns* sequences and most *Rns* sequences are complete genes. Sequences of both genes place strains in the same genotype clades.

† GAE, patients with granulomatous amebic encephalitis; AK, patients with *Acanthamoeba* keratitis.

‡ The strain tested is derived from the species type-strain.

§ Only *rns* has been sequenced.

‖ *Rns* sequences are incomplete, but strain genotypes can be identified.

With the exception of *rns*T1, which includes a single strain that has not been assigned a unique species name, most of the genotypes included a single species type-strain. When there was more than one type-strain, the strain with taxonomic precedence was identified. The resultant correlations between genotypes and type-strains based on the currently available data are as follows: *rns*T2 (*A. palestinensis* type-strain Reich, ATCC 30870), *rns*T3 (*A. griffini* type-strain S7, ATCC 30731), *rns*T5 (*A. lenticulata* type-strain PD_2S, ATCC 30841), *rns*T7 (*A. astronyxis* type-stain Ray and Hayes, ATCC 30137), *rns*T8 (*A. tubiashi* type-strain OC-15C, ATCC 30867), *rns*T9 (*A. comandoni* type-strain A1P, ATCC 30135), and *rns*T12 (*A. healyi* type-strain V013, ATCC 30866). *A. castellanii* type-strain Castellani (ATCC 50374) is the type-strain for *rns*T4, and it also is the genus type-strain. All these correlations are consistent with genotype clusters previously based on *Rns* sequences.[5,6] With the exception of the relative similarity of the morphologic group I species, the only other departure of the current *rns* data with the nuclear *Rns* data were the 4.4% sequence dissimilarity between genotypes T3 and T11 (Table 3). In the *Rns* analysis, we used a 5% sequence dissimilarity value to distinguish genotypes. Based on that criterion T3 and T11 were designated separate genotypes with a sequence dissimilarity of more than 5%. Although the selection of the 5% sequence dissimilarity was subjective, using the same criteria in the current *rns* study, we would fail to distinguish between the T3 and T11 genotypes, because the sequence dissimilarity is less than 5%. However, it should be noted that in the *Rns* study T3,

Table 2. PCR and Sequencing Primers

	Sequences (5′ to 3′) and Location in *rns**	Genome Location†
PCR primers		
Forward.mt1	CCGCGGGTCGAC/T^{1}TGTATAAACAATCGTTGGGT^{21}	6184–6204
Forward.tALA	TCGATTCTGATTGCGTCC	
Reverse.mt1541	CCCGGGGGATCC/A^{1541}AAATTTGTCCAGCAGCA^{1523}	7706–7724
Sequencing primers		
Reverse.mt243	^{260}CAAACCAGCTAAGCATCG^{243}	6426–6443
Forward.mt400	^{277}CATTGGGACTGAAAACGG^{294}	6460–6477
Reverse.mt515	^{532}AACCACCTACGCACCCTT^{515}	6698–6715
Reverse.mt900	^{895}CAAATTAAACCACATACT^{878}	7061–7078
Forward.mt600	^{622}AAGTGTAAAGGTGAAATT^{639}	6805–6822
Forward.mt1037	^{1037}TGTCGGCAGTTCGTGTTG^{1054}	7220–7237
Reverse.mt1230	^{1224}GCTTCACATTGTAATTAC^{1207}	7390–7407
Reverse.mt1180	^{1197}ACGTGTGTAGCCCAACCT^{1180}	7363–7380
Forward.mt1353	^{1353}CTTTGTACACACCGCCCG^{1370}	7536–7553

* Base pair positions in *rns* of the Neff strain of *A. castellanii.*[32]

† Base pair positions within the mitochondrial genome as determined by Lonergan and Gray.[32]

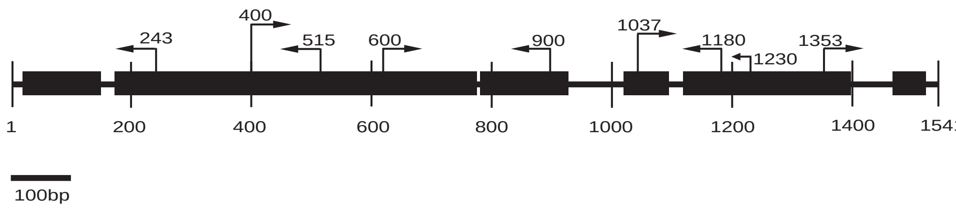

FIGURE 1. Location of primers and aligned regions in the mitochondrial 16S rDNA used in this study. Regions that were included in the alignment correspond to six regions of *A. castellanii*, Neff.[18] The base positions of our alignment in the reference *A. castellanii* Neff sequence are as follows: 23 to 150, 172 to 774, 788 to 928, 1021 to 1094, 1120 to 1399, and 1468 to 1522. These regions are shown by the *black boxes* in the figure. Primer locations (see Table 2 for primer details) and direction of extension (direction of *arrowhead*) are shown *above* the schematic of the gene.

T4, and T11 were phylogenetically very closely related to one another, and the relationship between T3 and T11 was therefore not unexpected. In fact, it supports the results of the *Rns* study that suggested a close relationship between these three genotypes.

Although there was very good agreement between the nuclear and mitochondrial rDNA trees in the distribution of isolates among the previously described genotypes, the same level of agreement was not seen in the distribution of isolates in clades within genotype T4. Because the T4 clades are distinguished with a higher level of significance in the *rns* tree compared to the *Rns* tree, and because two of the eight clades in this genotype are associated with single type-strains, these clades may serve as the best available molecular basis for differentiating among species within this genotype. A more conclusive position on this matter awaits determination of *rns* sequences from the remaining species type-strains for which sequences are not yet available.

DISCUSSION

Mitochondrial and Nuclear Small-Subunit rRNA Gene Phylogenies and the Genotype Trees

The similarity of the genotype clusters identified on the basis of sequences of both mitochondrial and nuclear small subunit rRNA genes suggests that they represent the main branches in the phylogeny of the genus. There are minor differences in the branching orders in the various phylogenetic analyses, but the major clades are remarkably consistent. The dissimilarity of only 4.4% in the *rns* tree (Table 3) between strains classified previously as T3 and T11 in nuclear *Rns* trees is not surprising. The dissimilarity between these genotypes was only 5.6% to 6.6% in the *Rns* trees, and the 5% cutoff point we have used for genotypes is arbitrary.[5] It remains to be determined whether there are any other characteristics that justify the distinction between T3 and T11 strains. In every other case where both *Rns* and *rns* sequences were available, isolates are assigned to the same genotype cluster by either gene sequence, although placement on different branches within a genotype has been observed. This becomes important only if these branches become useful as stable taxonomic units. At the present time, this appears to be a viable possibility, because species type-strains from 9 of the more than 20 described species correlate with individual genotypes or clades within genotypes. Sequences from the remainder of the species type-strains are currently being studied (Booton GC, Kelly DJ, unpublished results, 2002).

The close agreement between the nuclear and mitochondrial gene trees strongly supports the conclusion that they reflect the evolutionary history of the genus rather than being the result of anomalous distributions. At present, then, it appears likely that any acanthamoebae with the *Rns*T4 or *rns*T4 genotype would have the potential to cause keratitis. Whether they are the primary cause of encephalitis and other manifestations of infection is under study. Isolated cases suggest that other *Acanthamoeba* genotypes may also be pathogenic in

TABLE 3. Percent Dissimilarity between Genotypes

	T1	T2	T3	T4	T5	T7	T8	T9	T10	T11
T2	8.0* (13.9)	**2.4†** **(7.1)**								
T3	7.8 (13.0)	6.1 (12.1)	**2.4** **(6.9)**							
T4	6.1 (11.5)	5.4 (10.9)	5.6 (11.1)	**2.0** **(5.2)**						
T5	8.0 (15.8)	7.1 (14.6)	7.9 (14.6)	6.4 (13.0)	**0.2** **(0.2)**					
T7	13.2 (18.6)	13.4 (18.2)	13.5 (18.4)	12.2 (17.0)	13.2 (20.0)					
T8	13.7 (19.4)	13.6 (18.5)	14.0 (18.7)	12.4 (17.6)	13.1 (19.4)	2.8 (5.0)				
T9	13.1 (19.3)	13.3 (18.9)	13.2 (17.9)	12.0 (17.3)	12.8 (18.9)	2.2 (4.7)	2.7 (4.3)			
T10	7.6 (12.9)	6.9 (13.1)	6.5 (12.3)	6.6 (12.8)	7.7 (14.2)	13.7 (19.5)	13.4 (19.2)	13.3 (19.3)		
T11	7.5 (12.8)	6.1 (12.7)	4.4 (9.9)	5.7 (11.7)	7.0 (14.0)	13.7 (18.8)	14.0 (19.2)	13.5 (18.4)	6.2 (12.6)	
T12	7.4 (11.6)	6.1 (13.1)	6.5 (11.5)	5.8 (11.4)	6.9 (13.2)	13.5 (18.3)	13.3 (18.3)	13.0 (17.9)	5.3 (10.6)	6.3 (12.9)

* Average percentage dissimilarity between genotypes based on pair-wise comparisons of strains, using only sequences that are alignable for all the strains (used for the phylogenetic reconstruction in Fig. 2). Values in parentheses are average dissimilarities between genotypes based on pair-wise comparisons of complete gene sequences.

† Percentage dissimilarities between strains within individual genotypes are shown in bold type.

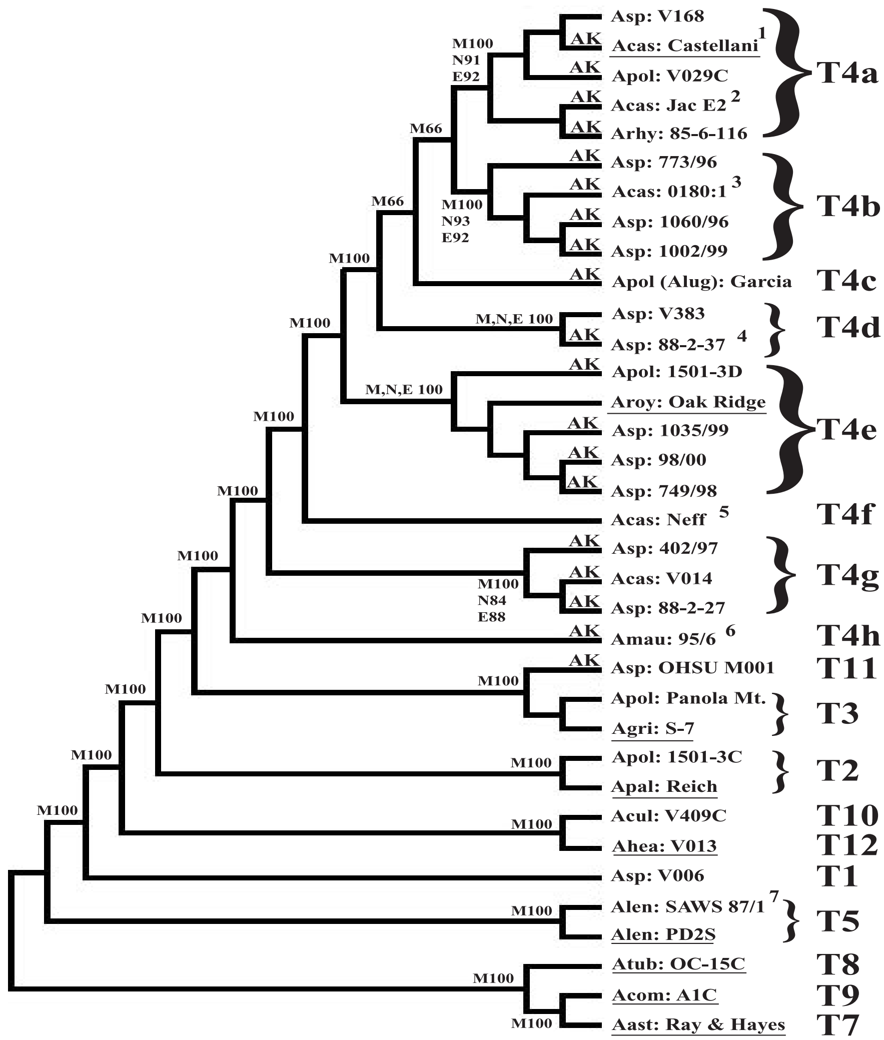

FIGURE 2. 16S mitochondrial rDNA (*rns*) bootstrapped maximum parsimony phylogenetic tree. Strain details are in Table 1. Branches having several strains with identical *rns* sequences are indicated on the tree by strain labels with superscript numbers 1 to 7 (see Table 4). A consensus maximum parsimony tree is presented, and numbers at nodes preceded by M are bootstrap percentages based on 100 bootstraps in maximum parsimony. The other two numbers at the nodes are bootstrap values (1000 bootstraps) from neighbor joining (N) and minimum evolution (E) analyses. Terminal lineages that contain one or more isolates from cases of *Acanthamoeba* keratitis are shown on the tree by the abbreviation AK. *Acanthamoeba* species type-strains are *underscored*. Aast, *A. astronyxis*; Acas, *A. castellanii*; Acom, *A. comandoni*; Acul, *A. culbertsoni*; Agri, *A. griffini*; Ahea, *A. healyi*; Alen, *A. lenticulata*; Alug, *A. lugdunensis*; Amau, *A. mauritaniensis*; Apal, *A. palestinensis*; Apol, *A. polyphaga*; Arhy, *A. rhysodes*; Aroy, *A. royreba*; Asp, *Acanthamoeba* species; Atub, *A. tubiashi.*

non-AK diseases, including clinical manifestations of *Acanthamoeba* infections of the brain (granulomatous amebic encephalitis [GAE]), skin, and lung.

Relative Diagnostic Values of *Rns* and *rns* Sequences

Sequences of either the nuclear or mitochondrial gene are suitable for identifying and classifying isolates. *Rns* sequences have the disadvantage of a larger size with highly variable insertions that are difficult or impossible to align reliably and thus are not included in comparisons. Moreover, some strains categorized in two of the genotypes, *Rns* T3 and *Rns* T5, have been shown to have introns that make the genes even larger.[33,34] As discovered by Chung et al.,[35] the introns can present diagnostic problems for genotype determinations based on the riboprinting of *Rns*, which has been suggested as an alternative to sequencing for identification of specimens. However, *Rns* sequences have a significant advantage when it is important to identify a strain more specifically than at the genotype level. This is the case, for example, when attempting to identify decisively the environmental source of a particular clinical isolate, or when trying to determine whether the same strain is present before and after drug treatment of an infection.[36–38] The nuclear sequences are preferable in these situations because they are more likely to be unique than the mitochondrial sequences. The 68 strains listed in Table 1 include 40 (59%) strains with *rns* sequences that occur in more than one strain (Table 4). Thus, only 41% of the sequences are unique. When the 66 *Rns* sequences that are available from the same group of strains are considered (data not shown), 53 (80%) sequences are unique. In addition, because the presence of introns in *Rns* is relatively uncommon, they also have the potential to serve as unique markers.[35] Use of the nuclear gene also has the present advantage that much more is known about the genotypic and generic specificity of various PCR amplimers and about their use in clinical diagnostics. An example is the use of the *Rns* sequence region designated diagnostic fragment (DF)-3 as a marker for exploring relationships between corneal scrapes of patients with *Acanthamoeba* keratitis, their contact lens paraphernalia, and their home water supplies.[38]

As demonstrated herein, however, the mitochondrial gene also has important advantages for strain comparisons. It has a more consistent length averaging approximately 1540 bp compared with the approximately 2300 to 3000 bp for *Rns* and a larger proportion of the base pairs can be aligned for comparisons of sequences. We are attempting to design genus-specific primers for the amplification of *rns* amplicons, but the very limited availability of suitable *rns* sequence information for organisms closely related to *Acanthamoeba* has been a hindrance. The apparent absence of introns in *rns* makes this gene more suitable for the use of restriction fragment length polymorphisms (RFLP) for the identification of isolates when DNA sequencing is not readily available.[39] Another advantage of using *rns* for sequencing is the larger proportion of isolates

Table 4. Clusters of Strains with Identical *rns* Sequences

Cluster 1 (*rns*T4a)*		**Cluster 2 (*rns*T4a)**		**Cluster 3 (*rns*T4b)**	
A. castellanii: Castellanii†		*A. castellanii:* JAC E2†	AK‡	*A. castellanii:* 0180:1†§	
A. castellanii: V042	AK	*A. castellanii:* JAC E3	AK	*Acanthamoeba* species: V125§	AK
A. polyphaga: JAC S2		*A. polyphaga:* UNAM HC-2		*A. culbertsoni:* Diamond	AK
Acanthamoeba species: JAC 473U	AK	*Acanthamoeba* species: 82-12-324	AK	*A. polyphaga:* TV8	
Acanthamoeba species: JAC E7	AK	*Acanthamoeba* species: Liu-E1	AK	*Acanthamoeba* species: V017	
Acanthamoeba species: JAC Kamph	AK	*Acanthamoeba* species: V328		*Acanthamoeba* species: V504	
A. castellanii: Ma	AK	*Acanthamoeba* species: V382			
		Acanthamoeba species: V390			
		Acanthamoeba species: OHSU M002	AK		
		Acanthamoeba species: 324.jpn	AK		
Cluster 4 (*rns*T4d)		Cluster 5 (*rns*T4f)		Cluster 6 (*rns*T4h)	
A. castellanii: JAC E4†	AK	*A. castellanii:* Neff†		*A. mauritaniensis:* SAWE 90/1	AK
Acanthamoeba species: JAC 9E	AK	*A. castellanii:* Pussard 425 (partial *Rns*)		*A. mauritaniensis:* SAWE 92/2	AK
Acanthamoeba species: 88-2-37	AK			*A. mauritaniensis:* SAWE 93/3	AK
				A. mauritaniensis: SAWE 94/4	AK
				A. mauritaniensis: SAWE 94/5	AK
Cluster 7 (*rns*T5)				*A. mauritaniensis:* SAWE 95/6†	AK
A. lenticulata: SAWS 87/1†				*A. mauritaniensis:* SAWL 91/3	AK
A. lenticulata: SAWS 87/2				*A. mauritaniensis:* SAWL 91/4	AK
A. lenticulata: SAWS 87/3				*A. mauritaniensis:* SAWL 93/1	AK

* Strain designations follow the colon. All strains included in a cluster share the same *rns* sequence. Abbreviations are defined in Table 1.
† This strain identifies the sequence of the cluster in Fig. 2.
‡ AK identifies isolates from cases of *Acanthamoeba* keratitis.
§ One of two strains with the same *Rns* and *rns* sequences.

with identical sequences. This is an advantage, because discovery of a sequence that is the same as one that already has been placed in a phylogenetic reconstruction eliminates the need to repeat these more complex evaluations. The clusters of isolates with identical *rns* sequences also may provide a consistent basis for establishing associations between morphologic species and sequence variants within the main genotypes that have been described.

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