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Acanthamoeba mitochondrial 16S rDNA sequences: inferred phylogeny and support of nuclear ribosomal 18S rDNA gene sequence types

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ABSTRACT

DNA sequence variation in the nuclear small subunit ribosomal RNA gene *(Rns;* 18S rDNA) and the inferred phylogenetic relationships are being used increasingly for the identification and classification of clinical and environmental isolates of *Acanthamoeba.* As a test of the validity of conclusions from this approach, we have examined the sequence variation, and inferred phylogeny, of a second gene. Complete sequences of ~ 1,540 bp were obtained for mitochondrial small subunit ribosomal RNA genes *(rns;* 16S rDNA) from 68 strains. These included 35 unique sequences and represented 11 of 12 *Rns* genotypes. Phylogenetic reconstruction identified 11 corresponding *rns* genotypes (mT1-5 and mT7-12). Also, the large group designated T4 in *Rns* sequencing and containing nearly all *Acanthamoeba* keratitis (AK) isolates was strongly supported in the current study. Seven groups within mT4 included strains with identical mitochondrial sequences. In three cases, *Rns* sequences from the same strains were different. It is proposed that these strains may have originated from possibly ancient parasexual or sexual interactions previously unrecognized in this genus. The close agreement between the *rns* and *Rns* phylogenies suggests that they represent evolutionary history of both genes and genus and that either gene appears suitable for identification and classification at the genotype level.

Introduction

The genus *Acanthamoeba* has a worldwide distribution and inhabits a wide variety of environmental niches. It has been isolated from soil, fresh- and saltwater, air, humans and various domestic and feral animals [1]. The genus includes opportunistic pathogens responsible for the sight-threatening disease *Acanthamoeba* keratitis (AK) that occurs in otherwise healthy humans and for life-threatening infections of patients with immune defense deficiencies (IDD). Infections of animal tissues that appeared to be harmless as well as those that were fatal have been described [2]. Clear variations in pathogenicity of *Acanthamoeba* strains have been observed in various studies, but the relevance of these results to human disease are unclear. Until recently, the lack of a reliable subgeneric classification system for the genus has been a further complicating factor.

Stothard *et al.* (1998) introduced a genotypic classification based on nuclear 18S rDNA *(Rns)* sequence variations that appears to have promise for studies of pathogenicity [3]. This study observed that a single *Rns* genotype (T4) is associated with the large majority of AK cases. This genotype encompasses at least five species that have been differentiated largely on the basis of morphology. However, because it is generally agreed that morphology alone is unreliable for classification of this organism, there is a need for other markers that are more reliable in attempts to determine whether human AK is preferentially associated with particular subsets of *Rns* genotype T4. Effective chemotherapy is available for AK, but a more specific identification of the pathogenic agents could lead to more effective therapy, especially for disease involving IDD patients. An earlier study with a smaller sample of *rns* sequences demonstrated that *rns* could be used to identify sublineages within *Rns* genotype T4 [4]. We now have expanded this study using a much larger sample of isolates, for many of which both *Rns* and *rns* sequences are now available.

Materials and methods

Cultures

All strains used for this study were grown axenically in 5 ml of growth medium at 30° C as described previously [5]. About 1 x 106 amoebae were obtained per flask by harvesting as soon as a confluent monolayer was observed.

Isolation, amplification and sequencing of DNA

Nucleic acids were isolated using a scaled down version of the UNSET method described by Hugo *et al.* [6]. Phenol and chloroform extraction was performed on the UNSET Iysate, and the nucleic acid was precipitated with ethanol and resuspended in 30 ul of distilled water. Mitochondrial *rns* were amplified by PCR using forward and reverse primers that spanned the entire gene, and 1 to 5 µl of whole cell DNA extract. Amplification and sequencing primers were based on sequences of *A. castellanii* Neff [7]. PCR internal primers were designed to sequence across the gene in both directions. Sequencing of direct, or cloned, PCR products was done by manual or automated fluorescent sequencing methods.

Sequence alignment and phylogenetic analysis

Sequences were aligned using XESEE [8]. Alignments were based on both primary sequence and secondary structure [9]. Sequence similarities were calculated by subtracting the number of differences in a pairwise comparison from the total number of bases and then dividing this number by the total number of bases. Dissimilarities were calculated by subtracting the similarity value from one *(Table 1)*. For the 68 different sequences examined, 1,313 sites, about 85% of the total sites, could be aligned unambiguously. Variation was at least ditypic at 148 sites, and therefore these sites were considered phylogenetically informative. Distances were calculated from the 1,313 bp alignment in MEGA2 using the Kimura 2 parameter model [10]. Neighbor-joining gene tree reconstruction was performed in MEGA 2. Bootstrapping of the data (1,000 bootstrap replications) was performed as a test of the reliability of the data. Cladistic reconstruction was done using the program PAUP [11].

Results

DNA sequence heterogeneity of *rns*

Sequences were obtained for the *rns* coding region from all 68 isolates of *Acanthamoeba.* The gene ranged from 1,514-1,578 bp in length and averaged ~ 1,540 bp. There were 35 different *rns* sequences among the 68 isolates. No introns were present in any of the genes. Each of seven sequences was found in more than one isolate. No evidence was found for more than one allele in any of the strains examined. High interstrain sequence variation was observed in seven of nine regions identified by Lonergan and Gray as being variable among different organisms. The highly variable regions include about 27% of the gene. A lesser level of sequence variation also occurs throughout the rest of the gene.

Table 1. *Sequence dissimilarities between Acanthamoeba sequence types. Dissimilarities within sequence types are averages and are shown in bold font.*

Seq Type	T1	T2	T3	T4	T5	T7	T8	T9	T10	T11
T1	–									
T2	8.0	**8.0**								
T3	10.5	10.0	**5.8**							
T4	9.5	9.0	9.0	**4.5**						
T5	12.5	11.7	11.4	10.5	**0.4**					
T7	14.7	14.4	14.6	13.8	15.4	–				
T8	14.9	14.4	14.5	13.8	14.8	4.4	–			
T9	15.0	14.8	13.9	13.6	14.6	4.3	3.8	–		
T10	10.2	10.6	9.6	10.0	11.0	15.0	14.5	14.6	–	
T11	10.2	10.4	8.1	9.4	11.0	14.6	14.6	14.2	9.9	–
T12	9.4	10.7	9.3	9.2	10.4	14.3	14.0	13.7	8.6	10.3

Phylogeny and genotypes

Phylogenetic relationships among isolates were examined using neighbor joining and parsimony analyses contained in MEGA2 and PAUP, respectively. Neighbor joining analysis identified a major clade, designated *rns* genotype mT4, that included 52 different strains with 22 different, but closely related *rns* sequences. The clade was supported with a bootstrap value of 99%. Sequence dissimilarities within mT4 strains ranged from 0-7% *(Table 2).* The clade included 18 strains currently classified into 6 different species plus 35 unclassified strains. A second clade (mT2), formed by A. *palestinensis* Reich and A. *polyphaga* 1501/3c, also was identified. The two strains had a dissimilarity value of 6%. The two T3 isolates (A. *polyphaga* Panola mt. and A. *griffini* S7) had a dissimilarity between them of 5.8%. The remaining sequence type with multiple strains was T5 and these sequences were very similar with four identical sequences and a dissimilarity value of 0.4% between the strains. The morphological group I taxa (A. *astronyxis,* A. *comandoni* and A. *tubiashi)* were much more similar to one another in the *rns* sequences than they were in the *Rns* study. They differed from one another in the present study by an average dissimilarity of 2.5%. However, these three taxa were very different from all other sequence types with an average dissimilarity to the remaining sequence

Table 2. *Summary of* Acanthamoeba *strains used in phylogenetic reconstruction using 16S mtDNA gene* (rns).

Species	Number of Strains	Morphological Group	*Rns* Type	*rns* Type
A. astornyxis	1	1	T7	mT7
A. castellanii	9	2	T4	mT4
A. comandoni	1	1	T9	mT9
A. culbertsoni	2	3	T4, T10	mT4, mT10
A. griffini	1	2	T3	mT3
A. healyi	1	3	T12	mT12
A. lenticulata	4	3	T5	mT5
A. mauritaniensis	9	2	T4	mT4
A. palestinensis	1	3	T2	mT2
A. polyphaga	8	2	T2, T3, T4	mT2, mT3, mT4
A. rhysodes	1	2	T4	mT4
A. royreba	1	3	T4	mT4
A. species	27	not done (n.d.)	1 T1, 10 T4, 1 T11, 15 n.d.	1 mT1, 25 mT4, 1mT11
A. terricola	1	2	T4	mT4
A. tubiashi	1	1	T8	mT8

types of 14.4%. Cladistic analysis in PAUP produced a phylogenetic gene tree that also included the majority of clades determined in the neighbor-joining analysis.

Morphological groups

The three *Acanthamoeba* morphological groups *(Table* 2) each included several *rns* genotypes. The three Group I strains, A. *astronyxis* Ray and Hayes (mT7), A. *comandoni* Comandon & DeFonbrune (mT8) and A. *tubiashi* OC-15C (mT9) were the most distinct from the other sequence types. The 17 isolates identified as Group 2 strains had four different genotypes, mT 1 -mT4, but all except A. *griffini* S-7 (mT3), A. *polyphaga* 1501/3C (mT2) and A. species V006 (mT1) had the mT4 genotype. The five Group 3 strains also included four different genotypes. A. *palestinensis* Reich, A. *healyi* V013, and A. *lenticulata* PD2S had genotypes mT2, mT12 and mT5, respectively, whereas, A. *culbertsoni* Diamond and A. *royreba* Oak Ridge both had mT4 genotypes.

Discussion

Nuclear and mitochondrial small subunit rRNA gene phylogenies and taxonomy

The phylogeny based on mitochondrial *rns* sequences was mostly consistent with that observed elsewhere for nuclear *Rns* DNA [3]. The exceptions were several mT4 strains with identical mitochondrial 16S rDNA sequences, but different nuclear sequences. There were a total of seven *rns* sequences, each from 2-10 strains, in which all strains shared the same sequence. One was a set of three A. *lenticulata* sewage isolates from S. Africa that were identical (mT5). In this case their *Rns* sequences were identical as well, supporting the conclusion that these were the same strain collected at various times in different places. The remaining six sets of strains with identical *rns* sequences were all mT4 genotypes. One set was analogous to the S. African sewage samples. These were nine samples of AK corneal scrapes and lens case samples that were also all identical in their *Rns* sequences. Another pair of identical *rns* sequences from A. *terricola* and A. *castellanii* Neff cannot be compared at this time because only a partial *Rns* sequence is available for A. *terricola.* Another set of strains, A. species V125 and A. *castellanii* 180:1, and A. *culbertsoni* Diamond have identical *rns* sequences. The first two strains were collected from an AK case in California and a lung infection in Pennsylvania, respectively, and also have identical *Rns* sequences. A. *culbertsoni* Diamond differs from the other two strains by 18 nucleotides in the *Rns* gene.

An examination of the four remaining sets of strains with identical *rns* sequences is more interesting. Complete nuclear rDNA sequences are not available for all of these strains, but in some cases at least, strains with identical *rns* sequences also had different *Rns* sequences. For example, A. *castellanii* V042 had a mitochondrial sequence identical to A. *polyphaga* Jac/S2, A. *castellanii* Ma and A. *castellanii* Castellanii. However A. *castellanii* Castellanii and A. *castellanii* V042 are in different clades in the *Rns* analysis and separated from A. *polyphaga* Jac/S2 and A. *castellanii* Ma by up to 18 nucleotides. Another group with identical *rns* sequences includes A. *polyphaga* MC-2, A. sp. 82-12-324 and A. sp. Liu E1. These three strains have also been examined by *Rns* analysis and A. *polyphaga* HC-2 is found in a different clade from A. sp. 82-12-324 and A. sp. Liu E-1. In the *Rns* analysis they differ from A. *polyphaga* HC-2 by an average of 17.5 nucleotides. Partial *Rns* sequences available for other strains within the *rns* clusters suggest that additional cases will be found in which invariant mitochondrial sequences are coupled with variable nuclear sequences.

Does Acanthamoeba have genetic exchange?

The association of identical mitochondrial 16S rDNA genes with variable nuclear 18S rDNA genes could be due to relatively faster rates of evolution in the nuclear genes. However, this seems unlikely because mitochondrial genes usually have faster rates of evolution than nuclear genes. Several alternative explanations for the clusters of amoebae with genetically identical mitochondrial rDNA sequences, but different *Rns* sequences, are possible. In some cases the nuclear genes might evolve faster than the mitochondrial genes and in other cases, the opposite might be true. This seems unlikely, but can't be ruled out. A more attractive possibility is that strains of amoebae with identical mitochondrial rDNAs and different nuclear rDNAs resulted from some form of genetic exchange that has not been observed previously in this genus [4]. Reproduction in *Acanthamoeba* generally is thought to be asexual, but this has not been proven. Chromosomes in this genus tend to be very small and the ploidy level is uncertain. Therefore, a true sexual process followed by mitotic sorting out of mitochondria is a possible explanation. Alternatively, some kind of parasexual nuclear process, or cytoplasmic exchange in the absence of nuclear exchange, could explain the association of invariant mitochondrial genes with variable nuclear genes.

Relationships between rDNA sequence types and species

We have raised the possibility that some form of genetic exchange might occur in *Acanthamoeba,* but in the absence of definitive proof, we continue to assume that reproduction is asexual. In asexual reproduction, the assignment of species names becomes more arbitrary and totally depends on the ability to distinguish subgroups on the basis of other characteristics that are consistently reliable. Until the present time, efforts to classify *Acanthamoeba* species by use of morphology and other methods have been only partially successful. For that reason, we have used a cladistic method based on sequence variation in *Rns* genes that we believe will be more consistent and quantitatively useful for classification. The present study using *rns* analysis has supported the *Rns* conclusions and shows that subgenotype classification can be accomplished using either gene.

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