

**MULTIPLE ALLELES OF *Acanthamoeba* NUCLEAR SMALL  
SUBUNIT RIBOSOMAL DNA GENE: FURTHER EVIDENCE  
OF POSSIBLE GENETIC EXCHANGE BETWEEN  
CLOSELY RELATED STRAINS.**

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*Gregory C. Booton*<sup>1</sup>, *Fernando Lares-Villa*<sup>2</sup>, *Daryl J. Kelly*<sup>1</sup>, *Paul A. Fuerst*<sup>1</sup> and *Thomas  
J. Byers*<sup>3</sup>

**ABSTRACT**

The nuclear small subunit ribosomal RNA (*Rns*) gene has been used in the last decade to establish genotypes of the genus *Acanthamoeba* based upon these sequences. In some cases direct sequencing of the *Rns* gene has resulted in mixed sequences, suggesting either a mixed culture of more than one *Acanthamoeba* strain or the presence of multiple alleles of this gene in a single *Acanthamoeba* strain. A previous study has established that multiple alleles in a single *Acanthamoeba* strain were possible. Here we examine an *Acanthamoeba* isolate from a keratitis case that produced mixed sequences when PCR products were directly sequenced. A single *Acanthamoeba* trophozoite was directly isolated from an agar plate to produce a clonal culture derived from this single troph. Following PCR amplification *Rns* products were cloned and sequenced. This produced two distinct sequences, indicating the presence of two alleles in this isolate. One allele, *Rns* 02-039/1, is identical to the *Rns* gene of *A. sp.* strain Galka. The second allele, *Rns* 02-039/2, has 99.7% similarity with the *Rns* from *A. castellanii* strain V042. Both of these isolates are genotype T4 based on *Rns* sequence analysis. Further, both are in sub-genotype T4a identified by mitochondrial *rns* analysis, and both are in clusters of identical sequences in *rns* analysis. These data suggest that transfer of alleles (genetic exchange) may be possible between closely related strains of *Acanthamoeba*. Specifically, the results suggest a possible transfer of one allele (02-039/2) after the divergence of *A. sp.* Galka from *A. sp.* 02-039.

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<sup>1</sup>Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, Columbus, Ohio, USA.

<sup>2</sup>Technological Institute of Sonora, Cd. Obregon, Sonora, Mexico,

<sup>3</sup>Department of Molecular Genetics, The Ohio State University, Columbus, Ohio, USA.

**Corresponding Author:** Gregory C. Booton

Dept. of Evolution, Ecology, and Organismal Biology. The Ohio State University. 388 Aronoff Laboratory. 318 W. 12th Ave., Columbus, Ohio 43210. Phone: 614-292-4570. Email: booton.1@osu.edu

## **INTRODUCTION**

The genus *Acanthamoeba* is a ubiquitous assemblage of free-living amoeba species inhabiting a wide range of environments, having been isolated from soil, freshwater, saltwater, sewage sites, etc [8,11,12]. The genus is also an opportunistic pathogen that is pathogenically most commonly found in the sight-threatening infection of the eye *Acanthamoeba* keratitis (AK) [16]. Less often, members of this genus are found in infections of the skin, lung, and in the brain where they lead to granulomatous amoebic encephalitis (GAE) infections, which are typically fatal [12,13,18]. In the past, morphological analyses of this genus based largely on cyst morphology, has led to the proposal of three morphological groups of this genus, and over twenty species have been proposed [14]. However, the taxonomy of *Acanthamoeba* based on morphology has been problematic because of the plasticity of many of the characters.

The advent of molecular methods to examine questions regarding the evolutionary relationships of organisms has permitted an examination of many of these species using objective data obtained from phylogenetically informative genes. Over the last decade examination of the nuclear small subunit ribosomal RNA (*Rns*) gene in *Acanthamoeba* has provided support for the three morphological groups of *Acanthamoeba*, and supported many of the named species [3,4,5,6,17]. However, it has also shown that some of the named species are polyphyletic, and not distinguishable from one another using molecular methods. These analyses have resulted in the discrimination of *Acanthamoeba* isolates based on the dissimilarity of *Rns* sequences. This led to the description of *Acanthamoeba* isolates as belonging to different sequence types, or genotypes, with up to fourteen genotypes proposed at this time [3,4,5,6,7,17]. Significantly, it has been shown that the overwhelming majority of *Acanthamoeba* keratitis isolates are from a single genotype: T4. Further, it has been observed that *Acanthamoeba* isolates from other types of infections come from a wider variety of genotypes. Further, one genotype, T5, which corresponds to *Acanthamoeba lenticulata*, is often found in sewage and other polluted sites, but there are no genotype determinations available for isolates reported from infections. Genotype T4 also represents the most common genotype of *Acanthamoeba*. However, even with the large number of samples analyzed using the nuclear *Rns* gene, results thus far have largely failed to identify sub-clades within this genotype. By contrast, recent analyses using an organellar gene, the mitochondrial small subunit RNA (*rns*) gene has not only supported the results based on the *Rns* studies but has shown discrimination within genotype T4, subdividing this genotype into eight clades. Some of these sub-clades of genotype T4 correspond to type-strains of *Acanthamoeba* species.

In an attempt to create methods that will more rapidly identify *Acanthamoeba* isolates with regard to genotype, we have shown that PCR amplification and sequence analysis of a small portion of the *Rns* gene (designated diagnostic fragment three or DF3) can robustly identify an isolate with regard to genotype [1,15]. This method has been used successfully to identify parasitic isolates from freshwater fish, isolates from AK cases, lens case solutions, and from home water supplies (1,2,15). Specifically, when the genotype of an *Acanthamoeba* isolate has been identified using this fragment of the *Rns* gene, it has always been supported when the entire gene was sequenced. This has, in most cases, allowed for much more rapid identification of clinical and environmental samples. However, in some *Acanthamoeba* isolates PCR amplification and direct sequencing of the PCR products has led to a mixture of sequences observed in the electropherograms. This occurs in repeated experiments from these particular isolates.

The observation of a mixture of sequences following PCR amplification and direct sequencing of these amplimers can be explained most easily as being caused by two possibilities. In the first possibility, an *Acanthamoeba* isolate has not been clonally purified, and is instead a mixture of two strains. The second possibility is that it is indeed a pure clonal isolate, but that there are multiple alleles of the *Rns* gene. Multiple alleles of the *Rns* gene are thought to be rare, even though there are multiple copies of the gene in the genome of most eukaryotic organisms, due to the phenomenon of concerted evolution, which is thought to homogenize the *Rns* to a single allele. Nonetheless, we have previously documented a case of multiple *Rns* alleles in *Acanthamoeba* in which three alleles were observed [9]. One of these alleles (designated *RnsA*) was identical to the *Rns* gene of *A. sp.* Rawdon. The other two alleles were closely related to *A. sp.* Rawdon. Also, most of the observed substitutions in the different alleles were concentrated in specific stems and loops of the *Rns* gene. The observation of a mixture of sequences has not been observed to date in the mitochondrial *rns* gene. Here we examine another case of an *Acanthamoeba* keratitis isolate that produced multiple sequences following PCR amplification and direct sequencing of the DF3 fragment. Results show that this is not the case of a mixture of *Acanthamoeba* isolates, rather a single isolate of *Acanthamoeba* with two distinct alleles, which are related to alleles previously obtained from other *Acanthamoeba* isolates. These differences between the alleles are greater than observed in the previous study, but are again concentrated in specific stems and loops of the *Rns* gene. Hypotheses regarding the acquisition of these alleles in this isolate are presented.

## **MATERIALS AND METHODS**

*Acanthamoeba* keratitis isolate (OSU 02-039) was grown on non-nutrient agar with *Enterobacter* sp. as a food source. Liquid growth of 02-039 was done in Bacto-Casitone/Serum (BCS) media in 25 cm<sup>2</sup> culture flasks. DNA was extracted using a commercial DNA extraction kit: DNeasy (Qiagen, Inc., Valencia, CA). Initial *Rns* genotyping of *A. sp.* 02-039 was done by amplification of *Rns* fragment ASA.S1, which contains DF3 [1,15]. PCR was done using genus-specific primers JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGCTAGGGGAGTCA-3'). These primers are genus-specific and are not affected by co-extraction of bacterial DNA from *Acanthamoeba* cultures that contain *Enterobacter* as a food source. PCR conditions were the following: an initial denaturing step of 7 min at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 55 °C, and 2 min at 72°C. This was followed by a final extension of 15 min at 72°C. Amplicons of approximately 470 bp from *Acanthamoeba* cultures were visualized on a 1 % agarose gel following electrophoresis of 20 % of the PCR reaction and staining with ethidium bromide. The remaining PCR product was prepared for sequencing by using the Qiaquick PCR clean-up kit (Qiagen, Inc., Valencia, CA). Samples were typically eluted in 30-50µl of Qiagen elution buffer and 3.5 µl of purified sample was routinely used for sequencing reactions. In later PCR reactions, after a mixture of sequences was observed in ASA.S1 amplicons, we employed a different primer set that we have previously used to amplify the whole *Rns* gene in *Acanthamoeba* [17]. It was this large fragment that was used in later cloning and sequencing procedures so that the entire gene could be examined in this study.

When initial direct sequence analysis showed a mixture of sequences, two different methods were used to clonally isolate *A. sp.* 02-039. The first method was a direct physical isolation of a single trophozoite onto a fresh non-nutrient agar (NNA) plate. After outgrowth,

this was repeated 2X to insure a clonal isolation. This method was repeated twice so that results obtained by this method could be compared. The second method was serial dilution of a known quantity of *Acanthamoeba* isolates to a theoretical concentration of one ameba or less. The starting culture for the serial dilution experiment was derived from the liquid culture at the endpoint of the physical clonal isolation experiment, described above. This was done to further enhance the probability of selection of a single ameba cell as the starting point for this method. The lowest concentration serial dilution that produced growth was then grown in BCS media as described above. Following clonal isolation and growth, DNA extraction and PCR (using primers to amplify the entire *Rns* gene) were as described above.

Sequencing of PCR products was done on an ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, CA) using *Rns* conserved primers that were previously used to determine the primary DNA sequence of DF3 of *Rns*. Also, other primers that determined the entire sequence of the *Rns* gene were used on the full *Rns* PCR amplicons [15,17]. Following observation of mixed sequences using fluorescent sequencing the entire gene was cloned using a T/A cloning kit (Invitrogen, Inc.). One microliter of a 1:10 and one microliter of a 1:100 dilution of the PCR product were ligated into the T/A cloning vector. Ten positive clones were picked from the plates, grown overnight in Luria Broth (LB) media with Kanamycin for antibiotic selection, and prepped using the Qiaprep kit (Qiagen Inc, Valencia, CA). These clones were sequenced as described above using conserved primers within the *Rns* and primers that are in the vector, outside of the *Rns* insert, to determine the entire primary sequences of these clones.

In addition to the nuclear *Rns* gene studied here, we also amplified and sequenced the mitochondrial *rns* gene, which has been a target in our previous studies [10]. This gene was amplified and sequenced using conserved primers that we have previously used to amplify this gene in *Acanthamoeba* spp. [10]. Following amplification, the *rns* was directly sequenced (i.e. without T/A cloning of the PCR product) using conserved primers. No mixture of sequences was observed in direct sequencing of *rns* in repeated experiments.

The obtained sequences of the nuclear *Rns* and mitochondrial *rns* were compared with previous sequences in the Ohio State University Ameba DNA Sequence Database for comparison and identification.

## **RESULTS**

### **Nuclear *Rns***

Initial PCR amplification of the *Rns* gene in *Acanthamoeba* sp. isolate 02-039 followed by direct sequencing of these *Rns* amplicons produced mixed sequence results on electropherograms. Therefore, the methods described above were employed to determine if the results were because of a mixture of two cultures or the presence of two alleles in a single strain. Following direct isolation of a single trophozoite and growth in BCS media, DNA was extracted, PCR was performed, and direct sequencing was again done. This also resulted in a mixture of sequences on the electropherograms. Therefore, the PCR amplicon was T/A cloned and ten positive clones were prepped and sequenced. This resulted in two separate sequences that were determined by this sequence analysis. These sequences, hypothesized to be two separate alleles of *Rns*, were designated 02-039/1 and 02-039/2. This experiment was repeated

in a separate isolation of a single trophozoite, with confirmatory results. That is, the same two alleles were again produced following sequencing. A different method, serial dilution, was then employed to hypothetically isolate a single trophozoite. The starting culture used for the serial dilution experiment was obtained from the end point of the physical dilution experiments described above, that is the serial dilution experiments were begun from a culture already thought to be clonal. Following serial dilution and growth of a culture derived from a calculated starting point of a single amoeba, the direct sequencing of *Rns* PCR products using this method again produced a mixture of sequences. Therefore, this PCR product was also T/A cloned and subsequently sequenced, and again produced the same two alleles: 02-039/1 and -2-039/2.

All experiments carried out in the current study resulted in the observation of two sequences (presumed alleles) following sequence analysis. These alleles share similarity with previously analyzed isolates, following comparison of these sequences to our database. One allele, *Rns* 02-039/1, is identical to the *Rns* gene of *A. sp. Galka*. The second allele, *Rns* 02-039/2, has 99.7% similarity with the *Rns* from *A. castellanii* V042. Similar to that observed in the study by Ledee, Seal, and Byers 1998, all but one of the changes of *A. sp. 02-039/2* vs *A. castellanii* V042 are in one stem loop region of the *Rns* gene. Both of these isolates are genotype T4 based on *Rns* sequence analysis. Lastly, the close relationship of the two *A. sp. 02-039* alleles to these two isolates' *Rns* genes shows that *A. sp. 02-039* is a genotype T4 *Acanthamoeba*.

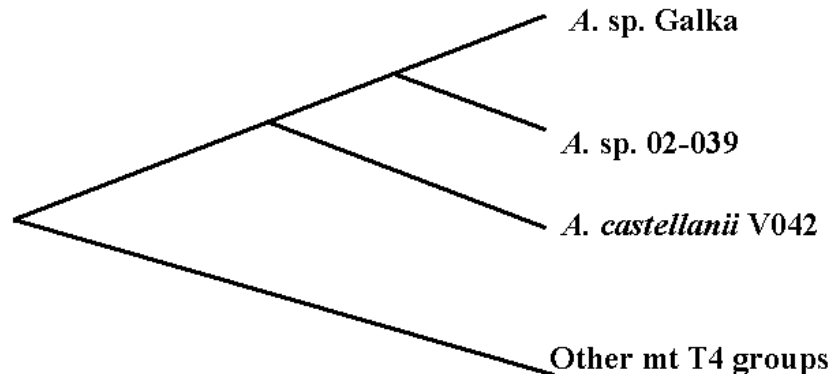
#### **Mitochondrial *rns***

A single mitochondrial sequence of *rns* was observed by direct sequencing of the PCR product of this gene. This was observed in repeated experiments and, as has been the case in all of our previous experience, a mixture of products was not observed in the mitochondrial *rns*. As discussed above, previous analysis of the mitochondrial small subunit RNA (*rns*) gene from representative samples of the genotypes proposed using *Rns* analysis has supported subdivision of the T4 genotype into eight separate clades (T4a-T4h). *A. sp. Galka* and *A. castellanii* V042 are both found within one of these subdivisions, T4a, reflecting their close relationship to one another, and to *A. sp. 02-039*. When the substitutions of *A. sp. 02-039* are compared to *A. sp. Galka* and *A. castellanii* V042, there are six differences vs. *A. sp. Galka*, and 26 differences vs. *A. castellanii* V042. Similar to the results of the *Rns* gene, half of the changes in the *rns* vs *A. sp. Galka* are in a single loop of the transcribed *rns* gene.

## **DISCUSSION**

The analysis of the *Rns* gene in this study clearly indicates that *Acanthamoeba* AK isolate 02-039 is a genotype T4 isolate. This is indicated by the close phylogenetic relationship of *A. sp. 02-039* with *A. sp. Galka* and *A. castellanii* V042 based on the *Rns* gene. Both of these other isolates were previously determined to be genotype T4. Further, *A. sp. 02-039* is closely related to these two isolates based on the mitochondrial *rns* gene. In earlier mitochondrial analyses, *A. sp. Galka* and *A. castellanii* V042 are found in one of the sub-clades of genotype T4, specifically T4a [10]. The conclusion that can be drawn is that *A. sp. 02-039* is closely related to both of these isolates, but based on the mitochondrial analysis, more closely related to *A. sp. Galka*. This relationship is shown in *figure 1*. This close relationship between *A. sp. 02-039* and *A. sp. Galka* is further supported by the 100% similarity of one of the alleles found in

02-039 and the *Rns* gene of *A. sp. Galka*. But what of the other allele in *A. sp. 02-039*, which is 99.7% similar to the *Rns* gene from *A. castellanii* V042. Two alternative hypotheses can be posited to explain the presence of this allele. First, these two alleles could have existed in the ancestor of all three isolates: *A. sp. 02-039*; *A. sp. Galka*, and *A. castellanii* V042. The second allele has since been lost in the lineage leading to *A. sp. Galka* and in the lineage leading to *A. castellanii* V042. This scenario requires two losses of the allele. An alternative hypothesis posits that *A. sp. 02-039* has obtained this allele following the divergence of *A. sp. 02-039* and *A. sp. Galka* by genetic exchange with (figure 2). Further, it is hypothesized that this acquisition event was followed by the subsequent divergence of this gene from the *A. castellanii* V042 (explaining the differences in this allele versus that found in *A. castellanii* V042). This hypothesis requires one gain of an allele by genetic exchange between closely related strains. As discussed previously *A. sp. Galka* and *A. castellanii* V042 are found within a sub-clade of genotype T4 identified in the mitochondrial *rns* analysis. This scenario would suggest that genetic exchange is possible between closely related strains of this genus. Furthermore, it appears that the acquired allele acquires changes in a non-random manner since most of the observed substitutions are found in specific stem/loop regions of the RNA transcript of this gene. Most of the substitutions between *A. sp. 02-039/2* and *A. castellanii* V042 are found in a single stem/loop (45-1), which is in a highly variable region of the *Rns* gene (figure 3). It may be that these regions have some role in pathogenicity or drug resistance in strains that maintain these alternative alleles. It is interesting that this phenomenon was observed in a case obtained from the Bascom-Palmer Eye Institute. Many of the cases sent to this world renowned eye center are those that have been difficult to treat, and may represent especially harsh infections. We have also observed this mixed sequence phenomena in other samples from this institute and these are being further investigated. In summary, the observation of multiple alleles in the nuclear *Rns* gene of *Acanthamoeba*, which may cause problems for direct sequencing methods, may provide insight into possible genetic exchange in close relatives of this genus, and further may offer insight into the relative levels of pathogenicity of closely related isolates.



**Figure 1. Phylogenetic relationship of *A. sp. Galka*, *A. castellanii* V042, and *A. sp. 02-039* based on mitochondrial *rns* analysis. Other mitochondrial genotype T4 subdivisions are used as an outgroup in this tree.**

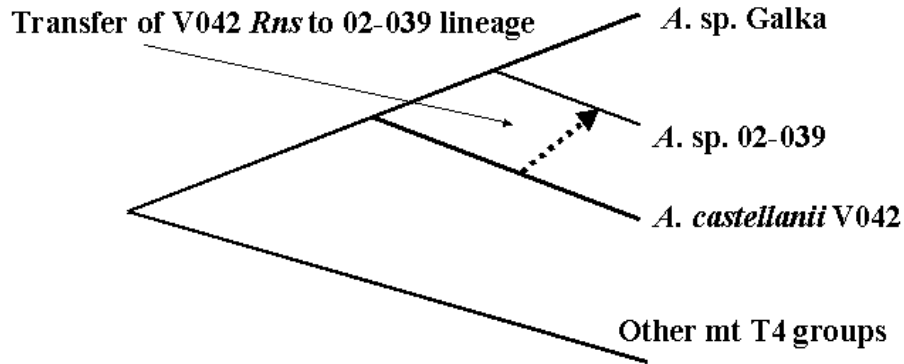


Figure 2. Proposed transfer of allele 02-039/2 from *A. castellanii* V042 to *A. sp.* 02-039 shown as dotted line, superimposed upon the phylogenetic tree based upon *rns* analysis presented in figure 1.

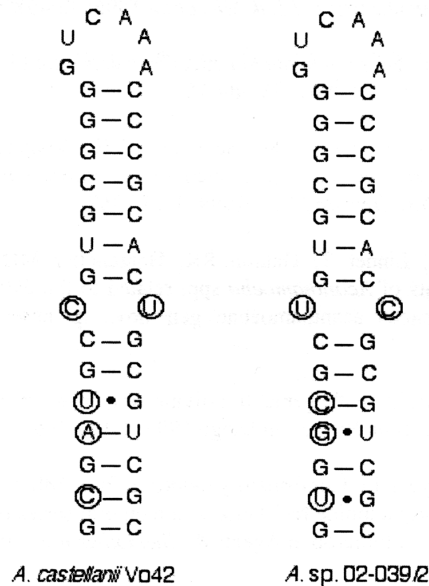


Figure 3. Stem-loop 45-1 of *Acanthamoeba Rns* gene. Stem on the left is the proposed secondary structure of Stem 45-1 of *A. castellanii* V042. On the right is the proposed secondary structure of Stem 45-1 from *A. sp.* 02-039/2.

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