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REVIEW ARTICLE

Phylogenetic Analysis and the Evolution of the 18S rRNA Gene Typing System of *Acanthamoeba*[1]

Paul A. Fuerst[a,b], Gregory C. Booton[b] & Monica Crary[b]

a Department of Evolution, Ecology and Organismal Biology, The Ohio State University, Columbus, Ohio,
b Department of Molecular Genetics, The Ohio State University, Columbus, Ohio,

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Correspondence
P.A. Fuerst, Department of Ecology, Evolution and Organismal Biology, Department of Molecular Genetics, The Ohio State University, 388 Aronoff Laboratory, 318 West 12th Avenue, Columbus, OH 43210, USA
Telephone number: +614-292-6403;
FAX number: +614-292-2030;
e-mail: fuerst.1@osu.edu

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ABSTRACT

Species of *Acanthamoeba* were first described using morphological characters including cyst structure and cytology of nuclear division. More than 20 nominal species were proposed using these methods. Morphology, especially cyst shape and size, has proven to be plastic and dependent upon culture conditions. The DNA sequence of the nuclear small-subunit (18S) rRNA, the *Rns* gene, has become the most widely accepted method for rapid diagnosis and classification of *Acanthamoeba*. The Byers–Fuerst lab first proposed an *Rns* typing system in 1996. Subsequent refinements, with an increasing DNA database and analysis of diagnostic fragments within the gene, have become widely accepted by the *Acanthamoeba* research community. The development of the typing system, including its current state of implementation is illustrated by three cases: (i) the division between sequence types T13 and T16; (ii) the diversity within sequence supertype T2/T6, and (iii) verification of a new sequence type, designated T20. Molecular studies make clear the disconnection between phylogenetic relatedness and species names, as applied for the genus *Acanthamoeba*. Future reconciliation of genetic types with species names must become a priority, but the possible shortcomings of the use of a single gene when reconstructing the evolutionary history of the acanthamoebidae must also be resolved.

TRADITIONAL CLASSIFICATION OF ACANTHAMOEBA

The genus *Acanthamoeba* is classified within the phylum Amoebozoa, subphylum Lobosa, and the order Centramoebidae (Rogerson and Patterson 2002; Smirnov et al. 2011). The other recognized genera of the Order Centramoebida are *Balamuthia* and *Protacanthamoeba*. Some isolates that have been classified to *Comandonia* also appear to belong to this Order, even being placed inside of *Acanthamoeba*, but the nominal identification of these forms is suspect (Smirnov et al. 2011). The classification of Centramoebida with respect to other taxa of the Amoebozoa is supported by DNA sequence similarity of the nuclear 18S rRNA genes, as well as the DNA sequences of several protein genes, including α-tubulin, β-tubulin and actin (Lahr et al. 2013).

Descriptions of amoebae that are now placed in the genus *Acanthamoeba* date from the early part of the 20th century. Puschkarew (1913) was the first to isolate an amoeba that is plausibly now considered a member of *Acanthamoeba*, when he isolated a cyst-forming amoeba from dust, naming the form *Amoeba polyphagus*. Unfortunately, this isolate no longer appears to exist in any culture collection. A second amoeba, now certainly a representative of *Acanthamoeba* was isolated by Aldo Castellani as a contaminant in a culture of the fungus *Cryptococcus pararoseus* (Castellani 1930a,b,c,d). Douglas (1930) examined this amoeba and categorized it as a member of the genus *Hartmanella*. Castellani's isolate still exists in several culture collections, being listed as ATCC 30011 in the American Type Culture Collection (ATCC) and as CCAP 1501/10 in the Culture Collection of Algae and Protozoa (CCAP).

The taxonomic classification of free-living amoebae has always been challenging, given the pliable nature of amoebic cell size and shape. Since the early part of the

last century, taxonomic classification of small free-living amoebas was based primarily on morphological criteria. These included the type of locomotion of the trophozoites, the morphology of the cysts and the type of nuclear division of the organism. Castellani's isolate was formally described by Volkonsky, based on cytological examination of cell division and cyst form (Volkonsky 1931). He considered the diversity of forms that were being placed within the genus *Hartmanella* and suggested that the genus contained a heterogenous grouping of taxa. As such he proposed a new genus, *Acanthamoeba* gen. nov., and identified Castellani's isolate as *Acanthamoeba castellanii*. He also suggested that Pushkarew's isolate be renamed *Acanthamoeba polyphagus*. The new genus was not initially universally accepted, and classification of many free-living forms still favoured placement in the genus *Hartmanella*. Over the next few decades additional free-living amoebas were described that showed sufficient similarities with the isolates described by Castellani and Volkonsky to be considered to be members of the same genus. These included amoebas isolated from soil by Reich (1933), Comandon and de Fonbrune (1937), Singh (1952), and Neff (1957), from fungal cultures and environmental fungal sites by Hewitt (1937), and from water in a laboratory termite culture by Ray and Hayes (1954). However, even as late as 1970, the question of whether these forms represented anything more than species within the genus *Hartmanella* remained a point of contention (Singh and Das 1970). The morphological criteria that were important to distinguish *Acanthamoeba* from other forms continued to be debated (Pussard 1966; Singh 1952). Page (1967a,b) provided the morphological analysis that argued most persuasively for recognition of *Acanthamoeba* as a taxon separate and distinct from *Hartmanella*. In the following decade the definitive study of cyst morphology by Pussard and Pons (1977) cemented the authenticity of the identity of *Acanthamoeba* as a genus separate from *Hartmanella* and other free-living amoebas.

The paper of Pussard and Pons (1977) also marked a significant additional observation concerning morphological variability between isolates within the genus *Acanthamoeba*. They proposed that three morphological groups could be identified on the basis of cyst size and shape. Group 1 consisted of species with large trophozoites and cysts. The average diameter of cysts is greater than 18 μm. The ectocyst and endocyst are widely separated. The outer cyst wall is smooth or gently wrinkled. The endocyst is roughly stellate. The endocyst meets the ectocyst at the ends of arms or rays. Species placed in *Acanthamoeba* Group 2 have cysts with mean diameter usually less than 18 μm. This group turned out to include the most common and widespread forms such as "*A. castellanii*." The ectocyst and the endocyst are either close together or widely separated. The ectocysts of this group may be thick or thin and are usually wrinkled or mamillated. The endocyst may be stellate, polygonal, triangular, or sometimes round or oval, but usually does not have well-developed arms or rays. The final group, Group 3 has cysts with a mean diameter usually less than 18 μm. The ectocysts of this group are thin and either gently rippled or unrippled. The endocysts are usually round but may have three to five gentle corners. This identification of morphological groups has proved very useful in interpreting molecular clustering of isolates of *Acanthamoeba*.

By the mid-1970s consideration of morphology had advanced to the point that Sawyer and Griffin (1975) proposed that these amoebas represented a new family, the Acanthamoebidae. Other workers were rapidly reporting new isolations, and the number of nominal species within the genus rapidly grew to more than 20 (Visvesvara 1991). This also marked the time when the pathogenic potential of *Acanthamoeba* began to be better understood (Ma et al. 1981). *Acanthamoeba* had already been identified as the cause of rare cases of meningoencephalitis. However, when reports of amoebic keratitis began to accumulate (Ma et al. 1990), the importance of *Acanthamoeba* as a health concern was greatly increased.

By the late 1970s, however, new methods of analysis began to impact taxonomy. The categorization of species, especially for simple microbial organisms such as the free-living amoebae, began to come under scrutiny. Previously accepted morphological criteria began to be questioned as biochemical and, later, molecular criteria indicated inconsistencies in the patterns of classification. New biochemical methods for systematic classification used allozyme similarity to cluster individuals and to identify the boundaries of putative species on the basis of similar protein patterns. A series of studies used multiple isozyme systems to examine 30–71 different isolates representing most of the named species (Daggett et al. 1982, 1985; De Jonckheere 1983). These studies came to the conclusion that the species assignments used at that time did not, in general, correspond to biochemically distinguishable lineages. Biochemical groups usually included members assigned to multiple nominal species, and isolates assigned to the same nominal taxa were often found in different biochemical lineages.

By 1980, DNA analysis had begun to replace isozymes as the methods of choice for evolutionary genetics. These new methods were employed in studies of the relationship among isolates of *Acanthamoeba*. In conjunction with Tom Byers, my lab applied DNA based RFLP phylogenetic analysis of mitochondrial DNA in an effort to further clarify the relationships among different *Acanthamoeba* strains (Bogler et al. 1983; Byers et al. 1983). The method provided strong evidence of clustering of different isolates, but similar to the findings based on isozymes, failed to strongly support previous, morphologically based assessments of isolate similarity. Questions concerning the relationship between underlying DNA sequence changes and changes in isozyme or RFLP similarity continued to be raised. In addition, the comparability of population based RFLP analyses between labs was raised. Further improvement in methodology was required.

That improvement was provided by methods that obtained direct information concerning DNA sequences of

isolates of *Acanthamoeba*. The seminal contribution that permitted DNA sequencing to be applied extensively to the study of *Acanthamoeba* was the report of the sequence of the nuclear small-subunit (18S) rRNA gene for the Neff strain of *A. castellanii* (Gunderson and Sogin 1986). This represented the first 18S rRNA gene sequence from any member of *Acanthamoeba.* Ultimately, however, the most important finding of this paper was the observation that *A. castellanii* Neff possessed an 18S rRNA gene that had unusually long length and (possibly) unusual characteristics. The gene exceeded the length of a typical eukaryotic 18S rRNA gene by almost 500 nucleotides. As sequences from other isolates of *Acanthamoeba* began to be obtained, it became clear that the increased length of the sequences was due to the occurrence of a series of regions that were dispersed within the gene, and that showed high levels of variation between isolates, especially when compared to variation in those regions of the gene that were part of the "normal" eukaryotic 18S rRNA gene.

With the development of the polymerase chain reaction (PCR) in the late 1980s, DNA sequencing, especially using the Sanger dideoxy chain termination method, began to expand rapidly. By 1990, our research group had begun to pursue molecular evolutionary analyses that involved obtaining the sequences of ribosomal RNA genes in a variety of prokaryotic and eukaryotic systems. As part of these studies the Byers–Fuerst laboratory collaboration began to sequence the nuclear 18S rRNA genes from several free-living amoebae, emphasizing, but not restricted to, *Acanthamoeba* (Gast, Fuerst and Byers, 1994; Weekers et al. 1994). In the study of *Acanthamoeba*, these studies were motivated by an interest in developing rapid and accurate diagnostic procedures to be used to foster better clinical outcomes for patients suffering from infections by *Acanthamoeba*. Because the 18S rRNA gene, designated the *Rns* gene, or its homologues, is universally present in living organisms, it presented a good target to investigate for the development of taxa-specific DNA diagnostics.

Allow a momentary digression on topics of gene nomenclature. Most studies of *Acanthamoeba* have emphasized sequences of the gene referred to as the 18S rRNA gene. More correctly, this gene would be identified as the nuclear ribosomal small-subunit rRNA gene, or the SSU gene. Geneticists would designate this gene, labelling it the *Rns* gene. In eukaryotes this would differentiate it from the gene coding for the nuclear ribosomal large subunit or LSU gene, designated *Rnl*. It is also differentiated from the mitochondrial ribosomal small-subunit gene, designated *rns*. This latter gene is also referred to as the 16S-like rRNA gene, acknowledging the prokaryotic origin of the mitochondrial genome.

It immediately became clear that the 18S rRNA genes of *Acanthamoeba* were much more informative about interstrain relationships than would usually be the case when sequencing the typical eukaryotic *Rns* gene. In most situations involving eukaryotes, the various strains of a particular species are likely to have very similar or even identical nucleotide sequences. In fact, it is now often stated that the *Rns* gene is only of use in identifying organisms to the genus, and does not provide species information. For instance, in a leading text on evolutionary biology (Barton et al. 2007; online chapter 27) the authors state "Perhaps the most significant limitation of rRNA for phylogenetic analysis is that even the most rapidly evolving regions generally do not evolve fast enough for this molecule to be used to study relatively recent evolution (e.g., relationships among species within genera or within species)." Natural selection acts to maintain the functioning of the rRNA genes and their products, because they are vital for the life of the cell. As a consequence, changes in rRNA gene sequences over evolutionary time are usually slow. Within-species polymorphism in these genes is usually restricted (Hillis and Dixon 1991). Observations in which different conspecific individuals differ by more than a few nucleotides (out of ~1,800 nucleotides in a typical eukaryotic 18S rRNA) are rare (Eickbush and Eickbush 2007). However, that did not seem to be the case for "species" of *Acanthamoeba*.

It was clear that the extra sequence regions discovered by Gunderson and Sogin (1986) in *Acanthamoeba* were not being constrained by purifying natural selection in the same way that the rest of the gene was; these segments varied much more extensively than most nucleotide sites within the gene. These hypervariable regions allow investigation of many different aspects of isolate differences in *Acanthamoeba*. For instance, do different genotypes of *Acanthamoeba* have the same pathogenic potential? Is the frequency of genotypes in the genus the same in different geographic regions around the world? What is the relationship between the genotype(s) involved in an infection and possible sources of contamination in the environment? All of these questions, and many others that could be posed, require the acquisition of the sequences from many different isolates of the putative disease agent. Fortunately, for *Acanthamoeba*, the past two decades have seen the accumulation of an extensive collection of sequences that have only begun to be exploited.

DEVELOPMENT OF A GENOTYPE CLASSIFICATION SYSTEM FOR *ACANTHAMOEBA*

Following the publication of the first full sequence of the 18S rRNA gene from *A. castellanii* Neff (Gunderson and Sogin 1986) additional sequences began to be determined, and by the 1990s *Rns* sequences from various isolates of *Acanthamoeba* allowed a sequence based analysis of genotype diversity within *Acanthamoeba,* identifying patterns of phylogenetic relationships. The first seminal paper on this approach by our lab (Gast et al. 1996) proposed that subgenus classification was possible using *Rns* sequences. Sequence types were defined using criteria that provided significant separation of types, while uniting isolates that were substantially similar, but not identical. The original definition was "types are defined as sequences or groups of sequences that differ from all other sequences by at least 6%, have a minimum of 134

base differences, or an evolutionary distance greater than 0.8% in the current database." (Gast et al. 1996). In choosing this level of divergence, we were influenced by suggestions being made in prokaryotic phylogenetics that 5% sequence divergence between 16S rRNA sequences could be used as a defining criterion for species. We never assumed that our original definition would become a hard rule, as the number of sequences analysed (18) was quite small. In the study, this criterion was applied to sequences that were substantially complete, roughly as long as the original sequence of Gunderson and Sogin (1986), and we did not envision that it would be applied to sequence fragments that represented a small fraction of the total gene. From a phylogenetic perspective, the most comprehensive information will be contained in *Rns* sequences that are as complete as possible, preferably 2,000 nucleotides or longer. We will refer to such sequences using the term "complete" sequences, although they usually lack the terminal 5′ and 3′ segments of the gene. All of the sequences that we included in our initial analysis of *Rns* variation exceeded 2,250 nucleotides in size.

Four genotype clusters were initially identified from among the collection of 18 isolates (Gast et al. 1996). These were designated type T1 through T4. One-third of the isolates in this study were provided by Govinda Visvesvara from his collection of isolates at the CDC labs. The analysis included the sequences of the original *Acanthamoeba* isolate of Castellani (ATCC 30011, subsequently resubmitted by our lab to the ATCC as ATCC 50374), and the isolate "*A. castellanii* Neff" (ATCC 30010, resubmitted as ATCC 50373). Both were classified within the sequence type T4. Among the 18 isolates, no identical sequences occurred, although 15 of the 18 isolates were classified as Type T4. Type T4 has remained the predominant type as the number of sequenced isolates has expanded. For some time following the proposal of this genotyping system, we and others thought that the unusual nature of the sequence variability for *Rns* in *Acanthamoeba* might result in every isolate having a unique sequence. This has not proven to be true, especially as the number of sequences in the DNA databases has increased, and especially when sequence fragments are compared. Gast et al. (1996) identified twelve variable regions of the *Acanthamoeba Rns* gene, most corresponding to sequence segments without obvious homologues in the *Rns* genes of other eukaryotes. Many of these regions have become the basis for further subdivision of the genus, for analysis of gene fragments, and for attempts to coordinate species names with sequence genotype analysis.

Following the Gast et al. (1996) paper, sequence analysis of *Rns* genes expanded rapidly and within a short time we reported a significant enlargement of the scale of genotype diversity. Eight new sequence types within *Acanthamoeba* were identified using an additional 35 isolates (Stothard et al. 1998), representing all three morphological groups within *Acanthamoeba.* Species designations were studied by including isolates representing 16 named species of *Acanthamoeba*. All of the new sequences exceeded 2,200 nucleotides in length. Isolates identified from *Acanthamoeba* morphological groups II and III possessed *Rns* sequences that were roughly similar in size. However, the sequences representing three isolates that were members of *Acanthamoeba* morphological Group I had sequences that exceeded 2,500 bases in length. The analysis of Stothard et al. (1998) is also included a sequence that we hoped would represent a close outgroup that could be used to identify the root of the phylogenetic tree in the analysis of *Acanthamoeba*. The outgroup that we studied was *Balamuthia mandrillaris*, a free-living amoeba that had been identified originally as a leptomixid amoeba (Visvesvara et al. 1990), but subsequently was identified as closely related to *Acanthamoeba* (Visvesvara et al. 1993). The *Rns* sequence of *B. mandrillaris* lacks the expansion regions seen in *Acanthamoeba*, with the *B. mandrilaris* sequence being more than 250 nucleotides smaller than any *Acanthamoeba* sequence, even though it spanned the entire gene region examined in *Acanthamoeba*. Nevertheless, it has proven very useful in identifying the direction of phylogenetic change within *Acanthamoeba*. The use of the *B. mandrillaris Rns* sequence allowed us to be very confident that the first split within the genus (as it is currently defined) was between the group I *Acanthamoeba*, with large trophozoites and cysts represented, and other (group II and group III) forms.

Our definition of sequence types was revised in the Stothard et al. (1998) study based on our ability to identify monophyletic lineages that roughly corresponded to the level of diversity observed in the original sample of T4 isolates (Gast et al. 1996). In 1998, we observed that sequence differences between types were at least 5%, and were always greater than sequence differences within types. However, we again never formally proposed the 5% difference criterion that has shaped considerations by ourselves and others of subsequent sequence discoveries. Given the much greater dataset of sequences from *Acanthamoeba* that now exists (Fuerst 2014), an approach similar to that originally employed (i.e., identifying significant monophyletic lineages) suggests that formal subtypes within our previous sequence types can also be identified. In such a reassessment, formal sequence subtypes would have similarities that differ by less than 5%, but would differ from other subtypes by some newly defined level of sequence divergence.

Some care must be taken in interpreting the values of sequence dissimilarity. When large phylogenetic analyses are undertaken on the most complete partial *Rns* sequences from *Acanthamoeba* (those sequences that exceed 2,000 nucleotides in length), the difficulty of identifying homologous nucleotide sites within the variable regions of the gene becomes a significant issue. In this instance, a question can be raised concerning what is meant by 5% sequence difference. Is it a 5% difference between sequences as they are aligned in a multi-isolate alignment including many (or all) sequence types? Or is it 5% difference when two sequences are aligned pairwise against each other in the absence of a multisequence

alignment? These two approaches can lead to very different values, because of the difficulty of defining how sites of insertion or deletion are included in calculations of sequence similarity, especially in a multisequence alignment. In addition, care must be taken in deciding whether sites that involve insertions or deletions (in/dels) are also counted in the sequences being compared. In many phylogenetic comparisons only sites that have a nucleotide present in all sequences being compared, whether for a pair of sequences or for a multisequence alignment, will be counted. We would propose that dissimilarity measured in pairwise alignments of sequences, including in/del positions, should be the appropriate criterion, combined with a consideration of the phylogenetic significance of sequence clades. This is the approach that has been taken in this paper, and in other papers that are being prepared for the complete analysis of various genotype groups of *Acanthamoeba*.

As Stothard et al. (1998) and other investigators have identified sequences that do not fall into the original 12 sequence types that we described (Corsaro and Venditti 2010; Gast 2001; Hewett et al. 2003; Horn et al. 1999; Lanocha et al. 2009; Magnet et al. 2014; Nuprasert et al. 2010; Qvarnstrom et al. 2013). There are about 20 sequence types that can be confidently identified; there will be more if a lower level of sequence divergence is applied. Some confusion exists in the literature, with divergent sequences having been assigned the same new type number (Corsaro and Venditti 2010; Lanocha et al. 2009). There have also been new types proposed based only on partial *Rns* sequences (Hewett et al. 2003 Lanocha et al. 2009). While these may be validated, we strongly recommend that no new type be proposed without a sequence that spans essentially the entire *Rns* gene. This would necessitate *Rns* sequences of at least 2,000 nucleotides in length. However, the T15 genotype proposed by Hewett et al. (2003) and assigned to *Acanthamoeba jacobsi* illustrates a possible problem. No "complete" *Rns* sequence has been reported for any representative of *A. jacobsi*. Our own lab attempted to obtain such a sequence, but we were able to obtain only ~1,400 nucleotides, roughly the same length as for isolates studied by Hewett et al. (2003). It is not clear why the 3′ end of the gene resists analysis. Are the PCR primer sites changed? Is there an intron, as has been found for some isolates of *Acanthamoeba* (Gast, Fuerst & Byers, 1994)? These sequences require further analysis.

Following the development of the system of genotypic types in 1998, many researchers began to apply our proposed system to describe the frequency of sequence types in local collections of *Acanthamoeba* clinical or environmental isolates around the world. Fortunately, also at about this time, many journals began requiring that papers in which sequence descriptions or analysis were being reported must deposit the final versions of sequences in one of the International DNA databases (Genbank, DDBJ, or EMBL) to make the data available to the scientific community. Consequently, the number of *Acanthamoeba Rns* sequences in these databases began to expand rapidly. DNA sequences are much more amenable to comparison between laboratories than are data sets such as isozymes or RFLP digestion data. Some issues exist with sequences in the DNA databases, especially with respect to the accuracy of DNA sequencing. Several studies have suggested that GenBank entries may have error rates which range from 0.1% to over 3% (Bandelt et al. 2002; Clayton et al. 1995; Harris 2003; Hill et al. 2000; Karlin et al. 2001). Others have suggested that sequence error may be especially a problem with some rRNA genes (Noor and Larkin 2000). Given that the criterion for the identification of new types has been set at > 5% sequence divergence, the probability of incorrect taxonomic identification is most likely low, but not negligible. Inaccuracies could become more important if and when the criterion for type difference is reduced. Although some level of sequencing error certainly exists in the databases, it is hoped that real sequence divergence, and consequently real phylogenetic signal, will be greater than the noise from error. We will consider these problems further in this paper when appropriate.

THE IDENTIFICATION OF DIAGNOSTIC FRAGMENTS AND THEIR EFFECT ON ISOLATE SCREENING

While obtaining long, almost complete *Rns* sequences was an ideal goal, smaller parts of the *Rns* sequence might contain a substantial proportion of the phylogenetic signal of the entire gene. This would speed the transmission of diagnostic information back to a clinician treating possible *Acanthamoeba* infections. The use of fragments required several criteria to maintain accuracy. First, the fragment must be highly specific for the genus *Acanthamoeba*. Second, it must be available from all known *Acanthamoeba* genotypes. Finally, it should identify individual genotypes. We were able to identify specific regions of the *Rns* sequence that met these goals, and which could be obtained easily by PCR by focusing on areas of the gene that had been previously identified as regions of high variability (Stothard et al. 1998). One PCR amplimer clearly satisfied the first two requirements. This amplimer, designated ASA.S1 (*Acanthamoeba*-specific amplimer S1), was slightly greater than 400 nucleotides in length in most sequence types (Schroeder et al. 2001). The region of the gene identified by ASA.S1 is also often referred to in the literature as the JDP, the JDP1-JDP2 or the JDP-PCR product, because it is amplified using primers JDP1 and JDP2 (Schroeder et al. 2001). The product was shown to be diagnostic for the presence of *Acanthamoeba*. However, this amplimer did not appear to distinguish between all sequence types. A second set of PCR primers were identified that would provide genotype identification, producing an amplimer GTSA.B1 (genotype specific amplimer B1), which was ~1,475 bases in length and included the sequences within ASA.S1. We further identified three amplimers internal of GTSA.B1 that we felt should be routinely obtained and designated these diagnostic fragments DF1, DF2, and DF3 (Schroeder et al. 2001). It has turned out that the most informative of these, DF3, which is a

fragment of only about 240 nucleotides, was also included within the bounds of amplimer ASA.S1.

As our lab and others began to study these diagnostic amplimers, it soon became obvious that much information could be obtained by examining only the region of amplimer ASA.S1, or even just by studying the fragment represented by DF3. Often, questions in which we were interested did not require us to obtain "complete" *Rns* sequences. Although fragments represented by ASA.S1 or DF3 do not contain all of the information encoded by a "complete" *Rns* sequence, most genotypes can be accurately identified. It cannot be argued, however, that diagnostic fragments that differ by more than 5% in sequence would necessarily be characteristic of different sequence types. Potentially variable positions make up a greater proportion of the nucleotides in the diagnostic fragments. It is our contention that identification of potentially new sequence types in *Acanthamoeba* must usually be accomplished by the analysis of *Rns* sequences that are greater than 2,000 nucleotides in length. Further, we feel that the identification of new sequence types is essentially a phylogenetic question, and should occur in a context of appropriate phylogenetic analysis.

THE DESIGNATION OF ALLELE TYPES; IDENTIFICATION USING INFORMATION BELOW THE LEVEL OF DIAGNOSTIC FRAGMENTS

In a study of local geographic variability among *Acanthamoeba* isolates from Hong Kong, we found it useful to designate isolates on the basis of allelic sequence differences (Booton et al. 2002), based on only a portion of the DF3 region. We used this approach because all of the isolates being studied fell within a sequence type, into either the T3 or the T4 sequence types. The number of sequences in the DNA databases had risen to about 250 and evidence was increasing that we were not adequately accounting for diversity that existed between isolates within sequence types. In the Hong Kong study, we identified 10 different "alleles" present among the 17 T4 isolates, while all 5 of the T3 isolates in the study had different "alleles". We did not attempt to go back to our earlier studies to designate alleles, nor did we systematically analyse the taxonomic significance of the alleles. Subsequently, however, this allelic designation system has been sporadically used by others to identify variation within type T4 (Abe and Kimata 2010; Ledee et al. 2009; Magnet et al. 2013; Risler et al. 2013; Zhao et al. 2010). This has resulted in a confusing set of designations in which at least 38 "alleles" have been identified, often with the same number from different studies identifying different alleles. These designations are very incomplete, given the availability of close to 1,800+ *Acanthamoeba Rns* DNA sequences in the databases, of which more than 1,300 lie within the T4 type (Fuerst 2014). There is relatively little indication how these alleles relate to the original types or to "complete" sequences. There has also been no attempt to provide a comprehensive list of alleles. The allele types might be useful in the future to provide some indication of how variability is partitioned within *Acanthamoeba*, but presently they are of limited use. We plan to further examine this aspect of intratype variation in a future report.

MULTIGENE ANALYSIS OF *ACANTHAMOEBA*

As the reader will be aware, the sequence of the *Rns* gene has been unusually informative in differentiating and categorizing isolates of *Acanthamoeba*. However, it is important to realize in the genomics era of 2014 that a classification based on more than a single gene is more appropriate. We have been endeavouring to produce multigene analyses when examining multiple isolates to understand the levels of genetic variation and degree of phylogenetic information in other gene sequences. These began with the sequences of the mitochondrial 16S-like small-subunit rRNA gene, designated *rns* (Ledee et al. 2003), and is proceeding to analyses of the mitochondrial cytochrome oxidase subunit I gene (COI) (Crary 2012), and in partial sequences of a set of five nuclear encoded enzyme loci (Crary 2012). For the remainder of the paper, we will emphasize the use and limitation of *Rns* data to provide insights into the identification of new sequence types and to the problem of heterogeneity within previously defined sequence types.

THE PHYLOGENETIC RELATIONSHIP BETWEEN SEQUENCE TYPES

From the time of the first sequence report by Gunderson and Sogin (1986) to the present has seen a remarkable increase in the number of sequence reports from workers around the world concerning the genetic diversity of *Acanthamoeba* that have been found in clinical, nonclinical and environmental settings. The breadth and growth of information on *Acanthamoeba* in the sequence databases has been reviewed elsewhere (Fuerst 2014). However, by the end of February 2014, data on nearly complete or partial *Rns* sequences had been reported for over 1,880 isolates. Such an extensive dataset allows many questions to be investigated. For identification of sequence types, the most phylogenetically informative subset of this data is represented by *Rns* sequences that are 2,000 nucleotides or longer. By February 2013, 335 sequences of greater than 2,000 bases had been collected.

Using these long sequences, we continue to re-examine the phylogenetic relationship within and among sequence types. In this paper, we will present details concerning some specific types. Given the large size of the dataset and the many questions that it represents, our intention is to present results in a series of papers in this journal and elsewhere. The phylogenetic relationships encoded within this set of sequences has been analysed using several different methods, including neighbour joining (Saitou and Nei 1987), Maximum Likelihood (developed following Felsenstein 1981) and Bayesian analysis (Huelsenbeck and Ronquist 2001). All of these methods resulted in essentially the same general insights into the relationship

among genotypes, although specific relationships between individual isolates were less likely to be the same when different methods were used. *Acanthamoeba Rns* sequences also bring unique challenges for phylogenetic analysis because of numerous insertion/deletion events that have occurred in the most rapidly evolving, and phylogenetic interesting portions of the molecule.

The general relationships between various sequence types within *Acanthamoeba* are shown in the phylogenetic tree in Fig. 1. This tree is the same as that presented in Fuerst (2014). The tree does not show relationships between all 335 sequences available, but rather includes only a representative small subset of isolates, in order to provide a general indication of the patterns of divergence.

Note that Genotype T15 (*A. jacobsi*) is not included on the tree, as no "complete" *Rns* sequence is available. T15 appears to be related to the branch in Fig. 1 that includes types T10, T12, and T14. In Fig. 1, T99 is an ambiguous clade based on "complete" *Rns* sequences from an environmental survey of soil microbiodiversity (Lesaulnier et al. 2008). These sequences show divergence levels from established types of *Acanthamoeba* equal or less than that observed between the Group I *Acanthamoeba* and Groups II and III. Although T99 sequences show reasonably low levels of divergence compared to those from acanthamoebae, they do not share the hypervariable expansion sequences of the *Acanthamoeba Rns*. To our knowledge, no organism carrying a T99 sequence is currently in culture that would clarify the nature of this group. Nevertheless, sequences that are T99-like have been reported in at least 7 environmental surveys (including our own work) that focused specifically on free-living amoebae from many different geographic localities.

Examination of Fig. 1 provides some insight into the relationship of sequence type with phylogenetic placement in the genus. At the top of the tree, type T4 is subdivided into a number of subgroups. It has been known since our first definition of sequence types (Gast et al. 1996) that type T4 is the most frequent type encountered either clinically or environmentally. It represents more than 70% of all *Acanthamoeba* isolates that have been analysed and deposited in the DNA databases (Fuerst 2014). Together with type T3 and T11, T4 appears to represent a derived set of sequences that correspond generally to the Group II morphological group of *Acanthamoeba*. We would hypothesize that evolution of Group II morphology occurred when the T4 and associated types diverged from other small *Acanthamoeba*. This hypothesis needs to be tested carefully, as much information on morphological type has become associated with species names, and species names appear to be very unreliable as an indicator of the position of an isolate within the evolutionary framework represented by the hypothetical tree shown in Fig. 1.

The taxa shown at the bottom of the tree, branching down from the root, represent those forms that comprise morphological Group I acanthamoebae. These are the largest members of *Acanthamoeba*, physically. They comprise types 7, 8, 9 in the analysis of Stothard et al. (1998), together with additional types T17 and T18 that have been recently identified (Nuprasert et al. 2010; Qvarnstrom et al. 2013). With respect to the proportion of isolates in the DNA databases, they represent about 2% of *Acanthamoeba* isolates. However, diversity of the forms in this group, their divergence from other members of the genus and the fact that some are potentially pathogenic, combined with the relative paucity of isolates, actually suggests that future work on them is likely to yield considerable insight into the overall biology of the acanthamoebidae.

In Fig. 1, the majority of recognized sequence types are found between the part of the tree that contains types T3-T4-T11, with Group II cyst morphology, and the point on the tree where the small cyst acanthamoebae and the large cyst Group I forms diverge (essentially above the root of the tree at the left in Fig. 1). As a generality, these other parts of the tree seem to represent many (or most) forms that have been classified as morphological Group III of the genus. If this is true, the evolution of cyst type would have been from an ancestral form leading to group III and then to the Group II forms found in T4. The taxa in the central part of the tree constitute numerous genetic types that have been proposed, including T1, T2, T5, T6, T10, T12 (each proposed by our group in Gast et al. 1996 or Stothard et al. 1998), T13 (Horn et al. 1999), T14 (Gast 2001), T15 (Hewett et al. 2003), and T16 (Corsaro and Venditti 2010).

Examination of the literature indicates, however, that proposal of sequence type is not without confusion. Horn et al. (1999) proposed to identify two types T13 and T14, whose sequences were ultimately found not to be sufficiently different, resulting in a redundant T14 claim. Similarly, multiple claims on the number T16 have been made, first by Lanocha et al. (2009) with respect to partial sequences and later by Corsaro and Venditti 2010, with respect to sequences from a completely different genotype. There have also been "claims" of new sequence types that appear as parts of isolate names associated with sequences in the DNA databases. Finally, there is the question of whether the 5% sequence divergence cut-off is a truly appropriate landmark.

To examine these problems with the definition of sequence types, we will use the remainder of the paper to examine three cases: The case of T13 and T16 (Corsaro and Venditti), an examination of claims of Lanocha et al. (2009) in concert with findings that we made in collaboration with Govinda Visvesvara that also raises issues about the use of partial sequences (Visvesvara et al. 2007), and finally, the question of the separation of our original T2 and T6 sequence types as data has accumulated in the DNA databases.

CASE STUDIES OF SEQUENCE TYPES:

Case 1: differences between T13 and T16 (Corsaro and Venditti)

The placement of the sister clades types T13 and T16 is shown in Fig. 1, where four of the five isolates for which

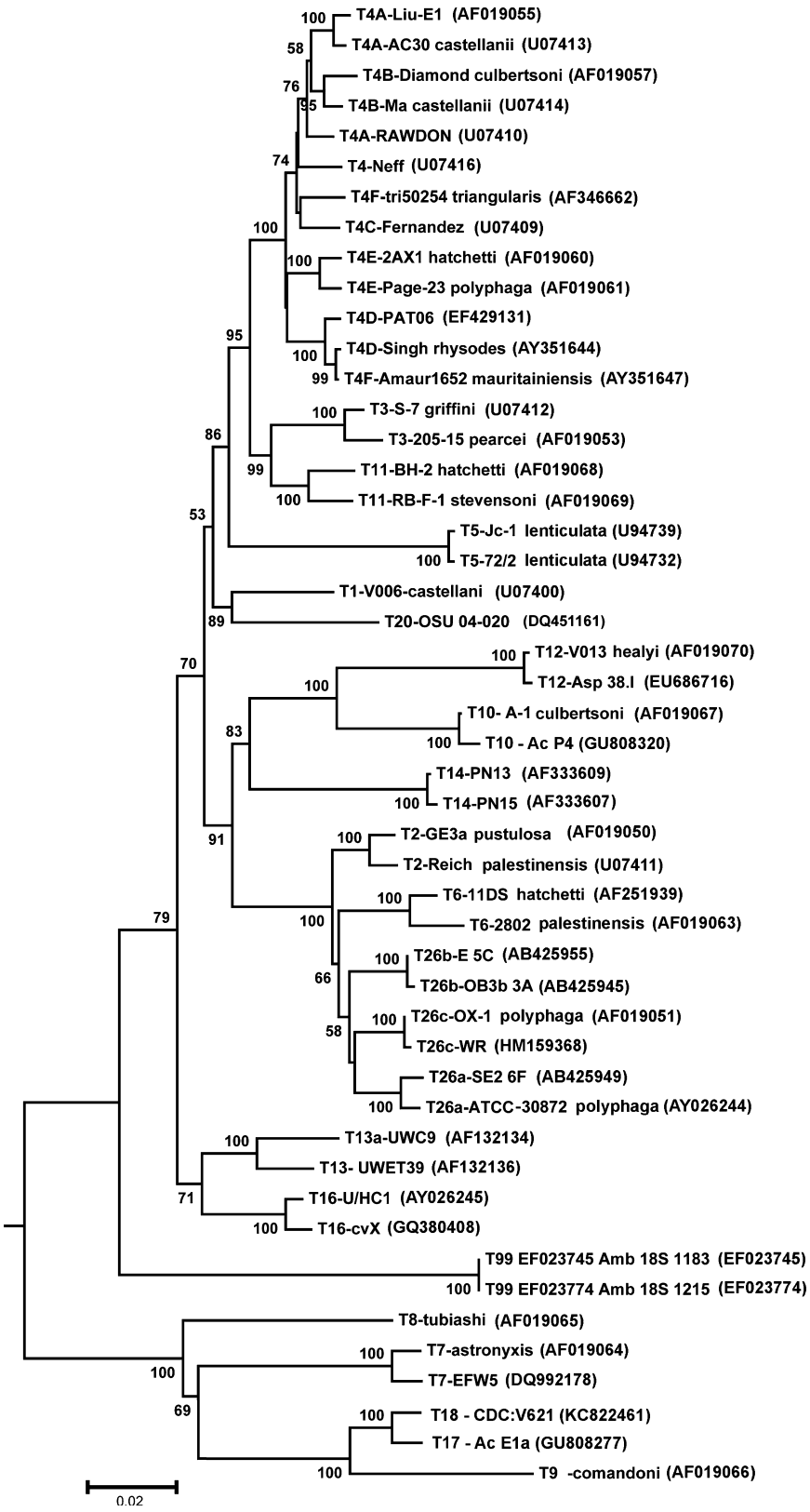

Figure 1 Phylogenetic relationships between representative isolates of various sequence types or subtypes of *Acanthamoeba* based on "complete" *Rns* sequences. The tree was constructed using Maximum likelihood in the program MEGA5 (Tamura et al. 2011).

"complete" sequences are available are shown. Genotype T13 was first described in a study of the bacterial endosymbionts of isolates of *Acanthamoeba* (Horn et al. 1999). Three isolates of *Acanthamoeba* were examined in their study. Two of these isolates, UWC9, and UWE39, had *Rns* sequences that differed significantly from the genotypes that had previously been reported by our lab (Stothard et al. 1998). In the initial report of Horn et al. (1999), a comparison indicated that the two isolates differed by 8–9% from other *Acanthamoeba* isolates and by almost 8% from each other as well (table 2 in Horn et al. 1999). Two new sequence types were proposed. However, subsequent analysis and correction of the reported sequences indicated that the level of divergence between the two isolates was only about 3.7%. Since the first isolation of the original T13 strains, a third isolate has been reported (Beier et al. 2002) based on a "complete" *Rns* sequence, while no more than 10 additional isolates appear to have been identified based on the ASA.S1 fragment. This makes T13 among the rarer types known.

Subsequent to the description of T13, two additional isolates were reported that had relatively close phylogenetic relationships with the T13 isolates (U/HC1, Alves et al. 2000; and cvX, Corsaro and Venditti 2010). Examination of the placement of T13 and these new isolates in Fig. 1 shows that they may represent one of the first divergences of a lineage from the small *Acanthamoeba* evolutionary line after the split from Group I *Acanthamoeba*. The new isolates were sufficiently diverse to warrant the proposal that they represented a new sequence type, the T16 type. Careful analysis indicates that these two isolates are between 5% and 6% divergent from the T13 sequences. Five additional isolates have been reported that would also be placed in T16 by a partial *Rns* sequence, indicating that it is even less frequent than T13. Pairwise differences among T13 and T16 sequences are listed in Table 1. It should be noted; however, that examination of the five "complete" T13 and T16 *Rns* sequences suggests that there may be sequencing errors in several of the entries that might push sequence differences beyond 5%. While accepting the likelihood that these rare forms represent defined sequence types, further identification and study is clearly warranted.

No species names have yet to be proposed for any of the isolates that belong to either group T13 or T16; the two types appear to correspond to two separate species. Their association with unusual bacterial endosymbionts (Beier et al. 2002; Horn et al. 1999) makes them important parts of our knowledge about *Acanthamoeba*. The classification of types T13 and T16 according to cyst morphological groups is generally lacking. Corsaro and Venditti indicate that examination of the cysts of isolate cvX would suggest that it falls into Group II. We hypothesize, however, that given their very early divergence from other small *Acanthamoeba*, they will ultimately be shown to be morphologically Group III.

Table 1. Percentage sequence divergence between "complete" *Rns* sequences for *Acanthamoeba* isolates that are classified into the T13 or T16 sequence types

	Isolate acc #	1	2	3	4
1. T13	UWET39 AF132136	–			
2. T13	UWC9 AF132134	0.0368	–		
3. T13	TUMK-23 AY102615	0.0221	0.0400	–	
4. T16	U/HC1 AY026245	0.0617	0.0603	0.0670	–
5. T16	cvX GQ380408	0.0669	0.0656	0.0731	0.0080

Case 2: the heterogeneity of types T2 and T6

It has been evident for some time that the differentiation between *Acanthamoeba* genotypes T2 and T6 is the smallest between any of the original 12 recognized types (Corsaro and Venditti 2010; Fuerst and Booton 2007; Stothard et al. 1998). In the DNA databases and related materials, 25 "complete" *Rns* sequences that fall into the T2/T6 joint clade have been reported. This allows an analysis within and between close genotypes that is only exceeded by analysis of T4 sequences. A close examination of isolates that are potential members of either Type T2 or T6 shows that, within the entire class of isolates, pairwise differences range from a single nucleotide difference up to 5.6% divergence.

Our estimation of the placement of the T2/T6 joint clade is shown in Fig. 1. This placement differs from that suggested by Corsaro and Venditti (2010) and is undoubtedly the result of the difficulty of determining alignment and assigning homology. At the present time we attempt as far as possible to assign homology by considering secondary structure, but a thorough analysis will always be problematic given the high rate of change in the hypervariable regions of the *Acanthamoeba Rns*. However, such problems are less important when closely related sequences are being compared. In this case we have performed a specific phylogenetic analysis to determine the relationships among the 25 T2/T6 isolates. Other sequences are used only to provide the placement of the root with the joint clade. The analysis shows the presence of five phylogenetically significant subclades within the T2/T6 family of sequences (Fig. 2). The figure represents the maximum-likelihood tree computed using MEGA5 (Tamura et al. 2011), and using the best-fit model for the data (the T92 + G+I model: Tamura 3-parameter model with rates among sites following a gamma distribution including a class for invariant sites). The five subclades are identified as T2 and T6, represented by *Acanthamoeba palestinensis* Reich (ATCC 30870) and *A. palestinensis* 2802 (ATCC 50708), respectively, and three intermediate clades designated T2/6A (represented by *Acanthamoeba polyphaga* CCAP 1501/3b; ATCC 30872), T2/6B (represented by isolate OB3b_3A, as no culture collection isolate is known for this subclade), and T2/6C (represented by *A. palestinensis* OX-1, CCAP 1501/3c, formerly designated *A. polyphaga* OX-1). The average pairwise sequence difference within or

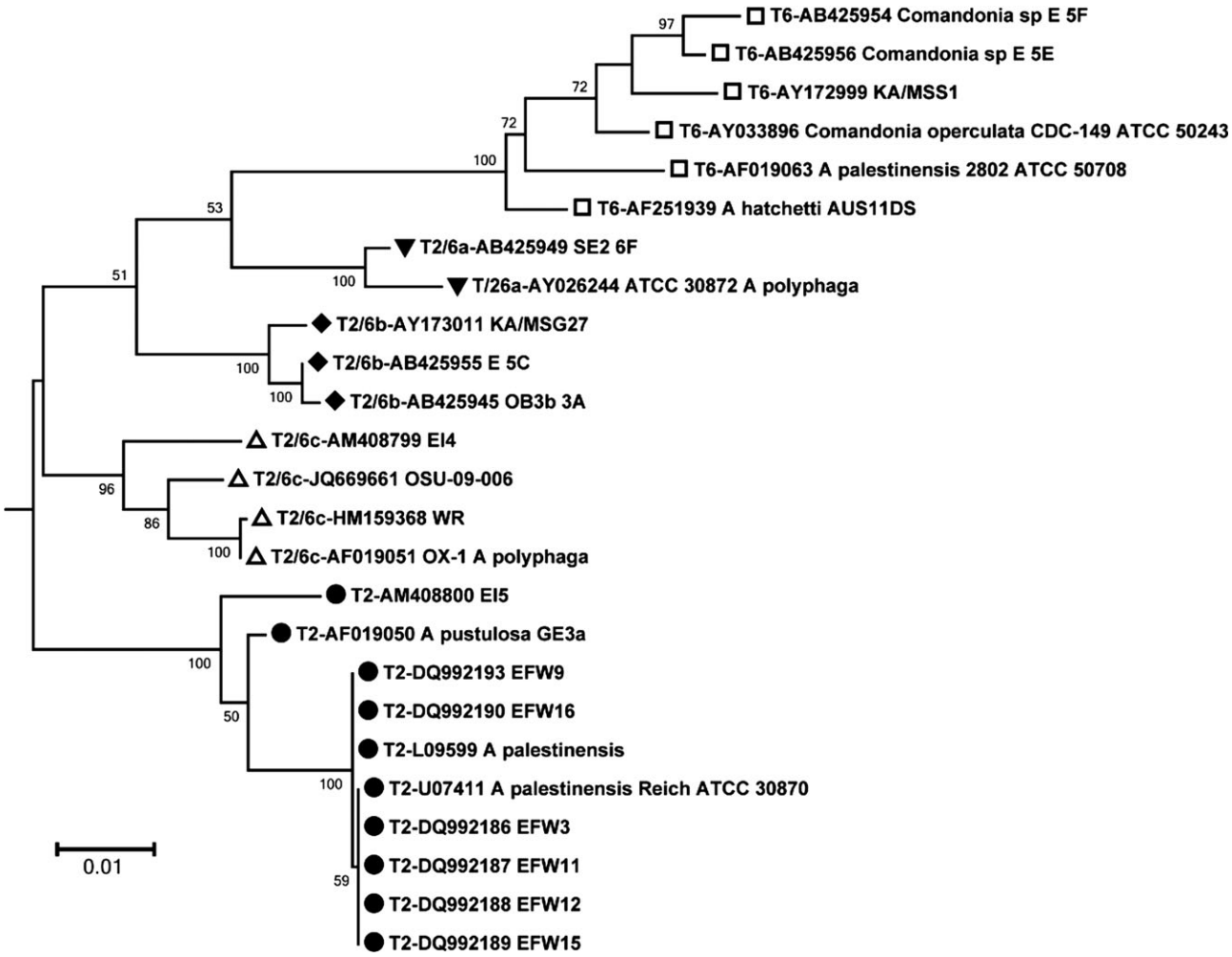

Figure 2 Phylogenetic relationships between isolates of *Acanthamoeba* classified into the sequence type T2/T6 joint clade based on "complete" *Rns* sequences. The tree was constructed using Maximum likelihood in the program MEGA5 (Tamura et al. 2011). ● members of type T2; ▼ members of type T2/T6A; ◆ members of T2/T6B; △ members of type T2/T6C; □ members of type T6.

Table 2a. Average per cent sequence divergence between "complete" *Rns* sequences for *Acanthamoeba* isolates that have been classified into sequence types T2 or T6 or defined subtypes T2/6A, T2/6B, and T2/6C

Complete	T2	T2/6A	T2/6B	T2/6C	T6
T2	0.0135				
T2/6A	0.0531	0.0096			
T2/6B	0.0464	0.0402	0.0044		
T2/6C	0.0409	0.0395	0.0367	0.0137	
T6	0.0540	0.0483	0.0536	0.0474	0.0196

Table 2b. Average per cent sequence divergence between sequences of the ASA.S1 fragment within the *Rns* for the same isolates of *Acanthamoeba*

ASA.S1	T2	T2/6A	T2/6B	T2/6C	T6
T2	0.0010				
T2/6A	0.0778	0.0050			
T2/6B	0.0613	0.0672	0.0114		
T2/6C	0.0604	0.0478	0.0635	0.0202	
T6	0.0657	0.0550	0.0700	0.0514	0.0271

between each of the five subclades for the "complete" *Rns* sequence is shown in Table 2a.

Comparison of Table 2a and Fig. 2 shows how genetic divergence is spread across this category of isolates. Genotype T6 is the most divergent of the subclades, and is most evolved from the basal split at the root of the grouping. This basal split involved a separation of T2 from the remainder of the T2/T6 joint clade. The other subclades are spaced between T2 and T6 and show intermediate differentiation from each other and from the other members of the greater T2/T6 clade. Pairwise

differences in the *Rns* sequences within each subclade, as shown in Table 2a,b, are generally less than 2%. Isolate EI5 within T2 shows the greatest difference from other members of its own subclade, with some values approaching 2.9%. The overall picture is one of a shading of differences from the two extreme subclades, T2 and T6, as the entire category evolves from their common ancestral type.

In addition to the 25 "complete" sequences that have been assigned to the T2/T6 category, there have been almost 100 further isolates for which a partial *Rns* sequence was obtained and the isolate is then assigned to the T2/T6 grouping on the basis of similarity within the partial comparison to this clade. Close examination of these sequences suggests that very few of these partial sequences clearly belong to one of the three intermediate subclades (only eight partial sequences are assigned to T2/6A, 2 to T2/6B and 5 to T2/6C). The low number of isolates assigned to the intermediate subclades may indicate that these subclades represent isolates that are in the process of transient diversifications from the basic dichotomy between types T2 and T6. As such, they may provide an insight into how these protist forms evolve. Among the remaining partial sequences, T6 type isolates outnumber T2 type isolates about 2:1. There is also evidence to suggest that variation within type T6 is greater than within the other subclades, also suggesting diversification from the standard types. Table 2a,b shows greater average differences between the T6 isolates. In addition, examination of partial *Rns* sequences assigned to T6 shows that many share oligonucleotide patterns that are not observed in the 6 "complete" T6 sequences that are currently available. From an evolutionary viewpoint, types may simply represent intermediate starting points for further divergence.

Some caution should be applied when partial sequences are analysed. In general, the concordance between the placements within a clade based on a "complete" Rns sequence and based on a partial sequence is good. However, percentage of sequence similarity can be different when partial and "complete" sequences are compared. Table 2b shows the sequence similarities for the 25 "complete" sequences when only the ~400 bases corresponding to the sequence of the ASA.S1 fragment are considered. In general, there is an increase of sequence divergence between subclades within ASA.S1. However, some comparisons can show reduced divergence. More analysis in other sequence types, especially within the more complex T4 group, will be necessary to determine whether any rules exist correlating partial and complete sequence divergences for the *Rns* of *Acanthamoeba*.

In our opinion, this T2/T6 clade category exemplifies the evolutionary dynamics of *Acanthamoeba*, given the possibility that genetic exchange between different isolates may be extremely limited. The genus is characterized by a large number of almost independent evolutionary units accumulating genetic differences as they evolve from a common ancestor. It would be appropriate to consider the T2/T6 clade as representing five species. However, at present, the species names "*palestinensis*" and "*polyphaga*" have been applied to members that occur in multiple subclades. The name *hatchetii* is also found in this grouping of isolates. All three of these species names, however, have also been applied to isolates that occur in other sequence types, suggesting that all these names must be considered unreliable. The only species name that appears to be restricted to the T2/T6 clade is *pustulosa*, a name which has, however, been viewed as a synonym to *palestinensis.* It is also of note that several isolates assigned to type T6 have been assigned morphologically as belonging to the genus *Comandonia*. This generic label seems to be inappropriate given the genetic information, but may indicate the difficulties of morphological typing within both *Acanthamoeba* and other amoebozoan species (Kudryavtsev et al. 2009).

What are the species that make up the T2/T6 joint clade? In examining the question of species names and associated sequence types, several options can be considered. Sequence type T2 was originally defined by the sequence of the Reich isolate *of A. palestinensis* (ATCC 30870) (Gast et al. 1996). Subsequently, Stothard et al. (1998) identified two additional isolates as belonging to T2. This muddied the species association of T2. The two isolates were the GE3a strain (ATCC:50252) of *A. pustulosa*, and the OX-1 strain (CCAP 1503/3a) of *A. polyphaga*. The latter may not be as relevant now, as it appears that the OX-1 strain is more appropriately placed in subclade T2/6C. As the original definition of T2 was with the Reich isolate *of A. palestinensis*, this strain should be considered as the type strain of a T2 species. Given the issue of precedence, since *palestinensis* predates *pustulosa*, the former would be favoured. Further, since the Reich isolate *A. palestinensis* was described earliest (Reich 1933) of any isolate given that species nominal, the name *A. palestinensis* should be restricted only to the members of the T2 genotype.

Restriction of the name *A. palestinensis* to T2 does present a problem, because the species name *A. palestinensis* was also associated with the original T6 classification of Stothard et al. (1998). *Acanthamoeba palestinensis* strain 2802 (ATCC 50708) was the original T6 representative (Stothard et al., 1998), making that isolate the type strain for T6. However, by precedence, a new species name for members of T6 is required. Other "complete" sequences that have been determined for isolates in the T6 clade have been labelled as *Acanthamoeba hatchetti* (isolate 11DS, accession #AF251939), a name usually associated with type T3, and *Comandonia operculata* (accession # AY033896), a debatable name associated with an alternative amoebic group. Neither species name seems appropriate without much additional consideration.

Note that there is an additional problematic aspect that should be noted about the sequence for isolate *A. palestinensis* 2802. In the ATCC description of the strain, it is described as having been isolated from a swimming

pool, in Strasbourg, France. The strain is also listed by ATCC as being synonymous with the strain CCAP 1501/3c. CCAP 1501/3c no longer appears in the catalogue of the CCAP (http://www.ccap.ac.uk/). Examination of previous hard copy material of the CCAP catalogue from 2001 indicates that CCAP 1501/3c is an strain isolated by Sawyer in 1967 from an old distilled water carboy in the U.S. and originally designated as the OX-1 strain of *A. palestinensis*. This is the strain (OX-1) that was analysed by Stothard et al. (1998) (Genbank acc #AF019051). If strain OX-1 still exists within the culture collections, or if it can be reseeded from a sample in culture, it would represent the type strain of the defined group T2/6C, which is likely to represent a new species distinct from either "species" T2 (*A. palestinensis*) or "species" T6 (no name yet assigned).

Assignment of species names with the other three subclades is also unsettled, as these subclades appear only to be associated with the name "*A. polyphaga*", which would not be an appropriate nominal as the name is likely to have precedence in other genotypes. New species names for four of the five genotype subclade in the T2/T6 assemblage are thus needed.

With respect to the specific cyst/trophozoite group designation of the taxa in the T2/T6 clade, it has long been recognized that several isolates nominally classified as *A. palestinensis* belong to group III. It is assumed that careful analysis of members of the subclades would provide a similar classification, but that remains to be determined. Some reports indicate that cyst morphology may not be group III-like for all of the isolates assigned to the T2/T6 joint clade (Walochnik et al. 2000). This further emphasizes the problems with morphological classification of species. If cyst/troph morphology is closely tied to evolutionary history, we expect that there would be homogeneity in the classification of this clade with respect to group III. Alternatively, the taxonomic hypothesis represented by the tree in Fig. 1 provides a basis to test whether cyst morphology has changed multiple times with *Acanthamoeba*. Either way, it provides further information that can be used to plan studies concerning the genetic control of encystment/excystment.

Case 3: Determination of a new sequence type, T20

As part of the ongoing collaboration between Govinda Visvesvara and the *Acanthamoeba* group at The Ohio State University, a paper was published detailing a case of a lethal *Acanthamoeba* infection in a keel-billed toucan (*Ramphastos sulfuratus*) (Visvesvara et al. 2007). The analysis included determination of the sequence of an *Acanthamoeba* (CDC:V459) that was present in several tissue samples. We obtained four independent sequences for most of the ASA.S1 segment of the *Rns* gene and determined at the time (late 2005) that the sequences appeared to be a T4-like sequence. We did find small differences between the sequences from samples from different tissues, but felt that they were insufficient to require further study. However, the sequences, while similar to each other, did not match any of the T4 sequences that were present in the database. As our analyses of other sequences from our own work and sequences from the database continued to advance, these "generic T4" sequences were always troubling, as they seemed not to be greatly divergent from other T4 sequences, while never matching them. Was our analysis subject to sequencing error, or was something further at play?

In 2009, several sequences were submitted to the DNA databases that began to make the situation more interesting. These were associated with a paper (Lanocha et al. 2009) reporting the results of environmental sampling for amoebae in western Poland. Their analysis produced a fragment of the *Rns* that was about 300 bases longer than but overlapped our sequences. They suggested that the sequences came from an organism that was related but not identical to sequence type T1, represented by *A. castellanii* CDC:0981:V006 (ATCC 50494). They subsequently designated it T16. This is a problem for two reasons. First, designation of a new sequence type from only a part of the *Rns* is questionable, as the criterion for designating types depends on pairwise comparisons of long, nearly complete sequences. Secondly, their claim of a new type was ignored by Corsaro and Venditti (2010) when the latter proposed that T16 be used to designate the sister group to T13.

By 2009, our lab had already begun to re-examine *Acanthamoeba* CDC:V459. It quickly became clear as we obtained additional sequence that the isolate was not in fact a divergent member of T4. We have subsequently obtained the nearly complete sequence of two *Rns* forms from CDC:V459 that correspond to two sequences reported in our original analysis. These are two very closely related "allelic" forms of the *Rns* that share most type features, but have some differences from each other. This is not an unprecedented observation (Booton et al. 2003; Stothard et al. 1998). When compared in a pairwise analysis, the two alleles show 1.5% difference in sequence. When compared with three "complete" sequences from the T1 genotype (the *A. castellanii* CDC:0981:V006 and two alleles from an isolate that we have studied in our lab) the alleles from CDC:V459 show an average of 6.7% difference from the T1 sequences. The phylogenetic position of the sequences from CDC:V459 is shown in Fig. 1, where it clusters loosely with the T1 sequence type.

Since our original report and including the report of Lanocha et al. (2009); partial sequences have been reported for 15 independent isolates, showing a worldwide distribution of the new type. Isolates have been mostly from environmental surveys, but include samples from contact lens cases and nasal mucosa. It is important to note that the placement of this new type as a sister clade to type T1 and outside of T4 is also supported by sequence analysis of the cytochrome oxidase subunit I gene (COI) of the mitochondrial DNA and by four nuclear encoded protein sequences that we have

been studying to move the study of *Acanthamoeba* towards multilocus sequence typing (Crary 2012) Given the "complete" *Rns* sequences from the isolate CDC:V459 OSU-04-020, the evidence supports the proposal of a new sequence type, T20, represented by these isolates, with the CDC:V459 OSU-04-020 as the type isolate (18S rRNA gene accession # DQ451161). Further, cytological evidence indicates that it represents a Group III form (Visvesvara et al. 2007).

THE ISSUE OF SINGLE GENE CATEGORIZATION OF *ACANTHAMOEBA* SEQUENCE TYPES

The story of the identification of the new sequence type T20 presents a cautionary tale about the use of a partial sequence from a single gene to classify isolates of *Acanthamoeba*. We believe strongly that the increasingly large database of *Acanthamoeba Rns* sequences (Fuerst 2014) provides a powerful tool for the identification and classification of isolates. This will be the foundation of much additional research on the biology of *Acanthamoeba*. However, the nature of the data that makes up the DNA database results in the fact that most isolate identification has been effected via a single partial *Rns* sequence. Fuerst (2014) showed that only about 18% of isolates have been characterized using "complete" *Rns* sequences. Often the typing of an isolate is made by analyzing sequences in the DNA database using BLAST (Altschul et al. 1990), and then applying the sequence type identification that has been given to the sequence of closest match. Partial sequences often yield matches that may differ by only one or two nucleotides. Are these differences real? Is the designation of sequence type on the basis of a partial sequence accurate? Polymorphism within a type certainly is present. There are clearly multiple, very closely related sequences that represent different alleles that can exist either within an isolate, or occur when isolates of the same sequence type are compared. In one of our original papers on sequence types (Stothard et al. 1998), we observed multiple sequences in 7 of 53 isolates that we categorized to type based on "complete" *Rns* sequences. The fact that we originally classified the CDC:V459 OSU-04-020 as a T4 isolate indicates the caution that must be applied. It is important that our data be as clear and accurate as possible.

USING PHYLOGENY TO TEST EVOLUTIONARY QUESTIONS

The chronological order of evolutionary divergence, as pictured in Fig. 1, provides a hypothesis with which to test numerous questions about *Acanthamoeba*. For instance, it provides a basis to test questions about changes in cyst morphology. Has cyst form changed multiple times within *Acanthamoeba*, or just twice related to morphological groups. Either way, the tree provides information that can be used to plan studies concerning the genetic control of encystment/excystment. Alternatively, searches for pathogenic mechanisms of *Acanthamoeba* can be furthered by examining forms from different parts of the tree that seem to have different propensity to cause keratitis or GAE (Booton et al. 2005). But to be most useful, the tree must also be as accurate as possible.

The existence of the large number of almost complete sequences within the *Acanthamoeba Rns* database provides an additional tool to separate true polymorphisms from sequencing errors. Sequences can be compared to the large number of aligned sequences to ascertain the accuracy of any differences. Unusual sequence differences should be compared by examining the electropherogram that is produced from any DNA sequencing run. When the same rare sequence change occurs in several different independent isolates, especially when reported by multiple investigators, it suggests the presence of a true new variant, or even a new sequence type. For example, in the analysis of *Acanthamoeba Rns* sequences from the DNA databases, we have noticed a number of partial sequences that have been reported several times, that also do not fit neatly into the described sequence types, and for which a "complete" *Rns* sequence is not currently available. Do these represent additional types (which undoubtedly will continue to be discovered), or are they sequencing artefacts which could have multiple causes? Only more extensive analysis can resolve such questions. However, our emphasis on the information from a single gene (the nuclear *Rns* gene) hampers our ability to resolve these issues.

We strongly advocate analyses that make use of multiple gene sequences to better compare forms, especially as we attempt to resolve the species name conundrum. A start has been made by the introduction of sequences from the *Acanthamoeba* mitochondrial genome. These include the mitochondrial small-subunit rRNA gene (rns) (Ledee et al. 2003) and the CO-I gene (Crary 2012), the nuclear ribosomal ITS sequence (Kohsler et al. 2006) and several other nuclear protein coding genes (Crary 2012). Various analyses based on a limited number of isolates using each of these products indicates significant correspondence in the information from each gene, but also suggests that different genes do not give exactly the same placement for every isolate. It should be noted that some level of discordance is expected because of the presence of various evolutionary forces, such as mutation rate and natural selection, which could be acting differently on different genes in different evolutionary lineages. Further information concerning the phylogenetic analysis of *Acanthamoeba*, the status of sequences types, and a summary of the state of *Acanthamoeba* sequences in the international DNA databases can be found at http://u.osu.edu/acanthamoeba/.

Much remains to investigate. We have much to be grateful to Govinda Visvesvara for in providing us an invitation to take part in this journey with him. Thanks Vish.

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[1]This paper has been prepared in part to honour our friend Govinda Visvesvara. Interactions between Vish and the *Acanthamoeba* researchers at The Ohio State University extend back over almost to the start of Vish's career, spanning almost 40 yr. This means that OSU and Vish have been intertwined since before any of the current three authors knew Vish. These interactions predate the first International Meeting on Free-Living Amoeba, which took place in Columbus, Ohio in 1978 through the efforts of Tom Byers and others. Subsequently, Tom sought out a newly arrived (1980) OSU colleague (P.A.F.), a population geneticist with no knowledge of *Acanthamoeba*, to collaborate on studies concerning RFLP variation between isolates. That collaboration evolved and lasted until Tom's untimely death. Greg Booton was a Ph.D. student with Paul Fuerst, who became interested in *Acanthamoeba* while a post-doc with Tom Byers, and before joining the OSU faculty. Both Paul and Greg came to know Vish through Tom, and together with Tom formed part of an OSU team that continued to collaborate often and very productively with Vish. Monica Crary was the latest Ph.D. student in the Fuerst Lab. We have all been influenced by Vish. Vish's paper that appeared as part of the International Symposium on *Acanthamoeba* and the Eye (Visvesvara 1991) was an important motivation for much of our thinking about rapid diagnosis and defining species in *Acanthamoeba*. When we collected the original DNA data that resulted in our first proposal of sequence types, about one-third of the isolates we studied were provided by Vish from his collection of isolates at the CDC labs. Overall, almost 25% of the more than 300 isolates that we have analysed in our lab have originated either directly or indirectly in some way from Vish's work. Our ability to define the typing system would have been greatly reduced without his generous support. Investigations on the difference between isolates obtained from pathogenic and nonpathogenic conditions, and our studies of the ability to recover isolates after many years in storage represent some of the highlights of many collaborative papers with Vish and his CDC colleagues. Vish's fingerprints are all over our work, and we thank him greatly for his friendship and patience.