Acta Protozool. (2003) 42: 325 - 329

ACTA
PROTOZOOLOGICA

Identification of a New *Acanthamoeba* 18S rRNA Gene Sequence Type, Corresponding to the Species *Acanthamoeba jacobsi* Sawyer, Nerad and Visvesvara, 1992 (Lobosea: Acanthamoebidae)

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Summary. Since 1996, a new classification system has been used to assign members of the genus *Acanthamoeba* to different sequence types. This study aimed to place the thermophilic species *Acanthamoeba jacobsi* into the sequence type classification by sequencing part of the 18S rRNA gene for six isolates of this species. Phylogenetic analysis of the sequences showed the six strains to be closely related and confirmed their identification as *A. jacobsi*. This species is well separated from all previously typed *Acanthamoeba* strains, and is allocated to a new sequence type, T15.

Key words: 18S rRNA gene, *Acanthamoeba jacobsi*, sequence type.

INTRODUCTION

The protozoan genus *Acanthamoeba* contains free-living organisms, some of which are clinically significant. Many strains cause the corneal infection *Acanthamoeba* keratitis, while some strains can also cause granulomatous amoebic encephalitis (GAE), a potentially fatal brain infection. Traditionally, identification beyond the genus level of *Acanthamoeba* was based on morphological characteristics. The cyst size and shape, as well as the growth temperature range, allow the Acanthamoebae to be distinguished into three subgeneric groups (Pussard and Pons 1977), encompassing more than 20 nominal species. However, characterisation at the species level has been inconsistent in the past, with several 'type strains' not genetically distinct from earlier-named species and many clinical and environmental isolates now judged to be incorrectly assigned (Stothard *et al.* 1998). A very confused binomial classification has resulted.

More recently, a subgeneric identification system has been described which involves sequence analysis of the 18S rRNA gene to segregate the *Acanthamoeba* strains into sequence types. To date, fourteen separate sequence types have been reported (Gast *et al.* 1996,

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Stothard *et al.* 1998, Horn *et al.* 1999, Gast 2001). The largest and most diverse sequence type, T4, contains many of the strains that are infectious to humans, causing keratitis and occasionally GAE.

In this study, 18S rRNA gene sequencing was employed to determine the affinities of six *Acanthamoeba* strains from diverse geographic origins, identified as *Acanthamoeba jacobsi* by isozyme analysis and physiological characteristics.

MATERIALS AND METHODS

Cultures. The *Acanthamoeba* strains that were analysed in this study were the type strain of *A. jacobsi*, ATCC 30732 (AC005 in the culture collection of the Australian Water Quality Centre (AWQC)), isolated from a marine source in the United States of America; and five environmental isolates from the AWQC collection. Strains AC080 (from an Australian water supply), AC194 (fresh water sediment, England) and AC227 (fresh water, Fiji) were selected from an isozyme study (Flint *et al.* 2003) to represent the wide geographic distribution of *A. jacobsi*. AC304 and AC305, which originated from an untreated water system in Australia, were included as they are natural hosts of bacterial endosymbionts (Todd *et al.* 2001) and their responses to temperature suggested that they may also belong to *A. jacobsi*. The cultures were grown at 35°C in 25 cm^2 and 75 cm^2 culture flasks containing 1/4 strength Ringer solution (Oxoid, Hampshire, England) seeded with approximately 10^9 cells/ml *Escherichia coli* (ATCC 11775).

DNA isolation, PCR and sequencing. Amoeba cells were harvested from culture flasks, concentrated by centrifugation, then lysed by treatment with lysozyme and freeze-thawing (using liquid nitrogen and heating to 65°C). The samples were then treated with proteinase K, SDS and RNAse. The DNA was purified using the QIAamp DNA mini kit (Qiagen) according to the manufacturers instructions.

PCR primers CRN5 and 1137 as described by Schroeder *et al.* (2001) were used for amplification of 1475 bp of the 18S rRNA gene. PCR amplifications were performed in 50 µl volumes, containing 20 pmol of each of the primers, 2.5 mM $MgCl_2$, Buffer II, 200 µM deoxynucleoside triphosphates (Promega), 2.5 units of Ampli *Taq* Gold (Applied Biosystems), 5% DMSO and 1-10 ng of template DNA. PCR was carried out using the Perkin Elmer GeneAmp 2400 PCR system. Thermal cycling conditions for the PCR were: 94°C for 10 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; followed by a final extension at 72°C for 7 min. PCR products were purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturers instructions.

Oligonucleotide primers used for sequencing were: CRN5, 1137, 373, 570C, 892C and 892 (Schroeder *et al.* 2001). DNA was sequenced using the BigDye v2.0 Terminator mix and an automated model 3700 capillary sequencer (Applied Biosystems). Sequences were analysed using SeqMan software (DNASTAR), and homology searches were performed using the BLAST network service on the database of the National Centre for Biotechnology Information.

Phylogenetic analysis. The six *Acanthamoeba* sequences from this study were aligned with 17 published sequences representing all previously characterised *Acanthamoeba* sequence types, and including three representatives of sequence type T4, the most diverse sequence type. Table 1 details the GenBank accession numbers of the *Acanthamoeba* sequences that were analysed, as well as strain details. Sequence alignments were performed using Clustal X version 1.64b software (Thompson *et al.* 1997). Phylogenetic relationships were inferred by neighbour-joining analysis of Kimura two-parameter distance estimates using Mega version 2b3 software (Kumar *et al.* 2000). The data were also analysed by maximum parsimony analysis (heuristic search) using Paup* version 4.0b10 software (Swofford 1998). The robustness of the distance-based and parsimony trees was determined by bootstrap analysis (1,000 replicates).

GenBank accession numbers. The 18S rRNA gene sequences of the six *A. jacobsi* isolates were submitted to the GenBank database with the following accession numbers: AY262360 (AC005); AY262361 (AC080); AY262362 (AC194); AY262363 (AC227); AY262364 (AC304) and AY262365 (AC305).

RESULTS

A portion of the 18S rRNA gene (between 1443 bp and 1475 bp in length) was amplified and sequenced for all six of the *Acanthamoeba* strains studied. This region of the 18S rRNA gene was studied as it has been shown to contain eight segments of variable sequence for members of the genus *Acanthamoeba* (Schroeder *et al.* 2001). Previous studies have differentiated *Acanthamoeba* strains into different sequence types by sequencing the complete 18S rRNA gene, which is between 2,300 and 2,700 bp (Stothard *et al.* 1998). However, the smaller fragment analysed in the current study contains enough information to differentiate all of the sequence types (Schroeder *et al.* 2001).

The neighbour-joining and maximum parsimony analyses (neighbour-joining tree illustrated in Fig. 1) showed that the six *Acanthamoeba* strains AC005, AC080, AC194, AC227, AC304 and AC305 were all closely related to one another, sharing greater than 99.4% sequence similarity (calculation based on the p-distance, Mega version 2b3). The distances between the sequences were very small, much less than the distances between the three representative sequences of the sequence type T4. Therefore, all six of these strains are considered to belong to the same sequence type. As a group, the six *Acanthamoeba* strains were well separated from members of all other sequence types (Fig. 1). The bootstrap support for the *A. jacobsi* group is highly significant, as it shows 100% support with both neighbour-joining and maximum parsimony analysis. The six *A. jacobsi* strains share at most 92.1% sequence similarity with members of the most closely related sequence type, T13, and so the *A. jacobsi* strains can be consid-

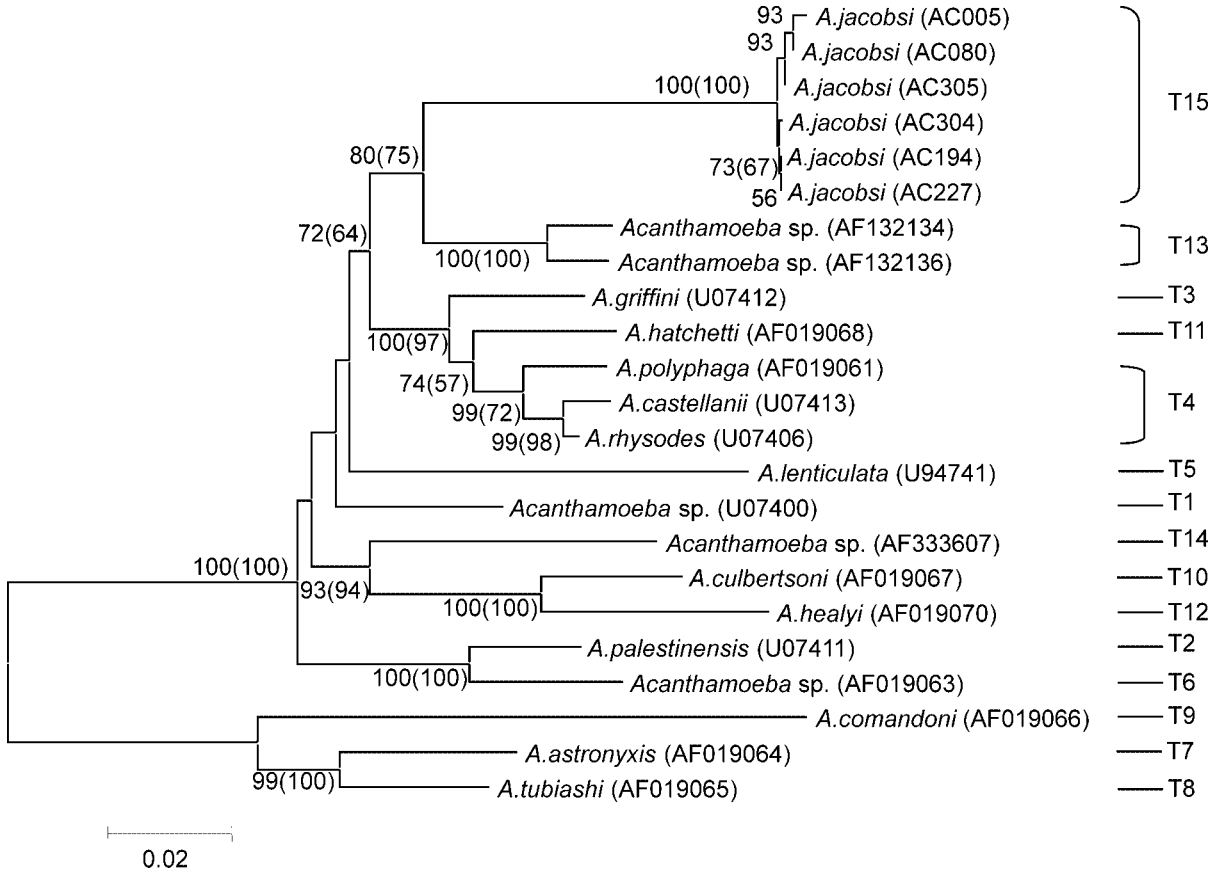

Fig. 1. Neighbour-joining tree showing the position of six *A. jacobsi* isolates (AC005, AC080, AC194, AC227, AC304, AC305) in relation to representatives of the other *Acanthamoeba* sequence types, based on their 18S rRNA gene sequences. Numbers on branch points depict the percent occurrence of a given branch during 1,000 bootstrap replicates. Numbers in brackets are bootstrap values based on maximum parsimony analysis, with 1,000 replicates. Values below 50 are not shown.

ered a new sequence type. We therefore propose the new sequence type T15, which corresponds to the species *A. jacobsi*.

DISCUSSION

The assignment of *Acanthamoeba* species to a series of 18S rRNA gene sequence types is gaining wide acceptance as a genetically-sound classification system and corresponds partially with the existing binomial taxonomy (Stothard *et al.* 1998). However, it does not yet encompass all previously described species. In particular, *A. jacobsi* is a widely distributed thermophilic species for which sequence data have not previously been published.

In this study, comparison of a substantial part of the 18S rRNA gene confirmed that the *Acanthamoeba* strains AC080, AC167, AC227, AC304 and AC305, are closely related to *A. jacobsi* AC005, consistent with their isozyme profiles, growth temperature range and cyst morphology (Flint *et al.* 2003). Furthermore, the *A. jacobsi* strains are distinctly separated from members of the existing sequence types, with high bootstrap support (Fig. 1). The degree of sequence uniformity among the six strains and their divergence from other Acanthamoebae represent strong agreement with the isozyme analysis (Flint *et al.* 2003). We conclude therefore that *A. jacobsi* is a sound genetic entity and a new sequence type.

Previous analysis of the 18S rRNA gene of *Acanthamoeba* strains by Gast *et al*. (1996) and Stothard *et al*. (1998) identified twelve discrete sequence types, T1-T12. Horn *et al*. (1999) described two additional sequence types, T13 and T14, corresponding to the strains *Acanthamoeba* sp. UWC9 and *Acanthamoeba*

Table 1. *Acanthamoeba* strains analysed in this study

Sequence type	Species classification	Strain identification	GenBank accession number	Reference (GenBank sequence)
T1	*Acanthamoeba* sp.	CDC:0981:V006	U07400	Gast *et al.* 1996
T2	*A. palestinensis*	ATCC:30870	U07411	Gast *et al.* 1996
T3	*A. griffini*	ATCC:30731	U07412	Gast *et al.* 1996
T4	*A. castellanii*	ATCC:50374	U07413	Gast *et al.* 1996
T4	*A. polyphaga*	ATCC:30871	AF019061	Stothard *et al.* 1998
T4	*A. rhysodes*	ATCC:50368	U07406	Gast *et al.* 1996
T5	*A. lenticulata*	ATCC:30841	U94741	Stothard *et al.* 1998
T6	*Acanthamoeba* sp.	ATCC:50708	AF019063	Stothard *et al.* 1998
T7	*A. astronyxis*	ATCC:30137	AF019064	Stothard *et al.* 1998
T8	*A. tubiashi*	ATCC:30867	AF019065	Stothard *et al.* 1998
T9	*A. comandoni*	ATCC:30135	AF019066	Stothard *et al.* 1998
T10	*A. culbertsoni*	ATCC:30171	AF019067	Stothard *et al.* 1998
T11	*A. hatchetti*	Sawyer:NMFS	AF019068	Stothard *et al.* 1998
T12	*A. healyi*	CDC1283:V013	AF019070	Stothard *et al.* 1998
T13	*Acanthamoeba* sp.	Horn:UWC9	AF132134	Horn *et al.* 1999
T13	*Acanthamoeba* sp.	Horn:UWE39	AF132136	Horn *et al.* 1999
T14	*Acanthamoeba* sp.	Gast:PN15	AF333607	Gast 2001
T15	*A. jacobsi*	ATCC:30732 (AWQC:AC005)	AY262360	This study
T15	*A. jacobsi*	AWQC:AC080	AY262361	This study
T15	*A. jacobsi*	AWQC:AC194	AY262362	This study
T15	*A. jacobsi*	AWQC:AC227	AY262363	This study
T15	*A. jacobsi*	AWQC:AC304	AY262364	This study
T15	*A. jacobsi*	AWQC:AC305	AY262365	This study

sp. UWE39 respectively. Subsequently Gast (2001) described a new sequence type, also named T14, which included the near identical strains *Acanthamoeba* sp. PN13 and *Acanthamoeba* sp. PN15. In the current study, the sequences of *Acanthamoeba* sp. UWC9, *Acanthamoeba* sp. UWE39 and *Acanthamoeba* sp. PN15 (obtained from GenBank) were included in the phylogenetic analysis to determine whether they represented distinct sequence types. In our analysis, the sequence similarity of the full length 18S rRNA gene sequence of *Acanthamoeba* sp. UWC9 and *Acanthamoeba* sp. UWE39 was 98.2%, while *Acanthamoeba* sp. PN15 showed less than 92% sequence similarity to these two strains. Stothard *et al.* (1998) established the criterion that strains must be at least 5% different based on their 18S rRNA gene sequence to belong to different sequence types. Since *Acanthamoeba* sp. UWC9 and *Acanthamoeba* sp. UWE39 are less than 5% different and show less separation than some representatives of the sequence type T4, we conclude that *Acanthamoeba* sp. UWE39 should be allocated to the sequence type T13. The distinctiveness of sequence type T14, represented by the strain *Acanthamoeba* sp. PN15 (Gast 2001), is not in doubt. The new sequence type described in this study, corresponding to the species *A. jacobsi*, is therefore designated T15.

The position of *A. jacobsi* in Fig. 1 provides further evidence that the morphological groups II and III are not monophyletic. Beyond its affinity with sequence type T13 (not assigned to a morphological group), *A. jacobsi* (which has typical group III cyst morphology) shows the greatest affinity to the T3, T4 and T11 *Acanthamoeba* species, all of which belong to morphological group II. Therefore cyst morphology can provide criteria for preliminary sorting of strains without indicating their true affinity.

The 18S rRNA gene analysis presented here confirms the conclusion of the isozyme analysis, that *A. jacobsi* is a distinct genetic entity, widely distributed and relatively uniform (Flint *et al.* 2003). Since there is also strong concordance of sequence and isozyme identities for *A. culbersoni* (T10), *A. healyi* (T12), *A. lenticulata* (T5) and *A. palestinensis* (T2) (Flint *et*

al. 2003), we predict that other *Acanthamoeba* lineages recognised from isozyme criteria will prove to represent new sequence types.

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Received on 22nd May, 2003; accepted on August 27th, 2003