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More *Acanthamoeba* Genotypes: Limits to Use rDNA Fragments to Describe New Genotype

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Summary. Strains of the genus *Acanthamoeba* are usually assigned to sequence types or genotypes according to pair-wise similarity values of the nuclear gene for the small subunit of ribosomal RNA. This classification system was established by comparing full or nearly full gene sequences, > 2000 bp. For practical reasons, diagnostic fragments of smaller lengths have been identified and used for rapid and economic identification of large number of strains. While the use of these small fragments in diagnostics applications remains valid when and only if the reference full sequence-type is available, we contest their use to identify and describe new genotypes. We report herein the case of a new genotype described on the basis of solely a small partial sequence and discuss the poor reliability of this fragment to correctly infer phylogenetic relationships, and its limits in the description of new genotypes of *Acanthamoeba*.

Key words: *Acanthamoeba*; genotype; full gene sequence; partial gene sequence

INTRODUCTION

Acanthamoeba spp. (Amoebozoa) are free-living naked amoebae, ubiquitous in the environment as microbial predators. They are able to cause opportunistic infections in humans and other vertebrates, as well as to harbor pathogenic microorganisms. The various species were classified on the basis of cyst features into three major morphogroups, numbered I to III (Pussard and Pons 1977, Page 1988). However, biomolecular approaches have permitted an easier and more coherent classification system on the basis of pair-wise similarity values of the nuclear gene for the small subunit ribosomal RNA (18S rDNA). Sequence-types or genotypes, named T1 to T12, were defined on a 5% dissimilarity value considering full gene sequences, i.e. more than 2200 bp (Gast *et al.* 1996, Stothard *et al.* 1998).

For practical reasons, diagnostic tests targeting smaller but variable regions of the gene have been developed. Primer sets JDP1-JDP2 produces 500-bp amplicons highly specific for *Acanthamoeba* containing the diagnostic region ASA.S1 (Dykova *et al.* 1999, Schroeder *et al.* 2001). Schroeder *et al.* (2001) showed that sequencing three inner portions of GTSA.B1, of about 500–600 bp that includes six variable regions, among which ASA.S1, might be a useful substitute for the full gene sequence for a rapid and low-cost identifi-

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cation of known genotypes of *Acanthamoeba*. However, GTSA.B1 fragment comprises in total eight variable regions, and the complete sequencing produces about 1,500 bp, i.e. about 65% of the 18S rRNA gene. By performing full sequencing of this fragment, Hewett *et al.* (2003) showed the uniqueness of strains belonging to *Acanthamoeba jacobsi*, and proposed this species as the new genotype T15.

The Ami fragment, of about 850 bp, i.e. 38% of the 18S rRNA gene, overlaps five variable regions of GTSA.B1 fragment, lacking three variable regions at the 5'end. The Ami fragment is obtained by the eukaryotic primers ami6F and ami9R, wrongly designed as specific for "amoebae" (sic!) since they also allow an amplification from unrelated amoeboid organisms like *Acanthamoeba* (Amoebozoa) and *Naegleria* (Excavata). Amplification and sequencing of Ami fragment may be a useful strategy for rapid identification of isolated strains of almost any eukaryotes, including amoebae at the genus level (Thomas *et al.* 2006). We also used this fragment to screen large numbers of amoebae, with successful identification down to species or genotype levels depending on available databases (Corsaro *et al.* 2009, 2010). However, when new putatitive taxa were recovered, full sequences were obtained.

Lanocha *et al.* (2009) recently reported on a new genotype of *Acanthamoeba* from human clinical samples, designed as T16 but characterized on the basis of the Ami fragment. We also proposed a new genotype T16, but on the basis of full sequence (Corsaro and Venditti 2010). Herein we analysed the relationship between these strains and we discuss the validity of the various molecular fragments to focus on in order to determine *Acanthamoeba* genotype.

We showed these strains as belonging to distinct lineages and we proposed minimal requirements to satisfy for the claim of a new *Acanthamoeba* genotype.

MATERIALS AND METHODS

Partial and full sequences of representative members of each *Acanthamoeba* genotypes were obtained from GenBank, and a closest relative search was carried out for particular strains with the BLAST server. Multiple alignments were performed with clustalX and edited with BioEdit. Introns were excluded from the analysis. Phylogenetic reconstructions were built on the partial Ami and GTSA.B1 fragments, using neighbour-joining (NJ, p-distance) and maximum parsimony (MP) with MEGA3 (Kumar *et al.* 2004), and maximum likelihood (ML, GTR, gamma+I:4 model) with Treefinder (Jobb *et al.* 2004), with bootstrap test of 1000.

Pair-wise similarity values were calculated with BioEdit, using all the sites and indels, but excluding introns, and by removing common and terminal gaps. Values were calculated for both full and partial sequences and compared.

RESULTS AND DISCUSSION

Lanocha *et al.* (2009) proposed the genotype T16 for two strains AM22 and AM38, from human clinical samples, but on the basis of the 850-bp Ami fragment. In our previous study, when defining genotype T16 (Corsaro and Venditti 2010) these strains were not included because we considered the 1450-bp GTSA.B1 of *A. jacobsi* T15 (Hewett *et al.* 2003) to be the minimum sequence length to define genotype.

In order to evaluate the relatedness between AM22/AM38 and our genotype, as well as the reliability of Ami and GTSA.B1 fragments to provide good information to infer phylogenetic relationships and more particularly to diagnose for new genotypes, we performed phylogenetic analysis based on both fragments.

Overall tree topologies based on the gene fragments are similar, but several nodes are best supported when the larger GTSA.B1 fragment is used (Fig. 1). More particularly, GTSA.B1 strongly supported the relationship ((T10, T12) T14) and the sister-group of T13 and T16 (Fig. 1A). Also T1 and T15 emerged as intermediate lineages within a moderately supported clade from which the T4/T3/T11 (T4 complex) are excluded. This is largely in accordance with tree topology obtained from full gene (Corsaro and Venditti 2010, Nuprasert *et al.* 2010). By contrast, Ami fragment changed the branching order and lowered the bootstrap values (Fig. 1B). The T4 complex emerged nested to a poorly supported T14 plus T10/T12 clade, with T3 within the T4. The sister-group relationship between genotypes T13 and T16, highly supported in GTSA.B1 (Fig. 1A) and full gene trees (Corsaro and Venditti 2010) as well as by higher pair-wise values (Table 1), is not observed by using the smaller Ami fragment (Fig. 1B). T16 emerged weakly as sister to T1 and both sequences AM22 and AM38. However, the two groups of sequences designed as T16, i.e. AM22 and AM38 by Lanocha *et al.*. (2009) and cvX and U/H-C1 by us (Corsaro and Venditti 2010), constitute two distinct lineages, pair-wise values for the common Ami portion being only of 91–91.2%. Relative position of T15 also appeared unstable in Ami tree.

Pair-wise similarity values calculated on the Ami and GTSA.B1 fragments are lower when compared

with values obtained from full gene sequences (Table 1). However, distances between genotypes are clearly increased with the Ami fragment, of 1.1 to 5.9%. By contrast, the GTSA.B1 fragment gives values closer to the ones obtained with full sequence, with a maximum of difference 0.9%.

In the particular case of interest here, by using the Ami portion, distance between T13 and T16 increased of 3% with respect to reference values obtained with full gene, and T16 was slightly more closely related to T1 (Table 1). Such a modification in pair-wise values among T13, T16 and T1 is well compensated by GTSA.B1 portion, whit a limited increase of only 0.5% for T1 and T16. This explains why T13 and T16 emerge as sister-groups, as expected with full gene tree, only with GTSA.B1 tree (Fig. 1).

Additionally, the Ami fragment could fail to show the eventual presence of group I introns. In fact, many strains of *A. lenticulata* (T5) have 18S rDNA with introns at position 1485 (Gast *et al.* 1994), thus falling within the Ami fragment. But in a few strains, the introns occur at two different positions, 1498 and 2111 (Schroeder-Diedrich *et al.* 1998), thus falling out both from Ami and GTSA.B1 fragments. In some strains of genotype T3 (Gast *et al.* 1994, Ledee *et al.* 1996, Nagyova *et al.* 2010) and more rarely in strains of genotype T4 (Liu *et al.* 2006, Xuan *et al.* 2007, Nagyova *et al.* 2010), introns occur at positions 628 and 636, i.e. about 20 nt before the forward Ami primer. Group I introns of *Acanthamoeba* have different lengths (ranging from 490 to about 1 000 nt) and they are absent from mature rRNA. They show poor relatedness with one another, and possibly originated by lateral transmission from corresponding introns of green algae (Schroeder-Diedrich *et al.* 1998). These sequences are omitted from master alignments through which molecular phylogenetic trees are inferred. However, examining the presence/absence of such introns and recording their position would be of interest, especially when a putative new genotype is described.

Pair-wise similarity value determination, and proposition of the sequence-type/genotype for the 18S rDNA of *Acanthamoeba*, was established on full or nearly full gene sequences, i.e. more than 2,200 bp (Stothard *et al.* 1998). The 850-bp Ami and the 1450-bp GTSA.B1 fragments represent approximatively 38% and 65% of the 18S rRNA gene, respectively.

The GTSA.B1 fragment contains enough information to allow identification of all the currently known *Acanthamoeba* genotypes (Schroeder *et al.* 2001). With

Table 1. Differences in 18S rDNA pair-wise values, as obtained on the near full gene sequence (> 2,200 bp) and compared with values calculated by considering only the portions Ami (850 bp) and the GTSA.B1 (1,450 bp).

Compared genotypes (strains)		Full gene	Ami	Diff	GTSA.B1	Diff
T1 (V006)	T14 (PN15)	90.7	86.5	4.2	89.8	0.9
T1 (V006)	T10 (V013)	87.6	85.1	2.5	87.9	0.3
T1 (V006)	T12 (Lilly A1)	89.07	85.7	3.3	88.6	0.47
T1 (V006)	T13 (UWC9, UWET39)	92.1–92.3	90.6–90.8	1.5	91.7–92.0	0.35
T1 (V006)	T16 (U/H-C1, cvX)	92.6–92.9	91.5–91.7	1.1	92.2–92.3	0.5
T12 (Lilly A1)	T10 (V013)	91.01	89.5	1.5	90.7	0.31
T12 (Lilly A1)	T14 (PN15)	89.5	86.1	3.4	88.8	0.7
T13 (UWC9, UWET39)	T14 (PN15)	88.5–89.0	85.0–85.5	3.5	87.7–88.6	0.6
T13 (UWC9, UWET39)	T16 (U/H-C1, cvX)	93.6–94.6	91.1–91.6	3	93.3–94.2	0.35
T15 (ATCC 30732)	T1 (V006)	86.9	83.6	3.3	87.1	–0.2
T15 (ATCC 30732)	T10 (V013)	83.7	78.5	5.2	83.9	–0.2
T15 (ATCC 30732)	T12 (Lilly A1)	83.9	78	5.9	84.1	–0.2
T15 (ATCC 30732)	T13 (UWC9, UWET39)	86.5–87.1	82.8–83.7	3.5	86.7–87.2	–0.15
T15 (ATCC 30732)	T14 (PN15)	84.4	79.5	4.9	84.6	–0.2
T2 (Reich, GE3a)	T6 (2892, 11DS)	94.9–95.2	91.9–93.8	2.2	94.0–95.0	–0.5

For T15, partial sequence only is available, about 1,450 bp for full gene, and 820 bp for Ami fragment.
For T16, we consider our genotype described on the basis of full gene sequence from two strains.
Diff: Difference of Percentage of similarity values with full sequence.

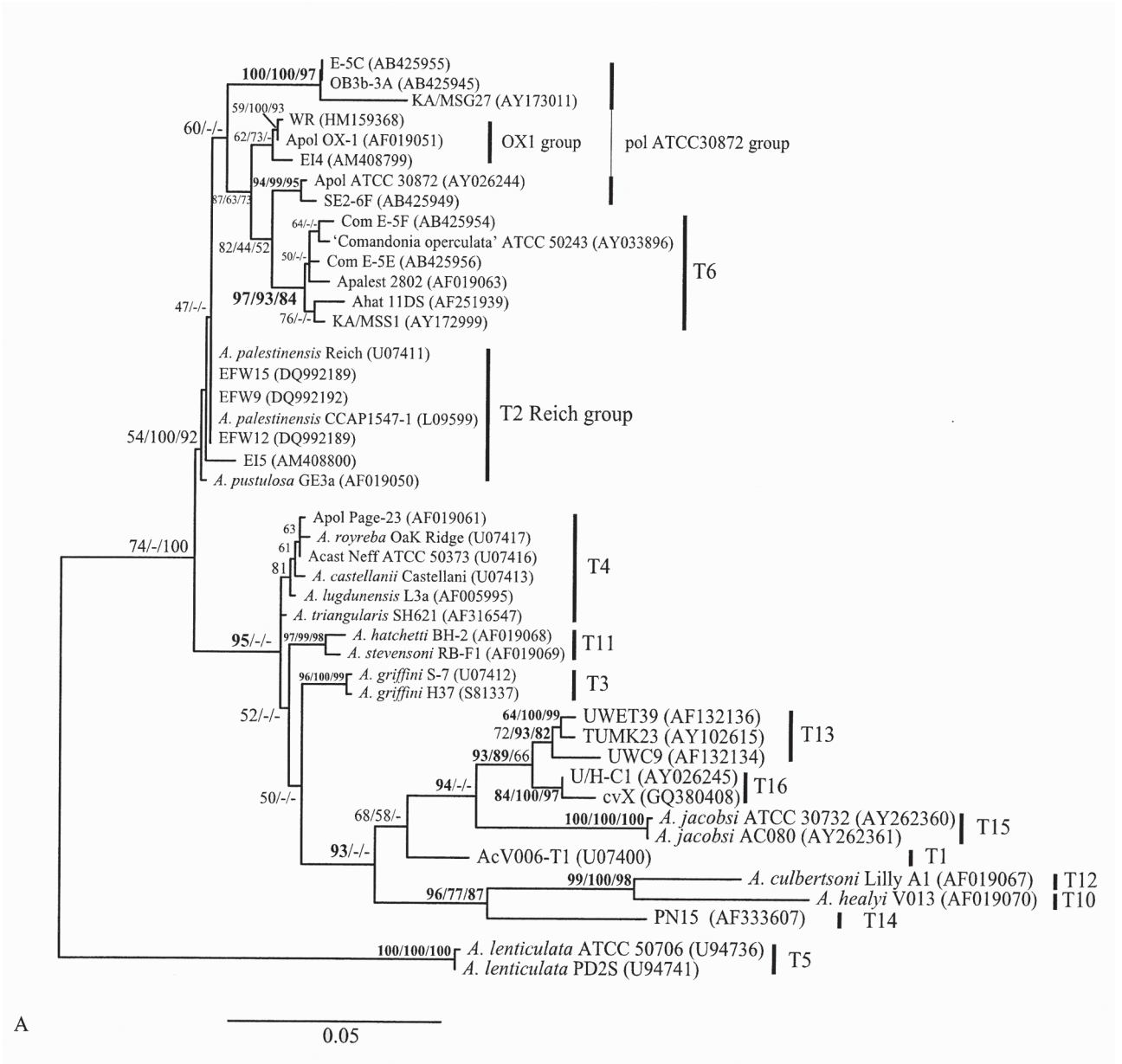
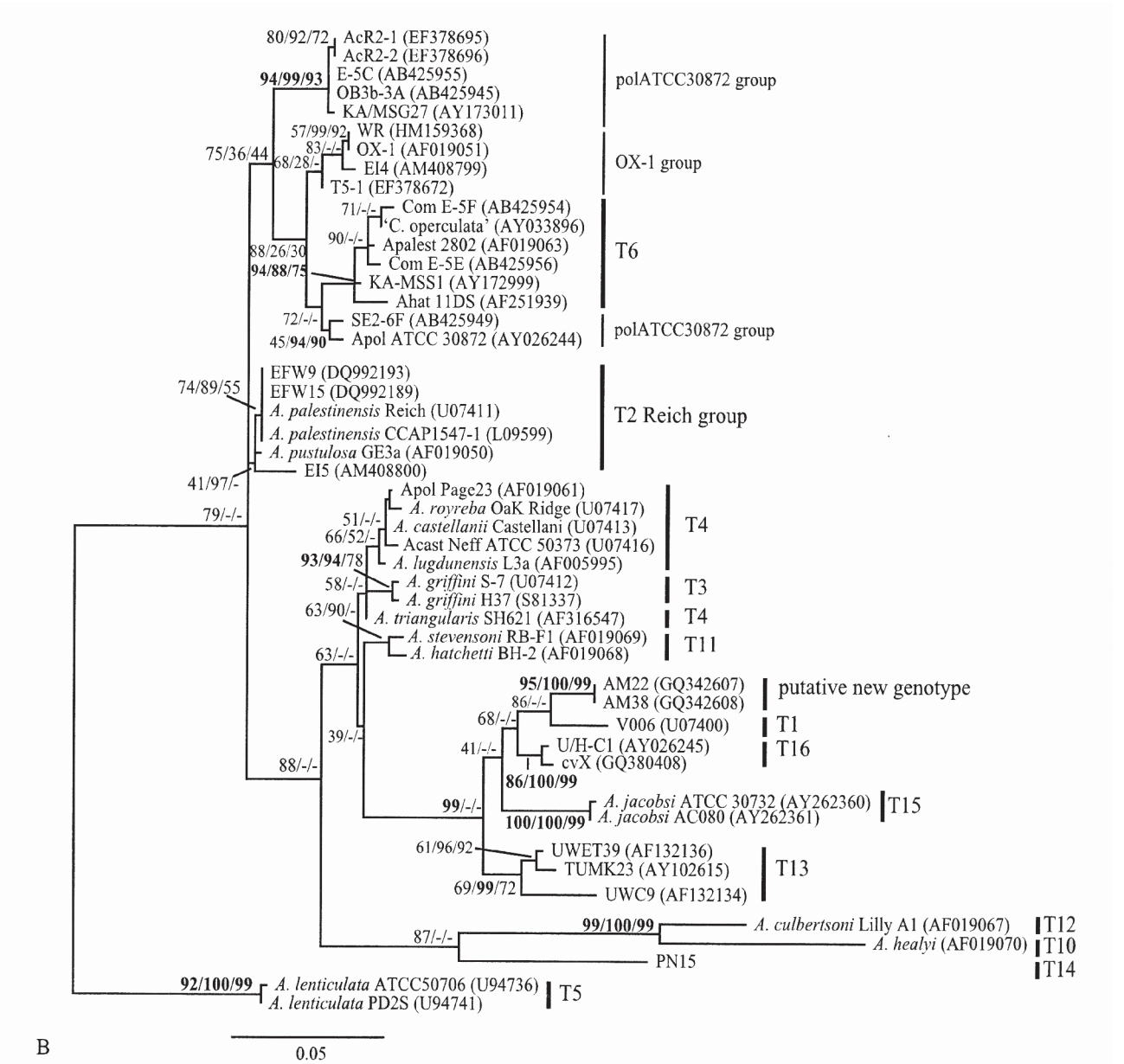


Fig. 1. Maximum likelihood 18S rDNA tree of *Acanthamoeba* genotypes based on 1,450-bp GTSA.B1 (**A**) and 850-bp Ami (**B**) fragments. Bootstrap values (1000 replications) for ML/NJ/MP are shown at nodes. Morphogroup I (genotypes 7/8/9, not showed) was used as outgroup.

the exception of genotype T15, defined solely on this fragment (Hewett *et al.* 2003), all the other genotypes have been proposed on the basis of full sequence (Gast *et al.* 1996, Stothard *et al.* 1998, Horn *et al.* 1999, Gast 2001, Corsaro and Venditti 2010, Nuprasert *et al.* 2010).

By contrast, the Ami fragment, which contains only five out eigth variable regions of *Acanthamoeba* gene, has generally been used for rapid genus-level identification of large numbers of taxa. High identity values in BLAST with reference sequences could permit to assign strains down to species or genotype levels, but full sequences are needed in case of new taxa (Corsaro *et al.* 2009). Nevertheless, data reported herein showed that phylogenetic analyses conducted on the Ami fragment



appear clearly biased by artifacts in the tree building and increases in genetic distances. The two sequences AM22 and AM38 reported by Lanocha *et al.*. (2009) showed 91% and 92.8% pair-wise values with T16 and T1 and emerge weakly related with T1. We contest the usefulness of Ami fragment to strictly define a genotype, but rather suggest that these data are highly suggestive for such a definition. Therefore, it is necessary to obtain the full sequence of these strains, as they very likely represent a new genotype, distinct from T1 and T16 (described successively, but on the basis of full sequences), and as they are of clinical origin and thus potentially pathogenic.

Description of a new *Acanthamoeba* genotype should require ideally full gene sequences, i.e. > 2,200 bp, which would serve as reference sequences for either phylogenetic analyses or diagnostic searches based on smaller fragments like Ami. The GTSA.B1 fragment should be retained as the minimum length size acceptable to identify new genotypes, since *A. jacobsi* T15 is

presently defined solely on this gene portion. However, the full T15 sequence should be completed to ensure reliable description.

REFERENCES

Corsaro D., Venditti D. (2010) Phylogenetic evidence for a new genotype of *Acanthamoeba* (Amoebozoa, Acanthamoebida). *Parasitol. Res.* **107:** 233–238

Corsaro D., Feroldi V., Saucedo G., Ribas F., Loret J. F., Greub G. (2009) Novel *Chlamydiales* strains isolated from a water treatment plant. *Environ. Microbiol.* **11:** 188–200

Corsaro D., Saucedo Pages G., Catalan V., Loret J. F., Greub G. (2010) Biodiversity of amoebae and amoeba-associated bacteria in water treatment plants. *Int. J. Hyg. Environ. Health* **213:** 158–166

Dykova I., Lomn J., Schroeder-Diedrich J. M., Booton G. C., Byers T. J. (1999) *Acanthamoeba* strains isolated from organs of freshwater fishes. *J. Parasitol.* **85:** 1106–1113

Gast R. J. (2001) Development of an *Acanthamoeba*-specific reverse dot-blot and the discovery of a new ribotype. *J. Eukaryot. Microbiol.* **48:** 609–615

Gast R. J., Fuerst P. A., Byers T. J. (1994) Discovery of group I introns in the nuclear small subunit ribosomal RNA genes of *Acanthamoeba*. *Nucleic Acids Res.* **22:** 592–596

Gast R. J., Ledee D. R., Fuerst P. A., Byers T. J. (1996) Subgenus systematic of *Acanthamoeba*: four nuclear 18S rDNA sequence types. *J. Eukaryot. Microbiol.* **43:** 498–504

Hewett M. K., Robinson B. S., Monis P. T., Saint C. P. (2003) Identification of a new *Acanthamoeba* 18S rRNA gene sequence type, corresponding to the species *Acanthamoeba jacobsi* Sawyer, Nerad and Visvesvara, 1992 (Lobosea: Acanthamoebidae). *Acta Protozool.* **42:** 325–329

Horn M., Fritsche T. R., Gautom R. K., Schleifer K. H., Wagner M. (1999) Novel bacterial endosymbionts of *Acanthamoeba* spp. related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus*. *Environ. Microbiol.* **1:** 357–367

Lanocha N., Kosik-Bogacka D., Maciejewska A., Sawczuk M., Wilk A., Kuzna-Grygiel W. (2009) The occurrence *Acanthamoeba* (free-living amoeba) in environmental and respiratory samples in Poland. *Acta Protozool.* **48:** 271–279

Ledee D. R., Hay J., Byers T. J., Seal D. V., Kirkness C. M. (1996) *Acanthamoeba griffini*, molecular characterization of a new corneal pathogen. *Invest. Ophthalmol. Vis. Sci.* **37:** 544–550

Liu H., Ha Y. R., Lee S. T., Hong Y. C., Kong H. H., Chung D. I. (2006) Genetic diversity of *Acanthamoeba* isolates from ocean sediments. *Korean J. Parasitol.* **44:** 117–125

Nagyova V., Nagy A., Janecek S., Timko J. (2010) Morphological, physiological, molecular and phylogenetic characterization of new environmental isolates of *Acanthamoeba* spp. from the region of Bratislava, Slovakia. *Biologia* **65:** 81–91

Nuprasert W., Putaporntip C., Pariyakanok L., Jongwutiwes S. (2010) Identification of a novel T17 genotype of *Acanthamoeba* from environmental isolates and T10 genotype causing keratitis in Thailand. *J. Clin. Microbiol.* **48:** 4636–4640

Page F. C. (1988) A new key to freshwater and soil Gymnamoebae. Freshwater Biological Association, Ambleside, Cumbria, 92–97

Pussard M., Pons R. (1977) Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (*Protozoa*, *Amoebida*). *Protistologica* **8:** 557–598

Schroeder J. M., Booton G. C., Hay J., Niszl I. A., Seal D. V., Markus M. B., Fuerst P. A., Byers T. J. (2001) Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of Acanthamoebae from humans with keratitis and from sewage sludge. *J. Clin. Microbiol.* **39:** 1903–1911

Schroeder-Diedrich J. M., Fuerst P. A., Byers T. J. (1998) Group-I introns with unusual sequences occur at three sites in nuclear 18S rRNA genes of *Acanthamoeba lenticulata*. *Curr. Genet.* **34:** 71–78

Stothard D. R., Schroeder-Diedrich J. M., Awwad M. H., Gast R. J., Ledee D. R., Rodriguez-Zaragoza S., Dean C. L., Fuerst P. A., Byers T. J. (1998) The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *J. Eukaryot. Microbiol.* **45:** 45–54

Thomas V., Herrera-Rimann K., Blanc D. S., Greub G. (2006) Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl. Environ. Microbiol.* **72:** 2428–2438

Xuan Y. H., Yu H. S., Jeong H. J., Seol S. Y., Chung D. I., Kong H. H. (2007) Molecular characterization of bacterial endosymbionts of *Acanthamoeba* isolates from infected corneas of Korean patients. *Korean J. Parasitol.* **45:** 1–9