SHORT COMMUNICATION

Phylogenetic evidence for a new genotype of *Acanthamoeba* (Amoebozoa, Acanthamoebida)

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Abstract *Acanthamoeba* are widespread free-living amoebae, able to cause infection in animals, with keratitis and granulomatous encephalitis as major diseases in humans. Recent developments in the subgenus classification are based on the determination of the nucleotide sequence of the 18S rDNA. By this mean, *Acanthamoeba* have been clustered into 15 sequence types or genotypes, called T1 to T15. In this study, we analysed near full 18S rDNA of an *Acanthamoeba* recovered from an environmental sample and various unidentified *Acanthamoeba* sequences retrieved from GenBank. We provided phylogenetic evidence for a new genotype, which we proposed to name T16.

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Introduction

The genus *Acanthamoeba* comprises several species of free-living amoebae, widespread in many types of habitats, playing a role of major microbial predators. Many strains naturally harbour endosymbionts or may act as vector/reservoir for various microbial pathogens. Furthermore, trophozoites can be used as host cells to isolate intracellular organisms (Horn and Wagner 2004; Corsaro and Greub 2006).

Acanthamoeba spp. may cause diseases in animals, including humans, like keratitis, encephalitis and disseminated tissue infections; thus, great interest is brought to identification of pathogenic species or strains (Marciano-Cabral and Cabral 2003; Visvesvara et al 2007).

Acanthamoeba species have been traditionally clustered into three morphological groups, on the basis of cyst features (Pussard and Pons 1977). However, successive studies based on biochemical and molecular methods have pointed out in many cases incoherences in strain/species clustering, proving also that some species, e.g. *Acanthamoeba polyphaga*, are polyphyletic. Gast et al. (1996) and Stothard et al. (1998) proposed to classify *Acanthamoeba* strains on the basis of 18S ribosomal RNA gene (18S rDNA) sequence similarities. By analysing more than 50 strains from the three morphogroups, they identified 12 sequence types or genotypes, named T1 to T12, where each genotype should correspond to natural species or species complex (Gast et al. 1996; Stothard et al. 1998). This approach has been largely adopted, and three new genotypes have been established, called T13 to T15 (Horn et al. 1999; Gast 2001; Hewett et al. 2003).

This study focuses on the phylogenetic analysis of an *Acanthamoeba* strain, isolated from a freshwater pond, and of other *Acanthamoeba* sequences, identified as candidates for novel genotypes.

Materials and methods

Sample origin and DNA extraction

Amoebae were recovered from a freshwater pond containing decaying vegetable matter (South Italy) onto bacterised 1.5% non-nutritive agar during a study focusing on the search for chlamydiae in the environment (Corsaro and Venditti, 2009). Fungal overgrowth persisted in subcultures even after 24-h HCl treatment of cysts; thus, agar plates were eliminated. Prior to discharge plates, a small quantity of amoebae was recovered from the least contaminated agar and centrifuged in Page's amoeba saline buffer (three times at 200×*g*, 5 min). Total DNA was extracted by repeated passages through a 24-gauge needle after freezing–thawing. PCR for the amoeba 18S rDNA was performed by using several primer sets: CAT1 (5′-CAT GCA TGT CTA AGT ATA AGC-3′) with GSPr (5′-TTC AC <G/A> GTA AAC <G/A> ATC TGG GC-3′) (modified from Stothard et al. 1999), or 1137R (5′-GTG CCC TTC CGT TCA AT-3′), and 892cF (5′-GTC AGA GGT GAA ATT CTT GG-3′) with Br (5′-GAT CCT TCT GCA GGT TCA C-3′). Primers 1137R and 892cF were from Schroeder et al. (2001), and Br was modified from Gast (2001). Reaction conditions were 5 min at 94°C, followed by 40 cycles of 94°C 45 s, 56°C 30 s, 72°C 1 min, with final extension of 72°C 5 min. PCR products were purified and sequenced by using the same PCR primers, and screened at BLAST for their identity.

Phylogenetic analysis

The obtained sequence was aligned with closest relatives, retrieved after BLAST (www.ncbi.nlm.nih.gov), and with representatives of each *Acanthamoeba* genotype. Multiple alignments were obtained with clustalX and edited with BioEdit. Introns were excluded from the analysis. Phylogenetic reconstructions were made on a partial ~1,450-bp diagnostic region of the 18S rDNA, in order to include also the genotype T15 (Hewett et al. 2003; Schroeder et al. 2001), and on full sequences (T15 excluded). Treeing methods used were neighbour-joining, minimum evolution (NJ, ME; 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 1,000. Following Stothard et al. (1998), genetic similarity between full sequences (~2,200 bp) was calculated with BioEdit by pairwise comparison, under optimal global alignment, using all sites and indels but excluding introns, and by removing common and terminal gaps.

Results and discussion

Heavy fungal overgrowth prevented subculture and isolation of the strain cvX. Cysts onto agar appeared wrinkled, with a polygonal endocyst; therefore, we considered this strain as belonging to group II. DNA analyses were then performed on extracts of amoebae scraped directly from a contaminated agar plate. Amoebae resulted negative for chlamydiae.

Amplicons obtained with CAT1/1137R (~1,400 bp) and 892cF/Br (~1,120 bp), starting from both DNA extracts and after PCR with CAT1/Br (~2,200 bp), give identical sequences. Thus, a unique *Acanthamoeba* intronless sequence of 2,157 bp was obtained. At BLAST, our sequence revealed almost identical (99.2) to the strain U/HC1, isolated from a human keratitis in Brazil, of undefined genotype (Alves et al. 2000). Other closest relatives were strains of genotype T13, showing sequence identity of 94%, while following entries in the BLAST all showed sequence identity values <93%.

Figure 1 shows a phylogenetic tree based on a partial ~1,450-bp portion of the 18S rDNA, in order to include also the genotype T15, corresponding to the species *Acanthamoeba jacobsi* (Hewett et al. 2003), for which only this part of the gene is available. However, this gene portion is a diagnostic region comprising eight variable sequence regions able to differentiate among all the 15 genotypes (Schroeder et al. 2001). In this partial tree, our sequence emerged in a highly supported holophyletic clade with the strains of genotype T13 and clustered unambiguously with the strain U/HC1. The genotype T15, presented as the sister group of the genotype T13 in the original report of Hewett et al. (2003), emerged as an independent clade in the present analysis.

To identify genotypes, pairwise similarity values were determined by considering available full sequences, i.e. >2,100 bp. Pairwise similarity value between the strains U/HC1 and cvX was of 99.17% (dissimilarity 0.8%). Sequence from cvX strain showed 16 point mutations from that of U/HC1, 10 of which are in the diagnostic region, and two nt insertions. T13 genotype was represented by three strains, showing values of 96.0–97.7% (dissimilarity 2.3–4%): UWC9 isolated from contact lens case (Fritsche et al. 1993), and TUMK-23 and UWET39 from soils (Horn et al. 1999; Hewett et al. 2003). Pairwise similarity value between the group U/HC1 and cvX and the T13 strains was 93.6–94.6% (dissimilarity 5.4–6.4%). Following the criterion of a 5% of dissimilarity (Stothard et al. 1998), the
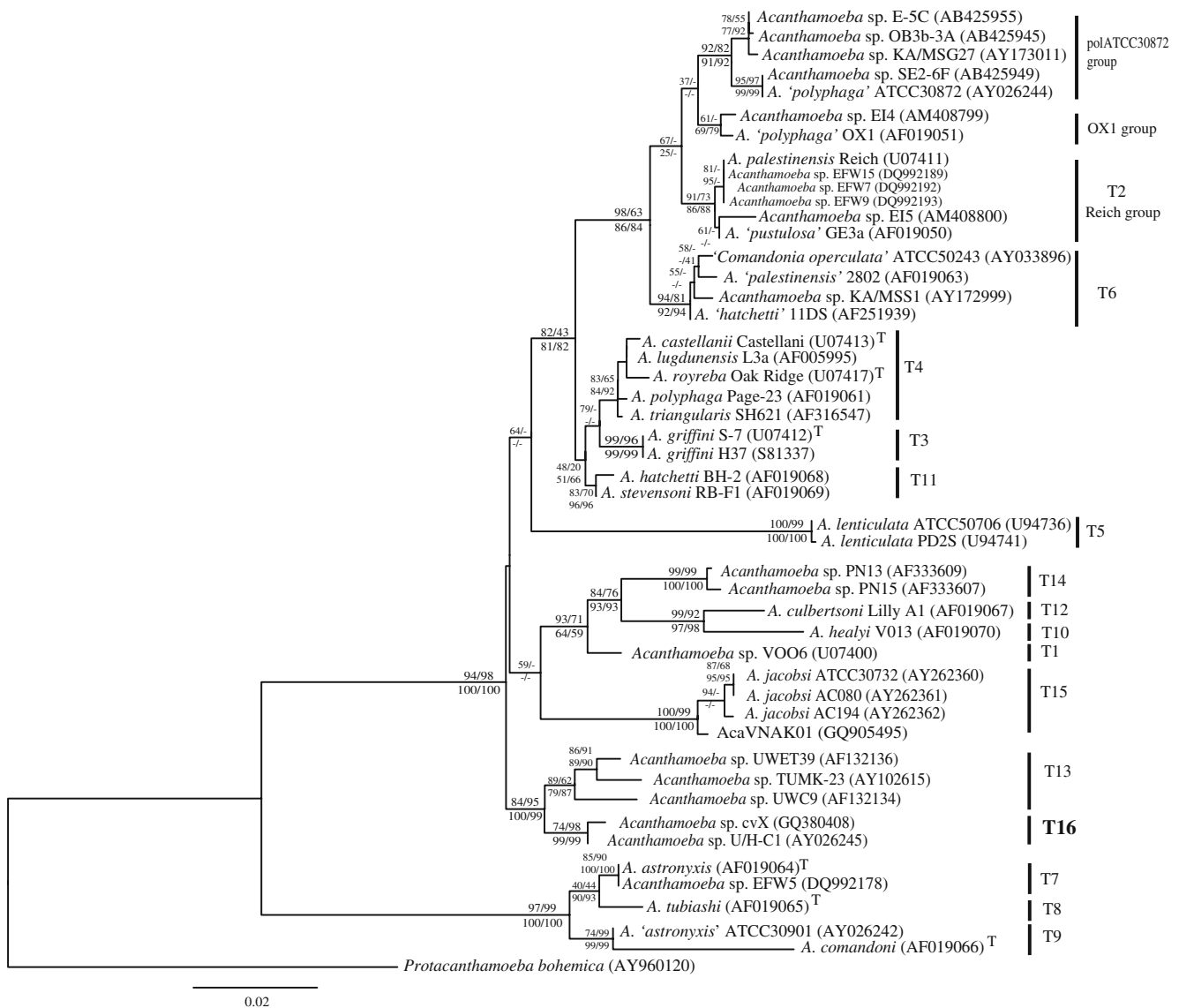
Fig. 1 Maximum likelihood 18S rDNA tree of the genus *Acantha-moeba* based on the diagnostic region of 1,450 bp including all the genotypes. Bootstrap values (1,000 replications) for ML/MP and NJ/ME are shown above and below the nodes, respectively. GenBank accession numbers are in *parentheses*. Upper *T* after GenBank indicates type strain for the species. *T1–T15* recognised *Acantha-moeba* genotypes; *T16*, in bold, new genotype proposed in this study. *Protacanthamoeba bohemica* (Acanthamoebida) is used as outgroup

group U/HC1 and cvX represents a new genotype, from keratitis sample (Alves et al. 2000) and freshwater (this study), for which we proposed the label T16. The T13 is closely related to but clearly distinct from T16, and comprises both ocular (Fritsche et al. 1993) and environ-mental strains (Horn et al. 1999; Edagawa et al. 2009).

In our study, we retrieved from GenBank other five sequences of environmental strains of undefined genotype: *A. 'polyphaga'* ATCC 30872, from freshwater (Alves et al. 2000); *Acanthamoeba* sp. strains SE2-6F, E-5C and OB3b-3A, from rice soil (Murase and Frenzel 2008); and *Acanthamoeba* sp. strain MSG27, from marine sediment (Liu et al. 2006).

These five strains, 'pol30872 group', emerged as a relatively robust holophyletic clade in phylogeny performed on the 1,450-bp diagnostic region (Fig. 1), very weakly linked in ML tree to a paraphyletic T2 lineage, and sister group to a holophyletic T6 lineage. In NJ and ME, T2 emerged as holophyletic and separated from these five strains, while under MP significant bootstrap values (>65%) support independent emergence for each 'pol30872', 'OX-1' and 'Reich' groups in a large T2–T6 clade, but with 'pol30872' sister to T6. In full gene (>2,000 bp, Fig. 2) trees, the five 'pol30872 group' strains clustered together, but with lower bootstrap values (%BV 74/70 and 75/60 for ML/MP and NJ/ME), and the T2 split
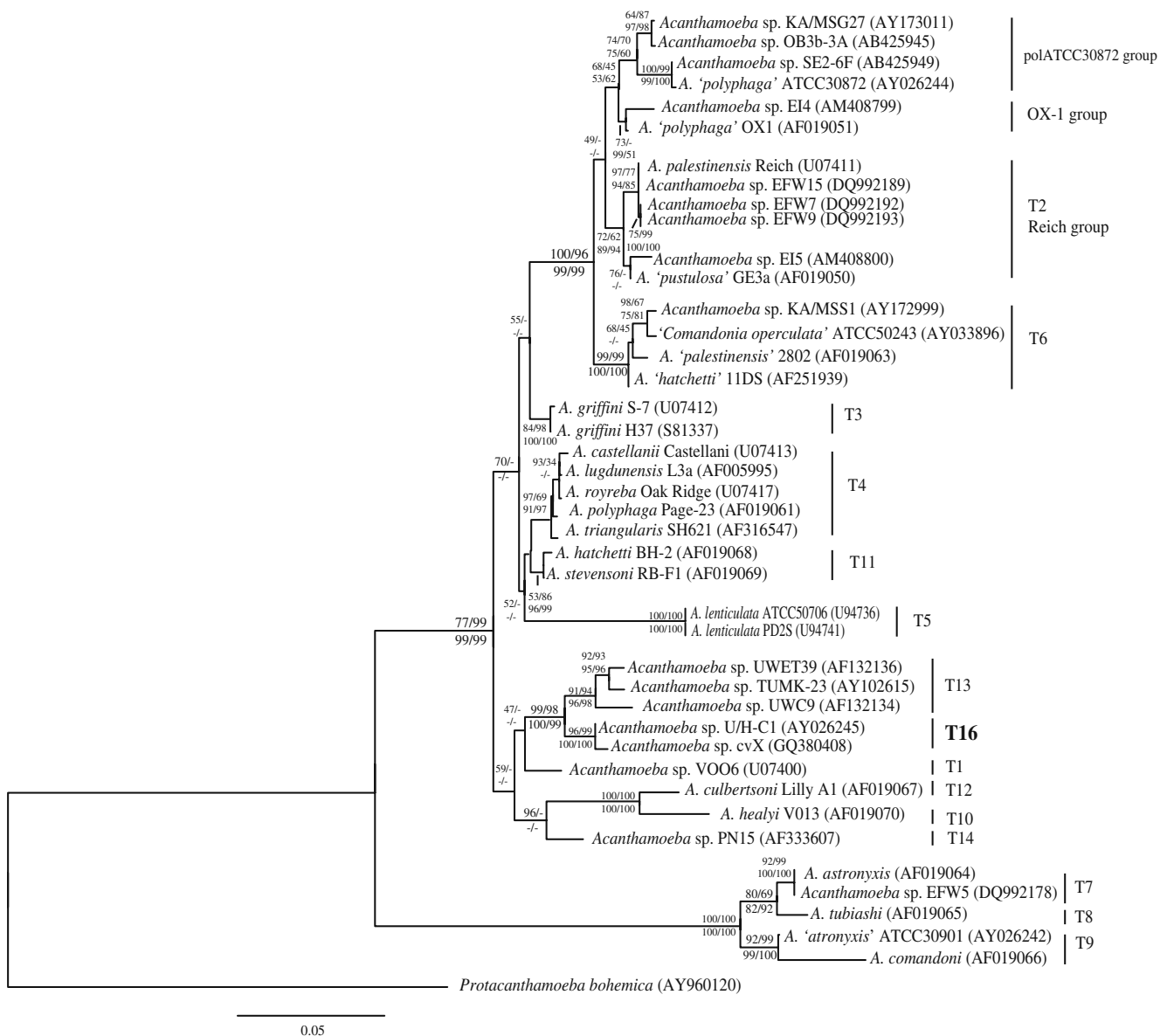
Fig. 2 Maximum likelihood 18S rDNA tree of the genus *Acanthamoeba* based on full sequences. Bootstrap values (1,000 replications) for ML/MP and NJ/ME are shown above and below the nodes, respectively. GenBank accession numbers are in *parentheses*. *T1–T14* recognised *Acanthamoeba* genotypes; *T16*, in bold, new genotype proposed in this study. *Protacanthamoeba bohemica* (Acanthamoebida) is used as outgroup

into apparently distinct 'OX-1' and 'Reich' lineages, with the 'OX-1' as sister to the 'pol30872'.

Intra-group pairwise dissimilarity values, based on full sequences (>2,000 bp), were of 0.6–4.8% for T2, 0.7–4.4% for the 'pol30872' group and 1.5–2.5% for the T6. For T2, dissimilarity values were 0.6–3.5% and 2.3% within the Reich and the OX-1 groups, respectively, and 3.4–4.8% between the groups.

Mean inter-group dissimilarity values were >4.5%. T6 strains differed in mean by 5.2% (4.6–5.6%) from the 'pol30872' group, and by 5.3% (4.6–6.5%) from T2. Mean

dissimilarity value between the 'pol30872' group and T2 was 4.6% (3.5–5.9%), with slightly higher value for the Reich group, 4.9% (3.9–5.9%), with respect to OX-1 group, 4.1% (3.5–4.7%).

T2 genotype seemed to be of environmental source (Stothard et al. 1998; Lorenzo-Morales et al. 2006); however, recently, strains of this genotype have been recovered in human cases of keratitis and encephalitis (Maghsood et al. 2005; Walochnik et al. 2008). Pathogenic strains seems more closely related to the strain OX-1, and Maghsood et al. (2005) proposed to split the genotype T2

into two groups T2A and T2B, on the basis of 4% dissimilarity, and eventually to differentiate between pathogenic and non-pathogenic strains.

The 'pol30872' group could represent an additional subclade of the genotype T2; however, in phylogenetic reconstructions, this group appeared distinct from both Reich and OX-1 groups. The sister group relationship between the 'pol30872' and the OX-1 group found in full sequence tree is very weakly supported (BV 68% and 62% in ML and ME trees, respectively), and there is no support unifying all 'pol30872', OX-1 and Reich groups in a large clade T2 (Fig. 2). In phylogenetic reconstructions using the genotypes T7–T8–T9 as outgroup instead of *Protacanthamoeba* (not shown), the 'pol30872' emerged as holophyletic (70% in ML tree) but as sister to T6 (52%), and OX-1 and Reich groups followed as paraphyletic well-supported (89% and 79%, respectively) clades. All putative T2 subgoups (Reich, OX-1 and 'pol30872') and T6 were supported by 100%.

Stothard et al. (1998) proposed a minimum dissimilarity value of 5% for new genotypes in *Acanthamoeba*. However, programmes used for sequence alignment and analysis as well as number and variety of strains analysed influenced the results, explaining slight discrepancies between studies. We analysed eight T2 and four T6 strains, for which only three and one, respectively, were available on the study of Stothard et al., as well as other five closely related strains. However, position of OX-1 group was unclear, and we consider only the Reich group as true members of the T2.

Molecular phylogeny shows the 'pol30872' group emerging as a distinct clade within the T2/T6 lineage. A future redefinition of the arbitrary 5% dissimilarity value, or its less rigid application, would possibly place this clade as a new genotype. *Acanthamoeba* sp. ATCC 30872 could be the reference strain, without the specific name 'polyphaga'. This strain is sometimes used as lab organism, e.g. to study interactions with bacteria or pathogenicity on human cell cultures (Steinert et al. 1998; Rocha-Azevedo et al. 2006), but always with the confusing name *A. polyphaga*, showing significant differences in its properties, as compared to other *A.* 'polyphaga' belonging to other genotypes like T4.

In conclusion, we presented genetic and phylogenetic data supporting the establishment of a new genotype T16, with representatives from clinical and environmental samples, and proposed to consider a cluster of five environmental strains as a distinct group within the T2/T6 lineage.

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