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ACANTHAMOEBA STRAINS ISOLATED FROM ORGANS OF FRESHWATER FISHES

Iva Dyková, Jiří Lom, Jill M. Schroeder-Diedrich*†, Gregory C. Booton*, and Thomas J. Byers*‡
Institute of Parasitology, Academy of Science of the Czech Republic, Branišovská 31, 370 05 Ceské Budejovice, Czech Republic

ABSTRACT: Contrary to data on *Acanthamoeba* infections in humans, little is known about infections in fishes. The present study combines the description of strains isolated from fishes with presentation of an improved method for subgeneric classification. *Acanthamoeba* spp. were isolated aseptically from tissues of 14 (1.7%) of 833 asymptomatic fishes collected in rivers and streams in the Czech Republic. Acanthamoebae successfully cloned from 10 of the 14 isolated strains were examined here. Morphology of these isolates was evaluated using light optics plus scanning and transmission electron microscopy. Cyst morphology, which varied extensively within and among clones, was most like morphological group II, but species-level classification was considered impossible. A distance analysis based on 442 bases in an 18S rDNA polymerase chain reaction fragment of about 460 bp placed the isolates in a clade composed of sequence types T3, T4, and T11, the 3 subdivisions of morphological group II. Fluorescent in situ hybridization (FISH) using oligonucleotide probes indicated that all isolates belong to a single subdivision of group II, the T4 sequence type. It has been concluded that the fish isolates are most closely related to strains commonly isolated from human infections, especially *Acanthamoeba* keratitis. The shorter diagnostic fragment sequences have proved nearly as useful as complete 18S rDNA sequences for identification of *Acanthamoeba* isolates.

The ubiquitous distribution of free-living amoebae of *Acanthamoeba* spp. in aquatic habitats has been well established and a voluminous literature has been accumulated on this topic. Taylor (1977) first demonstrated that some strains of this genus could colonize organs of freshwater fishes as facultative parasites. He isolated amoebae, identified as *Acanthamoeba polyphaga,* from organs of asymptomatic freshwater fishes of 11 species. The presence of trophozoites within tissues of apparently healthy individuals was demonstrated histologically. Host-to-host transmission of the infection was demonstrated by intramuscular injection of 1 of the amoeba isolates into uninfected fishes. *A. polyphaga* also has been isolated from the intestinal contents of apparently healthy specimens of white sucker *Catostomus commersoni,* and common shiner *Notropis cornutus* (Franke and Mackiewicz, 1982). One of the isolated strains, identified as *A. polyphaga,* was capable of invading tissues of uninfected fishes after intramuscular injection. Derr-Harf and Monteil (1983) isolated *Acanthamoeba* spp. from organs of asymptomatic trout in 3 locations in the Alsatian water system. Křepelová (1984) isolated *A. polyphaga* from the liver of a feral specimen of roach *Rutilus rutilus.* When injected into the dorsal musculature of laboratory-bred goldfishes, acanthamoebae could be reisolated from liver, spleen, and kidney tissues up to 7 mo postinfection without any observable pathological changes. Isolation of *Acanthamoeba culbertsoni* from fish gills by J. Yang also has been reported (Im, 1990; Moura et al., 1992).

Under some conditions, infections of fish by *Acanthamoeba* spp. are clearly associated with disease and can be fatal. *A. polyphaga* was repeatedly isolated from the peritoneal fluid, intestines, and gills of blue tilapias *Sarotherodon aureus,* sampled during a large fish kill in an experimental pond at Auburn University in Alabama (Taylor, 1977). *Acanthamoeba* also has been suggested as a possible agent of systemic amoebiasis in European catfish *Silurus glanis,* (Nash et al., 1988) and of spontaneous intracranial amoebiasis of goldfish *Carrasius auratus* (Wilson et al., 1995). Although the transmission of *Acanthamoeba* infections from infected to uninfected fishes has been demonstrated experimentally by intramuscular injection (Taylor, 1997; Franke and Mackiewicz, 1982; Křepelová, 1984), the ability of *Acanthamoeba* strains isolated from human infections to induce pathogenic effects in fish and cytopathic effects in fish cell cultures only rarely has been tested (Taylor, 1977; Derr-Harf et al., 1986).

The genus and subgenus identification of amoebae infecting fish are of fundamental interest for fish pathology as well as for the epidemiology of free-living amoeba infections. The identities of 2 of the *Acanthamoeba* strains isolated from fish tissues have been based on isoenzyme profiles and antigenic properties. One of these, isolated by Křepelová (1984) in the Czech Republic, was identified as *Acanthamoeba griffini* (De Jonckheere, 1987), and the other one, isolated by J. Yang (Im, 1990) in South Korea, was identified as *Acanthamoeba royreba* (Moura et al., 1992). Identification of the other *Acanthamoeba* strains mentioned above was based on morphological criteria that were not always accompanied by appropriate documentation. Furthermore, recent work on the classification of *Acanthamoeba* using ribosomal RNA gene sequences (Stothard et al., 1998) has resurrected old concerns (Visvesvara, 1991) about the reliability of morphological classification at the species level.

The present report is part of a larger study on the occurrence of amoebae in the organs of freshwater fishes collected in the Czech Republic (Dyková et al., 1995, 1996, 1997, 1998). Amoebae, including *Acanthamoeba* (Fig. 1), were repeatedly found in tissues of feral fishes. We report here on 10 *Acanthamoeba* strains isolated from brain, liver, spleen, or kidney tissue from asymptomatic individuals of 6 species of fish collected in 5 different rivers or streams. Both morphology and, for the first time, ribosomal RNA gene sequences have been used to classify fish-infecting isolates.

MATERIALS AND METHODS

Isolation and cloning of *Acanthamoeba* strains

Strain isolations were performed as previously described (Dyková et al., 1997) using standard methods (Page, 1988; Kalinina and Page, 1992). Aseptically sampled small pieces of tissues were placed on non-nutrient agar (NNA). Primary isolates were then subcultured on NNA seeded with heat-killed *Escherichia coli* or *Bacillus subtilis.* After sev-

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* Department of Molecular Genetics, The Ohio State University, 484 W. 12th Avenue, Columbus, Ohio 43210-1292.
† Present address: Department of Medicine, Division of Infectious Diseases, Indiana University School of Medicine, 545 Barnhill Drive, Indianapolis, Indiana 46202.
‡To whom correspondence should be addressed.

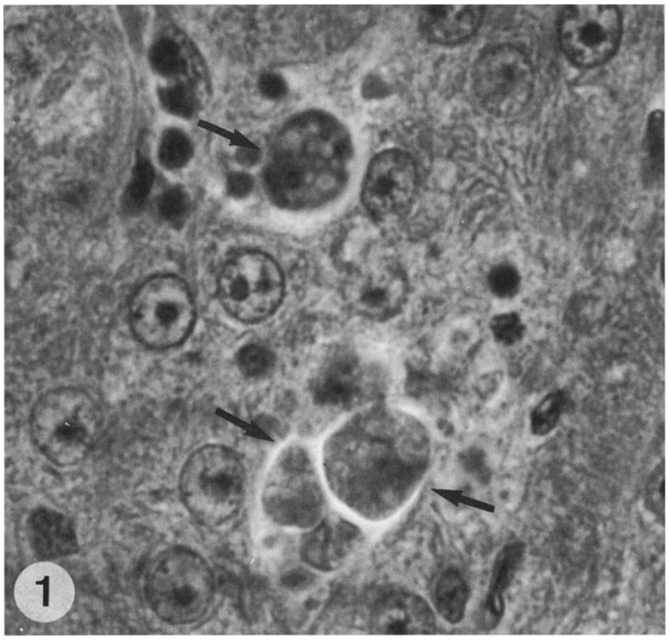


FIGURE 1. Spontaneous infection of *Acanthamoeba* in the liver of carp fingerling (arrows). Hematoxylin and eosin, ×1,400.

eral subsequent passages, well established strains were cryopreserved and stored in liquid nitrogen. Two strains (nos. 4465 and G1) were cloned from culture in liquid medium. All other strains were cloned from cryopreserved agar plate cultures after thawing followed by several successive subcultures. One clone from each strain was selected as representative. Clonal strains were transferred to liquid media (bactocasitone [BCS; Červa, 1969] or Chang's serum–casein–glucose–yeast extract medium [SCGYEM; Page, 1988] with bovine fetal serum) supplemented with antibiotics (4,000 i.u. penicillin and 100 μg streptomycin/ml of media). Cultures were axenized, then the amoebae were mass-cultured in tissue culture flasks at room temperature. A total of 10 clones derived from fish strains and 1 environmental clone have been studied (Table I).

Morphological characterization of isolates

The morphology of clones was characterized using light and transmission electron microscopy. Translucent light, phase-contrast, and Nomarski optics were used to observe and photograph living trophozoites in hanging drops. Silver-staining methods (Pussard and Pons, 1977) were used for a better understanding of cyst morphology. In 7 clones, the diameters of cysts formed on bacterized agar plates were compared with those formed in liquid media and stored in water. Selected *t*-tests and 2-way analysis of variance (Sokal and Rohlf, 1994) were used to compare strains. Cultures of individual strains washed several times in phosphate-buffered saline were harvested by centrifugation in the log phase of growth. The cell pellets were fixed for transmission electron microscopy in sodium cacodylate buffered 2% osmium tetroxide. Epon–Araldite was used for embedding.

Molecular characterization of isolates

Live cultures ordinarily would be the preferred source of DNA for sequencing. However, fixed amoebae were used here to minimize complications with the international transport of potential pathogens. The fixed specimens proved satisfactory both for sequencing and for fluorescent in situ hybridization (FISH) analyses in the present study. Samples containing 1.8–8.6 × 10^7 amoebae were fixed and stored in 80% ethanol or were fixed in 12% freshly depolymerized paraformaldehyde and stored in 70% ethanol. Total nucleic acids were isolated from both types of fixed amoebae using the UNSET procedure. (Hugo et al., 1992).

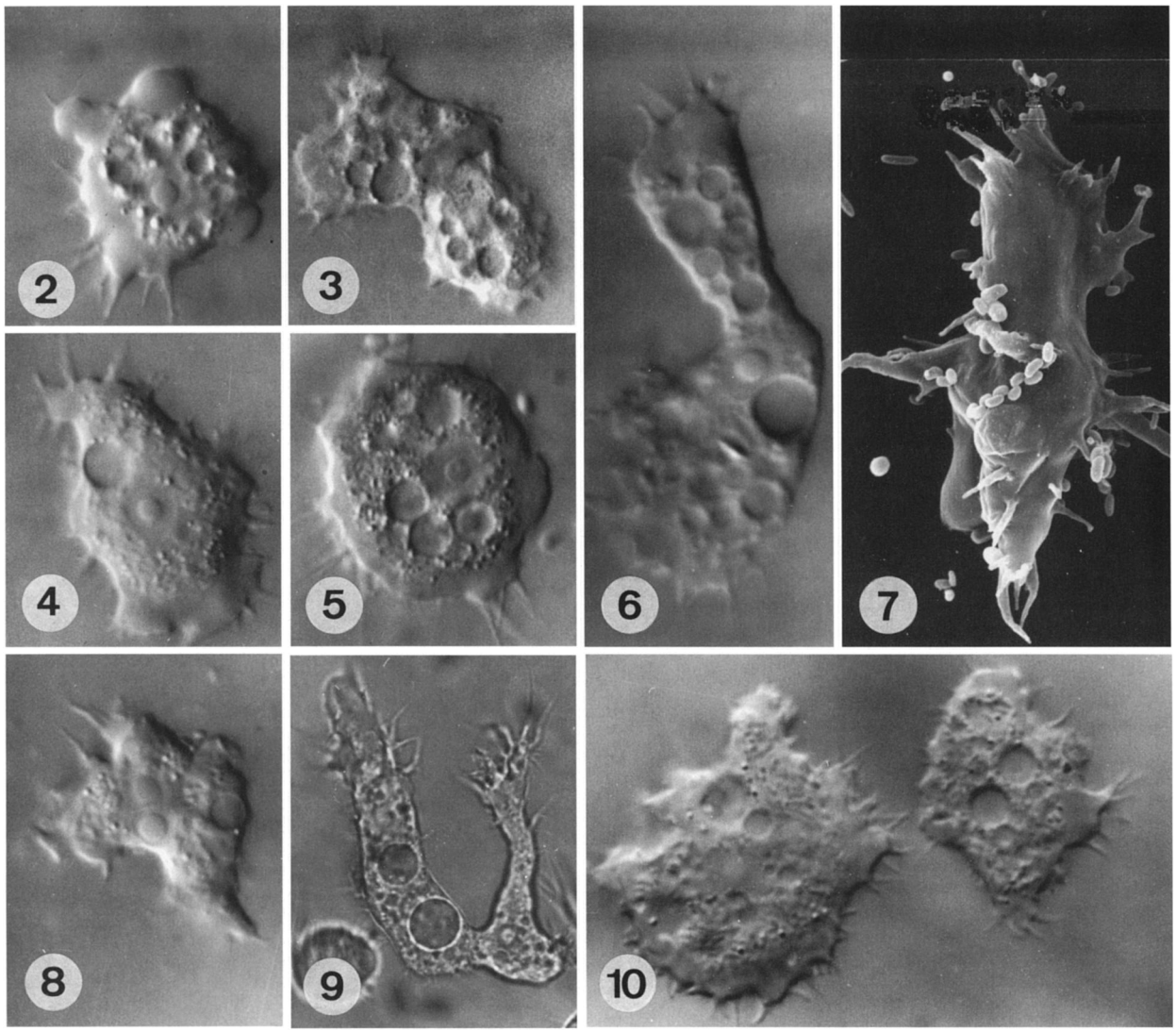
For polymerase chain reaction (PCR) studies, the nucleic acid pellet was resuspended in 60 μl of sterile double distilled water. One microliter was used for PCR amplification of an approximately 460-bp portion of the 18S ribosomal RNA gene (18S rDNA) located between rRNA secondary structure stems E23-2 and E30 (Stothard et al., 1998). The forward primer used (JDP1) was 5′-GGGCCCAGATCGTTTACCGTGAA and the reverse primer (JDP2) was 5′-CTCACAAGCTGCTAGGGGAGTCA. The genus specificity of the PCR products obtained with these primers has been demonstrated in our laboratory (J. M. Schroeder-Diedrich, unpubl. obs.). The BRL dsCycle Sequencing Kit (Gibco/BRL, Gaithersburg, Maryland) was used for sequencing of PCR and cloned products as previously described (Stothard et al., 1998). DNA sequences were obtained for PCR products from both ethanol- and paraformaldehyde-fixed amoebae. Sequences from highly conserved regions were identical to those obtained from unfixed amoebae in prior studies. Thus, there was no evidence that the fixation protocols altered sequences.

Phylogenetic distances were determined for the new sequences plus those from homologous regions from more than 50 other *Acanthamoeba* strains (Stothard et al., 1998). Nucleotide sequence alignments required were determined by eye using the program ESEE (Cabot and Beckenbach, 1989) and the master alignment of Stothard et al. (1998) in which homologous positions are identified based on secondary structure (Neefs et al., 1993; Gutell, 1994). The alignment is available at www.biosci.ohio-state.edu/tbyers/byers.htm. The phylogenetic tree illustrated was obtained using MEGA, version 1.01 (Kumar et al., 1993). This analysis

TABLE I. *Acanthamoeba* strains included in the study.

Clone	Fish species; local origin; organs sampled*	Strain isolated/cloned
4465/I	*Perca fluviatilis* Linnaeus, 1758; Vltava River, South Bohemia; **brain**, liver, kidney, gills	Dec 1990/May 1995
4706/I	*P. fluviatilis* Linnaeus, 1758; Černovický potok Brook, South Bohemia; brain, **liver**, kidney, spleen, gills	Jul 1991/Aug 1995
4339/II	*P. fluviatilis* Linnaeus, 1758; Malše River, South Bohemia; **liver**, gills	Sep 1990/Jan 1996
4800/V	*P. fluviatilis* Linnaeus, 1758; Černovický potok Brook, South Bohemia; brain, **liver**, kidney, spleen, gills	Dec 1991/Dec 1996
4178/II	*Blicca bjoerkna* Linnaeus, 1758; Vltava River, South Bohemia; **spleen**	Jun 1990/Oct 1996
4422/II	*Gymnocephalus cernuus* Linnaeus, 1758; Vltava River, South Bohemia; **brain**, liver, gills	Dec 1990/Nov 1994
4337/II	*Leuciscus cephalus* Linnaeus, 1758; Malše River, South Bohemia; **brain**, liver, spleen, kidney, gills	Sep 1990/Jan 1996
4528/II	*L. cephalus* Linnaeus, 1758; Černovický potok Brook, South Bohemia; **kidney**, brain, liver, spleen, gills	Feb 1991/Oct 1996
4436/III	*Rutilus rutilus* Linnaeus, 1758; Lužnice River, South Bohemia; **kidney**, brain, liver, spleen, gills	Dec 1990/Nov 1995
3668/IV	*Silurus glanis* Linnaeus, 1758; Skalice River, Central Bohemia; **spleen**, liver, brain	Sep 1989/Aug 1996
G1	Environmental strain; water sample, unknown locality in Czech Republic	Apr 1995

* Clonal strains were isolated from the organs indicated in bold type.



FIGURES 2–10. Representative trophozoites of clones isolated from fish tissues. Figure 7 is a scanning electronmicrograph and Figure 9 is a phase-contrast micrograph. All the rest are Nomarski interference pictures ×1,630. **2.** 4465/I. **3.** 4422/II. **4.** 4800/V. **5.** 4339/I. **6.** 4528/II. **7.** 4706/I. **8.** 3668/IV. **9.** G1. **10.** 4178/I.

included 44 bases of which 121 were variable and 91 were phylogenetically informative.

All isolates also were examined by FISH using a 30-base-long rhodamine-labeled oligonucleotide (5′-rhodamine-GCTGCCAAAACCAACTGAAAATAGGAGGAC-3′) that is very specific for the 18S rDNA sequence type T4 (D. R. Stothard, J. M. Schroeder-Diedrich, J. Hay, D. V. Seal, and T. J. Byers, unpubl. obs.). The probe, synthesized by Amitof Biotech, Inc. (Boston, Massachusetts), hybridizes to both 18S rRNA and 18S rDNA.

RESULTS

Isolation of *Acanthamoeba*

Tissue samples were obtained from a total of 833 individual fishes. Naked amoebae of several genera were isolated from tissues of 73 asymptomatic fishes. *Acanthamoeba* was found in 14 of these fishes, i.e., the prevalence was 1.7%. The present study reports on 10 strains of *Acanthamoeba* spp. that were successfully cloned and cultured, both on agar plates and in liquid media (Table I). One environmental isolate of *Acanthamoeba* (G1) also was included.

Morphological studies

The distinctive trophozoite and cyst morphology permitted easy identification of cultured *Acanthamoeba* strains at the genus level, but morphological differences were of limited use for classifying individual clones at the subgeneric level.

In general, trophozoite morphology at the light microscope and electron microscope levels did not differ noticeably among the clones. The same types of trophozoites that are demonstrated in Figures 2–10 could be observed in all clones when prepared for observation in the same way. The same applied to trophozoites whether cultured on agar plates or in liquid media. Trophozoites from liquid media and those washed from bacterized agar plates observed in hanging drops did not differ after being placed for a short period of time in a drop of amoeba saline or water on coverslips.

TABLE II. Cyst diameters of *Acanthamoeba* clones isolated from fishes.

Clone	Culture medium	Mean* (μm)	Median (μm)	SD (μm)	Range (μm)	*t*	*P*	Months in storage
3668/IV	Agar	12.95	13.00	1.28	10–15			
	BCS	13.65	13.00	2.46	11–20	−1.13	0.27	6
4178/II	Agar	11.20	11.00	1.44	9–14			
	BCS	14.35	14.00	2.60	11–20	−4.74	0.000029	12
4337/II	Agar	13.85	14.00	1.35	11–16			
	BCS	20.85	21.00	1.95	17–24			
4339/II	Agar	13.55	13	1.28	12–16			
	BCS	16.65	16.50	2.21	13–21			
4422/II	Agar	9.85	10.00	1.66	7–13			
	BCS	12.45	12.50	2.19	9–16	−4.20	0.00014	11
4436/III	Agar	17.65	17.5	2.46	13–22			
	SCGYEM	21.60	21.50	2.48	17–26			
4465/I	Agar	13.20	13.00	1.74	10–16			
	BCS	13.85	13.00	2.81	10–20	−1.49	0.144	12
4528/II	Agar	11.50	11.00	1.88	9–15			
	BCS	13.35	12.50	3.03	9–20	−2.32	0.025	6
4706/I	Agar	13.90	14.00	1.41	12–17			
	BCS	16.45	15.50	3.91	11–23	−2.74	0.009	11
4800/V	Agar	12.10	12.00	1.65	9–15			
	BCS	15.10	15.00	2.86	11–21	−4.05	0.0002	0.5

* Average of 20 cyst diameters.

Cyst morphology separated our clones from morphological groups I and III and identified them as morphological group II (MG2) as defined by Pussard and Pons (1977), De Jonckheere (1987), and Visvesvara (1991). Cyst morphometrics are summarized in Table II. The average cyst diameters of clones were relatively small (≤21.6 μm). In individual clones, diameters were different for cysts formed on agar plates and in liquid media. The mean diameters of cysts formed both in liquid BCS medium and on agar surfaces were determined for 7 clones. The diameters of BCS cysts were significantly larger ($P \leq 0.05$) for all of the 7 clones except 3668/IV and 4465/I. The cysts formed in BCS had greater diameters even after up to 12 mo of storage in water. Two-way analysis of variance demonstrated that the culture medium has a constant effect on cyst size, whereas variation among clones is random.

Cyst morphology observed in the fresh state (Figs. 11–20) and after silver staining divided the clones into 2 subgroups of MG2. The more numerous subgroup (MG2a), clones 4465/I, 4706/I, 4339/I, 4178/I, 4337/II, 4436/III, 4422/II, and 4528/II, had slightly wrinkled ectocysts (Figs. 11–18). No sign of reticulation appeared when silver impregnation methods were applied. When focused in a single plane, the endocyst was mostly polygonal, occasionally appearing triangular or quadrangular with corners that indicated the location of cyst pores. The second subgroup (MG2b), clones 3668/IV and 4800/V, was characterized by stellate endocysts with 6–9 arms seen in a single plane (Figs. 19–20). The outline between arms was gently concave. The silver staining methods revealed endocyst arms and cyst pores at various levels.

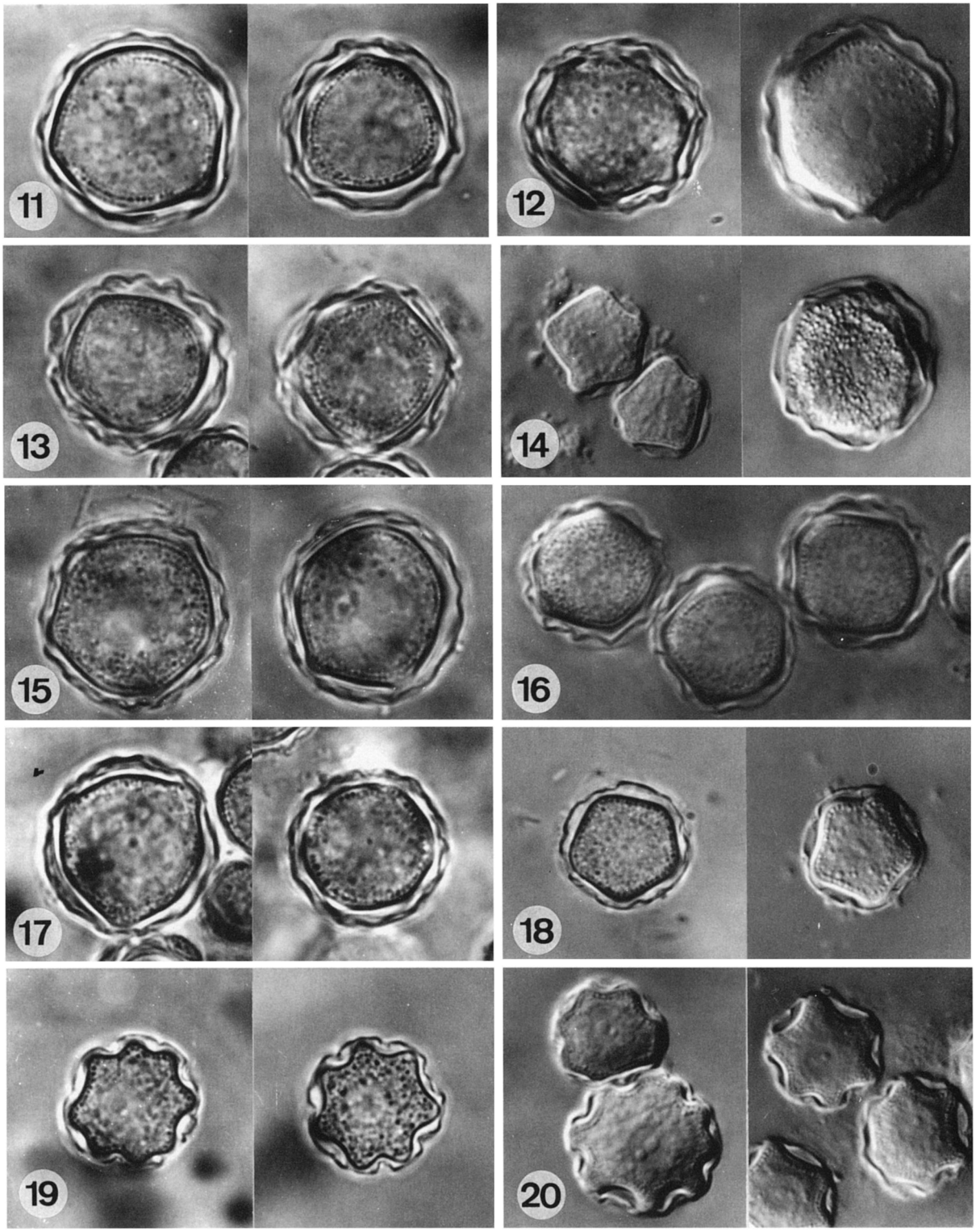
The cysts of the 8 clones forming MG2a, with the polygonal endocyst shape, resembled those of the Neff strain of *Acanthamoeba castellanii* (CCAP 1505/1a) and of *A. polyphaga* as depicted by Page (1988). The 2 clones of MG2b, with the stellate endocysts, were similar to the type strain of the genus, *A. castellanii* (CCAP/1501/2a) as depicted by Page (1988), as well as to *Acanthamoeba griffini* H37 (Ledee et al., 1996). Thus, morphology of the cysts gave no clear answer as to species identification within MG2.

Molecular studies

Analysis of nuclear small subunit rRNA gene sequences (18S rDNA) has previously succeeded in subdividing MG1–3 into 12 rDNA sequence types (Stothard et al., 1998). Three of these types, i.e., T3, T4, and T11, are included in MG2. Thus,18S rDNA sequence analysis was used in an attempt to determine whether all 3 MG2 sequence types were represented. All the fish isolates except 4800, which was unavailable, were used for the analysis and sequences were determined for cloned PCR products from each of these strains.

Evolutionary distances among the clonal strains were determined using comparisons of partial sequences of 18S rDNA rather than the much longer sequences that were the basis of previous phylogenetic
analyses (Gast et al., 1996; Stothard et al., 1998). Primers specific for the genus *Acanthamoeba* were used to amplify sequences coding for the region from stem E23–2 to stem 30 of the 18S rRNA (Stothard et al., 1998). This sequence segment, the diagnostic fragment (DF), encompasses both conserved and highly variable regions totaling about 460 bp but has less phylogenetically useful information than the sequences previously used by Stothard et al. (1998).

Three different DF sequences (DFS) were obtained for the fish isolates, i.e., DFS1, 4436 (Fig. 16); DFS2, 4178 (Fig. 14), 4528 (Fig. 18), 3668 (Fig. 19); and DFS3, 4465 (Fig. 11), 4706 (Fig. 2), 4339 (Fig. 13), 4337 (Fig. 15), and 4422 (Fig. 17). Relationships among DF sequences from the fish isolates, the environmental isolate G1, and 50 previously sequenced *Acan-*



FIGURES 11–20. Representative cysts from clones isolated from fish tissues. Each clone is represented by 2 micrographs ×1,630. **11.** 4465/I. **12.** 4706/I. **13.** 4339/I. **14.** 4178/I. **15.** 4337/II. **16.** 4436/III. **17.** 4422/II. **18.** 4528/II. **19.** 3668/IV. **20.** 4800/V.

thamoeba strains (Stothard et al., 1998) were then examined by a neighbor-joining distance analysis. Sequences from T7, T8, and T9 strains used in the previous study were excluded from this analysis because they are very different from the other sequences in the DF region. Their inclusion would add excessive ambiguity to interpretation of strain relationships. Figure 21 indicates that all the fish-infecting strains belong in a clade that includes T3, T4, and T11 isolates and has a bootstrap value of 96%. The clade is equivalent to MG2. The analysis suggests that the fish isolates all have T4 sequences, but low bootstrap

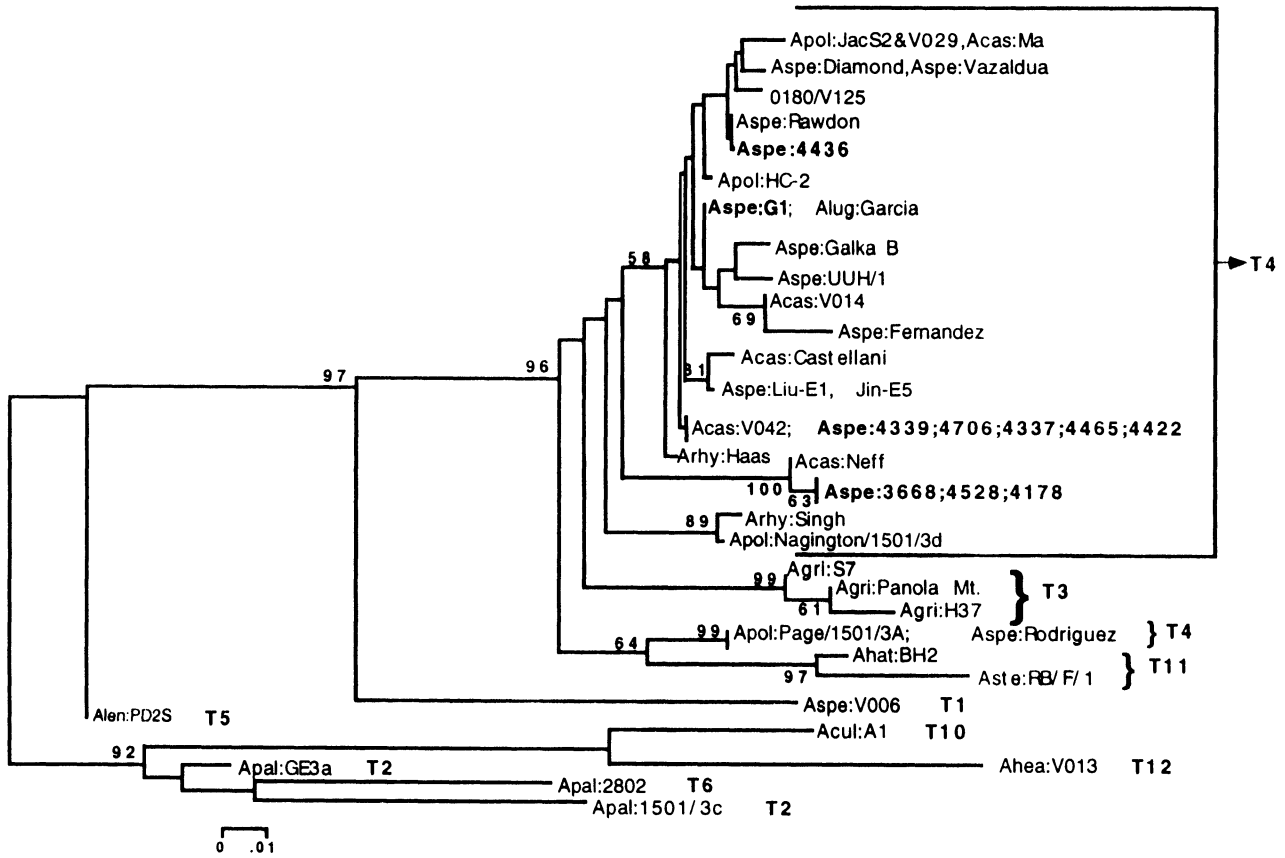


FIGURE 21. Phenogram of relationships between the fish clones and 53 other *Acanthamoeba* strains based on sequences of the 18S rDNA diagnostic fragment. The phenogram represents a neighbor-joining analysis calculated using MEGA (Kumar et al., 1983) with the Kimura 2-parameter correction. The tree is unrooted and the distance scale is the number of base pair substitutions calculated per base pair. Bootstrap values were determined from 1,000 runs and only are indicated if they are >50%. Species names have been abbreviated as follows: Acas (*A. castellanii*), Acul (*A. culbertsoni*), Agri (*A. griffini*), Ahat (*A. hatchetti*), Ahea (*A. healyi*), Alen (*A. lenticulata*), Alug (*A. lugdunensis*), Apal (*A. palestinensis*), Apol (*A. polyphaga*), Arhy (*A. rhysodes*), Aspe (*Acanthamoeba* species), and Aste (*A. stevensoni*). The fish isolates and the environmental isolate used in this study are indicated by bold font. The other isolates are described in Stothard et al. (1998). GenBank accession numbers for the DF sequences of the fish isolates are AF140711–13 for 4339, 4706, and 4337, respectively, and AF140715–21 for 4528, 4178, 3668, 4465, 4422, G1, and 4436, respectively. All DF sequences other than those of the fish isolates are extracted from the complete 18S rDNA sequences used by Stothard et al. (1998). That reference includes GenBank accession numbers for all the additional sequences used in this study.

values indicate that branching patterns within the T3/T4/T11 clade are ambiguous. The ambiguity could have been reduced by sequencing of additional sites, but this turned out to be unnecessary in this particular case due to the availability of a sequence-type-specific fluorescent oligonucleotide probe. Fluorescent in situ hybridization with this probe that repeated tests have demonstrated hybridizes only to acanthamoebae with the T4 sequence type (D. R. Stothard, J. M. Schroder-Diedrich, J. Hay, D. V. Seal, and T. J. Byers, unpubl. obs) strongly stained the 9 clonal strains from fishes and the G1 isolate. Thus, the fish isolates and the G1 strain all belong to the T4 subset of MG2.

DISCUSSION

The acanthamoebae that could be isolated from tissues by the methods used here were found in feral fishes with a prevalence of 1.7% (14/833). Morphological analysis of cysts and sequencing of the 18S rDNA DF from trophozoites both indicate that the fish-infecting acanthamoebae belong to MG2. The objective of this study was to use a minimal amount of DNA sequence to determine which lineage or lineages the fish isolates belonged to. Although DF sequence variation was sufficient to confirm the conclusion from morphological studies that the fish isolates all were from the MG2 clade, it was insufficient for determining which of the 3 MG2 sequence types were involved. The specific type(s) involved could have been achieved with additional sequencing. However, the availability of a highly specific T4 FISH probe made it possible to demonstrate that all isolates belonged to T4 without the need for further sequencing. Of course, if the fish isolates had belonged to T3 or T11, the available probe would only have been able to rule out T4. The availability of a complete panel of FISH probes specific for the different sequence types could have entirely eliminated the need for any sequencing. However, this panel is still in development and highly reliable probes will not be available until more information on sequence variation within the 12 18S rDNA sequence types has been obtained.

Identification of the 3 distinct T4 sequences DFS1–3 among the fish isolates suggests subdivisions differing somewhat from those identified by cyst morphology that distinguishes only the

2 subdivisions MG2a and MG2b. Further clarification of relationships between sequence type and morphological subdivisions awaits further resolution of phylogenetic branching patterns within T4.

Relationships between subgroups of T4 and named species also are unresolved (Stothard et al., 1998). Previous investigators most frequently identified *Acanthamoeba* isolates from fish as *A. polyphaga.* The type strain of this species is no longer available, but rDNA sequences for various other isolates of this species indicate that it belongs in T4. Thus, the observations of these prior workers are in agreement with the present results. In several cases, *A. griffini* (De Jonckheere, 1987), *A. culbertsoni* (Im, 1990) and *A. royreba* (Moura et al., 1992) also have been isolated from fish. The type strains for *A. griffini* and *A. culbertsoni* currently are classified in T3 and T10, respectively, whereas, classification of *A. royreba* is being reevaluated (G. Booton, unpubl. obs.). Confirmed T4 sequence types also have been found in the large majority of isolates from human *Acanthamoeba* keratitis (Stothard et al., 1998). As in the case of fishes, *A. griffini* (Ledee et al., 1996) and *A. culbertsoni* (Mannis et al., 1986) have been reported infrequently in human infection. It remains to be seen whether the predominance of the T4 strains in fish tissues and human eye infections is due to greater invasiveness of this group or, simply, to greater abundance in nature.

The shorter diagnostic fragment sequences used here have proved nearly as useful as complete 18S rDNA sequences for identification of *Acanthamoeba* isolates. Although the DF sequences failed to differentiate reliably the closely related MG2 strains T3, T4, and T11, they did clearly differentiate this complex from representatives of each of the other known sequence types (Fig. 21). Differentiation among the 3 sequence types within the complex can be achieved, however, by sequencing of the near-complete 18S RNA gene as previously demonstrated (Stothard et al., 1998). We also have shown here the utility of FISH as a diagnostic tool for *Acanthamoeba.* The T4 probe is complementary to a sequence that lies within the DF. We have shown elsewhere that it is highly specific for T4 isolates based on analysis of sequences from more than 50 *Acanthamoeba* strains and on hybridization tests using representatives of all sequence types (D. R. Stothard, J. M. Schroeder-Diedrich, J. Hay, D. V. Seal, and T. J. Byers, unpubl. obs.). Development of similar specific FISH probes for other sequence types or subtypes could make this approach more convenient than DNA sequencing for most diagnostic work.

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