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Genetic Analysis of Forty Isolates of *Acanthamoeba* Group III by Multilocus Isoenzyme Electrophoresis

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Summary. Two clinical and 32 environmental *Acanthamoeba* isolates belonging to Group III were compared with reference strains of *A. culbertsoni*, *A. healyi*, *A. jacobsi*, *A. lenticulata*, *A. royreba* and *A. palestinensis* by multilocus isozyme analysis. Genetically useful data were produced for 21 loci. The two principle clusters corresponded to *A. jacobsi* (20 isolates) and *A. lenticulata* (up to 9 isolates). *A. jacobsi* was the least variable species and was widely distributed, with isolates from Australia, Fiji, Japan, New Guinea, New Zealand and Britain. Most other isolates occurred as single zymodemes, not closely related to each other. No new isolate could be assigned to *A. culbertsoni*, *A. healyi*, or *A. royreba*. A clinical isolate from an Australian GAE case, AC118, was the only 44°C-tolerant isolate not referable to *A. jacobsi* and appears to be a new species.

Key words: *Acanthamoeba* species, biogeography, isozyme analysis.

INTRODUCTION

Acanthamoeba species are small, generally bactiverous amoebae with robust cysts, which occur widely in soil, freshwater and marine environments. While they are undoubtedly freeliving, they are responsible for several distinct human diseases, including granulomatous amoebic encephalitis (GAE), the corneal infection amoebic keratitis and disseminated cutaneous ulcerations (Martinez and Janitsche 1985, Visvesvara and Stehr-Green 1990). *Acanthamoeba* species may also be indirectly significant for health through their ability to harbour and assist in the dissemination of pathogenic bacteria such as *Legionella pneumophila* (Rowbotham 1986, Berk *et al.* 1998).

The genus *Acanthamoeba* includes about 25 nominal species, in three sub-generic groups distinguished on the basis of cyst morphology (Pussard and Pons 1977). Most species were originally described from single strains and few of the descriptions adequately distinguished interspecific differences from intraspecific variation. The morphological characteristics used in many of the descriptions overlap considerably and may vary with culture conditions (Sawyer 1971). New clinical and environmental isolates are therefore difficult to assign to species. In any case, genetic analysis, by isozymes and more recently by 18s rDNA sequencing, has shown that

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many of these organisms are not sufficiently distinct to be treated as species (De Jonckheere 1983, Stothard *et al.* 1998). A classification of *Acanthamoeba* species into a series of sequence types (Gast *et al.* 1996, Stothard *et al.* 1998) is growing in acceptance.

In the study reported here, we examined two clinical isolates and 32 environmental isolates of *Acanthamoeba* Group III, using multi locus isoenzyme electrophoresis to compare them with reference strains representing six species or sequence types. By concentrating on a putative subgeneric group and characterising a large number of new isolates, we hoped to discern patterns that have not been apparent in other studies. The following specific questions were addressed: Is genetic diversity discontinuous within *Acanthamoeba* group III, so that the boundaries between species are clear? Is there agreement between isozyme and sequence analysis, particularly regarding the genetic soundness of 'species' represented by the reference strains? Finally, can the clinical and environmental isolates be identified with previously characterised lineages, or do they include new divergent groups?

MATERIALS AND METHODS

Origin and cultivation of strains. All *Acanthamoeba* group III isolates came from the culture collection of the Australian Water Quality Centre, with the reference strains of six currently recognised Group III species acquired originally from the American Type Culture Collection (ATCC). Thirty-two environmental isolates from nine countries were chosen to represent diverse geographic sources and habitats, for comparison with two Australian clinical isolates (Table 1). The type strain of *A. palestinensis*, the species with the lowest temperature tolerance and already known to be divergent from other Group III species, was used as an outgroup.

Isolates were grown initially on non-nutrient agar plates spread with a lawn of penicillin- and streptomycin-sensitive *Escherichia coli* (*NNA/E. coli*) and incubated at 30°C (*A. palestinensis*) or 37°C. To establish axenic cultures, actively growing trophozoites were transferred into 10 ml of Peptone Yeast extract Glucose medium (PYG, ATCC Medium 712) incorporating penicillin and streptomycin to eliminate the *E. coli*, and incubated at the same temperature. For isozyme extracts, amoebae were harvested in late log phase (10^5 to 10^6 cells per ml). Strains that were difficult to grow axenically were enriched on plate cultures by re-spreading with additional *E. coli* and incubating for a further 16-20 h before harvesting.

Temperature tolerance. *NNA/E. coli* plates were inoculated with trophozoites from active cultures and incubated at temperature intervals of one or two degrees between 35°C and 44°C for 48-72 h. The highest temperature at which growth occurred was recorded as the temperature tolerance of the isolate.

Isozyme extracts. Amoebae from plate cultures, suspended in quarter-strength Ringer solution (Oxoid), or from PYG medium were concentrated by centrifugation at 12,000 rcf for 5 min. The pellet was resuspended in an equal volume of homogenising solution (14 mM 2-mercaptoethanol, 120 µM NADP) and subjected to three 0.3 s pulses of sonication (Branson 250 sonicator). Unwanted cell debris was removed by centrifugation for 5 min at 12,000 rcf and the supernatant transferred to haematocrit tubes in 5-10 µl aliquots for storage at -20°C.

Electrophoresis. Enzyme extracts were separated on cellulose acetate gels (Cellogel, Chemetron, Milan, Italy) and stained using a range of conditions found suitable for various enzymes from *Naegleria* species (Table 2; Adams *et al.* 1989, Robinson *et al.* 1992). The resulting zymograms were interpreted in a Mendelian framework. That is, the smallest number of loci consistent with diploidy was assumed, and allelic states were designated in light of the known tertiary structures of particular enzyme families.

Data analysis. The allelic data were converted to a matrix of pairwise differences (% of loci without shared alleles) from which neighbor-joining and UPGMA dendrograms were derived using the PHYLIP package (Felsenstein 2002). A second matrix was calculated for differences weighted to take account of the genetic diversity at each locus (i.e. placing greater weight on less variable loci). The frequency distribution of all pairwise distances (weighted and unweighted) was plotted to aid recognition of any genetic discontinuity that might correspond to species boundaries.

RESULTS

Among the reference strains, only *A. palestinensis* could not grow at least at 40°C (Table 1). The environmetal isolates included a large set that grew at 44°C and others that could not grow above 40°C or 42°C. The strain from the keratitis case grew at 40°C, while the strain from the GAE case could grow at 44°C. Of the 44°C-tolerant isolates, only AC118 would grow in PYG medium. *A. palestinensis* and all 40°C and 42°C-tolerant isolates grew axenically.

Allelic profiles for the 40 *Acanthamoeba* group III isolates are presented in Table 3. The isolates fell into 33 distinguishable zymodemes, with patterns that could be interpreted as heterozygous at 12 of the 21 loci. Two zymodemes consisted of four isolates each (AC088, AC194, AC178, AC201 and AC062, AC085, AC087, AC138) and one of two isolates (AC080, AC229). The relationships among the zymodemes are presented as a neighbor-joining dendrogram (Fig. 1), with the indistinguishable isolates represented by AC088, AC062 and AC080. Twenty replications of the neighbor-joining computation with randomised input order gave dendrograms with identical topology. The UPGMA dendrogram (not shown) was more sensitive to input order, with replicates varying principally in the branching order of the closer relationships within *A. jacobsi* and *A. lenticulata*.

Table 1. *Acanthamoeba* isolates employed in this study.

AWQC No	Genus	Species	TT[a] (°C)	Geographic origin	Habitat	Cross reference
Reference strains						
AC-001	*Acanthamoeba*	*culbertsoni*	40	India	Cell culture contam.	ATCC 30171
AC-005	*Acanthamoeba*	*jacobsi*	44	USA	Marine	ATCC 30732
AC-006	*Acanthamoeba*	*lenticulata*	40	France	Swimming pool	ATCC 30841
AC-014	*Acanthamoeba*	*palestinensis*	35	Israel	Soil	ATCC 30870
AC-020	*Acanthamoeba*	*healyi*	40	USA	Fresh water	ATCC 30866
AC-023	*Acanthamoeba*	*royreba*	42	USA	Cell culture contam.	ATCC 30884
Clinical isolates						
AC-118	*Acanthamoeba*		44	Australia	Clinical (GAE)	
AC-230	*Acanthamoeba*		40	Australia	Clinical (keratitis)	
Environmental isolates						
AC-062	*Acanthamoeba*		44	Australia	Water supply	
AC-080	*Acanthamoeba*		44	Australia	Water supply	
AC-081	*Acanthamoeba*		44	Australia	Sediment	
AC-082	*Acanthamoeba*		44	PNG	Well	
AC-083	*Acanthamoeba*		44	Australia	Sediment	
AC-084	*Acanthamoeba*		44	Australia	Sediment	
AC-085	*Acanthamoeba*		44	Australia	Sediment	
AC-087	*Acanthamoeba*		44	Australia	Water supply	
AC-088	*Acanthamoeba*		44	Australia	Water supply	
AC-089	*Acanthamoeba*		40	Australia	Water supply	
AC-090	*Acanthamoeba*		40	Australia	Soil	
AC-091	*Acanthamoeba*		40	Australia	Soil	
AC-092	*Acanthamoeba*		42	Australia	Soil	
AC-126	*Acanthamoeba*		40	Australia	Air	
AC-137	*Acanthamoeba*		44	Australia	Fresh water	
AC-138	*Acanthamoeba*		44	Australia	Sediment	
AC-140	*Acanthamoeba*		42	Madagascar	Fresh water	
AC-141	*Acanthamoeba*		40	Australia	Airborne dust	
AC-148	*Acanthamoeba*		40	Madagascar	Fresh water	
AC-153	*Acanthamoeba*		40	Madagascar	Fresh water	
AC-159	*Acanthamoeba*		44	Japan	Fresh water	
AC-167	*Acanthamoeba*		40	Bangladesh	Sediment	
AC-168	*Acanthamoeba*		42	Bangladesh	Sediment	
AC-178	*Acanthamoeba*		44	NZ	Sediment	
AC-180	*Acanthamoeba*		40	Bangladesh	Sediment	
AC-181	*Acanthamoeba*		44	Indonesia	Sediment	
AC-194	*Acanthamoeba*		44	UK	Sediment	
AC-201	*Acanthamoeba*		44	Australia	Fresh water	
AC-227	*Acanthamoeba*		44	Fiji	Fresh water	
AC-229	*Acanthamoeba*		44	Australia	Fresh water	
AC-235	*Acanthamoeba*		44	Australia	Sediment	
AC-236	*Acanthamoeba*		40	Australia	Sediment	

[a] - Temperature tolerance

The genetic diversity at individual loci varied from 0.1623 to 0.7034 (Table 4). The frequency distribution of genetic distances was bimodal in nature, whether or not the distance calculations were weighted for the diversity of individual loci (Fig. 2), though a significant number of pairwise comparisons fell in the 'trough' from 20 to 50%. Dendrograms based on weighted distances (not shown) were nearly identical in topology, although some branch lengths were changed.

All 44°C-tolerant environmental isolates cluster closely with the reference strain of *A. jacobsi* (AC005), with fixed differences among the 20 isolates ranging from 0-19% (average 6%). Intraspecific variation was observed at ADH, HK, LAP and ME, while GOT1 ap-

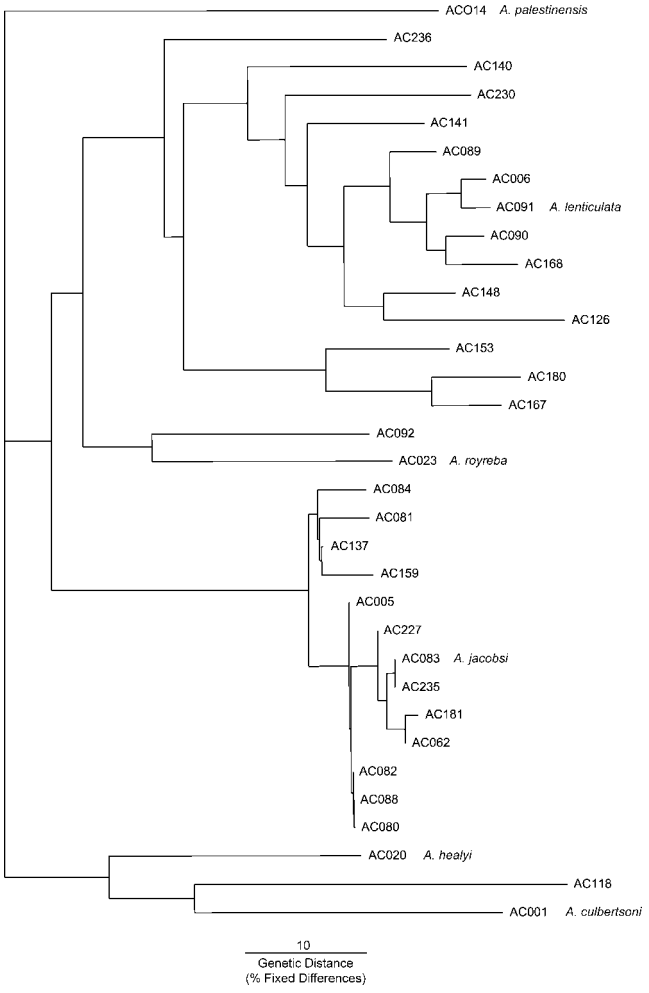

Fig 1. Neighbour-joining tree of genetic relationships among 40 *Acanthamoeba* isolates using isozyme data for 21 loci. 20 replicates of randomised input order gave identical topology.

peared to be universally heterozygous. The only other 44°C-tolerant isolate, the clinical strain AC118, is not closely related to *A. jacobsi*, nor to any other reference strain or environmental isolate.

A looser clustering occurs with *A. lenticulata*, in which four new isolates cluster within 15% of the type strain; further isolates clustered at progressively greater distances, including a discernible sub-group consisting of AC148 and AC126. The only other significant clustering is AC167 with AC180 (14%), then with AC153 (average 28%). The remaining isolates, including reference strains of described species, occur as single zymodemes isolated by distances greater than 40%. The fixed differences among the reference strains, excluding the outgroup *A. palestinensis*, ranged from 55-80% (mean 67%).

DISCUSSION

Relationships among *Acanthamoeba* strains

A number of published studies of *Acanthamoeba* have used isozymes to determine the variation among strains, usually without formal genetic analysis (Tyndall *et al.* 1979, De Jonckheere 1983, Costas and Griffiths 1984, Daggett *et al.* 1985). All but the De Jonckheere study relied strongly on peptidases, esterases and phosphatases, enzymes known to exhibit intraspecific polymorphism in metazoan and other protozoan groups. Not surprisingly, several authors noted the difficulty of recognising affinities within their sets of strains, to the extent that there has been a proposal to abandon the formal binomial nomenclature for this genus (Costas and Griffiths 1984). Other authors have erected new *Acanthamoeba* species partly on the basis of polymorphic isozymes (Sawyer *et al.* 1992, Nerad *et al.* 1995). At least two such 'species' are among those now considered synonyms of better established names, as they fall in the same sequence type (Stothard *et al.* 1998).

By contrast, in the unrelated amoeba genus *Naegleria*, the species-level taxonomy has proved extremely robust. As in *Acanthamoeba*, more than 20 species have been named since pathogenic strains were first isolated in the 1960s, but most species characterisations have included extensive isozyme and/or rDNA sequence data. The most thorough isozyme studies have been extensive in two dimensions (large number of strains and numerous loci) and employed formal genetic interpretation to distinguish polymorphic loci from fixed differences between species (Adams *et al.* 1989, Pernin and Cariou 1989, Robinson *et al.* 1992, Dobson *et al.* 1997). As a consequence, there is almost complete congruence between the clusters recognised on isozyme and sequence criteria.

In the present study, we used the multi-locus approach that has succeeded in studies of *Naegleria* to collect information about the diversity and divergence of strains and the relative scales of intra- and interspecific variation. The analysis suggests that genetic variation among Group III *Acanthamoeba* is at least partly discontinuous. In the dendrogram drawn from the data (Fig. 1), the tight cluster of isolates that group with

Table 2. Enzymes examined and conditions used.

Abbreviation	Enzyme	E.C. No.[a]	Loci scored	Buffer[b]	Run time (min)
Acn	Aconitase	4.2.1.3	1	B	90
Adh	Alcohol dehydrogenase	1.1.1.1	1	A	90
Enol	Enolase	4.2.1.11	1	B	90
Est	Esterase	3.1.1.1	1	C	105
Fdp	Fructose-diphosphatase	3.1.3.11	2	B	90
Gapd	Glycerald-phosphate dehydrogenase	1.2.1.12	1	B	90
Got	Aspartate aminotransferase	2.6.1.1	2	B	90
Gpi	Glucose-phosphate isomerase	5.3.1.9	1	C	120
Hex	Hexosaminidase	3.2.1.30	1	A	120
Hk	Hexokinase	2.7.1.1	1	B	90
Idh	Isocitrate dehydrogenase	1.1.1.42	1	A	120
Lap	Leucine aminopeptidase	3.4.11.1	1	A	75
Mdh	Malate dehydrogenase	1.1.1.37	1	A	105
Me	Malic enzyme	1.1.1.40	1	B	90
Mpi	Mannose-phosphate isomerase	5.3.1.8	1	C	75
Np	Nucleoside phosphorylase	2.4.2.1	1	B	60
PepB	Peptidase Leucine-leucine-leucine	3.4.11/13	1	C	75
6Pgd	6-Phosphogluconate dehydrogenase	1.1.1.44	1	B	90
Pgm	Phosphoglucomutase	5.2.4.2	1	B	75

[a] Enzyme commission number (E.C.)
[b] Running buffers from Richardson *et al.* 1986.

AC005 confirm that *A. jacobsi* is a sound genetic entity, deserving species status. Strains corresponding to *A. lenticulata* cluster less tightly: the four new isolates within 15% of the type strain certainly belong to this species but its boundary is difficult to define with confidence, considering the repeated branching beyond 25% difference. We assign the clinical isolate AC230 tentatively to *A. lenticulata*, to be confirmed by sequence data. Several corneal isolates from North America have been identified as *A. lenticulata* (Visvesvara and Stehr-Green 1990), as have isolates from nasal swabs collected from healthy soldiers in Turkey (De Jonckheere and Michel 1988), in each case by different criteria from those developed in this paper. The new cluster represented by AC153, AC167 and AC180 is also rather poorly defined, since there is no major discontinuity in the internode distances linking it ultimately to *A. lenticulata*.

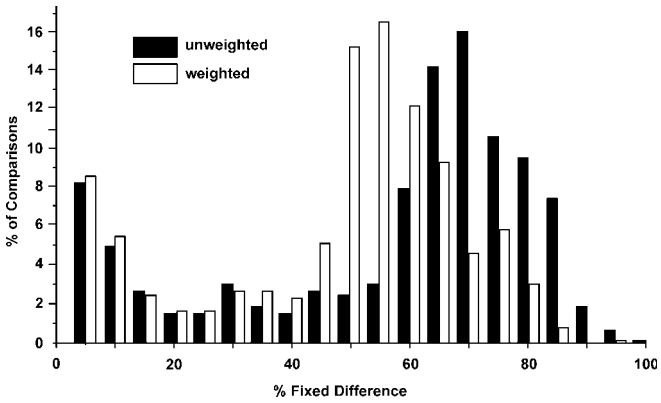

Fig. 2. Frequency distribution of all pairwise genetic distances between *Acanthamoeba* isolates, measured as the percentage of loci without shared alleles. Histograms based on un-weighted distances and on distances weighted using the genetic diversity at each locus.

While the frequency distribution of genetic distances is certainly bimodal (Fig. 2), the low-difference (left-hand) peak is composed almost entirely of interstrain comparisons within *A. jacobsi* and *A. lenticulata*. The high-difference (right-hand) peak is inflated by comparisons among the large number of single, isolated zymodemes and between these and individual strains of the species with multiple representatives. Comparisons in the trough, which is narrowed but not deepened by weighting of individual loci, reflect the continuous branching in some regions of the dendrogram.

In the genus *Naegleria*, all described species for which more than one strain is available cluster in the manner of *A. jacobsi*, as do numerous un-named species (Adams *et al.* 1989, Robinson *et al*. 1992). Internode distances are generally long (greater than 50% difference) and few intermediate relationships have

Table 3. Allelic profiles for *Acanthamoeba* zymodemes.

ZYMODEME	ACN2	ADH	ENOL	EST2	FDP1	FDP2	GAPD	GOT1	GOT2	GPI	HEX	HK	IDH	LAP	MDH	ME	MPI	NP	PEPB	6PGD	PGM
ACO14	cc	dd	—	dd	aa	aa	bb	dd	cc	ee	dd	bb	dd	ff	dd	bb	cc	ee	—	dd	dd
AC001	cc	aa	aa	cc	bb	aa	aa	aa	cc	bb	cc	bc	aa	cc	aa	cc	—	bb	bb	cc	cc
AC006	cc	ee	bb	ee	ee	dd	—	cc	bb	dd	ee	cc	cc	cc	bb	cc	bb	cc	cc	dd	dd
AC020	aa	cc	bb	ee	bb	dd	—	dd	cc	cc	cc	bb	aa	aa	bb	cc	aa	bb	ae	dd	cc
AC126	cc	ee	bb	ee	ee	cc	bb	cc	bb	ff	aa	dd	cc	cc	bb	cc	cc	cc	cc	cc	dd
AC141	dd	cc	bb	ee	ee	dd	—	cc	bb	ee	aa	dd	cc	cc	cc	cc	cc	cc	cc	dd	dd
AC148	dd	ee	bb	ee	ee	bd	bb	cc	bb	ff	cc	dd	cc	cc	dd	cc	cc	cc	cc	bd	dd
AC153	dd	cc	bb	ee	ee	dd	bb	dd	bb	ee	cc	ab	ff	ce	dd	cc	dd	cc	ee	cc	cc
AC167	dd	ee	bb	ee	ee	aa	—	cc	bb	dg	cc	ab	ff	cc	dd	cc	dd	cc	cd	ac	cc
AC180	dd	ee	bb	ee	ee	aa	bb	cc	bb	ff	cc	bb	ff	cc	dd	cc	dd	dd	cc	cc	bb
AC230	bb	cc	bb	ee	ee	dd	—	cc	bb	ee	ee	cc	cc	dd	bb	aa	cc	cc	cc	dd	dd
AC236	bd	cc	bb	dd	ee	dd	bb	bb	bb	dd	cc	dd	ee	ee	cc	cc	dd	dd	cc	dd	dd
AC089	cc	ee	bd	ee	ee	—	bb	cc	bb	dd	ee	bb	cc	cc	cc	cc	cc	cc	cc	dd	dd
AC090	cc	ee	bd	ee	ee	—	—	cc	bb	dd	ee	bb	cc	cc	bb	cc	cc	cc	cc	dd	dd
AC091	cc	ee	bb	ee	ee	dd	bb	cc	bb	dd	ee	cc	cc	dd	bb	cc	bb	cc	cc	dd	dd
AC023	aa	cc	aa	ad	dd	dd	—	aa	bb	dd	aa	dd	bb	ce	cc	dd	aa	aa	df	dd	dd
AC092	dd	dd	bd	dd	—	—	—	bb	—	dd	—	dd	bb	bb	cc	dd	aa	aa	cc	dd	cc
AC140	dd	ee	cc	ee	ee	bd	bb	cc	bb	ff	bb	dd	cf	dd	cc	ee	dd	cc	cc	bd	dd
AC168	cc	ee	bb	ee	ee	dd	—	cc	bb	dd	ee	cc	cc	cc	bb	aa	cc	cc	cc	dd	dd
AC005	bb	ce	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	bb	cc	cc	ee	bb	aa	dd	dd	—
AC062	bb	ce	dd	ee	cc	dd	bb	bb	ac	ee	cc	cc	bb	cc	cc	ee	bb	aa	dd	dd	bb
AC080	bb	cc	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	bb	cc	cc	ee	bb	aa	dd	dd	bb
AC081	cc	cc	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	bb	bd	cc	ee	bb	aa	dd	dd	bb
AC082	bb	ce	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	bb	cc	cc	ee	bb	aa	dd	dd	bb
AC083	bb	cc	dd	ee	cc	dd	bb	bb	ac	ee	cc	cc	bb	cc	cc	ee	bb	aa	dd	dd	bb
AC084	bb	cc	dd	ee	cc	dd	bb	bb	ac	ee	cc	dd	bb	bd	cc	ee	bb	aa	dd	dd	bb
AC137	bb	ce	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	bb	bd	cc	ee	bb	aa	dd	dd	bb
AC159	bb	—	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	bb	dd	cc	dd	bb	aa	dd	dd	bb
AC181	bb	ee	dd	ee	cc	dd	bb	bb	ac	ee	cc	cc	—	cc	cc	ee	bb	aa	dd	dd	bb
AC227	bb	ce	dd	ee	cc	dd	—	bb	ac	ee	cc	bc	bb	cc	cc	ee	bb	aa	dd	dd	bb
AC235	bb	cc	dd	ee	cc	dd	bb	bb	ac	ee	cc	cc	bb	cc	cc	ee	bb	aa	dd	dd	bb
AC088	bb	ce	dd	ee	cc	dd	bb	bb	ac	ee	cc	bb	—	cc	cc	ee	bb	aa	dd	dd	bb

Table 4. Genetic diversity of *Acanthamoeba* Group III isolates at 21 loci.

Locus	No. alleles	Genetic diversity
ACN2	4	0.6876
ADH	5	0.6210
ENOL	4	0.6166
EST2	5	0.3221
FDP1	5	0.6429
FDP2	4	0.3557
GAPD	2	0.1623
GOT1	5	0.6583
GOT2	3	0.6383
GPI	7	0.6606
HEX	5	0.5655
HK	4	0.6937
IDH	6	0.7034
LAP	6	0.6000
MDH	4	0.5949
ME	5	0.6620
MPI	4	0.6885
NP	5	0.6340
PEPB	6	0.6339
6PGD	4	0.3557
PGM	4	0.6548

been observed. Most strains which appeared initially as single, isolated zymodemes proved to be representatives of cohesive clusters, when additional strains were characterised (Robinson *et al*. 1992, Dobson *et al*. 1997). By analogy, isolates in this study that occur as single zymodemes separated by large internode distances could be treated as sole representatives of distinct species, on the criterion of distance alone. Indeed, they include the reference strains of the described species *A. culbertsoni, A. healyi* and *A. royreba*. Whether, on study of additional strains, these species prove to be as cohesive as *Acanthamoeba jacobsi* or as loosely defined as *A. lenticulata* remains to be determined.

Concordance of isozyme and sequence data

There is growing acceptance of a classification that uses the sequence of the 18s rDNA gene to assign *Acanthamoeba* strains to a series of sequence types or ribotypes (currently numbering 14), encompassing most of the reference strains for named species (Gast *et al*. 1996, Stothard *et al*. 1998). For the traditional binomial taxonomy to be reconciled with this classification, many 'species' would become synonyms, but *A. culbertsoni, A. healyi, A. lenticulata* and *A. palestinensis* comprise discrete sequence types. These same strains were also genetically distant from each other in the present study, suggesting that there will be a general concordance of isozyme and rDNA sequence data in *Acanthamoeba*, as there is in *Naegleria*. As a corollary, we predict that other clusters and divergent single zymodemes in our dendrogram will prove to be discrete sequence types. New sequence data for *Acanthamoeba jacobsi* (Hewett *et al*. 2003) fulfill this expectation.

Among the single zymodemes likely to represent new sequence types, the clinical strain AC-118, isolated from a GAE infection (Harwood *et al*. 1988), deserves special mention. A large number of 44°C-tolerant strains were analysed here, in anticipation that an environmental source of this virulent pathogen would be found. Instead, all such isolates were identified unambiguously as *A. jacobsi*. AC-118 appears therefore to represent a new, rare and virulent *Acanthamoeba* species and will be described in detail elsewhere. It seems likely that one of the other single zymodemes will correspond to the recently described sequence type T14, which was not available to us for comparison.

Biogeography and ecology

An interesting debate is developing over the global geographic distribution of protozoan species and their diversity. On one side, Finlay and others argue that the small size of free-living microorganisms, their high numbers and growth potential make opportunities for dispersal to new habitats so frequent that their occurrence should invariably be cosmopolitan (Finlay and Fenchel 1999). Limited opportunities for local speciation would constrain the total number of protozoan species. On the other side, Foissner has described numerous ciliate species from Australia and South America whose distributions appear to correspond with biogeographic regions based on ancient geological events (Foissner 1999). The 'cosmopolitan school' argues that such reports of endemic species reflect a sampling bias, resulting from the intense study of a particular area and sometimes of specialised habitats. The 'endemicity school' argues that in Europe, the ciliate fauna in particular has been so well studied that the apparent absence of certain species is likely to be genuine. It is also argued that reliance on morphological criteria for species identification obscures genuine geographic patterns (De Jonckheere 2002). The two schools differ vastly in their estimates of the total number of protozoan species (Finlay and Fenchel 1999).

The data presented here show that *A. jacobsi* is a widely distributed and rather uniform species, occurring in Asia, Australia, Europe, the Pacific region and in

North America. Strains corresponding closely to *A. lenticulata* occur in Australia and Asia, as well as in Europe and North America. These species clearly fit the cosmopolitan model. Beyond these species, the current study raises questions about the distribution of *A. culbertsoni*, *A. healyi*, *A. royreba* and the species represented by AC118 that will only be answered with further environmental studies. Whichever of these models eventually prevails, it appears from the organisms studied here that the species diversity of *Acanthamoeba* is still not completely described.

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