

Phylogenetic relationships among *Acanthamoeba* spp. based on PCR-RFLP analyses of mitochondrial small subunit rRNA gene

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Abstract: We investigated the value of mitochondrial small subunit rRNA gene (mt SSU rDNA) PCR-RFLP as a taxonomic tool for *Acanthamoeba* isolates with close inter-relationships. Twenty-five isolates representing 20 species were included in the analysis. As in nuclear 18S rDNA analysis, two type strains (*A. astronyxis* and *A. tubiashi*) of morphological group 1 diverged earliest from the other strains, but the divergence between them was less than in 18S riboprinting. *Acanthamoeba griffini* of morphological group 2 branched between pathogenic (*A. culbertsoni* A-1 and *A. healyi* OC-3A) and nonpathogenic (*A. palestinensis* Reich, *A. pustulosa* GE-3a, *A. royreba* Oak Ridge, and *A. lenticulata* PD2S) strains of morphological group 3. Among the remaining isolates of morphological group 2, the Chang strain had the identical mitochondrial riboprints as the type strain of *A. hatchetti*. AA2 and AA1, the type strains of *A. divionensis* and *A. paradivionensis*, respectively, had the identical riboprints as *A. quina* Vil3 and *A. castellanii* Ma. Although the branching orders of *A. castellanii* Neff, *A. polyphaga* P23, *A. triangularis* SH621, and *A. lugdunensis* L3a were different from those in 18S riboprinting analysis, the results obtained from this study generally coincided well with those from 18S riboprinting. Mitochondrial riboprinting may have an advantage over nuclear 18S rDNA riboprinting because the mt SSU rDNAs do not seem to have introns that are found in the 18S genes of *Acanthamoeba* and that distort phylogenetic analyses.

Key words: *Acanthamoeba*, phylogenetic relationship, PCR-RFLP, mitochondrial SSU rDNA

INTRODUCTION

Acanthamoeba spp. are the most ubiquitous protozoa among environments such as soil,

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air, freshwater and ocean sediment (De Jonckheere, 1991). Sometimes the amoebae can be carriers or vectors in the dispersion and dissemination of pathogenic microbes such as *Legionella* spp., *Listeria* spp., *Mycobacterium* spp., and *Vibrio* spp. (Jadin, 1973; Ly and Muller, 1990; Field, 1991; Thom et al., 1992; Kong and Chung, 1996; Chung et al., 1997). Furthermore, several members of the genus *Acanthamoeba* play direct roles as human pathogens causing life-threatening granulomatous amebic encephalitis and vision-threatening keratitis (Sisson et al.,

1995).

Recent molecular systematic studies provided a new classification of the genus *Acanthamoeba*. Stothard et al. (1998) classified 53 strains of *Acanthamoeba* spp. into 12 *Rns* sequence types based on sequence analysis of 18S rDNA. The results obtained were inconsistent with some previous species designation and indicated that the taxonomy of *Acanthamoeba* should be revised. Although 18S rDNA sequence data are useful for identification and differentiation of *Acanthamoeba* isolates, generation of sequence data is too labor-intensive and expensive for routine identification and classification of *Acanthamoeba*. Riboprinting, PCR-RFLP analysis of 18S rDNA, is a simpler and less expensive substitute for 18S rDNA sequence analysis (Clark and Diamond, 1997). Chung et al., (1998) applied riboprinting to subgenus classification of *Acanthamoeba*. The results were congruent with those of sequence analysis. However, 18S rDNA of some *Acanthamoeba* strains contains an intron, which therefore can generate misleading results (Chung et al., 1998).

Mitochondrial DNA (mtDNA) is a molecular clone within mitochondriate eukaryotic cells like *Acanthamoeba*. Although variation of the mitochondrial genome size limited the availability of the RFLP analysis of whole mitochondrial genome as a phylogenetic tool, usefulness of mitochondrial small subunit (SSU) rDNA remains unknown. In this study, we evaluated PCR-RFLP of *Acanthamoeba* mitochondrial SSU rDNA as a phylogenetic tool and compared the results with those from nuclear riboprinting analysis.

MATERIALS AND METHODS

Acanthamoeba

Twenty-five strains including 20 type strains of the genus *Acanthamoeba* which were previously assigned to 20 species, were obtained from ATCC (Table 1). They were cultured axenically in Proteose peptone-Yeast extract-Glucose (PYG) or Proteose peptone-Yeast extract-Glucose-Cysteine (PYGC) media at 25°C or 37°C (Chung et al., 1996).

Extraction of mtDNA

mtDNA of *Acanthamoeba* was extracted using phenol and phenol/chloroform (1:1) and recovered by precipitation with cold absolute ethanol in the presence of sodium acetate (Yagita and Endo, 1990). The DNA was stored at -20°C until used as template DNA for PCR reactions.

PCR amplification of mitochondrial SSU rRNA coding DNA (SSU rDNA)

The primers for the PCR, FP16; 5'-TTGTATAAACAATCGTTGGGTTTATT-3', RP16; 5'-GTCCAGCAGCAGGTTCCCCTACCGCTA-3', are designed to hybridize to highly conserved sequences at the extreme 5' (P3) and 3' (P4) termini of mitochondrial SSU rDNA (Lonergan and Gray, 1994). The PCR was done using a kit of premixed PCR reagents (Bioneer, Chongwon, Korea) and a thermal cycler (Perkin Elmer Cetus, CA, USA). Fifty μ l scale premix was dissolved in 47 μ l of distilled water and vortexed vigorously. One μ l of template DNA and 1 μ l of each primer (25 nmol concentration) were added to the premix and mixed thoroughly. The whole mixture was covered with 20 μ l of mineral oil. Each PCR process was performed through 30 cycles at 94°C for 1 min, 58°C for 30 sec, and 72°C for 2 min followed by an extension time of 10 min. After amplification, the mineral oil was removed by treating with chloroform and the amplified DNA was stored at -20°C until used (Chung et al., 1998).

RFLP of mitochondrial SSU rDNA

The PCR products of 25 strains were electrophoresed in a 1.5% agarose gel with DNA size standards (Amplisize, Biorad, CA, USA). Eight kinds of restriction endonucleases (*Hae* III, *Hsp92* I, *Hinf* I, *Dde* I, *Mbo* I, *Sau96* I, *Rsa* I, and *Taq* I; Poscochem, Seoul, Korea), which have recognition sequences of four nucleotides, were used to generate comparative riboprints. The amplified DNA (1 μ g) was digested with 5-10 units of the enzymes for 2 hr with recommended buffers at 37°C, except for *Taq* I (67°C), and electrophoresed in a 2.5% agarose gel (agarose 3: Nusieve 1) for 1.5 hr. To differentiate small DNA fragments, which

Table 1. List of 25 strains of *Acanthamoeba* spp. analysed in this study

Number	Strain	ATCC No.	Virulence	Environmental source	Geographic source	Reference	Former species designation
1	Castellani	30011	+	yeast culture	England	Douglas (1930)	<i>A. castellanii</i>
2	L3a	50240	+	swimming pool	France	Pussard & Pons (1977)	<i>A. lugdunensis</i>
3	Vil3	50241	nd	swimming pool	France	Pussard & Pons (1977)	<i>A. quina</i>
4	Jones	30461	+	keratitis	USA	Jones et al. (1975)	<i>A. polyphaga</i>
5	SH621	50254	nd	human feces	France	Pussard & Pons (1977)	<i>A. triangularis</i>
6	Nagington	30873	+	keratitis	England	Nagington et al. (1974)	<i>A. polyphaga</i>
7	Singh	30973	-	soil	England	Singh (1952)	<i>A. rhyssodes</i>
8	1652	50253	-	soil	Morocco	Pussard & Pons (1977)	<i>A. mauritanensis</i>
9	AA2	50238	-	soil	France	Pussard & Pons (1977)	<i>A. divionensis</i>
10	AA1	50251	-	soil	France	Pussard & Pons (1977)	<i>A. paradvionensis</i>
11	Neff	30010	-	soil	USA	Neff (1957)	<i>A. castellanii</i>
12	Ma	50370	+	keratitis	USA	Ma et al. (1981)	<i>A. castellanii</i>
13	P23	30871	-	fresh-water	USA	Page (1967)	<i>A. polyphaga</i>
14	Chang	30898	+	fresh water	USA	Byers et al. (1990)	<i>A. castellanii</i>
15	BH-2	30730	+	ocean sediment	USA	Sawyer et al. (1977)	<i>A. hatchetti</i>
16	RB-F-1	50388	nd	ocean sediment	USA	Sawyer et al. (1993)	<i>A. stevensoni</i>
17	S-7	30731	+	beach-bottom	USA	Sawyer (1971)	<i>A. griffini</i>
18	A-1	30171	+	tissue culture	USA	Singh & Das (1970)	<i>A. culbertsoni</i>
19	OC-3A	30866	+	GAE	USA	Moura et al. (1992)	<i>A. healyi</i>
20	GE-3a	50252	-	swimming pool	France	Pussard & Pons (1977)	<i>A. pustulosa</i>
21	Reich	30870	-	soil	Israel	Reich (1933)	<i>A. palestinensis</i>
22	PD2S	30841	+	swimming pool	France	Molet & Ermoloeff-Braun (1976)	<i>A. lenticulata</i>
23	Oak Ridge	30884	+	cell culture	USA	Willært et al. (1978)	<i>A. royreba</i>
24	Ray & Hayes	30137	nd	soil	USA	Ray & Hayes (1954)	<i>A. astronyxis</i>
25	OC-15C	30867	nd	river	USA	Lewis & Sawyer (1979)	<i>A. tubiashi</i>

were unclear in the agarose gel, digested samples were electrophoretically separated in 12% polyacrylamide gels (Chung et al., 1998). The gels were stained with ethidium bromide and photographed under an UV transilluminator.

Sequence divergence estimates were calculated by the Nei and Li equation (1979) from a fragment comigration dataset which was obtained by comparison of the riboprints of 25 *Acanthamoeba* strains with each other. A phylogenetic tree was reconstructed by the unweighted pair group method with arithmetic averages (UPGMA) using the computer program Phylip, version 3.5 (Felsenstein, 1989).

RESULTS

Only a single PCR product was amplified from each *Acanthamoeba* strain (Fig 1). The PCR products of 25 *Acanthamoeba* strains were approximately 1,550 nucleotides in length as predicted.

Mitochondrial riboprint patterns of 25 *Acanthamoeba* strains using eight restriction endonucleases are shown schematically in Fig 2. The patterns of *A. divionensis* AA2 and *A. paradivionensis* AA1 were identical with all enzymes used. The Chang strain which was previously assigned to *A. castellanii* had identical riboprints with BH-2, the type strain of *A. hatchetti*. Vil3, the type strain of *A. quina* had identical mitochondrial riboprints with the Ma strain of *A. castellanii*.

Proportions of co-migrating fragments and estimated genetic distance between amoebae strains are shown in Table 2, and the dendrogram constructed based on the estimated genetic distance is presented in Fig 3. *Acanthamoeba astronyxis* Ray & Hayes and *A.*

tubiashi OC-15C of morphological group 1 (Pussard and Pons, 1977) were the earliest branching strains. Among the remaining strains, the most divergent were *A. culbertsoni* A-1 and *A. healyi* OC-3A, which belong to morphological group 3 and are known to be highly virulent. *Acanthamoeba griffini*, with an intron in its nuclear 18S rDNA (Gast et al., 1996; Ledee et al., 1996) branched between virulent strains A-1 and OC-3A and avirulent strains GE-3a, Reich Oak Ridge and PD2S of morphological group 3. Strain P23 which is the neotype strain of *A. polyphaga* of morphological group 2 formed a cluster with the Oak Ridge strain (the type strain of *A. royreba*; morphological group 3). RB-F-1, the type strain of *A. stevensoni* was the earliest branching strain among the remaining 15 strains of morphological group 2. The Castellani, Ma, Vil3, and Jones strains formed a cluster separated from the remaining ten strains. The Nagington, 1652, L3a, Singh, AA2 and AA1 strains formed another cluster.

DISCUSSION

Analysis of mtDNA, using either sequence or RFLP data, may permit the identification of problematic specimens (Wells and Sperling, 1999). Among mitochondrial genes, the SSU rRNA gene has been widely and successfully applied to investigation of relationships at subgeneric levels as well as among very distantly related groups (Flook and Rowell, 1997). Although RFLP analyses of the mitochondrial genome have been used in phylogenetic and taxonomic studies of *Acanthamoeba* spp. (Byers et al., 1983; Yagita and Endo, 1990), the results were somewhat ambiguous partly because of the quite

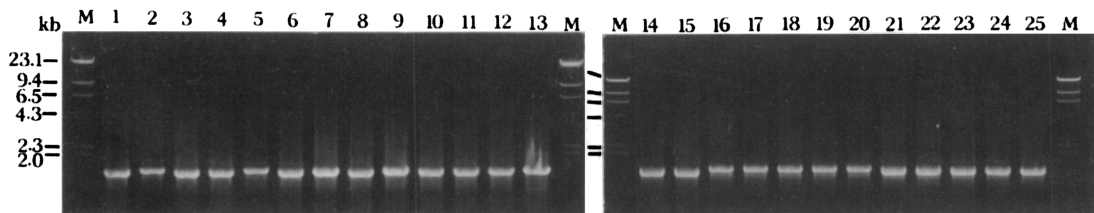


Fig. 1. Agarose gel electrophoretic patterns of PCR products from mitochondrial small subunit rRNA gene of 25 *Acanthamoeba* strains. *Hind* III digested λ phage DNA was used as the DNA size marker (M).

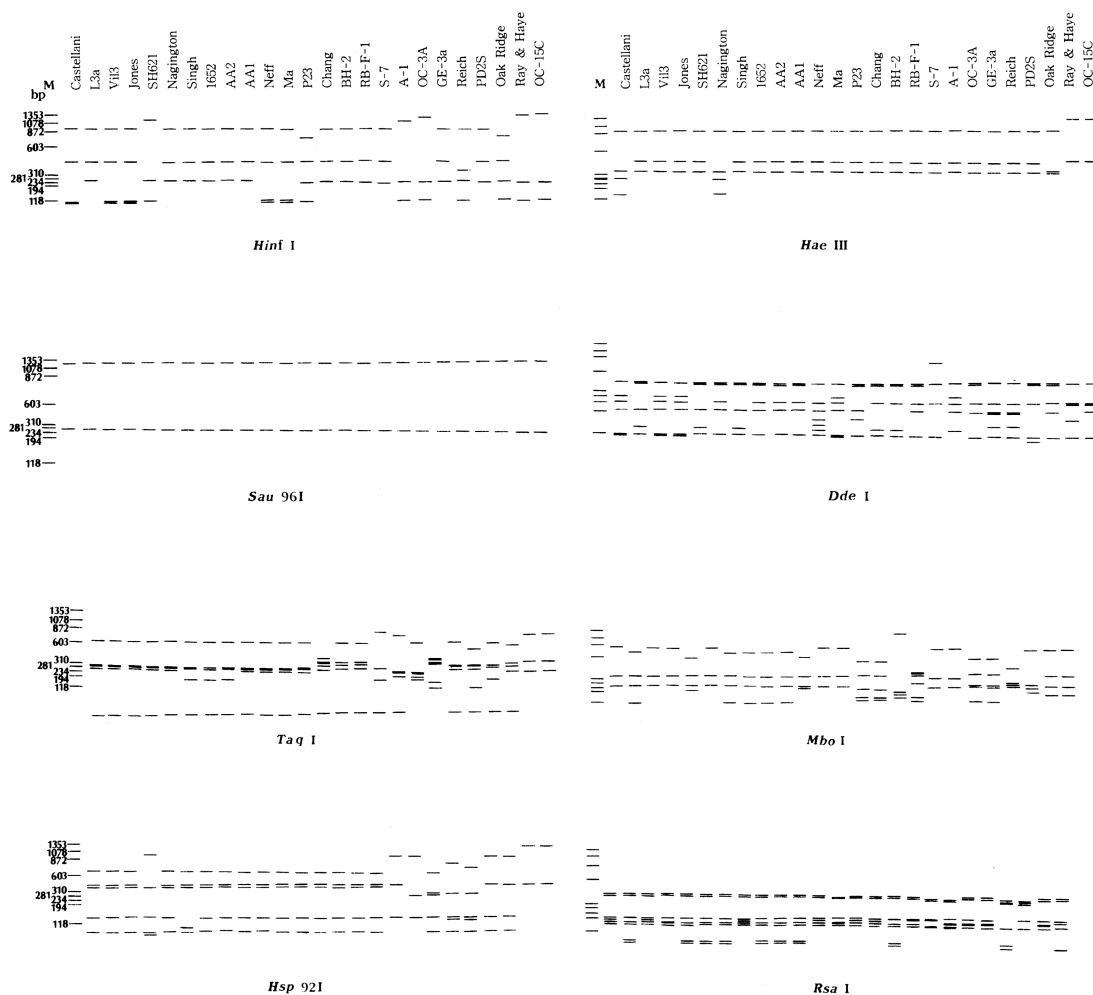


Fig. 2. Schematic representation of mt riboprints of 25 strains of *Acanthamoeba* by eight restriction enzymes. *Hae* III digested Φ X174 DNA was used as the size marker (M).

difference of genome size among strains. Recently, riboprinting of nuclear 18S rDNA has been widely applied to classification of morphologically indistinguishable protozoa including *Acanthamoeba* and recognized as an inexpensive and rapid tool for taxonomic and phylogenetic studies (Clark et al., 1995; Clark and Diamond, 1997; Chung et al., 1998). However, some strains of *Acanthamoeba* have introns in their nuclear 18S rDNA (Stothard et al., 1998) and identification of these strains can be problematic with nuclear riboprinting. PCR-RFLP analysis of mt SSU rDNA (mt riboprinting) has not been used previously for phylogenetic and taxonomic studies of *Acanthamoeba*.

In this study, we obtained similar but not identical results from mt riboprinting as we obtained previously with nuclear 18S riboprinting. A dendrogram constructed in this study (Fig 3) coincided generally with the grouping of Pussard and Pons (1977) based on the morphological features of cysts. Some of the results strongly support the observations of Chung et al. (1998) based on nuclear 18S riboprinting. For example, AA2 and AA1, the type strains of *A. divionensis* and *A. para-divionensis* respectively, showed the identical mt riboprints with all restriction enzymes used. Both strains were isolated simultaneously from soil of France by the same group of investigators (Pussard and Pons,

Table 2. Proportional homologous fragments (values above the diagonal) and estimates of genetic divergence (values below the diagonal) among 25 *Acanthamoeba* strains based on the mitochondrial SSU rDNA PCR-RFLP

No.	Species	Strain	II																							I		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		24	25
1	<i>A. castellanii</i>	Castellani	—	52/68	64/67	58/67	50/68	56/68	50/68	54/67	54/68	54/68	58/71	64/67	48/68	52/68	52/68	52/68	44/67	40/66	38/64	40/68	44/69	42/71	38/66	42/66	30/60	30/60
2	<i>A. lugdunensis</i>	L3a	0.067	—	54/67	50/67	60/68	58/68	66/68	60/71	54/67	50/68	58/68	60/71	54/67	50/68	58/68	58/68	52/67	42/66	34/64	36/68	50/69	46/71	46/66	42/62	28/60	30/60
3	<i>A. quina</i>	Vil3	0.011	0.054	—	60/66	52/66	52/67	56/66	56/67	56/67	60/70	66/66	60/66	50/67	54/67	46/66	42/65	40/63	42/67	46/68	44/70	40/65	42/65	42/65	32/59	32/59	
4	<i>A. polyphaga</i>	Jones	0.036	0.073	0.024	—	48/67	48/67	48/67	50/66	52/67	52/67	56/70	60/66	48/67	48/67	48/67	42/66	40/65	42/63	42/67	48/68	46/70	40/65	42/65	30/59	30/59	
5	<i>A. trisrigularis</i>	SH621	0.077	0.031	0.060	0.083	—	56/68	62/68	56/67	62/68	62/68	64/71	54/67	50/68	56/68	56/68	52/67	40/66	34/64	36/68	50/69	46/71	46/66	44/66	28/60	30/60	
6	<i>A. polyphaga</i>	Nagington	0.049	0.049	0.063	0.083	0.049	—	62/69	60/68	62/69	62/69	54/72	50/68	44/69	52/69	52/69	50/68	40/67	30/65	38/69	44/70	40/72	46/67	38/67	26/61	28/61	
7	<i>A. rhysodes</i>	Singh	0.077	0.007	0.063	0.023	0.027	—	62/67	64/68	64/68	64/68	58/71	52/67	48/68	56/68	56/68	52/67	40/66	32/64	38/68	48/69	44/71	48/66	38/66	26/60	28/60	
8	<i>A. mauritanensis</i>	1652	0.054	0.028	0.041	0.069	0.045	0.031	0.019	—	62/67	62/67	54/70	56/66	48/67	58/67	58/67	50/66	44/65	32/63	42/67	48/68	44/70	46/65	40/65	28/59	30/59	
9	<i>A. ditionensis</i>	AA2	0.058	0.007	0.045	0.063	0.023	0.027	0.015	0.019	—	68/68	60/71	56/67	50/68	58/68	58/68	54/67	44/66	34/64	40/68	50/69	46/71	48/66	44/66	30/60	32/60	
10	<i>A. parathionensis</i>	AA1	0.058	0.007	0.045	0.063	0.023	0.027	0.015	0.019	0	—	60/71	56/67	50/68	58/68	58/68	54/67	44/66	34/64	40/68	50/69	46/71	48/66	44/66	30/60	32/60	
11	<i>A. castellanii</i>	Nelf	0.051	0.042	0.039	0.056	0.026	0.072	0.051	0.065	0.042	0.042	—	60/70	50/71	54/71	54/71	50/70	40/69	38/67	40/71	50/72	48/74	44/69	44/69	32/63	30/63	
12	<i>A. castellanii</i>	Ma	0.011	0.054	0	0.024	0.054	0.077	0.063	0.041	0.045	0.045	0.039	—	50/67	54/67	54/67	46/66	42/65	40/63	42/67	46/68	44/70	40/65	42/65	32/59	32/59	
13	<i>A. polyphaga</i>	P23	0.087	0.077	0.073	0.083	0.077	0.112	0.087	0.083	0.077	0.077	0.088	0.073	—	50/67	50/67	44/66	38/65	36/63	48/67	40/68	40/70	36/65	48/65	32/59	30/59	
14	<i>A. castellanii</i>	Chang	0.067	0.040	0.054	0.083	0.049	0.071	0.049	0.036	0.040	0.040	0.068	0.054	0.073	—	68/68	50/67	48/66	30/64	38/68	50/69	46/71	40/66	42/66	30/60	30/60	
15	<i>A. hachetti</i>	BH-2	0.067	0.040	0.054	0.083	0.049	0.071	0.049	0.036	0.040	0.040	0.068	0.054	0.073	0	—	50/67	48/66	30/64	38/68	50/69	46/71	40/66	42/66	30/60	30/60	
16	<i>A. stevensoni</i>	RB-F-1	0.105	0.063	0.090	0.113	0.063	0.077	0.063	0.069	0.054	0.054	0.084	0.090	0.101	0.073	0.073	—	42/65	28/63	38/67	40/68	38/70	42/65	42/65	30/59	32/59	
17	<i>A. griffithi</i>	S-7	0.125	0.113	0.109	0.121	0.125	0.129	0.125	0.098	0.101	0.101	0.136	0.109	0.148	0.080	0.080	0.109	—	34/62	30/66	42/67	38/69	36/64	36/64	28/58	28/58	
18	<i>A. culbertsoni</i>	A-1	0.130	0.158	0.114	0.101	0.158	0.193	0.173	0.169	0.158	0.158	0.142	0.114	0.140	0.189	0.189	0.203	0.150	—	36/64	30/65	30/67	28/62	36/62	26/56	26/56	
19	<i>A. headlyi</i>	OC-3A	0.133	0.159	0.117	0.117	0.159	0.149	0.145	0.117	0.133	0.133	0.143	0.117	0.083	0.145	0.145	0.142	0.197	0.144	—	36/69	40/71	34/66	40/66	30/60	30/60	
20	<i>A. pushtulosa</i>	GE-3a	0.112	0.081	0.098	0.087	0.081	0.116	0.091	0.087	0.081	0.081	0.091	0.098	0.133	0.081	0.081	0.133	0.117	0.193	0.163	—	64/72	40/67	36/67	24/61	26/61	
21	<i>A. palestinensis</i>	Reich	0.131	0.109	0.116	0.105	0.109	0.147	0.120	0.116	0.109	0.109	0.112	0.116	0.140	0.125	0.109	0.153	0.149	0.201	0.143	0.029	—	36/69	36/69	26/63	26/63	
22	<i>A. lenticalata</i>	PD2S	0.138	0.090	0.121	0.121	0.090	0.094	0.080	0.086	0.080	0.080	0.108	0.121	0.148	0.125	0.109	0.144	0.199	0.166	0.129	0.163	—	40/64	18/58	22/58	22/58	
23	<i>A. royceba</i>	OakRidge	0.113	0.113	0.109	0.109	0.101	0.142	0.138	0.121	0.101	0.101	0.112	0.109	0.076	0.113	0.113	0.109	0.144	0.136	0.125	0.155	0.163	0.118	—	30/58	32/58	
24	<i>A. astronyxus</i>	Ray & Hayes	0.173	0.191	0.153	0.169	0.191	0.213	0.209	0.186	0.173	0.173	0.169	0.153	0.153	0.173	0.173	0.169	0.182	0.192	0.173	0.233	0.221	0.293	0.165	—	46/52	
25	<i>A. tubiashii</i>	OC-15C	0.173	0.173	0.153	0.169	0.173	0.195	0.191	0.169	0.157	0.157	0.185	0.153	0.169	0.173	0.173	0.153	0.182	0.192	0.173	0.213	0.221	0.242	0.149	0.031	—	

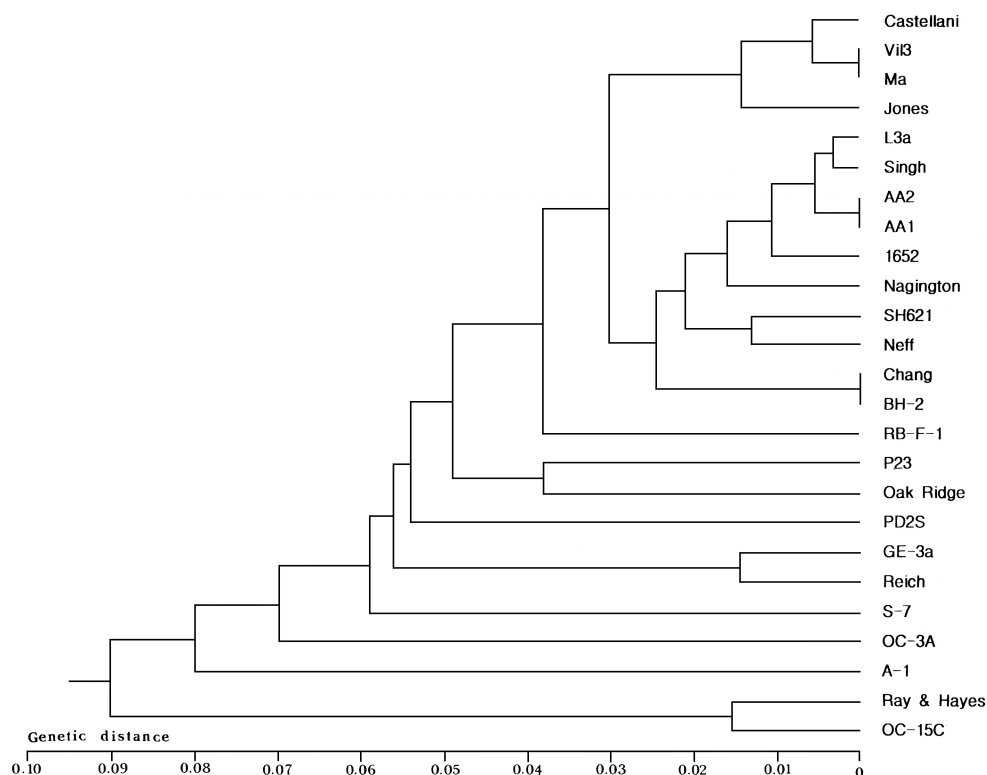


Fig. 3. Dendrogram of 25 *Acanthamoeba* strains constructed by UPGMA method using Phylip ver. 3.5 based on genetic divergence estimates.

1977), and differed slightly in morphology. Furthermore, they showed the same mt genome RFLP, alloenzyme (unpublished data) and nuclear riboprint patterns (Chung et al., 1998). As Chung et al. (1998) proposed, both strains should be a species and closely related with the Singh strain, the type strain of *A. rhysodes*. Also, the Chang strain, previously assigned to *A. castellanii* revealed the identical mt riboprints with BH-2, the type strain of *A. hatchetti*. Both strains were pathogenic for mice (unpublished data) and were found to be closely related based on nuclear riboprinting. In addition, these strains had lots of co-migrating DNA fragments on mtDNA RFLP analyses and revealed very similar alloenzyme patterns (unpublished data).

In contrast to our previous studies, L3a, the type strains of *A. lugdunensis*, which clustered with the Castellani, Vi13, Jones, and Ma strains in a dendrogram reconstructed by 18S riboprinting, formed a cluster with the Nagington, 1652, Singh, AA2, and AA1 strains

here. The Neff, SH621, and P23 strains also branched at different positions in this dendrogram. Estimated divergence between *A. astronyxis* and *A. tubiashi* was lower in this study than in 18S riboprinting analysis. These differences may result from a difference in evolutionary rate between the mitochondrial and nuclear genes.

In spite of the likely difference in evolutionary rate between the mitochondrial and nuclear genomes, similar results with mt and nuclear riboprint patterns were observed with several strains assigned to different species. These results suggest that the taxonomic status of some species of the genus *Acanthamoeba* should be revised. This study has shown that mt riboprinting is another tool for the phylogenetic study of *Acanthamoeba*. In addition, the mt riboprinting confirms the basic features of the evolutionary history of the genus as revealed in studies of the 18S rDNA. One important advantage is that it avoids problems caused by isolates with introns

within nuclear 18S rDNA.

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