The Korean Journal of Parasitology
Vol. 36, No. 2, 69-80, June 1998

Subgenus classification of *Acanthamoeba* by riboprinting

Dong-Il CHUNG*, Hak-Sun YU, Mee-Yul HWANG, Tae-Ho KIM, Tae-Ook KIM, Ho-Cheol YUN and Hyun-Hee KONG

Department of Parasitology, Kyungpook National University School of Medicine, Taegu 700-422, Korea

Abstract: Subgenus classification of *Acanthamoeba* remains uncertain. Twenty-three reference strains of *Acanthamoeba* including 18 (neo)type-strains were subjected for classification at the subgenus level by riboprinting, PCR/RFLP analysis of 18S rRNA gene (rDNA). On the dendrogram reconstructed on the basis of riboprint analyses, two type-strains (*A. astronyxis* and *A. tubiashi*) of morphological group 1 diverged early from the other strains and were quite distinct from each other. Four type-strains of morphological group 3, *A. culbertsoni*, *A. palestinensis*, *A. healyi* were considered taxonomically valid, but *A. pustulosa* was regarded as an invalid synonym of *A. palestinensis*. Strains of morphological group 2 were classified into 6 subgroups. Among them, *A. griffini* which has an intron in its 18S rDNA was the most divergent from the remaining strains. *Acanthamoeba castellanii* Castellani, *A. quina* Vil3, *A. lugdunensis* L3a, *A. polyphaga* Jones, *A. triangularis* SH621, and *A. castellanii* Ma strains belonged to a subgroup, *A. castellanii* complex. However, *A. quina* and *A. lugdunensis* were regarded as synonyms of *A. castellanii*. The Chang strain could be regarded as *A. hatchetti*. *Acanthamoeba mauritaniensis*, *A. divionensis*, *A. paradivionensis* could be considered as synonyms of *A. rhysodes*. Neff strain was regarded as *A. polyphaga* rather than as *A. castellanii*. It is likely that riboprinting can be applied for rapid identification of *Acanthamoeba* isolated from the clinical specimens and environments.

Key words: *Acanthamoeba*, subgenus classification, PCR/RFLP, 18S rRNA gene

INTRODUCTION

Acanthamoeba spp. are ubiquitous among human environments such as soil, air, fresh- and sea-water (De Jonckheere, 1991). Some species of the genus *Acanthamoeba* are recognized as human pathogens causing life-threatening granulomatous amebic encephalitis and vision-threatening keratitis (Sisson *et al.*, 1995). Sometimes, the amoebae can play roles as carriers or vectors in the dispersion and dissemination of pathogenic microbes such as *Mycobacterium* spp., *Legionella* spp., *Vibrio* spp. and *Listeria* spp. (Jadin, 1973; Ly and Muller, 1990; Field, 1991; Thom *et al.*, 1992).

In spite of the medical importance of the genus *Acanthamoeba*, subgenus classification of *Acanthamoeba* spp. is still problematic. Although identification at the genus level could be easily accomplished by morphology, profound variation of the cyst morphology within a clone (Page, 1988) limited the availability of morphology alone as a taxonomic tool (Visvesvara, 1991). Alloenzyme and mitochondrial DNA RFLP analyses have

• Received 20 March 1998, accepted after revision 16 May 1998.
• This study was financially supported by a Grant-in Aid for Korea Science and Engineering Foundation in 1996 (No. 961-0716-100-1).
* Corresponding author (e-mail: dichung@bh.kyungpook.ac.kr)

been applied, but the results were highly polymorphic among strains assigned to the same species (Kong *et al.*, 1995; Chung *et al.*, 1996).

Comparison of highly conserved sequences, which have a central function, can reveal phylogenetic relationships between organisms (Sogin *et al.*, 1989). The nuclear small-subunit rRNA gene is a good example and has been the most widely used for phylogenetic study of organisms. Recently, after sequence analysis of 18S rDNA, Stothard *et al.* (1998) classified 53 strains of *Acanthamoeba* spp. into 12 Rns sequence types. The results obtained were inconsistence with some previous species designations and indicated that the taxonomy of *Acanthamoeba* should be revised. The taxonomic validity of many *Acanthamoeba* strains at the species level remains unclear.

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*. Therefore, a simpler and less expensive method is needed.

Riboprinting, PCR/RFLP analysis of 18S rRNA gene, was recently used for subgenus classification of morphologically indistinguishable protozoan genera such as *Trypanosoma* (Clark *et al.*, 1995) and *Tetrahymena* (Jerome and Lynn, 1996) and the results were very satisfactory. Thus, in this study, we analyzed the riboprinting patterns of 23 reference strains including 18 (neo)type strains for subgenus classification of *Acanthamoeba* spp. and discussed taxonomic validity of some taxa.

MATERIALS AND METHODS

Acanthamoeba

Twenty three strains including 18 (neo) type strains of the genus *Acanthamoeba* which were previously assigned to 18 species, were obtained from ATCC (Table 1). They were cultured axenically in Proteose peptone-Yeast extract-Glucose (PYG) medium or Proteose peptone-Yeast extract-Glucose-Cysteine (PYGC) medium at 25°C or 37°C.

Extraction of genomic DNA

Genomic DNA of *Acanthamoeba* was obtained by the method described by Kong and Chung (1996). Briefly, *Acanthamoeba* trophozoites (5×10^6) washed with phosphate-buffered saline (PBS) were boiled with 0.1 ml of 0.1 N NaOH for 3 min. Supernatant collected after centrifugation at 800 *g* for 2 min at room temperature was mixed with 0.2 ml of distilled water. The genomic DNA was extracted using phenol and phenol/chloroform (1:1) and recovered by precipitation with cold absolute ethanol in the presence of sodium acetate. The DNA was stored at –20°C until used.

PCR amplification of small subunit ribosomal RNA coding DNA (ssu rDNA)

The primers for the PCR, P3; 5'-CCGAATTCGTCGACAACCTGGTTGAT CCTGCCAGT-3', P4; 5'-GGATCCAAGCTTGA TCCTTCTGCAGGTTCACCTAC-3', are designed to hybridize to highly conserved sequences at the extreme 5' (P3) and 3' (P4) termini of eukaryotic ssu rDNA (Bhattacharya *et al.*, 1995). The PCR was done using a kit of premixed PCR reagents (Bioneer, Korea) and a thermal cycler (Perkin Elmer Cetus, USA). Fifty μl scale premix was dissolved in 47 μl of distilled water and vortexed vigorously. One μl of template DNA and 1 μl of each primer (25 nmol concentration) were added to the premix and mixed thoroughly. The whole mixture was covered with 20 μl 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.

Riboprinting

The PCR products of 23 strains were electrophoresed in a 2.5% agarose gel with DNA size standards (*Hind* III digested λ phage DNA, Poscochem, Korea; Amplisize, Biorad, USA). Ten kinds of restriction endonucleases (*Hae* III, *Hha* I, *Hinf* I, *Msp* I, *Dde* I, *Mbo* I, *Sau*96 I, *Rsa* I, *Taq* I and *Tru*9 I; Poscochem, Korea) which have recognition sequences of

Table 1. List of 23 strains of *Acanthamoeba* analysed in this study

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

[a]Same species name retained. [b]Not determined. [c]Further studies may be needed for accurate species designation.

four nucleotides were used to generate comparative riboprints. The amplified DNA (1 μg) was digested with 5-10 units of the enzymes for 2 hr with recommended buffers at 37°C, except for *Taq* I and *Tru*9 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 were unclear in the agarose gel, digested samples were electrophoretically separated in 12% polyacrylamide gels. 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 co-migration dataset (an average of 15.8% of the SSU-rDNA sequence) which was obtained by comparison of the riboprints of 23 *Acanthamoeba* strains each other. A phylogenetic tree was reconstructed by the unweighted pair group method with arithmetic average (UPGMA) using a computer program Phylip version 3.5 (Clark, 1992; Felsenstein, 1993).

RESULTS

Only a single PCR product was amplified from each *Acanthamoeba* strain (Fig. 1). The PCR products of *Acanthamoeba* strains were approximately 2,300 nucleotides in length as predicted, except for *A. astronyxis* (2.7 kb), *A. tubiashi* (2.6 kb), and *A. griffini* (2.8 kb).

Riboprint patterns of 23 *Acanthamoeba* strains by ten restriction endonucleases are shown schematically in Fig 2. The patterns of *A. rhysodes*, *A. mauritaniensis*, *A. divionensis* and *A. paradivionensis* were identical with all enzymes. The Chang strain which was previously assigned to *A. castellanii* showed very similar riboprints to the BH-2 strain, the type strain of *A. hatchetti*. The GE-3a strain which was assigned to *A. pustulosa* revealed very similar riboprints to the Reich strain, the type strain of *A. palestinensis*.

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

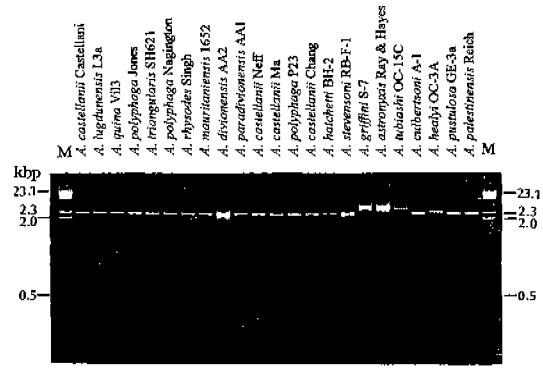

Fig. 1. Agarose electrophoretic pattern of PCR products from *Acanthamoeba* 23 strains. *Hind* III digested λ phage DNA was used as DNA size marker (M).

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 18S rRNA gene (Gast *et al.*, 1996; Ledee *et al.*, 1996), branched between virulent (strains A-1 and OC-3A) and avirulent subgroups (strains GE-3a and Reich) of morphological group 3. The 17 strains belonging to morphological group 2 were classified into six subgroups by 2% estimated genetic distance. The first subgroup of strains consisted of *A. castellanii* Castellani and Ma, *A. quina* Vil3, *A. lugdunensis* L3a, *A. polyphaga* Jones, and *A. triangularis* SH621. Among these, the Vil3 and L3a strains were found to be most closely related with the Castellani strain. The second subgroup of strains included *A. polyphaga* Nagington, *A. mauritaniensis* 1652, *A. rhysodes* Singh, *A. divionensis* AA2, and *A. paradivionensis* AA1. *Acanthamoeba polyphaga* P23 formed a subgroup with *A. castellanii* Neff strain. *Acanthamoeba hatchetti* BH-2 clustered with *A. castellanii* Chang to form another subgroup. *Acanthamoeba stevensoni* RB-F-1 and *A. griffini* S-7 were placed individually.

DISCUSSION

The present study revealed that a dendro-

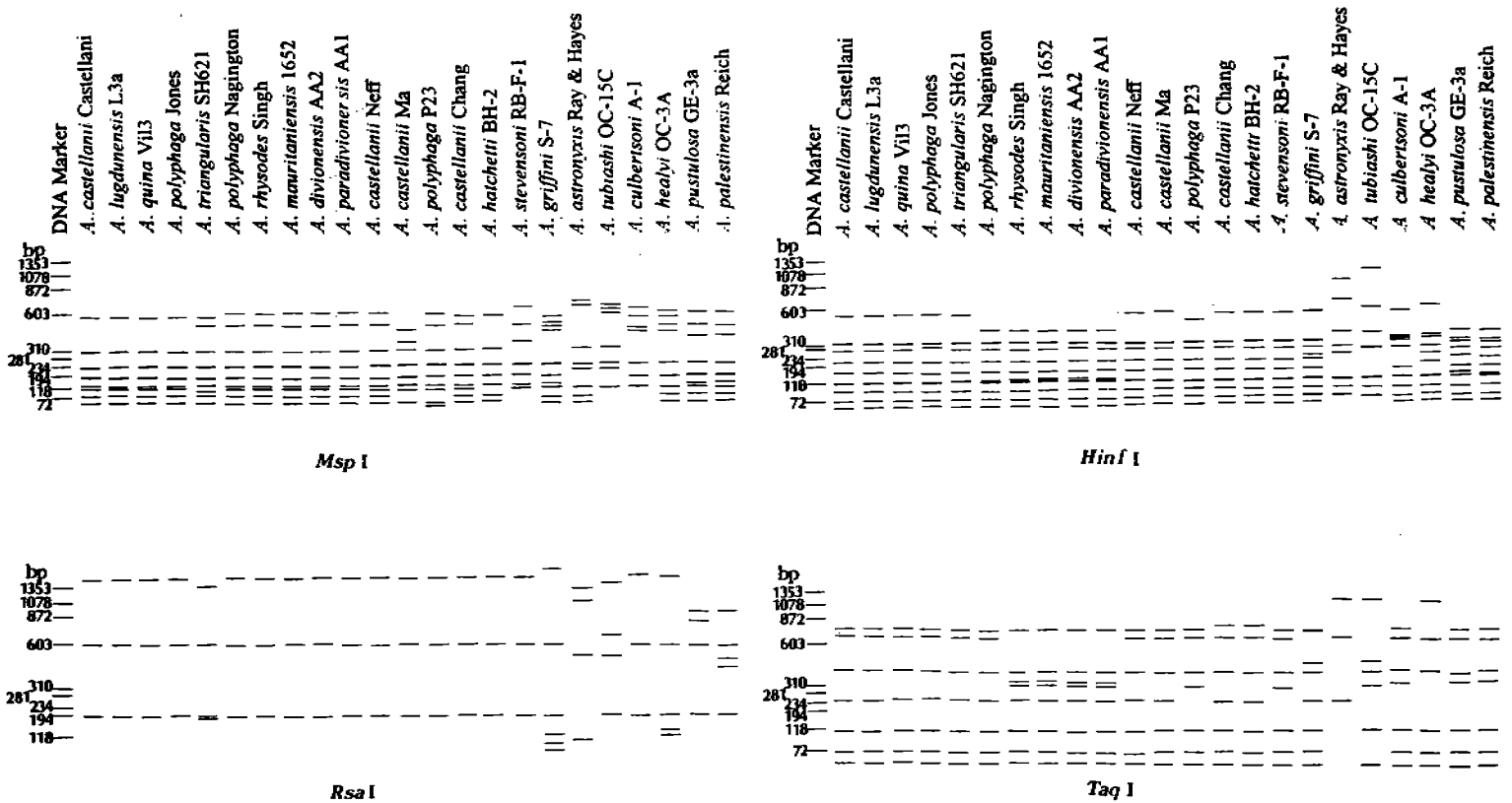

Fig. 2. Schematic representation of riboprints of 23 strains of *Acanthamoeba* by 10 kinds of restriction enzymes. *Hae* III digested ΦX174 DNA was used as the size marker.

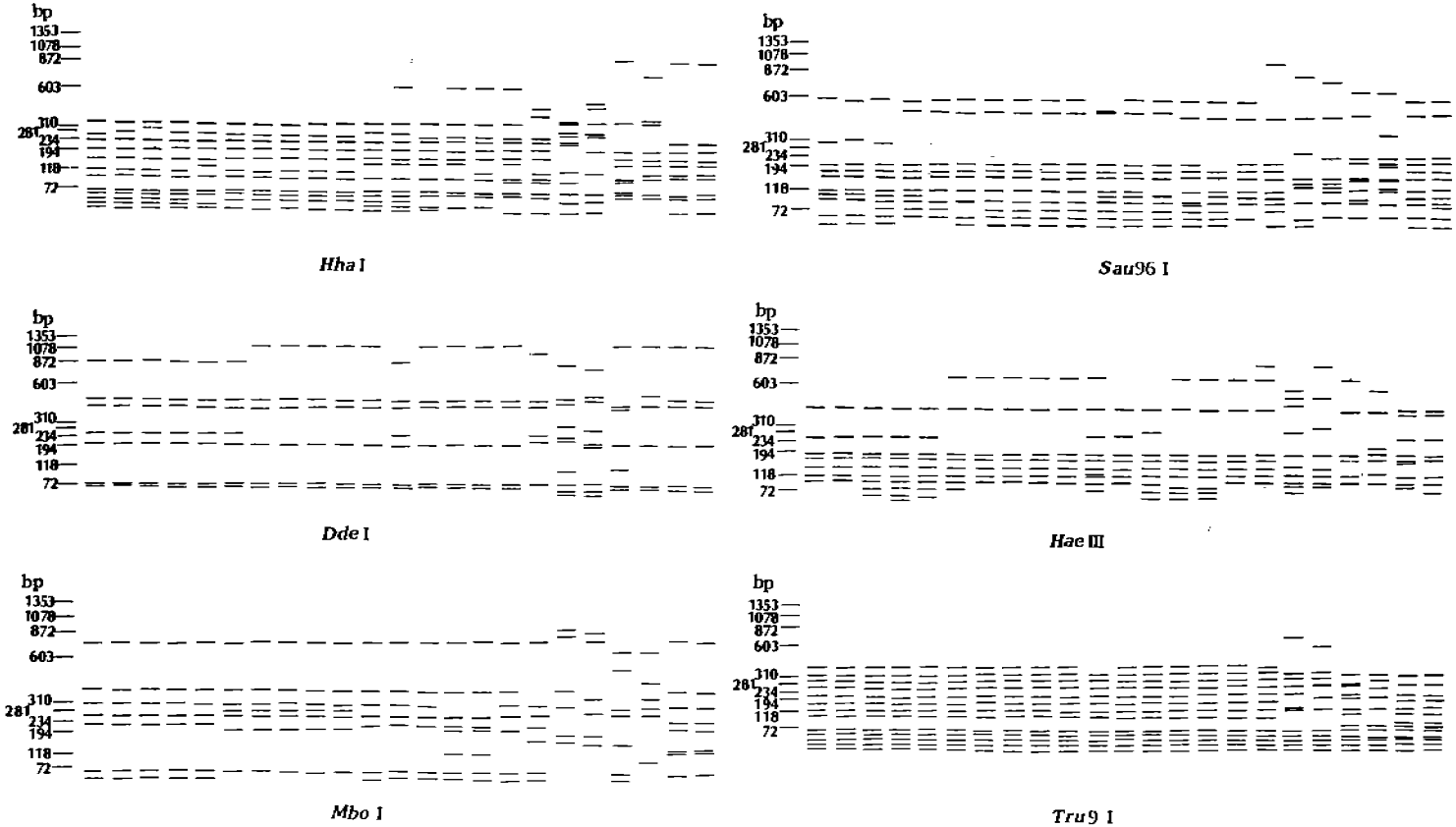

Fig. 2. Continued.

Table 2. Proportional homologous fragments (values above the diagonal) and estimates of genetic divergence (values below the diagonal) among 23 strains of *Acanthamoeba*

	Group		II										
No.	Species	Strain	1	2	3	4	5	6	7	8	9	10	11
1	*A. castellanii*	Castellani	—	186/196	190/197	176/195	168/195	164/191	158/192	158/192	158/192	158/192	164/195
2	*A. lugdunensis*	L3a	0.013	—	186/197	178/195	168/195	162/191	156/192	156/192	156/192	156/192	170/195
3	*A. quina*	Vil3	0.009	0.014	—	178/196	174/196	164/192	158/193	158/193	158/193	158/193	168/196
4	*A. polyphaga*	Jones	0.026	0.023	0.024	—	170/194	158/190	152/191	152/191	152/191	152/191	164/194
5	*A. triangularis*	SH621	0.037	0.037	0.030	0.033	—	164/190	158/191	158/191	158/191	158/191	166/194
6	*A. polyphaga*	Nagington	0.038	0.041	0.039	0.046	0.037	—	178/187	178/187	178/187	178/187	162/190
7	*A. rhysodes*	Singh	0.049	0.052	0.050	0.057	0.047	0.012	—	188/188	188/188	188/188	162/191
8	*A. mauritaniensis*	1652	0.049	0.052	0.050	0.057	0.047	0.012	0	—	188/188	188/188	162/191
9	*A. divionensis*	AA2	0.049	0.052	0.050	0.057	0.047	0.012	0	0	—	188/188	162/191
10	*A. paradivionensis*	AA1	0.049	0.052	0.050	0.057	0.047	0.012	0	0	0	—	162/191
11	*A. castellanii*	Neff	0.043	0.034	0.039	0.042	0.039	0.040	0.041	0.041	0.041	0.041	—
12	*A. castellanii*	Ma	0.024	0.027	0.028	0.038	0.038	0.048	0.056	0.056	0.056	0.056	0.044
13	*A. polyphaga*	P23	0.045	0.055	0.043	0.053	0.047	0.045	0.043	0.043	0.043	0.043	0.038
14	*A. castellanii*	Chang	0.054	0.057	0.049	0.056	0.049	0.047	0.045	0.045	0.045	0.045	0.046
15	*A. hatchetti*	BH-2	0.052	0.058	0.050	0.054	0.060	0.055	0.053	0.053	0.053	0.053	0.057
16	*A. stevensoni*	RB-F-1	0.050	0.057	0.058	0.066	0.052	0.047	0.045	0.045	0.045	0.045	0.049
17	*A. griffini*	S-7	0.091	0.094	0.095	0.108	0.104	0.103	0.100	0.100	0.100	0.100	0.093
18	*A. astronyxis*	Ray & Hayes	0.228	0.221	0.223	0.240	0.226	0.203	0.223	0.223	0.223	0.223	0.208
19	*A. tubiash*	OC-15C	0.210	0.210	0.217	0.235	0.215	0.197	0.192	0.192	0.192	0.192	0.202
20	*A. culbertsoni*	A-1	0.127	0.127	0.128	0.135	0.130	0.148	0.125	0.126	0.126	0.126	0.126
21	*A. healyi*	OC-3A	0.150	0.155	0.152	0.164	0.149	0.139	0.140	0.140	0.140	0.140	0.149
22	*A. pustulosa*	GE-3a	0.104	0.112	0.105	0.111	0.107	0.056	0.083	0.083	0.083	0.083	0.103
23	*A. palestinensis*	Reich	0.108	0.112	0.101	0.107	0.099	0.056	0.084	0.084	0.084	0.084	0.099

Table 2. Continued.

	Group		II						I		III			
No.	Species	Strain	12	13	14	15	16	17	18	19	20	21	22	23
1	*A. castellanii*	Castellani	176/194	162/194	154/191	156/192	152/186	144/207	78/194	80/185	112/186	102/186	126/191	126/194
2	*A. lugdunensis*	L3a	174/194	156/194	152/191	152/192	148/186	142/207	80/194	80/185	112/186	100/186	122/191	124/194
3	*A. quina*	Vil3	174/195	164/195	158/192	158/193	148/187	142/208	80/195	78/186	112/187	102/187	126/192	130/195
4	*A. polyphaga*	Jones	166/193	156/193	152/190	154/191	142/185	134/206	74/193	72/184	108/185	96/185	122/190	126/193
5	*A. triangularis*	SH621	166/193	160/193	156/190	150/191	150/185	136/206	78/193	78/184	110/185	102/185	124/190	130/193
6	*A. polyphaga*	Nagington	156/189	158/189	154/186	150/187	150/181	134/202	84/189	82/180	100/181	104/181	132/186	136/190
7	*A. rhysodes*	Singh	152/190	160/190	156/187	152/188	152/182	136/203	78/190	84/181	110/182	104/182	134/187	136/190
8	*A. mauritaniensis*	1652	152/190	160/190	156/187	152/188	152/182	136/203	78/190	84/181	110/182	104/182	134/187	136/190
9	*A. divionensis*	AA2	152/190	160/190	156/187	152/188	152/182	136/203	78/190	84/181	110/182	104/182	134/187	136/190
10	*A. paradivionensis*	AA1	152/190	160/190	156/187	152/188	152/182	136/203	78/190	84/181	110/182	104/182	134/187	136/189
11	*A. castellanii*	Neff	162/193	166/193	158/190	152/191	152/185	142/206	84/193	82/184	112/185	102/185	126/190	130/193
12	*A. castellanii*	Ma	—	156/192	154/189	150/190	158/184	144/205	72/192	74/183	114/184	96/184	126/189	126/192
13	*A. polyphaga*	P23	0.052	—	156/189	150/190	158/184	136/205	76/192	76/183	110/184	102/184	132/189	134/192
14	*A. castellanii*	Chang	0.051	0.048	—	178/187	154/181	138/202	72/189	76/180	108/181	100/181	120/186	126/189
15	*A. hatchetti*	BH-2	0.059	0.059	0.012	—	148/182	132/203	70/190	74/181	108/182	98/182	116/187	122/190
16	*A. stevensoni*	RB-F-1	0.038	0.038	0.040	0.052	—	138/197	62/184	78/175	114/176	100/176	124/181	124/184
17	*A. griffini*	S-7	0.088	0.103	0.095	0.108	0.089	—	72/205	76/196	104/197	90/197	112/202	112/205
18	*A. astronyxis*	Ray & Hayes	0.245	0.232	0.241	0.250	0.272	0.262	—	96/183	58/184	64/184	68/189	72/192
19	*A. tubiash*	OC-15C	0.226	0.220	0.216	0.224	0.202	0.237	0.161	—	70/175	68/175	70/180	68/183
20	*A. culbertsoni*	A-1	0.120	0.129	0.129	0.130	0.109	0.160	0.289	0.229	—	120/176	116/181	118/184
21	*A. healyi*	OC-3A	0.163	0.147	0.148	0.155	0.141	0.196	0.264	0.236	0.096	—	104/181	108/184
22	*A. pustulosa*	GE-3a	0.101	0.090	0.110	0.119	0.095	0.147	0.256	0.236	0.111	0.139	—	174/189
23	*A. palestinensis*	Reich	0.105	0.090	0.101	0.116	0.099	0.151	0.245	0.247	0.111	0.133	0.021	—

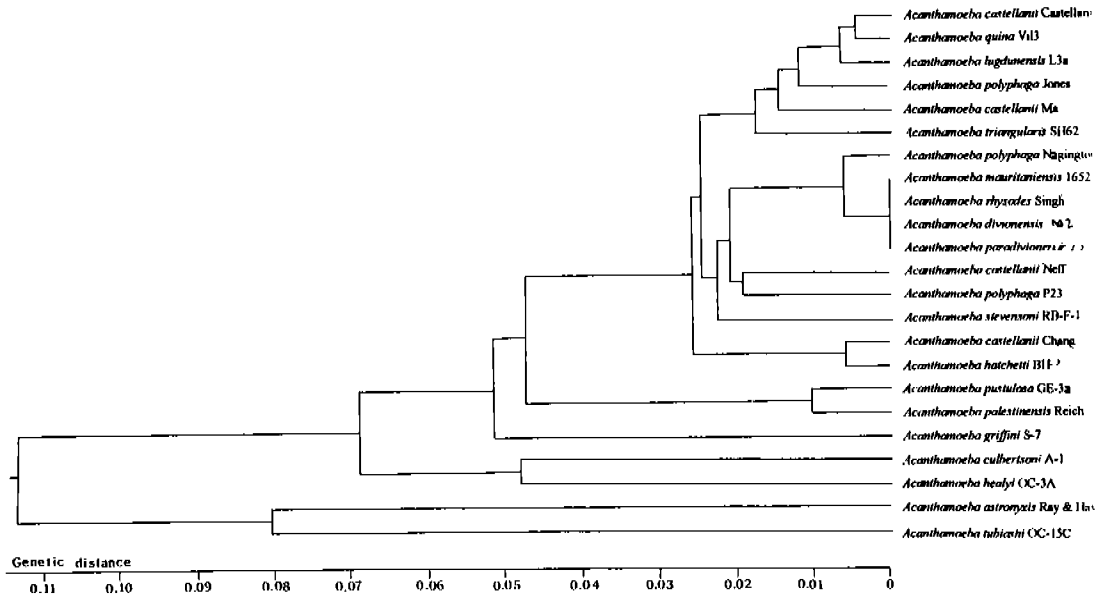

Fig. 3. Dendrogram of 23 strains of *Acanthamoeba* constructed by UPGMA method using Phylip ver. 3.5 based on genetic divergence estimates.

gram based on riboprints coincided well with the grouping of Pussard and Pons (1977) based on the morphological features of cysts and with the dendrogram reconstructed by Stothard *et al.* (1998) based on the 18S rRNA gene sequences. Two strains of morphological group 1, *A. astronyxis* Ray & Hayes and *A. tubiashi* OC-15C, were the most divergent (estimated genetic divergence, d>0.2) from the Castellani strain of *A. castellanii*, the type strain of the type species. Those two strains showed larger PCR products as reported by Stothard *et al.* (1998). Four strains of morphological group 3, *A. culbertsoni* A-1, *A. healyi* OC-3a, *A. palestinensis* Reich, and *A. pustulosa* GE-3a were the second most divergent (0.15>d>0.10) from the Castellani strain. Among 17 strains of morphological group 2, *A. griffini* was the most divergent (d = 0.091) from the Castellani strain. It was placed between virulent and avirulent subgroups of morphological group 3 amoebae on the dendrogram in this study. However, when the intron was removed from its 18S rDNA by computer-aided analysis, the branching order of the amoeba was next to the other group 2 amoebae (Data not shown). *Acanthamoeba griffini* previously has been shown to have an intron within the 18S rRNA gene (Gast *et al.*, 1996; Ledee *et al.*, 1996), thus, explaining the gene's larger size. The large size of the genes of *A. astronyxis* and *A. tubiashi* were due to insertions, but not to introns (Stothard *et al.*, 1998).

Strains AA1, AA2, and 1652 were previously assigned to *A. paradivionensis*, *A. divionensis*, and *A. mauritaniensis*, respectively. However, these 3 strains showed the identical riboprints with Singh strain, the type strain of *A. rhysodes*. In particular, AA1 and AA2 strains were identical in mitochondrial (mt) DNA RFLP patterns (unpublished data) and alloenzyme patterns (De Jonckheere, 1983). These four strains were originally isolated from soil and were regarded avirulent. De Jonckheere (1983) analyzed electrophoretic patterns of alloenzymes and total proteins and suggested that *A. paradivionensis* and *A. divionensis* were closely related to *A. rhysodes* R4c strain. However, he couldn't conclude that the species names are synonyms of *A. rhysodes*, since he failed to analyze the type strain of *A. rhysodes*. In the present study using Singh strain, the type strain of *A. rhysodes*, we confirmed that *A. divionensis* and *A. paradivionensis* are synonyms of *A. rhysodes*. *Acanthamoeba mauritaniensis* should also be a synonym of *A. rhysodes*. The Nagington strain was found to

be closely related with these strains.

Vil3 and L3a strains, previously assigned as *A. quina* and *A. lugdunensis* respectively, should be regarded as synonyms of *A. castellanii*. The genetic distance of these 2 strains from *A. castellanii* were less than 0.015 which can be considered as intraspecific variation. For example, *Entamoeba moskovskii* which consisted of 6 ribodemes showed intraspecific variation between 0.007 and 0.028 (Clark and Diamond, 1997). The mean genetic distance among these free-living *Enatmoeba* ribodemes was 0.016. Furthermore, the Vil3 and L3a strains showed very similar alloenzymes IEF and mt DNA RFLP patterns to those of the Castellani strain of *A. castellanii* (data not shown).

The Chang strain, which had been isolated from freshwater and assigned to *A. castellanii*, was closely related with the type strain of *A. hatchetti* isolated from ocean sediments. Both strains are pathogenic to animal models. Furthermore, these strains had lots of co-migrating DNA fragments on mt DNA RFLP analyses. It is suggested that Chang strain should be regarded as *A. hatchetti*.

Strains in a subgroup showed more similar patterns of alloenzymes and mt DNA RFLP to one another than to strains belonging to the other subgroups (data not shown). Results obtained from this study confirmed close relatedness of *A. pustulosa* with *A. palestinensis* proposed by De Jonckheere (1983) by alloenzyme analyses and Kim *et al.* (1996) by PCR RFLP analysis of a conserved portion of rDNA.

This study has shown that riboprinting is useful for subgenus classification of *Acanthamoeba* and for estimating relatedness among strains. Furthermore, riboprinting can be used for rapid identification of unknown *Acanthamoeba* isolates.

REFERENCES

Bhattacharya D, Helmchen T, Melkonian M (1995) Molecular evolutionary analyses of nuclear-encoded small subunit ribosomal RNA identify and independent rhizopod lineage containing the Euglyphina and the Chlorarachniophyta. *J Euk Microbiol* **42:** 65-69.

Byers TJ, Hugo ER, Stewart VJ (1990) Genes of *Acanthamoeba*: DNA, RNA and protein sequences (A Review). *J Protozool* **37:** s17-s25.

Chung DI, Kong HH, Yu HS, Oh YM, Yee ST, Lim YJ (1996) Biochemical and molecular characterization of a strain KA/S2 of *Acanthamoeba castellanii* isolated from Korean soil. *Korean J Parasitol* **34:** 79-85.

Clark CG (1992) Riboprinting: a molecular approach to the identification and taxonomy of protozoa. *In* Protocols in Protozoology, Lee JJ and Soldo AT (eds), 1: D-4.1-D-4.4. Allen Press, Lawrence, Kansas, USA.

Clark CG, Diamond LS (1997) Intraspecific variation and phylogenetic relationships in the genus *Entamoeba* as revealed by riboprinting. *J Euk Microbiol* **44:** 142-154.

Clark CG, Martin DS, Diamond LS (1995) Phylogenetic relationships among anuran trypanosomes as revealed by riboprinting. *J Euk Microbiol* **42:** 92-96.

De Jonckheere JF (1983) Isoenzyme and total protein analysis by agarose isoelectric focusing, and taxonomy of the genus *Acanthamoeba*. *J Protozool* **30:** 701-706.

De Jonckheere JF (1991) Ecology of *Acanthamoeba*. *Rev Infect Dis* **13**(s5): s385-s387.

Douglas M (1930) Notes on the classification of the amoeba found by Castellani in cultures of a yeast-like fungus. *J Trop Med London* **33:** 258-259.

Felsenstein J (1993) PHYLIP manual, version 3.5. Department of Genetics, University of Washington.

Field BS (1991) The role of amoebae in legionellosis. *Clin Microbiol Newsletter* **13:** 92-93.

Gast RJ, Ledee DR, Fuerst PA, Byers TJ (1996) Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. *J Euk Microbiol* **43:** 498-504.

Jadin JB (1973) De la meningoencephalite amibienne et du pouvoir pathogene des amibes limax. *Annee Biologique* **12:** 305-342.

Jerome CA, Lynn DH (1996) Identifying and distinguishing sibling species in the *Tetrahymena pyriformis* complex (Ciliophora, Oligohymenophorea) using PCR/RFLP analysis of nuclear ribosomal DNA. *J Euk Microbiol* **43:** 492-497.

Jones DB, Visvesvara GS, Robinson NM (1975) *Acanthamoeba polyphaga* keratitis and

Acanthamoeba uveitis associated with fatal meningoencephalitis. *Trans Ophthalmol Soc UK* **95:** 221-232.

Kim YH, Ock MS, Yun HC, *et al.* (1996) Close relatedness of *Acanthamoeba pustulosa* with *Acanthamoeba palestinensis* based on isoenzyme profiles and rDNA PCR-RFLP patterns. *Korean J Parasitol* **34:** 259-266.

Kong HH, Chung DI (1996) PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. *Korean J Parasitol* **34:** 127-134.

Kong HH, Park JH, Chung DI (1995) Interstrain polymorphisms of isoenzyme profiles and mitochondrial DNA fingerprints among seven strains assigned to *Acanthamoeba polyphaga*. *Korean J Parasitol* **33:** 331-340.

Ledee DR, Hay J, Byers TJ, Seal DV, Kirkness CM (1996) *Acanthamoeba griffini*: Molecular characterization of a new corneal pathogen. *Invest Ophthalmol Vis Sci* **37:** 544-550.

Lewis EJ, Sawyer TK (1979) *Acanthamoeba tubiashi* n. sp. a new species of fresh-water Amoebida (Acanthamoebidae). *Trans Am Microsc Soc* **98:** 543-549.

Ly TMC, Muller HE (1990) Ingested *Listeria monocytogenes* survive and multiply in protozoa. *J Med Microbiol* **33:** 51-54.

Ma P, Willaert E, Juechter KB, Stevens AR (1981) A case of keratitis due to *Acanthamoeba* in New York, and features of 10 cases. *J Inf Dis* **143:** 662-667.

Moura H, Wallace S, Visvesvara GS (1992) *Acanthamoeba healyi* n. sp. and the the isoenzyme and immunoblot profiles of *Acanthamoeba* spp., Groups 1 and 3. *J Protozool* **39:** 573-583.

Nagington J, Watson PG, Playfair TJ, McGill J, Jones BR (1974) Amebic infection of the eye. *Lancet* **ii:** 1537-1540.

Neff RJ (1957) Purification, axenic cultivation, and description of a soil amoeba, *Acanth-amoeba* sp. *J Protozool* **4:** 176-182.

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proc Nat Acad Sci USA* **76:** 5269-5273.

Page FC (1967) Re-definition of the genus *Acanthamoeba* with descriptions of three species. *J Protozool* **14:** 709-724.

Page FC (1988) A new key to freshwater and soil gymnamoebae with instructions for culture. Freshwater Biological Association. Ambleside, Cumbria.

Pussard M, Pons R (1977) Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). *Protistologica* **13:** 557-598.

Ray DL, Hayes RE (1954) *Hartmannella astronyxis*: a new species of free-living amoeba. Cytology and life cycle. *J Morphol* **95:** 159-188.

Reich K (1933) Studien er die Bodenprotozoen palstinos. *Arch Protistenkd* **79:** 76-98.

Sawyer TK (1971) *Acanthamoeba griffini*, a new species of marine amoeba. *J Protozool* **18:** 650-654.

Sawyer TK, Nerad TA, Lewis EJ, McLaughlin SM (1993) *Acanthamoeba stevensoni* n. sp. (Protozoa: Amoebida) from sewage-contaminated shellfish beds in Raritan bay, New York. *J Protozool* **40:** 742-746.

Sawyer TK, Visvesvara GS, Harke BA (1977) Pathogenic amoebas from brackish and ocean sediments, with a description of *Acanthamoeba hatchetti*, n. sp. *Science* **196:** 1324-1325.

Singh BN (1952) Nuclear division in nine species of small free-living amoebae and its bearing on the classification of the order Amoebida. *Phil Trans Roy Soc London, Ser B* **236:** 405-461.

Singh BN, Das SR (1970) Studies on pathogenic and nonpathogenic small free-living amoebae and the bearing of nuclear division on the classification of the order Amoebida. *Philos Trans Roy Soc London, Ser Biol Sci* **259:** 435-476.

Sisson JP, Kemper CA, Loveless M, McShane D, Visvesvara GS, Deresinski SC (1995) Disseminated *Acanthamoeba* infection in patients with AIDS: Case reports and review. *Clin Infect Dis* **20:** 1207-1216.

Sogin ML, Gunderson JH, Elwood HJ, Alonso RA, Peattie DA (1989) Phylogenetic meaning of the Kingdom concept: An unusual ribosomal RNA from *Giardia lamblia*. *Science* **243:** 75-77.

Stothard DR, Schroeder-Diedrich JM, Awwad MH, *et al.* (1998) The evolutionary history of the genus *Acanthamoeba* and the identifi-cation of eight new 18S rRNA gene sequence types. *J Euk Microbiol* **45**(1): 45-54.

Thom S, Warhurst D, Drasar BS (1992) Association of *Vibrio cholerae* with fresh water amoebae. *J Med Microbiol* **36:** 303-306.

Visvesvara GS (1991) Classification of *Acanthamoeba*. *Rev Inf Dis* **13**(Suppl. 5): s369-s372.

=초록=

Riboprinting에 의한 가시아메바속의 분류

정동일, 유학선, 황미열, 김태호, 김태욱, 윤호철, 공현회

경북대학교 의과대학 기생충학교실

가시아메바 (*Acanthamoeba*) 속의 종 동정과 분류 체계를 확립하기 위해 18종의 대표주를 포함한 23 분리주들의 18S rRNA 유전자를 PCR로 증폭하고, RFLP를 비교 분석하는 riboprinting을 시행하였다. 이에 근거한 dendrogram은 형태학적 grouping과 잘 부합하였다. 형태학적 제1군에 속한 별가시아메바와 *A. tubiashi*는 형태학적 제2군 및 제3군의 분리주들로부터 가장 먼저 분지해 나왔으며, 서로간에도 매우 큰 유전적 거리를 나타내었다. 형태학적 제3군의 4분리주 중 *A. culbertsoni*, *A. healyi* 및 *A. palestinensis*는 서로간에 큰 유전적 거리를 나타내어 합당한 분류로 인정할 수 있었으나, *A. pustulosa*는 *A. palestinensis*와 유전적으로 매우 가깝게 나타나 *A. palestinensis*로 재동정되어야 할 것으로 판단되었다. 형태학적 제2군의 분리주들은 0.2%의 유전적 거리를 기준으로 하여 6개의 분지군으로 나누어졌다. 그 중 18S rRNA 유전자 내에 intron을 포함하고 있는 *A. griffini*가 나머지 분리주들과 가장 큰 유전적 거리를 나타내었다. 카스텔라니가시아메바 Castellani주, Ma주, *A. quina* Vil3주, 담수가시아메바 L3a주, 대식가시아메바 Jones주 및 *A. triangularis* SH621주가 하나의 분지군을 형성하였으며, 그 중 *A. quina* Vil3와 담수가시아메바 L3a주는 카스텔라니가시아메바로 재동정되어야 할 것으로 판단되었다. Chang주는 *A. hatchetti*로, *A. mauritaniensis*, *A. divionensis*와 *A. paradivionensis*는 *A. rhysodes*로 재동정되어야 할 것으로 판단되었다. Neff주는 카스텔라니가시아메바보다는 대식가시아메바와 유전적으로 훨씬 가까웠다. Riboprinting은 임상 및 환경에서 분리되는 가시아메바 분리주의 빠른 동정에도 유용할 것으로 사료된다.

〔기생충학잡지 36(2): 69-80, 1998년 6월〕