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Acanthamoeba sohi, n. sp., a pathogenic Korean isolate YM-4 from a freshwater fish

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Abstract: A new species of *Acanthamoeba* was isolated from a freshwater fish in Korea and tentatively named *Acanthamoeba* sp. YM-4 (Korean isolate YM-4). The trophozoites were 11.0-23.0 μm in length and had hyaline filamentous projections. Cysts were similar to those of *A. culbertsoni* and *A. royreba*, which were previously designated as *Acanthamoeba* group III. *Acanthamoeba* YM-4 can survive at 40°C, and its generation time was 19.6 hr, which was longer than that of *A. culbertsoni*. In terms of the in vitro cytotoxicity of lysates, *Acanthamoeba* YM-4 was weaker than *A. culbertsoni*, but stronger than *A. polyphaga*. On the basis of the mortality of experimentally infected mice, *Acanthamoeba* YM-4 was found to be highly virulent. The isoenzymes profile of *Acanthamoeba* YM-4 was similar to that of *A. royreba*. An anti-*Acanthamoeba* YM-4 monoclonal antibody, McAY7, was found to react only with *Acanthamoeba* YM-4, and not with *A. culbertsoni*. Random amplified polymorphic DNA marker analysis and RFLP analysis of mitochondrial DNA and of 18S small subunit ribosomal RNA, placed *Acanthamoeba* YM-4 in a separate cluster on the basis of phylogenetic distances. Thus the *Acanthamoeba* Korean isolate YM-4 was identified as a new species, and assigned as *Acanthamoeba sohi*.

Key words: *Acanthamoeba*, pathogenicity, restriction fragment length polymorphism, phylogeny

INTRODUCTION

The ubiquitous distribution of small free-living amoebae in nature, and the ability of certain species to survive and grow especially in freshwater, suggesting a role in human and animal disease (Ma et al., 1990). The small free-living amoebae, *Acanthamoeba* spp., *Naegleria* spp. and *Balamuthia mandrillaris*, are distributed worldwide, and thus provide a potential source of meningoencephalitis or keratoconjunctivitis infection for humans and other mammals.

Based on differences in size as well as on the morphologic characters of the cysts, in 1977 Pussard and Pons recognized three groups within the genus *Acanthamoeba*. However, species differentiation based on morphology alone may not always be correct. Thus, many attempts have been made to include other features, such as isoenzyme profiles and restriction fragment length polymorphisms (RFLP) of mitochondrial DNA (mtDNA), in the classification of *Acanthamoeba* (Bogler et al., 1983; De Jonckheere, 1983). The classification of *Acanthamoeba* spp. at the subgenus level has been attempted by riboprinting, e.g., by RFLP analysis of the 18S small subunit ribosomal RNA (srRNA) gene by polymerase chain reaction (PCR) (Chung et al, 1998). Kong and Chung (2002) described a riboprinting scheme for the identification of unknown *Acanthamoeba* Korean

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isolates at the species level by using the PCR-RFLP of 18S srRNA using four kinds of restriction enzymes. In addition, the interstrain polymorphisms of the isoenzyme profiles and the RFLP patterns of mtDNA were observed in seven strains of *Acanthamoeba polyphaga* and four strains of *Acanthamoeba castellanii* (Kong et al., 1995).

This paper described the identification of the *Acanthamoeba* Korean isolate YM-4 at the species level based on morphologic characteristics, biochemical and molecular genetic criteria, by reviewing previous reports and adding some new figures and experimental data for 18S srDNA RFLP. Moreover, we propose a new species, *Acanthamoeba sohi* (Korean isolate YM-4), isolated from the gills of a freshwater fish collected in a fish market in Seoul, Korea, as presented in the 36th annual meeting of The Korean Society for Parasitology in 1994.

MATERIALS AND METHODS

Isolation of Korean isolate YM-4

Acanthamoeba Korean isolate YM-4 was isolated from the gills of a fresh water fish collected in a fish market in December 10, 1977. The gills of the freshwater fish, *Carassius auratus*, were dissected and separated. The sample obtained was cut into small pieces and aseptic sterilized physiologic saline was added. Small amounts of the sediment obtained by centrifugation were transferred to the surface of a non-nutrient agar plate previously streaked with killed *Escherichia coli*. Amoeba colonies were selected from the agar plates. The strain CDC-Fish-SK submitted to Dr. G. S. Visvesvara (Division of Parasitic Diseases, Center for Disease Control, Atlanta, USA) by Dr. J. Yang (Moura et al., 1992) was the original Korean isolate YM-4.

Cultivation

The culture medium was composed of 1.5% Bacto-agar containing 14 mg of neomycin, 3,750 units of nystatin and 2.0 ml of heat-killed *E. coli* suspended in 100 ml of distilled water. Amoeba colonies were maintained in continuous culture on the surface of a non-nutrient agar plate, and transferred aseptically to axenic CGV medium in June 1984. Pure ameba cultures were established by aseptically placing a portion of a single colony on agar medium and by preparing subcultures at weekly intervals. These were then placed in axenic liquid CGV medium at 37°C in a CO_2 incubator. CGV medium was composed bactocasitone 20 gm, glucose 1gm, folic acid 2 mg, biotin 20 μg, NaCl 5 mg, penicillin (50×10^4 units), streptomycin (5×10^4), and distilled water (1,000 ml).

Biological, biochemical, immunological, molecular and genetic characteristics of *Acanthamoeba* YM-4

The morphologic characteristics, biochemical and molecular genetic criteria are described by reviewing previous reports. In this report, some new figures and experimental data for 18S srDNA RFLP has been added. The 18S-srDNAs (or srRNA) of *Acanthamoeba* YM-4 and of reference amoebae were amplified by PCR with primer encoding the 18S-srRNA gene and digested with six restriction endonucleases, i.e., *Dde* I, *Hae* III, *Hinf* I, *Msp* I, *Rsa* I and *Sau* I (Sigma). Amplified DNAs were loaded on 1% agarose horizontal gel. A phylogenic tree based on the RFLPs of the 18 srDNA of *Acanthamoeba* spp. was constructed as described previously (Shin et al., 1998).

RESULTS

1. Descriptions of *Acanthamoeba sohi*, n. sp.

Synonym: *Acanthamoeba* YM-4, *Acanthamoeba* sp. YM-4 and *Acanthamoeba* Korean isolate YM-4.

Type host: Fresh water fish and experimental animals.

Habitats: Fresh water.

Locality: South Korea.

Type specimens: Trophozoites are deposited in the Department of Parasitology, Yonsei University, College of Medicine, Seoul 120-752, Korea.

Morphology: The dimentions of living trophozoites and cysts from culture media were determined with an ocular micrometer and photographed under a phase-contrast microscope. The trophozoite of *A. sohi*

is a small filose amoeba with features typical of the genus of *Acanthamoeba*; i.e., locomotive form, elongate to broad, measuring 11.0-23.0 μm long when grown in liquid medium; hyaline and filamentous projections; usually uninucleate; no flagellate stage and no detectable centrosome-like organelles; numerous food vacuoles (Fig. 1A, B). The feeding form is variable in shape and size; refractive, and without a wide, clear, hyaline anterior zone of protoplasm. Nuclear division is accompanied by the disappearance of the nuclear membrane and of nucleoli during the late prophase. *A. sohi* has a small and refractive cyst with a nearly circular ectocyst and oval or some irregular endocyst (Fig. 1C); of diameter, 7.0-14.0 μm (measured on agar). Based upon the morphological characteristics of trophozoites and cysts, *Acanthamoeba* YM-4 (*A. sohi*) was found to resemble *A. culbertsoni* and *A. royreba* (Shin et al., 1992a; Shin et al., 1997). Cysts of *Acanthamoeba* YM-7 (Korean isolate serial number) were similar to those of *A. polyphaga*, which was designated as a member of *Acanthamoeba* group II by the classification key of Pussard and Pons (1977). Morphologically *Acanthamoeba* YM-4 and YM-5 belong to *Acanthamoeba* group III.

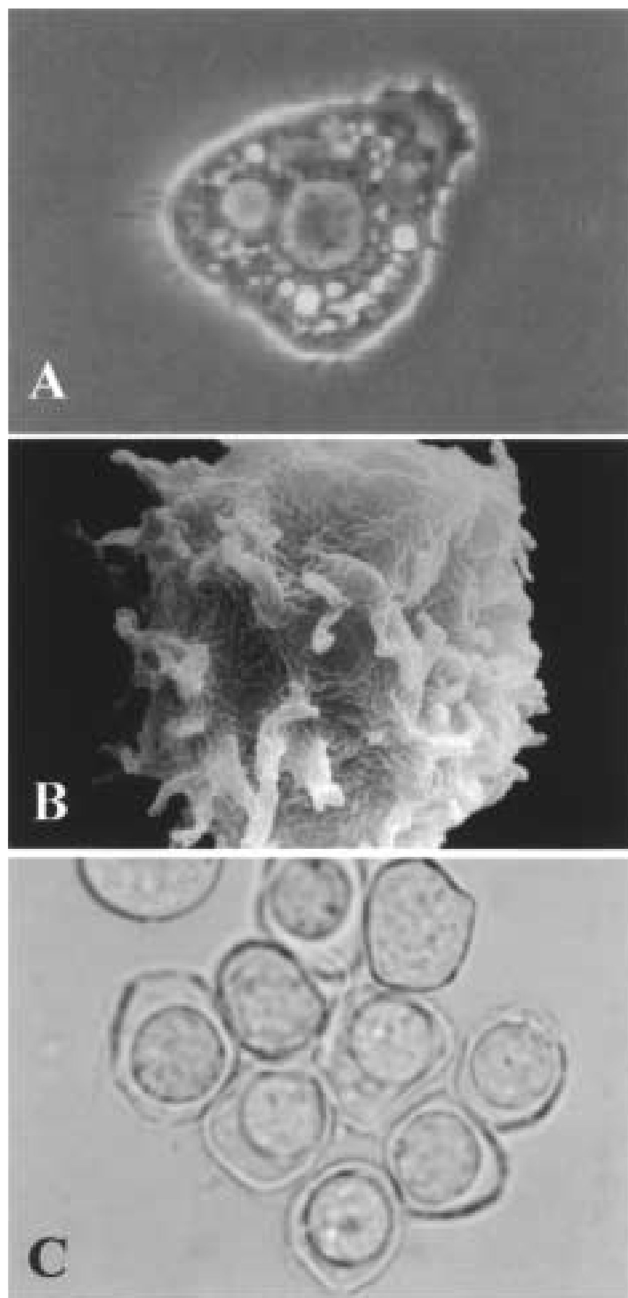

Fig. 1. Trophozoites of *Acanthamoeba* Korean isolate YM-4 in locomotion by a light miroscope (A) and scanning electronic microscope (B), the animals are slightly broad, and have numerous fine spike-like micropseudopodia. Cysts have a nearly circular ectocyst and oval or some irregular endocyst (B).

Generation time: The generation time of *Acanthamoeba* YM-4 was compared with *A. culbertsoni* and *A. polyphaga* AP strain (Shin et al., 1993). At 37°C, the generation time of YM-4 was 19.1 hr, (*A. culbertsoni* 18.7 hr, *A. polyphaga* 19.4 hr). At 25°C, the generation times of *Acanthamoeba* YM-4, *A. culbertsoni* and *A. polyphaga* were 25.7 hr, 24.2 hr and 18.2 hr. and at 40°C, the generation time of YM-4 (19.6 hr) was longer than that of *A. culbertsoni* (17.8 hr).

2. Biological characteristics

Cytotoxicity: *Acanthamoeba* YM-4 was compared with *A. culbertsoni* and *A. royreba* with respect to its cytotoxic effect upon Chinese hamster ovary cells (Shin et al., 1993). The in vitro cytotoxicity of *Acanthamoeba* YM-4 using trophozoite lysate was weaker than that of *A. culbertsoni*, but stronger than that of *A. polyphaga* (Table 1).

Pathogenicity and virulence: *Acanthamoeba* Korean isolates and reference *Acanthamoeba* spp. have been demonstrated to be pathogenic to experimental mice, and to induce death when inoculated intranasally (Im et al., 1999). Based on infected mouse mortality, *Acanthamoeba* YM-4 and *A. culbertsoni* were found to

Table 1. Cytotoxicity of *Acanthamoeba* lysates on Chinese hamster ovarian cells

Groups	Cytotoxicity activity (%)				
	1:1[a)]	1:2	1:4	1:8	1:64
Acanthamoeba YM-4	100	99.9	88.5	67.4	38.7
A. culbertsoni	100	100	100	100	99.3
A. polyphaga	60.7	31.5	5.1	5.1	4.1

[a)]Ratio of target cells and amoeba lysate (2 mg/ml) (Shin et al., 1993).

Table 2. Mortality of the mice infected intranasally with *Acanthameoba* spp. (Im et al., 1999)

Amoeba strains	No. of mice	Number of dead mice in each post-inoculation day																			Mortality (%)
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22 ············	30	
YM-2	19				3	1		1			1		1				2				9 (47.4)
YM-3	20																				0 (0.0)
YM-4	29	3	5	4				2	2	2		1									19 (65.5)
YM-5	32		1	3	4	2				1		1	1			1					14 (43.8)
YM-7	18	2	1	1		2						1		1							7 (38.9)
A. culbersoni	30	2	1	2	2	1		1	1		4	1	1							2	18 (60.0)
A. hatchetti	20		1	3	7		2		1												15 (75.0)
A. royreba	18				1	1				1											3 (16.7)

be highly virulent, *Acanthamoeba* YM-2, YM-5, *A. royreba* and *A. castellanii* to have low virulence, and *Acanthamoeba* YM-3 to be nonpathogenic (Table 2).

Biochemistry: The zymodeme patterns of glucose-6-phosphate dehydrogenase (G6PD), malate dehyrogenase (MDH), hexokinase (HK), glutamate oxaloacetate transaminase (GOT) and malic enzyme (ME) of *Acanthamoeba* spp. differed for the Korean isolates (Fig. 2), especially between *Acanthamoeba* YM-4 and *A. royreba* (Han et al., 1982; Im et al., 1999). According to the result of Moura et al. (1992), the isoenzymes profile of *Acanthamoeba* YM-4 (CDC-Fish-SK in this paper) is similar to *A. royreba.*

Immunology: As a result of the SDS-PAGE profile of the lysate of *Acanthamoeba* YM-4, 16 major protein fractions were found to be similar to those of *A. culbertsoni*, but *Acanthamoeba* YM-4 exhibited distinctly different protein fractions patterns when compared with *A. royreba* and *A. polyphaga* (Fig. 3). Cross-reactivity experiments were performed in the various amoebae by ELISA using anti-*Acanthameoba* YM-4 monoclonal antibody, McAY 7 (IgG1, 43 kDs by EITB), which was found to react only with *Acanthamoeba* YM-4, and not with *A. culbertsoni* (Shin et al., 1992b).

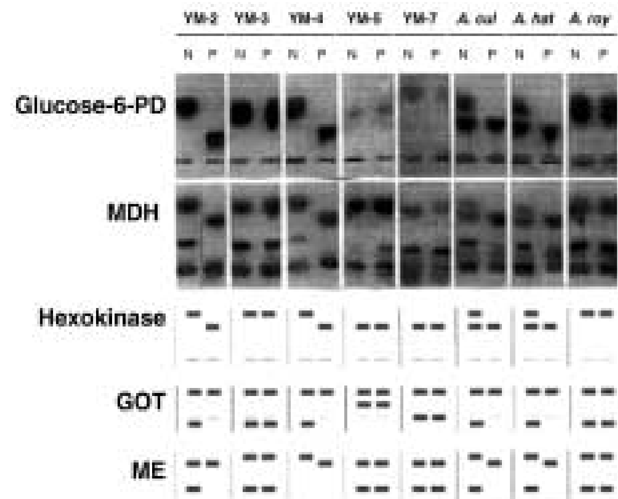

Fig. 2. Isoenzyme zymographic profiles of the *Acanthamoeba* Korean isolate YM-4, and other *Acanthamoeba* spp. (Im et al., 1999).

3. Molecular and genetic phylogeny

Random amplified polymorphic DNA marker analysis: The genetic status of *Acanthamoeba* spp. was investigated by random amplified polymorphic DNA marker analysis (Fig. 4). Four *Acanthamoeba* Korean isolates and other reference *Acanthamoeba* spp. were analyzed by RAPD-PCR using arbitrary decamer

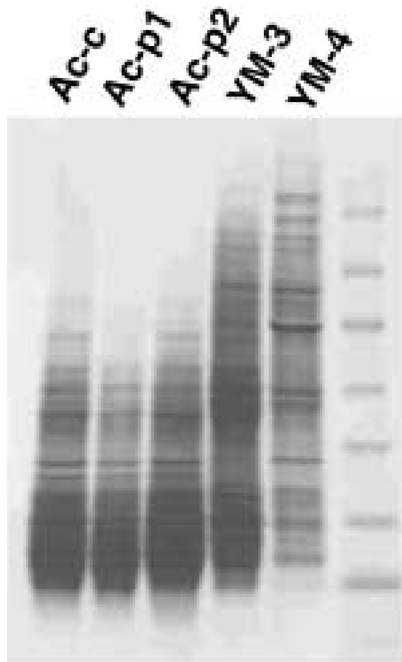

Fig. 3. Protein profiles by SDS-PAGE of trophozoite lysates of the *Acanthamoeba* Korean isolate YM-4 and of *A. culbertsoni* obtained before (Ac-c) and after (Ac-p1,2) mouse brain passage.

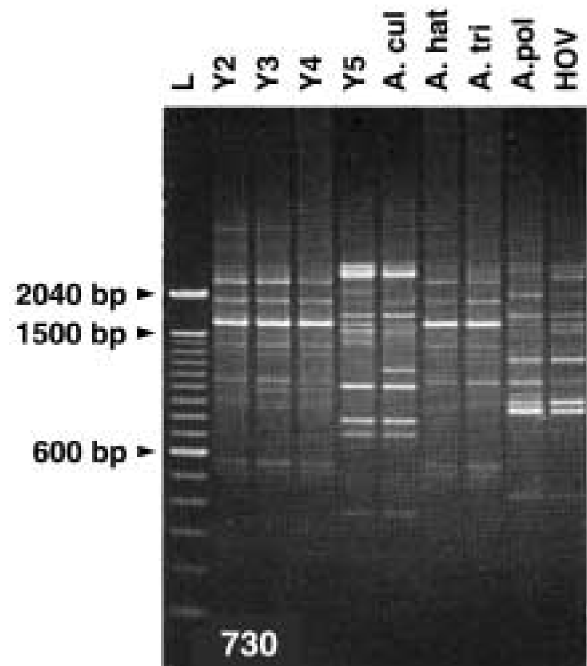

Fig. 4. Amplified DNA profiles of *Acanthamoeba* spp. using a primer of 730. Y4, *Acanthamoeba* YM-4; A. cul, *Acanthamoeba culbertsoni*; A. hat, *Acanthamoeba hatchetti*; A. tri, *Acanthamoeba triangularis*; A. pol, *Acanthamoeba polyphaga*. L, DNA ladder marker (Hong et al., 1995).

primers (Hong et al., 1995). The similarity index between *A. hatchetti* and *A. triangularis* was found to be 0.833. The mean similarity indices between three Korean isolates (*Acanthamoeba* YM-2, YM-3, YM-4) and two other species (*A. hatchetti* and *A. triangularis*) were 0.959 and 0.832. The mean similarity index between *Acanthamoeba* YM-5 and the other Korean isolates (*Acanthamoeba* YM-2, YM-3, YM-4) was 0.237. A phonogram reconstructed using the UPGMA method revealed the presence of two groups; one group of *A. hatchetti*, and *A. triangularis*, and the three Korean isolates (*Acanthamoeba* YM-2, YM-3, YM-4), and the other of *A. culbertsoni*, *A. polyphaga*, and *Acanthamoeba* YM-5.

RFLP analysis of mtDNA: The analysis of the interstrain variability of nucleotide sequences of mitochondrial DNA (mtDNA) offers a molecular means of determining overall genotype differences; restriction endonuclease analysis has proven a powerful tool for revealing mtDNA phylogenic relationships among closely related organisms. RFLP analysis of mtDNA was found to be useful in the classification of members to the genus *Acanthamoeba* (Shin and Im, 1996; Shin et al., 1997). The RFLP analysis of *Acanthamoeba* mtDNA was accomplished using five restriction enzymes; *Hae* III, *Hind* III, *Cla* I, *Pvu* II and *Sal* I. Each restriction enzyme produced approximately 3-15 fragments (Fig. 5). The mtDNA genome size, calculated by the summation of restriction fragments, averaged 46.4 kbp in *Acanthamoeba* YM-4, 48.3 kbp in *A. culbertsoni* and 48.8 kbp in *A. polyphaga*. The estimated genetic divergence was 10.1% between *Acanthamoeba* YM-4 and *A. culbertsoni*, and 9.9% between *Acanthamoeba* YM-4 and *A. polyphaga*.

RFLP analysis of 18S srDNA: The 18S-srDNAs (or srRNA) of *Acanthamoeba* YM-4 was amplified by PCR with primer encoding the 18S-srRNA gene, and the products obtained were digested with restriction endonucleases (Fig. 6). RFLP analysis was applied to the classification of the *Acanthamoeba* Korean isolates (YM-4, YM-5 and YM-7), and compared with the reference amoebae, *A. culbertsoni*, *A. polyphaga* and *A.*

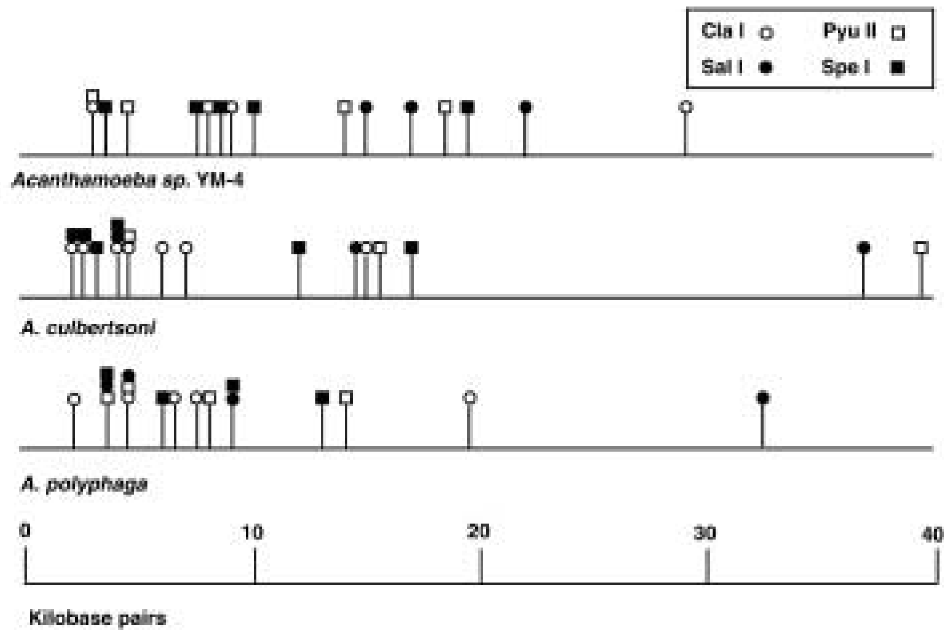

Fig. 5. Unique profile of the mitochondrial DNA map of *Acanthamoeba* Korean isolate YM-4 (Shin and Im, 1996).

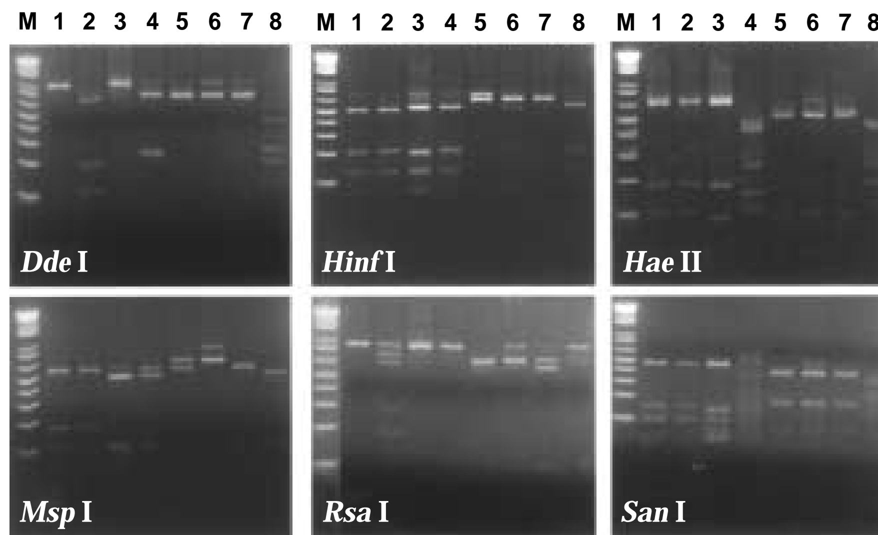

Fig. 6. RFLP patterns of 18S srDNA of *Acanthamoeba* spp. digested with six restriction endonucleases. Lanes 1-8, *Acanthamoeba culbertsoni*, *A. healyi*, *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. royreba*, *Acanthamoeba* YM-4 and *Acanthamoeba* sp. KA/LS5. M, DNA size marker (1 kb ladder).

royreba (Shin et al., 1998). The genetic distance between *A. culbertsoni* and *Acanthamoeba* YM-5 was 0.070, and between *Acanthamoeba* YM-4 and *A. polyphaga* 0.364. Moreover, genetic divergence was not observed between *A. culbertsoni* and *Acanthamoeba* YM-4. According to this result, *Acanthamoeba* YM-7 is a similar species to *A. polyphaga*, and *Acanthamoeba* YM-5 and *A. polyphaga* are different species, but *Acanthamoeba* YM-5 is related closely to *A. culbertsoni.* Our RFLP result gave a genetic distance of 0.105 between *Acanthamoeba* YM-4 and *A. royreba* (Fig. 7), which were closer species among the reference amoebae. The genetic distances between *Acanthamoeba* YM-4 and *A. polyphaga*, *A. culbertsoni* and *A. castellanii*

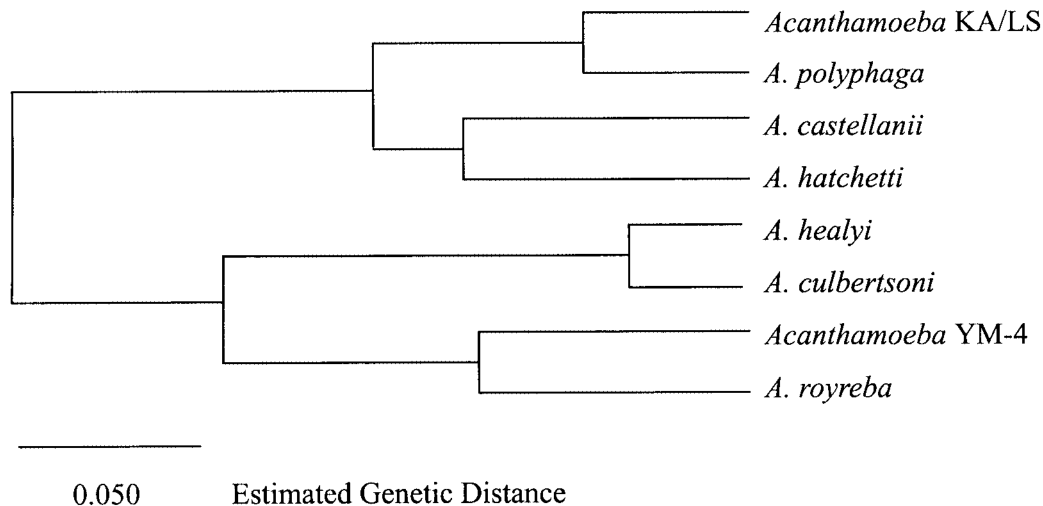

Fig. 7. Dendrogram of the estimated genetic distances of the 18S srDNA of *Acanthamoeba* spp. by UPGMA cluster analysis.

were 0.341, 0.232 and 0.201, respectively. Phylogenetic relationships placed *Acanthamoeba* YM-4 in a separate cluster (Fig. 7).

DISCUSSION

Acanthamoeba spp. is an amphizoic amoebae, which belongs to the genus *Acanthamoeba* Volkonsky 1931, emend Page (1967), and was incorporated into the family Acanthamoebidae by Sawyer and Griffin (1975). Pathogenic free-living amoebae belong to the genera *Acanthamoeba* and *Naegleria.* Group taxonomy, including pathogenic free-living amoebae, was clarified and updated at the generic level by P.C. Page (1967), who published a taxonomic key (Page, 1976). The genus *Naegleria* Alexeieff 1912, emend Calkins 1913, belongs to the family Vahlkampfiidae. The genus *Acanthamoeba* Volkonsky 1931, emend Page (1967), was included in the family Acanthamoebidae Sawyer and Griffin (1975). The genus *Acanthamoeba* was established in 1931, and until recently considerable confusion existed in terms of its taxonomic status. In 1966, Pussard stated that spindle shape is not a satisfactory feature for intergeneric differentiation but considered the characteristic morphology of the cyst to be a decisive character at the generic level and recognized the genus *Acanthamoeba.* In 1967, Page considered the presence of acanthopodia and the structure of the cyst to be sufficiently distinctive and concluded that the generic designations of *Acanthamoeba* and *Hartmannella* were justified. In 1975, Visvesvara and Balamuth identified clearly demonstrable differences not only in the trophozoite and cyst stages of *Acanthamoeba* and *Hartmannella*, but also in their nutritional requirements and serologic responses. Sawyer and Griffin created the family Acanthamoebidae in 1975, and Page created the suborder Acanthopodina under the order Amoebida. However, Page separated *Acanthamoeba* and *Hartmannella* at the ordinal level by creating a new order, Acanthopodida, which includes the family Acanthamoebidae and placed *Hartmannella* in the family Hartmannellidae, order Euamoebida.

As a result of experiments and reviews based on morphological characteristics, pathogenicity to mice, in vitro cytotoxicity, isoenzyme patterns and RFLP analysis of mtDNA and 18S srRNA, *Acanthamoeba* YM-4 was found to differ significantly from *A. culbertsoni*, *A. polyphaga*, and *A. royreba* and other reference *Acanthamoeba* spp. Thus, the *Acanthamoeba* Korean isolate YM-4 should be identified as a new species, assigned as *Acanthamoeba sohi.* The Korean isolate YM-4 was proposed as new species, *Acanthamoeba sohi,* at the 36th annual meeting of The Korean society for Parasitology in 1994. This new species is named in honor of Dr. Chin-Thack Soh, a

Emeritus Professor of Yonsei University College of Medicine, Seoul, Korea.

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