Ajou Medical Journal
Vol. 1, No. 1, 1996

Restriction Site Maps of Mitochondrial DNA for *Acanthamoeba* Strain in Korea

Ho-Joon Shin and Kyung-il Im*

Department of Microbiology, Ajou University School of Medicine, Suwon, 442-749, Korea
*Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul 120-752, Korea

The study of taxonomic relationships among members of *Acanthamoeba* has been based upon morphological, physiological, biochemical and immunological criteria. Studies of above methods including isoenzyme electrophoresis, however, were not consistent with various species assignments based on morphological criteria. Analysis of interstrain variability in nucleotide sequences of mitochondrial DNA(mtDNA) should be better molecular method of measuring overall genotype differences; restriction endonuclease analysis have proven to be quite powerful tool in revealing mtDNA phylogenetic relationships among closely-related organisms. In the present study, we performed the restriction endonuclease digestions of mitochondrial DNAs in order to make restriction site maps. Fragments of mtDNAs of *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* digested with six restriction endonucleases, Cla I, Hae III, Hin dIII, Pvu II, Sal I and Spe I, have shown approximately 3-13 bands. Total mtDNA genome size by summation of six restriction enzymes averaged 42.4 kbp in *Acanthamoeba* sp. YM-4, 43.9 kbp in *A. culbertsoni* and 43.8 kbp in *A. polyphaga*, respectively. Fragment sizes ranged between 0.6 kbp and 39.3 kbp. Based on these data, restriction site maps were established.

Key Words: *Acanthamoeba*, Restriction endonuclease, Mitochondrial DNA, Restriction site map

INTRODUCTION

Free-living amoebae from genus *Acanthamoeba* and *Naegleria*, widespread in soil and freshwater, have been known to cause disease in human and other mammals. A rapidly fatal disease, most often called primary amoebic meningoencephalitis(PAME), is usually caused by *Acanthamoeba culbertsoni* and *Naegleria fowleri*[10,12]. Human infection accompanying PAME has been estimated as 200 cases, 56 of these cases have been due to *Acanthamoeba* spp. and 144 cases due to *Naegleria fowleri* in the United States[27]. Another cases, Acanthamoeba keratitis have been reported in the United States and Japan[25,29].

In Korea, one probable PAME case due to *Acanthamoeba* sp. happened in 5-year-old child[22], acanthamoebic pneumonitis combined with immunodeficient syndrome[16] and four cases of acanthamoebic keratitis were noticed[6,17]. And *Acanthamoeba* spp. and *Naegleria* spp. had been isolated from various sources and had been proved their pathogenicity in experimental animals[14,15].

The study of taxonomic relationships among members of *Acanthamoeba* has been based upon morphological and physiological criteria[13,23,24]. Other species differences such as antigenic diversity[26], starch-gel electrophoresis for serologic analysis[7], agarose isoelectric focusing[11] and isoenzyme electrophoretic analysis[8,18], has proven to be useful tools in that genetic distances between the various species and strains can be estimated. Studies of immunological methods including isoenzyme electrophoresis, however, were not consistent with various species assignments based on morphological criteria[9,21].

In theory, analysis of interstrain variability in nucleotide sequences of organelle DNA should be a better molecular method of measuring overall genotype differences. In addition, restriction endonuclease analysis have proven to be quite powerful Fool in revealing mitochondrial DNA(mtDNA) phylogenetic relationships among closely-related organi-

*This study was performed by the Grant of Ajou University, 1995.

Reprint requests to: *Corresponding Author:* Ho-Joon Shin Department of Microbiology, School of Medicine, Ajou University, Suwon, 442-749, Korea

sms[3,19]. This molecular approach has been used to examine the relationships between various isolates of *Acanthamoeba*[2,4,9,20,21,29]. In the present study, we performed the restriction endonuclease digestions of mitochondrial DNAs for making the restriction site maps, expecting more apparent sights on diversity of restriction fragments, as an aid to refer informations to taxonomy of *Acanthamoeba* isolate in Korea.

MATERIALS AND METHODS

Acanthamoeba strains

Pathogenic *A. culbertsoni, A. polyphaga* (donated from Dr. JB Jadin, Belgium) and *Acanthamoeba* isolate in Korea (designed as *Acanthamoeba* sp. YM-4) were axenically subcultured in CGV medium[28] at 37℃ in incubator.

Preparation of mitochondrial DNA of *Acanthamoeba*

Mitochondrial DNA of *Acanthamoeba* were obtained by Alkaline lysis method of Birnbiom and Doly(1979). Midlog phase amoebae (0.5×10^7) were collected from media, washed 3 times in PBS(pH 7.2) and centrifuged at 300 g for 5 min. Pelleted amoebae were washed again 3 times in a Eppendorf tube in TES buffer(pH 8.0, 50 mM Tris-HCl, 10 mM EDTA, 50 mM Sucrose), resuspended in 100 μl of chilled TES and added to 200 μl of freshly prepared 1% SDS solution in 0.2 N NaOH. Suspension was gently mixed by inverting the tubes and incubated at 0℃ for 5 min. It was added to 150 μl of chilled 3 M potassium acetate buffer(pH 6.0, 5 M Potassium acetate 60 ml, Glacial acetate 11.5 ml, D.W. 28.5 ml). Suspension was mixed, incubated for 30 min. at 0℃ and centrifuged 12,000rpm for 10 min. at 4℃. Supernatant fluid was transferred to a fresh Eppendorf tube. Equal volume of phenol/chloroform(1 : 1) solution was added and centrifuged 12,000rpm for 10 min. at 4℃. Supernatant fluid was collected, 1.0 ml of cold absolute ethanol and 35 μl of 3 M sodium acetate solution were added, stood at −70℃ and centrifuged 15,000rpm for 15 min at 4℃. Supernatant fluid was dried with a centrifugal evaporator at 230g for 2~3 min at 42℃. Sediment was dissolved in 30~50 μl of TE buffer(pH 8.0, 5 mM Tris-HCl, 1 mM EDTA). DNA isolates were monitored by agarose gel electrophoresis and stored at −20℃ until use.

Restriction enzyme digestion of mtDNA

0.5 ug of the mtDNAs were incubated with six restriction endonucleases, Cla I, Hae III, Hin dIII, Pvu II, Sal I and Spe I (Stratagene Co. USA) at 37℃ overnight in a total volume of 10 μl using buffers specified by the enzyme supplier. The digests were electrophoresed in 1.0% agarose gel at 3 V/cm for 6 hrs in TBE buffer(89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA). Gel was stained with ethidium bromide and observed with a short wave ultraviolet transilluminator. Hind III digests of λ phage was included in each gel run as size standards.

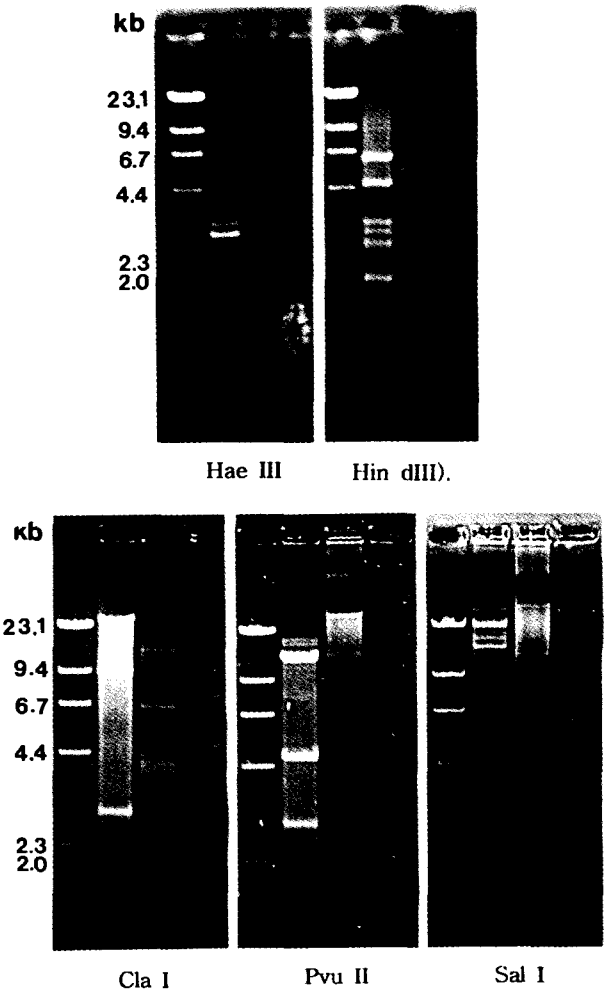

Fig. 1. Mitochondrial DNAs gel electrophoretic patterns of *Acanthamoeba* YM-4 (each lane 2), *A. culbertsoni* (each lane 3) and *A. polyphaga* (each lane 4) after digestion with Hal III, Hin dIII, Cla I, Pvu II and Sal I respectively. Each lane 1 is the size marker (λ DNA digested with Hin dIII).

Table 1. Mitochondrial DNA sizes in *Acanthamoeba* spp. calculated by summation of six endonucleases

Amoebae	Kinds of restriction endonucleases						Average size*
	Cla I	Hae III	Hin dIII	Pvu II	Sal I	Spe I	
Acanthamoeba sp. YM-4	41.0±4.71	24.3±0.95	38.5±0.58	48.0±0.32	54.3±1.34	48.3±1.54	42.4±10.5
A. culbertsoni	39.2±2.27	28.0±1.08	43.3±1.41	58.8±2.27	54.2±2.93	40.0±1.57	43.9±11.1
A. polyphaga	40.1±0.68	30.7±1.32	48.8±1.59	47.4±0.52	50.2±2.81	46.3±1.16	43.8± 7.35

*summed fragment-size in kilobase pairs (mean±S.D)

Table 2. Fragment-size* estimates for *Acanthamoeba* spp.

Endonuclease fragments		Kinds of *Acanthamoeba*		
		Acanthamoeba YM-4	*A. culbertsoni*	*A. polyphaga*
Cla I	1	29.0±4.09	14.3±0.97	19.7±0.35
	2	9.0±0.51	6.8±0.35	7.6±0.23
	3	2.9±0.17	5.8±0.29	6.1±0.15
	4		4.2±0.29	4.6±0.06
	5		4.0±0.30	2.1±0.06
	6		2.2±0.11	
	7		1.9±0.06	
Hae III	1	3.2±0.21	5.3±0.30	7.2±0.50
	2	2.9±0.17	4.7±0.25	4.4±0.20
	3	2.6±0.11	3.1±0.23	3.3±0.21
	4	2.2±0.06	2.6±0.11	3.1±0.23
	5	1.8±0.06	2.3±0.10	2.7±0.11
	6	1.7±0.06	2.0±0.06	2.3±0.06
	7	1.6±0.06	1.7±0.11	2.2±0.06
	8	1.3±0.11	1.5±0.00	2.1±0.06
	9	1.1±0.11	1.4±0.00	1.8±0.00
	10	1.0±0.06	1.2±0.00	1.7±0.00
	11	0.9±0.06	1.1±0.00	
	12	0.8±0.00	1.0±0.00	
	13	0.7±0.00		
	14	0.6±0.00		
Hin dIII	1	9.8±0.30	13.3±0.78	9.4±0.70
	2	6.1±0.25	6.6±0.30	7.7±0.45
	3	4.4±0.06	6.1±0.25	6.6±0.30
	4	3.2±0.26	5.5±0.06	6.1±0.25
	5	2.8±0.10	4.4±0.00	5.3±0.21
	6	2.5±0.10	2.5±0.11	3.7±0.15
	7	2.4±0.10	2.2±0.11	2.5±0.10
	8	1.8±0.11	1.5±0.06	2.2±0.11
	9	1.5±0.06	1.3±0.06	2.1±0.10
	10	1.3±0.06		1.8±0.06
	11	1.1±0.00		1.5±0.06
	12	0.9±0.00		
	13	0.7±0.00		
Pvu II	1	18.3±0.15	39.3±1.45	13.8±0.30
	2	13.8±0.30	15.0±1.08	8.2±0.25
	3	8.2±0.25	4.5±0.15	4.5±0.15
	4	4.7±0.10		3.5±0.10
	5	2.9±0.10		
Sal I	1	21.9±0.95	36.4±2.05	32.6±3.38
	2	17.2±0.25	13.8±1.88	8.8±0.43
	3	15.2±0.35	4.0±0.10	4.5±0.15
	4	2.2±0.00		3.5±0.06
	5	1.8±0.00		
Spe I	1	19.4±0.30	16.8±0.35	13.2±3.38
	2	9.9±0.40	12.1±0.47	8.8±0.43
	3	8.4±0.26	4.0±0.38	5.4±0.30
	4	7.3±0.46	2.8±0.21	3.4±0.30
	5	3.3±0.26	2.3±0.23	
	6		2.0±0.23	

* : kilobase pairs(mean±S.D.)

RESULTS

Fragments of mtDNA by restriction enzymes

Fragments of mtDNAs of *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* digested with six restriction endonucleases showed approximately 3-13 bands, ranged from 0.6 kilobase pairs(kbp) to 39.3 kbp. Figure 1 shows the fragments digested with Hal III, Hin dIII Cla I, Pvu II and Sal I. Total mtDNA sizes by summation of six restriction enzymes ranged 24.3 kbp to 54.3 kbp (average 42.4 kbp) in *Acanthamoeba* sp. YM-4, 28.0 kbp to 58.8 kbp (average 43.9 kbp) in *A. culbertsoni* and 30.7 kbp to 50.2 kbp (average 43.8 kbp) in *A. polyphaga*, respectively (Table 1). Each fragment sizes are shown in table 2.

Restriction site maps of mtDNA of *Acanthamoeba*

Restriction site maps were established by the models of Byers *et al.*(1990), by simply arranging the ratricton fragmetns on linear line that mt DNA sizes were marked, in order to compare only the ditterince of restriction sites digested with various restriction endonucleases. Figure 2 showed the restriction site maps of mtDNAs *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* digested with Cla I, Pvu II, Sal I and Spe I endonuclease, respectively.

DISCUSSION

It had been known that mtDNA of *Acanthamoeba* consists of circular molecules. Estimates of genome size based on the summation of restriction fragment size and measurements of circular molecules by electron microscopy give an average of 42.5 kbp for 17 strains representing seven to nine different species.[5] In this study, the mtDNA genome size of *Acanthamoeba* sp. YM-4 determined by summation of fragment sizes was an average 42.4 kbp, which was slightly smaller than *A. culbertsoni* and *A. polyphaga*, 43.9 kbp and

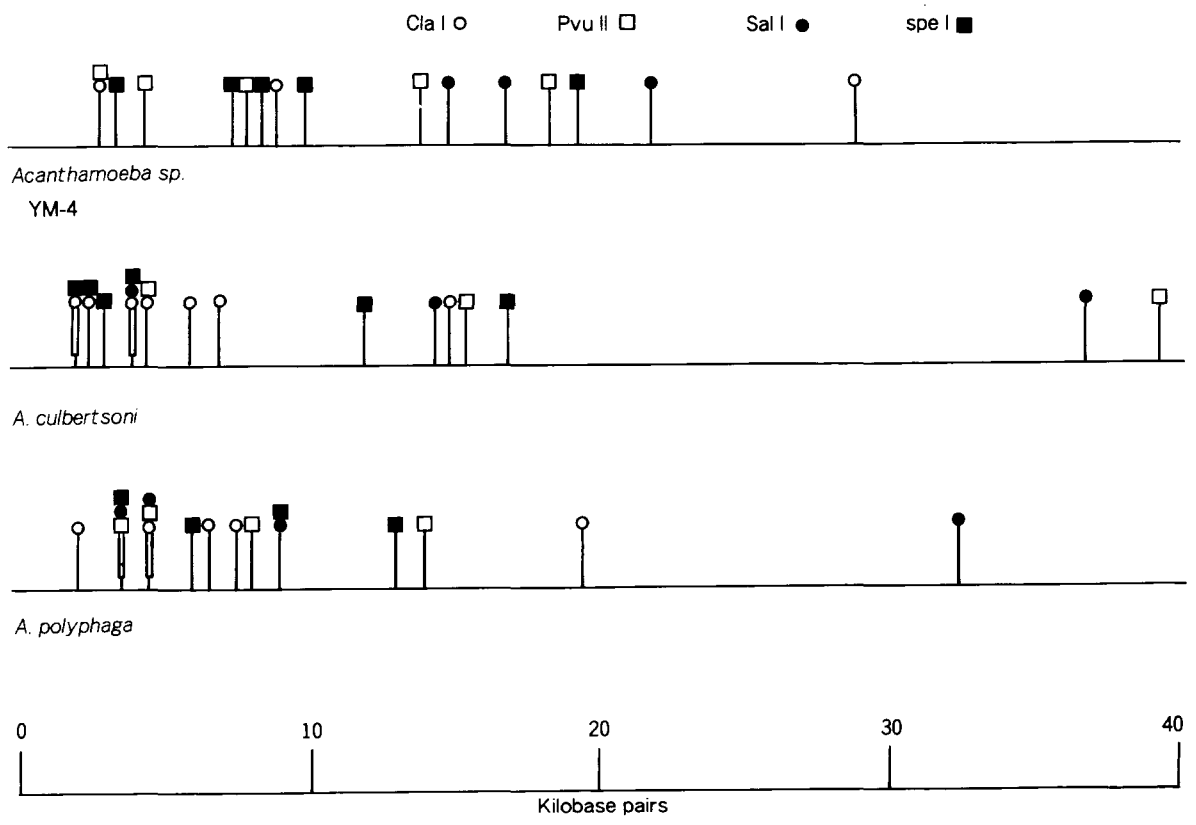

Fig. 2. Restriction site maps of mtDNA of *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* digested with four restriction endonucleases.

43.8 kbp, respectively. Fragment sizes of *Acanthamoeba* were various by ranged from 0.6 kbp to 39.3 kbp. In previous report[5], the mtDNA genome sizes of *A. culbertsoni* and *A. polyphaga* were 44.3 kbp and 43.6 kbp, respectively. Of course, the mtDNA genome size, based on the summation of fragment sizes digested with restriction enzymes, may not be same as the true sizes obtained by whole sequencing of mtDNA. Because it was not ruled out that the fragments were digested partially or been doublet, in spite of the digestion for overnight or mean value by repeat experiments. In addition, high molecular marker should have been used to estimate large fragments above 23 kbp.

Byers *et al.*,(1990) showed the restriction site maps of *A. castellani* strains-Boyce, Chang, Neff and Ray-digested with Eco RI, Bcl I, Bgl II and Pst I. In that paper, the mtDNA genome size by summation of restriction fragments was reported and restriction site maps were shown by simply arranging the restriction fragments in order to compare only the difference of restriction sites digested with various restriction endonucleases. It was speculated that these resticition site mapping was basically different from the gene mapping. Unfortunately, since the species and endonuclease used in experiment were not the same as those of present study, their results could not be compared. In present study, restriction site maps of mtDNA of *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* was established from data obtained with 4 endonuclease digestins, Cla I, Pvu II, Sal I and Spe I. This is because the difference of restriction sites among three species can be easly compared. Restriction site maps using Hae III and Hin dIII enzyme were not designed, due to the fact that their fragment sizes were relatively small and closely concentrated onto low part, 10 more fragments being smaller than 10 kbp.

In order to elucidate the phylogenetic diversity among members of *Acanthamoeba*, restriction fragments length polymorphism(RFLP) analysis is undoubtfully a powerful tool, and its applications have been reported in phylogenetic studies of *Acanthamoeba*[2,4,9,20,21,29]. Of course, in another study, the RFLP of mtDNA of *Acanthamoeba* isolate in Korea have been observed, including *A. culbertsoni* and *A. polyphaga* as reference amoeba. However more easily comparing tool is needed for a glance on the difference of restriction fragments.

As previous review paper showed the restriction site maps of *Acanthamoeba* strains[5], their schematic maps lead us to more apparent sites on diversity of restriction fragments digested with various endonucleases. And it is suggested that restriction site maps of mtDNA of *Acanthamoeba* used in present study serve as a guide for identification of *Acanthamoeba* isolate in Korea.

CONCLUSION

In the present study, we performed the restriction endonuclease digestions of mitochondrial DNAs for making the restriction site maps. Fragments of mtDNAs of *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* digested with six restriction endonucleases, Cla I, Hae III, Hin dIII, Pvu II, Sal I and Spe I, were showed approximately 3-13 bands. Total mtDNA sizes by summation of six restriction enzymes were average 42.4 kbp in *Acanthamoeba* sp. YM-4, 43.9 kbp in *A. culbertsoni* and 43.8 kbp in *A. polyphaga*, respectively. Fragment sizes ranged between 0.6 kbp and 39.3 kbp. Based on these data, restriction site maps were established.

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