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Restriction endonuclease analysis of mitochondrial DNA of *Acanthamoeba* sp. YM-4 (Korean isolate)

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Abstract: *Acanthamoeba* sp. YM-4 is similar to *A. culbertsoni* based upon morphological characteristics of trophozoites and cysts. However, based on other characteristics, pathogenicity to mice, *in vitro* cytotoxicity and isoenzyme patterns, *Acanthamoeba* sp. YM-4 was quite different from *A. culbertsoni*. Restriction fragment length polymorphism (RFLP) analysis of mtDNA is useful in the classification of members belonging to the genus *Acanthamoeba*. Therefore, in this study, RFLP analysis of *Acanthamoeba* mtDNAs 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 (range: from 0.6 kbp to 34.4 kbp). The mtDNA genome size, calculated by the summation of restriction fragments, averaged 46.4 kbp in *Acanthamoeba* sp. YM-4, 48.3 kbp in *A. culbertsoni* and 48.8 kbp in *A. polyphaga*, respectively. Digested mtDNA fragments of *Acanthamoeba* sp. YM-4 contained nine and seven same size fragments, respectively, from a total of 67 and 69 fragments observed in *A. culbertsoni* and *A. polyphaga*. An estimate of the genetic divergence was 10.1% between *Acanthamoeba* sp. YM-4 and *A. culbertsoni*, and 9.9% between *Acanthamoeba* sp. YM-4 and *A. polyphaga*.
Key words: *Acanthamoeba*, mtDNA, RFLP, genetic divergence

INTRODUCTION

Members of the genus *Acanthamoeba* are small free-living amoebae, are wide-spread and commonly found in soil and freshwater, and are known to cause clonic glanulomatous amoebic encephalitis (GAE) in humans and experimental animals (Visvesvara and Stehr-Green, 1990). Several hundred cases of Acanthamoeba keratitis, caused by *A. polyphaga*, have been reported (Stehr-Green *et al.*, 1989; Cho *et al.*, 1992). In Korea, an *Acanthamoeba* sp. was isolated from sewage-water and a fish's gill (designated as *Acanthamoeba* sp. YM-4), and in experimental studies, cause GAE in mice (Hwang *et al.*, 1976).

Taxonomic relationships among members of *Acanthamoeba* have been based mostly upon morphological and physiological criteria (Singh, 1952; Page, 1967). Other characteristics, i.e., antigenic diversity (Stevens *et al.*, 1977), agarose isoelectric focusing (De Johnckheere, 1983) and isoenzyme electrophoretic analysis (Jacobson and Band, 1987), have proven to be useful tools for estimating genetic distances between the various species and strains. Studies of immunological methods as well as isoenzyme electrophoresis, however, were not consistent with various species assignments based on

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morphological criteria.

As DNA technology has improved, the restriction endonuclease analysis has proven to be a quite powerful tool for determining mitochondrial DNA (mtDNA) phylogenetic relationships among closely related organisms. Restriction fragment length polymorphism (RFLP) analysis of whole-cell DNA and mtDNA are useful for the classification of members of the genus *Acanthamoeba* (McLaughlin *et al.*, 1988; Yagita and Endo, 1990; Kong *et al.*, 1995). Thus, a relatively high number of intra and interspecfic comparisions of mtDNA RFLP has been conducted for *Acanthamoeba* spp.

Acanthamoeba sp. YM-4 was similar to *A. culbertsoni* based on morphological characteristics of trophozoites and cysts (Shin *et al.*, 1992). However, it was less pathogenic in experimental animals (Park *et al.*, 1989) and demonstrated decreased cytotoxicity against mammalian cell line-CHO cells (Shin *et al.*, 1993), when *Acanthamoeba* sp. YM-4 compared to *A. culbertsoni* parasites. Their isoenzyme patterns were different from each other (Hwang *et al.*, 1980) and the monoclonal antiboby specific for *Acanthamoeba* sp. YM-4 was produced as an aid for immunological taxonomy (Shin *et al.*, 1992). Although *Acanthamoeba* spp. have been studied intensively, their taxonomy has not been clearly defined.

In this study, we performed RFLP analysis of mtDNA to estimate the total mtDNA genome size of *Acanthamoeba* sp. YM-4 and also to compare the mtDNA RFLP between *A. culbertsoni* and *A. polyphaga* for reference information of *Acanthamoeba* sp. YM-4 identification.

MATERIALS AND METHODS

Acanthamoeba strains

Pathogenic *A. culbertsoni*, *A. polyphaga* (donated by Dr. Jadin JD, Belgium) and *Acanthamoeba* sp. YM-4 were subcultured axenically at 37°C and 25°C in PYG medium [2% proteose peptone, 0.2% yeast extract, 0.1 M glucose, 4 mM $MgSO_4.7H_2O$, 0.4 mM $CaCl_2$, 3.4 mM sodium citrate.$2H_2O$, 50 mM $Fe(NH_4)_2(SO_4)_2.6H_2O$, 2.5 mM KH_2PO_4, 2.5 M $Na_2HPO_4.7H_2O$ in distilled water, pH 6.5].

Preparation of mtDNA

Mitochondrial DNA of *Acanthamoeba* spp. were extracted by the modified method described by Yagita and Endo (1990). Approximately 5×10^7 trophozoites of each strain of *Acanthamoeba* were used for mtDNA isolation. Trophozoites were collected in a centrifuge tube and washed three times in 20mM Tris-HCl (pH 7.4) by centrifuging at 400 g for 1 min. The pellet was washed once in Eppendorf tube containing TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The pelleted amoebae were resuspended in 100 μl of chilled TEG buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) and mixed gently. 200 μl of freshly prepared 0.2N NaOH solution containing 1% sodium dodecyl sulfate (SDS) were added. The suspension was gently mixed by inverting the tubes several times and incubated on wet ice for 5 min. 150 μl of ice-cold 5M potassium acetate buffer (5M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of distilled water, pH 6.0) was added into each tube, incubated for 30 min, and then centrifuged at 12,000 g for 5 min at 4°C. The supernatants were extracted once with an equal volume of buffered-phenol solution. The aqueous phase removed after centrifugation was extracted once with an equal volume of chloroform solution. The supernatant was precipitated by adding 0.1 volume of 3M sodium acetate (pH 7.5) and 2 volumes of cold ethanol. The tube was maintained for 30-60 min at –20°C and then spun for 10 min in a microfuge. The pellet was dissolved in 50 μl of TE buffer. RNAse (5 mg/ml) was added up to a final concentration of 50 μg/ml, and the mixture incubated at 37°C for 1 hr and stored at –20°C until used.

Restriction enzyme digestion and electrophoresis

The endonucleases-*Hae*III, *Hind*III, *Cla*I, *Pvu*II and *Sal*I, provided with appropriate reaction buffers, were purchased from Stratagene Co. (USA). DNA samples (2-3 μg in 3 μl) were digested overnight at 37°C with 2 units (0.5 μl) of the restriction endonuclease in appropriate reaction buffer. The digested DNAs were loaded onto horizontal 0.7% agarose gels

prepared in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) and separated at 2 V/cm for 4 hrs. Subsequently, the electrophoresed gels were stained with ethidium bromide (0.4 g/ml in TAE buffer) for 30 min and then briefly rinsed with water. DNA fractions were observed with a short wave ultraviolet transilluminator and photographed with a Polaroid 667 film and a Kodak Wratten 23A orange filter. The *Hind*III digested λDNA was run as DNA size standards.

Genetic divergence

Interspecies genetic divergence was estimated by the method of Nei and Li (1979) based on the comparisions of the electrophoretic patterns of mtDNA digested by restriction enzymes.

RESULTS

Digestion of restriction endonucleases

Acanthamoeba mtDNAs were digested with five restriction enzymes: *Hae*III, *Hind*III, *Cla*I, *Pvu*II and *Sal*I. Each enzyme produced approximately 3-15 fragments, ranging from 0.6 kilobase pairs (kbp) to 34.4 kbp. MtDNA restriction fragment patterns are shown in Fig. 1 and summarized in Table 1. Total mtDNA sizes, calculated as the summation of five restriction enzymes, ranged from 38.5 kbp to 54.3 kbp (average 46.4 kbp) in *Acanthamoeba* sp. YM-4, 39.2 kbp to 56.2 kbp (average 48.3 kbp) in *A. culbertsoni* and 40.1 kbp to 57.8 kbp (average 48.8 kbp) in *A. polyphaga*, respectively (Table 2).

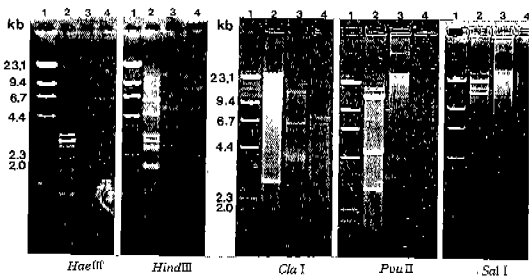

Fig. 1. DNA gel electrophoretic patterns of *Acanthamoeba* YM-4 (lane 2), *A. culbertsoni* (lane 3) and *A. polyphaga* (lane 4) after digestion with *Hae*III, *Hind*III, *Cla*I, *Pvu*II and *Sal*I, respectively. Lane 1 is the size marker (λ DNA digested with *Hind*III).

Genetic divergence

The genetic divergence among the three *Acanthamoeba* species examined was estimated using the RESTSITE program (Nei and Li, 1979) based on collected data from the five restriction enzymes (Table 3). In camparison with *A. culbertsoni* and *A. polyphaga*, digested mtDNA fragments of *Acanthamoeba* sp. YM-4 shared nine and seven homologous fragments among a total 67 and 69 fragments, respectively. Between *Acanthamoeba* sp. YM-4 and *A. culbertsoni*, the genetic divergence was 10.1% (this means that the estimated sequence substitution was 0.101 per nucleotide position surveyed in this study). Also, interspecies divergence between *Acanthamoeba* sp. YM-4 and *A. polyphaga* was 9.9%. Using the UPGMA method, the schematic diagram of genetic distances among *Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. polyphaga* is shown in Fig. 2.

DISCUSSION

RFLP is a powerful tool in revealing mtDNA phylogenetic relationships among closely-related organisms, and has been used to examine the relationships between various isolates of *Acanthamoeba* (Byers *et al.*, 1990). It is known that the mtDNA of *Acanthamoeba* is a circular molecule. Estimated genome size, based on the summation of restriction fragment size and measurements of circular molecules by electron microscopy, is an average of 42.5 kbp for 17 strains representing seven to nine different species (Byers *et al.*, 1990). In this study, the mtDNA genome of *Acanthamoeba* sp. YM-4, determined as the summation of restriction fragments sizes, was an average 46.4 kbp, which was slightly smaller than *A. culbertsoni* (48.3 kbp) and *A. polyphaga* (48.8 kbp). MtDNA genome size, based on the summation of fragment sizes

Table 1. Fragment-size estimates for *Acanthamoeba* spp.

Endonuclease fragments		Kinds of *Acanthamoeba*		
		Acanthamoeba sp. 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	26.0 ± 0.16	28.1 ± 0.70	27.1 ± 0.62
	2	3.2 ± 0.21	5.3 ± 0.30	7.2 ± 0.50
	3	2.9 ± 0.17	4.7 ± 0.25	4.4 ± 0.20
	4	2.6 ± 0.11	3.1 ± 0.23	3.3 ± 0.21
	5	2.2 ± 0.06	2.6 ± 0.11	3.1 ± 0.23
	6	1.8 ± 0.06	2.3 ± 0.10	2.7 ± 0.11
	7	1.7 ± 0.06	2.0 ± 0.06	2.3 ± 0.06
	8	1.6 ± 0.06	1.7 ± 0.11	2.2 ± 0.06
	9	1.3 ± 0.11	1.5 ± 0.00	2.1 ± 0.06
	10	1.1 ± 0.11	1.4 ± 0.00	1.8 ± 0.00
	11	1.0 ± 0.06	1.2 ± 0.00	1.7 ± 0.00
	12	0.9 ± 0.06	1.1 ± 0.00	
	13	0.8 ± 0.00	1.0 ± 0.00	
	14	0.7 ± 0.00		
	15	0.6 ± 0.00		
*Hind*III	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	31.3 ± 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	34.4 ± 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		

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

Table 2. Mitochondrial genome sizes in *Acanthamoeba* spp. calculated by summation of five endonucleases

Amoebae	Kinds of restriction endonucleases					Average size*
	*Cla*I	*Hae*III	*Hind*III	*Pvu*II	*Sal*I	
Acanthamoeba sp. YM-4	41.0 ± 4.71	50.3 ± 1.12	38.5 ± 0.58	48.0 ± 0.32	54.3 ± 1.34	46.4 ± 6.55
A. culbertsoni	39.2 ± 2.27	56.2 ± 1.72	43.3 ± 1.41	50.8 ± 2.27	52.2 ± 2.93	48.3 ± 6.92
A. polyphaga	40.1 ± 0.68	57.8 ± 1.47	48.8 ± 1.59	47.4 ± 0.52	50.2 ± 2.81	48.8 ± 6.32

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

Table 3. Proportions of homologous fragments. Estimates of interspecies genetic divergence in paired comparisons of *Cla*I, *Hae*III, *Hind*III, *Pvu*II and *Sal*I digestion fragment patterns

	Acanthamoeba sp. YM-4	*A. culbertsoni*	*A. polyphaga*
Acanthamoeba sp. YM-4	*Cla*I	0/10[a]	0/8
	*Hae*III	4/20	2/22
	*Hind*III	4/14	3/18
	*Pvu*II	1/6	2/5
	*Sal*I	0/8	0/9
		(9/58)	(7/62)
A. culbertsoni	0.102 ± 0.050	—	0/12
			3/18
			5/10
			2/3
			0/7
			(10/50)
A. polyphaga	0.099 ± 0.028	0.093 ± 0.053	—

[a]the number of common fragments per the number of heteromorphic fragments (summation from five enzymes)

[b]genetic divergence based on collected data ( ) from five enzymes

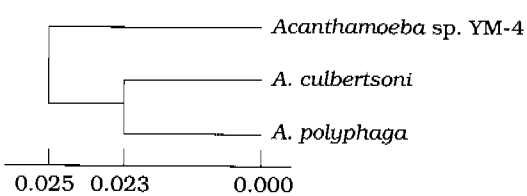

Fig. 2. Dendrogram showing estimated genetic divergence among *Acanthamoeba* spp. using UPGMA cluster analysis.

digested with restriction enzymes, could not be the same as the real size obtained by whole sequencing of mtDNA. In previous report (Byers *et al.*, 1990), the mtDNA genome sizes of *A. culbertsoni* and *A. polyphaga* were 44.3 kbp and 43.6 kbp, repectively. It was hypothesized that their differences resulted from the number and kinds of restriction enzymes used, based on the results of Milligan and Band (1988) and the estimation techniques for determining fragment size in agarose gels. It was not ruled out that the fragments were digested partially or were doublets, in spite of the overnight digestion and mean value by repeat experiments. In addition, high weight molecular markers are not reliable for estimating large fragments above 23 kbp.

RFLP analysis is undoubtfully a powerful tool for elucidating the phylogenic relationships among the members of *Acanthamoeba*, and its applications have been

reported in phylogenetic studies of *Acanthamoeba* (Milligan and Band, 1988; Yagita and Endo, 1990). Fragments obtained by digestion of *Acanthamoeba* spp. mtDNA were campared intraspecifically with *A. polyphaga* (Kong *et al.*, 1995), Milligan and Band (1988) had used to analyse restriction patterns of mtDNA on relatedness to *Naegleria* and other vahlkampfid amoebae. The authors obtained interstrain divergence of 3-14%, while intraspecific divergence of 3-7% was significantly less than the interspecific divergence of 12-14%. In this study, the genetic divergence was 10.1% between *Acanthamoeba* sp. YM-4 and *A. culbertsoni*, and 9.9% between *Acanthamoeba* sp. YM-4 and *A. polyphaga*. These data indicate that *Aanthamoeba* sp. YM-4 is a distinct species from *A. culbertsoni* and *A. polyphaga* based on RFLP analysis of mtDNAs.

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=초록=

Acanthamoeba sp. YM-4의 미토콘드리아 DNA의 RFLP 분석

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Acanthamoeba sp. YM-4는 영양형 및 포낭의 형태학적 특징이 *A. culbertsoni*와 비슷하지만, 마우스에 대한 병원성, *in vitro* 세포독성, isoenzyme pattern 비교 분석 및 종-특이성 단세포군 항체 교차반응 등에 의하면 *A. culbertsoni*와는 조금 다르다. 많은 아메바들이 다양한 환경에서 다양한 형태로 분리됨으로써 종 동정에 있어서 좀더 다양한 정보를 얻고자 분자유전학적 접근을 시도하였다. 본 실험은 *Acanthamoeba* sp. YM-4(한국 분리주)에 대한 미토콘드리아 DNA(mtDNA)를 분리하여, 여러 종류의 제한효소를 처리함으로써 mtDNA의 단편들을 얻은 다음, 전체 크기 및 제한효소 절단 단편길이 다형성(RFLP) 분석을 하였다. 5가지의 제한효소 즉, *Hae*Ⅲ, *Hind*Ⅲ, *Cla*Ⅰ, *Pvu*Ⅱ 및 *Sal*Ⅰ으로 처리된 *Acanthamoeba*의 mtDNA는 최소 3개의 단편들로부터 많은 것은 15개의 단편들로까지 나뉘어 졌다. 단편들을 합산한 mtDNA의 전체 크기는 *Acanthamoeba* sp. YM-4가 평균 46.4 kbp였으며, *A. culbertsoni* 및 *A. polyphaga*는 각각 48.3 kbp 및 48.8 kbp로 관찰되었다. 제한효소 단편길이들은 0.6 kbp로부터 34.4 kbp까지 다양하였으며, *Acanthamoeba* sp. YM-4의 mtDNA 단편들을 *A. culbertsoni* 및 *A. polyphaga*와 비교해 볼 때, 각각 총 67개 및 69개 중에서 공통으로 갖은 단편들이 각각 9개 및 7개로 관찰되었다. 그것을 토대로 genetic divergence를 계산한 결과 *Acanthamoeba* sp. YM-4와 *A. culbertsoni* 간에는 10.1%였으며, *A. polyphaga*와는 0.99%였다. 이런 다형성의 결과는 *Acanthamoeba* sp. YM-4가 *A. culbertsoni* 및 *A. polyphaga*와는 종이 다를 수 있다는 것을 보여준다고 하겠다.

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