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Interstrain polymorphisms of isoenzyme profiles and mitochondrial DNA fingerprints among seven strains assigned to *Acanthamoeba polyphaga*

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Abstract: Interstrain polymorphisms of isoenzyme profiles and mitochondrial (Mt) DNA fingerprints were observed among seven strains of *Acanthamoeba* isolated from different sources and morphologically assigned to *A. polyphaga*. Mt DNA fingerprints by eight restriction endonucleases (*Bgl* II, *Sca* I, *Cla* I, *EcoR* I, *Xba* I, *Kpn* I, *Sal* I, and *Sst* I) revealed considerable interstrain polymorphisms. Isoenzyme profiles revealed considerable interstrain polymorphisms for acid phosphatase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase while those for glucose phosphate isomerase, leucine aminopeptidase, and malate dehydrogenase showed similarity. Despite of the interstrain polymorphisms, the isoenzyme profiles and Mt DNA fingerprints of the strain Ap were found to be identical with those of the strain Jones. Mt DNA fingerprinting was found to be highly applicable for the strain identification, characterization, and differentiation.

Key words: *Acanthamoeba polyphaga*, interstrain polymorphism, isoenzyme profiles, Mt DNA fingerprints, strain differentiation, strain identification.

INTRODUCTION

Acanthamoeba spp. are widespread in environments such as soil, air, fresh-water, and ocean sediments (Ma *et al.*, 1990). Recently, medical protozoologists have paid attention to *Acanthamoeba* spp. since several species of the genus had been known to cause serious human infections. Some of the amoebae cause the amoebic keratitis, especially in contact lens wearers (Moore *et al.*, 1987) and the granulomatous amoebic encephalitis (GAE) in debilitated patients, chronic alcoholics, or patients under immunosuppressive therapy (Martinez, 1987; Helton *et al.*, 1993).

The identification of *Acanthamoeba* at the generic level can be easily accomplished by morphologic characters (Visvesvara, 1991). However, there have been disputes over species identification of *Acanthamoeba*. Pussard and Pons (1977) classified *Acanthamoeba* spp. into three groups according to the cyst size and morphologic features. The grouping has been widely used before species identification of the amoebae. The morphology of the cyst, however, can be changed with the culture conditions (Stratford and Griffiths, 1978) and highly variable within a single strain. Therefore, species identification by morphology alone can hardly be possible (Visvesvara, 1991).

Investigators have used several kinds of non-morphological methods for the taxonomy of *Acanthamoeba* spp. Among the methods, isoenzyme analysis and restriction fragment length polymorphism (RFLP) of mitochondrial (Mt) DNA have been recently applied (Byers *et*

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al., 1990; Kilvington *et al.*, 1991; Moura *et al.*, 1992; Yagita, 1993; Gautom *et al.*, 1994).

The authors observed the interstrain polymorphisms of isoenzyme profiles and Mt DNA fingerprints among the strains morphologically assigned to *A. polyphaga*, and propose the applicability of the isoenzyme analysis and Mt DNA fingerprinting as tools for strain differentiation, identification, and characterization of *Acanthamoeba* spp.

MATERIALS AND METHODS

1. *Acanthamoeba* isolation and cloning

One gram of soil was inoculated on an 1.5% agar plate which had been covered with heat-inactivated *Escherichia coli* (ATCC 25922, free of plasmid) at 60°C for 1 hour. The plates were incubated at 25°C for 1 week and examined for the presence and growth of *Acanthamoeba* under an inverted microscope. The *Acanthamoeba* cysts encysted on the plate were grouped by their size and morphological features according to Pussard and Pons (1977). Each cyst isolated by micromanipulation was inoculated and incubated on a new agar plate for one more week.

2. Axenic culture

A piece of agar plate (0.5 × 1 cm) covered with the cysts of a clone was treated with 0.1 N HCl for 24 hours for axenization and washed with glass distilled water three times. The agar plate was placed and incubated in PYG (10 g Proteose Peptone, 10 g Yeast extract, 50% Glucose 10 ml, 0.5 M Na_2HPO_4 10 ml and 0.5 M KH_2PO_4 10 ml in 970 ml of glass distilled water) or PYGC (10 g Proteose Peptone, 10 g Yeast extract, 1 g Glucose, 0.95 g L-Cystein, 5 g NaCl, K_2HPO_4 0.87 g and KH_2PO_4 0.68 g in 1,000 ml of distilled water) media at 25°C and 37°C.

3. Strains of *Acanthamoeba* used

Three strains (KA/S3, KA/S6, KA/S7) of *Acanthamoeba* isolated from Korean soil were morphologically assigned to *A. polyphaga* according to the keys described by De Jonckheere (1987) and Page (1988). Three strains of *A. polyphaga* (ATCC #30461, the Jones strain, #30871, the P23 strain, & #30873, the Nagington strain) were obtained from American Type Culture Collection (ATCC, Rockville, MD). The Ap strain, was donated by Professor Kyung-Il Im, Department of Parasitology, Yonsei University College of Medicine.

4. Preparation of amoeba lysate

The trophic amoebae cultured axenically were harvested at the end of the logarithmic growth phase by chilling on ice water under sterile condition. The absence of bacterial contamination was ascertained. The amoebae collected were washed by centrifugation (5 min at 2,000 rpm) three times with cold phosphate-buffered saline (PBS, pH 7.4) and resuspended in cold PBS at a concentration of 1 mg wet weight of amoebae per 5 μl. The amoeba suspension was lysed by freezing at –70°C for 24 hours and rapid thawing at 37°C. The frozen-thawed homogenate was centrifuged for 1 hour at 40,000 g at 4°C. The supernatant was collected and stored in 15 μl aliquots at –70°C until use.

5. Extraction of Mt DNA

Mt DNA of *Acanthamoeba* was extracted by the method described by Yagita and Endo (1990). *Acanthamoeba* trophozoites harvested at the end of logarithmic growth phase were washed with cold PBS. The pellet was resuspended in 100 μl of chilled TEG buffer (25 mM Tris-HCl, 10 mM EDTA, 50 mM Glucose; pH 8.0) and incubated on ice for 5 minutes. Amoebae were lysed by adding 200 μl of chilled fresh 1% sodium dodecyl sulfate solution in 0.2 N NaOH, and gently mixing by inversion, and then incubated subsequently on ice for 5 minutes again. Then 150 μl of 3 M chilled potassium acetate buffer (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetate, 28.5 ml glass distilled water, pH 6.0) was added to the suspension and mixed by inverting the tube. The mixture was incubated on ice for 15 minutes and centrifuged at 12,000 rpm for 5 minutes at 4°C. The collected supernatant fluid was mixed with equal volume of phenol saturated with 10 mM Tris-1 mM EDTA (pH 8.0) and centrifuged at 12,000 rpm for 5 minutes at 4°C (somethimes this step was repeated twice). The collected

supernatant was added with equal volume of phenol/chlorform (1:1) solution and centrifuged at 12,000 rpm for 5 minutes at 4°C. Mt DNA was precipitated by adding 1.0 ml of absolute ethanol and 40 μl of 3 M sodium acetate solution and by being placed at –70°C for at least 15 minutes. After centrifugation at 15,000 rpm for 20 minutes at 4°C, the precipitated DNA was washed with 70% chilled ethanol. The DNA sediment was dried in a vacuum desiccator and dissolved 15-25 μl of TE buffer (5 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at –20°C until used.

6. Isoelectric focusing (IEF) and isoenzyme development

Novex Pre-cast vertical IEF gel, pH 3-10 and 1 mm thick, was used for isoenzyme isoelectric focusing. Phosphoric acid 7 mM was used as anode buffer. Cathode buffer consisted of 20 mM lysine and 20 mM arginine. The composition of sample buffer was 20 mM arginine, 20 mM lysine and 15% glycerol. The amoebic lysate was mixed with equal volume of the sample buffer. Isoelectric focusing was performed at 100 V for 1 hour, 200 V for 1 hour and 500 V for 30 minutes at 4°C. After focusing, six kinds of isoenzymes, acid phosphatase (AcP), glucose 6-phosphate dehydrogenase (G6PD), glucose phosphate isomerase (GPI), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), and malate dehydrogenase (MDH), were developed by conditions summarized in Table 1. The gel in the developing solution was incubated in darkness at 37°C with gentle shaking. After development of isoenzymes, the reaction was stopped and the gel was fixed by 7.5% acetic

Table 1. Developmental conditions of enzymes tested (Final conc./100 ml)

Enzyme	Substrate	Coenzyme	Linking enzyme	Other reagents	Buffer	Staining time
AcP[a] (EC[b]2.1.2.1)	β-naphthyl acid phosphate (Na) 100 mg			Black K salt 100 mg	0.05 M Acetate pH 5.0	40 min
G6PD[c] (EC1.1.1.49)	D-glucose-6-phosphate (Na) 45 mg	NADP[d] 45 mg		MTT[e] 16 mg PMS[f] 8 mg $MgCl_26H_2O$ 170 mg	0.1 M Tris-HCl pH 7.4	40 min
GPI[g] (EC 5.3.1.9)	D-fluctose-6-phosphate 80 mg	NADP 20 mg	G6PD 50 units	MTT 16 mg PMS 6 mg $MgCl_26H_2O$ 150 mg	0.1 M Tris-HCl pH 8.0	1 h
LAP[h] (EC 3.4.1.1)	L-leucine β-naphthyl amide 40 mg	NAD[i] 32 mg		Black K salt 50 mg	0.2 M Tris-maleate pH 6.0	1 h
LDH[j] (EC 1.1.1.27)	DL-lactate Li 3 g	NADP 45 mg		NBT[k] 20 mg PMS 4 mg	0.1 M Tris-HCl pH 7.4	2 h
MDH[l] (EC 1.1.1.37)	1 M L-malic acid pH 7.3 1 ml	NAD 15 mg		MTT 12 mg PMS 4 mg	0.1 M Tris-HCl pH 8.5	30 min

[a]AcP, acid phosphatase; [b]EC, Enzyme Commission number; [c]G6PD, glucose-6-phosphate dehydrogenase; [d]NADP, nicotinamide adenine dinucleotide phosphate; [e]MTT, 3-(4,5-dimethyl thiazolyl)2-5-diphenyl tetrazolium bromide; [f]PMS, phenazine methosulfate; [g]GPI, glucose phosphate isomerase; [h]LAP, leucine aminopeptidase; [i]NAD, Nicotinamide adenine dinucleotide; [j]LDH, lactate dehydrogenase; [k]NBT, nitroblue tetrazolium; [l]MDH, malate dehydrogenase.

acid solution. The gel was then differentiated in 10% acetic acid and 25 % ethanol until a clear background was obtained.

7. Total proteins IEF

Novex Pre-cast vertical IEF gel, pH 3-7 and 1 mm thick, was used for total proteins IEF. The same amoeba lysate as the isoenzyme study was used as sample. Instead of enzyme development, the gel was stained with Coomassie Brilliant Blue R.

8. Mitochondrial (Mt) DNA fingerprinting

Mt DNAs of seven strains were digested with eight kinds of restriction enzyme at 37°C for 2 hours (sometimes overnight) in 20 μl reaction volume with the buffers specified for each restriction enzyme (*Bgl* II, *Sca* I & *Xba* I obtained from Promega, USA; *Sst* I from Gibco BRL, USA; *Cla* I, *EcoR* I, *Sal* I & *Kpn* I from Poscochem, Korea). Digested DNA was electrophoresed in 0.7% agarose gel at 4 V/cm for 1~2 hours and stained with ethidium bromide for 15 minutes. The Mt DNA fingerprints of seven strains were observed and photographed under UV transilluminator. The *Hind* III digests of λ phage was used as the size marker.

RESULTS

1. Morphology

The photomicrographs of a trophozoite and a typical cyst of *Acanthamoeba ploryphaga* are presented in Figure 1. The cyst morphology of seven strains of *A. polyphaga* analysed in the present study is summarized in Table 2.

2. Isoenzyme profiles.

Photographs and diagrammatic representations of zymograms of seven strains for six isoenzymes are shown in Fig. 2. Six zymograms of the strain Jones (lane 3) were found to be identical to those of the strain Ap (lane 7). Except for these two strains,

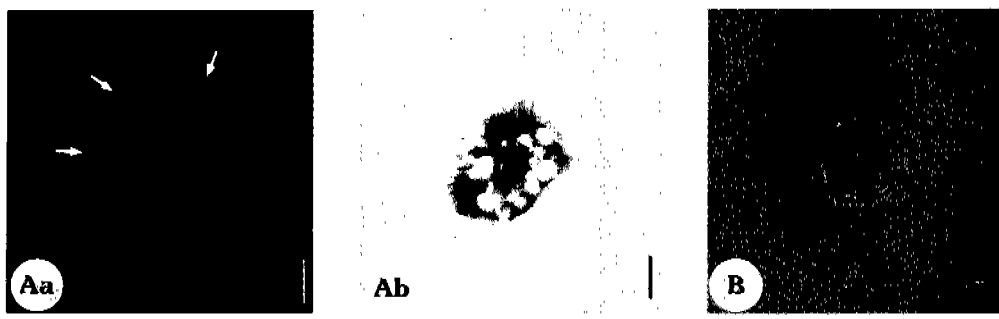

Fig. 1. Photomicrographs of the trophozoites and the typical cyst of *A. polyphaga*. **A.** Trophozoites **a.** Differential interference contrast, characteristic acanthpodia (arrows); **b.** Trichrome strained, bull's eye shaped nucleus (arrow head); **B.** Cyst of strain Jones. The ectocyst is relatively thin and close to the endocyst. Bars indicate 10 μm.

Table 2. Morphology of *Acanthamoeba polyphaga* strains analysed in this study

	Strain	Source	Cyst diameter (μm)		Number of arms		References
			mean	range	mean	range	
1	P23	freshwater	15.3	13-18	6.1	4-8	Page (1967)
2	Nagington	keratitis	11.0	10-12	4.3	3-6	Nagington *et al.* (1975)
3	Jones	keratitis	13.8	12-16	6.0	4-8	Jones *et al.* (1975)
4	KA/S3	soil	12.7	11-16	5.3	5-6	—
5	KA/S6	soil	14.6	12-18	4.6	3-6	—
6	KA/S7	soil	12.1	11-13	4.2	4-5	—
7	Ap	—	14.2	13-17	5.4	3-6	—

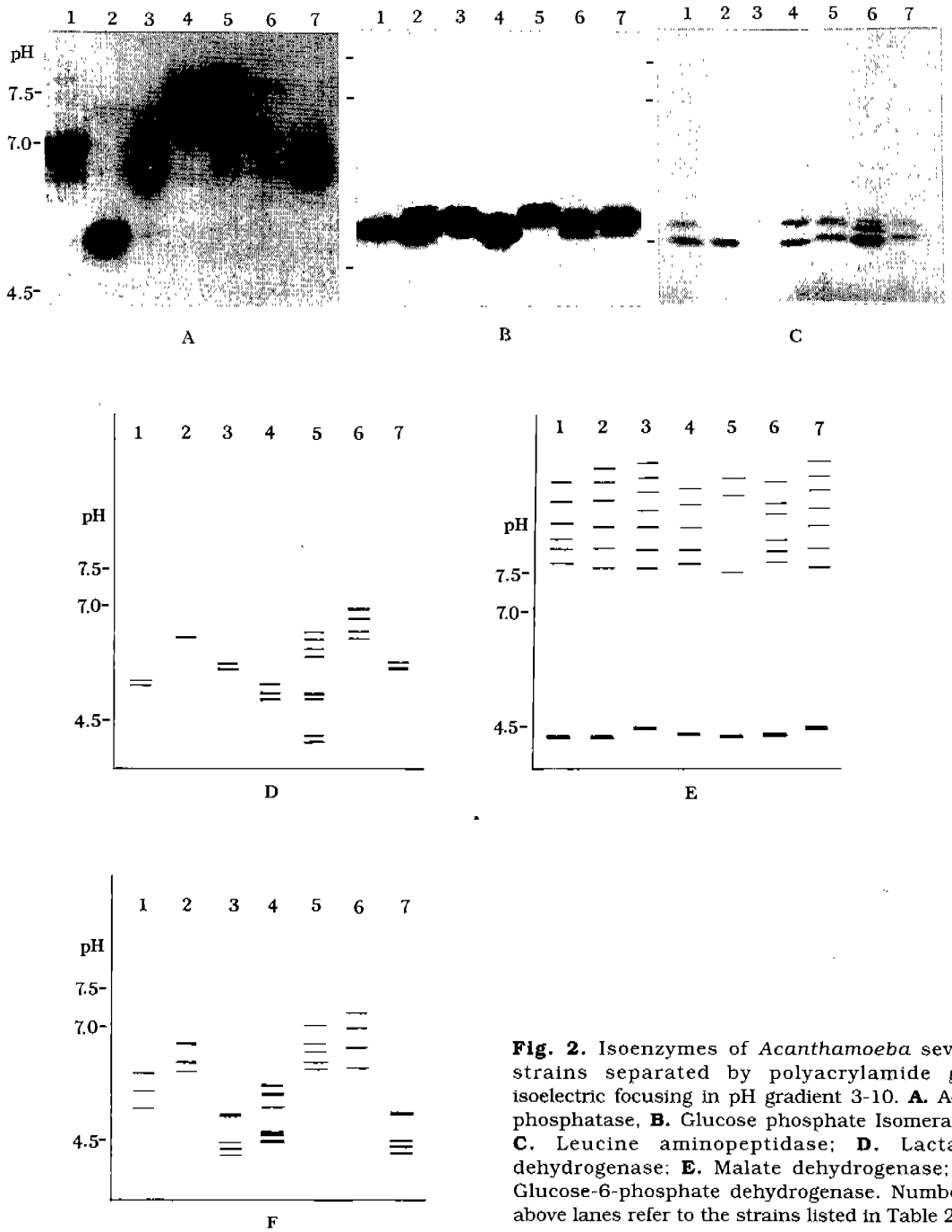

Fig. 2. Isoenzymes of *Acanthamoeba* seven strains separated by polyacrylamide gel isoelectric focusing in pH gradient 3-10. **A.** Acid phosphatase, **B.** Glucose phosphate Isomerase; **C.** Leucine aminopeptidase; **D.** Lactate dehydrogenase; **E.** Malate dehydrogenase; **F.** Glucose-6-phosphate dehydrogenase. Numbers above lanes refer to the strains listed in Table 2.

considerable interstrain polymorphisms of the zymograms were observed for acid phosphatase (AcP), lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G6PD). The zymograms for glucose phosphate isomerase (GPI), leucine aminopeptidase (LAP), and malate dehydrogenase (MDH) revealed similarity regardless of the strain. In spite of the considerable interstrain polymorphisms in AcP zymogram, the pattern of the strain KA/S3 (lane 4) for AcP was found to be similar to that of the strain KA/S6 (lane 5).

3. Total protein profiles analysed via IEF

The total protein patterns of the strains of *Acanthamoeba* produced in pH 3.0 to 7.0 are shown in Figure 3. The majority of the bands were condensed between pH 4.0~6.5 regardless of the strain, and showed similar patterns. Above the level of pH 6.5, a few bands were shown. The banding patterns of the strain Jones (lane 3) was found to be identical with that of the strain Ap (lane 7). Those of the remaining 5 strains showed mild interstrain diversity.

4. Mitochondrial (Mt) DNA fingerprints

Figure 4 shows the digestion phenotypes of seven strains of *Acanthamoeba*. The Mt DNA digestion phenotypes of strains Jones (lane 3) and Ap (lane 7) by eight of restriction endonucleases were found to be identical to each other. The phenotypes of the remaining five strains were quite heterogeneous from strain to strain.

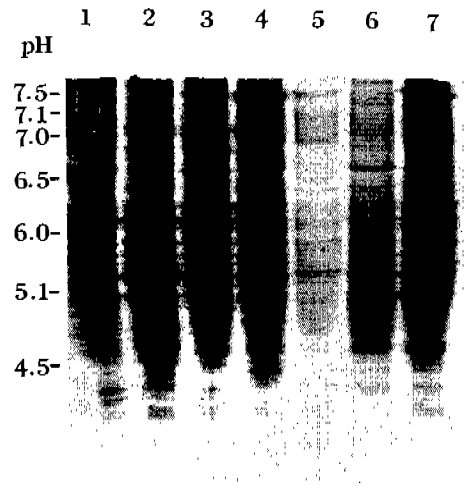

Fig. 3. Total proteins of *Acanthamoeba* separated by polyacrylamide gel isoelectric focusing in pH gradient 3-7. Numbers above lanes refer to the strains listed in Table 2.

DISCUSSION

Isoenzyme analysis has been applied to resolve the taxonomic problems of the genus *Acanthamoeba* (De Johnckheere, 1983; Visvesvara *et al.*, 1983; Costas and Griffiths, 1985; Moura *et al.*, 1992). More recently, mitochondrial (Mt) DNA fingerprinting has been substituted (Yagita and Endo, 1990; Kilvington *et al.*, 1991; Yagita, 1993; Gautom *et al.*, 1994). The researchers suggested the applicability of the both methods as tools for epidemiology, identification, and systematics of *Acanthamoeba*.

Profound polymorphisms were observed in Mt DNA fingerprints among strains analysed in this study. These results coincided with the reports of Byers *et al.* (1990) and Yagita (1993). As for isoenzyme profile, considerable interstrain differences of the zymograms were observed for acid phosphatase (AcP), lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G6PD) whereas zymograms for glucose phosphate isomerase (GPI), leucine aminopeptidase (LAP), and malate dehydrogenase (MDH) revealed similarity regardless of the strain. Six zymograms of the strain Jones (lane 3) were found to be identical with those of the strain Ap (lane 7). The polymorphisms suggest that the characteristics of any strain can hardly be the representatives of those of *A. polyphaga*. Interestingly, authors observed that the isoenzyme profiles and Mt DNA fingerprints of the Jones strain were identical with those of Ap strain. It is so far unclear whether these two strains are the closely related clones or the same clone. Even if the strains were originated from a single clone, the strain Ap has been cultivated in the different laboratory for over 10 years. The strains have been maintaining the restriction sites, at least, for the endonucleases employed in this study. Further study using ribosomal DNA analysis should be done to illucidate the relationship between the strains Jones and Ap.

The present study confirmed the applicability of both methods, especially Mt DNA fingerprinting, as tools for strain identification, differentiation, and characterization. However, as tools for taxonomy or systematics of *Acanthamoeba* spp., the usefulness of the methods seems to be limited. First, according to the endosymbiont hypothesis (Butow *et al.*, 1988), Mt DNA might not be the genomic DNA of

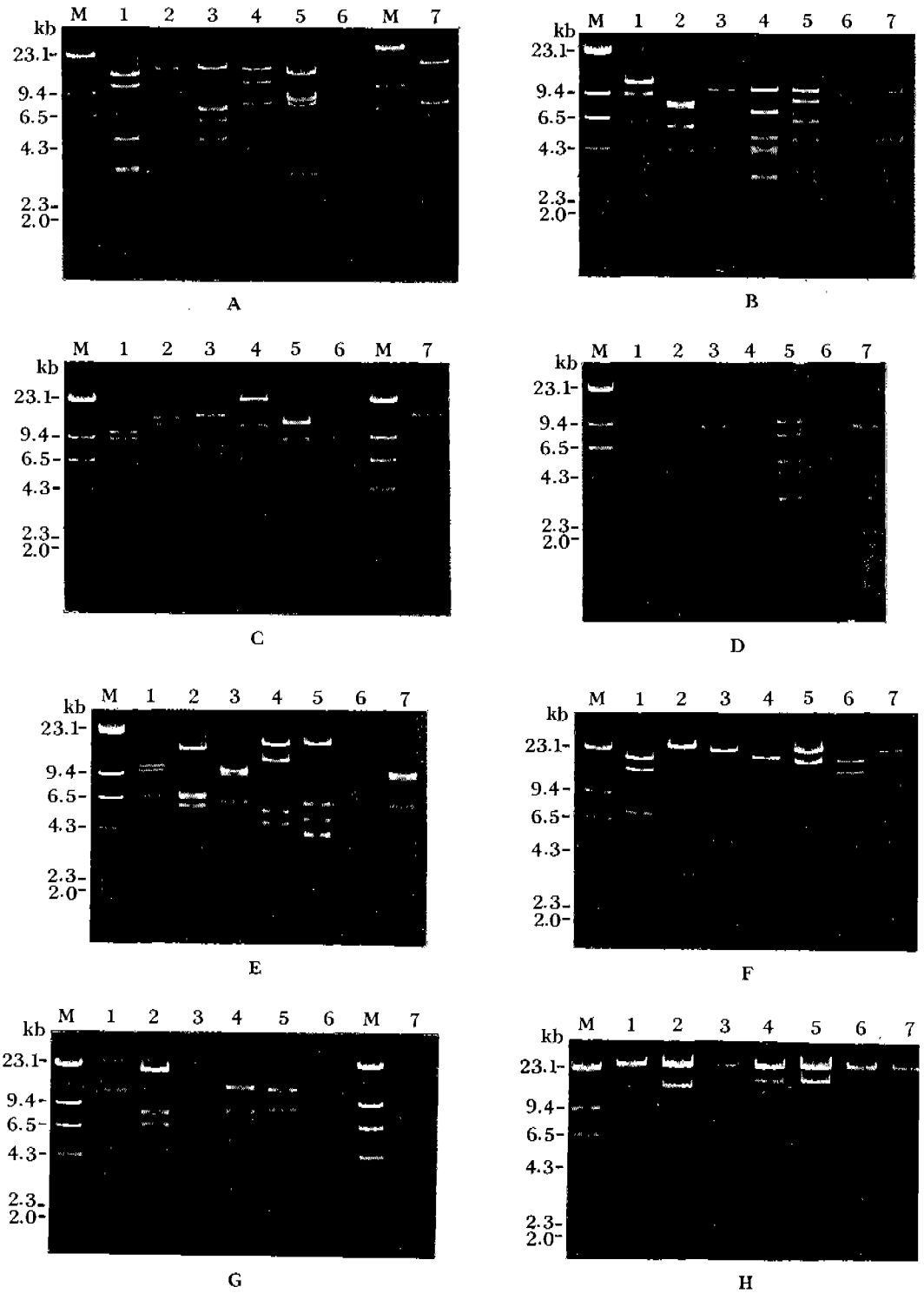

Fig. 4. Agarose gel electrophoretic fingerprints of mitochondrial DNA from seven strains assigned to *A. polyphaga*. A. *Cla* I digests; B. *Sca* I digests; C. *Bgl* II digests; D. *Hpa* I digests; E. *EcoR* I digests; F. *Sst* I digests; G. *Xba* I digests; H. *Sal* I digests. Numbers above lanes refer to the strains listed in Table 2. Size marker is *Hind* III digested λ phage DNA (M).

Acanthamoeba itself, but be that of symbiont within the amoeba. Moreover, as Byers *et al.* (1990) indicated, certain two strains of *Acanthamoeba*, which have very similar restriction phenotypes with several enzymes, can reveal very different phenotypes with the other enzymes. Therefore, if sufficient number of the restriction enzymes were not used for the analysis, the results would be different depending on the choice of the enzymes used. Organelle DNA sequences, in general, evolve five to ten times faster than nuclear DNA sequences do (Ferris *et al.*, 1981). That means the heterogeneity of Mt DNA fingerprints may be several times profound compared with that of nuclear genome. Secondly, the usefulness of isoenzyme analysis as a tool for taxonomy seems also questionable. Like Mt DNA fingerprinting, certain two strains may reveal very similar zymograms for some isoenzymes, but may produce different zymograms for the other isoenzymes.

In the present study, the authors confirmed the high resolution of polyacrylamide gels in isoenzyme isoelectric focusing (IEF). We observed some minor bands of the strain P23 for AcP which were not observed in previous studies on isoenzyme IEF using agarose gel (De Jonckheere, 1983). The minor bands were reproducibly observed in repeatitive IEF.

In conclusion, the authors presented the interstrain polymorphisms of isoenzyme profiles and Mt DNA fingerprints among the strains assigned to *A. polyphaga*. It is suggested that the isoenzyme analysis and Mt DNA fingerprinting should be used for the strain identification, differentiation, and characterization, rather than for species identification or construction of phylogenetic tree of *Acanthamoeba* spp.

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=초록=

대식가시아메바(*Acanthamoeba polyphaga*) 일곱 분리주간의 동위효소 profile과 Mitochondria DNA fingerprint의 다양성

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형태적으로 *Acanthamoeba polyphaga*로 동정된 일곱 분리주의 동위효소 profile과 Mitochondria (Mt) DNA fingerprint를 비교 분석하였다. 8가지 제한효소(*Bgl* II, *Sca* I, *Cla* I, *EcoR* I, *Xba* I, *Kpn* I, *Sal* I, 및 *Sst* I)에 의한 Mt DNA fingerprint는 주간의 심한 다양성을 나타내었다. 3가지 동위효소(acid phosphatase, lactate dehydrogenase 및 glucose-6-phosphate dehydrogenase)는 주간의 심한 다양성을 나타내었으나 다른 3가지 동위효소(glucose phosphate isomerase, leucine aminopeptidase 및 malate dehydrogenase)는 비슷한 양상으로 나타났다. Ap 주의 동위효소 양상과 Mt DNA fingerprint는 Jones 주와 동일하였다. Mt DNA fingerprinting은 대식가시아메바 주의 동정과 분리에 매우 유용함을 알았다. *Acanthamoeba polyphaga*의 우리말 학명을 대식가시아메바로 제안한다.

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