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PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*

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Abstract: Twelve isolates of *Acanthamoeba* spp. assigned to either *A. castellanii* or *A. polyphaga*, and type strains of *A. culbertsoni*, *A. healyi*, *A. palestinensis*, and *A. astronyxis* were examined by restriction fragment length polymorphism (RFLP) of a conserved region of small subunit ribosomal RNA gene (ssu rDNA) amplified by polymerase chain reaction (PCR). The PCR products of the isolates measured approximately 910-930 bp, except for that of *A. astronyxis* which was extraordinarily long, approximately 1,170 bp. Average of estimated sequence divergence of the amplified DNA among the isolates assigned to *A. castellanii* was 9.8% whereas that among the isolates assigned to *A. polyphaga* 9.6%. The maximum intraspecific sequence divergence among the isolates assigned to *A. castellanii* was observed between the Chang and Ma strains (17.3%) while that among the isolates assigned to *A. polyphaga* was observed between KA/S3 and KA/S7 strains (16.1%). The both maximum sequence divergences were much greater than the minimum interspecific sequence divergence between *A. castellanii* and *A. polyphaga* (2.6%) which appeared between the Castellani (or CCAP 1501/2 g) and KA/S3 strains. The PCR-RFLP patterns of *A. culbertsoni*, *A. healyi*, *A. palestinensis*, and *A. astronyxis* were quite diverse from one another and from those of isolates assigned to either *A. castellanii* or *A. polyphaga*. It is suggested that taxonomic validity of the isolates assigned to either *A. castellanii* or *A. polyphaga* should be reevaluated.

Key words: *Acanthamoeba*, PCR-RFLP, ssu rDNA, sequence divergence

INTRODUCTION

The taxonomy of the members of the genus *Acanthamoeba* Volkonsky, 1931 has yet to be established. Although species identification of the genus by cyst morphology has been extensively used (Page, 1967; Pussard and Pons, 1977; Singh and Hanumaiah, 1979), high variability of the morphology within a clone (Page, 1988) limited the availability of the morphology alone as a taxonomic tool (Visvesvara, 1991). New, refined and reproducible methods have been applied to resolve the problems of the taxonomy. Isoenzyme studies using isoelectric focusing (IEF) and mitochondrial (Mt) DNA RFLP have been applied, but the results appeared highly polymorphic among the strains assigned to the same species (Kong *et al.* 1995; Chung *et al.*, 1996). In addition to the high intraspecific heterogeneity, the fact that both methods require considerable number of trophozoites may be another shortcoming.

Comparisons of highly conserved sequences, which have a central function and therefore

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change only slowly during evolution, can reveal phylogenetic relationships between organisms (Sogin *et al.*, 1989). The small subunit ribosomal RNAs (ssu rRNAs) have recognized to be particularly well suited for estimating phylogenetic relationships between even the most divergent taxa (Woese, 1987). However, the generation of sequence data is labor intensive and expensive and, as a result, two or more isolates of a species were seldom analysed (Clark *et al.*, 1995). Therefore, interstrain or intraspecific variation of the sequence can be missed. In order to analyse lots of strains, less expensive and easier methods were needed to be developed.

The examination of restriction fragment length polymorphism (RFLP) of ssu rDNA amplified by polymerase chain reaction (PCR), has recently been used to establish the taxonomic relationships among *Entamoeba* spp. (Clark and Diamond, 1991 a & b), among *Naegleria* spp. (Clark *et al.*, 1989; De Jonckheere, 1994) and among anuran trypanosomes (Clark *et al.*, 1995). With as few as ten trophozoites, the PCR-RFLP can characterize the strain or species of the protozoa (Vodkins *et al.*, 1992).

A. polyphaga (Puschkarew 1913) Page, 1967, was a species recently separated from *A. castellanii* (Douglas 1930) Volkonsky, 1931, the type species of the genus, mainly by morphology (Page, 1967). However, based on Mt DNA studies, it was suggested that *Acanthamoeba* spp. of morphological group II including *A. castellanii* and *A. polyphaga* might be a single species complex (Bogler *et al.*, 1983). Recently, Yagita and Endo (1990) reported that some isolates from contact lens container and Japanese soil, which had been morphologically assigned to *A. polyphaga*, showed the same restriction patterns of Mt DNA as those of *A. castellanii* Ma strain.

In the present study, the authors amplified enzymatically the highly conserved sequence within ssu rRNA genes of twelve isolates assigned to either *A. castellanii* or *A. polyphaga*, and type strains of *A. culbertsoni*, *A. healyi*, *A. palestinensis*, and *A. astronyxis*. The amplified DNAs were digested with eight restriction endonucleases (REs) and the restriction patterns were compared to analyse phylogenetic relationship among *Acanthamoeba* isolates.

MATERIALS AND METHODS

Acanthamoeba strains analysed

Sixteen *Acanthamoeba* isolates used in this study are listed in Table 1 with their characteristics and origins.

Table 1. Sixteen isolates of *Acanthamoeba* analysed in this study

	Group	Species	Strain	Origin
1	II	*A. castellanii*	Castellani	yeast culture
2			Ma	human eye
3			Neff	soil
4			Chang	fresh water
5			CCAP 1051/2 g	human cornea
6			KA/S2	soil
7		*A. polyphaga*	P23	fresh water
8			Nagington	human cornea
9			Jones	human cornea
10			KA/S3	soil
11			KA/S7	soil
12			Ap	human cornea
13	III	*A. culbertsoni*	A-1	tissue culture
14		*A. healyi*	OC-3A	GAE
15		*A. palestinensis*	Reich	soil
16	I	*A. astronyxis*	Ray & Hayes	soil

Extraction of nuclear DNA

Amoebae harvested at the end of logarithmic growth phase were washed with cold phosphate buffered saline (PBS) three times and boiled with 0.1 ml of 0.1 N NaOH at 100°C for 3 minutes. The supernatant was collected after centrifugation at 2,000 rpm for 2 minutes at room temperature and mixed with 0.2 ml of glass distilled water. Equal volume of phenol was added to the solution and vortexed for 1 minute. The mixture was centrifuged at 12,000 rpm for 2 minutes at 4°C. The resulting aqueous phase was centrifuged again with 300 μl of phenol/chloroform (1:1) solution. The nuclear DNA was precipitated by adding 750 μl of cold absolute ethanol and 30 μl of 3 M sodium acetate (pH 5.2) and incubating at -70°C for 15 minutes and centrifuged at 15,000 rpm for 20 minutes at 4°C. The sediment was washed with 70% ethanol, vacuum dried and dissolved in 30~50 μl of glass distilled water. The DNA was stored at -20°C until used.

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

The sequences of primers for polymerase chain reaction (PCR) were originated from that of ssu rRNA of *A. castellanii* Neff strain reported by Gunderson and Sogin (1986). The sequences of the primers were 5'-TTT GAA TTC GCT CCA ATA GCG TAT ATT AA-3' and 5'-TTT GAA TTC AGA AAG AGC TAT CAA TCT GT-3'. The premix of 50 μl scale for PCR (Koreabiotech, Korea) was used for the ssu rDNA amplification by PCR. The premix was disolved in 47 μl of glass distilled water and vortexed vigorously. Template DNA 1 μl and 1 μl of each primer (25 pmol concentration) were added to the premix. The whole mixture was covered with 20 μl of mineral oil. PCR consisted of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C in a thermal cycler (Perkin Elmer Cetus, USA). After 30 cycles, ten minutes of extension time was given. The mineral oil was removed by adding chloroform and the DNA amplified was stored at -20°C until used.

Restriction Fragment Length Polymorphism of amplified fragments of ssu rDNA

After amplification, the PCR products of 16 *Acanthamoeba* isolates were checked by electrophoresis in a 1.5% agarose gel at 4 V/cm for 1.5 hours. The amplified DNA was examined by digestion with eight different restriction endonucleases (*Hae* III, *Hha* I, *Hinf* I, *Msp* I, *Dde* I, *Sau* 96 I, *Rsa* I, and *Taq* I; Kosco, Korea) for 2 hours. The digested DNA was electrophoresed in a 2.5% agarose gel (agarose 3: Nusieve 1) for 1.5 hours. The gel was stained with ethidium bromide for 15 minutes and washed in distilled water for 30 minutes. The gel was examined and photographed under an UV transilluminater. To differentiate small DNA fragments, which were unclear in the agarose gel, digested samples were electrophoretically separated in 15% polyacrylamide gels using TBE buffer.

Estimation of sequence divergence and reconstruction of phylogenetic tree

Sequence divergence estimates were calculated from a fragment comigration dataset (Nei and Li, 1979). A phylogenetic tree was produced by unweighted pair group method with arithmetic average (UPGMA) using a computer program Phylip version 3.5.

RESULTS

PCR products of 16 isolates of *Acanthamoeba* spp. were approximately 910-930 basepairs in length except for that of *A. astronyxis*, which was extraordinarily long, approximately 1,170 bp (Fig. 1). Schematic representation of RFLP patterns by eight kinds of restriction endonucleases (REs) are shown in Fig. 2. Estimates of genetic divergence among the isolates are presented in Table 2. Among six strains previously assigned to *A. castellanii*, the Neff strain showed identical RFLP patterns with KA/S2, and the Castellani strain showed the same RFLP patterns as the CCAP 1501/2 g strain. Among six strains assigned to *A. polyphaga*, the Jones and Ap strains showed the same RFLP patterns. Average sequence divergence of the amplified

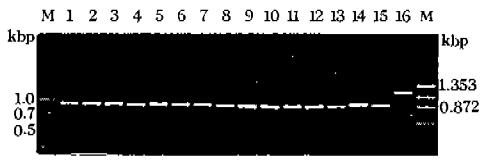

Fig. 1. PCR products of 16 isolates of *Acanthamoeba* analysed in this study. Numbers above the lanes refer to the strains of Table 1. *Hae* III digested Φ X174 DNA and PCR marker (Promega, U.S.A.) were used as the size marker.

DNA among the isolates assigned to *A. castellanii* was 9.8% whereas that among the isolates assigned to *A. polyphaga* 9.6%. Average interspecific sequence divergence (10.0%) was not significantly different from the both average intraspecific divergences. The maximum intraspecific sequence divergence among the isolates assigned to *A. castellanii* was observed between the Chang and Ma strains (17.3%) while that among the isolates assigned to *A. polyphaga* was observed between KA/S3 and KA/S7 strains (16.1%). The both maximum sequence divergences were much greater than the minimum interspecific sequence divergence between *A. castellanii* and *A. polyphaga* (2.6%) which appeared between the Castellani (or CCAP 1501/2g) and KA/S3 strains. The PCR RFLP patterns of *A. culbertsoni, A. healyi, A. palestinensis*, and *A. astronyxis* were quite diverse from one another (mean sequence divergence, 23.6%) and from those of isolates assigned to either *A. castellanii* or *A. polyphaga*.

DISCUSSION

In the present study, PCR-RFLP patterns of *A. culbertsoni* (Singh and Das, 1970), *A. healyi* (Moura, Wallace, and Visvesvara, 1992), *A. palestinensis* (Reich, 1933), and *A. astronyxis* (Ray and Hayes, 1954) were found to be distinct from *A. castellanii*, the type species of the genus, and *A. polyphaga*. Therefore, they could be recognized as a separated species. However, some isolates assigned to *A. castellanii* or *A. polyphaga* showed significant sequence divergence within a single species. The maximum intraspecific sequence divergences in the amplified DNA were observed between Ma and Chang strains of *A. castellanii* (17.3%), and between KA/S3 and KA/S7 strains of *A. polyphaga* (16.1%). The both maximum intraspecific sequence divergences within either *A. castellanii* and *A. polyphaga* were much greater than the minimum interspecific sequence divergence (2.6%) between *A. polyphaga* and *A. castellanii*. Average sequence divergence of isolates assigned to *A. castellanii* (9.8%) and that of the isolates assigned to *A. polyphaga* (9.6%) was not significantly different from average interspecific sequence divergence (10.0%). Moreover, the maximum interspecific sequence divergence (18.6%) between *A. castellanii* Chang strain and *A. polyphaga* Jones (or Ap) strain was not significantly different from the both maximal intraspecific sequence divergences (17.3% in *A. castellanii*; 16.1% *A. polyphaga*).

A phylogenetic tree reconstructed on the basis of present data by UPGMA method (Fig. 3) corresponded well with the grouping by Pussard and Pons (1977) who classified the members of genus *Acanthamoeba* into 3 morphologic groups. However, subgroups within Group II *Acanthamoeba* spp, to which *A. castellanii* and *A. polyphaga* belong, did not coincide with the previous species assignment. Based on the present data, twelve isolates of *A. castellanii* or *A. polyphaga* were divided into two subgroups. Each subgroup consisted of isolates assigned to *A. castellanii* and *A. polyphaga*. This might indicate, as Pussard and Pons (1977) already mentioned, that *A. castellanii* and *A. polyphaga* should be an assemblage of heterogeneous organisms.

It is very interesting that Chang and Ma strains were significantly different from the remaining isolates of *A. castellanii* in their cyst morphology, cyst diameter and number of arms, analysed by one way ANOVA (Chung *et al.*, 1996). More data should be accumulated to elucidate the correlation between the morphological difference and the sequence divergence.

The PCR-RFLP patterns between *A. castellanii* KA/S2 and Neff strains, and between Castellani and CCAP 1501/2 g strains were identical. These results coincide well with the results of Chung *et al.* (1996) and Gautom *et al.* (1994) who analysed the Mt DNA

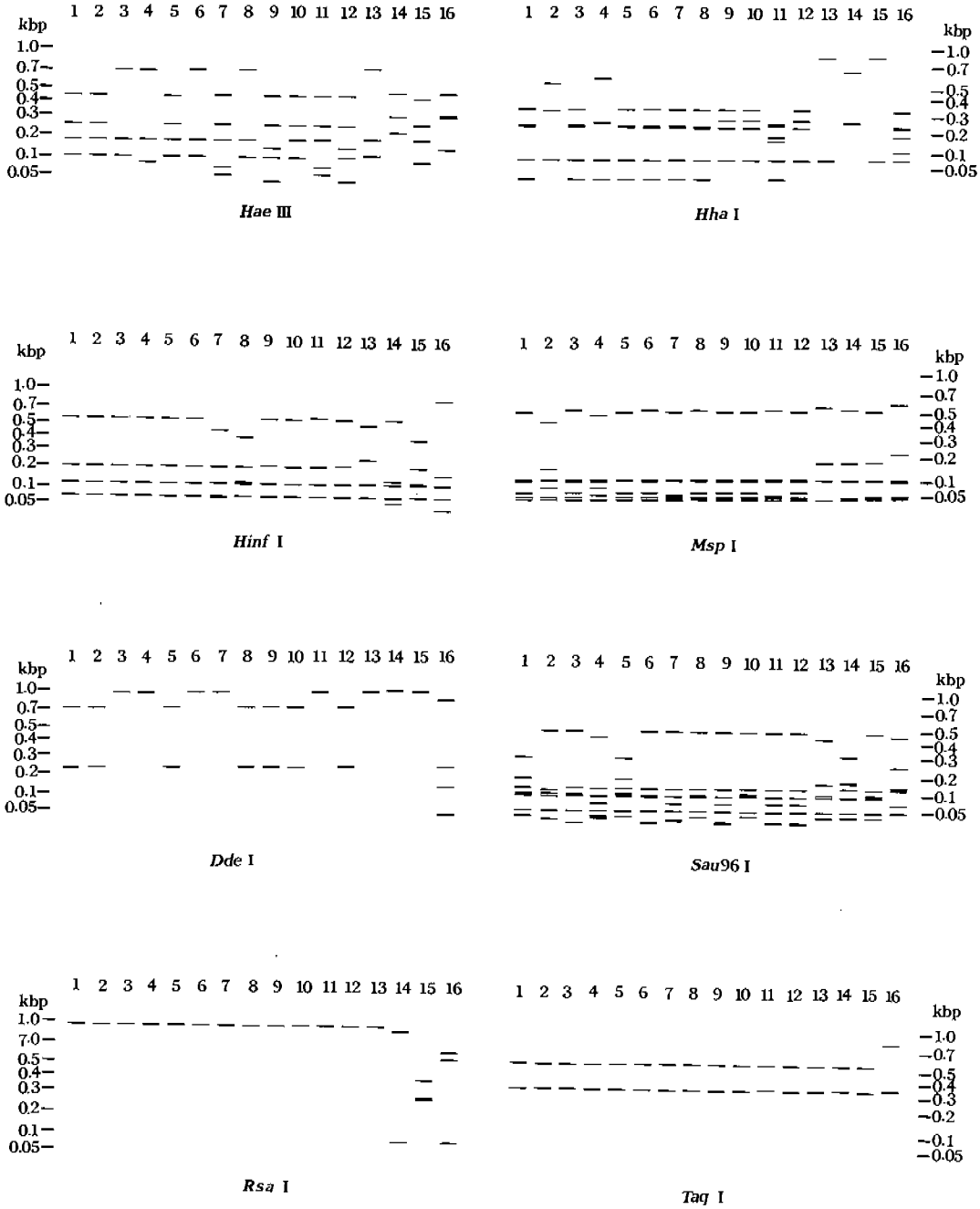

Fig. 2. Schematic representation of PCR-RFLP patterns of *Acanthamoeba* rDNA conserved region obtained with eight kinds of restriction endonucleases. Numbers above the lanes refer to the strains of Table 1. Amplisize® (Biorad, U.S.A.) was used as a size marker.

RFLP patterns. *A. castellanii* Castellani strain, which showed the identical patterns of Mt DNA RFLP and PCR-RFLP of ssu rDNA as CCAP 1501/2 g, a clinical isolate, has to be evaluated for its pathogenicity as Yagita and Endo suggested (1990). The PCR-RFLP patterns of *A. polyphaga* Jones strain were found to be identical with those of Ap strain of

Table 2. Proportions of homologous fragments (values above the diagonal) and estimates of genetic divergence[a](values below the diagonal) among isolates

Species		*A. castellanii*					*A. polyphaga*							*A. culbertsoni*	*A. healyi*	*A. palestinensis*	*A. astronyxis*
	Strain	Castellani	Ma	Neff	Chang	CCAP 1501/2g	KA/S2	P23	Nagington	Jones	KA/S3	KA/S7	Ap	A-1	OC-3A	Reich	Ray & Hayes
A. castellanii	Castellani	—	40/59	44/59	32/59	62/62	44/59	44/63	46/61	46/62	54/60	36/63	46/62	26/54	24/58	32/59	28/67
	Ma	0.097	—	32/56	28/56	40/59	32/56	34/60	34/58	36/59	44/57	32/60	36/59	24/51	20/55	28/56	18/64
	Neff	0.073	0.140	—	36/56	44/59	56/56	42/60	50/58	40/49	42/57	40/60	40/59	24/51	26/55	28/56	26/64
	Chang	0.153	0.173	0.110	—	32/59	36/56	36/60	34/58	28/59	32-57	40/60	28/59	24/51	26/55	26/56	20/64
	CCAP 1501/2g	0	0.097	0.073	0.153	—	44/59	44/63	48/61	46/62	54/60	36/63	46/62	26/54	24/58	30/59	28/67
	KA/S2	0.073	0.140	0	0.110	0.073	—	42/60	50/58	40/59	42-57	40/60	40/59	24/51	26/55	28/56	26/64
A. polyphaga	P23	0.090	0.142	0.089	0.128	0.090	0.089	—	40/62	40/63	44/61	48/64	40/63	22/55	22/59	32/60	30/68
	Nagington	0.075	0.134	0.037	0.134	0.075	0.037	0.110	—	40/61	46/59	36/62	40/61	24/53	24/57	32/58	28/66
	Jones	0.075	0.124	0.097	0.186	0.075	0.097	0.114	0.105	—	50/60	36/63	62/62	20/54	24/58	28/59	30/67
	KA/S3	0.026	0.065	0.076	0.144	0.026	0.076	0.082	0.062	0.046	—	32/61	50/60	26/52	26/56	34/57	26/65
	KA/S7	0.140	0.157	0.101	0.101	0.140	0.101	0.072	0.136	0.140	0.161	—	36/63	20/55	24/59	30/60	24/68
	Ap	0.075	0.124	0.097	0.186	0.075	0.097	0.114	0.105	0	0.046	0.140	—	20/54	24/58	28/59	28/67
A. culbertsoni	A-1	0.183	0.188	0.188	0.168	0.183	0.188	0.229	0.198	0.248	0.173	0.253	0.248	—	24/50	30/51	14/59
A. healyi	OC-3A	0.221	10.253	0.187	0.187	0.221	0.187	0.247	0.216	0.221	0.192	0.225	0.221	0.183	—	26/55	22/63
A. palestinensis	Reich	0.153	0.173	0.173	0.192	0.153	0.173	0.157	0.149	0.186	0.129	0.173	0.186	0.133	0.187	—	20/64
A. astronyxis	Ray & Hayes	0.218	0.317	0.225	0.291	0.218	0.225	0.205	0.214	0.201	0.229	0.260	0.218	0.360	0.263	0.291	—

[a]genetic divergence: estimated average number of changes per nucleotide position.

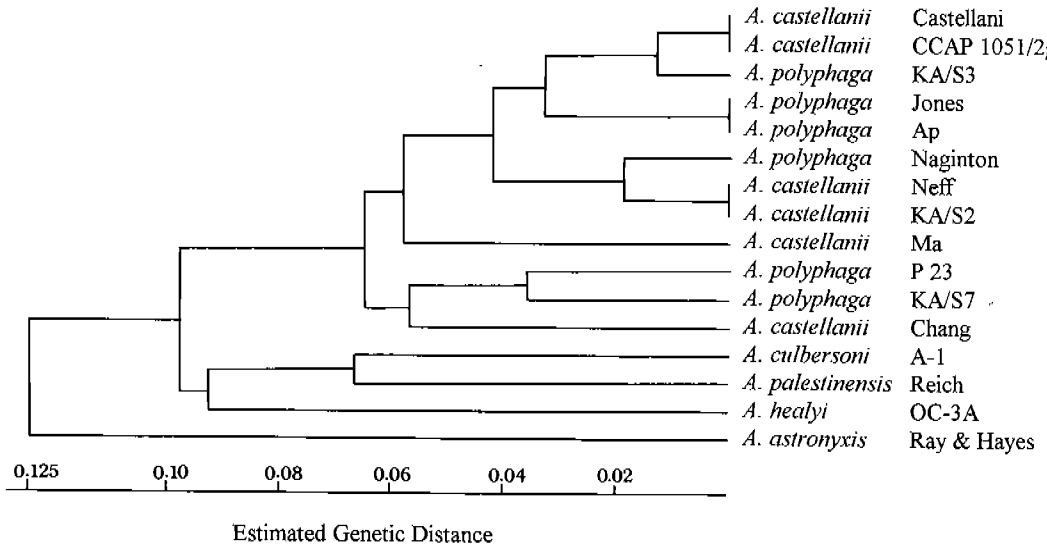

Fig. 3. Phylogenetic tree of *Acanthamoeba* isolates based on genetic divergence estimates. The matirx of divergence estimates in Table 2 was used to construct this tree using UPGMA.

the species. This result supports Kong *et al.*, (1995) who reported the identical patterns of Mt DNA RFLP and isoenzymes from the both strains.

Clark *et al.* (1995) omitted small DNA fragments (< 90 bp) from the analysis of phylogenetic relationship among anuran trypanosomes. However, the small DNA fragments should be included in the analysis, because their significance is equal to that of larger ones in PCR-RFLP. Electrophoretic separation in 15% polyacrylamide gels applied in the present study were highly efficient in confirmation of accurate numbers of the small DNA fragments, which were unclear in 2.5% agarose gel (data not shown).

This study presented the applicability of PCR-RFLP of rDNA as a tool for reconstructing the phylogeny of *Acanthamoeba* spp. It is recommended that all the type strains of *Acanthamoeba* spp. should be analysed for confirmation of their taxonomic validity.

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=초록=

카스텔라니가시아메바 혹은 대식가시아메바로 분류된 분리주간의 ribosomal DNA conserved region의 PCR-RFLP의 다양성

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형태학적으로 카스텔라니가시아메바 혹은 대식가시아메바로 동정된 12 분리주들과 쿨버트손가시아메바, 힐리가시아메바(*Acanthamoeba healyi*), 팔레스타인가시아메바(*A. palestinensis*), 별가시아메바의 small subunit ribosomal RNA유전자(ssu rDNA) 중 conserved region을 PCR로 증폭하여 제한효소절단부위를 비교하였다. 별가시아메바의 PCR 증폭 산물의 크기는 1,170 bp였고 나머지 분리주들의 것은 910-930 bp 사이였다. 카스텔라니가시아메바로 분류된 여섯 주간의 추정 염기치환율의 평균은 9.8%였고, 대식가시아메바로 분류된 분리주간의 그 평균은 9.6%였다. 카스텔라니가시아메바로 분류된 여섯 분리주들 사이의 최대 염기치환율은 Chang주와 Ma주 사이의 17.3%였고, 대식가시아메바로 분류된 여섯 분리주간의 최대 염기치환율은 KA/S3주와 KA/S7주 사이의 16.1%였다. 이들 종내 최대 염기치환율은 Castellani주 혹은 CCAP 1501/2g주와 KA/S3주 사이에서 나타난 카스텔라니가시아메바와 대식가시아메바간의 종간 최소 염기치환율(2.6%)보다 훨씬 컸다. 쿨버트손가시아메바, 힐리가시아메바, 팔레스타인가시아메바 및 별가시아메바의 PCR-RFLP 양상은 카스텔라니가시아메바 또는 대식가시아메바로 동정된 분리주들의 것들과, 그리고 상호간에서도 높은 염기치환율(평균 23.6%)을 보였다. 이상의 성적으로 미루어 보아 가시아메바속의 분류는 재평가 해 보아야 할 것으로 생각된다. *A. healyi*와 *A. palestinensis*의 우리말 이름을 각각 힐리가시아메바와 팔레스타인가시아메바로 제안한다.

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