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Close relatedness of *Acanthamoeba pustulosa* with *Acanthamoeba palestinensis* based on isoenzyme profiles and rDNA PCR-RFLP patterns

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Abstract: The taxonomic validity of morphological group III *Acanthamoeba* spp. is uncertain. In the present study, six type strains of group III *Acanthamoeba* spp., *A. culbertsoni*, *A. healyi*, *A. pustulosa*, *A. palestinensis*, *A. royreba* and *A. lenticulata* were subjected for the evaluation of their taxonomic validity by comparison of the isoenzyme patterns by isoelectic focusing on polyacrylamide gels, mitochondrial DNA (Mt DNA) restriction fragment length polymorphism (RFLP), and small subunit ribosomal DNA (ssu rDNA) PCR-RFLP patterns. The Mt DNA RFLP patterns were heterogeneous between the species. The type strains of *A. palestinensis* and *A. pustulosa* showed almost identical patterns of isoenzymes and rDNA PCR-RFLP with an estimated sequence divergence of 2.6%. The other species showed heterogeneous patterns of isoenzymes and rDNA PCR-RFLP. It is likely that *A. pustulosa* is closely related with *A. palestinensis* and that the former may be regarded as a junior synonym of the latter.

Key words: ERelatedness, *A. pustulosa*, *A. palestinensis*, isoenzyme, IEF, rDNA PCR-RFLP

INTRODUCTION

Pussard and Pons (1977) classified *Acanthamoeba* spp. into 3 groups based on the cyst size and morphologic features. Group I *Acanthamoeba* spp. have relatively large cysts (> 18 μm) with dictinctly stellate endocysts and smooth and somewhat spherical ectocysts. Group II consists of *Acanthamoeba* spp. with polygonal to stellate endocysts and irregular or wrinkled ectocysts. Group III *Acanthamoeba* spp. have rounded or slightly angular endocysts and thin ectocysts (Pussard and Pons, 1977; Visvesvara, 1991; Moura *et al.*, 1992).

The grouping of Pussard and Pons (1977) has been widely used before species identification of the amoebae. However, the morphology of the cyst can be changed by modifying culture conditions (Stratford and Griffiths, 1978) and are highly variable within a clone. Therefore, species identification of *Acanthamoeba* spp. by morphology alone is uncertain (Visvesvara, 1991). Studies using non-morphological methods, such as isoenzyme analysis, restriction fragment length polymorphism (RFLP) of mitochondrial DNA (Mt DNA), RFLP of small subunit ribosomal DNA (ssu rDNA) and arbitary primers-polymerase chain reaction (AP-PCR), have been applied for the taxonomy of

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Acanthamoeba spp.

The type strain of *A. palestinensis* (Reich, 1933), isolated from the Palestine soil, had been originally described as *Mayorella palestinensis* (Reich, 1933), but was later assigned to the genus *Hartmanella* (Adam, 1964), finally redefined as *Acanthamoeba* (Page 1967). The type strains of *A. pustulosa* Pussard and Pons, 1977 had been isolated from a swimming pool in France. Based on isoenzyme and total protein analysis by isoelectric focusing on agarose gel, De Jonckheere (1983) suggested that *A. pustulosa* should be a synonym of *A. palestinensis*. In contrast, Costas and Griffiths (1984) reported that different isoenzyme patterns by starch gel electrophoresis were observed between two species. Therefore, the taxonomic validity of *A. pustulosa* still remains uncertain.

The type strain of *A. culbertsoni* (Singh and Das, 1970), isolated from the culture of monkey kidney cell, was proven for the first time that *Acanthamoeba* can be pathogenic in experimental infections. The species designation of the type strain has never been disputed since this strain has shown to be the most aberrant. The type strain of *A. lenticulata* Molet and Ermolieff-Braun, 1977 was isolated from a medicinal pool. The morphology and pathogenicity of *A. lenticulata* resembles *A. culbertsoni*. The type strain of *A. royreba* Willaert, Stevens and Tyndall, 1978 was isolated from malignant cell culture and known to be virulent for mice. Recently, Moura *et al.* (1992) isolated a strain from the brain tissue of an AIDS patient who died of encephalitis and recorded the isolate as a new species, *A. healyi* on the basis of isoenzyme and antigenic difference. However, taxonomic validity of *A. healyi* remains uncertain.

Biochemical and molecular biological methods have been applied mainly to the taxonomy of morphological group II *Acanthamoeba* spp. In the present study, we compared the isoenzyme profiles, the RFLP patterns of Mt DNA and a conserved region of the ssu rDNA among six type strains of morphological group III *Acanthamoeba* spp. with reference to their taxonomic validity.

MATERIALS AND METHODS

Type strains of *A. culbertsoni*, *A. healyi*, *A. palestinensis*, *A. pustulosa*, *A. royreba* and *A. lenticulata* were analyzed in the present study (Table 1). The type strains were purchased from the American Type Culture Collection. The cysts were morphologically examined under a Nomarski (DIC: differential interference contrast) microscope. The significance of difference in cyst diameter among the strains was statistically analyzed by one-way analysis of variance (ANOVA).

Isoenzyme IEF was performed as previously described by Kong *et al.* (1995). Mt DNA of the *Acanthamoeba* isolates was extracted by the method described by Yagita and Endo (1990). RFLP analysis of Mt DNA was conducted as previously described by Chung *et al.* (1996). Extraction of nuclear DNA, PCR amplification of a portion of the small subunit ribosomal RNA coding DNA (ssu rDNA), and RFLP of amplified DNA were performed as previously described by Kong and Chung (1996).

Sequence divergence was estimated by a fragment comigration dataset (Nei and Li, 1979). A phylogenetic tree was constructed by unweighted pair group method with arithmetic average (UPGMA) using a computer program, Phylip version 3.52.

Table 1. Morphology and sources of *Acanthamoeba* strains analyzed in this study

Species	Strain	Source	cyst diameter (μm)	References
1. *A. culbertsoni*	A-1	cell culture	14.0 (11~18)	Singh & Das (1970)
2. *A. healyi*	OC-3A	GAE	14.2 (12~16)	Moura *et al.* (1992)
3. *A. pustulosa*	GE 3a	swimming pool	14.6 (11~19)	Pussard & Pons (1977)
4. *A. palestinensis*	Reich	soil	17.6 (14~23)	Reich (1993)
5. *A. lenticulata*	PD2S	swimming pool	14.1 (12~19)	Molet & Ermolieff-Braun (1976)
6. *A. royreba*	Oak Ridge	cell culture	14.2 (12~17)	Willaert *et al.* (1978)

RESULTS

Morphology of the cysts

Fig. 1 shows the photomicrographs of cysts of 6 *Acanthamoeba* isolates. Cysts of *A. palestinensis* were significantly larger ($p < 0.05$ by one way ANOVA) than those of the other species (Table 1). All species had cysts with typical morphology of group III (Pussard and Pons, 1977).

Isoenzyme profiles

Zymograms for acid phosphatase (AcP), alcohol dehydrogenase (ADH), leucine amino peptidase (LAP), malate dehydrogenase (MDH), and glucose phosphate isomerase (GPI) from the lysates of the six strains are shown in Fig. 2. The bands for AcP were multiple and widely distributed at pI range from approximately 4.5 to 7.5. The zymograms for ADH were quite variable between strains, except for *A. palestinensis* and *A. pustulosa* which showed the similar ADH zymogram patterns. Bands of *A. culbertsoni* for ADH were very faint but the enzyme activity was confirmed by the present study. The bands for GPI of all strains were positioned at pI between approximately 4.8 and 5.2 without regard to species. The major bands for LAP of all strains were positioned at pI between approximately 4.5 and 5.0. A major band for MDH was observed at pI approximately 4.5 with several minor bands over pI 7.5. The banding patterns for 5 isoenzymes of *A. pustulosa* and *A. palestinensis* were almost identical while those of the other isolates varied.

Mt DNA RFLP patterns

Fig. 3 shows the digestion phenotypes of six isolates of *Acanthamoeba* spp. studied. The Mt DNA digested by *Eco*R I, *Bgl* II, *Sca* I, *Sal* I, *Sst* I, and *Cla* I produced a discrete RFLP pattern for each strain.

RFLP of conserved region of the small subunit ribosomal RNA coding DNA (ssu rDNA)

Fig. 4 presents amplified DNA and RFLP patterns. The size of PCR product was approximately 910 to 930 bp, except for that of *A. lenticulata* which showed multiple bands. The RFLP patterns of PCR products of *A. pustulosa* and *A. palestinensis* were homologous, except for those by *Rsa* I and *Sau* 96 I (some data not shown). The other species

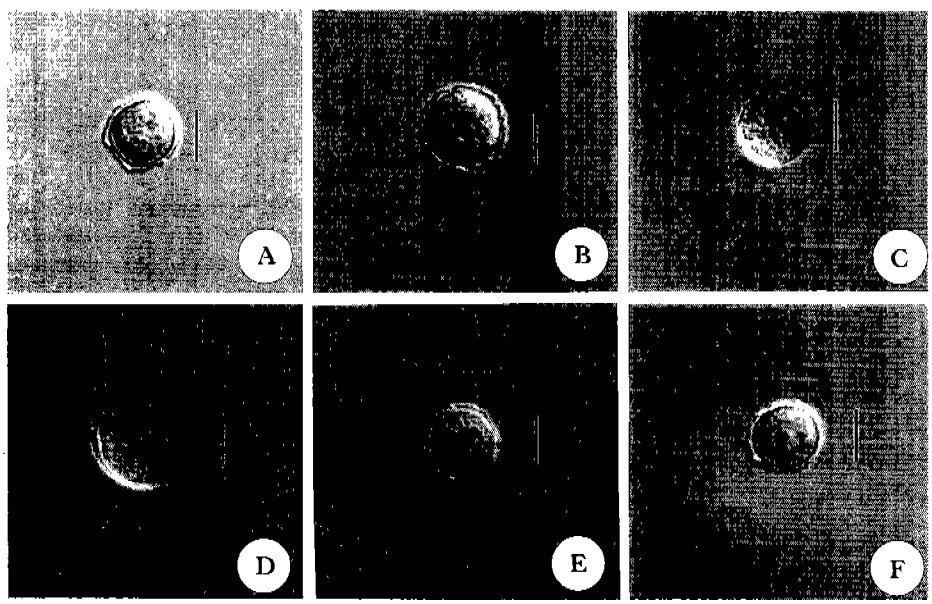

Fig. 1. Micrographs of *Acanthamoeba* cysts. A, *A. culbertdoni*; B, *A. healyi*; C, *A. pustulosa*; D, *A. palestinensis*; E, *A. royreba*; F, *A. lenticulata*. Bar = 10.0 μm.

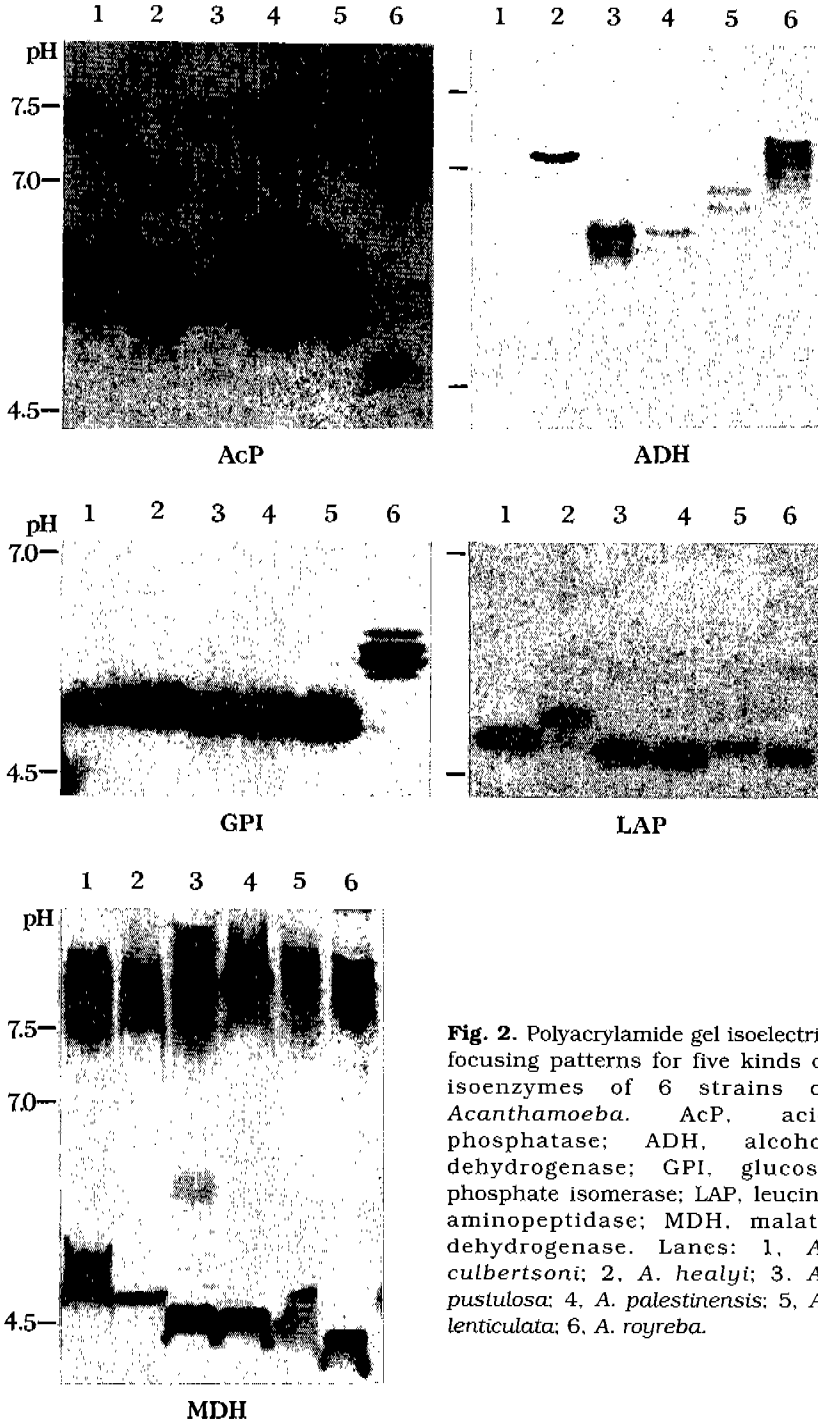

Fig. 2. Polyacrylamide gel isoelectric focusing patterns for five kinds of isoenzymes of 6 strains of *Acanthamoeba*. AcP, acid phosphatase; ADH, alcohol dehydrogenase; GPI, glucose phosphate isomerase; LAP, leucine aminopeptidase; MDH, malate dehydrogenase. Lanes: 1, *A. culbertsoni*; 2, *A. healyi*; 3, *A. pustulosa*; 4, *A. palestinensis*; 5, *A. lenticulata*; 6, *A. royreba*.

showed heterogeneous restriction patterns by eight restriction endonucleases.

Table 2 shows proportions of homologous fragments and estimated average numbers of changes per nucleotide position. The estimated sequence substitution was the lowest, 0.026 per nucleotide position, between *A. pustulosa* and *A. palestinensis*. Fig. 5 presents a

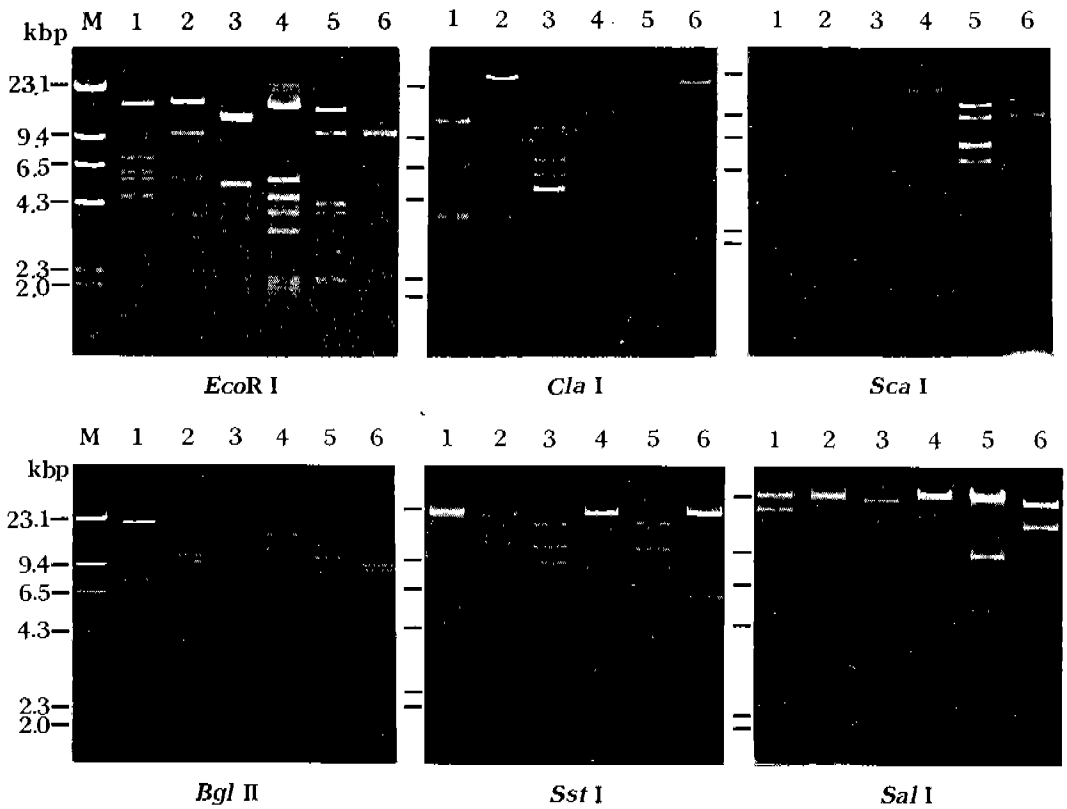

Fig. 3. Agarose gel electrophoretic restriction fragment patterns for mitochondrial DNA from 6 strains of *Acanthamoeba*. Lanes: 1, *A. culbertsoni*; 2, *A. healyi*; 3, *A. pustulosa*; 4, *A. palestinensis*; 5, *A. lenticulata*; 6, *A. royreba*; M, *Hind* III digested lambda DNA for molecular size standard.

phylogenetic tree reconstructed based on genetic divergence estimates.

DISCUSSION

The present study demonstrates that *A. palestinensis* and *A. pustulosa* were almost identical in isoenzyme patterns (Fig. 2) and total proteins (data not shown). Furthermore, the RFLP patterns of the amplified rDNA of the two species were also almost identical, with an estimated sequence of divergence 2.6% (Table 2). Considering average sequence divergence among strains assigned to a single species, *A. castellanii* (9.8%) or *A. polyphaga* (9.6%) calculated by Kong and Chung (1996), this divergence could be regarded as intraspecific variation. Both type strains of *A. palestinensis* and *A. pustulosa* were originated from environmental sources and are not pathogenic for mice. These results support De Jonckheere (1983 & 1987) who suggested *A. pustulosa* as a junior synonym of *A. palestinensis* based on isoenzyme analysis. The remaining four species were regarded as separated species based on rDNA PCR RFLP analysis.

The present study confirmed the profound variability in Mt DNA digestion phenotype of morphological group III *Acanthamoeba* spp. Mt DNA RFLP patterns were quite different between even *A. pustulosa* and *A. palestinensis*, both of which showed similarity in isoenzyme profiles and rDNA RFLP patterns. As for the interstrain sequence diversity of Mt DNA of *Acanthamoeba*, Chung *et al.* (1995) and others (Bogler *et al.*, 1983; Byers *et al.*, 1990; Yagita and Endo, 1990; Kilvington *et al.*, 1991; Yagita, 1993; Gautom *et al.*, 1994; Kong *et al.*, 1995) reported similar results.

The authors expected that the size of PCR

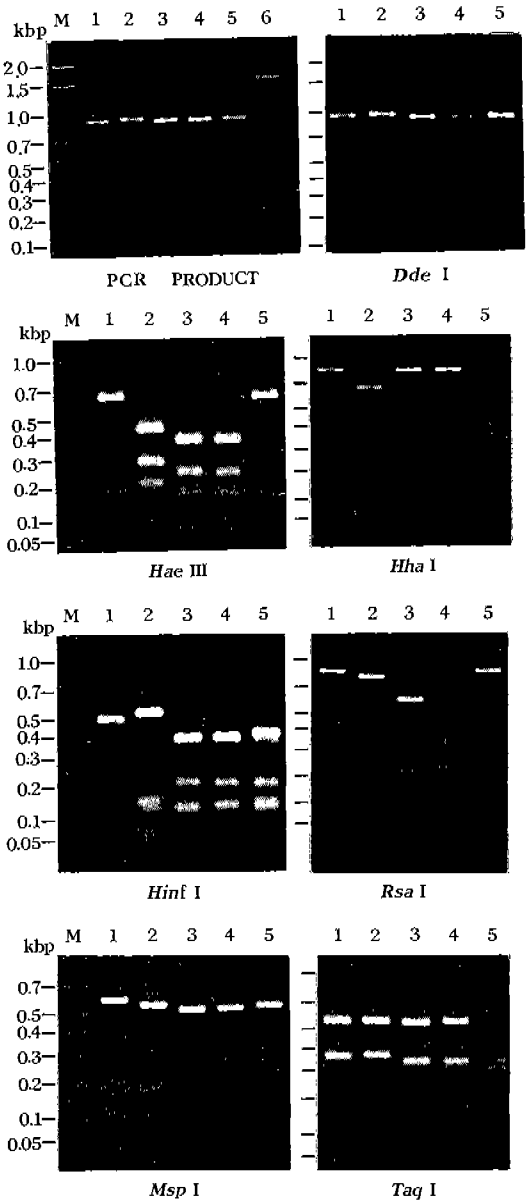

Fig. 4. Agarose gel electrophoretic restriction fragment patterns for PCR amplified ssu rDNA from 6 strains of *Acanthamoeba*. lanes: 1, *A. culbertsoni*; 2, *A. healyi*; 3, *A. pustulosa*; 4, *A. palestinensis*; 5, *A. royreba*; 6, *A. lenticulata*; M, DNA molecular size standard.

products would be approximately 920 bp. The size of all the products amplified was between 910 and 930 bp as expected, except for *A. lenticulata*, which showed multiple bands after amplification. We are trying to determine the cause of this abnormal finding. Interestingly, Gast and Byers (1995) reported similar findings for the same strain of *A. lenticulata*. They observed RPG 2/3 probe, which was designed to hybridize with genomic DNA (ssu rDNA) of morphological groups II and III, hybridized with all 21 group II and III amoebae, but not with *A. lenticulata*. Their results and those of the present study might suggest that *A. lenticulata* should be quite different from the other species of group III. Moreover, most strains of *A. lenticulata* have unique ssu rDNA introns (Gast *et al.*, 1994; Diedrich, unpublished observation). Gast and Byers (1995) proposed that *A. lenticulata* probably should be reclassified into a different group.

De Jonckheere (1983) reported the absence of alcohol dehydrogenase (ADH) activity in *A. culbertsoni* after agarose gel IEF of isoenzymes. While, in the present study, ADH activity was confirmed in all of the strains analyzed, ADH activity of *A. culbertsoni* was very low. If the gel background was not clear, the bands for ADH were not observed. This result supports the opinion of Kong *et al.* (1995) who indicated the superiority of the acrylamide gel IEF to agarose gel IEF for isoenzyme analyses.

Regardless of the genetic diversity among *Acanthamoeba* spp. of morphological group III confirmed in the present study, *A. pustulosa* was closely related with *A. palestinensis* based on isoenzymes and rDNA RFLP patterns and might be regarded as a synonym of *A. palestinensis*. Cumulative data for more strains, especially of *Acanthamoeba* spp. belonging to this group, will help to resolve the discrepancy of *Acanthamoeba* taxonomy.

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Table 2. Proportions of homologous fragments and estimated average numbers of changes per nucleotide position

	A. culbertsoni	*A. healyi*	*A. pustulosa*	*A. palestinensis*	*A. royreba*
A. culbertsoni	—	26/63	32/64	32/65	38/65
A. healyi	0.221	—	20/69	26/70	26/70
A. pustulosa	0.173	0.310	—	64/71	32/71
A. palestinensis	0.177	0.248	0.026	—	36/72
A. royreba	0.134	0.248	0.199	0.173	—

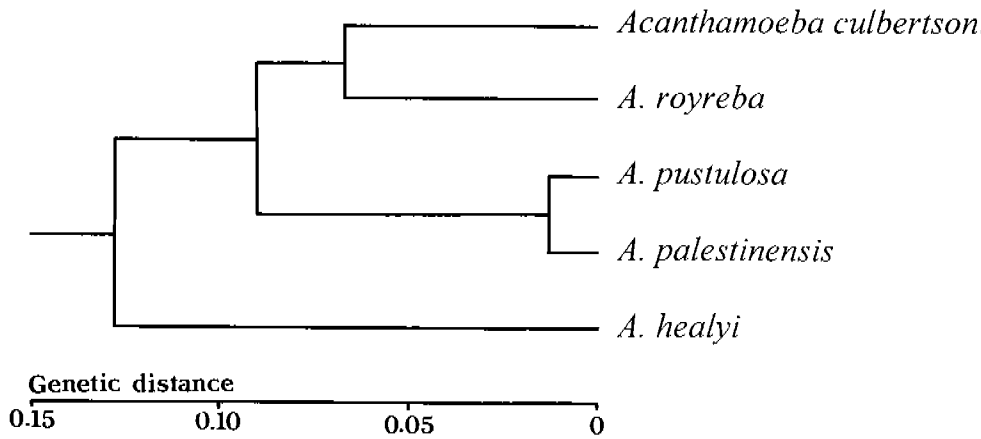

Fig. 5. Phylogenetic tree of *Acanthamoeba* isolates based on genetic divergence estimates. The matirx of divergence estimates in Table 2 was used to construct this tree using UPGMA.

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=초록=

*Acanthamoeba pustulosa*와 *A. palestinensis*의 동위효소 및 rDNA PCR-RFLP 양상의 유사성

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형태학적 제3군 가시아메바의 taxonomic validity는 아직 확실하지 않다. 이번 연구에서 제3군에 속하는 6종의 가시아메바 즉 *A. culbertsoni*, *A. healyi*, *A. palestinensis*, *A. pustulosa*, *A. royreba* 및 *A. lenticulata*의 type strain들의 동위효소, 미토콘드리아 DNA 및 small subunit(ssu) rDNA의 Restriction Fragment Length Polymorphism(RFLP) 양상을 비교하여 이들의 taxonomic validity를 검토하였다. 미토콘드리아 DNA의 RFLP 양상은 분리주 간에 서로 심한 차이를 보였다. *A. palestinensis*와 *A. pustulosa*는 거의 동일한 rDNA RFLP(추정 염기 치환율, 2.6%) 및 동위효소의 양상을 보여 *A. palestinensis*와 *A. pustulosa*는 같은 종으로 판단되었다. 그외의 종들은 서로 아주 다양한 rDNA RFLP 및 동위효소의 양상을 나타내어 독립종으로 인정되었다.

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