

## Isoenzyme patterns and phylogenetic relationships in *Acanthamoeba* spp. isolated from contact lens containers in Korea

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**Abstract:** In order to refer to the basic information regarding the identification of isolates obtained from a contact lens container in Korea, the isoelectric focusing gel electrophoresis was employed to compare the isoenzyme band patterns among *Acanthamoeba* spp. including eight isolates and the simple pairwise dissimilarity analysis was carried out. For an alkaline phosphate development, isolate 7 and *Acanthamoeba polyphaga* showed homologous band patterns, and isolates 1, 2, and 3 showed the same patterns. For lactate dehydrogenase, similar patterns were observed in isolates 2 and 3. Isolates 3 and 5 showed homologous band patterns for malate dehydrogenase and glucose phosphate isomerase. For hexokinase, isolates 4, 7, and *A. hatchetti* showed the same band patterns. In others, a considerable number of interstrain polymorphisms was observed in nine isoenzyme band patterns. In *Acanthamoeba* group II, genetic distances among isolates 1, 2, 3, 4, and 5 ranged from 0.104 to 0.200. In comparison to *A. castellanii*, *A. hatchetti*, and *A. polyphaga*, genetic distances of isolates 7 and 8 were 0.254 and 0.219, respectively. In *Acanthamoeba* group III, including *A. culbertsoni*, *A. healyi*, and *A. royreba*, isolate 6 had genetic distances which ranged from 0.314 to 0.336. Finally, when comparing to the six reference *Acanthamoeba*, it was possible to classify isolates 1, 2, 3, 4, and 5, as genetically close-related species and as independent species group. Furthermore, isolates 6, 7 and 8 were identified as independent species as well.

**Key words:** *Acanthamoeba*, acanthamoebic keratitis, isoelectric focusing, isoenzyme patterns, phylogeny

### INTRODUCTION

*Acanthamoeba* spp. inhabit in soil, pond, sewage, and in the atmosphere. *Acanthamoeba*

*culbertsoni* and *A. healyi* cause chronic glaucomatous amoebic encephalitis (GAE), and *A. polyphaga* and *A. castellanii* are a causative agent of the acanthamoebic keratitis (Stehr-Green et al., 1989; Visvesvara and Stehr-Green, 1990; Moura et al., 1992; van Klink et al., 1992). In Korea, two probable GAE cases and one acanthamoebic pneumonia case, and several acanthamoebic keratitis cases have been reported (Ringsted et al., 1976; Kim et al., 1995; Im and Kim, 1998).

The basic classification of *Acanthamoeba*

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spp. has depended upon the morphological characteristics of trophozoites and cysts (Page, 1976; Pussard and Ponds, 1977). In addition, the comparison of isoenzyme patterns was a useful tool (De Jonckheere, 1983; Costas and Griffiths, 1985) and molecular techniques were applied for the classification of *Acanthamoeba* spp. which include the restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA and 18S SSU RNA (small subunit ribosomal RNA) using the polymerase chain reaction (PCR) technique (McLaughlin et al., 1988; Kilvington et al., 1991; Hinkle and Sogin, 1993; Chung et al., 1998).

For the past two decades, biological characterization of amoebae has been focused on isoenzymes. On the basis of starch gel electrophoresis of isoenzymes, pathogenic and nonpathogenic strains of *Entamoeba* and *Naegleria* could be separated (Nerad and Daggett, 1979; Sargeaunt and Williams, 1979; De Jonckheere, 1983). Although *N. fowleri* could be distinguished from other species of the same genus, it was not clear whether such distinction could be made for other members of the family or not (Daggett and Nerad, 1983). Nevertheless, Costas and Griffiths (1985) reported the taxonomy of 32 strains of *Acanthamoeba* using starch gel electrophoresis.

In this study, in order to refer to some basic

information on the identification of *Acanthamoeba* spp. isolated from contact lens containers in Korea, isoelectric focusing gel electrophoresis was attempted to compare band patterns of nine isoenzymes, and the simple pairwise dissimilarity analysis was carried out.

## MATERIALS AND METHODS

### *Acanthamoeba*

Eight *Acanthamoeba* were isolated from contact lens containers in Korea (in 1997, by Prof. Dong-Il Chung and their collaborators), and reference amoebae such as *A. culbertsoni*, *A. healyi*, *A. royreba*, *A. castellanii*, *A. polyphaga*, and *A. hatchetti* (Table 1) were cultured in Proteose Peptone-Yeast extract-Glucose (PYG) medium (Visvesvara and Balamuth, 1975) at 25°C and 37°C.

### Lysate preparation of *Acanthamoeba*

Cultured trophozoites ( $1 \times 10^8$ ) of each amoebae were harvested and washed with phosphate buffered saline (PBS, pH 7.4). Each suspension of trophozoites was in 1 ml of PBS and was frozen (-70°C) and thawed (37°C) three times, and centrifuged at 10,000 g for 2 hr. After the supernatants were filtered with 0.25 µm disk filter, the protein concentration (adjusted to 10 mg/ml) was determined.

**Table 1.** Eight isolates and six reference *Acanthamoeba* spp.

Isolates and species		Origin	Culture temperature	Group <sup>a)</sup>
1	<i>Acanthamoeba</i> KA/LS1	contact lens container	25°C	II
2	KA/LS2	"	"	"
3	KA/LS5	"	"	"
4	KA/LS4	"	"	"
5	KA/LS18	"	"	"
7	KA/LS37	"	"	"
8	KA/LK59	"	"	"
Ca	<i>A. castellanii</i> Chang	fresh water	37°C	"
Ha	<i>A. hatchetti</i>	brackish water, 1977	"	"
Po	<i>A. polyphaga</i> Ap	eye infection	"	"
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6	<i>Acanthamoeba</i> KA/LS7	contact lens container	25°C	III
Cu	<i>A. culbertsoni</i>	human brain, 1976	37°C	"
He	<i>A. healyi</i> OC-3A	GAE	"	"
Ro	<i>A. royreba</i> OR	tissue culture, 1977	"	"

<sup>a)</sup>Grouping by Pussard and Ponds (1977)

Samples were stored at  $-70^{\circ}\text{C}$  until used.

### Isoelectric focusing

For isoenzyme developments, the isoelectric focusing was done by using Novex Pre-cast vertical IEF gel, pH 3-10 and 1 mm thick, with the Vertical Electrophoresis Kits (Novex, San Diego, USA). The amoeba lysates were prepared by adding one part sample to one part sample buffer (10x cathode buffer 2.0 ml, glycerol 3.0 ml and D.W. to 10.0 ml), and added to each well. Then, both cathode buffer (arginine 3.5 g, lysine 2.9 g and D.W. to 1,000 ml) and anode buffer (8.5% phosphoric acid 4.7 g and D.W. to 5,000 ml) were added to each corresponding chamber. Isoelectric focusing was performed at 100 V (volt) for 1 hr, 200 V for 1 hr and 500 V for 30 min.

### Isoenzyme developments

After isoelectric focusing, gels were put into each chamber containing several kinds of substrate solutions for the isoenzyme development. Nine kinds of isoenzymes, acid phosphatase (AcP), glucose phosphate isomerase (GPI), glucose-6-phosphate dehydrogenase (G-6-PD), Hexokinase (HK), malate dehydrogenase (MDH), malic enzyme (ME), leucine aminopeptidase (LAP), lactate dehydrogenase (LDH) and propionyl esterase (PE) were developed. Each substrate with appropriate experimental condition was prepared according to the previous papers (Costas and Griffiths, 1985; Shin et al. 1993). After each development of isoenzymes, gels were pretreated with 7.5% acetic acid solution, and differentiated in 10% acetic acid and 25% ethanol until the background was clear.

### Phylogenetic relationships among *Acanthamoeba*

The developed isoenzyme band patterns of *Acanthamoeba* spp. were compared to the pairwise dissimilarity value (Nei and Li, 1979), and dendrograms of genetic relationships among *Acanthamoeba* spp. were produced with the unweighted pair-group method with the arithmetic mean analysis (UPGMA) using PHYLIP program (Felsenstein, 1993).

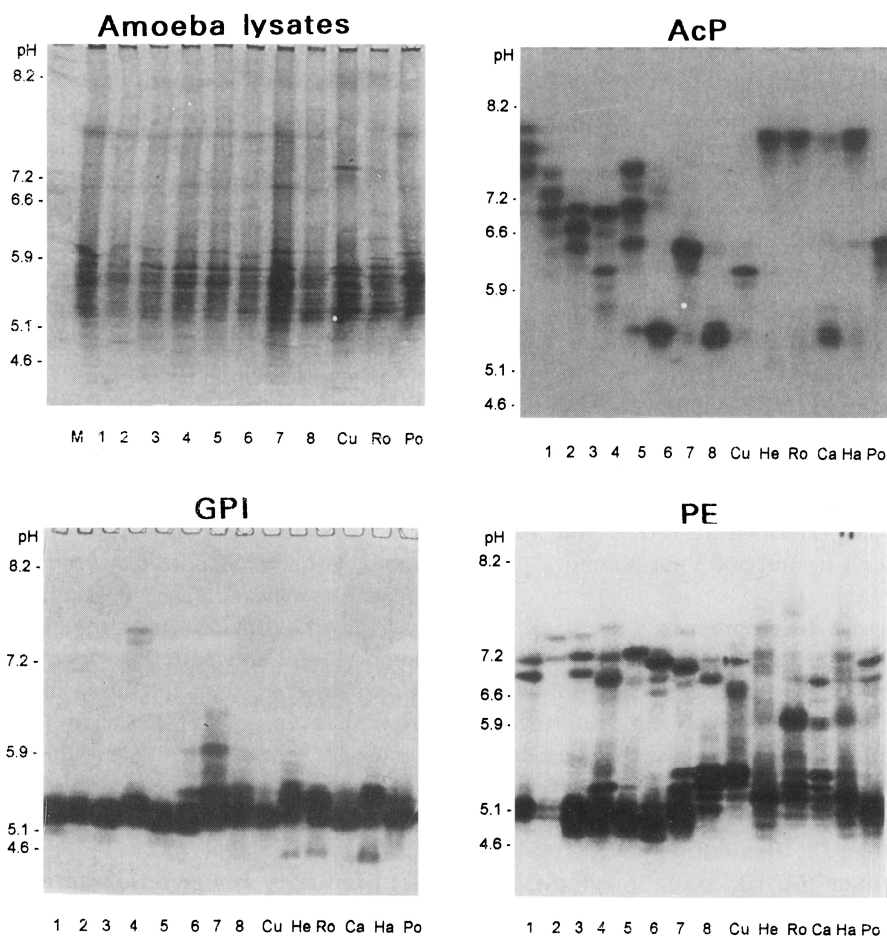
## RESULTS

### Isoenzyme patterns of *Acanthamoeba*

Various band patterns of nine isoenzymes for fourteen strains of *Acanthamoeba* were observed (Figs. 1, 2). A considerable amount of interstrain polymorphisms of zymograms was observed for nine isoenzymes, except for the isolates described below. Being assigned to *Acanthamoeba* group II, isolates 1, 2, and 3 showed the same patterns for GPI, MDH, and LAP developments. For an LDH development, similar patterns were shown for isolates 2 and 3. Isolates 3 and 5 showed homologous band patterns for MDH and GPI developments. Isolate 7 and *A. polyphaga* showed some homologous band patterns for AcP development. For a hexokinase development, isolates 4, 7, and *A. hatchetti* showed the same homologous band patterns. Being assigned to *Acanthamoeba* group III, the isolate 6 showed interspecies polymorphisms in nine isoenzymes developments, although *A. healyi* and *A. royreba* showed somewhat homologous band patterns for AcP development.

### Similarity and genetic relationships

As a result of the summation of nine isoenzyme band patterns, the values of the pairwise dissimilarity were calculated among *Acanthamoeba* group II including seven isolates and isolate 6 of *Acanthamoeba* group III (Table 2, 3). In *Acanthamoeba* group II, the dissimilarity values varied from 0.207 to 0.806 with genetic distances ranging from 0.14 to 0.323. In isolates 1, 2, 3, 4, and 5, the dissimilarity values ranged mostly from 0.207 to 0.458, which the genetic distances were from 0.104 to 0.200. In other *Acanthamoeba* spp. including isolates 7 and 8, the dissimilarity values ranged from 0.397 to 0.806, which the genetic distances ranged from 0.199 to 0.336. Between isolates 7 and 8, the genetic distance was 0.254 (Fig. 3). In *Acanthamoeba* group III including isolate 6, the dissimilarity values varied from 0.400 to 0.695 with genetic distances ranging from 0.200 to 0.336 (Fig. 4).



**Fig. 1.** Photographic findings of three isoenzyme band patterns of eight isolates (1-8), *A. culbertsoni* of *Acanthamoeba* spp. (Cu), *A. healyi* (He), *A. royreba* (Ro), *A. castellanii* (Ca), *A. hatchetti* (Ha) and *A. polyphaga* (Po). AcP, acid phosphatase; GPI, glucose phosphate isomerase; PE, propionyl esterase. Left-upper figure shows protein profiles of amoeba lysate stained by Coomassie Brilliant Blue R.

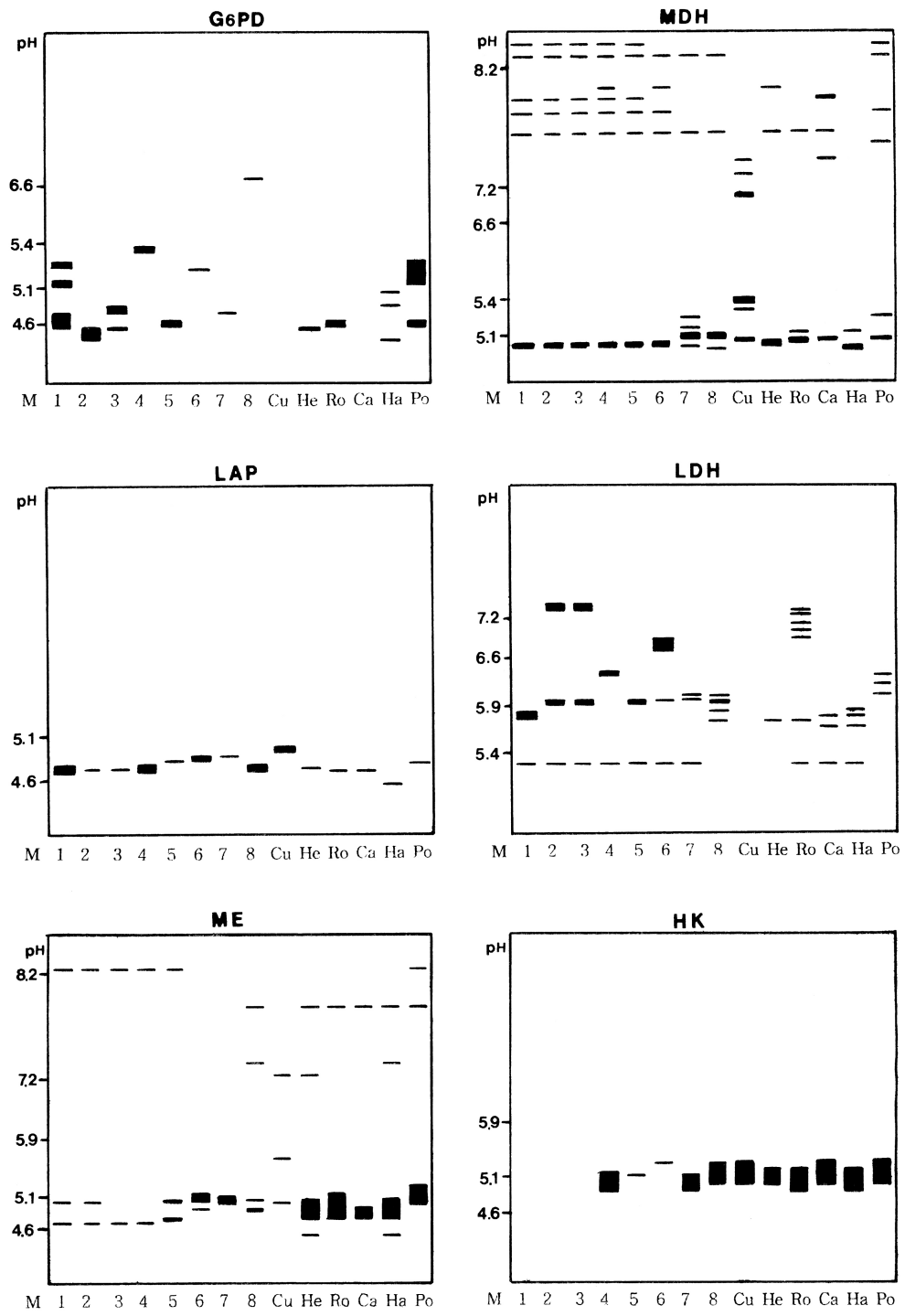
### DISCUSSION

In the taxonomy of 32 strains of *Acanthamoeba*, Costas and Griffiths (1985) reported on the application of starch gel electrophoresis. Strains that had originated from the same isolate had similar zymograms. Strains that had been assigned to the same species—especially *A. castellanii* and *A. polyphaga*—did not always have similar enzyme patterns. De Jonckheere (1983) reported the isoenzyme analysis by agarose isoelectric focusing for the taxonomy of the genus *Acanthamoeba*. Especially, strains of *A. castellanii*, two strains, 76-2252 and A-1,

showed different zymograms from Neff strain. Although it appears to be possible to distinguish different isoenzymes, Ma et al. (1990) reviewed that the application of the techniques to other amoebae does not yield clear-cut results.

Most previous studies were performed by starch gel electrophoresis which only revealed 2 to 5 band patterns. Pernin et al. (1985) reported the phylogenetic relationships in the genus *Naegleria*, with sixteen isoenzymes analysis using the calculation of genetic distances based upon Nei's standard index, which could be applied to both diploid and haploid organisms whether they reproduce sexually or not. In their studies, there were





**Fig. 2.** Diagrammatic representations of six isoenzyme band patterns of eight isolates of *Acanthamoeba* spp. (1-8), *A. culbertsoni* (Cu), *A. healyi* (He), *A. royreba* (Ro), *A. castellanii* (Ca), *A. hatchetti* (Ha) and *A. polyphaga* (Po). G-6-PD, glucose-6-phosphate dehydrogenase; MDH, malate dehydrogenase; LAP, leucine aminopeptidase; LDH, lactate dehydrogenase; ME, malic enzyme; HK, hexokinase.

**Table 2.** Proportions of homologous fragments and pairwise dissimilarity calculated by summation of nine isoenzyme patterns in *Acanthamoeba* group II

	Isolates							Group II		
	1	2	3	4	5	7	8	Ca <sup>a)</sup>	Ha <sup>b)</sup>	Po <sup>c)</sup>
1	—	20/58 <sup>d)</sup>	17/60	19/61	21/61	12/65	10/63	9/64	10/69	15/66
2	0.310 <sup>e)</sup>	—	23/58	21/64	16/59	12/63	10/61	6/62	7/67	12/64
3	0.433	0.207	—	20/66	19/61	12/65	10/63	9/64	7/69	15/66
4	0.377	0.344	0.394	—	19/67	18/71	14/69	14/70	15/75	15/72
5	0.311	0.458	0.377	0.433	—	17/66	12/64	12/65	12/70	16/77
7	0.631	0.619	0.631	0.493	0.485	—	17/68	16/69	19/74	17/71
8	0.682	0.672	0.682	0.584	0.625	0.500	—	19/67	20/72	15/69
Ca	0.719	0.806	0.719	0.600	0.631	0.536	0.433	—	22/73	13/70
Ha	0.710	0.791	0.797	0.600	0.657	0.486	0.444	0.397	—	13/75
PO	0.545	0.625	0.535	0.583	0.583	0.521	0.565	0.629	0.653	—

<sup>a)</sup>*A. castellanii*; <sup>b)</sup>*A. hatchetti*; <sup>c)</sup>*A. polyphaga*; <sup>d)</sup>Proportions of homologous band numbers per total band numbers obtained by summation of nine isoenzyme band patterns. <sup>e)</sup>Dissimilarity estimated by the method of Nei and Li (1979).

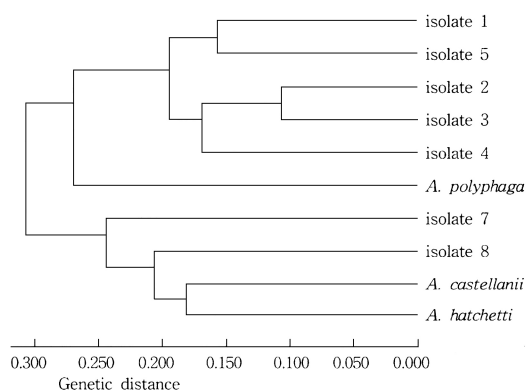
**Table 3.** Proportions of homologous fragments and pairwise dissimilarity calculated by summation of nine isoenzyme patterns in *Acanthamoeba* group III

	isolate 6	Cu <sup>a)</sup>	He <sup>b)</sup>	Ro <sup>c)</sup>
6	—	9/59 <sup>d)</sup>	12/65	13/69
Cu	0.695 <sup>e)</sup>	—	11/60	10/64
He	0.631	0.633	—	21/70
Ro	0.623	0.687	0.400	—

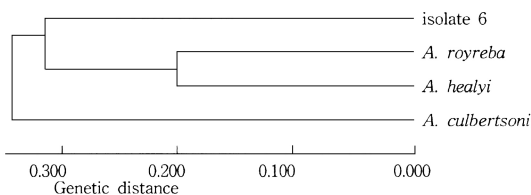
<sup>a)</sup>*A. culbertsoni*; <sup>b)</sup>*A. healyi*; <sup>c)</sup>*A. royreba*; <sup>d)</sup>Proportions of homologous band numbers per total band numbers obtained by summation of nine isoenzyme band patterns. <sup>e)</sup>Dissimilarity estimated by the method of Nei and Li (1979).

some problems regarding the presence of allozymes as well as the existence of considerable number of parameters involving isozymes.

In this paper, we employed isoelectric focusing gel electrophoresis and the simple pairwise dissimilarity analysis of isoenzyme band patterns which could be detected in a few isoenzyme developments. Mostly, a considerable amount interstrain and interspecies polymorphism of zymograms were observed for nine isoenzymes in this study. Being assigned to *Acanthamoeba* group II, nevertheless, isolates 1, 2, and 3 showed the same patterns for GPI, MDH and LAP



**Fig. 3.** Dendrogram of seven isolates and *Acanthamoeba* group II based on the dissimilarity estimates by UPGMA methods.



**Fig. 4.** Dendrogram of isolate 6 and *Acanthamoeba* group III based on the dissimilarity estimates by UPGMA methods.

developments. For an LDH development, similar patterns were shown for isolates 2 and

3. And isolates 3 and 5 showed homologous band patterns for MDH and GPI. In comparison to the other reference amoebae which showed considerable interspecies polymorphisms, isolates 1 to 5, at least, could be classified as closely-related strains; thus, being assigned to a new species with genetic distances ranging from 0.104 to 1.200. Also, isolates 7 and 8 could be assigned to an independent species, as shown in the dendrogram based upon the pairwise dissimilarity analysis, which the genetic distances ranged from 0.219 to 0.254. In previous study by Kong et al. (1995), the interstrain polymorphism of isoenzyme profiles by isoelectric focusing among seven strains assigned to *A. polyphaga* was observed: *A. polyphaga* Ap strain was similar to Jones strain, but other strains including the Korean isolates (KA/S3, KA/S6 and KA/S7) did not show similar band patterns, especially for AcP, LDH, and G-6-PD developments. In this paper, the interspecies polymorphism was not observed among reference amoebae. Being assigned to *Acanthamoeba* group III including *A. culbertsoni*, *A. healyi* and *A. royreba*, the isolate 6 should be classified as a new species, since its genetic distance ranged from 0.314 to 0.336.

Ultimately, in many previous studies as well as the present study, it was emphasized that the isoenzyme analysis may be a strong tool in assigning *Acanthamoeba* strains to species, but different band patterns may be detected within a species. Thus, first of all, a type of strains and/or a type species should be established. Therefore, the isoenzyme band patterns analysis must not be used as the sole criterion to assign new species. Also, a variety of different criteria should be considered in describing new species.

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