

# Evaluation of taxonomic validity of four species of *Acanthamoeba*: *A. divionensis*, *A. paradivionensis*, *A. mauritaniensis*, and *A. rhyodes*, inferred from molecular analyses

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**Abstract:** The taxonomy of *Acanthamoeba* spp., an amphizoic amoeba which causes granulomatous amoebic encephalitis and chronic amoebic keratitis, has been revised many times. The taxonomic validity of some species has yet to be assessed. In this paper, we analyzed the morphological characteristics, nuclear 18s rDNA and mitochondrial 16s rDNA sequences and the Mt DNA RFLP of the type strains of four *Acanthamoeba* species, which had been previously designated as *A. divionensis*, *A. parasidionensis*, *A. mauritaniensis*, and *A. rhyodes*. The four isolates revealed characteristic group II morphology. They exhibited 18S rDNA sequence differences of 0.2-1.1% with each other, but more than 2% difference from the other compared reference strains. Four isolates formed a different clade from that of *A. castellanii* Castellani and the other strains in morphological group II on the phylogenetic tree. In light of these results, *A. paradivionensis*, *A. divionensis*, and *A. mauritaniensis* should be regarded as synonyms for *A. rhyodes*.

**Key words:** *Acanthamoeba*, taxonomy, 18s rDNA sequence analysis

## INTRODUCTION

During the last few decades, the taxonomy of the genus *Acanthamoeba* has been revised several times (Marciano-Cabral and Cabral, 2003). Morphological characteristics, including the size of trophozoites and cysts, arm numbers of cysts, and the shape of the cyst wall, constituted the principal criteria by which the species of the genus have classically been identified (Page, 1967, 1976; Pussard and Pons, 1977). However, morphological characteristics alone have proven unre-

liable as taxonomic criteria, due to the high degree of observed variation within a clone (Page, 1988; Visvesvara, 1991). Several non-morphological methods have been applied to the taxonomy of the genus *Acanthamoeba*. Alloenzyme profiles and mitochondrial DNA RFLP (restriction fragment length polymorphism) have been reported to be suitable with regard to strain identification, differentiation, and characterization, rather than species identification, due to the high polymorphism observed between individual isolates (Kong et al., 1995). Small subunit rDNA PCR-RFLP (riboprinting) and sequence analysis have been the most recently applied, and the promising methods for the identification of *Acanthamoeba* species (Gast et

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al., 1996; Chung et al., 1998; Stothard et al., 1998).

Some *Acanthamoeba* species, which were assigned as a species based on their morphologic criteria alone, have been proposed to be, in actuality, invalid synonyms or junior synonyms, based on the data generated by new techniques which analyze the biochemical and molecular characteristics of the amoeba (De Jonckheere, 1983; Kim et al., 1996). De Jonckheere (1983) reported that *A. paratuberculosis* is considered to be an invalid synonym for *A. tuberculosis*, as the former exhibits identical isoenzyme and total protein patterns with the latter. These two species originated from the same isolate, and *A. tuberculosis* was differentiated from *A. paratuberculosis* on the basis of a difference in the arm numbers of the endocyst. Among *Acanthamoeba* species classed under morphological group III, *A. pustulosa* is proposed to be synonym of *A. palestinensis*, based on the isoenzyme and 18S rDNA RFLP patterns determined by Kim et al. (1996). Therefore, it is necessary to reevaluate the taxonomic validity of species names which are assigned based on morphological criteria alone, via the application of novel identification techniques.

In the present paper, we evaluated the taxonomic validity of four *Acanthamoeba* isolates by comparing their morphological characteristics, nuclear 18s rDNA and mitochondrial 16s rDNA sequences, and by conducting Mt DNA RFLP. Ultimately, we propose three species, which were previously designated as *A. divionensis*, *A. paratuberculosis*, and *A. mauritaniensis*, to be synonyms for *A. rhyssodes*.

## MATERIALS AND METHODS

### *Acanthamoeba*

Four strains of *Acanthamoeba*, *A. rhyssodes* Singh strain (ATCC#30973), *A. divionensis* AA2 strain (ATCC#50238), *A. paratuberculosis* AA1 strain (ATCC#50251) and *A. mauritaniensis* 1652 strain (ATCC#50253), were purchased from the American Type Culture Collection (ATCC), and incubated in PYG media at 25°C. The size and number of arms of the cysts of four *Acanthamoeba* isolates were determined, in order to assign morphological grouping.

The reference strain for Mt DNA RFLP, *A. castellanii* Castellani (ATCC#30011), was also purchased from the ATCC, and cultured in PYG media at 25°C.

### Chromosomal DNA extraction and 18S rDNA amplification

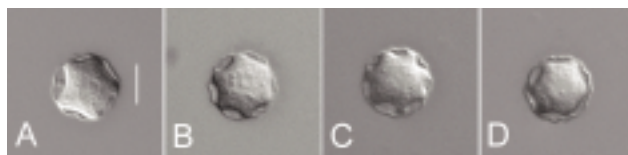
The chromosomal DNA of *Acanthamoeba* isolates was obtained by the methods described by Kong and Chung (1996). In brief, *Acanthamoeba* trophozoites ( $5 \times 10^6$ ) were washed with PBS, then boiled with 0.1 ml of 0.1 N NaOH for 3 min. Supernatants collected after 2 min of centrifugation at 800 g at room temperature were mixed with 0.2 ml of distilled water. Genomic DNA was then extracted with phenol and phenol/chloroform (1:1), and recovered by precipitation with cold absolute ethanol, in the presence of sodium acetate. The DNA was then used as a template for the PCR amplification of 18S rDNA. The primers, P3; 5'-CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT-3', and P4; 5'-GGATCCAAGCTTGATCCTTCTGCAGGTTACCTAC-3', were designed to hybridize to highly conserved sequences at the extreme 5' (P3) and 3' (P4) termini of eukaryotic 18s rDNA (Bhattacharya et al., 1995). The PCR products of the four isolates were electrophoresed on 2.5% agarose gel, with DNA size standards (Hind III digested  $\lambda$  phage DNA, Poscochem, Korea; Amplisize, Biorad, U.S.A.).

### Sequencing and phylogenetic analysis

PCR products were sequenced after being cloned into pBSK+ (Stratagene, La Jolla, CA) or PCR II (Invitrogen, San Diego, CA). The sequences of the four strains were compared pair-wise, then multi-aligned with those of 16 *Acanthamoeba* strains available in the GenBank database, using Clustal X. The phylogenetic tree was then constructed via the bootstrap method with the morphological group I species, *A. astronyxis* and *A. tubiashi*, used as an out-group. The intron sequence of *A. griffini* was omitted for this analysis.

### Extraction of Mt DNA and RFLPs

We extracted *Acanthamoeba* mtDNA via the method described by Yagita and Endo (1990). *Acanthamoeba*



**Fig. 1.** Micrographs of *Acanthamoeba* 4 strains analyzed in this study. A, *A. rhyssodes* Singh; B, *A. divionensis* AA2; C, *A. paradiionensis* AA1; D, *A. mauritaniensis* 1652. Bar represents 10  $\mu$ m.

trophozoites, harvested at the end of the logarithmic growth phase, were washed in cold PBS. The amoebae were then lysed with fresh 1% sodium dodecyl sulfate solution in 0.2 N NaOH, after which, potassium acetate buffer was added, and the whole mixture was incubated for 30 min on ice. The mtDNA was extracted using phenol and phenol/chloroform (1:1), and recovered via precipitation with cold absolute ethanol in the presence of sodium acetate. We digested the mtDNA of the *Acanthamoeba* isolates with two kinds of restriction enzymes, *EcoR* I and *Cla* I (Promega, USA), for 2 hr at 37°C in a 20  $\mu$ l reaction volume with the buffers specified for each restriction enzyme. The digested DNA was electrophoresed in 0.7% agarose gel at 4 V/cm for 1-2 hrs, then stained with ethidium bromide for 15 min. The mtDNA RFLP patterns were observed and photographed under an UV transilluminator.

#### Mt DNA 16S rDNA sequence analysis

The primers for the PCR, FP16; 5'-TTGTATAAA-CAATCGTTGGGTTTTATT-3', RP16; 5'-GTCCAGCAGCAGGTTCCCTACCGCTA-3', are designed to hybridize to the highly conserved sequences at the extreme 5' (P3) and 3' (P4) termini of the mitochondrial ssu rDNA (Lonergan and Gray, 1994). PCR was carried out as was described in the previous paper (Yu et al., 1999). The PCR products were cloned into pBSK+ (Stratagene, La Jolla, CA) or PCR II (Invitrogen, San Diego, CA). The sequencing of the PCR products was outsourced to Macrogene (Korea). The sequences of the 16s rDNA of four isolates were compared in a pair-wise manner.

**Table 1.** Cyst diameter and the number of arms of the four strains of *Acanthamoeba* assessed in the present study

Species	Cyst diameter ( $\mu$ m)	Arm number
<i>A. rhyssodes</i>	12.3-22.0 (16.5)	5-7 (5.5)
<i>A. divionensis</i>	12.0-21.5 (16.5)	4-7 (5.6)
<i>A. paradiionensis</i>	12.5-22.3 (16.9)	5-8 (6.5)
<i>A. mauritaniensis</i>	10.3-19.8 (15.6)	4-7 (5.6)

## RESULTS

### Morphological examination

The four strains of *Acanthamoeba* analyzed in this study exhibited cyst morphology characteristic of the *Acanthamoeba* group II, as defined by the work of Pussard and Pons (1977). All strains exhibited double-walled cyst morphology consisting of thick wrinkled ectocysts and polygonal or satellite endocysts (Fig. 1). The cyst diameter and the number of arms are shown in Table 1.

### Nuclear 18S rDNA sequence analysis

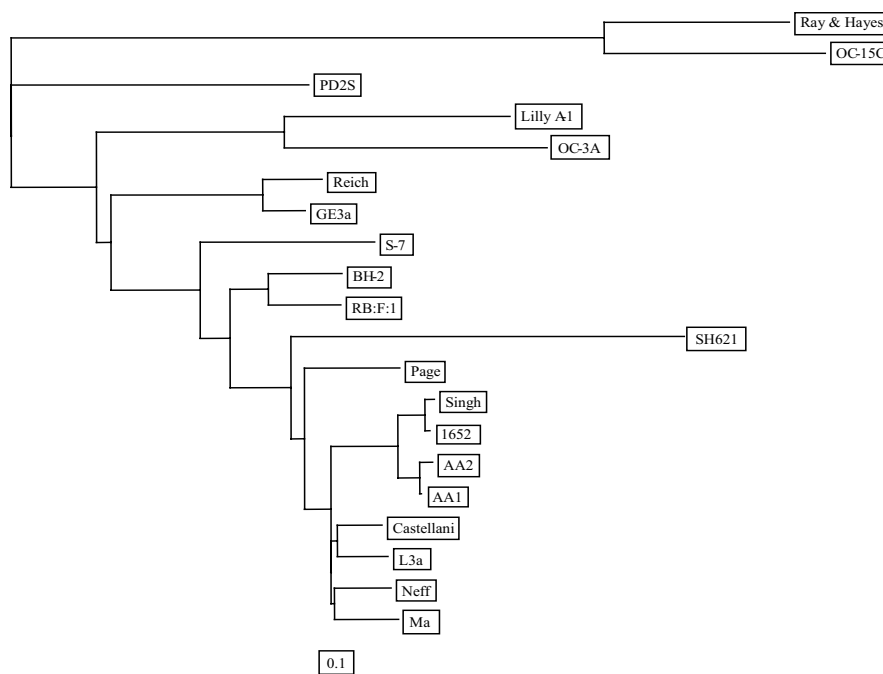
All four of the strains yielded PCR products which were approximately 2.3 kb in size, when amplified with specific primers for 18S rDNA. The nucleotide sequences of the 18s rDNA of all four strains were submitted to Genbank, and were allocated the following accession numbers: AF005998 for *A. rhyssodes* Singh, AF316545 for *A. divionensis* AA2, AY148961 for *A. paradiionensis* AA1, and AY148962 for *A. mauritaniensis* 1652. We compared the 18S rDNA sequences of the four isolates with each other, as well as those of several reference strains stored in GenBank, and have presented the percentage differences of the sequences in Table 2. The four isolates exhibited 0.2-1.1% of sequence difference with each other, but over 2% of difference from all other reference strains compared. As expected, AA2 and AA1 evidenced the closest measure of relatedness among the strains. Interestingly, the 1652 strain was determined to be genetically similar to the Singh strain, exhibiting a sequence difference of only 0.2%. The phylogenetic tree of the *Acanthamoeba* 20 strains, based on analyses of their 18S rDNA sequences, is presented in Fig. 2. Four isolates formed a different clade from *A. castel-*

**Table 2.** Percentage differences between the 18S rDNA sequence for *A. rhyodes* Singh, *A. divionensis* AA2, *A. paradvionensis* AA1 and *A. mauritaniensis* 1652 strains, and other *Acanthamoeba* strains representing the three morphological groups

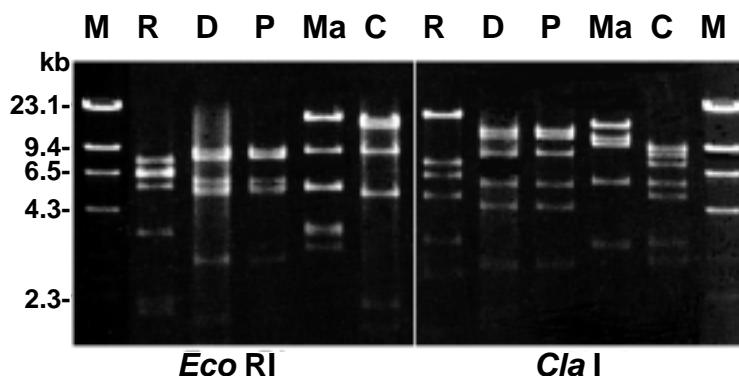
Species and strain	Group	Percent difference <sup>a)</sup>			
		Singh	AA2	AA1	1652
<i>A. astronyxis</i> Ray & Hayes (AF019064) <sup>b)</sup>	I	26.7 (2681:2242)	26.6 (2681:2243)	26.7 (2681:2243)	26.7 (2681:2243)
<i>A. tubiashi</i> OC-15C (AF019065)	I	23.1 (2516:2242)	23.4 (2516:2243)	23.3 (2516:2243)	23.1 (2516:2243)
<i>A. castellanii</i> Castellani (U07413)	II	2.7 (2243:2242)	2.8 (2243:2243)	2.7 (2243:2243)	2.7 (2243:2243)
<i>A. polyphaga</i> Page (AF019061)	II	2.9 (2252:2242)	3.3 (2252:2243)	3.1 (2252:2243)	2.8 (2252:2243)
<i>A. palestinensis</i> Reich (U07411)	III	8.5 (2242:2242)	8.6 (2242:2243)	8.4 (2242:2243)	8.6 (2242:2243)
<i>A. healyi</i> OC-3A (AF019070)	III	15.1 (2322:2242)	14.9 (2322:2243)	14.9 (2322:2243)	15.1 (2322:2243)
<i>A. rhyodes</i> Singh	II	-	1.1 (2242:2243)	0.8 (2242:2243)	0.2 (2242:2243)
<i>A. divionensis</i> AA2	II	-	-	0.2 (2243:2243)	0.9 (2243:2243)
<i>A. paradvionensis</i> AA1	II	-	-	-	0.7 (2243:2243)

<sup>a)</sup>Percent differences are the percent of mismatches between aligned sequences but excluding PCR primer regions.

<sup>b)</sup>Genbank accession number of the 18s rDNA sequence of the strain.



**Fig. 2.** Phylogenetic tree of *Acanthamoeba* 20 isolates based on 18S rDNA sequences. The scale bar represents an evolutionary distance equivalent to 1%. The species names of six reference strains are presented in Table 2. The others are *A. castellanii* Ma, *A. castellanii* Neff, *A. lugdunensis* L3a, *A. triangularis* SH621, *A. stevensoni* RB:F:1, *A. hatchetti* BH-2, *A. griffini* S-7, *A. pustulosa* GE3a, *A. lenticulata* PD2S, and *A. culbertsoni* Lilly.



**Fig. 3.** Agarose gel electrophoretic restriction fragment patterns for mitochondrial DNA from four *Acanthamoeba* strains. Lanes: R, *A. rhyodes* Singh; D, *A. divionensis* AA2; P, *A. paradivionensis* AA1; Ma, *A. mauritaniensis* 1652; C, *A. castellanii* Castellani; M, Hind III digested lambda DNA for molecular size standard.

*lanii* Castellani, as well as the other strains in morphological group II.

**Mitochondria (Mt) DNA RFLP and 16S rDNA sequence analysis**

The Mt DNA RFLP of the four strains, as well as that of *A. castellanii* Castellani (used as a reference strain) is presented in Fig. 3. The RFLP pattern of *A. paradivionensis* AA1 was almost identical to that of *A. divionensis* AA2, with both restriction enzyme tested. The other strains exhibited very different Mt DNA RFLP patterns from each other.

Sequence dissimilarities in the 16S rDNA results from the four strains are shown in Table 3. Percent sequence differences ranged from 0.8% between AA1 and AA2, to 2.9% between AA2 and 1652, or between AA1 and 1652. AA2 and AA1 were found to be the most closely related among the four strains, with a sequence difference of 0.8%.

**DISCUSSION**

*Acanthamoeba* investigators have long disagreed over the taxonomy of the genus *Acanthamoeba*. In the present paper, we evaluated the taxonomic validity of four species of *Acanthamoeba*, namely, *A. divionensis*, *A. paradivionensis*, *A. mauritaniensis*, and *A. rhyodes*, by analyses of nuclear 18s rDNA and mitochondrial 16s rDNA sequences analyses, as well as Mt DNA RFLP analysis. The four strains exhibited 0.2-1.1% 18S

**Table 3.** Percentage differences between the 16S rDNA sequence of *A. rhyodes* Singh, *A. divionensis* AA2, *A. paradivionensis* AA1 and *A. mauritaniensis* 1652 strains

Strain	Percent difference <sup>a)</sup>		
	AA2	AA1	1652
Singh	2.5 (1526:1528)	2.5 (1526:1531)	2.1 (1526:1527)
AA2	-	0.8 (1528:1531)	2.9 (1528:1527)
AA1		-	2.9 (1531:1527)

<sup>a)</sup>Percent differences are the percent of mismatches between aligned sequences but excluding PCR primer regions.

rDNA nucleotide dissimilarity, and 0.8-2.9% mitochondrial 16S rDNA nucleotide dissimilarity. AA1 and AA2, however, evidenced identical mitochondrial RFLP patterns. These results suggested that the AA1, AA2, and 1652 strains of *Acanthamoeba*, formerly designated *A. paradivionensis*, *A. divionensis*, and *A. mauritaniensis*, are actually synonyms for *A. rhyodes*.

Several common characteristics have been reported among the four strains analyzed in this study. They were isolated from soil, and proved to be either avirulent, or weakly virulent (De Jonckheere, 1980). The Singh strain was isolated from English soil (Singh, 1951), AA1 and AA2 were isolated from French soil (Pussard and Pons, 1977), and 1652 was isolated from Moroccan soil (Pussard and Pons, 1977). The AA1, AA2, and 1652 strains have proved incapable of grow-

ing at 40°C, and are almost completely avirulent in mice (De Jonckheere, 1980). In the following experiment, De Jonckheere reported that *A. paradiuionensis* AA1 and *A. diuionensis* AA2, both of which exhibited identical isoenzymes profiles, were closely related to the *A. rhyodes* R4c strain (De Jonckheere, 1983). However, because in the case of the Singh strain, the *A. rhyodes* type strain was not included in the experiment, he was unable to assign the species names AA1 and AA2.

Via the 18s rDNA PCR-RFLP analysis of 23 *Acanthamoeba* strains, Chung et al. (1998) reported identical riboprints for AA1, AA2, and 1652, with the Singh strain, and suggested that *A. diuionensis*, *A. paradiuionensis*, and *A. mauritaniensis* were synonyms for *A. rhyodes*. The four strains were also closely related according to the phylogenetic tree, based on the PCR-RFLP of MtDNA 16s rDNA (Yu et al., 1999).

Stothard et al. (1998) placed *A. rhyodes* Singh in the T4 sequence type of 18S rDNA, where *A. castellanii* Castellani also belonged, and suggested that the various species in T4 might all be reclassified as *A. castellanii*. However, *A. rhyodes* Singh exhibited a 2.7% sequence dissimilarity with *A. castellanii* Castellani (Table 2). Although strains in the T4 sequence type are closely genetically related, and form a cluster which is distinct from the other strains in morphological group II, they would appear to constitute more of a species complex than a species, due to their diversity.

Gene sequences for nuclear 18S rRNA and mitochondrial 16S rRNA of AA1 and AA2 isolated from one strain (Pussard and Pons, 1977), were not identical. Long periods of separated isolation may result in microchanges in the gene sequences of both isolates.

Although the differences in the mitochondrial 16S rDNA and nuclear 18S rDNA sequences of the four strains were not identical, the four isolates were determined by both analyses to be quite closely related. The higher degree of sequence differences with regard to mitochondrial 16s rDNA than were seen in the 18s rDNA among the four strains may be attributable to the fact that organellar DNA evolves at a more rapid rate than does the nuclear genome (Ferris et al., 1981).

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