

Random amplified polymorphic DNA profiles as a tool for the characterization of Brazilian keratitis isolates of the genus *Acanthamoeba*

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Abstract

The genus *Acanthamoeba* comprises free-living amoebae identified as opportunistic pathogens of humans and other animal species. Morphological, biochemical and molecular approaches have shown wide genetic diversity within the genus. In an attempt to determine the genetic relatedness among isolates of *Acanthamoeba* we analyzed randomly amplified polymorphic DNA (RAPD) profiles of 11 Brazilian isolates from cases of human keratitis and 8 American type culture collection (ATCC) reference strains. We found that ATCC strains belonging to the same species present polymorphic RAPD profiles whereas strains of different species show very similar profiles. Although most Brazilian isolates could not be assigned with certainty to any of the reference species, they could be clustered according to pattern similarities. The results show that RAPD analysis is a useful tool for the rapid characterization of new isolates and the assessment of genetic relatedness of *Acanthamoeba* spp. A comparison between RAPD analyses and morphological characteristics of cyst stages is also discussed.

Key words

- *Acanthamoeba*
- Keratitis
- RAPD typing
- Genetic variation

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Introduction

Free-living amoebae of the genus *Acanthamoeba* are opportunistic pathogens of humans and other animal species and have been isolated from a wide variety of natural and man-made environments (1,2). In humans, some species have been implicated in different pathologies, including granulomatous amebic encephalitis (GAE), which occurs in immunologically depressed individu-

als, and keratitis, a severe and painful corneal infection mainly associated with the use of contact lenses (3,4).

The identification of *Acanthamoeba* spp at the genus level is relatively easy since the trophozoite and cyst stages show some features, especially the double-walled cyst shape, that are unique to the genus. Since a wide variability of cyst sizes and shapes can be detected among different isolates, 18 different species were described and assigned to

three distinct morphological groups (I, II and III) based primarily on endo- and ectocyst features (5). However, since the shape of cyst walls can be altered by growth conditions (6), various studies have shown that division of *Acanthamoeba* sp isolates into different groups and/or species is often inconsistent with the classical group designations mentioned above (7-9). Morphological parameters alone are thus not suitable for an accurate identification at the species level.

Among the molecular approaches that have been employed to discriminate species within the genus are isoenzyme patterns (8,10), restriction analysis of mitochondrial DNA (7,11), whole-cell DNA (12) or ribosomal DNA small subunit (SSU rDNA) (13). Based on SSU rDNA sequences, at least 12 different sequence types (T1-T12) were detected (14). Despite the demonstration of a high genetic variability within the genus, and nonetheless contributing to the classification of *Acanthamoeba* sp, most of these methods require large amounts of DNA, relatively complex procedures and even sequencing of the target DNA. On the other hand, the randomly amplified polymorphic DNA (RAPD) method does not require large amounts of DNA, time-consuming procedures or any *a priori* knowledge about the target sequences (15,16). This method has been extensively used to evaluate genetic differences between related organisms (17-20).

In the present study we investigated by RAPD analysis the genetic relatedness among 19 *Acanthamoeba* spp, including 8 reference species from the American type culture collection (ATCC) and 11 not yet characterized Brazilian keratitis isolates. The results were used to construct data matrices and phenograms. RAPD profiles showed highly polymorphic patterns among most of the isolates studied but revealed some groups of more related organisms. The results of RAPD analysis were compared with those of morphological and morphometric analysis of cysts from the different isolates.

Material and Methods

Organisms, growth conditions and DNA extraction

The origin and characteristics of the organisms used are listed in Table 1. For cloning purposes, isolates were grown on 2%-soy extract agar plates previously seeded with heat-killed, plasmidless *Escherichia coli*. Two to 5 trophozoites were picked from each isolate and grown on fresh soy agar plates. After growing and encystment, one cyst of each isolate was cultured, unless otherwise specified. For large axenic cultures, cysts or trophozoites were inoculated in Neff medium (21) containing ampicillin (100 µg/ml) and/or gentamicin (40 µg/ml) and incubated at 28°C with shaking. DNA was purified from late-log-phase cultured cells by SDS lysis, phenol-chloroform extraction, and isopropanol precipitation, basically by the method of Kilvington et al. (22).

Determination of cyst features

To determine their size and structural features, a total of 50 cysts of each corneal scraping isolate and ATCC reference strains were photographed under the phase microscope and the main diameters were measured. The images were scanned and digitally processed to enhance quality. The average measurements of each isolate are presented in Table 1.

RAPD reactions and product analyses

A total of 35 decameric oligonucleotides of arbitrary sequence were used in a previous screening of some *Acanthamoeba* spp isolates. According to the number and intensity of the resulting bands, the reproducibility and discriminating potential of the amplified products, three oligonucleotides were selected for further analyses: 606 (5'-CGGTCCGCCA-3'), 688 (5'-GCAGGAGC

GT-3') and 694 (5'-GGTTTGGAGG-3'). Several of the remaining oligonucleotide-derived patterns, while still useful to confirm the results of genetic similarity estimates, were not used in the computer analyses.

The DNA amplification reactions were performed in 50 µl volumes containing 100 ng of template DNA, 250 µM of dNTPs, 100 pmol of a single primer, 2 units of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD, USA), and 1.5 mM MgCl₂, in a Progene thermocycler (Techne, Cambridge, UK). The cycling conditions used were: initial denaturing phase of 94°C for 3 min and 35 repetitions at 94°C for 1 min, 37°C for 2 min and 72°C for 2 min. The primer extension phase was prolonged for 10 min at 72°C in the last cycle. Amplification products were fractionated by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light with an Eagle Eye II (Stratagene, La Jolla, CA, USA) image processor. The molecular marker used for fragment sizing was a 100-bp DNA ladder (Pharmacia Biotech, Uppsala, Sweden). For data analysis, amplification products obtained with each primer from each *Acanthamoeba* spp isolate were fractionated in the same gel.

Computer-assisted analysis

The digitized gel images were analyzed with the use of the RFLPscan Plus software (version 3.0, Scanalytics CSP Inc., Billerica, MA, USA) in order to size and compare the DNA fragments amplified from the various strains. The discrete character matrix (absence or presence of bands) was analyzed by the RAPDistance software version 1.03 (23) for the calculation of genetic distances between all compared isolates, using the Jaccard index ($A/A + B + C$, where A is the number of shared bands among two organisms x and y , and B and C are the number of bands present only in organism x or y , respectively). To graphically represent the inferred groups, a

phenogram was constructed by the UPGMA method using the PHYLIP 3.5 Neighbor program (24). In order to assess the robustness of the branching, a hundred bootstrap replicates were constructed and only branches presenting bootstrap values of at least 50 were considered as supported clusters.

Results and Discussion

A total of 11 Brazilian keratitis isolates and 8 ATCC reference strains, the latter belonging to 4 different species representative of the three known morphological groups of *Acanthamoeba* (Table 1), were characterized based on cyst morphology and RAPD analysis.

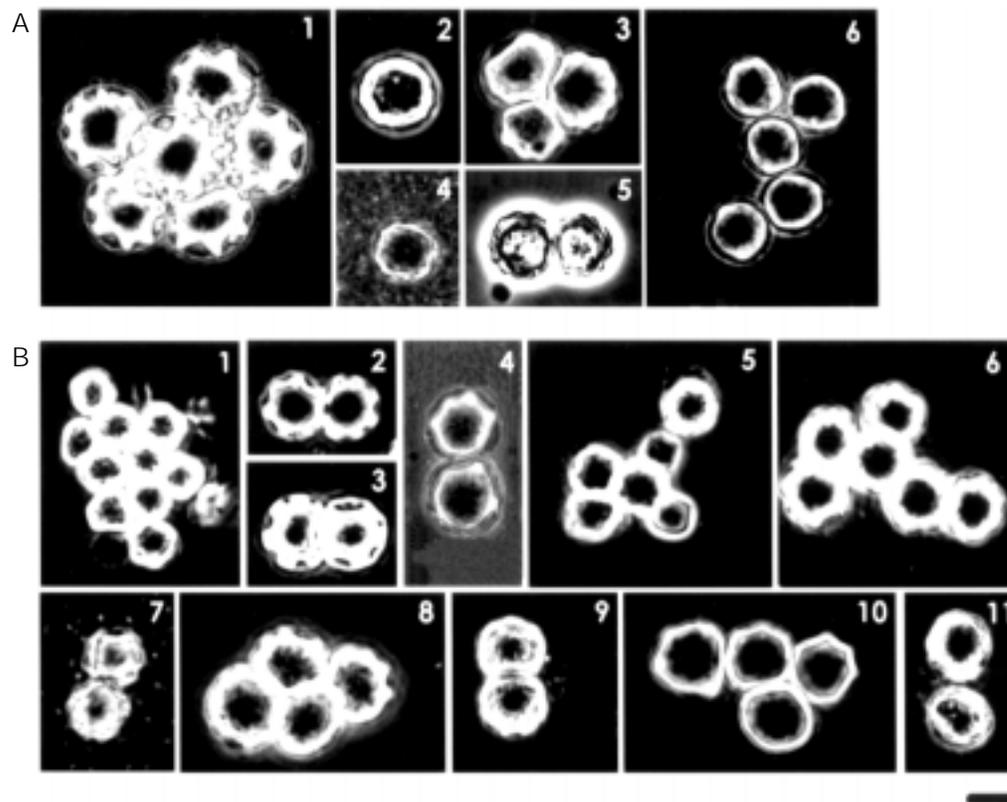
The classification of the isolates within the genus *Acanthamoeba* was based on morphological criteria (5). Figure 1 shows the highly characteristic double-walled morphology of cyst stages of Brazilian keratitis isolates and some representative ATCC refer-

Table 1 - Characteristics of *Acanthamoeba* isolates.

ATCC: American type culture collection; A. poly.: *Acanthamoeba polyphaga*; A. cast.: *A. castellanii*; HAK: human *Acanthamoeba* keratitis; CLW: contact lenses wearer; NCLW: noncontact lenses wearer; HCC: human choriocarcinoma cells; U/E: USP/EPM; U/HC: USP/HC; ^aaverage measurements of 50 cysts; ^btwo morphologically distinct clones isolated from the same corneal sample; ^cisolated from the right (R) and left (L) eye of the same patient.

Isolate	ATCC	Source	Cyst diameter (µm ± SD) ^a
A. astronyxis	30901	water	19.7 ± 1.6
A. poly. #1	30873	HAK	16.4 ± 1.5
A. poly. #2	30461	HAK	16.6 ± 2.2
A. poly. #3	30871	water	16.3 ± 2.7
A. poly. #4	30872	water	16.4 ± 1.5
A. cast. #1	30011	yeast culture	17.4 ± 1.6
A. cast. #2	30868	HAK	15.4 ± 1.6
A. royreba	30884	HCC	17.2 ± 2.4
U/E 1		HAK-CLW	17.0 ± 2.2
U/E 2		HAK-CLW	14.9 ± 1.4
U/E 3		HAK-CLW	16.7 ± 1.8
U/E 4		HAK-NCLW	18.3 ± 1.6
U/E 5		HAK-NCLW	14.2 ± 1.8
U/E 6		HAK-CLW	14.8 ± 1.9
U/E 7.1 ^b		HAK-CLW	14.0 ± 1.5
U/E 7.2 ^b		HAK-CLW	18.8 ± 2.0
U/E 8R ^c		HAK-CLW	16.5 ± 2.3
U/E 8L ^c		HAK-CLW	18.8 ± 1.4
U/HC1		HAK-CLW	10.8 ± 1.4

Figure 1 - Representative cysts of *Acanthamoeba* isolates photographed by phase microscopy and digitally processed. A, ATCC reference isolates - 1: *A. astronyxis*; 2: *A. cast.* #1; 3: *A. poly.* #3; 4: *A. cast.* #2; 5: *A. royreba*, and 6: *A. poly.* #4. B, Brazilian keratitis isolates - 1: U/HC 1; 2: U/E 1; 3: U/E 3; 4: U/E 4; 5: U/E 5; 6: U/E 6; 7: U/7.1; 8: U/E 7.2; 9: U/E 8R; 10: U/E 8L, and 11: U/E 2. Bar = 12.0 μ m. For abbreviations, see legend to Table 1.



ence strains. Except for *A. astronyxis*, which presents large cysts with a smooth ectocyst and a stellate endocyst typical of group I, and *A. royreba*, typical of group III, the shape features of cysts were closely similar among the different isolates (Figure 1). On the other hand, average cyst sizes obtained from our keratitis isolates showed wide variability, ranging from 10.8 to 18.8 μ m (Table 1). In spite of this high variability, the morphological criteria allowed us to classify keratitis isolates in the genus *Acanthamoeba*, although they proved insufficient to distinguish among most of them. Based on these criteria, they could be included in morphological group II which comprises the majority of species (25).

To determine the genetic relatedness of the isolates, DNA fingerprints were determined by RAPD analysis. Each reaction was repeated at least three times, and 100 stable bands were considered and computed in the matrices. In most cases, RAPD profiles al-

lowed detection of extensive polymorphism among isolates. Similar results were obtained with the three primers and an illustrative profile is shown in Figure 2.

Results from the RAPD analyses with the three primers were combined and used to calculate a matrix of pairwise similarities (Figure 3). This matrix was used to construct a phenogram, after determination of bootstrap values for 100 replicates (Figure 4). As expected from the RAPD profiles, some genetically more related Brazilian keratitis isolates could be detected. However, as a result of the high genetic variability, no major groups could be assigned. Moreover, none of our keratitis isolates could be surely grouped with any ATCC reference strains. Although minor differences in RAPD profiles were detected, four clusters of Brazilian keratitis isolates could be identified: a) U/E 1 and 3, b) U/E 4, 5 and 6, c) U/E 7.1 and 7.2, and d) U/E 8R and 8L. Isolates U/E 2 and U/HC 1 could not be assigned to any cluster

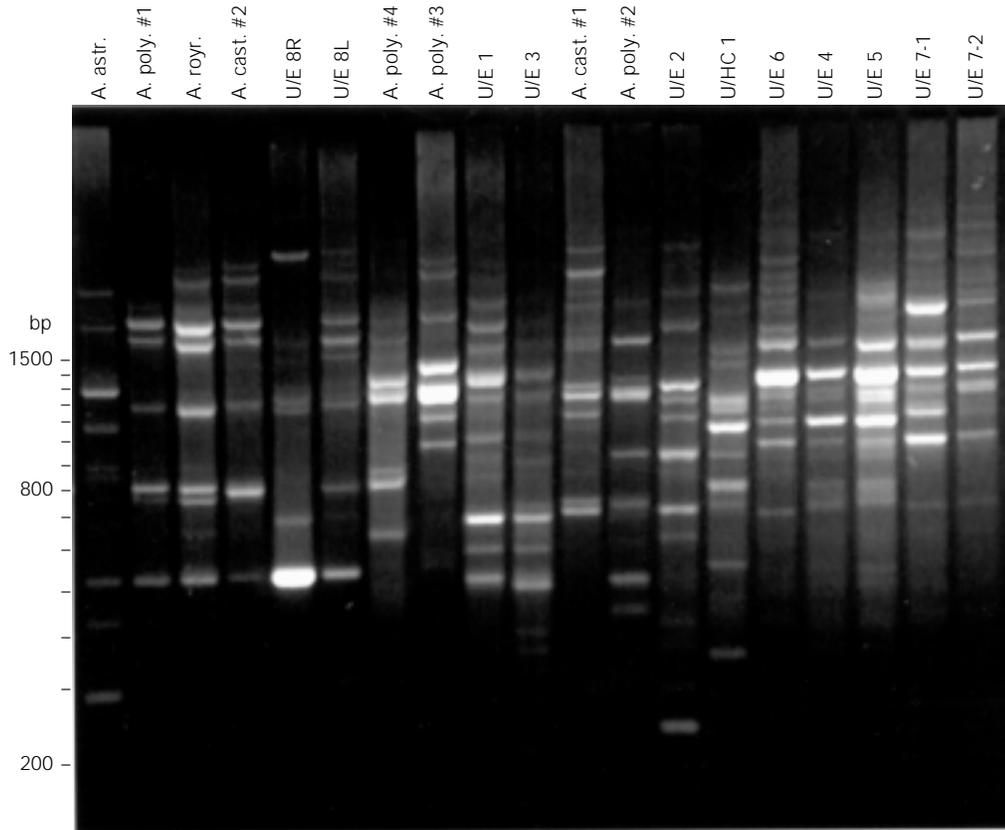
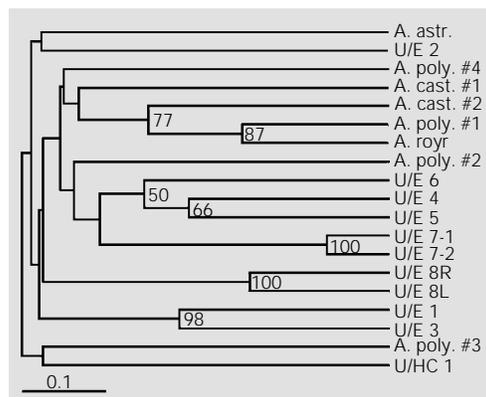


Figure 2 - RAPD profiles of 19 *Acanthamoeba* isolates obtained with primer 688 (5'-GCAGGA GCGT-3'). The PCR products were resolved by electrophoresis on 2% agarose gel. Designations of isolates are indicated on the top: A. astr.: *A. astronyxis*, A. poly.: *A. polyphaga*, A. cast.: *A. castellanii*, A. royr.: *A. royreba*, U/E: USP/EPM, U/HC: USP/HC. Molecular size markers (100-bp DNA ladder - Gibco BRL) are shown on the left.

	A. astr.	A. cast. #1	A. cast. #2	A. poly. #1	A. poly. #2	A. poly. #3	A. poly. #4	A. royr.	USP/EPM 1	USP/EPM 2	USP/EPM 3	USP/EPM 4	USP/EPM 5	USP/EPM 6	USP/EPM 7-1	USP/EPM 7-2	USP/EPM 8R	USP/EPM 8L
A. cast. #1	10.0																	
A. cast. #2	12.9	30.4																
A. poly. #1	26.7	26.9	45.8															
A. poly. #2	11.4	25.9	20.0	18.1														
A. poly. #3	6.4	16.7	11.1	13.8	13.3													
A. poly. #4	15.4	19.0	22.7	25.0	14.8	8.7												
A. royr.	26.7	22.2	40.0	65.2	18.2	13.8	25.0											
USP/EPM 1	18.2	13.3	20.0	21.9	21.2	6.2	19.2	18.2										
USP/EPM 2	17.6	20.7	19.4	17.6	17.1	12.9	10.3	14.3	17.1									
USP/EPM 3	16.7	15.4	14.3	20.7	16.1	7.1	12.5	16.7	50.0	15.6								
USP/EPM 4	13.3	26.1	19.2	21.4	25.0	16.0	30.0	25.9	20.7	12.5	19.2							
USP/EPM 5	20.0	24.0	26.9	33.3	23.3	14.8	27.3	38.5	19.4	11.8	17.9	52.4						
USP/EPM 6	19.4	33.3	17.2	19.4	26.7	14.3	20.8	23.3	22.6	18.2	17.2	43.5	40.0					
USP/EPM 7-1	8.8	23.1	17.2	15.6	26.7	23.1	20.8	15.6	18.7	18.2	9.7	26.9	40.0	28.6				
USP/EPM 7-2	8.6	22.2	16.7	15.2	25.8	22.2	20.0	15.2	18.2	17.6	9.4	25.9	38.5	27.6	85.0			
USP/EPM 8R	10.0	16.7	15.4	22.2	13.3	12.0	19.0	22.2	25.9	9.4	11.1	16.0	14.8	14.3	18.5	17.9		
USP/EPM 8L	9.4	20.0	18.5	25.0	12.5	15.4	22.7	25.0	20.0	8.8	6.7	19.2	17.9	13.3	17.2	16.7	66.7	
USP/HC 1	15.2	13.8	9.4	15.2	11.4	17.9	7.1	18.8	11.4	11.1	9.4	13.3	20.0	19.4	8.8	8.6	10.0	12.9

Figure 3 - Matrix showing pairwise similarities calculated for RAPD profiles obtained with primers 606, 688 and 694. For abbreviations, see legend to Figure 2.

Figure 4 - Unrooted UPGMA phenogram based on RAPD profiles of 19 *Acanthamoeba* isolates obtained with primers 606, 688 and 694. For abbreviations, see legend to Figure 2. The numbers at the nodes represent bootstrap values for a hundred replicates.



since they showed high genetic variability when compared to the others. Based on the analysis of the resulting clusters, we found both agreements and disagreements between RAPD profiles and morphological cyst features. In the clusters harboring U/E 1 and 3, and U/E 5 and 6, the isolates showed very similar cyst sizes (Table 1, Figure 1) and RAPD patterns (Figure 2), despite the fact that they were isolated from different patients. In contrast, the clusters to which U/E 4 and 5, U/E 7.1 and 7.2, and U/E 8R and 8L were assigned showed discordance between cyst sizes and RAPD patterns. The cyst sizes of isolates U/E 4 and 5 were 18.33 μm and 14.17 μm , respectively, whereas their RAPD profiles were very similar (Figure 2).

It is noteworthy that U/E 7.1 and 7.2, and U/E 8 L and R also showed discrepancies between cyst sizes and RAPD profiles. The two clones of U/E 7, which were simultaneously isolated from the same corneal sample, showed quite different cyst sizes (Table 1, Figure 1). These observations may suggest that the patient had been infected with genetically different amoebae. However, despite some detected differences, especially with primer 688 (similarity index of 0.87), very similar RAPD patterns were obtained (Figure 2), indicating that the two clones probably originated from the same organism. In fact, it has been reported that cyst morphology might vary depending on culture conditions, even within cloned strains (6,26), possibly explaining the polymorphism

of cyst sizes. Isolates U/E 8R and 8L, which were respectively obtained from the right and left eye of another patient, showed very similar cyst features (Table 1). However, the RAPD patterns with primer 688 (Figure 2) showed a lower similarity index (0.57) when compared with that of the two clones of U/E 7. Analyses with other primers showed similar results (data not shown), indicating that this patient could have been infected by genetically different organisms.

A remarkable feature of the RAPD-derived phenogram is the presence of strains assigned to the same species (*A. castellanii* and *A. polyphaga*) distributed through different branches. On the other hand, different species - *A. poly. #1*, *A. cast. #2* and *A. royreba* - were assigned to nearby branches. These observations do not agree with the morphological data, especially with cyst diameter. For example, the four strains designated as *A. polyphaga* (morphological group II) showed a narrow range of cyst sizes and *A. royreba* showed cyst shapes and measurements typical of morphological group III (Table 1). In contrast, *A. astronyxis*, a representative species of morphological group I, could not be clustered with any other reference species or our keratitis isolates, showing concordance between RAPD profiles and cyst features. Similar findings were also observed by other approaches (8,14,27). Therefore, it is clear that species definition in the genus *Acanthamoeba* - especially those assigned to either *A. polyphaga* or *A. castellanii* - must be reviewed, as recently proposed (14).

Our data demonstrate the ability of RAPD analysis to assign a specific genetic fingerprint to otherwise morphologically indistinguishable *Acanthamoeba* isolates. Furthermore, this approach proved to be useful in the characterization of new isolates and for the assessment of genetic relatedness and proved to be a fast and informative strategy to determine relationships among different strains. A general limitation of our technique

is its inability to properly infer phylogenetic relationships, because genetic distances calculated from RAPD could be affected by paralogy, which would make RAPD patterns inconsistent with speciation events. Even identification could be hampered by such problems, since some patterns could eventually mislead species assignment. However, our RAPD analyses are in agreement with preliminary SSU rDNA fingerprinting profile determination, which is in progress in our laboratory, indicating that this problem probably does not affect our analyses. This is nonetheless very serious and should be addressed with care in future studies.

Other approaches, such as sequencing of ribosomal DNA subunits, can be used for a more precise determination of the relationships among our strains. However, such relationships cannot be assessed if the approach used is unable to detect subtle differences between very closely related organisms, as recently found in the analysis of SSU rDNA sequences (14). Based on this last approach, at least 12 different sequence types (T1-T12) were detected, presenting dissimilarities ranging from 5.0 to 37.6% between sequence types. The detection of such divergent SSU rDNA sequences is in accordance with our data, since it also reflects the high polymorphism of these organisms. However, as stated by those authors, this strategy could not identify branching patterns within T4 sequence type strains, a

group to which 24 of 25 worldwide-distributed keratitis isolates were assigned. Although our keratitis isolates were not characterized by that approach, they showed a high degree of polymorphism and hence were distributed through different branches by our RAPD analyses. It would thus be interesting to investigate whether these isolates present the same SSU sequence types or, conversely, if RAPD patterns of the isolates designated as T4 would be so polymorphic as to allow the identification of their branching patterns. It should be noted that three ATCC reference strains - *A. poly.* #1 (30873), *A. poly.* #3 (30871), and *A. cast.* #1 (30011) - which were assigned to the same sequence type (T4), were also spread across different branches of the phenogram (Figure 4).

In conclusion, the RAPD technique can be useful to distinguish between closely related *Acanthamoeba* sp isolates and to possibly characterize additional isolates of this genus. On the other hand, more conserved sequences would still be required to determine major groups involving less related strains.

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