Sequence Variations in Small-Subunit Ribosomal RNAs of *Hartmannella vermiformis* and Their Phylogenetic Implications

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Evidence of associations between free-living amoebas and human disease has been increasing in recent years. Knowledge about phylogenetic relationships that may be important for the understanding of pathogenicity in the genera involved is very limited at present. Consequently, we have begun to study these relationships and report here on the phylogeny of *Hartmannella vermiformis,* a free-living amoeba that can harbor the etiologic agent of Legionnaires' disease. Our analysis is based on studies of small-subunit ribosomal RNA genes (srDNA). Nucleotide sequences were determined for nuclear srDNA from three strains of *H. vermiformis* isolated from the United Kingdom, Germany, and the United States. These sequences then were compared with a sequence previously obtained for a North American isolate by J. H. Gunderson and M. L. Sogin. The four genes are 1,840 bp long, with an average GC content of 49.6%. Sequence differences among the strains range are 0.38%–0.76%. Variation occurs at 19 positions and includes 2 single-base indels plus 14 monotypic and 3 ditypic single-base substitutions. Variation is limited to eight helix/loop structures according to a current model for srRNA secondary structure. Parsimony, distance, and bootstrap analyses used to examine phylogenetic relationships between the srDNA sequences of *H. vermiformis* and other eukaryotes indicated that *Hartmannella* sequences were most closely related to those of *Acanthamoeba* and the alga *Cryptomonas.* All ditypic sites were consistent with a separation between European and North American strains of *Hartmannella,* but results of other tests of this relationship were statistically inconclusive.

Introduction

Free-living amoebas such as *Hartmannella* and *Acanthamoeba* in the class Lobosea and *Naegleria* in the class Heterolobosea (Page 1988, pp. 14–15) are found widely in nature and are both ecologically and medically important. *Hartmannella* and *Acanthamoeba* play important roles in nitrogen mineralization in soils (Elliot et al. 1979; Clarholm 1981, 1985), and both genera can act as hosts for the bacterium *Legionella pneumophila,* which causes Legionnaires' disease (Fields et al. 1990; Visvesvara and Stehr-Green 1990; Moffat and Tompkins 1992). *Acanthamoeba* and *Naegleria* are opportunistic human pathogens causing eye and/or central nervous system disease (Visvesvara and Stehr-Green 1990; Anzil et al. 1991; Johns 1993).

The phylogenetic relationships of *Acanthamoeba* and *Naegleria* have been studied by comparison of small-subunit ribosomal RNA (srRNA) sequences (Sogin 1989, 1991; Johnson et al. 1990). These two genera are only very distantly related. *Naegleria* appears to be part of a group that diverged early in the eukaryotic line of descent (Hinkle and Sogin 1993). *Acanthamoeba,* which lacks a flagellated stage found in *Naegleria,* is more closely related to humans, green algae, fungi, and land plants than to many other protozoans (Baverstock et al. 1989; Sogin 1989, 1991; Wainwright et al. 1993). Since the phylogenetic position of *Acanthamoeba* is somewhat unexpected, we were interested in determining whether any other amoebas had similar phylogenetic affinities. *Hartmannella* was selected for study because it is similar to *Acanthamoeba* in morphology and life cycle. Both include trophozoite and cyst stages and lack the flagellated stage characteristic of *Naegleria.* Extensive work on nuclear and mitochondrial small-subunit ribosomal DNA (srDNA) sequences of *Acanthamoeba* has revealed relatively high levels of intraspecific variation in this genus (R. J. Gast, D. R. Ledee, P. A. Fuerst, and T. J. Byers, unpublished data). Thus, we also were interested in determining whether a similar degree of sequence diversity would occur in *Hartmannella.*

We have now amplified and sequenced nuclear srDNAs from three strains of *H. vermiformis,* one iso-

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Table 1
PCR Primer Sequences Used for Sequencing of srDNA

Primer	Sequence[a]	Base-Pair Location in Gene of *Hartmannella vermiformis* Nijmegen
5′ → 3′-directed PCR primers:		
SSU1	CCGCGGCCGCGTCGACTGGTTGATCCTGCCAGTAG	1–23
373C	GATTCCGGAGAGGGAGCCTGA	391–411
570C	GTAATTCCAGCTCCAATAGC	593–612
PCR2	GAACTTAAAGGAATTGA	1152–1168
1262C	GTGGTGCATGGCCGTTCTTA	1288–1307
1200C	CAGGTCTGTGATGCCC	1465–1480
3′ → 5′-directed PCR primers:		
170	GCATGTATTAGCTCTAGA	158–175
373	TCAGGCTCCCTCTCCGGAATC	391–411
570	GCTATTGGAGCTGGAATTAC	593–612
1137	GTGCCCTTCCGTCAAT	1164–1179
1262	GAACGGCCATGCACCAC	1288–1304
1200RE	GGGCATCACAGACCTG	1465–1480
SSU2	CCGCGGCCGCGGATCCTGATCCTTCT/CAGGTTCAC	1818–1840

[a] Written 5′ → 3′. Underlined regions in SSU1 and SSU2 are *Bam*HI and *Sal*I sites added to the primer 5′ ends to provide sites for cloning.

lated from the United Kingdom, one from Germany, and one from the United States. These sequences were compared with the sequence from a second North American isolate of *H. vermiformis* studied by J. H. Gunderson and M. L. Sogin (personal communication), as well as with sequences for several other protistans and higher eukaryotes. Sequence variation among the *H. vermiformis* strains was localized to eight helices and was less than that among *Acanthamoeba* strains. Phylogenetic analysis indicated that *Hartmannella* is most closely related to *Acanthamoeba* and the alga *Cryptomonas.*

Material and Methods

Hartmannella vermiformis strain Nijmegen, isolated in the United Kingdom, was obtained as a monoxenic culture from the Culture Collection of Algae and Protozoa (Ambleside, England; CCAP 1534/7B) and was cultured axenically in Nijmegen by P.H.H.W. *Hartmannella vermiformis* strain Koblenz, isolated in Germany, was obtained as an axenic culture from Dr. R. Michel (Ernst-Rodenwaldt Institut, Koblenz; strain OS-101). *Hartmannella vermiformis* strain Atlanta, isolated in the United States, was obtained as an axenic culture from Dr. B. S. Fields (Centers for Disease Control, Atlanta; strain CDC-19).

All amoebas were cultivated as static cultures at 30°C in 25-cm^2 tissue-culture flasks (Corning Glass Works, Corning, N.Y.) with 10 ml "PYNFH" (ATCC Medium 1034). Late-log-phase cultures were harvested by centrifugation at 2,000 *g*, and ~10^8 amoebas were resuspended and incubated at 65°C in 1.0 ml lysis buffer containing 200 μg proteinase K/ml, 0.2% sodium dodecyl sulfate, 10 mM Tris (pH 7.4), 10 mM NaCl, and 10 mM ethylenediaminetetraacetate (Burg et al. 1989). The mixture was extracted once with 1.0 ml phenol and once with 1.0 ml chloroform:isoamyl alcohol (25:1 [v/v]). After precipitation with ethanol at −80°C, the nucleic acids were recovered by centrifugation at 16,000 *g* for 15 min and then were resuspended to a final concentration of ~2–4 μg DNA/μl in double-distilled water. The DNA was stored at −20°C.

Eukaryotic primers and PCR were used to amplify complete srRNA genes as well as subfragments (table 1). The complete srDNA sequences of *H. vermiformis* strains Nijmegen, Koblenz, and Atlanta were amplified using primers SSU1 and SSU2, which are complementary to the strongly conserved 5′ and 3′ ends of eukaryotic srRNA genes. PCR conditions were 1 min at 95°C for denaturation, 2 min at 42°C for annealing, and 3 min at 72°C for extension, in a run of 35 cycles. PCR products were band-isolated on an 0.8% agarose gel and subsequently were purified with the GeneClean kit (Bio 101, LaJolla, Calif.). All samples were stored at −20°C.

The full-length srDNA was sequenced in triplicate in 5′→3′ and 3′→5′ directions by using the specific primers and with the products of PCR-amplified srDNA subfragments as templates. All sequencing was done with the dideoxynucleotide chain-termination method (Sanger 1981) and a Double Stranded DNA Sequencing Kit (BRL Life Technologies, Gaithersburg, Md.). Reactions were run on 6% acrylamide-urea sequencing gels, dried, and exposed to Kodak XRP-1 diagnostic film at room temperature. Complete overlapping sequence was obtained for both strands of the DNA.

Although posttranscriptional editing of other types of RNA has been described in other organisms, it is unknown for rRNA. Thus, we inferred the srRNA sequences for the *Hartmannella* strains on the basis of srDNA sequences and used them for secondary-structure and phylogenetic analyses. Sequences were aligned by eye, with the help of published primary- and secondary-structure alignments (Neefs et al. 1993) and the Eyeball Sequence Editor (ESEE; Cabot and Beckenbach 1989). The srDNA sequences that we determined were compared with the following sequences obtained from GenBank: *Acanthamoeba castellanii* Neff (M13435; Gunderson and Sogin 1986), *H. vermiformis* Balamuth (M95168; J. H. Gunderson and M. L. Sogin, personal communication), *Cryptomonas phi* (X57162; Douglas et al. 1991), *Oryza sativa* (X00755; Taikawa et al. 1984), *Zea mays* (K02202; Messing et al. 1984), *Chlamydomonas reinhardtii* (M32703; Gunderson et al. 1987), *Homo sapiens* (X03205; McCallum and Maden 1985), *Saccharomyces cerevisiae* (M27607; Rubtsov et al. 1980), and *Ochromonas danica* (M32704; Gunderson et al. 1987).

An initial global analysis of higher and lower eukaryotic organisms placed *Hartmannella* close to *Acanthamoeba.* Thus, further tests focused on this relationship. We first examined srDNA from a set of nine organisms including *Hartmannella,* seven organisms previously identified as relatively closely related to *Acanthamoeba* (Johnson et al. 1990; Sogin 1991; Douglas et al. 1991), and *Ochromonas,* which was the outgroup. We then examined a set including four strains of *H. vermiformis* plus *Acanthamoeba* and *Cryptomonas; Chlamydomonas* was used as the outgroup. The analyses in the first case were based on 190 informative sites (at least ditypic), of 1,381 total aligned sites. In the second case, we used 218 informative sites, of 1,650 aligned sites. Phylogenetic analyses used the following programs from version 3.4 of Felsenstein's (1989) PHYLIP package: DNAPARS (parsimony), DNADIST (distance), NEIGHBOR (neighbor joining), DNAINVAR (Lake's and Cavender's phylogenetic invariants), and DNABOOT (bootstrapping).

Results

srDNA Sequences and Secondary-Structure Predictions

We obtained complete unambiguous srDNA sequences for all three strains, except for the terminal regions complementary to the primers (fig. 1). For purposes of estimating gene lengths, these end sequences were inferred from the primer sequences. The coding sequence of the gene is 1,840 bp, and the average GC content is 49.7% ± 0.07% for the three strains and is 49.6% ± 0.14% when the Balamuth strain studied by J. H. Gunderson and M. L. Sogin (personal communication) is included.

Sequence differences among srDNAs from our three *Hartmannella vermiformis* strains plus the Balamuth strain were 0.38%–0.76% (table 2). Sequence heterogeneity included 17 base substitutions and 2 single-base indels, which were found in eight variable stems (fig. 1). The base substitutions included 11 transitions and 6 transversions.

The most recent secondary-structure model for srRNAs (Neefs et al. 1993) was used to identify base pairs in helical regions where base substitutions were observed. Compensatory substitutions occurred at three pairs of bases—670/696 and 671/695 in helix E23-1 and 714/752 in helix E23-2 (fig. 1)—thus lending support to the existence of these two stems. Base substitutions at positions 71, 287, 729, and 1734 have resulted in mismatches within stems, but base-paired or unpaired states have been retained at all other variable positions (fig. 1).

Phylogenetic Analyses

Parsimony analyses were used to explore the placement of *Hartmannella* srRNA sequences in (1) a "global" srRNA tree including 12 genera of eukaryotes, (2) a tree including a more limited group of nine eukaryotes, and (3) a tree limited to close relatives. The global tree, which included the protists *Naegleria, Plasmodium,* and *Euplotes* in addition to the nine species of the second tree, is not shown, but the branch order was similar to those of previously published trees, which did not include *Hartmannella* (Baverstock et al. 1989; Sogin 1989, 1991; Johnson 1990; Douglas et al. 1991). A single most-parsimonious tree was obtained for each of the other two analyses (fig. 2). The two trees are topologically consistent with each other. Bootstrap values >90% (fig. 2*A*) indicate that the analyzed data strongly support two clades—one including *Chlamydomonas, Zea,* and *Oryza* and a second including *Saccharomyces* and *Homo.* Bootstrap values of ~60% also show support for a clade that includes *Hartmannella* and *Acanthamoeba* as a sister group to *Cryptomonas.* Stronger support for the clustering of the amoebas was obtained by restricting the analysis to the two amoebas plus the alga *Cryptomonas,* with the alga *Chlamydomonas* as the outgroup. This restriction in the number of taxa increases the number of phylogenetically informative sites within the analysis by including positions that have ambiguous homology within the larger data set but that can be aligned with confidence in the smaller set. In this case, the clade linking the amoebas had a bootstrap value of 96% (fig. 2*B*).

Trees constructed using neighbor-joining on a distance matrix with distance values corrected using the Kimura two-parameter model had the same branching

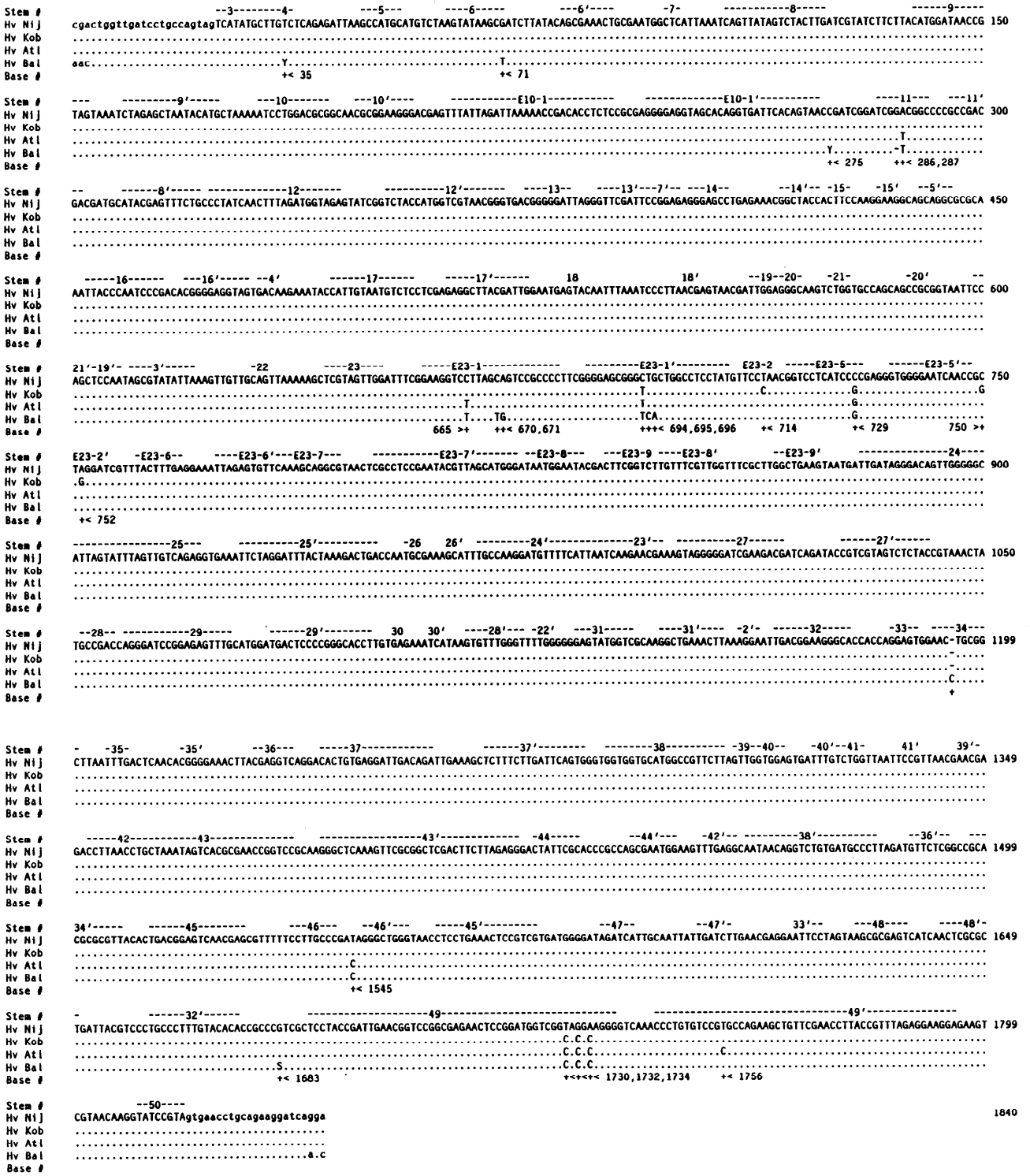

FIG. 1.—Comparison of srRNA sequences from four different *Hartmannella vermiformis* strains. The full sequence for the Nijmegen strain is given with variations in other strains listed below. Nucleotides indicated in lowercase are the PCR primer sequences used and may or may not be identical to the corresponding nucleotides in the gene amplified. The locations of helices that are predicted by Neefs et al. (1993) for the secondary structure of *Hartmannella* srRNA are indicated above the sequences. The helices are numbered in the order of occurrence, from 5′ to 3′ terminus. Helices bearing a composite number preceded by "E" are eukaryote specific and display extreme length variation among eukaryotes (Neefs et al. 1993). The base-pair numbers below the sequences correspond to the Nijmegen strain. The C inserted into the Balamuth strain occurs between 1194 and 1195. Positions designated "Y" or "S" in the Balamuth strain are numbered but are not considered variable sites in our analyses.

order as did the parsimony trees (not shown). Distances calculated between the four strains of *H. vermiformis* by using DNADIST and Kimura's two-parameter model indicate that they differ from each other by a maximum of 0.0049 substitutions/site, whereas they differ from *Acanthamoeba, Cryptomonas,* and *Chlamydonomas* by 0.15, 0.17, and 0.19 substitutions/site, respectively (table 2).

Bootstrap analysis with parsimony was used to determine if a statistically significant branching order among the four strains of *Hartmannella* could be identified, but the results were inconclusive. However, 3 of the 19 positions that varied among the four strains were ditypic, and all were consistent in clustering the North American and European strains separately (fig. 1).

Discussion

Sequence Variation and Secondary Structure

The srRNA sequences of the four strains of *Hartmannella vermiformis* differ by only 1.8–4.9 nucleotide changes/1,000 alignable positions, even though two strains are from North America and two are from Europe (table 2). In contrast, they vary from *Acanthamoeba castellanii* by 150 differences/1,000 positions (table 2). In addition, intraspecific variability is much greater in *Acanthamoeba* srRNA, even when the analysis is limited to informative sites and when a number of highly variable regions that are much less variable in *Hartmannella* are excluded (R. J. Gast, D. R. Ledee, and T. J. Byers, unpublished data). The reason for this difference is unknown.

The secondary structures predicted for *Hartmannella* and *Acanthamoeba* srRNAs by Neefs et al. (1993), and available from their srRNA data bank, are similar, with several exceptions. *Hartmannella* is missing helices that *Acanthamoeba* has in regions E23-4 and E45-1, and *Hartmannella* stems 10, 11, 29, 43, 49, and E23-1, 2, and 5 each are 20%–60% shorter in length than in the Neff strain. These differences account for most of the large difference, in overall lengths, between the srRNAs of *Hartmannella* (1,840 bp) and those of *Acanthamoeba* (2,303 bp).

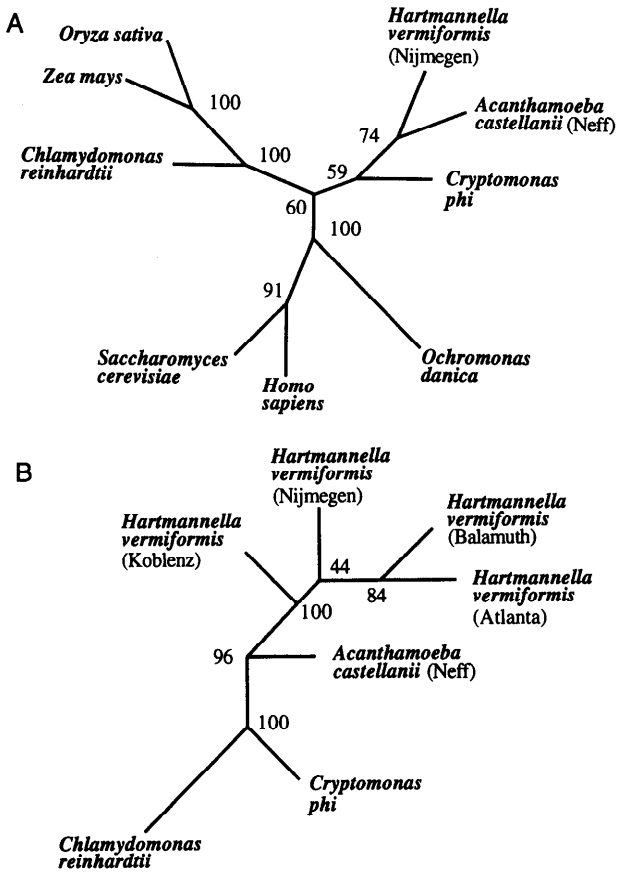

FIG. 2.—Parsimony trees showing phylogenetic relationships of *Hartmannella vermiformis,* inferred from srRNA sequences. Phylogenetic relationships of *H. vermiformis* to representative eukaryotes (*A*) and its closest relatives (*B*) are shown. Both trees are unrooted most-parsimonious trees. Numbers at the nodes are the number of times that a cluster appeared in a bootstrap test of 100 runs.

Table 2
Structural Distances Based on srDNA Comparisons of Four *Hartmannella vermiformis* Strains and Their Closest Relatives

	Hve(N)	Hve(K)	Hve(A)	Hve(B)	Aca	Cph
H. vermiformis (Nijmegen)						
H. vermiformis (Koblenz)	0.0018					
H. vermiformis (Atlanta)	0.0030	0.0024				
H. vermiformis (Balamuth)	0.0049	0.0043	0.0030			
Acanthamoeba castellanii	0.1503	0.1497	0.1512	0.1499		
Cryptomonas phi	0.1701	0.1710	0.1733	0.1705	0.1347	
Chlamydomonas reinhardtii	0.1913	0.1914	0.1922	0.1917	0.1658	0.1512

NOTE.—Values are substitutions per site. The data set for distance calculations included 271 informative sites of a total of 1,650 unambiguously aligned sites. The sequence for the Balamuth strain of *H. vermiformis* (ATCC 30966), sequenced by J. H. Gunderson and M. L. Sogin (personal communication), was obtained from GenBank (M95168).

Phylogeny

The tree topology that we found for organisms other than *Hartmannella* (fig. 2*A*) is in general agreement with previous analyses using srRNA sequences (Sogin 1989, 1991; Johnson et al. 1990; Douglas et al. 1991). Previous workers grouped *Acanthamoeba* with the alga *Cryptomonas phi* (Nu) (Douglas et al. 1991) and found that the amoeba branched off the plant lineage rather than off the human/yeast lineage (Schlegel 1991; Sogin 1991). In our analysis, *Hartmannella* also branched off the plant lineage with *Acanthamoeba* (fig. 2*A*). Recently, however, Wainwright et al. (1993) found *Acanthamoeba* branching off the animal/fungus lineage. One possible explanation for this difference is that the more global analysis of Wainwright et al. may exclude a number of sites that are informative for relationships among the more closely related set of organisms that we studied but that are uninformative in the global analysis.

The separation observed between *Hartmannella* and *Acanthamoeba* is relatively ancient and comparable to the separation between the green algae and the amoebas. A relative-rates test, which excluded expansion segments characteristic of *Acanthamoeba* but absent from *Hartmannella* and that used either *Chlamydomonas* or *Cryptomonas* as an outlier, suggested that the srRNA gene is evolving at a rate ~1.6 times faster in the lineage leading to *Hartmannella* than in the lineage leading to *Acanthamoeba.* Although suggesting some rate heterogeneity, this difference probably would not lead to any major distortion in the relative positions of *Hartmannella* and *Acanthamoeba,* because it is relatively small compared with rate differences that do seem to result in distortions (Felsenstein 1988).

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