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-
Table 1
PCR Primer Sequences Used for Sequencing of srDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Base-Pair Location in Gene of Hartmannella vermiformis Nijmegen</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' → 3'-directed PCR primers:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSU1</td>
<td>CCGGGGCGCGGCTCGAGTGATCGTATCCGTGCGAGTAG</td>
<td>1-23</td>
</tr>
<tr>
<td>373C</td>
<td>GATTCGGAGAGGGAGCCCTGA</td>
<td>391-411</td>
</tr>
<tr>
<td>570C</td>
<td>GTAATTCCAGCTCAATAGC</td>
<td>593-612</td>
</tr>
<tr>
<td>PCR2</td>
<td>GAACCTAAGGAAATTGA</td>
<td>1152-1168</td>
</tr>
<tr>
<td>1262C</td>
<td>GTGGTGACGGGCTTCTTA</td>
<td>1288-1307</td>
</tr>
<tr>
<td>1200C</td>
<td>CAGGTCGTTGATGCCC</td>
<td>1465-1480</td>
</tr>
<tr>
<td>3' → 5'-directed PCR primers:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>GCACTTGATTAGCTCTAGA</td>
<td>158-175</td>
</tr>
<tr>
<td>373</td>
<td>TCAGGCTCTCTCACGAACTC</td>
<td>391-411</td>
</tr>
<tr>
<td>570</td>
<td>GCTATGGACGGTGAATTAC</td>
<td>593-612</td>
</tr>
<tr>
<td>1137</td>
<td>GTGCCCTTCCGCTAAT</td>
<td>1164-1179</td>
</tr>
<tr>
<td>1262</td>
<td>GAAACGGCCATGCACCAC</td>
<td>1288-1304</td>
</tr>
<tr>
<td>1200RE</td>
<td>GGTCATACAGACCTG</td>
<td>1465-1480</td>
</tr>
<tr>
<td>SSU2</td>
<td>CCGGCGCGCGGACGTCTGATCTCATCCCAGTTGTACG</td>
<td>1818-1840</td>
</tr>
</tbody>
</table>

<sup>a</sup> Written 5' → 3'. Underlined regions in SSU1 and SSU2 are BamHI and SalI sites added to the primer 5' ends to provide sites for cloning.

lattened 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 protistanst 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-cm² 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 ~10⁸ 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 (M13433; 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. 2A) 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. 2B).

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.
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 *Chlamydomonas* 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

![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.](image)

Table 2

<table>
<thead>
<tr>
<th>Structural Distances Based on srDNA Comparisons of Four <em>Hartmannella vermiformis</em> Strains and Their Closest Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hve(N)</strong></td>
</tr>
<tr>
<td><em>H. vermiformis</em> (Nijmegen)</td>
</tr>
<tr>
<td><em>H. vermiformis</em> (Koblenz)</td>
</tr>
<tr>
<td><em>H. vermiformis</em> (Atlanta)</td>
</tr>
<tr>
<td><em>H. vermiformis</em> (Balamuth)</td>
</tr>
<tr>
<td><em>Acanthamoeba castellanii</em></td>
</tr>
<tr>
<td><em>Cryptomonas phi</em></td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
</tr>
</tbody>
</table>

**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 (M93108).
Acanthamoeba. Hartmannella and geneity, this difference probably would not lead to any off the human/yeast lineage (Schlegel 1991; Sogin 1991). Gene is evolving at a rate \( \sim 1.6 \) times faster in the lineage with the amoeba branched off the plant lineage rather than the separation between the green algae and the amoe-

workers grouped bas. A relative-rates test, which excluded expansion seg-

ments characteristic of 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 \( \sim 1.6 \) times faster in the lineage leading to Hartmannella than in the lineage leading to Acanthamoeba. Although suggesting some rate hetero-
genesis, 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).

Acknowledgments

The authors thank Drs. B. S. Fields and R. Michel for gifts of Hartmannella vermiformis strains Atlanta and Koblenz, respectively, and thank Drs. J. H. Gunderson and M. L. Sogin for sharing their unpublished srRNA sequence for H. vermiformis. P.H.H.W. submitted this work in partial fulfillment of the requirement for the Ph.D. degree at the University of Nijmegen and received a travel grant from the Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO). This research was supported by the Ohio State University and by NIH/NEI grant RO1-EY09073 to T.J.B.

LITERATURE CITED


BAVERSTOCK, P. R., S. ILLANA, P. E. CHRISTY, B. S. ROBINSON, and A. M. JOHNSON. 1989. srRNA evolution and phylogenetic relationships of the genus Naegleria (Protista: Rhi-


ford.

DOUGLAS, S. E., C. A. MURPHY, D. F. SPENCER, and M. W. GRAY. 1991. Cryptomonad algae are evolutionary chimera

don.


——. 1989. PHYLIP—phylogeny inference package, ver-
sion 3.2. Cladistics 5:164-166.


GUNDerson, J. H., and M. L. SOgin. 1986. Length variation in eukaryotic rRNAs: small subunit rRNAs from the protists Acanthamoeba castellanii and Euglena gracilis. Gene 44:

63-70.


Mitchell L. Sogin, reviewing editor

Received November 16, 1993

Accepted February 2, 1994