

ORIGINAL ARTICLE

The Mitochondrial Genome and a 60-kb Nuclear DNA Segment from *Naegleria fowleri*, the Causative Agent of Primary Amoebic Meningoencephalitis

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NAEGLERIA fowleri is a deadly human pathogen, and the causative agent of primary amoebic meningoencephalitis (PAM). Cases have been reported from Australia, New Zealand, Africa, Mexico, Venezuela, India, as well as the United States and Europe (Visvesvara and Stehr-Green 1990; Yoder et al. 2010). *Naegleria fowleri* may be more prevalent than reported, particularly in developing countries. It also exists in temperate regions in association with thermal waters; for example, in hot spring spas in

ABSTRACT

Naegleria fowleri is a unicellular eukaryote causing primary amoebic meningoencephalitis, a neuropathic disease killing 99% of those infected, usually within 7–14 days. *Naegleria fowleri* is found globally in regions including the US and Australia. The genome of the related nonpathogenic species *Naegleria gruberi* has been sequenced, but the genetic basis for *N. fowleri* pathogenicity is unclear. To generate such insight, we sequenced and assembled the mitochondrial genome and a 60-kb segment of nuclear genome from *N. fowleri*. The mitochondrial genome is highly similar to its counterpart in *N. gruberi* in gene complement and organization, while distinct lack of synteny is observed for the nuclear segments. Even in this short (60-kb) segment, we identified examples of potential factors for pathogenesis, including ten novel *N. fowleri*-specific genes. We also identified a homolog of cathepsin B; proteases proposed to be involved in the pathogenesis of diverse eukaryotic pathogens, including *N. fowleri*. Finally, we demonstrate a likely case of horizontal gene transfer between *N. fowleri* and two unrelated amoebae, one of which causes granulomatous amoebic encephalitis. This initial look into the *N. fowleri* nuclear genome has revealed several examples of potential pathogenesis factors, improving our understanding of a neglected pathogen of increasing global importance.

Japan (Izumiyama et al. 2003) or in Yellowstone National Park in the United States (Sheehan et al. 2003). While most of the 130 cases reported in the United States have occurred in the southern-tier states, a single case recently reported from Minnesota indicates that the geographic patterns in the occurrence of *N. fowleri* infection are changing (Kemble et al. 2012). Consistent with its isolation from thermal waters, *N. fowleri* is thermotolerant, growing preferentially at 37 °C, but surviving at temperatures up to

45 °C (Kadlec 1975). *Naegleria fowleri* can exist as a cyst, amoeba, or flagellate. The trophozoites range in size from 10 to 35 µm and are the primary infective stage of the amoeba, although the cyst form, as carried by wind currents, is associated with cases of PAM (Lawande et al. 1979). Recently, deaths from *N. fowleri* infection have been reported in the United States in association with the use of tap water in sinus irrigation devices (e.g. neti pots) (Yoder et al. 2012).

Infection by *N. fowleri* occurs when water containing the amoeba enters the nose (e.g. of swimmers), attaches to the olfactory mucosa, and passes through the cribriform plate to reach the olfactory bulb (Martinez and Visvesvara 1997). PAM symptoms include severe headache, nausea, vomiting, fever, stiff neck, and onset of coma and death within approximately 10 days (Carter 1972; Martinez and Visvesvara 1997). Disease manifestations are not restricted to immunocompromised patients. Infection in humans is rare, but rapid, with a mortality rate of approximately 99% (Visvesvara and Stehr-Green 1990). The majority of individuals infected with PAM fail to be diagnosed promptly or correctly, and thus most cases are diagnosed post-mortem (Heggie 2010). In diagnosed patients, the treatment involves the use of amphotericin B administered both intravenously and intrathecally along with miconazole or fluconazole and rifampin (da Rocha-Azevedo et al. 2009). However, to date, only eight cases of successful treatment have been reported (Vargas-Zepeda et al. 2005). A fundamental understanding of *N. fowleri* at the genomic level would constitute the first step in identifying its mechanisms of pathogenesis, which would guide the development of safer or more effective therapies, and would also facilitate more effective and rapid molecular diagnostics.

Naegleria fowleri is a single-celled microbial eukaryote, in the lineage Heterolobosea and within the supergroup *Excavata* (Dacks et al. 2008a). *Naegleria fowleri* has a non-pathogenic, nonthermotolerant relative *Naegleria gruberi*, for which the complete genome sequence has recently been determined (Fritz-Laylin et al. 2010). Several groups have identified differences between *N. fowleri* and *N. gruberi*, none of which fully define the pathogenic phenotype of *N. fowleri* (Marciano-Cabral and Cabral 2007; Marciano-Cabral and Fulford 1986; Serrano-Luna et al. 2007). Due to the unknown and likely multifactorial mechanisms of pathogenesis in *N. fowleri*, a comparative genomic approach may provide new insights into why *N. fowleri* causes a severe, quickly fatal disease, whereas *N. gruberi* is harmless. Specifically, we anticipate that genetic elements enabling pathogenesis that are unique to *N. fowleri* will be identified, including novel genes (i.e. ORFs with no known homologs), novel paralogs of known gene families, and genes obtained via horizontal gene transfer (HGT). An exploratory genomic approach may identify examples of these, even in the absence of a full genome analysis.

Here, we present the use of unbiased next-generation deep sequencing to sequence the 49,530-base pair (bp) mitochondrial genome of *N. fowleri* to an average of 2,732X coverage. The recently published mitochondrial genome of *N. gruberi* (Fritz-Laylin et al. 2010) permitted a detailed

comparative analysis of the mitochondrial genomes of *N. fowleri* and *N. gruberi*. In addition to the entire mitochondrial genome, we sequenced a 60,871-bp segment of the *N. fowleri* nuclear genome to an average of 501X coverage, and performed parallel analyses to those performed for the mitochondrial genome. These studies reveal genes uniquely found in *N. fowleri*, a cathepsin B homolog and a likely case of HGT, all examples of encoded factors that may play potential roles in *N. fowleri* pathogenesis.

MATERIALS AND METHODS

Genomic DNA sequencing library preparation

Naegleria fowleri (CDC:V212) was obtained from an existing collection at CDC. It was isolated from the cerebrospinal fluid (CSF) of a PAM patient from Alabama in 1990, for the purpose of diagnosis. All specimens received at CDC for diagnostic purposes are anonymized. Additionally, specimens from the deceased are exempt from IRB. Approximately 0.2 ml of the CSF was inoculated into monolayers of monkey kidney (E6) cell culture. Destruction of the cell culture from invasion of the monolayers by amoebae occurred within 3 days. Thereafter, amoebae were passaged ~12 times in E6 cell culture to maintain virulence and then established in modified Nelson's medium with 5% FBS and stored frozen in liquid nitrogen (John and John 1994). Prior to harvesting for DNA extraction, frozen amoebae were thawed and grown to a density of 1×10^{12} organisms/ml. DNA was extracted with 500:1 of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) (Invitrogen Inc., Carlsbad, CA) and purified with the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA) (Zhou et al. 2003). All manipulations of the organism and material extraction performed at the CDC was performed in Biosafety level 2 facilities as specified by CDC Biosafety guidelines, or in Biosafety level 2 facilities that have been specifically certified by the UCSF Biosafety Committee for research laboratories (BUA49187-BU-03-INC) for handling *Naegleria fowleri* and extracts derived from the organism.

Thirty micrograms of extracted DNA was sent to Macrogen (Seoul, Korea) for 454 GS FLX sequencing (25% 8-kb paired-end, 25% 1-kb paired-end, and 50% random shotgun sequencing), while 10 µg of extracted DNA was prepared for 100 bp paired-end Illumina HiSeq shotgun sequencing with the Illumina Paired-End Genomic DNA sample preparation kit, using nebulization for fragmentation. Genomic assembly was performed using Geneious Assembler (Geneious v. 5.5, Biomatters, Auckland, New Zealand) using Low Sensitivity/Fast options with no fine tuning (Drummond et al. 2010).

Mitochondrial ORF prediction and annotation

The *N. fowleri* mitochondrial genomic sequence was used as a BLASTx (Altschul et al. 1990) query to search all annotated *N. gruberi* mitochondrial proteins at the National Center for Biotechnology Information website (NCBI, <http://www.ncbi.nlm.nih.gov/>). The BLOSUM62 substitu-

tion matrix was used as the default scoring matrix in all BLAST searches. The maximum percent identity of the top hit was used in conjunction with the E-value for ORF prediction and annotation. Sequences retrieved with an acceptable E-value of $< 5 \times 10^{-2}$ were considered possible homologs in all BLAST searches.

Nuclear segment ORF prediction and annotation

Open reading frames (ORFs) were predicted using NCBI ORF Finder software (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and EMBOSS getorf software (<http://emboss.bioinformatics.nl/>), in combination with BLAST searching into the *N. gruberi* NCBI and/or Joint Genome Institute (JGI, <http://www.jgi.doe.gov/>) databases, and the NCBI nonredundant (nr) database. The minimum threshold size cut-off for an ORF was 300 bp. ORFs were annotated based on their retrieved candidate orthologs via BLAST searching of the nr database, at an E-value of $< 5 \times 10^{-2}$. Those that failed to retrieve any sequence with an acceptable E-value were designated as "putative ORFs". Candidate *N. fowleri* ORFs were retrieved from genomic sequence using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI), with efforts made to obtain sequences with start and stop codons, as well as being of a similar size to the *N. gruberi* homolog or the best top BLAST result.

Nuclear segment gene organization

The *N. fowleri* queries and the retrieved *N. gruberi* orthologs were used as tBLASTn (Altschul et al. 1990) queries to search the *N. gruberi* whole genome shotgun database (NCBI). The first criterion for an *N. gruberi* scaffold to be considered to contain an *N. fowleri* ortholog was that both the *N. fowleri* ORF and *N. gruberi* genes retrieve the same *N. gruberi* scaffold with an acceptable E-value. The second criterion was that when the *N. gruberi* protein database downloaded from NCBI was queried against the available *N. fowleri* nuclear genome sequence using tBLASTn, the *N. gruberi* gene considered orthologous must retrieve the *N. fowleri* ORF with an E-value at least ten orders of magnitude smaller (i.e. more significant) than the E-value corresponding to any other query sequence.

Horizontal gene transfer

To investigate possible cases of horizontal gene transfer (HGT), i.e. transfer of genetic material between distantly related organisms, the *N. fowleri* proteins were used as BLASTp (Altschul et al. 1990) queries to search genomes representing the least divergent and most complete available genome sequences from the major lineages of eukaryotes. Specifically, the following databases were queried: *Homo sapiens*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Dictyostelium discoideum*, *Entamoeba histolytica*, and *Tetrahymena thermophila*, all hosted by NCBI. For specific reasons having to do with taxonomic sampling points, the nr database was also searched for homologs in *Coprinopsis cinerea*, *Polysphondylium pallidum*, and *Mastigamoeba*

balamuthi. The *Cyanidioschyzon merolae* genome was searched (<http://merolae.biol.s.u-tokyo.ac.jp/>) as well as the *Ostreococcus tauri*, *Thalassiosira pseudonana*, and *Emiliana huxleyi* genomes hosted by JGI. The Eukaryotic Pathogen Database Resources (EuPathDB, <http://eupathdb.org/eupathdb/>) were used to search the following organisms: *Leishmania major*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Giardia intestinalis*, *Trichomonas vaginalis*, and *Toxoplasma gondii*. Additionally, the *Acanthamoeba castellanii* genome was searched using tBLASTn. The *A. castellanii* genome is jointly hosted by the Human Genome Sequencing Center and Baylor College of Medicine (<https://www.hgsc.bcm.edu/content/acanthamoeba-castellani-neff>). Again, the homology criterion was that the sequence must have an E-value of $< 5 \times 10^{-2}$. In the case of *A. castellanii*, from which only contigs ("contiguous sequences") were available for searching, genes were manually annotated using Sequencher 4.10.1.

Cathepsin identification

The genomes of *T. brucei*, *T. cruzi*, *Crithidia fasciculata* (<http://www.sanger.ac.uk>), *L. major*, and *N. gruberi*, were searched for cathepsin sequences using BLASTp, with an E-value cutoff of $< 5 \times 10^{-2}$. These were added to a previously established dataset modified from (Dacks et al. 2008b).

Domain identification

Open reading frames domains in the *N. fowleri* nuclear segment were identified using the Conserved Domain Database (Marchler-Bauer et al. 2010) at NCBI (at an E-value of $< 5 \times 10^{-2}$).

RNA prediction

tRNAscan-SE 1.21 (Schattner et al. 2005) was used to identify tRNAs, RNAweasel (<http://megasun.bch.umontreal.ca/RNAweasel/>) was used to predict group I and II type introns, tRNAs, and small subunit rRNA, and RNAmmer (Lagesen et al. 2007) was used to predict the large rRNA subunit.

Sequence mapping

The mitochondrial and linear DNA maps of *N. gruberi* and *N. fowleri* were created using the program XPlasMap 0.96 (<http://www.iayork.com/XPlasMap/>).

Signal sequence identification

The program SignalP (Dyrlov Bendtsen et al. 2004) (<http://www.cbs.dtu.dk/services/SignalP/>) was used to predict possible signal peptides and signal anchors.

Alignments and phylogenetics

Phylogenetic trees were run for the protein homologs of the *N. fowleri* putative cathepsin B gene (Con-

tig9_16_26041_25267). The dataset of cathepsin B and L homologs had 100 sequences and 156 positions. Sequences were aligned and trimmed to contain only unambiguously homologous positions. Alignment was performed using the sequence alignment program MUSCLE 3.5 (Edgar 2004), and masking and trimming were done by eye using MacClade 4.08 (Maddison and Maddison 1989). The alignment is available upon request. ProtTest 1.3 (Abascal et al. 2005) was used to determine that the WAG+G model of protein evolution best fit the data allowing for incorporating correction for a four-category gamma correction for rate variation when appropriate.

MR BAYES v. 3.2.1 (Ronquist and Huelsenbeck 2003) was used to search treespace using 1,000,000 generations. Consensus trees were generated using a burn-in value of 25%. This was validated by plotting likelihood vs. generations to ensure that no trees were included prior to the likelihood plateau. Two independent runs, each of four chains, were performed, with convergence of the results confirmed by ensuring a splits frequency of < 0.1. PhyML v. 2.4.4 (Guindon and Gascuel 2003) and RAxML-VI-HP v. 2.2.3 (Stamatakis 2006) were used for maximum-likelihood analyses, and to generate ML-bootstrap values based on 100 pseudo-replicates of each dataset. The tree diagram shown in phylogenetic figures is the best Bayesian topology, with support values listed in the order of Bayesian posterior probability values/PhyML bootstrap values/RAxML bootstrap values.

PCR confirmation of the *N. fowleri* 60-kb segment from the nuclear genome

For confirmation of organization of the initial contig assembly corresponding to the ~60-kb segment from the *N. fowleri* nuclear genome, PCR was performed using standard techniques. Briefly, 10 ng of genomic DNA was used as template in a 25 μ l PCR reaction containing 1X NEB Standard Taq buffer, 250 μ M dNTPs, 10 pmol of forward and reverse primer, and 5 units of purified recombinant Taq polymerase. Forward and reverse primer sequences are shown in Table S1. PCR conditions were 35 cycles of 95 °C denaturation for 30s, 50 °C annealing for 30s, and a 72 °C extension for 90s. 10 μ l of amplified material was run out on a 1.5% agarose gel stained with ethidium bromide. Amplified bands of the expected size were visualized under ultraviolet light.

RESULTS

To better understand the genetic makeup of *Naegleria fowleri* and gain insight into pathogenic mechanisms, we analyzed extracted amoebic DNA by unbiased deep sequencing using both 454 GS FLX and Illumina HiSeq technology. The goal of 454 pyrosequencing was to provide paired-end scaffolds to facilitate de novo assembly from short 100-bp Illumina reads. This enabled us to assemble the mitochondrial genome and a 60-kb segment from the nuclear genome of *N. fowleri*. For assembly of the mitochondrial genome of *N. fowleri*, ini-

tial contigs assembled via Geneious Assembler with greater than 100 \times coverage and > 75% nucleotide identity were aligned to the mitochondrial genome of *N. gruberi*. These initial contigs were then used to assemble the entire mitochondrial genome from 454 data using the Geneious assembler. In total, 393,244 reads assembled into a circular consensus mitochondrial genome of 49,519 nucleotides with an average coverage of 2,732 \times (range of 766–5,317 \times). The sequence of the mitochondrial genome of *N. fowleri* has been deposited into GenBank (accession number JX174181). De novo assembly of additional reads generated a 60-kb segment with an average coverage of 501X (range of 75–8,772 \times), which we chose for annotation and analysis. This sequence has also been deposited in GenBank (accession number JX827422).

To confirm the organization of the 60-kb assembly reported here, and to rule out the presence of gross indels or translocations, PCR of 11 regions spanning the nuclear genome, each ~1000 bp in length, was performed. Bands of expected size were seen for all 11 PCR amplicons (Fig. 1–2).

Statistics

The mitochondrial genome of *N. fowleri* is 49,519 bp and is AT-rich, having a GC content of only 25.2% (Table 1). Coding sequence comprises 90% of the genome, and no introns are present in the noncoding regions. The *N. gruberi* mitochondrial genome is slightly larger at 49,842 bp, with a GC content of 22% and coding content of 92%, and also does not contain introns (Table 1). The median exon length for *N. fowleri* is similar to that of *N. gruberi*, being shorter by only 32 bp. The *N. fowleri* mitochondrial DNA contains 70 genes, 46 of which encode proteins and 23 of which encode transfer and ribosomal RNAs.

The *N. fowleri* nuclear genome segment is 60,871 bp in length, with a GC content of 36.8%, and a coding content of 57.3% (Table 1). ORF prediction analysis identified 31 putative ORFs in the segment. Table 1 shows that there are 1.74 exons per gene with a median exon length of 432 bp. Thirty-five percent of the ORFs have introns, with 0.7 introns per gene and a median length of 87 bp. These statistics are largely similar to those for the entire *N. gruberi* genome (Table 1).

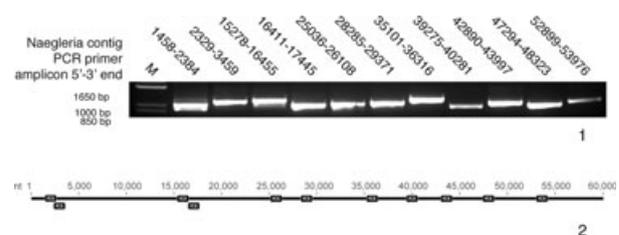


Fig. 1–2. PCR confirmation of assembly. **1.** Eleven ~1000 bp amplicons from across the 60-kb segment in the nuclear genome were successfully recovered by PCR amplification. M, ladder. **2.** Diagram of where the amplicons were located across the contig.

Table 1. Mitochondrial genome and nuclear segment statistics from *Naegleria gruberi* and *Naegleria fowleri*

Species	Size (bp)	%GC	%Coding	%Genes with introns	Introns per gene	Median intron length	Exons per gene	Median exon length
<i>Ng</i> mitochondria	49,842	22	92	0	0	N/A [†]	1	795 bp
<i>Nf</i> mitochondria	49,519	25.2	90	0	0	N/A [†]	1	766.5 bp
<i>Ng</i> nuclear genome*	~41M	33	57.8	36	0.7	60 bp	ND [‡]	ND [‡]
<i>Nf</i> nuclear segment	60,871	36.8	57.3	35	0.7	87 bp	1.74	432 bp

**Naegleria gruberi* nuclear genome data are from Fritz-Laylin et al. 2010.

[†]Not applicable.

[‡]Not determined.

Gene complement and organization

The mitochondrial gene complement of *N. fowleri* encodes products involved in reductive and oxidative phosphorylation, protein import and maturation, ribosomal proteins, rRNAs, tRNAs, and four ORFs of unknown function (Table S2). The majority of the genes are ribosomal proteins, tRNAs, or are involved in reductive and oxidative phosphorylation. In comparison with the mitochondria of other eukaryotes, as in Fig. 3, the proportions of genes in each category are standard among the diversity observed in other species.

Genes encoded by the *N. fowleri* mitochondrial genome are tightly packed; only 10% of the genome is non-coding sequence (Fig. 4). ORF prediction software failed to identify any protein-encoding regions in the *N. fowleri* genome corresponding to genes not present in *N. gruberi*, and homologs of all annotated *N. gruberi* genes were found in *N. fowleri*. The mitochondrial genome of *N. fowleri* retains the bacteria-like organization represented by a similar gene order of small and large ribosomal proteins to a contiguous alignment of the *str*, *S10*, *spc*, and α operons, seen in *N. gruberi* and a variety of related single-cell organisms.

Unlike the collinearity (exact corresponding gene order) observed between the mitochondrial genomes of *N. fowleri* and *N. gruberi*, the organization of the *N. fowleri* ORFs on the 60-kb nuclear segment is not maintained by the *N. gruberi* orthologs at this arbitrary locus (Fig. 5). Three *N. gruberi* scaffolds have two or more genes from the *N. fowleri* nuclear segment, and four genes from the segment are found separately on four additional *N. gruberi* scaffolds. PCR to amplify the predicted ORFs was used to confirm that this striking result is not due to misassembly of the *N. fowleri* nuclear segment (Fig. 1–2). Indeed, the organizational dissimilarity between the predicted *N. fowleri* ORFs and *N. gruberi* orthologs notably contrasts with the relative similarity in statistics and gene complement between the two organisms.

Thirty-one ORFs were identified in the 60-kb segment from the *N. fowleri* nuclear genome (Table S3; Fig. 5). Of the 31 ORFs, 12 appear to have homologs in multiple eukaryotic genomes, while nine of the 31 ORFs appear to be found only in *N. fowleri* and *N. gruberi*. Interestingly, 10 of the 31 ORFs were not identified as having a homolog in any other eukaryote, and therefore might be specific

to *N. fowleri*. Of the ORFs with homologs in other eukaryotic genomes, seven contain recognizable domains, such as Vps9 and ERV1, as identified by searching the conserved domain database (Table 2). Six ORFs with homologs in other eukaryotic genomes match functionally annotated proteins. There is possible evidence for a retrotransposon in the segment, as two adjacent ORFs have a reverse transcriptase or RNase H domain. One additional ORF of unknown identity was identified as having a signal anchor (Contig9_30_59176_60250), and was thus predicted to be a type II membrane protein.

Cathepsin identification

Using N-terminal peptide sequencing, Kim et al. (2009) identified two proteins secreted from *N. fowleri* that they annotated as cathepsin B and cathepsin B-like, proposing that these cysteine proteases might be involved in the invasion through the blood–brain barrier. Other pathogens, such as the liver fluke *Fasciola hepatica*, a multicellular parasite, have also been observed to secrete cathepsin B (Law et al. 2003). We identified a cathepsin homolog within the *N. fowleri* 60-kb nuclear segment (Contig9_16_26041_25267). Interestingly, this ORF was identified as having a signal peptide, raising the possibility of its transport out of the cell via the secretory pathway. Initial BLASTp searches suggested strong similarity to the human cathepsin B, as opposed to cathepsin L. Our phylogenetic analysis (Fig. 6) shows separation of the cathepsin B and L clades with strong statistical support, and groups the *N. fowleri* cathepsin homolog well within the cathepsin B clade. This analysis also revealed a large expansion of the cathepsin B family in *N. gruberi*, with multiple independent groups of duplications, two of which produce tubulointerstitial nephritis (TIN) antigen-like genes. While clearly a cathepsin B, the putative *N. fowleri* homolog is not closely related to any of the *N. gruberi* sequences.

Horizontal gene transfer

Horizontal gene transfer events that occurred in *N. fowleri*, but not in *N. gruberi*, could contribute to its pathogenic phenotype. In support of this possibility, we identified an ORF encoding a putative 121 amino acid protein (Contig9_23_40599_40961) in the 60-kb segment from the

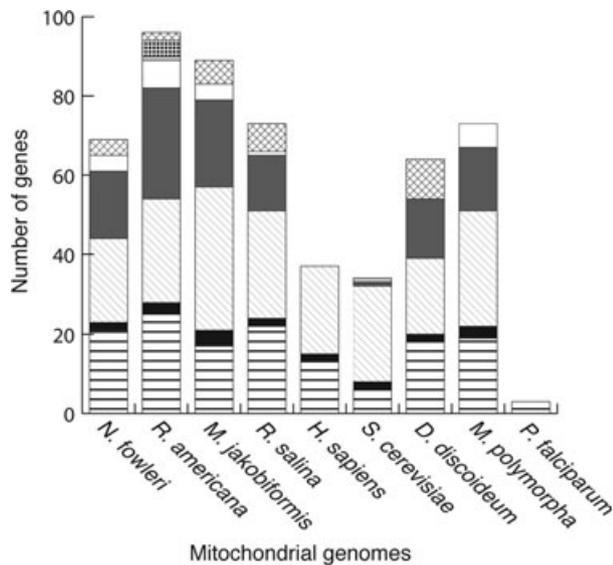


Fig. 3. Gene content of the *Naegleria fowleri* and *Naegleria gruberi* mitochondrial genomes in comparison with other eukaryotes. Genes are included in the classes in the following way, as in (Burger et al. 2003). Reduction and oxidative phosphorylation (horizontal bars): *atp1*, *atp3*, *atp4*, *atp6*, *atp8*, *atp9*; *cob*; *cox1-3*; *nad1-4*, *nad4L*, *nad6-11*, *sdh2-4*. rRNAs (solid black): *rnl*, *rns*, *rrn5*. tRNAs (diagonal dots): *trnA-Y*. Ribosomal proteins and EF-Tu (solid dark gray): *rps1-4*, *rps7*, *rps8*, *rps10-14*, *rps19*; *rpl1*, *rpl5*, *rpl6*, *rpl10*, *rpl11*, *rpl14*, *rpl16*, *rpl18-20*, *rpl27*, *rpl31*, *rpl32*, *rpl34*, *rpl36*; *tufA*. Protein import and maturation (solid white): *secY*, *ymf16*, *tatC*, *yejR* (*ccmF*), *yejU* (*ccmC*), *yejV* (*ccmB*), *yejW* (*ccmA*); *cox11*. RNA maturation (solid light gray): *rnpB*. Transcription (black squares): *rpoA-D*. Other (hatched): ORFs of unknown function. Data for all organisms except *N. fowleri* was retrieved from NCBI. In *Dictyostelium discoideum*, *cox1* and *cox2* are encoded as a single ORF, but are counted as two genes here. *N. fowleri*, *Naegleria fowleri*; *R. americana*, *Reclinomonas americana*; *M. jakobiformis*, *Malawimonas jakobiformis*; *R. salina*, *Rhodomonas salina*; *H. sapiens*, *Homo sapiens*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *D. discoideum*, *Dictyostelium discoideum*; *M. polymorpha*, *Marchantia polymorpha*; *P. falciparum*, *Plasmodium falciparum*.

nuclear genome that may have been involved in an HGT event. This sequence did not have a robustly identified *N. gruberi* homolog. However, upon expanding the search to a diversity of eukaryotes (via the nonredundant, plus organism-specific genomic databases, see Materials and Methods), we did retrieve a corresponding contig of 9,392 bp (high scoring segment pair of 384 amino acids) from the distantly related, amoebozoan, organism *A. castellanii* (E-value of 4×10^{-5}) and one of 311 amino acids from the amoebozoan slime mold *P. pallidum* (E-value of 3×10^{-5}).

DISCUSSION

We have obtained the full mitochondrial genome of *N. fowleri* and of a 60-kb segment of the *N. fowleri* nuclear genome, nearly doubling the number of *N. fowleri* protein entries in GenBank as of November 2012. In addition to

providing targets for future molecular parasitological work, these data have provided new insights into the organization and evolution of the *Naegleria* mitochondrial genome, and a glimpse into the genomic structure of *N. fowleri*.

The *N. fowleri* and *N. gruberi* mitochondrial genomes both reconstitute as circular and encode many of the protein-coding genes, rRNAs, and tRNAs required for mitochondrial function. While the mitochondrial genomic content is variable in eukaryotes (Burger et al. 2003), both *N. fowleri* and *N. gruberi* appear to have “classical” collinear sets of mitochondrial genes that are shared among bacteria and many single-celled eukaryotes. Conspicuous in their absence are the genes for RNA maturation. RNA maturation involves the *rnpB* gene, encoding the ribozyme RNase P, which has been found in the mitochondrial genomes of *Reclinomonas americana* and *S. cerevisiae*. It is not known what performs this function in *N. fowleri*, as an *rnpB* homolog has not yet been identified in either *N. fowleri* or *N. gruberi*, and eukaryotes related to *Naegleria* lack RNaseP (Burger et al. 2003), with the exception of *T. brucei* (Salavati et al. 2001).

With the abandonment of the bikont-unikont rooting for the tree of eukaryotes (Roger and Simpson 2009), several features of mitochondrial genomes have been brought into the spotlight. The Heterolobosea (of which *Naegleria* is a member) are related to two other lineages, most closely to the Euglenozoa and then to the jakobids. The latter prominently have the most bacteria-like mitochondrial genome organization described, both in possession of bacterial RNA polymerase subunits and in operon structure (Lang et al. 1997). No RNA polymerases are encoded in the mitochondrial genomes of *Naegleria*. Instead, the nuclear genome of *N. gruberi* encodes a bacteriophage T3/T7-like polymerase (Cermakian et al. 1996), and it is likely that this is the case in *N. fowleri*, as it is in the vast majority of eukaryotes. However, the *N. fowleri* and *N. gruberi* mitochondrial genomes do share a pattern of *rps* gene organization that is bacteria-like, as is found in at least one member of all six eukaryotic supergroups, which suggests the retention of a plesiomorphic state in these mitochondrial genomes rather than an indication of deeply branching status for any of the organisms in question (Hauth et al. 2005). Interestingly, no euglenozoans to date are identified as having mitochondrial genomes with bacteria-like organization. Trypanosomatids have maxicircles and minicircles (Westenberger et al. 2006), while the euglenid *Diplonema papillatum* has a small circular chromosome (Marande et al. 2005).

In general, there is great diversity in observed levels of mitochondrial genome synteny between species (Burger et al. 2003), even between closely related organisms. Within the genus *Candida*, for instance, there is extensive mitochondrial gene rearrangement between some members, although other members share a conserved gene order (Valach et al. 2011). Thus, it is notable that the *N. fowleri* and *N. gruberi* mitochondrial genomes have remarkably similar statistics, gene content, and gene order organization. This is in stark contrast with the gene organization of the 60-kb nuclear segment, which is marked by

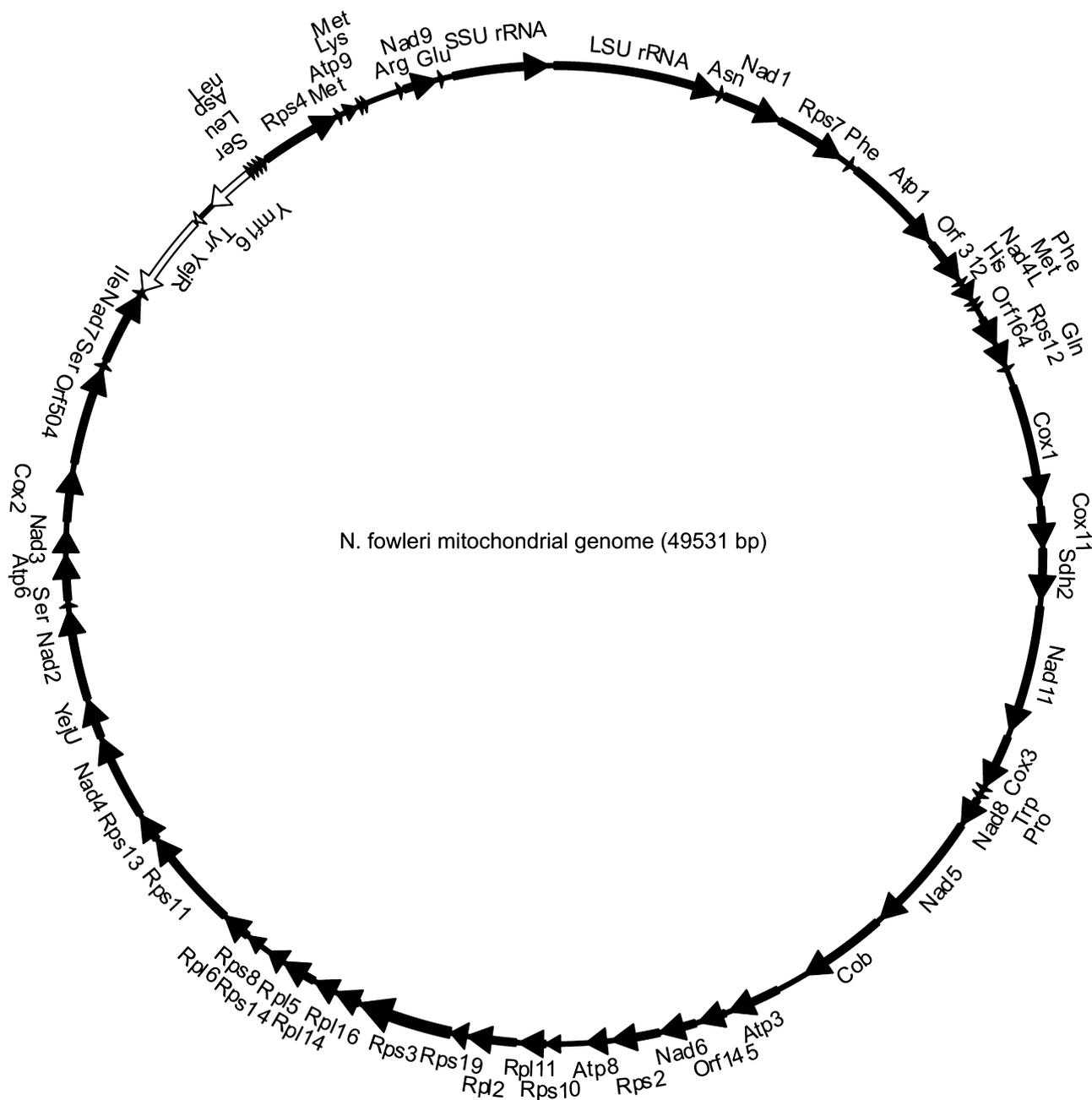


Figure 4. Circular map of the *Naegleria fowleri* mitochondrial genome. Genes encoding proteins are annotated based on BLAST results, and genes encoding RNA are annotated based on tRNA and rRNA scanning software predictions (see Materials and Methods section). For the full name of each gene, see Table S2. Black arrows with gene names on the outside of the map represent genes on one strand, and white arrows with gene names on the inside of the map represent genes on the alternate strand. The *N. fowleri* mitochondrial genome is identical in gene content and organization to the mitochondrial genome of *Naegleria gruberi*.

a conspicuous lack of collinearity between *N. fowleri* and *N. gruberi*, as confirmed by our PCR experimental testing of our *in silico* *N. fowleri* assembly. Although work with the phylum Apicomplexa has shown extensive genomic rearrangement between species, with little conserved collinearity and synteny between major lineages (DeBarry and Kissinger 2011), the most closely related comparison points to the Heterolobosea, the trypanosomatids, retain

highly conserved gene order (Ghedini et al. 2004). While there are several possible genetic mechanisms that lead to genomic re-arrangements, such as transposable elements, or chromosomal breakage, recombination events during meiosis prominently produce this effect. Thus, the lack of observed synteny between the *Naegleria* species may be due to sexual recombination events occurring after the evolutionary split from their last common *Naegleria*

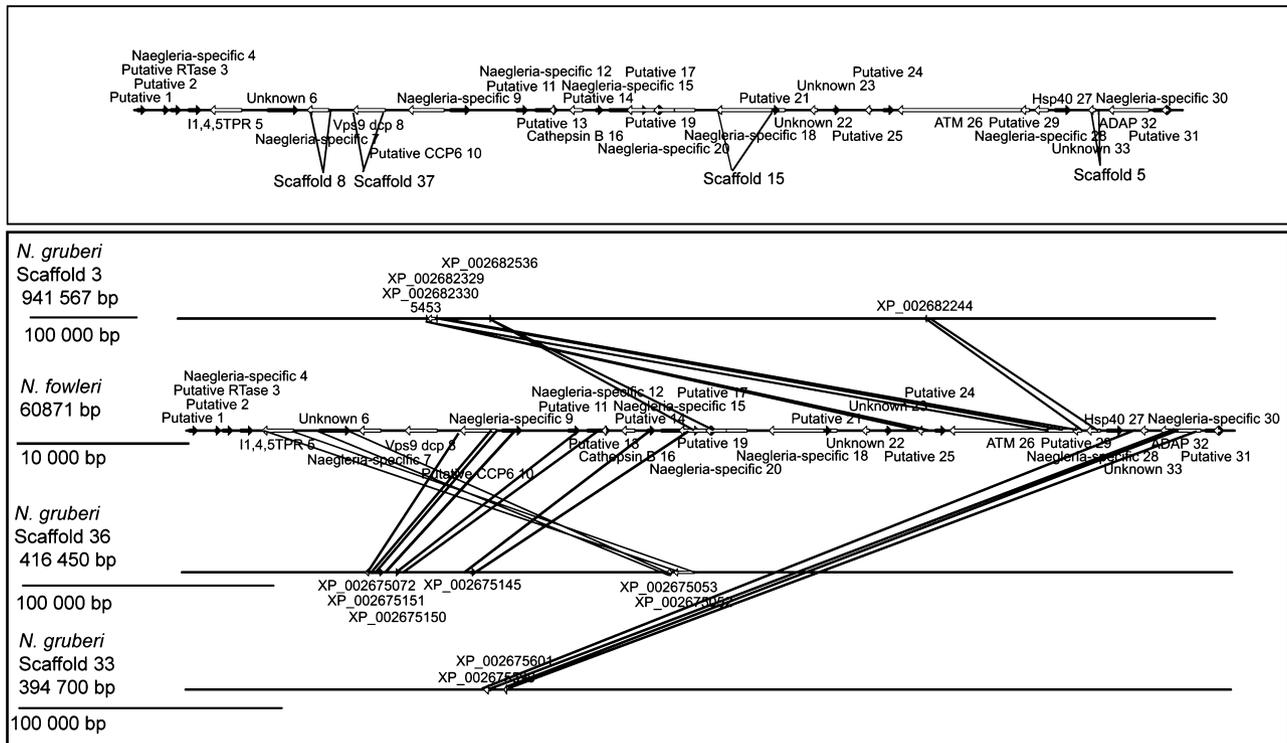


Fig. 5. Comparative collinearity analysis of the predicted *Naegleria fowleri* ORFs on the 60-kb nuclear segment and their homologs in the *Naegleria gruberi* nuclear genome. Black right arrows indicate ORFs on one strand, while white left arrows indicate ORFs on the other strand. (Top) Genes on the *N. fowleri* segment and the organization of homologs on individual scaffolds. (Bottom) The *N. fowleri* segment is in the middle, with the arrangements of homologous genes on *N. gruberi* scaffolds containing two or more genes above and below. Annotations of *N. fowleri* genes are based on BLAST results (see Materials and Methods), while those for *N. gruberi* are the GenBank protein accession for the homolog of each *N. fowleri* ORF. Scale bars are given for each map at 100,000 bp, with the exception of the *N. fowleri* segment, for which the scale bar indicates 10,000 bp. There is a distinct lack of collinearity between the two *Naegleria* species at this region of the *N. fowleri* genome.

ancestor. *N. gruberi* has maintained apparently functional copies of genes required for meiosis (Fritz-Laylin et al. 2011). Additionally, there is strong evidence for a sexual cycle in *N. lovaniensis* (Pernin et al. 1992). Based on these data, a sexual cycle is likely to be operating in *Naegleria* species, and acting as a source of genetic diversity. If the observed lack of synteny between *N. fowleri* and *N. gruberi* is indeed due to meiotic recombination events, this finding would have important implications for the development of drug resistance in *N. fowleri*.

GC content is widely variable in eukaryotic nuclear genomes. The *N. fowleri* 60-kb segment and *N. gruberi* genome are both GC-poor, at 36.8% and 33%, respectively. It is possible that the arbitrary *N. fowleri* segment that was sequenced might be part of an isochores, a long stretch of DNA homogenous in GC content, as related organisms *T. brucei* and *T. equiperdum* have isochores-like organization (Isacchi et al. 1993). On the other hand, assuming that the observed GC content in the 60-kb segment is representative of the overall *N. fowleri* genome, the slightly elevated GC content relative to *N. gruberi* might reflect an adaptation to its thermotolerant lifestyle. *N. fowleri* can grow at 45 °C (Visvesvara et al. 2007), while *N. gruberi* can only tolerate temperatures up to 37 °C (Griffin 1972). Indeed,

the red alga *Cyanidioschyzon merolae* lives in acidic hot springs at temperatures of 45 °C and has a GC content of 55% (Matsuzaki et al. 2004).

Our analysis of the 60-kb segment of the nuclear genome has furthermore identified examples of several of the anticipated sources of novel factors enabling pathogenesis in *N. fowleri*. Nine of 31 potential genes in the *N. fowleri* 60-kb segment were specific to *Naegleria* species, although the majority of ORFs had well-characterized and likely essential homologs in yeast or mammalian cells. Twenty of the 31 genes in the *N. fowleri* segment have a homolog in *N. gruberi*. Previously, the level of divergence between *N. fowleri* and *N. gruberi* has been estimated to be comparable to that between humans and frogs based on analyses using 18S ribosomal RNA (Baverstock et al. 1989). Our analyses of the mitochondrial genomes and nuclear genome segments of these two amoebae raise questions not only about the degree of their relatedness, but about how relatedness is best measured. While the lack of collinearity certainly highlights the unexpected diversity in genomes of *Naegleria* species, the identification of 20 of 31 homologs is evidence that many, if not the majority, of genes may be shared in common between *N. fowleri* and *N. gruberi*.

Table 2. *Naegleria fowleri* nuclear segment genes retrieving characterized homologs, or with domains in the Conserved Domain Database.

<i>Naegleria fowleri</i> gene	Annotation*	Notes†
Contig9_3_2113_2703	Putative reverse transcriptase	Reverse transcriptase domains
Contig9_5_6233_4411	Inositol 1, 4, 5-triphosphate receptor	Calcium channel glycoprotein activated by inositol triphosphate
Contig9_10_17987_15908	Possible cytosolic carboxypeptidase 6	Possible metallo-carboxypeptidase involved in deglutamylation
Contig9_16_26041_25267	Cathepsin B	Cysteine protease
Contig9_24_43494_44097	Erv1/Alr domain containing protein	Erv1/Alr family proteins involved in iron-sulfur cluster biogenesis
Contig9_26_51515_44333	ATM (ataxia telangiectasia mutated)	Cell cycle checkpoint protein, with serine/threonine kinase domains
Contig9_27_53454_54366	Hsp40	Heat shock protein, with DNAJ and RRM domains
Contig9_32_58903_56581	ADAP	ArfGAP with dual pH domains
Contig9_4_3139_3870	<i>Naegleria</i> -specific	Contains RNase H domain
Contig9_18_37776_33830	<i>Naegleria</i> -specific	Contains serine/threonine kinase domains
Contig9_6_7742_9509	Unknown	Contains leucine-rich repeats
Contig9_8_12703_14563	Unknown	Contains the Vps9 domain, found in vacuolar protein sorting protein 9
Contig9_33_55908_55399	Unknown	Contains PUB domain

*Annotations were assigned based on the annotation of homologous genes.

†Notes contain additional information about the gene.

Ten ORFs predicted in the 60-kb nuclear segment were not supported by BLAST searching in either the *N. gruberi* or the NCBI nonredundant database. Some of these ORFs may be mis-predicted, but others are likely specific to *N. fowleri* and may constitute a source of genetic novelty. Distinguishing real *N. fowleri*-specific ORFs is clearly crucial to identifying possible determinants of the pathogenic phenotype, as we expect that pathogenicity is a gain-of-function for *N. fowleri* (given that most *Naegleria* species that have been isolated are nonpathogenic). Future molecular biology work on these 10 ORFs and experimental validation on a genome-wide scale is ongoing and will be extremely important.

Another source of genetic novelty, and an example of a potential gain-of-function event through gene duplication, is the cathepsin B homolog (Contig9_16_26041_25267). Cathepsin B is highly expressed in both free-living (Villalobo et al. 2003) and parasitic protists (DuBois et al. 2006) post-excystation. It has been implicated in pathogenicity in several diverse and prominent microbial eukaryotic parasites (Dou et al. 2011; Somanna et al. 2002; Caffrey and Steverding 2009; Kisson-Singh et al. 2011 inter alia), including in *N. fowleri* itself (Kim et al. 2009). The cathepsin B homolog identified here is also predicted to have a signal peptide, and therefore has the potential to be secreted.

In the course of classifying Contig9_16_26041_25267, an expansion of the cathepsin B family in *N. gruberi* was uncovered with 24 cathepsin B members as compared with only four cathepsin L paralogs (Fig. 6). However, the kinetoplastids *T. brucei* and *L. major* have expanded their cathepsin L families to contain 11 and nine cathepsin L sequences respectively, while retaining a single cathepsin B homolog. Phylogenetic analysis showed that these expansions occurred independently in kinetoplastids and heteroloboseans (Fig. 6).

The third possible source of genetic novelty identified in *N. fowleri* is by HGT. The extent and scope of HGT and the role that this has played in adaptation to ecological niches in eukaryotes are controversial. However, exam-

ples of transfer across large evolutionary distances have been identified and some have implicated in pathogenesis (Fast et al. 2003; de Koning et al. 2000; Richards et al. 2006; Slot and Rokas 2011). Here, we have identified one likely case of inter-supergroup HGT in the *N. fowleri* nuclear segment, involving *N. fowleri* and the amoebozoans *A. castellanii* and *P. pallidum*, but not *N. gruberi*. We feel that HGT is the most likely explanation for the observed distribution of this gene, requiring only three evolutionary events, rather than the very high number of independent loss events required in a scenario of evolutionary conservation from a common ancestor. If this is truly HGT, it may be a significant case, as two of these three amoebae are potentially pathogenic to humans. *P. pallidum* is a nonpathogenic cellular slime mold that feeds on bacteria (Githens and Karnovsky 1973), as do *N. fowleri* and *A. castellanii* (Visvesvara et al. 2007) in their free-living stage. Given the common factor of predation of bacteria, it may be that the HGT gene product is involved in some shared response, possibly to phagocytosis of bacteria or an intracellular antibacterial response. However, it may also potentially be involved in pathogenesis, as both *N. fowleri* and *A. castellanii* cause amoebic encephalitis, albeit very different forms. The finding of an HGT event in an arbitrary segment comprising < 0.15% of the predicted nuclear genome suggests that other HGT events including the one described here may be common and essential macroevolutionary changes to the lifestyle and thus pathogenicity of *N. fowleri*. This possibility, however, awaits experimental investigation as the homologous ORFs in *A. castellanii* and *P. pallidum* have not been characterized and have no identifiable domains.

Overall, this snapshot of the *N. fowleri* genomic picture has revealed at the same time a highly conserved mitochondrial genome within *Naegleria* species and widely dissimilar nuclear genomes, with novel genes and candidates for elucidating pathogenic factors. As the field moves into an era of comparative high-throughput genomics, there is great promise that the data generated will yield new insights into the fight against this deadly human pathogen.

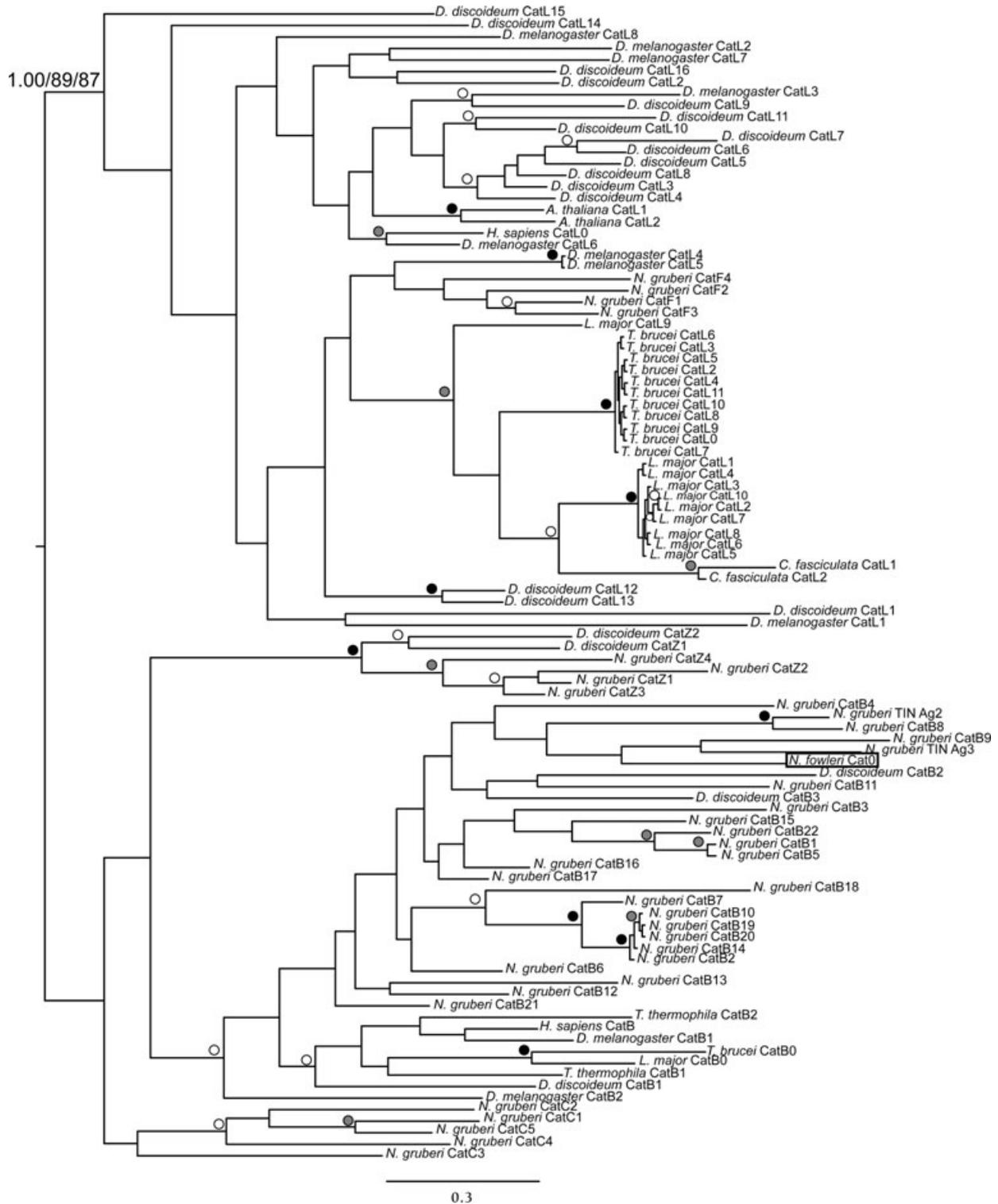


Fig. 6. Phylogeny of cathepsin family homologs. This phylogeny is a composite of MrBayes, PhyML, and RAxML results shown on the best Bayesian topology, arbitrarily rooted between the cathepsin B and L clades. The relevant nodes are marked using dots that represent Bayesian posterior probability/PhyML bootstrap/RAxML bootstrap values better than the following thresholds: 1.00/95/95 (black), 0.95/75/75 (gray), and 0.80/50/50 (white). The *N. fowleri* sequence is boxed. It is found within the cathepsin B clade, supporting its identity as part of the cathepsin B family.

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Forward and reverse primers used in PCR amplification of 11 regions of the 60-kb segment.

Table S2. *Naegleria fowleri* mitochondrial genome annotation based on BLAST searching and RNA scanning software.

Table S3. Annotation of *N. fowleri* 60-kb segment based on BLAST results.