

1 **Clinical metagenomic identification of *Balamuthia mandrillaris* encephalitis**
2 **and assembly of the draft genome: the critical need for reference strain**
3 **sequencing**

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36 **ABSTRACT**

37 Primary amoebic meningoencephalitis (PAM) is a rare, often lethal cause of
38 encephalitis, for which early diagnosis and prompt initiation of combination
39 antimicrobials may improve clinical outcomes. In this study, we present the first
40 draft assembly of the *Balamuthia mandrillaris* genome recovered from a rare
41 survivor of PAM, in total comprising 49 Mb of sequence. Comparative analysis of
42 the mitochondrial genome and high-copy number genes from 6 additional
43 *Balamuthia mandrillaris* strains demonstrated remarkable sequence variation,
44 with the closest homologs corresponding to other amoebae, hydroids, algae,
45 slime molds, and peat moss,. We also describe the use of unbiased
46 metagenomic next-generation sequencing (NGS) and SURPI bioinformatics
47 analysis to diagnose an ultimately fatal case of *Balamuthia mandrillaris*
48 encephalitis in a 15-year old girl. Real-time NGS testing of a hospital day 6 CSF
49 sample detected *Balamuthia* on the basis of high-quality hits to 16S and 18S
50 ribosomal RNA sequences present in the National Center for Biotechnology
51 Information (NCBI) nt reference database. Retrospective analysis of a day 1 CSF
52 sample revealed that more timely identification of *Balamuthia* by metagenomic
53 NGS, potentially resulting in a better outcome, would have required availability of
54 the complete genome sequence. These results underscore the diverse
55 evolutionary origins underpinning this eukaryotic pathogen, and the critical
56 importance of whole-genome reference sequences for microbial detection by
57 NGS.

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59

60 **BACKGROUND**

61 *Balamuthia mandrillaris* is a free-living amoeba that is a rare, almost
62 uniformly lethal, cause of primary amoebic encephalitis (PAM) in humans
63 (Visvesvara 1993). Originally isolated from the brain of a baboon at the San
64 Diego Zoo in 1986, *Balamuthia mandrillaris* has since been reported in over 100
65 cases of PAM worldwide (Visvesvara et al. 1990; Schuster et al. 2006; Matin et
66 al. 2008), and amoebae associated with fatal encephalitis in a child have been
67 cultured directly from soil (Schuster et al. 2003). The vast majority of cases are
68 fatal, although there are a few published case reports of patients surviving
69 *Balamuthia* encephalitis after receiving combination antimicrobial therapy and in
70 vitro data supporting the potential efficacy of several antimicrobial agents
71 (Schuster and Visvesvara 1996; Deetz et al. 2003; Schuster et al. 2006; Martinez
72 et al. 2010; Ahmad et al. 2013; Kato et al. 2013). Despite the availability of
73 validated RT-PCR assays for the detection of free-living amoebae (Yagi et al.
74 2005; Qvarnstrom et al. 2006), PAM is not often clinically suspected and the
75 diagnosis is most commonly made around the time of death or post-mortem on
76 brain biopsy (Schuster et al. 2004; Perez and Bush 2007).

77 Our lab has demonstrated the capacity of metagenomic next-generation
78 sequencing (NGS) to provide clinically actionable information in a number of
79 acute infectious diseases, most notably encephalitis (Wilson et al. 2014;
80 Naccache et al. 2015). This approach enables the rapid and simultaneous
81 detection of viruses, bacteria, and eukaryotic parasites in clinical samples

82 (Naccache et al. 2014). Encephalitis is a critical illness with a broad differential,
83 for which unbiased diagnostic tools such as metagenomic NGS can make a
84 significant impact (Schubert and Wilson 2015). However, the utility of diagnostic
85 NGS is highly dependent on the breadth and quality of databases that contain
86 whole-genome sequence information of reference strains needed for alignment
87 (Fricke and Rasko 2014).

88 In this study, we describe the first draft genome sequence of a strain of
89 *Balamuthia mandrillaris* from a rare survivor of PAM and comparative sequence
90 analysis of 6 additional mitochondrial genomes. We also demonstrate the ability
91 of metagenomic NGS to rapidly detect *Balamuthia mandrillaris* from the
92 cerebrospinal fluid (CSF) of a critically ill 15-year old, and highlight the
93 importance of genomic reference sequences by retrospective analysis of a
94 hospital day (HD) 1 sample.

95

96 **RESULTS**

97

98 **First mitochondrial genome of *Balamuthia mandrillaris***

99 We cultured *Balamuthia mandrillaris* strain 2046 in axenic media from a
100 brain biopsy corresponding to a rare survivor of PAM (Vollmer and Glaser,
101 manuscript in review). Sequencing of DNA extracted from the axenic culture
102 generated 3.8 million 75 base pair (bp) mate-pair reads with an average insert
103 size of 2,187 bp. *De novo* assembly yielded a circular mitochondrial genome of
104 41,656 bases that was comprised of 64.8% AT at 2,082X coverage (Fig. 1A).

105 The overall size and AT content of the *Balamuthia mandrillaris* mitochondrial
106 genome was closer to that of *Acanthamoeba castellani* (41,591 bp, 70.6% AT)
107 (Burger et al. 1995) than *Naegleria fowleri* (49,531 bp, 74.8% AT) (Herman et al.
108 2013), although overall average nucleotide identity with *Balamuthia* was found to
109 be low for both amoebae (~68%).

110 The *Balamuthia mandrillaris* 2046 mitochondrial genome contained 2
111 ribosomal RNA (rRNA) genes, 18 transfer RNA (tRNA) genes, and 38 coding
112 sequences, with 5 of those being hypothetical proteins. The organization of the
113 mitochondrial genome retained several syntenic blocks with the *Acanthamoeba*
114 *castellani* genome, including tnad3-9-7-atp6 and rpl11-rps12-rps7-rpl2-rps19-
115 rps3-rpl16-rpl14. However, many other features of the genome were unique,
116 such as the order of the remaining coding blocks, the lack of a combined
117 cox1/cox2 gene, as present in *Acanthamoeba castellani* (Burger et al. 1995), and
118 the lack of intron splicing in 23S rRNA. The *Balamuthia mandrillaris* mitochondrial
119 cox1 gene was interrupted by a LAGLIDADG endonuclease open reading frame
120 (ORF) containing a group I intron, as has been reported for a wide variety of
121 other eukaryotic species (Fukami et al. 2007; Zheng et al. 2012). The putative
122 rps3 gene was encoded within a 1290 bp ORF that, when translated, aligned by
123 hidden Markov model (HMM) analysis to rps3 proteins from *Escherichia coli*
124 (PDB 4TP8/4U26) and *Thermus thermophilus* (PDB 4RB5/4W2F), and only in
125 base positions 1-66 and 583-1290 of the ORF. This finding was consistent with
126 the presence of a putative >500 bp intron in the *Balamuthia mandrillaris* rp3 gene
127 that to date has only been described to in plants (Laroche and Bousquet 1999;

128 Regina et al. 2005). Alternatively, the ORF was also found to encode a putative
129 tRNA (Asn) such that the 5' end of the ORF could represent a large intergenic
130 sequence.

131

132 **Mitochondrial Genome Diversity of *Balamuthia mandrillaris***

133 To investigate the extent of sequence diversity in *Balamuthia mandrillaris*,
134 we sequenced the mitochondrial genomes from 6 additional *Balamuthia* strains
135 available at the California Department of Public Health and the American Tissue
136 Culture Collection (Table 1). The 7 total circular mitochondrial genomes
137 averaged 41,526 bp in size (range 39,996 to 42,217 bp), and shared pairwise
138 nucleotide identities ranging from 82.6% to 99.8%. The phylogeny revealed the
139 presence of at least 3 separate lineages of *Balamuthia mandrillaris*, with all of the
140 strains from California that had been submitted to the California Department of
141 Public Health clustering together in a single clade (Fig. 1B). Consistent with a
142 previous report (Booton et al. 2003), we found that the mitochondrial genome of
143 strain V451 was the most divergent among tested strains, and possessed an
144 additional 1,149 bp ORF downstream of the cox1 gene that did not align
145 significantly to any sequence in the NCBI nt or nr reference database.

146 Putative introns constituted the major source of variation among the
147 mitochondrial genomes (Fig. 1C). Four strains out of 7, including strain 2046
148 from the rare survivor of *Balamuthia* infection, contained a 975 bp LAGLIDADG-
149 containing intron in the cox1 gene, whereas no such intron was present in the
150 remaining 3 strains. Two of the remaining 3 strains, strains V451 and V188,

151 instead had an approximately 790 bp insert in the 23S rRNA gene (Figure 1A,
152 "rnl RNA") that contained a 530 bp or 666 bp LAGLIDADG-containing ORF,
153 respectively, and that coded for a putative endonuclease. The LAGLIGDADG-
154 containing endonucleases in the 2 strains shared 84% amino acid pairwise
155 identity with each other, but ~50% amino acid identity to a corresponding
156 LAGLIGDADG-containing endonuclease in the 23S rRNA gene of
157 *Acanthamoeba castellani*, and <12% amino acid identity to the LADGLIDADG-
158 containing cox1 introns in the four other *Balamuthia* strains. The final remaining
159 strain, ATCC-V039, lacked an intron in either the cox1 or 23S rRNA gene.

160 The ORF containing the rps3 gene, found to contain a possible rps3 intron
161 or intergenic region by analysis of the strain 2046 mitochondrial genome, varied
162 in length among the 7 sequenced mitochondrial genomes from 1,290 bp to 1,425
163 bp. Notably, the length of the putative intron or intergenic region accounted for
164 all of the differences in overall length of the rps3 gene. Confirmatory PCR and
165 sequencing of this locus using conserved outside primers revealed that each
166 strain tested had a unique length and sequence (Fig. 2), raising the possibility of
167 targeting this region for *Balamuthia mandrillaris* strain detection and genotyping.

168

169 **First draft genome of *Balamuthia mandrillaris***

170 Because of the high-copy number of mitochondrial sequences in
171 *Balamuthia*, as noted previously for *Naegleria fowleri* (Herman et al. 2013), we
172 performed an additional NGS run of 14.1 million 250 bp single-end reads, and
173 computationally subtracted reads aligning to the mitochondrial genome.

174 Assembly of the remaining 4.4 million high-quality reads yielded 31,194
175 contiguous sequences (contigs) with an N50 of 3,411 bp. Scaffolding and gap
176 closure using an additional 57.4 million NGS reads and computational removal of
177 exogenous sequence contaminants yielded a final assembly of ~44.3 Mb
178 comprised of 14,699 scaffolds with an N50 of 19,012 bp (Table 2). Direct
179 BLASTn alignment of the scaffolds to the National Center for Biotechnology
180 Information (NCBI) nt database revealed that the most common organism
181 aligning to *Balamuthia mandrillaris* was *Mus musculus* (house mouse)
182 (2,067/14,699 = 14.1% of scaffolds), nearly entirely due to low-complexity
183 sequences, followed by high-significance hits to *Acanthamoeba castellani*
184 (627/14,699 = 4.3% of scaffolds).

185 Analysis of individual genes from the *Balamuthia* mitochondrial genome
186 revealed the presence of significant diversity across all kingdoms of life (Fig. 3).
187 The 18S-28S rRNA locus in the *Balamuthia* mitochondrial genome corresponded
188 to a 12.5 kB contig sequenced at high coverage (>400X over rRNA regions).
189 The previously sequenced 18S gene (2,017 bp) demonstrated 99.5% identity to
190 existing *Balamuthia mandrillaris* 18S rRNA sequences in the NCBI nt database,
191 while the 28S gene (4999 bp) had homology across multiple diverse species,
192 with only 68.5% pairwise identity to its closest phylogenetic relative,
193 *Acanthamoeba castellani* (Fig. 3B). From the nuclear genome, one high-copy
194 contig contained a truncated 5,250 nucleotide ORF exhibiting only 33% amino
195 acid identity to *Rhizopus delemar* (pin mold), and harboring elements consistent
196 with a retrotransposon (Kordis 2005), including an RNase HI from Ty3/Gypsy

197 family retroelements, a reverse transcriptase, a chromodomain, and a
198 retropepsin. Two high-copy, ~1,600 bp ORFs that failed to match any sequence
199 by BLASTx alignment to the NCBI nr protein database were found to align
200 significantly to *Escherichia coli* site-specific recombinase by remote homology
201 HMM analysis.

202

203 **A case of *Balamuthia* encephalitis diagnosed by metagenomic NGS**

204 Concurrent with assembly of the *Balamuthia* genome, metagenomic NGS
205 was performed to investigate a case of meningoencephalitis in a 15-year-old girl
206 with insulin-dependent diabetes mellitus and celiac disease. The patient initially
207 presented to a community emergency room with 7 days of progressive symptoms
208 including right arm weakness, headache, vomiting, ataxia, and confusion. Her
209 diabetes was well controlled with an insulin pump, and she did not take any
210 additional medications. Exposure history was significant for contact with alpacas
211 at a family farm and swimming in a freshwater pond nine months prior. She had
212 no international travel, sick contacts, or insect bites. She was given 10 mg
213 dexamethasone with symptomatic improvement in her headaches, but was
214 subsequently transferred to a tertiary care children's hospital after a computed
215 tomography scan revealed left occipital and frontal hypodensities.

216 On HD 1, peripheral white blood count was 11.6×10^3 cells/ μ L (89%
217 neutrophils, 6% lymphocytes, 4% monocytes), erythrocyte sedimentation rate
218 was 13 mm/hr [normal range 0-20 mm/hr], C-reactive reactive was 3 mg/dL
219 [normal range 0-1 mg/dL], and procalcitonin 0.05 ng/mL [normal range 0-0.5

221 ng/mL]. CSF analysis demonstrated 377 leukocytes/ μ L (2% neutrophils, 53%
222 lymphocytes, 39% monocytes, and 6% eosinophils), glucose of 122 mg/dL
223 [normal range, 40-75 mg/dL], and protein of 59 mg/dL [normal range, 12-60
224 mg/dL]. Viral polymerase chain reaction (PCR) testing for herpes simplex virus
225 (HSV) and bacterial cultures were negative. Magnetic resonance imaging (MRI)
226 scan of the brain on HD 1 showed hemorrhagic lesions with surrounding edema
227 in the superior left frontal lobe and left occipital lobe with a small focus of edema
228 in the right cerebellum (Fig. 4A).

229 Given the patient's autoimmune predisposition and hemorrhagic
230 appearance of the brain lesions, acute hemorrhagic leukoencephalitis was
231 initially suspected and intravenous methylprednisolone (1,000 mg daily) was given
232 HD 2-5. The patient clinically deteriorated with worsening headache, increasing
233 weakness, and altered mental status on HD 5. Repeat MRI on HD 5
234 demonstrated enlargement of the previous hemorrhagic lesions with interval
235 development of multiple rim-enhancing lesions (Fig. 4B). Steroids were
236 discontinued and broad-spectrum antimicrobial therapy with vancomycin,
237 cefotaxime, metronidazole, amphotericin B, voriconazole and acyclovir was
238 initiated. On HD 6, she underwent craniotomy for brain biopsy, revealing partially
239 necrotic white matter, and had an external ventricular drain placed. CSF wet
240 mount and gram stain, bacterial and fungal cultures, PCR testing for HSV and
241 varicella-zoster virus (VZV), and oligoclonal bands were negative. Pathology of
242 the brain biopsy sample showed a hemorrhagic necrotizing process with
243 neutrophils, tissue necrosis, vasculitis and numerous amoebae. Parallel

244 metagenomic NGS testing of CSF and brain biopsy samples confirmed the
245 presence of sequences from *Balamuthia mandrillaris* (see below). She was
246 additionally started on azithromycin, sulfadiazine, pentamidine, and flucytosine on
247 HD 7. On HD 8, she developed intracranial hypertension, cardiac arrest and
248 died. Miltefosine had been requested and was en route from the CDC (Schuster
249 et al. 2006; Martinez et al. 2010; Centers for Disease and Prevention 2013);
250 however this medication did not arrive in time to administer before the patient
251 died. Autopsy was not performed according to the wishes of the family.

252

253 **Identification of *Balamuthia* in CSF and brain biopsy material**

254 Metagenomic NGS and SURPI bioinformatics analysis were used to
255 analyze the patient's HD 6 CSF and brain biopsy for potential pathogens.
256 Analysis of the viral portion of RNA or DNA derived reads revealed only phages
257 or misannotated sequences (Table S1), while most of the bacterial reads
258 mapped to common skin / environmental contaminants such as
259 *Propionibacterium* (12,926 reads) and *Staphylococcaceae* (6,028 reads) in the
260 RNA library. In contrast, 79% (20,145 of 25,631) of non-chordate (lacking a
261 backbone) eukaryotic reads that were taxonomically assigned at a species level
262 from the RNA library were assigned to available 16S and 18S sequences of
263 *Balamuthia mandrillaris* in the NCBI nt reference database (Booton et al. 2003)
264 (Table S1; Fig. 5A). A minority of the non-chordate eukaryotic reads aligned to
265 *Acathamoeba spp.* (145 reads). Reads to *Balamuthia* were also detected in the
266 DNA (13 reads) and brain biopsy RNA libraries (8 reads). The coverage of the

267 16S rRNA gene in the RNA library was sufficiently high to assemble a 1,405 bp
268 full-length contig sharing 99.9% identity with the 2046 strain of *Balamuthia*. In the
269 18S locus, mapped NGS reads from the patient spanned 98.1% of the gene and
270 were 99.1% identical by nucleotide. No NGS hits were detected to the RNaseP
271 gene, the only additional *Balamuthia* gene represented in the NCBI nt reference
272 database as of August 2015.

273 We then sought to determine in retrospect whether earlier detection and
274 diagnosis of *Balamuthia* infection in the case patient by NGS would have been
275 feasible. Metagenomic NGS of a day 1 CSF sample followed by SURPI analysis
276 using the June 2014 NCBI nt reference database generated no sequence hits to
277 *Balamuthia* (Fig. 5B; Table S1). However, repeating the analysis after adding the
278 draft genome sequence of *Balamuthia mandrillaris* to the reference database
279 resulted in the detection of many additional *Balamuthia* reads (Fig. 5B and C)
280 Importantly, 9 species-specific DNA reads were detected from day 1 CSF (Fig.
281 5B, boldface text; Table 3). Although only 2 of 9 putative *Balamuthia* reads had
282 identifiable translated nucleotide homology to any protein in the NCBI nr
283 database, one of those reads was found to share 77% amino acid identity to the
284 glutathione transferase protein from *Acanthamoeba castellani*, and hence most
285 likely represented a *bona fide* hit to *Balamuthia*. These findings also indicated
286 that the detection of *Balamuthia* reads was not due to errors in the draft genome
287 assembly from incorporation of contaminating sequences from other organisms.
288 Thus, detection of *Balamuthia* from the patient's day 1 sample and a more timely
289 diagnosis by metagenomic NGS would presumably not have been made without

290 the availability of the full draft genome as part of the reference database used for
291 alignment.

292

293 **DISCUSSION**

294 In this study, we describe a "virtuous cycle" of clinical sequencing in which
295 the continually increasing breadth of microbial sequences in reference databases
296 improves the sensitivity and accuracy of infectious disease diagnosis, in turn
297 driving the sequencing of additional reference strains. The assembly of the first
298 draft reference genome for *Balamuthia* not only enhances the potential sensitivity
299 of metagenomic NGS for detecting this pathogen, as shown here, but also
300 provides target sequences such as the rps3 intron / intergenic region or high-
301 copy number 28S rRNA gene that can be leveraged for the future development
302 of more sensitive and specific diagnostic assays. Given the lack of proven
303 efficacious treatments for *Balamuthia* encephalitis, it is unclear whether even a
304 much earlier diagnosis at HD 1 would have impacted the fulminant course of our
305 case patient's infection. However, it has been suggested that timely intervention
306 in cases of *Balamuthia* might lead to improved outcome (Bakardjiev et al. 2003).
307 In addition, promising new experimental treatments such as miltefosine (Schuster
308 et al. 2006; Martinez et al. 2010; Centers for Disease and Prevention 2013),
309 administered to the survivor infected by the sequenced 2046 strain (Vollmer and
310 Glaser, submitted), are now available.

311 Unbiased metagenomic NGS is a powerful approach for diagnosis of
312 infectious disease because it does not rely on the use of targeted primers and

313 probes, but rather, detects any and all pathogens on the basis of uniquely
314 identifying sequence information (Chiu 2013). Rapid and accurate bioinformatics
315 algorithms (Zaharia et al. 2011; Wood and Salzberg 2014; Buchfink et al. 2015;
316 Freitas et al. 2015) and computational pipelines (Naccache et al. 2014) have also
317 been developed, with the capacity to analyze metagenomic NGS data in clinically
318 actionable time frames. Nevertheless, we demonstrate here the critical role of
319 comprehensive reference genomes in the NGS diagnostic paradigm. The
320 availability of pathogen genomes with coverage of all clinically relevant
321 genotypes can maximize the utility of NGS in not only diagnosis of individual
322 patients (Wilson et al. 2014; Naccache et al. 2015), but also for public health
323 applications such as transmission dynamics (Grad and Lipsitch 2014) and
324 outbreak investigation (Briese et al. 2009; Greninger et al. 2015b).

325 In the field of amoebic encephalitis, draft genomes are now available for
326 *Acanthamoeba castellani* (Burger et al. 1995), *Naegleria fowleri* (Zysset-Burri et
327 al. 2014), and *Balamuthia mandrillaris*. However, more sequencing is certainly
328 necessary to better understand the genetic diversity of these eukaryotic
329 pathogens. In particular, shotgun sequencing and comparative analysis of
330 mitochondrial genomes from 7 *Balamuthia* strains uncovered at least 3 unique
331 lineages, one of which was comprised entirely of amoebae isolated from
332 California, revealing that geographic differences likely exist among strains (Fig.
333 1B). This study also identified a unique locus in a putative rps3 intron/intergenic
334 in the mitochondrial genome that is an attractive target for a clinical genotyping
335 assay (Figs. 1C and 3). Given the rarity of the disease, it is unknown whether

336 infection by different strains of *Balamuthia* would affect clinical course or
337 outcome, although the availability of routine genotyping could help in addressing
338 this question.

339 Limitations to this study include the small number of accessible clinical
340 samples of *Balamuthia mandrillaris* infection and assembly of a draft genome
341 with >14,000 scaffolds as a result of restricting the sequencing to short reads.
342 The use of long read technologies based on single molecular, real-time (SMRT)
343 or nanopore sequencing will likely be needed to achieve a highly contiguous,
344 haploid genome. Furthermore, additional RNA sequencing of *Balamuthia* will be
345 needed to predict transcripts, identify splice junctions, and enable complete
346 annotation of the genome.

347 In summary, we demonstrate here that the availability of pathogen
348 reference genomes is critical for the sensitivity and success of unbiased
349 metagenomic next-generation sequencing approaches in diagnosing infectious
350 disease. In hindsight, more timely and potentially actionable diagnosis at
351 hospital day 1 in a fatal case of PAM from *Balamuthia mandrillaris* would have
352 required the availability of the full genome sequence. Thus, in addition to
353 revealing a significant amount of evolutionary diversity, the draft genome of
354 *Balamuthia mandrillaris* presented here will improve the sensitivity of
355 sequencing-based efforts for diagnosis and surveillance, and can be used to
356 guide the development of targeted assays for genotyping and detection. The
357 draft genome also constitutes a valuable resource for future studies investigating
358 the biology of this eukaryotic pathogen and its etiologic role in PAM.

359

360

361 **METHODS**

362

363 **Ethics**

364 Informed consent was obtained from the patient's parents for analysis of her
365 clinical samples. This study was approved by the Colorado Multiple Institutional
366 Review Board (IRB). Coded samples were analyzed for pathogens by NGS
367 under protocols approved by the University of California, San Francisco IRB.

368

369

370 **Metagenomic Sequencing of CSF and Brain Biopsy**

371 Total nucleic acid was extracted from 200 µL of CSF using the Qiagen
372 EZ1 Viral kit. Half of the nucleic acid from CSF was treated with Turbo DNase
373 (Ambion). Total RNA was extracted from 2 mm³ brain biopsy tissue using the
374 Direct-zol RNA MiniPrep Kit (Zymo Research), followed by mRNA purification
375 using the Oligotex mRNA Mini Kit (Qiagen). Total RNA from CSF and mRNA
376 from brain biopsy was reverse-transcribed using random hexamers and randomly
377 amplified as previously described (Greninger et al. 2015b). The resulting double-
378 stranded cDNA or extracted DNA from CSF (the fraction not treated with Turbo
379 DNase) was used as input into Nextera XT, following the manufacturer's protocol
380 except with reagent volumes cut in half for each step in the protocol. After 14-18
381 cycles of PCR amplification, barcoded libraries were cleaned using Ampure XP

382 beads, quantitated on the BioAnalyzer (Agilent), and run on the Illumina MiSeq (1
383 x 160 bp run). Metagenomic NGS data were analyzed for pathogens via SURPI
384 using NCBI nt/nr databases from June 2014 (Naccache et al. 2014).

385 A rapid taxonomic classification algorithm based on the lowest common
386 ancestor was incorporated into SURPI, as previously described (Greninger et al.
387 2015b), and used to assign viral, bacterial, and non-chordate eukaryotic NGS
388 reads to the species, genus, or family level. For the SNAP nucleotide aligner
389 (Zaharia et al. 2011), an edit distance cutoff of 12 was used for viral reads
390 (Naccache et al. 2014), but adjusted to a more stringent edit distance of 6 for
391 bacterial and non-chordate eukaryotic reads to increase specificity.

392

393 **Propagation of *Balamuthia mandrillaris* in culture**

394 Trypsin-treated cultures from Vero or BHK cell monolayers and
395 *Balamuthia mandrillaris* 2046 strain amoebas were placed into T25 culture flasks
396 in Dulbecco's Modified Eagle Medium (DMEM) plus 10% bovine serum, 1% of
397 the antibiotics 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL
398 fungizone and incubated at 37° CO₂ for 7 to 10 days plus 2 days at room
399 temperature until the underlying cell sheet was completely destroyed and only
400 actively dividing amebae were seen floating and/or attached to the surface.
401 Attached amoebas were freed by gently tapping the side of the flask or putting
402 the flask on a bed of ice for 20 minutes. The amoebas were concentrated by
403 centrifugation for 5 minutes. After removal of supernatant, the amoeba pellet was
404 washed by addition of PBS, centrifuged again, and then placed in a flask with

405 Bacto-casitone axenic medium and allowed to grow for another 7-10 days, after
406 which the amoebas were concentrated again, washed, and placed into fresh
407 axenic medium. After a final centrifugation step, the amebae were collected,
408 washed 3X in PBS, pelleted, and stored at -80° C.

409 The fluid drained, the ameba pellet was washed by adding PBS,
410 centrifuged followed by placing the amoebas in a flask with Bacto-casitone
411 axenic medium and allowed to grow for 7-10 days, collected again and placed
412 into fresh axenic medium (Lares-Jimenez et al. 2015). The amebae were
413 collected, washed 3X in PBS, pelleted, stored at -80°C.

414

415 **Sequencing and annotation of cultured *Balamuthia mandrillaris* 2046 strain**

416 DNA from *Balamuthia mandrillaris* 2046 strain was extracted using the
417 Qiagen EZ1 Tissue Kit and used as input for the Nextera Mate Pair Kit (Illumina)
418 and Nextera XT Kit (Illumina), following the manufacturer's instructions. Mate-pair
419 libraries were sequenced on an Illumina MiSeq (2x80nt run and 2x300 nt), while
420 the Nextera XT library was sequenced on an Illumina HiSeq (2x250bp paired-end
421 sequencing) (Table 2). Mate-pair reads from run MP1 were adapter-trimmed with
422 NxTrim (O'Connell et al. 2015), and the mitochondrial genome of strain 2046 and
423 high-copy number contigs were assembled using SPAdes v3.5 (Bankevich et al.
424 2012; Greninger et al. 2015a). The average insert size of the mate-pair library
425 was 2,187 nucleotides. Prediction of tRNA and rRNA genes were performed
426 using tRNAscan-SE and RNAmmer v1.2, respectively (Lowe and Eddy 1997;
427 Lagesen et al. 2007). ORFs were predicted in translation code 4 with the

428 Glimmer gene predictor, and all predicted ORF sequences were confirmed using
429 BLASTx and HHpred (Altschul et al. 1990; Soding et al. 2005).

430 Reads from runs MP2 and MP3 were mate-pair adapter-trimmed using
431 NxTrim, while reads from all runs were quality-filtered (q30) and adapter-trimmed
432 using cutadapt (Martin 2011). Reads that aligned to the *Balamuthia*
433 mitochondrial genome and golden hamster (*Mesocricetus auratus*) were
434 identified using SNAP (Zaharia et al. 2011) and removed prior to *de novo*
435 assembly using platanus (Kajitani et al. 2014). Any scaffold of length less than
436 500 bp along with 62 scaffolds that aligned to *Mesocricetus auratus*, *Chlorocebus*
437 *sabaeus*, *Waddlia chondrophila*, and *Enterobacteria phage phiX174* (all likely
438 deriving from cell culture contamination), were removed.

439

440 **Quantitative RT-PCR**

441 qRT-PCR of the *Balamuthia mandrillaris* 28S gene was performed using 20 µL
442 total reactions of the Quantitect qRT-PCR Sybr Green kit (Qiagen) with 1 µL of
443 extracted nucleic acid. Conditions were 50°C x 30 min, 95°C x 15 min, followed
444 by 40 cycles of 95°C x 15 s, 60°C x 60 s using a final 0.5 µM concentration of
445 each primer Bal-28S-F (5'-CTAGCCGTGCTGTAGAGTCG-3') and Bal-28S-R (5'-
446 CGGTCTCGAGCTTTCCCTT-3').

447

448 **Rps3 PCR confirmation**

449 Genomic DNA was PCR-amplified using 0.5 µM final concentration of primers
450 rps3-F (5'-CTGYTCGATTTCGAAAAATAAAGTAG-3') and rps3-R (5'-

451 TGAAAGAACATTAGATCACGACT-3') using 2X iProof HF Master Mix
452 (Bio-Rad) in 20 µL total volume. Conditions were 95°C x 2 min, followed by 35
453 cycles of 95°C x 30 s, 52°C x 30 s, 72°C x 40 sec and a final incubation at 72°C
454 x 2 min. PCR amplicons were visualized by 3% agarose gel electrophoresis.

455

456 **DATA ACCESS**

457 The *Balamuthia mandrillaris* mitochondrial genomes have been deposited in
458 NCBI under the following accession numbers: 2046 axenic (KP888565), 2046-1
459 (KT175740), V451 (KT030670), OK1 (KT030671), RP-5 (KT030672), SAM
460 (KT030673), V188-axenic (KT175738), V188-frozen stock (KT175739), V039
461 (KT175741). The *Balamuthia mandrillaris* scaffolds have been deposited in NCBI
462 WGS under the accession number LEOU00000000. Metagenomic NGS data
463 from the brain biopsy and CSF fluid corresponding to non-human reads have
464 been submitted to the NCBI Sequence Read Archive (SRA). NGS reads were
465 filtered for exclusion of human sequences by both BLASTn alignment at an e-
466 value cutoff of 10^{-5} and Bowtie2 high-sensitivity local alignment to the human
467 hg38 reference database.

468

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475

476 AUTHOR CONTRIBUTIONS

477 ALG, KM, SD, and CYC conceived of and designed the study. ALG, TD,
478 JB, SY performed the experiments. KM, DM, YN, MV, CAP, BKK, and SR took
479 care of patients with *Balamuthia* infection and contributed clinical samples. KM,
480 DM, CG, MV, CAP, BKK, SR, and CYC analyzed the clinical and epidemiological
481 data. ALG, SNN, SF, JB, and CYC analyzed the genomic sequencing data. ALG,
482 SNN, SF, and CYC developed and contributed software analysis tools. ALG, KM,
483 and CYC wrote the manuscript.

484

485 DISCLOSURE DECLARATION

486 CYC is the director of the UCSF-Abbott Viral Diagnostics and Discovery
487 Center (VDDC) and receives research support in pathogen discovery from Abbott
488 Laboratories, Inc.

489

490 FIGURE LEGENDS

491

492 **Figure 1 – Sequencing and comparative phylogenetic analysis of the**
493 **mitochondrial genome of *Balamuthia mandrillaris*. (A)** *Balamuthia*
494 *mandrillaris* 2046 mitochondrial genome. Annotation of the 41,656 bp genome
495 was performed using RNAmmer, tRNAscan-SE, and Glimmer gene predictor,

496 with all ORFs manually verified using BLASTx alignment. **(B)** Phylogenetic
497 analysis of 7 newly sequenced genomes from different strains of *Balamuthia*
498 *mandrillaris*. An outgroup (e.g. *Acanthamoeba castellanii*) is not shown given the
499 lack of gene synteny. Branch lengths are drawn proportionally to the number of
500 nucleotide substitutions per position, and support values are shown for each
501 node. **(C)** Differences in individual gene features (cox1, 23S rRNA, and rps3),
502 among the 7 mitochondrial genomes, as detailed in the text.

503

504 **Figure 2 – PCR amplification of the *Balamuthia* rps3 mitochondrial gene.**

505 The variable length of the rps3 intron among 8 different *Balamuthia* strains (7
506 newly sequenced mitochondrial genomes and the case patient) suggests that
507 this gene may be an attractive target for development of a molecular genotyping
508 assay. Column 4 corresponds to the DNA ladder (faint appearance), while
509 columns 2 and 3 correspond to an additional clinical *Balamuthia* isolate whose
510 mitochondrial genome was not sequenced.

511

512 **Figure 3 – Phylogenetic trees of the mitochondrial cox1 protein and 28S**
513 **rRNA gene reveal the close phylogenetic relationship between *Balamuthia***
514 **and *Acanthamoeba*.** **(A)** Phylogeny of 7 *Balamuthia* cox1 amino acid
515 sequences along with the top complete sequence hits in NCBI nr ranked by
516 BLASTp E-score. **(B)** Phylogeny of 7 *Balamuthia* 23S rRNA nucleotide
517 sequences along with the top complete sequence hits in NCBI nt ranked by
518 BLASTn E-score. Sequences were aligned using MUSCLE and a phylogenetic

519 tree constructed using MrBayes. Branch lengths are drawn proportionally to the
520 number of nucleotide substitutions per position, and support values are shown for
521 each node.

522 .

523 **Figure 4 – Magnetic resonance imaging and histopathology from a 15-year**
524 **old patient with a fulminant acute encephalitis. (A)** A hospital day (HD) 1
525 coronal T2-weighted MR image, demonstrating a hemorrhagic lesion with
526 surrounding edema within the superior left frontal lobe (left panel, white arrow)
527 and left occipital lobe (right panel, white arrow). **(B)** A HD 5 contrast-enhanced
528 T1 weighted MR image, revealing enlargement of the pre-existing left frontal lobe
529 lesion (left panel, white arrow), as well as interval development of numerous
530 additional rim-enhancing lesions in multiple regions (right panel, white arrows).
531 **(C)** 20X (left and right panels) and 100X fields of view (right panel, inset) of a
532 brain biopsy specimen from the patient demonstrating numerous viable, large
533 ameba (black arrows), with abundant basophilic vacuolated cytoplasm, round
534 central nucleus, and prominent nucleolus, consistent with *Balamuthia*
535 *mandrillaris*. There were areas of extensive hemorrhagic necrosis accompanied
536 by a polymorphous inflammatory cell infiltrate including neutrophils and
537 eosinophils (right panel).

538

539 **Figure 5 – Identification of *Balamuthia mandrillaris* infection by**
540 **metagenomic next-generation sequencing (NGS). (A)** Coverage maps (blue
541 gradient) and pairwise identity plots (magenta gradient) of 2 of the 3 available

542 sequences from *Balamuthia* (16S/18S rRNA genes) in the NCBI nt reference
543 database as of August 2015 and prior to sequencing of the draft genome.
544 Shown are coverage maps corresponding to day 6 DNA and RNA libraries from
545 CSF and a day 6 mRNA library from brain biopsy. No hits to 16S and 18S
546 *Balamuthia* sequences were seen from day 1 samples. The asterisk denotes an
547 area with artificially low coverage after taxonomic classification of the NGS reads
548 due to high conservation among eukaryotic sequences (e.g. human, *Balamuthia*,
549 etc.) within that region **(B)** A bar graph of the number of species-specific NGS
550 reads aligning to *Balamuthia* 16S/18S rRNA (blue) or the *Balamuthia* genome
551 (orange) in day 1 or day 6 samples. Note that with the availability of the newly
552 assembled 44Mb *Balamuthia* genome, diagnosis of *Balamuthia mandrillaris*
553 encephalitis at day 1 would have been possible by detection of 9 species-specific
554 reads (red boldface). **(C)** Coverage maps of two large scaffolds, ~216 kB and
555 ~222 kB in size, from the *Balamuthia* draft genome, showing 8 out of 926 hits to
556 *Balamuthia* in the day 6 CSF DNA library that are identified by SURPI after the
557 draft genome sequence is added to the reference database (versus only 13 hits
558 previously).
559
560

561 **Table 1 – Strains used in this study**

Strain	Date	Location	Note	Citation
SAM**	Spring 2001	Rohnert Park, CA	3 yo girl, cultured on Vero cells	Bakardjiev A et al. 2002
RP-5**	Spring 2001	Rohnert Park, CA	environmental sample associated with SAm, cultured on Vero cells	Schuster FL et al. 2003
OK1**	2002	Oakland, CA	environmental sample in NorCal unrelated to SAm, cultured on Vero cells	Dunnebacke TH et al. 2004
V039*	1990	San Diego, CA	type strain isolated from pregnant mandrill at San Diego Zoo, cultured on Vero cells	Visvesvara GS et al. 1990
V188**	1989	Georgia	59 yo man, amoeba isolated from brain/skin lesion, cultured on Vero cells	Gordon SM et al. 1992
V451**	N/A	New York	6 yo girl, cultured on Vero cells	Yang XH et al. 2001
2046**	March 2010	Walnut Creek, CA	26 yo man, survivor, cultured on Vero cells	Vollmer and Glaser, in review
CSF108***	May 2015	Colorado	15 yo girl, direct metagenomic detection from CSF	

*strain obtained from ATCC (V39)

**strain obtained from California Department of Public Health

***strain obtained from recent clinical case in Colorado

562

563

564 **Table 2 – Sequencing runs and genome assembly details**

565

Sequencing run	Raw reads	Filtered reads	Read Length	Library Prep
MP1*	3845734	N/A	2x80bp	Nextera Mate-Pair
MP2*	31223454	12095792	2x80bp	Nextera Mate-Pair
MP3*	14121900	4397510	2x250bp	Nextera Mate-Pair
PE1**	213604902	29314946	2x250bp	Nextera XT
Assembly***	Contigs	N50 (bp)		
Mitochondria	1	41,656	41,656	
contigs	31,194	3,411	48938887	
scaffolds	26,811	19,415	49120517	
gap-fill	26,811	19,012	48939625	
Final assembly (minus <500bp, contaminants)	14,699	26,144	44270879	

*3 runs of a Nextera mate-pair library of strain 2046 from axenic culture sequenced on an Illumina MiSeq

**Nextera XT paired-end library of strain 2046 sequenced on an Illumina HiSeq

***assembly of mitochondrial genome from MP1 library, of whole-genome from all 4 libraries

566

567

568 **Table 3 - *Balamuthia mandrillaris* reads from the day 1 patient sample**

Read #	top NCBI NR match by BLASTx	% amino acid identity	E-value	matched scaffold in <i>Balamuthia</i> genome	% nucleotide identity (<i>Balamuthia</i> genome)	E-value (<i>Balamuthia</i> genome)
1	none	–	–	scaffold46	96%	5.00E-29
2	none	–	–	scaffold26764	96%	3.00E-27
3	none	–	–	scaffold45	100%	3.00E-36
4	none	–	–	scaffold353	98%	2.00E-24
	fumareylacetoacetase [Flavobacteriales					
5	bacterium BRH_c54]	82%	3.00E-05	scaffold203	100%	3.00E-36
6	none	–	–	scaffold106	96%	1.00E-26
	glutathione transferase protein					
	[Acanthamoeba					
7	castellani str. Neff]	77%	0.022	scaffold30	99%	8.00E-34
8	none	–	–	scaffold112	97%	7.00E-28
9	none	–	–	scaffold511	98%	5.00E-26
569						

570

571 **Table S1 – SURPI clinical metagenomic results**

572

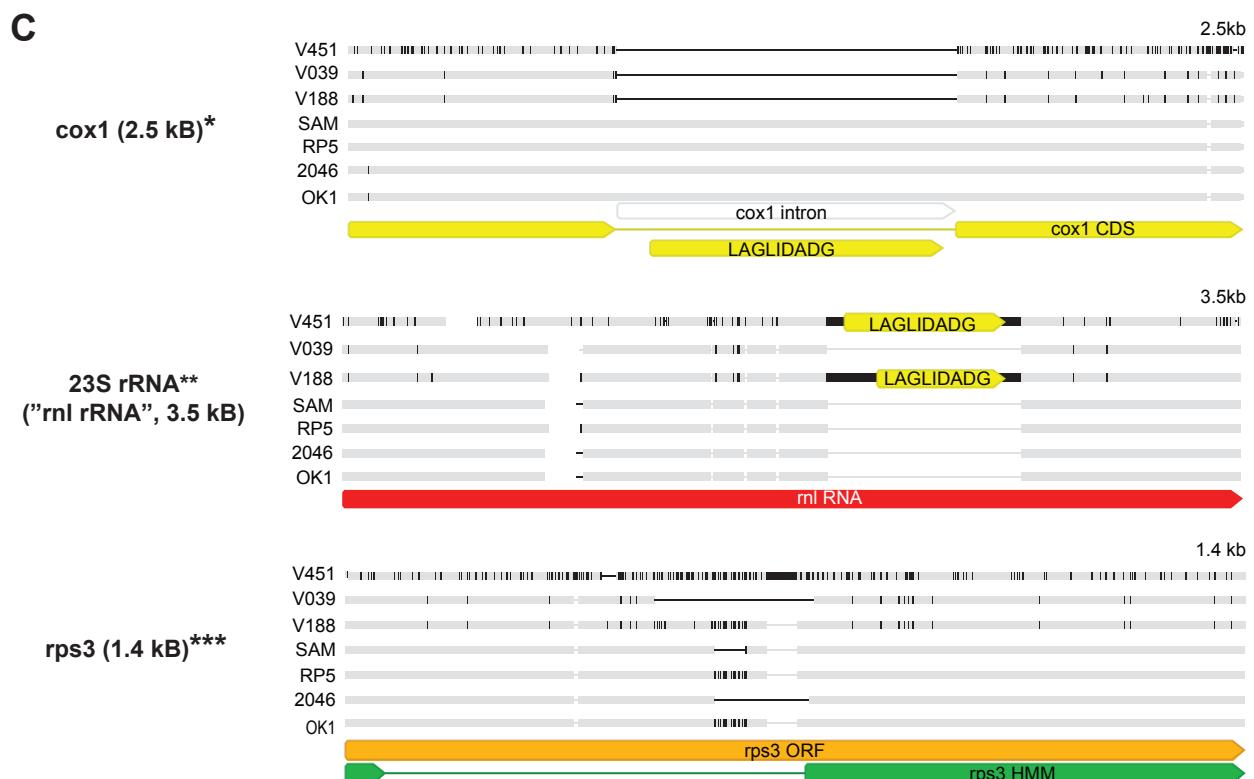
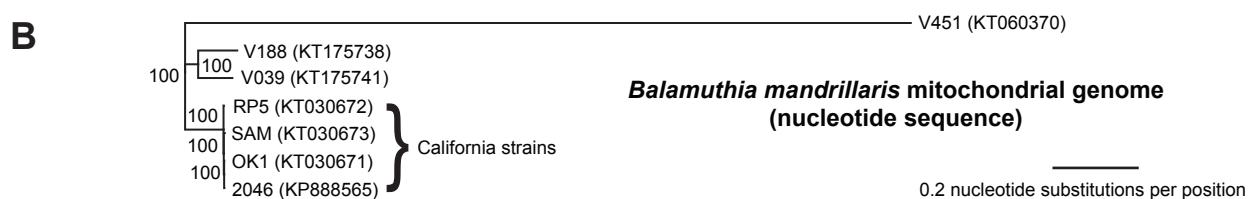
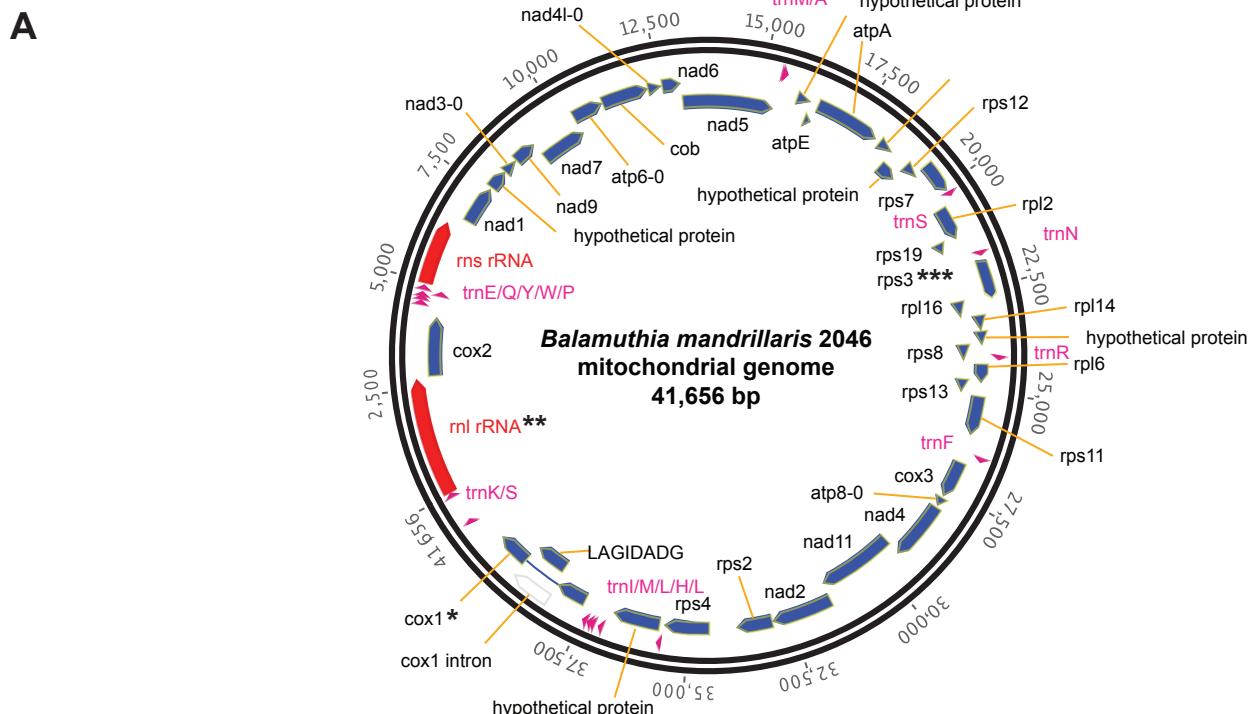
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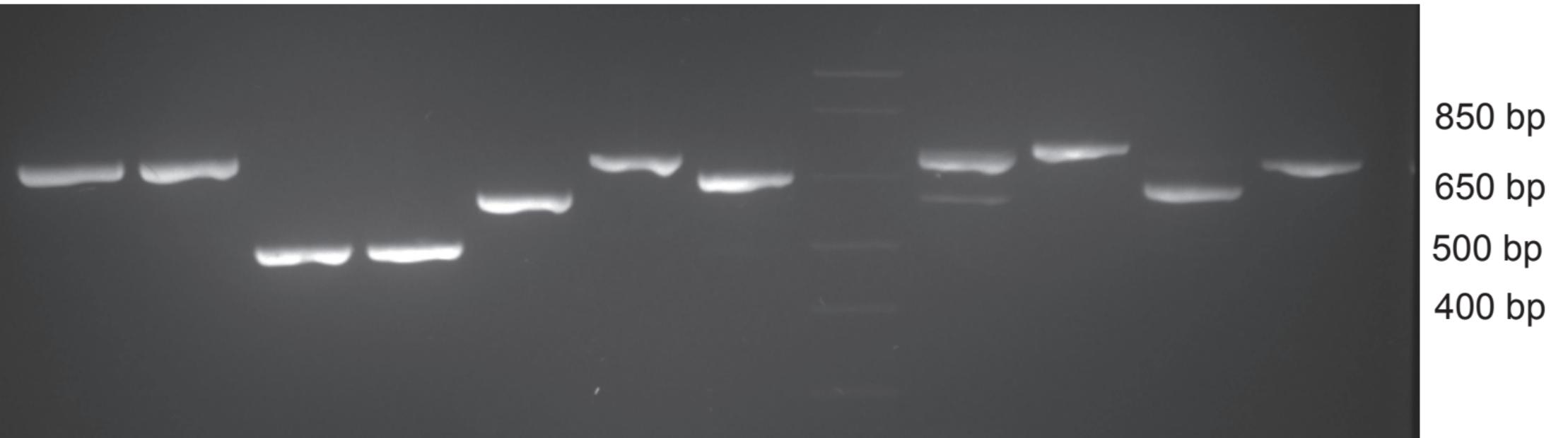
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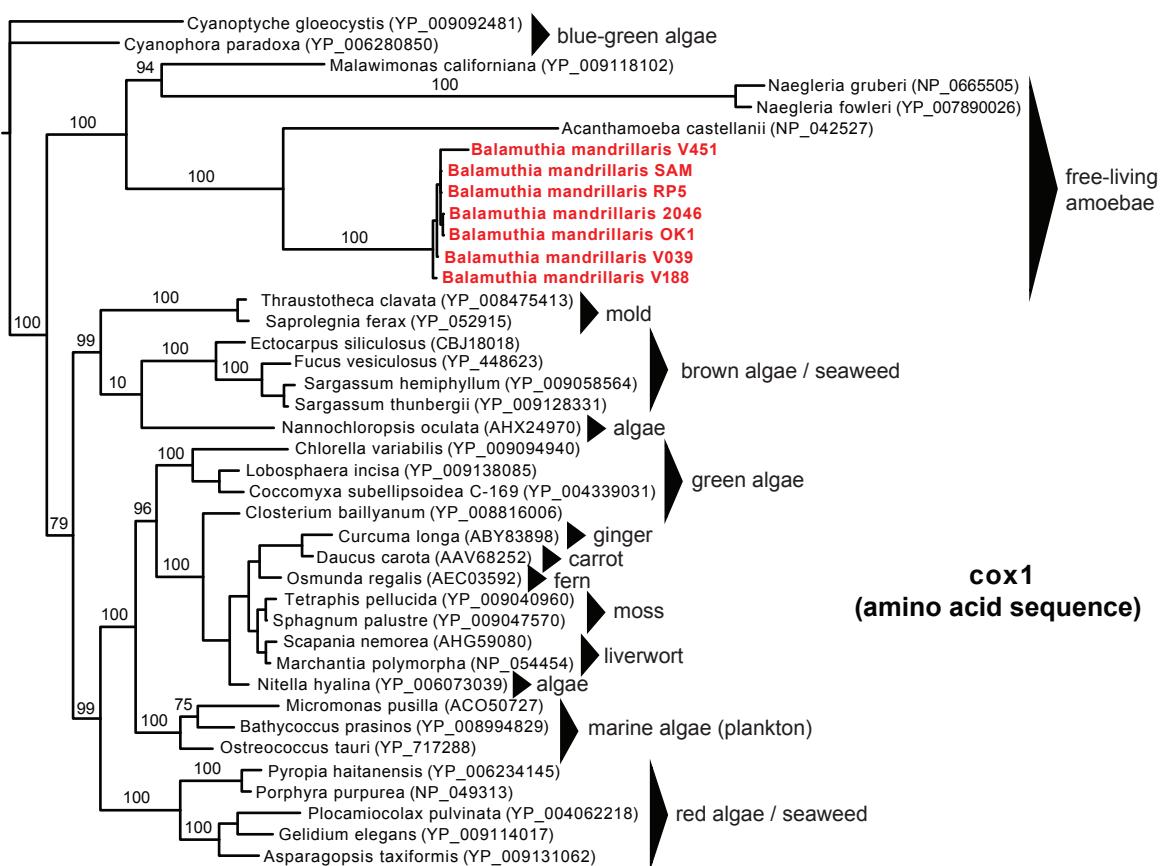
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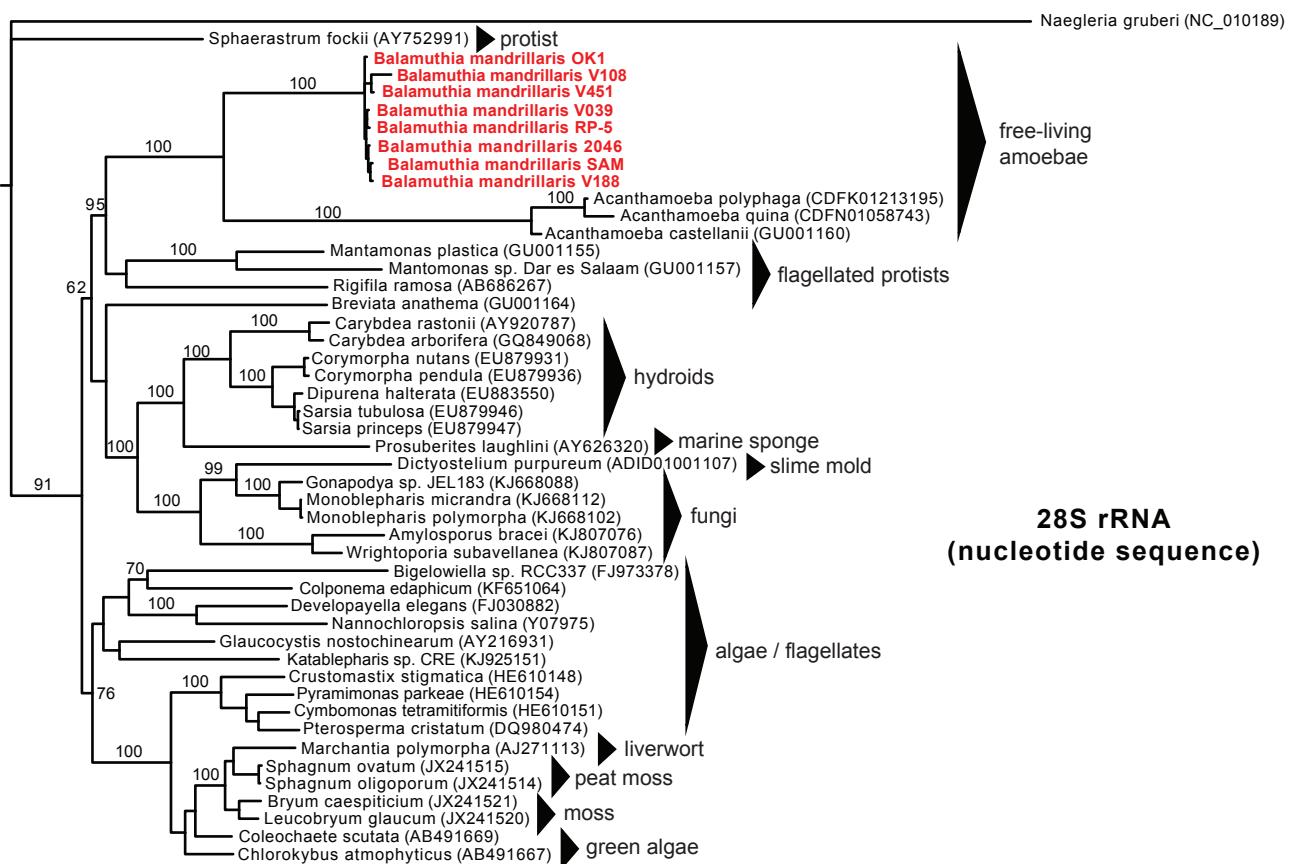
V188 *V188 repeat* clinical isolate clinical isolate repeat
2046 RRP5 SAM *OK₁* *V451* Case patient
V039

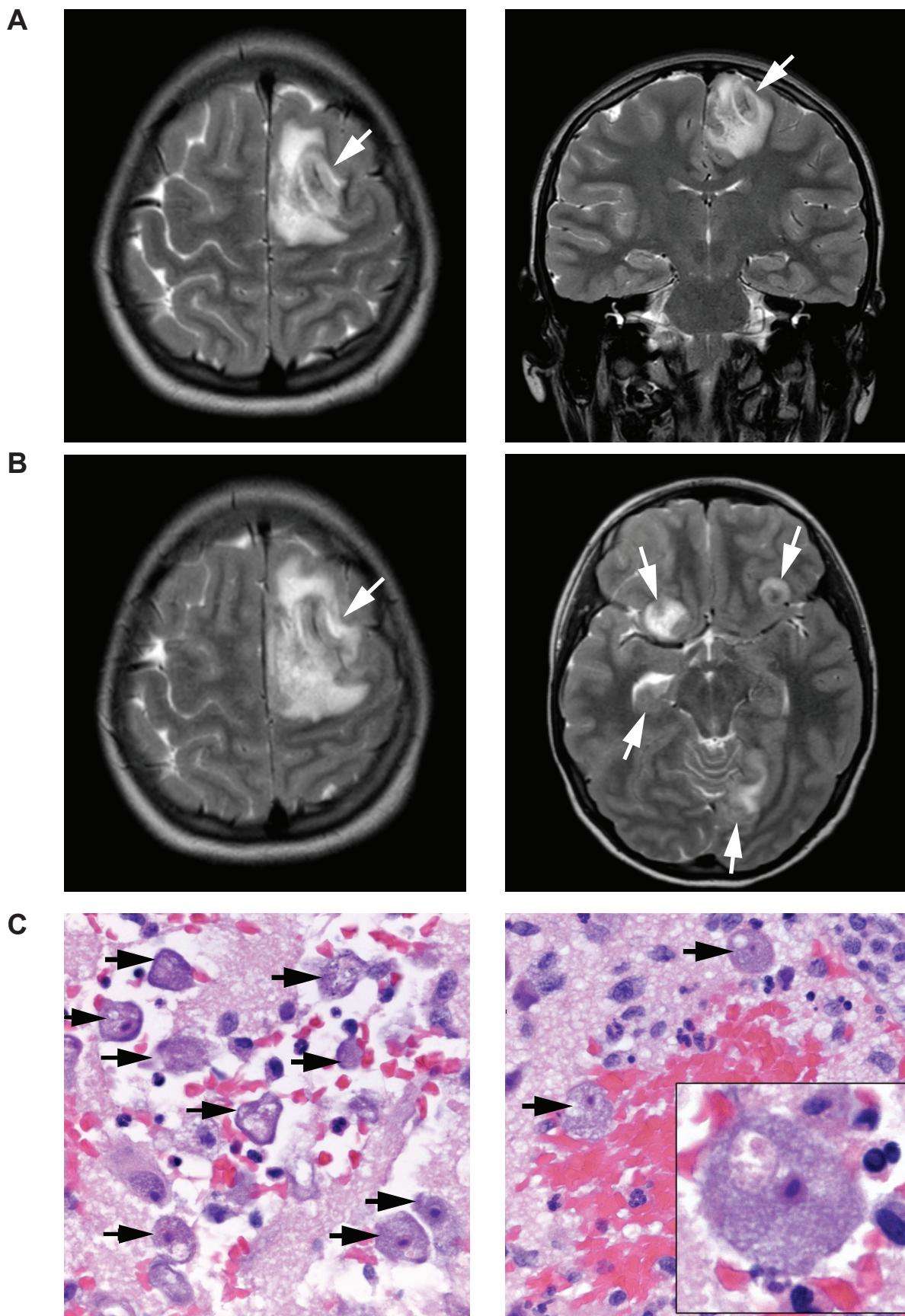


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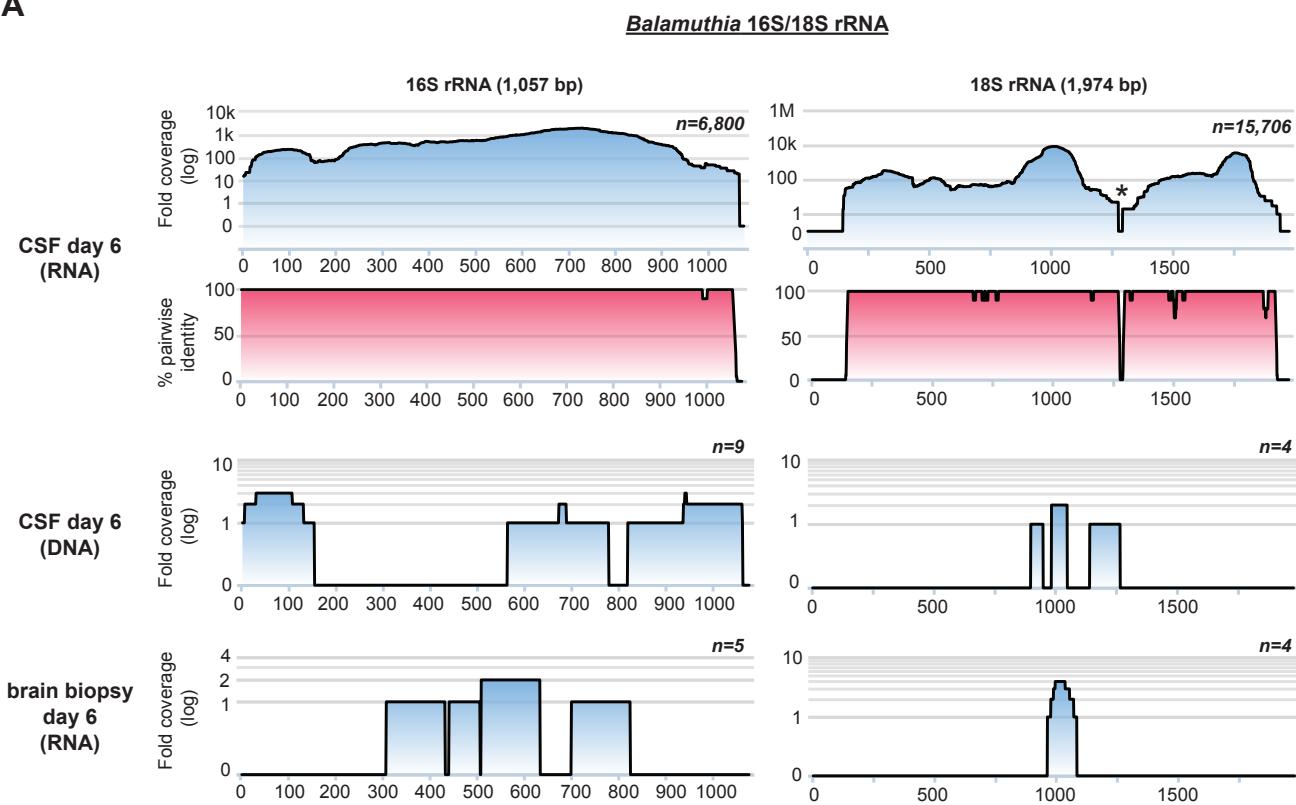


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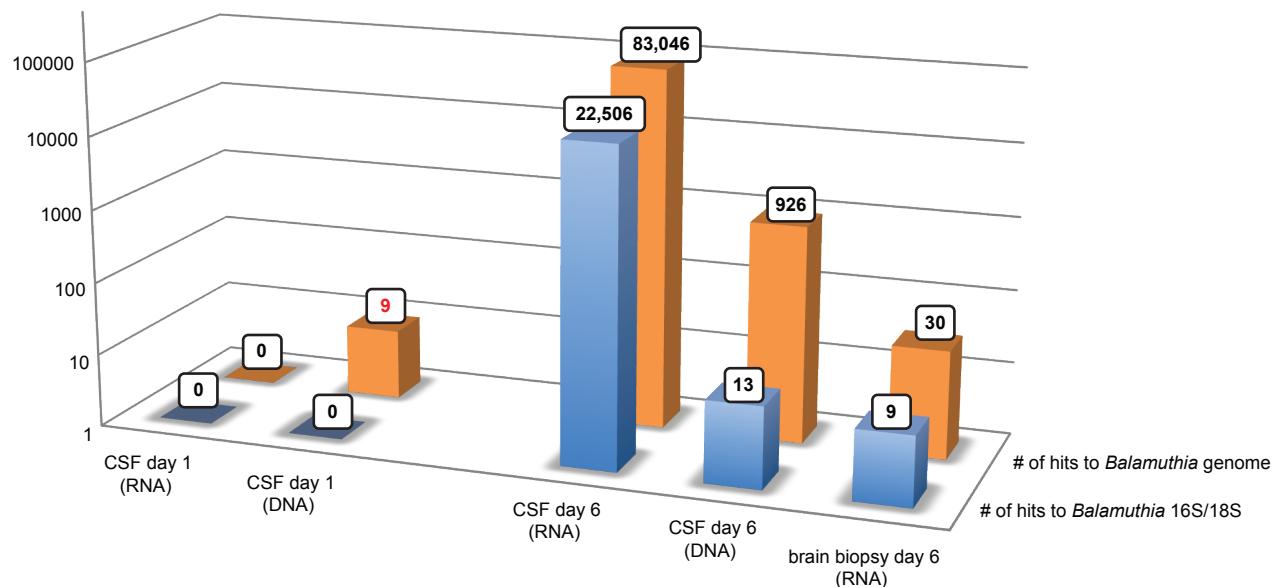




A



B



C

