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ORIGINAL ARTICLE

The Food Web of Boiling Springs Lake Appears Dominated by the Heterolobosean *Tetramitus thermacidophilus* Strain BSL

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ABSTRACT

We studied the protist grazers of Boiling Springs Lake (BSL), an acid geothermal feature in Lassen Volcanic National Park, using a combination of culture and genetic approaches. The major predator in BSL is a vahlkampfiid ameba closely related (95% 18S+ITS rRNA identity) to *Tetramitus thermacidophilus*, a heterolobose ameboflagellate recently isolated from volcanic geothermal acidic sites in Europe and Russia, as well as an uncultured heterolobosean from the nearby Iron Mountain acid mine drainage site. *Tetramitus thermacidophilus* strain BSL is capable of surviving the physical extremes of BSL, with optimal growth at 38–50 °C and pH 2–5. This bacterivore also ingested conidiospores of the ascomycete *Phialophora* sp., but ultrastructural observations reveal the latter may not be readily digested, and conidia were not separable from the ameboflagellate culture, suggesting a possible symbiosis. DGGE fingerprint transects studies showed the organism is restricted to near-lake environs, and we detected an average of ~500 viable cysts/cm^3 sediment on the shoreline. Other grazing protists were isolated from lakeshore environments, including the lobose amebae *Acanthamoeba* sp. and *Hartmannella* sp., and the kinetoplastid flagellate *Bodo* sp., but none could tolerate both low pH and high temperature. These appear to be restricted to cooler near lake geothermal features, which also contain other potential grazer morphotypes observed but not successfully cultured, including ciliates, euglenids, testate amebae, and possible cercozoans. We compare the food web of BSL with other acidic or geothermal sites, and discuss the impact of protists in this unique environment.

BOILING Springs Lake (BSL) is a flooded fumarole located in northern California's Lassen Volcanic National Park (LVNP). An acid-sulfate steam-heated feature (Janik and McLaren 2010) with pH 2.2 and seasonal temperature variation between 45 and 52 °C, BSL's microbial community has been characterized by genetic screens (Brown and Wolfe 2006; Siering et al. 2013; Wilson et al. 2008) and culture-based isolation (Arroyo et al. 2015). BSL phototrophs are dominated by Cyanidiales, but growth in the water column is limited due to light limitation by suspended clays (Siering et al. 2013); chemoautotrophic actinobacteria (*Acidomicrobium*) are the other primary producers. A variety of heterotrophs, both prokaryotes and eukaryotes (fungi), feed off allochthonous C inputs from the surrounding montane coniferous forest ecosystem (Wolfe et al. 2014).

As a NSF-funded Microbial Observatory, one goal was to characterize interactions among the eukaryotic and prokaryotic communities. Although a previous genetic screen found no evidence of phagotrophic microbial eukaryotes in BSL (Brown and Wolfe 2006), recent research has shown that microbial eukaryotes (protists and some fungi) are widely present, albeit with low diversity, in geothermal and/or acidic environments (Amaral-Zettler 2013). However, much less is known about ecological interactions, especially food webs and trophic structures in extreme environments. In most soil and aquatic environments, protists are the most important predators of bacteria, fungi (Foissner 1987; Sherr and Sherr 2002), or other protists (Bradley and Marciano-Cabral 1996; Chakraborty and Old 1986; Marciano-Cabral 1988; Matin et al. 2006; Rodríguez-Zaragoza 1994; Weekers et al. 1993), and can structure

the microbial community via top-down control (Jürgens and Güde 1994). Protists are also an important source of re-mineralized nutrients and colloidal and dissolved trace metals in aquatic systems, impacting systems biogeochemically via the "microbial loop" (Sherr and Sherr 2002).

Interestingly, protist grazers in thermal environments appear to be dominated by amebae. Among terrestrial volcanic geothermal sites, free-living amebae have mostly been investigated for their potential as human opportunistic pathogens, which can be found even in hospital hot-water systems (Rohr et al. 1998). The lobosean *Acanthamoeba* sp. has been isolated from several geothermal sites (Brown et al. 1983; Sheehan et al. 2005a), and can also harbor the bacterial pathogen *Legionella* (Kwaik et al. 1999; Lian-Yong Gao 2000), detected at YNP (Sheehan et al. 2005b) and in acid mine drainage (AMD) sites (Hao et al. 2010). More recently, *Acanthamoeba* sp. has also gained attention as the host of giant large nucleocytoplasmic viruses such as *Mimivirus* (La Scola et al. 2003) or *Marseillvirus* (Boyer et al. 2009). Although most *Acanthamoeba* taxa have been characterized as thermotolerant, growing up to 45 °C but with best growth at lower temperature, a true thermophile (*Echinamoeba thermaru*, growing to 57 °C) was isolated from sites in Italy, YNP, Kamchatka, and Costa Rica by Baumgartner et al. (2003). Other potential opportunistic amoebal pathogens include heterolobose ameboflagellates, a poorly studied group that alternates between ameboid and flagellate life stages. *Naegleria fowlerii*, the best-studied taxon – and the only heterolobosean to date with a sequenced genome (Fritz-Laylin et al. 2010, 2011) – can be found in diverse geothermal sites (Izumiyama et al. 2003; Moussa et al. 2013; Ramaley et al. 2001; Sheehan et al. 2003a,b, 2005a). *Naegleria fowlerii* and related species are likely thermotolerant cosmopolitan taxa, as they are also found in nonthermal and even sub-polar environments (de Jonckheere 2006). Heterloboseans that are truly adapted to thermal environments include *Marinamoeba thermophila* (de Jonckheere et al. 2009), found in marine hydrothermal vents, and *Fumarolamoeba ceborucoi*, found in volcanic fumaroles in Mexico (de Jonckheere et al. 2011b). Both grow at 50 °C, while *Oramoeba fumarolia* (de Jonckheere et al. 2011a), isolated from marine sediment near a fumarole in Italy, can grow to 50 °C. Acidic geothermal sites in Italy and Kamchatka have been found to harbor the thermo-acidophilic heterolobosean *Tetramitus thermacidophilus* (Baumgartner et al. 2009). This organism had a growth range of 28–52 °C, with an optimam of 44 °C, and a pH range of 1.2–6, with an optimum of pH 3. It grazed on a native thermo-acidophilic bacterium *Alicyclobacillus*. Gene sequences of highly similar organisms have been detected at Iron Mountain mine (Baker et al. 2003, 2004, 2009), a well-studied AMD system ~75 km west of LVNP.

The objectives of this study were to: (1) isolate and identify members of the eukaryotic grazing community using culturing and genetic techniques; (2) determine the distribution of these grazers and their relationship with environmental variables; and (3) understand the potential impact of the protest grazing community on native bacterial, fungal, and algal communities. We hypothesized that there is an endemic, specialized grazing community with low diversity, whose members will be well adapted to the extreme pH and temperature of the lake.

MATERIALS AND METHODS

Study site and sampling

Boiling Springs Lake is situated in the Warner Valley of LNVP; fig. 1 in Siering et al. (2013) presents a detailed map of the study site. Sampling was restricted to the summer and fall of years spanning 2008–2010 due to winter weather and road closures associated with snow. The pH at BSL was relatively constant at 2.2, and water temperatures varied between 48 and 52 °C during most sample periods (Siering et al. 2013). Water samples were collected in sterile 2–4 liter bottles from sites between the north end's outflow channel and western shoreline, and transported immediately to the lab, where they were stored at 4 °C. Other samples were taken from the dried clay of the seasonally exposed lake bed, various geothermal features surrounding the lake, and in transects from the lake shore to the surrounding forest. We stored these samples in sterile 50-ml Falcon tubes at room temperature until processed in the lab, which usually occurred within a few days.

Enrichment cultures

For culture medium, BSL water was vacuum-filtered through 47 mm glass-fiber filters (Whatman, GF/F; GE Healthcare Bio-Sciences, Pittsburgh, PA) to remove contaminating organisms and sediments and stored at 4 °C. Iron oxidation and precipitation during autoclaving could be prevented by addition of 0.01% w/v cysteine, but it appeared toxic to some grazers, so BSL filtrate was usually heated to 90 °C for 10 min to prepare semi-sterile media and plates.

We created primary enrichments using a sterile wheat berry as a nutrient source in 10–20 ml of BSL filtrate along with ~100 mg of sample material in 40 ml tissue flasks (Corning Inc., Corning, NY). To reduce obscuring sediments, these were subsequently diluted to secondary cultures in BSL filtrate, using 0.02% w/v peptone + yeast extract (Becton, Dickinson & Co., Franklin Lakes, NJ; hereafter abbreviated PYE) in as a nutrient source. Culture samples were examined with 100–400X phase microscopy using a variety of microtiter plates or deep-well slides. Due to their slow locomotion, amebae were observed without fixation, but the quick-moving flagellates required fixation with Lugol's iodine, added drop-wise to convert the culture to a light-yellow color.

Further enrichments and feeding tests were conducted with feeder cultures of bacteria and fungi were initially colony-isolated from BSL filtrate + PYE on 2% agar plates. After re-streaking for isolation, we grew the organisms in liquid broth (TSB for bacteria, potato dextrose broth for

fungi) to supply liquid cultures with prey cells. Additional native BSL bacterial strains, mostly *Firmicutes*, were provided by Drs Patricia Siering and Mark Wilson, Humboldt State University (Arroyo et al. 2015). Because the dominant Actinobacterium in BSL, *Acidomicrobium*, is a chemolithotroph that grows slowly in culture, we used standard lab strains of *Micrococcus* (*M. epidermidis, M. luteus*) grown in TSB at 37 °C. Prey cells were harvested by repeated centrifugation at 12,000 RPM for 10 min, washed 3X in phosphate-buffered saline before re-suspension in ~10 ml distilled water, and stored at 4 °C for use in liquid cultures and as lawns on solid media.

Isolation efforts

Isolation of ameba observed in enrichments was attempted by several methods. Actively feeding cultures were starved, or placed in a 50 °C water bath overnight to induce encystment, and then cysts were collected onto 45 mm 8 μm membrane filters, and the cyst-laden filters were used as an inoculum for subsequent cultures. Isolation on solid media used the walk-out method (Neff 1958) under aerobic conditions at 28–52 °C, or under anaerobic and microaerobic conditions using gas-generating pouches (Becton, Dickson & Co). A lawn of 500 μl concentrated prey cells (*Micrococcus* sp., BSL ascomycetes) was spread on the surface of the agar and allowed to dry overnight, and then small amounts of inoculum placed at the center of the lawn. Plates were incubated right side up at 37 °C until plaques appeared around the soil sample. The plaque was checked for amebae by suspending a loop of material in deionized water on a deep-well slide and observed under 400X phase microscopy. If amebae were present, they were re-cultured onto a separate plate with a lawn of feeder cells via sterile loop transfer, and subsequently into liquid cultures of BSL filtrate or DI water and a suspension of feeder cells. We also attempted to rid some ameba cultures of the consistent acid- and thermal-tolerant fungal contamination by filtration and serial dilutions, and also attempted isolation by dilution to extinction in BSL filtrate containing PYE. Three 4-fold serial dilutions were attempted, with ratios of 10:1, 20:1, and 100:1 in 24-well plates containing a *Micrococcus* sp. prey suspension (0.1% v/v concentrated feeder cells). Wells were monitored on an inverted scope with phase optics for the presence of amebae, and the lowest concentration with positive growth was used for subsequent work.

Genetic analysis

Genomic DNA was extracted from environmental and enrichment samples using bead-beating and SDS-chloroform extraction following Brown and Wolfe (2006), and in some cases purified by Wizard columns (Promega Corp., Madison, WI). DNA was quantified by gel electrophoresis and UV absorption to confirm DNA extraction.

For DGGE, we PCR-amplified the V8 region of the 18S rRNA using 1427GC/1616R primers (Table 1). PCR products were run on a 30–55% gradient gel (BioRad; DCODE, Life Science Research, Hercules, CA) at 58 °C (50 V for 960 min). Gels were stained with ethidium bromide for 15 min, destained for 30 min, and photographed under UV light, and bands excised using BandPick (Elchrom Scientific AG, Cham, Switzerland). Excised bands were placed in PCR tubes and stored at −20 °C until they could be re-amplified and sequenced.

Group-specific primers were obtained from various sources (Table 1), and used for screening DNA extracted from enrichments for specific organisms. *Tetramitus thermacidophilus* primers were designed using sequences

Table 1. rRNA gene primers used for group-specific and universal PCR

Primer	Target	Sequence (5′→3′)	Source
Universal			
20F	SSU	GTAGTCATATGCTTGTCTC	Brown and Wolfe (2006)
82F		GAAACTGCGAATGGCTC	Brown and Wolfe (2006)
516R	V3 SSU	AACCAGACTGCCCTCC	Brown and Wolfe (2006)
1427F	V8 SSU	TCTGTGATGCCCTTAGATGTTCTGGG	Brown and Wolfe (2006)
1616R		GCGGTGTGTACAAAGGGCAGGG	Brown and Wolfe (2006)
LSU-R1	LSU	TTAGTTTCTTTTCCTCCGCTTAGT	
Taxon-specific			
JITS-F	Vahlkampfiid amebae ITS	GTCTTCGTAGGTGAACCTGC	de Jonckheere and Brown (2005)
JITS-R	Vahlkampfiid amebae ITS	CCGCTTACTGATATGCTTAA	de Jonckheere and Brown (2005)
Tetramitus ITS-R	*Tetramitus* ITS	CACTCACAAGCAAGTACCCTTGCTACTCG	This study
JDP1	*Acanthamoeba* SSU	GGCCCAGATCGTTTACCGTGAA	Booton et al. (2005)
JDP2	*Acanthamoeba* SSU	TCTCACAAGCTGCTAGGGGAGTCA	Booton et al. (2005)
Cerco 25F	Cercozoan SSU	CATATGCTTGTCTCAAAGATTAAGCCA	Bass and Cavalier-Smith (2004)
Cerco 1256R	Cercozoan SSU	GCACCACCACCCAYAGAATCAAGAAAGAWCTTC	Bass and Cavalier-Smith (2004)
Kineto 14F	Kinetoplastid SSU	CTGCCAGTAGTCATATGCTTGTTTCAAG	von der Heyden and Cavalier-Smith (2005)
Kineto 2026R	Kinetoplastid SSU	GATCCTTCTGCAGGTTCACCTACAGCT	von der Heyden and Cavalier-Smith (2005)

obtained by aligning *Tetramitus* sp., ITS sequences found in the NCBI database, and finding a homologous region at the beginning of the ITS rRNA gene. Samples were sequenced by dye-termination (University of Washington High-Throughput Sequencing Center, Seattle, WA).

Sequences were added to the Silva Release 119 SSU ARB database (Quast et al. 2013) and aligned according to conserved secondary structure (Ludwig et al. 2004). An alignment of 1,634 homologous nucleotide positions from 30 representative reference Heterolobosea was used to build maximum likelihood phylogenetic trees using RAxML utilizing the GTR-GAMMA substitution method with 1,000 bootstrap runs (Stamatakis et al. 2005).

Transmission electron microscopy

Ten ml ameba cultures were concentrated by centrifugation for 40 min at low speed, then microwave-fixed with Karnovskey's fixative (1:1 v:v) for 20 s at 650 W under 20 mmHg vacuum (2X with a 30 s rest). Samples were cacodylate-buffered and microwaved for 30 s at 150 W, then postfixed with 100 µl 1% OsO_4 and microwaved at

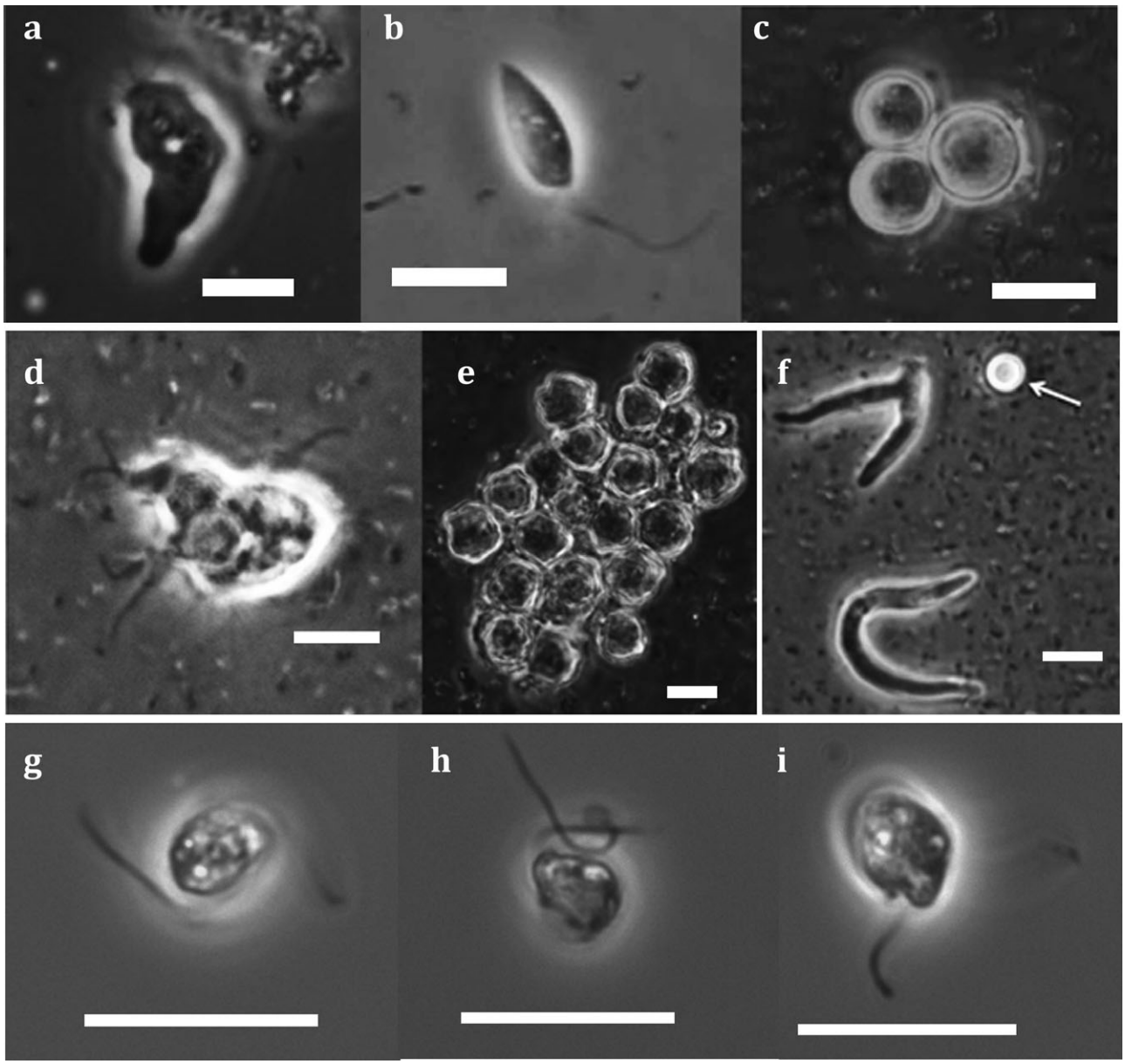

Figure 1 Dominant protists cultured from BSL hydrothermal features, as observed by 400X phase contrast. (**a**–**c**): Heterolobose ameba *Tetramitus thermacidophilus* strain BSL. **a.** Limax ameboid stage with tail fibers visible at top. **b.** Flagellate stage with one of two polar flagella visible. **c.** Smooth spherical cysts. **d–f.** Lobose amebae observed: *Acanthamoeba* sp. (d) and cysts (e); *Hartmannella* sp. and cyst (arrow) (f). **g, h.** Flagellates *Bodo* sp. Size bars = 10 µm.

450 W (3X 40 s/60 s on/off) and dehydrated with 50%, 70%, 90%, and 100% ethanol (150 W, 80 °C for 1 min). Samples were infiltrated with 300 µl Epon resin (350 W, 20 mmHg at 80 °C, for 1, 2, and 3 min per step), then centrifuged into BEEM capsules and incubated overnight at 50 °C before ultra-thin sectioning. Sample grids were stained with uranyl acetate and lead citrate for 10 min, and examined with a Philips CM120 (UC Davis Department of Pathology and Laboratory Medicine) or JEOL 2000FX (Center for Life in Extreme Environments, Portland State University).

pH and temperature tolerance

We tested isolates for growth and survival over a range of temperatures and pH using a custom-built temperature-gradient incubator (Wolfe et al. 2013). Samples were incubated in 20-ml glass scintillation vials with autoclavable phenolic lids (Wheaton, Millville, NJ), with growth and doubling times determined by triplicate cell counts every 24 h for 4–7 d. For temperature tests, typical sample temperatures were 50, 40, 34, 29, 24, 22, 20, and 16 °C. To test pH, we incubated cells in a basal salts medium (0.2% KH_2PO_4, 0.1% NaCl, 0.2% NH_4Cl, 0.05% $MgSO_4$, 0.0025% $FeSO_4$, 0.0025% $MnSO_4$, 0.01% $CaCl_2$), buffered with H_2SO_4 and NaOH to create a pH ranging from 1 to 8.

Environmental distribution of *T. thermacidophilus* cysts

Viable cysts were enumerated at six locations on the west side of BSL using the (Butler and Rogerson 1995)

Table 2. DGGE sequences of 2009 transect enrichment samples

Lane	Sample	*T* (°C)	Observed	Band #	PCR	BP	*I*%	*E*-val	Best BLAST match
A	TS-1 0 m	37	Heterolobose amebae, flagellates	1	+++	179	95	2E-73	*Acidomyce srichmondensis*
B	TS-1 3 m	37	Heterolobose amebae, flagellates	1	+++	197	92	4E-65	*Tetramitus thermacidophilus*
C	TS-1 9 m	37	Heterolobose amebae, flagellates, fungi	1	+++	199	93	9E-67	*Tetramitus thermacidophilus*
				2	+++	185	97	1E-69	*Trebouxiophyceae* sp., *Coccomyxa*
D	TS-1 34 m	37	Heterolobose amebae, flagellates, fungi	1	+++	203	93	1E-66	*Tetramitus thermacidophilus*
				2	+++	207	96	3E-77	Heterolobosea sp.
				3	+	210	82	5E-47	Heterolobosea sp.
				4	++	182	95	1E-74	*Trebouxiophyceae* sp., *Coccomyxa*
				6	++	184	97	3E-71	*Penicilliumpittii*
E	TS-1 46 m	37	Heterolobose amebae, flagellates	1	+	205	74	1E-09	*Lecanoromycetidae*
				2	++	185	97	9E-72	*Ochroconis gallopava*
				4	++	180	96	2E-68	Uncultured Sordariales clone
				5	+++	180	93	5E-69	Uncultured eukaryote isolate
				6	+++	180	93	2E-62	*Lopezaria versicolor*
F	WTA 2 m	37	Heterolobose amebae, flagellates	0	++	173	98	3E-71	*Acidomyces richmondensis*
				1	–	385	95	1E-63	*Penicillium pittii*

Lane	Sample	*T* (°C)	Band	PCR	BP	*I*%	*E*	BLAST Identity
I	A	37	1	++	186	90	9E-57	Uncultured ascomycete (Iron Mt. Mine)
			2	++	187	94	2E-63	*Ochroconis gallopava*
K	3	37	1	++	185	95	2E-67	*Acidomyces richmondensis*
			2	++	189	94	1E-65	*Aspergillus zonatus*
			3	++	187	95	3E-76	*Ochroconis gallopava*
L	3	13	1	+	186	98	1E-74	Uncultured fungi
			2	+	178	90	2E-57	*Phoma herbarum*
			3	+	191	97	9E-72	Uncultured fungi
			4	+	192	97	7E-73	*Acidomyces richmondensis*
M	4	37	1	++	185	91	5E-59	*Penicillium pittii*
			2	++	179	95	1E-65	Uncultured fungi
			4	++	192	96	4E-70	*Penicillium pittii*
N	4	50	1	+	187	81	5E-34	*Aspergillus* sp.
			2	+	188	86	2E-44	Uncultured fungi
			4	++	189	92	1E-69	*Galdieria partita/sulphuraria*
			5	+	186	87	5E-49	*Galdieria partita/sulphuraria*
			6	+	195	94	2E-53	Uncultured fungi
			7	++	185	92	3E-68	*Trebouxiophyceae* sp., *coccomyxa*

PCR = band strength (–, +, ++, +++); BP = PCR product length (bp); %*I* = identity. TS-1 = transect 1 (see Fig. 4), WTA = west transect A. See Supporting information for gel image and sequences.

method. Counting was performed by inoculating 24-well plates containing 2 ml BSL filtrate with PYE with an equal amount (mg) of suspended sediment, as determined by dry vs. wet weight. The wells were observed for positive or negative growth with an inverted microscope using Hoffman phase contrast optics (Olympus IX-70; 100–400X; America Inc., Center Valley, PA) after 14 d, and the presence or absence of amebae growth used to calculate viable cysts/cm^3 sediment present in the site sampled.

RESULTS

Enrichment of microbial eukaryotes from BSL

We set up enrichments from BSL water, sediments, and surrounding geothermal features. Samples were incubated with filtered BSL water, and all were provided 1–2 sterile wheat berries per 50 ml as a nutrient source. Within a few days of incubation at 27–50 °C, we observed a number of potentially phagotrophic microbial eukaryotes, including numerous amebae and small flagellates. The most frequent morphologies observed in the 37–45 °C range were limax amebae, including both vahlkampfiids (Schizopyrenidea), heterolobose amebae with pseudopodia produced in sudden bulges (Fig. 1a; Videos S1, S2**)**, whose flagellate life stages and cysts were also observed (Fig. 1b, c; Videos S2–S5), as well as lobose amebae (Fig. 1d–f). The heterlobose ameboflagellates were observed most frequently in enrichments directly from BSL water or lakeshore sediments, typically co-occurred with fungal conidia, and were especially numerous in culture flasks where macroscopic clearing of the sediment was observed (not shown). We also observed flagellates resembling *Bodo* sp. (Fig. 1g–i) in samples from cooler side pool features.

We used DGGE fingerprinting to determine diversity and complexity of environmental samples and enrichments. A total of 35 SSU rRNA V8 region bands (Fig. S1) were excised, re-amplified, and sequenced. Several phototrophic eukaryotes were detected: one sample incubated at 54 °C showed a number of bands corresponding to *Cyanidiales* (Table 2) and another showed chlorophyte *Chlamydomonas acidophila*, but most bands were similar to either fungi, nucleard, lobose or heterolobose amebae, or flagellates (Table 2). Although phylogenetic resolution was low, fungal rRNA seqeunces matched fungi later isolated from BSL, including *Aspergillus*, *Penicillium*, *Ochrochonis*, *Acidomyces*, and *Phialophora* (Ervin 2014), while heterolobose ameba bands showed high similarity to *T. thermacidophilus*, a valkampfiid taxon identified from similar acidic geothermal sites in Russia and Italy and published in 2009 (Baumgartner et al. 2009). A second DGGE PCR with 20-516GC primers to amplify the V3 region revealed the presence of diatoms, as well as possible cercomonads in a culture grown at 34 °C (not shown).

Cultures from transects and enrichments were also screened with group-specific primers for SSU and ITS rRNA. Targeted organisms included ciliates, kinetoplastids, cercozoans, and heteroloboseans (Table 1). The ciliate primers consistently failed to amplify, while the others gave strong bands in some samples (not shown). However, of those, only kinetoplastids were successfully sequenced to the target group (*Bodo* sp.), while cercozoan and heterolobosean JITS primers consistently amplified only fungal sequences. Upon further examination, we noted the JITS primers also matched ascomycete sequences, so we designed a new primer to specifically target the genus *Tetramitus* ITS region (Table 1). Both forward and reverse versions of this primer were constructed and used with other universal eukaryotic primers to achieve a 1,941 bp contiguous SSU-ITS rRNA sequence (Genbank accession #KM669765) that has 95–96% similarity to the *T. thermacidophilus* isolate CU8 (Baumgartner et al. 2009) as well as an uncultured Vahlkampfia sequence from Iron Mountain mine in nearby Redding, CA (Baker et al.

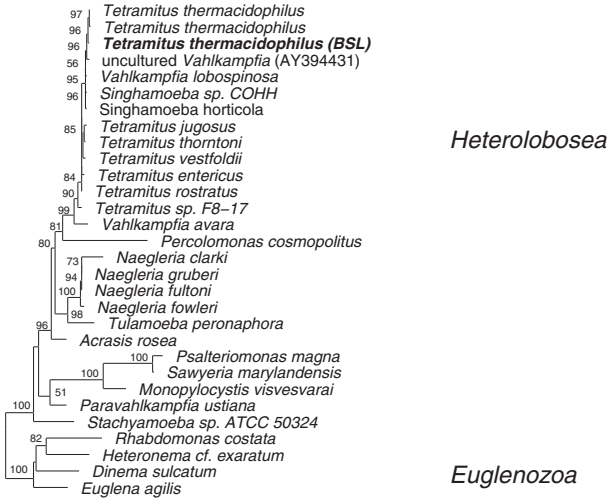

Figure 2 Alignment of *Tetramitus thermacidophilus* BSL 18S SSU with other Heterolobosea and Euglenozoa sequences. See Methods for details. Bootstrap analysis using RAxML are shown above the branches; only bootstrap values ≥ 50% are shown. Branch lengths are based on a scale of substitutions per site.

Table 3. Flagellates found before/after incubation at different temperatures

	Temperature (°C)		
Sample	13	37	40
BSL water from outflow site	–/–	–/–	–/–
Foam Sample + BSL filtrate #5	–/–	+/+	+/–
BSL Green Biofilm + BSL filtrate	+/+	+/+	+/–
Cyanidium biofilm from lake #9	+/–	+/–	+/+
Diatom Pool water and stick #10	+/–	+/+	+/–
Mud puddle sample (next to Green Pool) #8	–/–	+/+	+/+
W. Green Pool water (25 °C) #4	+/+	+/+	+/+

+ = presence; – = absence.

2009). Figure 2 shows an alignment of a 770-bp SSU rRNA sequence from our organism, hereby designated *T. thermacidophilus* strain BSL, with other Heterolobosea and Euglenozoa.

Isolation attempts

Repeated attempts to isolate heterolobose amebae, either from cysts, by plate walk-out, or from liquid dilutions, failed to separate contaminating fungi (Reeder IV 2011). On plate walk-outs, we were able to eventually free a lobose ameba with filose pseudopodia and large, irregular cysts, similar to *Acanthamoeba* sp., from contaminating fungi. The identity of this isolate was confirmed using *Acanthamoeba*-specific primers JDP (Table 1) (Edagawa et al. 2009). We were also able to isolate a limax amebae resembling *Hartmannella* sp., from warm (ca. 35 °C) forest soil above the south side of the lake after 5 d of incubation at 34 °C. This sample produced was later genotyped as *Hartmannella vermiformis*.

Efforts to enrich and isolate flagellates in filtered BSL water resulted in cultures resembling the flagellates *Bodo* or *Cafeteria* sp. Most of these were found in samples from cooler side pools from the western shelf, which are seasonally flooded by the lake in winter, but become isolated and gradually evaporate during summer (Table 3), and could be cultured successfully only at 37 °C or cooler. Of these, only *Bodo* was isolated from other protists and characterized genetically. Using the kinetoplastid 14F-2026R primers (Table 1), we amplified a ca. 2,000 bp PCR fragment (not shown). Sequences (ca. 760 bp) from both ends gave hits to *Bodo saltans*, but with only 91% identity, suggesting a novel species. A small scuticociliate was also occasionally observed in cooler samples, but was not successfully identified genetically or cultured (see Discussion).

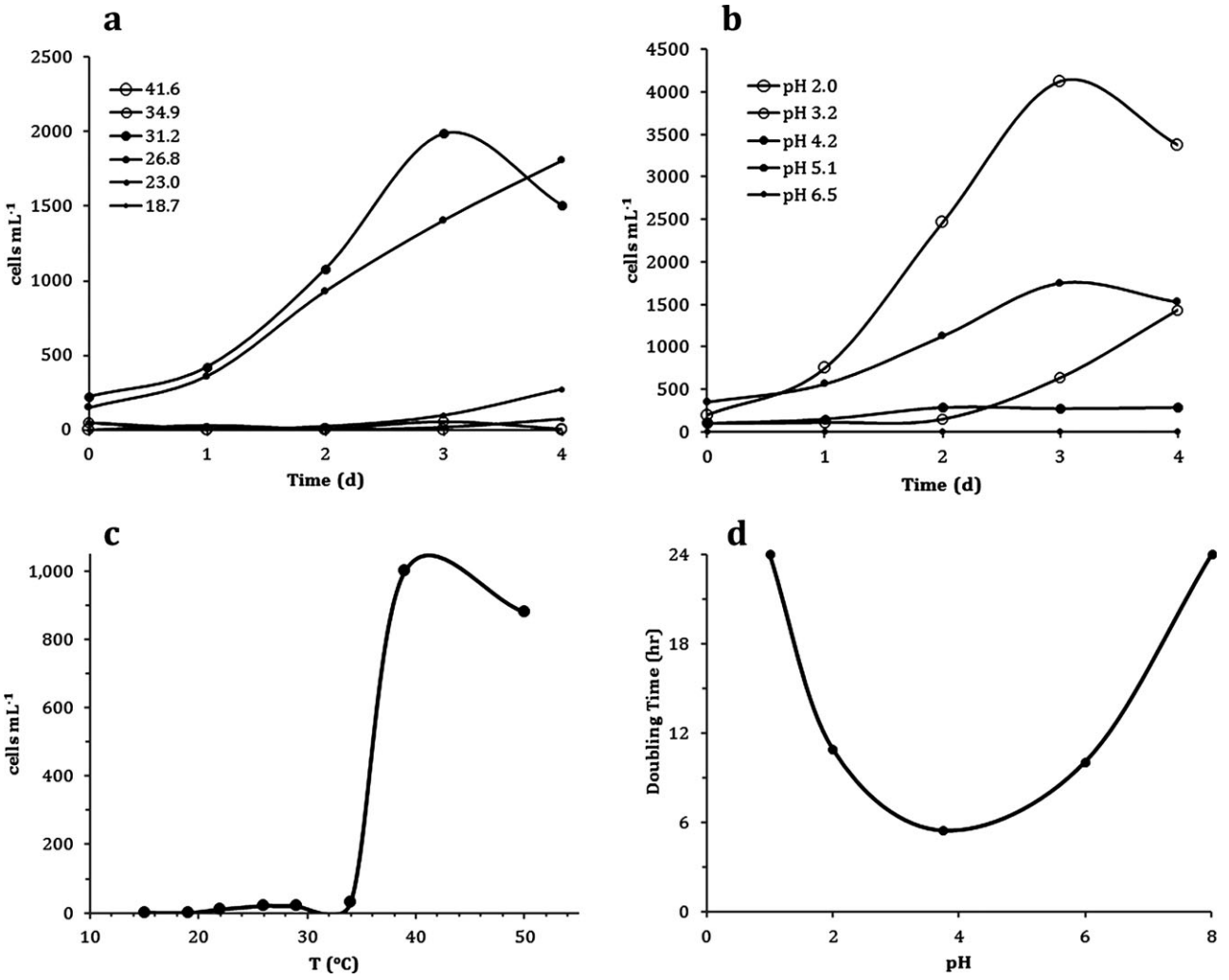

Figure 3 Growth vs. temperature and pH for BSL grazers. **a**, **b.** *Bodo* sp. **c**, **d.** *Tetramitus thermacidophilus* BSL. For temperature, 20 µl starter culture was added to 10 ml defined pH 2 media, and 200 µl *Micrococcus* feeder culture was added. Cells per 100 µl were counted after 48 h. For pH, the same setup was used, and cells per 100 µl were counted every 24 h for 4 d to find doubling times.

Growth at varying temperature and pH

The BSL *Tetramitus* culture was both thermo- and acidophilic, with a growth temperature range of 34–52 °C and an optimum of 40 °C, and a pH range of 2–6, with an optimum of pH 3.6 (Fig. 3a, b). Under optimal pH and temperature, the isolate grew with a doubling time of about 9 h. We not only observed mostly amebae but also flagellates (Video S4), although the factors that trigger the transition between these two life cycle stages are not known. Encystment could be induced by starvation or temperature above 52 °C, and we occasionally observed excystment into the ameboid form (Video S5). In support of this taxon being a true endemic grazer of BSL, DGGE analysis of transect samples showed the band associated with *T. thermacidophilus* strain BSL was strongest at the near lake region, but faded towards the forest (Fig. 4), while cysts were present at an average of 500 ml^{-1} at numerous sites along the western side of the lake (Fig. 4).

In contrast, all other cultures examined were not true acidothermophiles. *Acanthamoeba* and *Hartmannella* exhibited either acid tolerance or thermotolerance, but not both. *Acanthamoeba* was able to grow in both pH 2 BSL filtrate and neutral pH conditions. Growth initially occurred from 23 to 37 °C, with growth later observed as low as 4 °C (not shown). *Hartmannella* grew in distilled water, but not pH 2 BSL filtrate, and was capable of surviving temperatures ranging from 20 to 40 °C, suggesting it likely thrives in warm, neutral geological features. The kinetoplastid *Bodo* grew well from 27 to 31 °C, but not at 41 °C, and showed apparent biphasic pH growth (Fig. 3c, d). It grew best at pH 2, but also showed growth at pH 5.1, although less so at intermediate pH values, possibly reflecting a mixed culture population. All taxa were found in soil or water features surrounding BSL, and are likely restricted to cooler or less acidic sites.

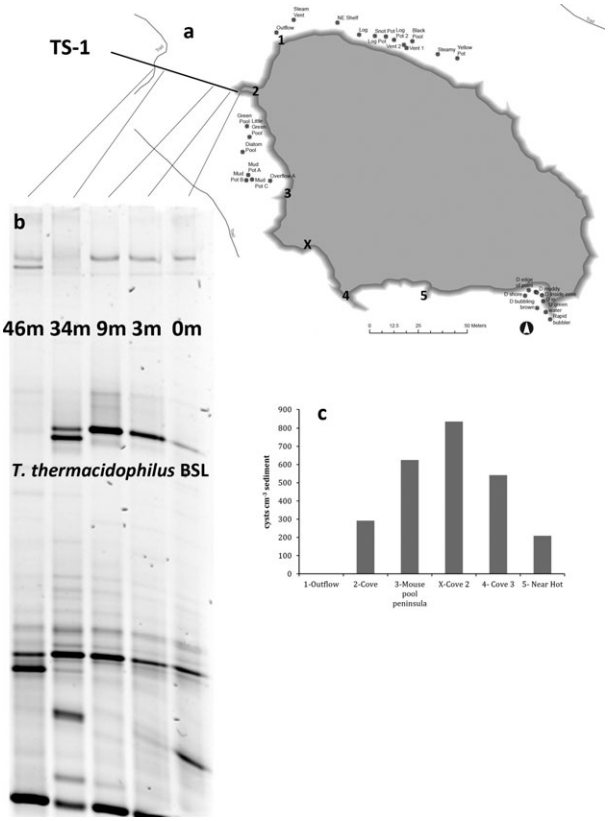

Figure 4 Distribution of *Tetramitus* near BSL. **a.** Map of BSL (courtesy Russell Shapiro, CSUC Geosciences) showing shoreline geothermal features, transect 1 (TS-1), and sampling sites for cysts. **b.** DGGE gel showing amplfiication of *Tetramitus thermacidophilus* BSL from samples along transect TS-1. Amplification is strongest 3–9 m from the lake, but also discernable in BSL water. **c.** Recovery of cysts from samples along the lake's western and southern shores.

Ameba feeding and ultrastructure

Tetramitus was initially observed ingesting *Phialophora* sp. conidia at 37 °C (Video S1, Fig. 5a). Although we initially suspected it to be mycophagous, after continued culturing amebae failed to remove *Phialophora* conidia despite their inability to grow at this temperature. Subsequently, TEM observations of *Tetramitus* with phagocytosized fungal conidia (Fig. 5b, c), showed most fungal conidia occurring singly, often enclosed by a minimal food vacuole, and appeared to be intact, with clearly visible septa. These all suggest an inability to digest conidia.

To evaluate potential prokaryotic prey in the cultures, we amplified the 16S rRNA V3 region using the bacterial 341GC/534 primer pair and used DGGE to separate amplicons. Sequencing indicated the presence of taxa known to be present in BSL and other AMD/geothermal sites, including the α-Proteobacteria *Acidiphilium* sp. and *Acetobacteraceae*, and the Firmicutes *Alicyclobacillus/Sulfobacillus* (Table 4). Conspicuously absent was the dominant chemoautotroph of BSL, the Actinobacterium *Acidomicrobium*. This may be in part because *Tetramitus* enrichments readily grazed this prey, as TEM observations of *Tetramitus* feeding on the Actinobacterium *Micrococcus* showed copious food vacuoles with 1–4 prey cells in multiple stages of digestion (Fig. 6). Detailed ultrastructure (Fig. 7) revealed typical heterolobosean structures such as tubular mitochondria, and lipid bodies surrounded by ribosomes, similar to those observed for or *Selenaion* (Park et al. 2012) or *Vahlkampfia* (Garstecki et al. 2005).

Acanthamoeba cultures showed typical ultrastructure and cysts, with a variety of ingested prey, including cyst (Fig. 8a), suggesting the potential for cannibalistic feeding. Intriguingly, possible Nucleocytoplasmic Large DNA Virus (NCLDV) particles were observed in several vegetative cells, hexagonal in shape and ~500 nm in size (Fig. 8), consistent with previous descriptions of *Mimivirus* and other NCLDVs found in *Acanthamoeba* (Boyer et al. 2009; La Scola et al. 2003). We attempted to confirm this by

PCR amplification, using four primer sets targeting relatively conserved regions of the *Mimivirus* genome. Two primer sets targeting a *Mimivirus* helicase and thiol oxidoreductase (Dare et al. 2008) gave no amplification, but the third pair, CP D13L, which targets the capsid protein (Azza et al. 2009), produced bands in all three mixed flagellate

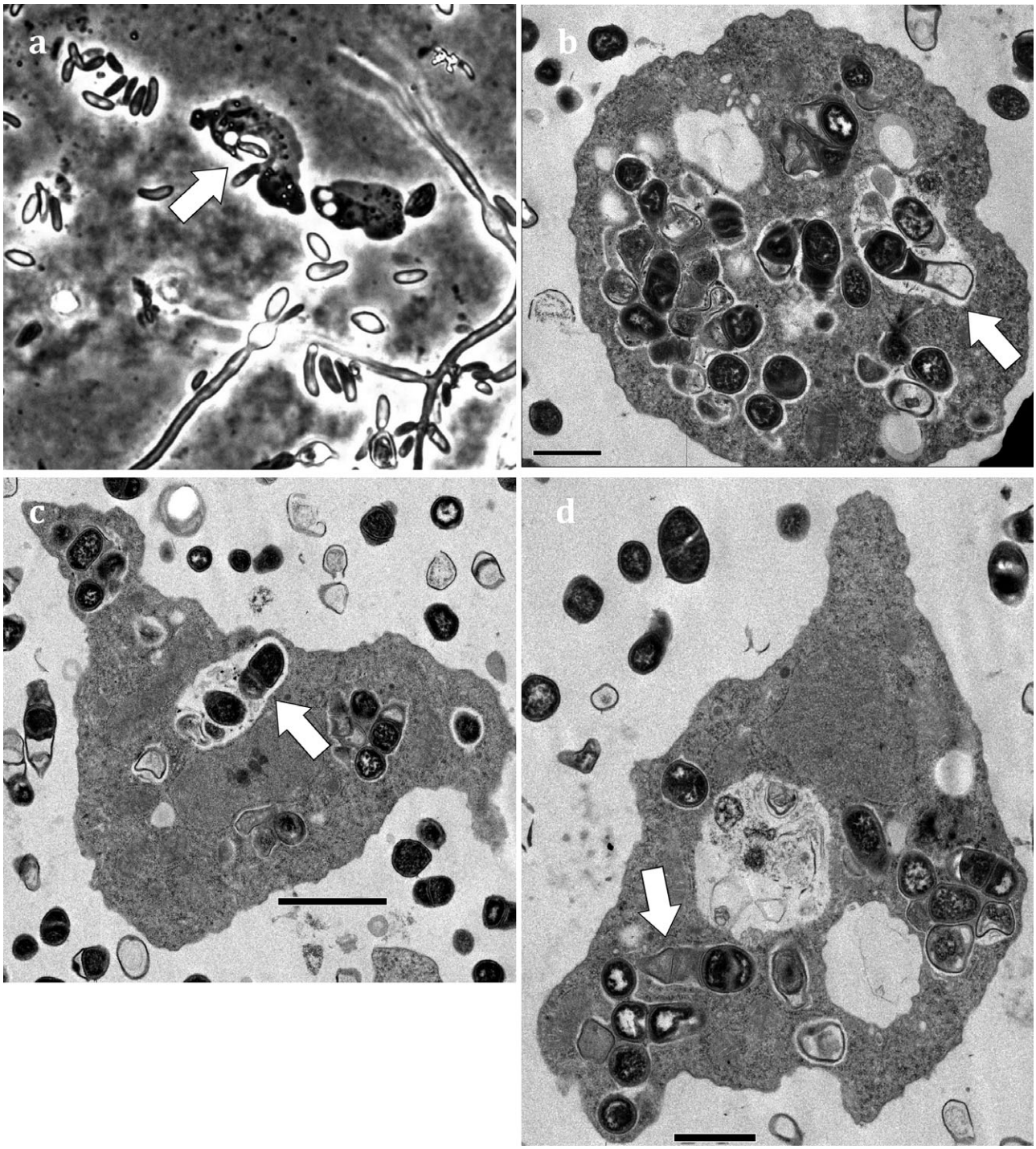

Figure 5 Observations of *Tetramitus thermacidophilus* strain BSL ingesting *Phialophera* conidia. **a.** phase contrast (400X); **b–d.** TEM showing gross ultrastructure. Ingested conidia are denoted by arrows. Note preservation of shape and conidial wall, even when in well-formed food vacuoles (b, c). Scale bars: (a) 10 μm; (b–d) 1 μm.

Table 4. Identification of prokaryotes in vahlkampfiid amebae enrichments following 16S rDNA V3 amplification and DGGE fingerprinting. See Supporting information for sequences

Sample	Culture	Food source	Closest BLAST hit (%identity)
α-*Proteobacteria*			
5a, 8a	F-3	BR002a, 9	*Acidiphilium* sp. clone CO_3 (95)
5b	F-3	BR002a, 9	*Acidiphilium* sp. BGR 75 (95)
2a	L-WD#2		*Acidiphilium* sp. DX1-13 (87)
14c			*Acidobacterium* sp. pan6 (96)
7a	F-2, F-9	SB-1	*Acetobacter* sp. clone HHband7 (79)
1a	LWD# EF		*Acetobacteraceae* sp. clone OTU0280 (94)
8b	F-3	BR009	*Acetobacteraceae* sp. clone OTU0280 (93)
10c	F-5	SB-2	*Acetobacteraceae* sp. clone OTU0569 (81)
10a	F-5	SB-2	*Rhodospirillaceae* sp. clone 9670 (90)
10b	F-5	SB-2	α-Proteobacterium clone SS31 (83)
Firmicutes			
6a	F-1	SB-3	*Alicyclobacillus* sp. (96)
14b	F-9	SB-1	Firmicutes, including Fe-oxidizing acidophile, *Alicycolbacillus* sp. (96)

cultures, while the fourth primer pair, MCP, which also targets the major capsid protein (Larsen et al. 2008), gave positive bands in *Acanthamoeba* sp. and *Hartmannella* sp. samples, as well as all flagellate cultures. However, when sequenced, none of the amplicons were similar to *Mimivirus* or other NCDVs.

DISCUSSION

Heterolobosea: key grazers in extreme environments?

Only the ameboflagellate *T. thermacidophilus* strain BSL was able to grow near the lake's typical temperature of 48–52 °C in our enrichment cultures. The lobose ameba *Acanthamoeba* sp., a cosmopolitan soil taxa that has been detected genetically in Yellowstone geothermal features (Sheehan et al. 2005a), was only able to tolerate temperatures up to 35 °C. In contrast, *Hartmannella* sp. was unable to multiply at low pH but was thermotolerant, and has been detected in hospital hot water systems from 24.7 to 59.5 °C (Rohr et al. 1998). The other consistently observed taxon was the kinetoplastid *Bodo* sp., which was detected in a previous genetic screen (Brown and Wolfe 2006), and inhabits cooler acidic AMD sites such as the Rio Tinto in Spain (Aguilera et al. 2007) and the Berkeley Pit Mine in Montana, USA (Mitman 1999). But it was unable to tolerate temperatures above ~31 °C (Fig. 3a).

Therefore, based on isolation attempts, BSL's grazing community appears to be dominated by a single heterlobosean taxon, which is genetically and phenotypically very similar to *T. thermacidophilus* isolated from volcanic geothermal sites in Italy and Kamchatka (Baumgartner et al. 2009). Previous studies have revealed a variety of heterolobose ameboflagellates (*Naegleria*, *Fumarolamoeba*) at volcanic acidothermal sites (de Jonckheere et al. 2011a; Sheehan et al. 2003a). Other heterolobosea occupy a range of extreme environments, including temperatures of 50–55 °C (Baumgartner et al. 2003; de Jonckheere et al. 2009, 2011b) or near freezing (Robinson et al. 2007),

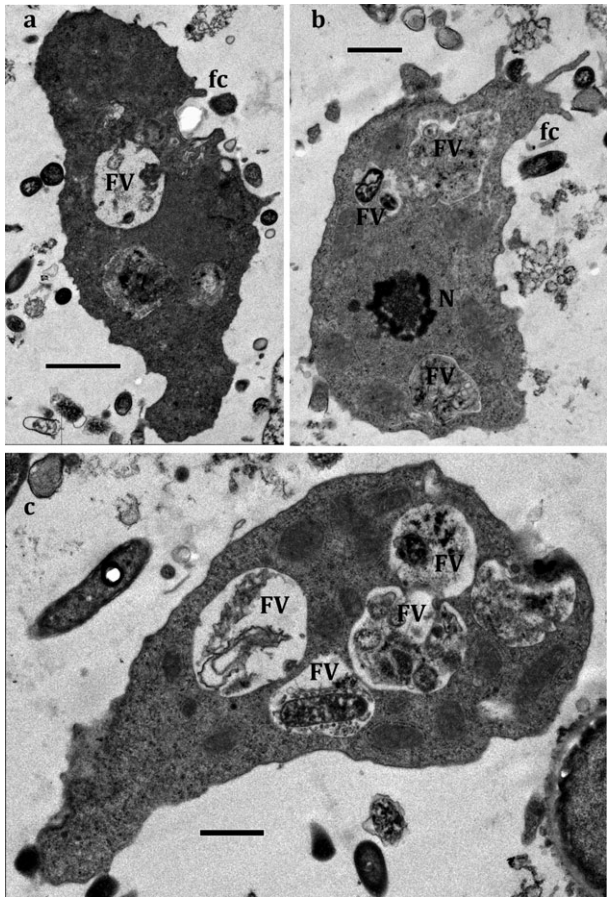

Figure 6 Gross ultrastructure of *Tetramitus thermacidophilus* strain BSL feeding on *Micrococcus* sp. and other bacteria. N = nucleus; FV = food vacuole; fc = feeding cup. Scale bars: (**a**) 2 μm; (**b**, **c**) 1 μm.

high-radiation (Zirnstein et al. 2012), high-salinity (Park et al. 2007, 2012), or low-oxygen (O'Kelly et al. 2003; Pánek et al. 2012; Smirnov and Fenchel 1996). This sug-

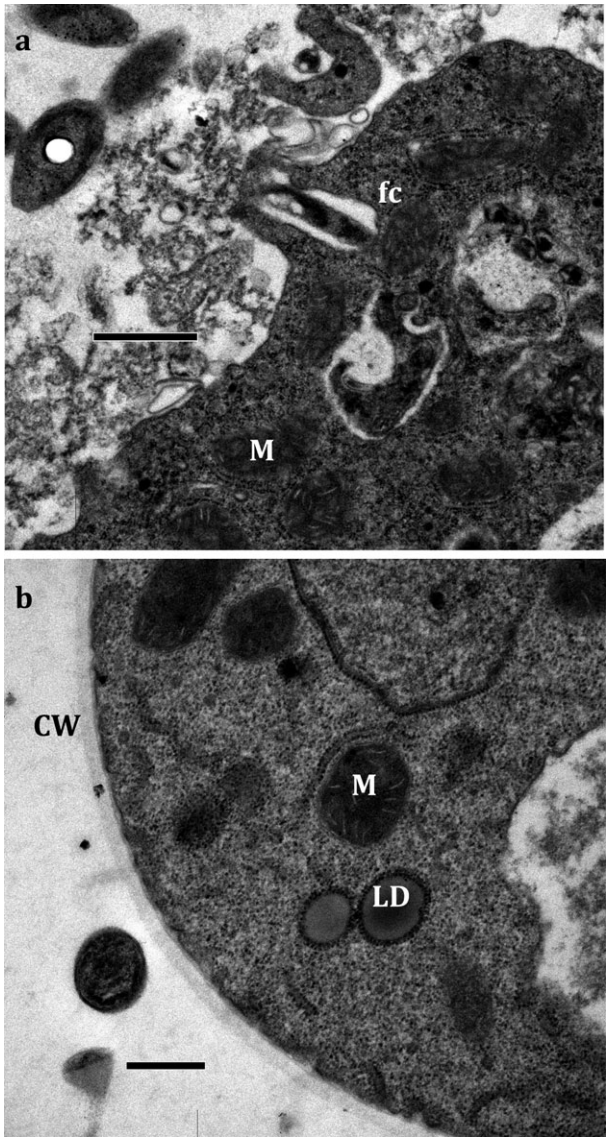

Figure 7 Fine ultrastructure of *Tetramitus thermacidophilus* BSL: **a**. ameboid cell with engulfed bacterium; **b**. cyst. fc = food cup; M = tubular mitochondria next to ER leaflets; CW = cell wall; LD = lipid droplet surrounded by ribosomes. Scale bars: (a) 1 μm; (b) 500 nm.

gests this group may be unusually successful consumers in extreme environments. Most thermal sites are also low in oxygen, and we were unable to grow our culture on solid media. However, this was not due to oxygen, as we observed no success on plates under microaerophilic or anaerobic conditions.

Comparison with other acidic and/or thermal environments

There have been surprisingly few studies of microbial food webs in natural geothermal environments, especially those that are also acidic. The closest analog to Lassen's geothermal sites, both physically and geochemically, is Yellowstone National Park (YNP; USA), in particular, Nymph Creek, which is slightly less acidic (pH 2.7) and is a shallow feature with a pronounced temperature gradient. Although published studies of YNP protists have been limited to specific taxa (e.g. *Naegleria*, Sheehan et al. 2005a), a collection of Nymph Creek protist images at the Micro*-Scope web site (http://pinkava.asu.edu/starcentral/microscope/portal.php?pagetitle=collectiondetails{00AMP00}collectionID=83{00AMP00}page=5) shows many images of taxa similar to those we observed in BSL enrichments, especially at cooler temperatures (Fig. 9).

In contrast, there are many studies of the protist food webs in acid mine sites. These include AMD sites, typically extremely acidic with very high concentrations of leached heavy metals, but usually in the 20–40 °C range. The best-studied of these include the Rio Tinto in Spain's Iberian pyrite belt, which has a high diversity of microbial eukaryotes (Aguilera et al. 2006, 2007; Amaral Zettler et al. 2002). *Tetramitus thermacidophilus* strain BSL is genetically very similar to a uncultured Vahlkampfiid from Iron Mountain mine, about 75 km west of LVNP (Baker et al. 2003, 2004, 2009). Both sites appear to have important bacterial and fungal components, including the highly acidophilic ascomycete *Acidomyces richmondensis* (Baker et al. 2004; Ervin 2014). Cooler acid environments, such as the lakes formed from coal mining sites in the former East Germany, include a greater diversity of protists, including heliozoans and mixotrophic chrysophytes (Packrof and Woelfl 2000). Therefore, temperature, rather than low pH, seems to be the limiting factor in BSL and other acidic environments, and the combination of the two likely imposes severe restrictions on diversity (Moser and Weisse 2011). How low pH or high temperature affect food web structure and function is still poorly known, but Gaedke and Kamjunke (2006) suggest that extreme environments have truncated food webs with relatively few functional groups. However, we note that there may still be BSL grazers that escaped cultivation and genetic detection. It took many efforts to detect *T. thermacidophilus* by PCR amplification, despite its dominance in enrichments, and we could have missed less-abundant taxa. Recently, 454 sequencing of 18S V9 rDNA from our BSL enrichments also failed to detect observed heterolobosea, but confirmed abundant *Dothideomycetes* and *Bodo* sp. (Dr. Bass, D., personal communication). Strangely, the most abundant taxon sequence was a Perkinsid, a sister group to dinoflagellates best known as marine endoparasites of mollusks. Furthermore, high-throughput sequencing of community rDNA or metagenomics would likely detect additional taxa.

Grazing impact of protists in BSL

The diversity of BSL prokaryotes is also low, with the chemolitotrophs *Acidimicrobium* and *Hydrogenobacter* dominating this highly oligotrophic environment (Arroyo

et al. 2015). Several BSL *Acidimicrobium* isolates have recently been described (Arroyo et al. 2015), but were not available at the time of our study for feeding trials, and we can only hypothesize it is also the main prey source in BSL, as Baumgartner et al. (2009) found their *T. thermacidophilus* isolate grazed on *Acidimicrobium,* and the BSL *Tetramitus* thrived on the Actinobacterium *Micrococcus*. Although BSL's prokaryote cell number and biomass are

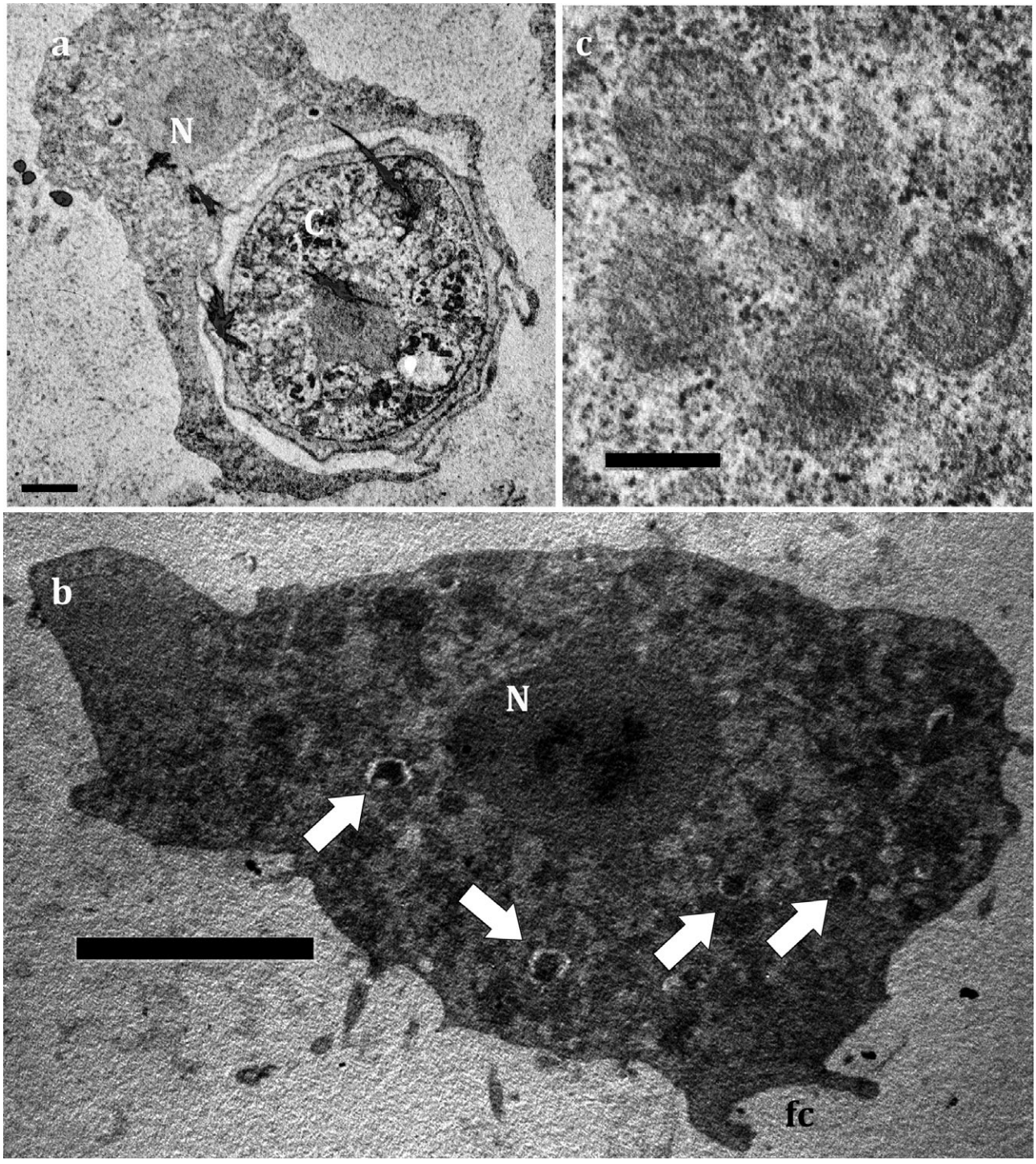

Figure 8 Gross and fine ultrastructure of *Acanthamoeba* sp. **a**. Cell engulfing *Acanthamoeba* cyst; **b.** low-magnification image of vegetative cell showing cytoplasmic inclusions resembling NCLDVs (arrows). C = cyst; N = nucleus; fc = feeding cup. **c**. High-magnification image of NCLDVs, showing hexagonal structure. Scale bars: (a) 2 μm; (b) 5 μm; (c) 500 nm.

low, many unusual or extreme environments are dominated by high biomass of chemoautotrophic prokaryotes, which are understudied as prey of protist grazers. The other major primary producers in BSL, the rhodophyte *Cyanidiales*, are common to many thermo-acidic environments (Lehr et al. 2007; Toplin et al. 2008) and were easily cultured and detected genetically in our warmer enrichments (Table 2). Although there is a report of *Naegleria* consuming cyanobacteria (Xinyao et al. 2006), we never observed chlorophyll-containing cells inside *Tetramitus*. However, we did occasionally observe testate amebae with ingested photoautotrophic *Cyanidiales* (Fig. 9). Some nucleariid amebae are also known to be algivorous (Amaral Zettler et al. 2001; Arndt 1993), and there is at

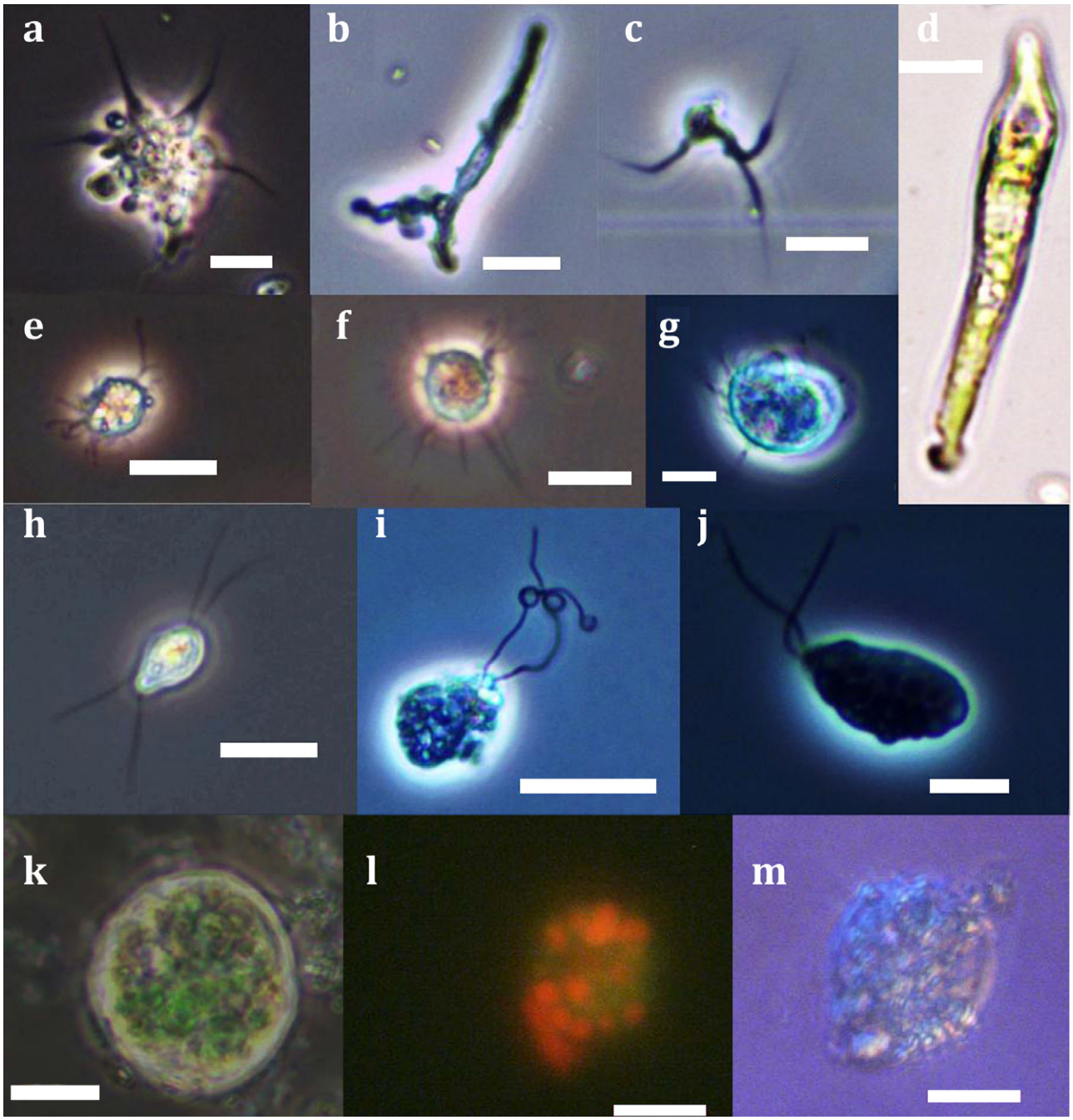

Figure 9 Other protist morphologies observed occasionally from BSL enrichments. **a–c.** uknown; **d.** euglenid; **e**, **f**. possible nucleariid amebae; **g.** scuticociliate; **h–j.** possible cercozoans or unknown flagellates; **k–m**. Testate ameba with ingested *Cyandiales* (k: brightfield; l: fluorescence; m: DIC). Size bars = 10 μm.

least one report of an isolate from moderate geothermal environments (30 °C) (Yoshida et al. 2009). These results suggest that there might still be other ameboid grazers in the BSL geothermal system that prey on photoautotrophs.

Recently, we also observed that additions of dissolved or particulate organic amendments results in the replacement of chemolithoautotrophs by heterotrophic *Firmicutes* such as *Alicyclobacillus*, which likely survive as endospores, or *Acetobacteraceae* (*Proteobacteria*), which are in part introduced from leaf litter (Wolfe et al. 2014). These taxa were detected genetically in our protist enrichments (Table 4), which were likely fueled by organics leaching from wheat berries. Clearly, heterotrophs can also sustain growth of *Tetramitus,* and these grazers may exert top-down pressure on growth of heterotrophs from BSL's highly episodic allochothonous C inputs.

Protists as possible refugia in extreme environments

Leaf litter inputs to BSL can also result in germination and growth of ascomycete fungi (Arroyo et al. 2015), although only at winter-time temperatures (Ervin 2014). There are a few reports of mycophagus amebae (Chakraborty and Old 1986; Ogden and Pitta 1990; Vohník et al. 2011) or flagellates (Ekelund 1998; Hekman et al. 1992), but none to our knowledge in extreme environments. Although we observed mycophagy of *Philaphora* conidia by *Tetramitus*, unlike ingested bacteria, fungal conidia appeared undigested and not always packaged into food vacuoles. Furthermore, *Phialophora* was later found to grow only below 30 °C (Ervin 2014), suggesting it could not proliferate in amebae cultures incubated at 37–45 °C. Although, we were consistently unable to purify the amebae away from the fungus, suggesting *Tetramitus* was not reducing conidia numbers. This raises the possibility that *Phialophora* conidia are ingested, but not used as food. The only known fungus to actively resist grazing by amebae is *Cryptococcus neoformans* (Greub and Raoult 2004; Steenbergen et al. 2001), which is ingested but later exocytosized from *Acanthamoeba* (Chrisman et al. 2010). Although we have not detected this taxon (or any yeasts) in BSL, it has been detected in several acidic sites (Gadanho and Sampaio 2004, 2006; Gadanho et al. 2006; Kawai et al. 2000; Russo et al. 2008) where similar amebae are found. We also do not know to what extent the spores or conidia of other endemic BSL fungi (*Acidomyces, Ochroconis*) are used as food. Interestingly, those fungi, like *Phialophora*, are highly melanized, which may confer grazing resistance (Hekman et al. 1992) and/or pathogenicity (Revankar and Sutton 2010).

Although we did not observe undigested bacteria in BSL *Tetramitus*, amebae are renowned for acting as refugia for some prokaryotes (Greub and Raoult 2004). A genetic screen of the heterolobosean *Naegleria* from Nymph creek in YNP also detected the intracellular pathogen *Legionella* (Sheehan et al. 2005a,b). This γ-*Proteobacterium* has been found in *Hartmannella* (Kuiper et al. 2004; Kwaik et al. 1999), but is particularly associated with *Acanthamoeba*, and both have been detected in YNP thermo-acidic environments (Sheehan et al. 2005a). Baker et al. (2003) detected *Rickettsiales* (α-*Proteobacteria*) sequences in the extremely low pH environment of Iron Mountain mine. They estimated from fluorescent in situ hybridization that about 4% of eukaryotes contained similar taxa, designated *Candidatus Captivus acidiprotistae*, which they suggested might take advantage of the near neutral intracellular pH of amebae. While we did not observe intracellular bacteria symbionts in the BSL *Acanthamoeba* isolate, we did observe NCLDV-like particles. We were unable to confirm these by PCR of *Mimivirus*-specific genes, but *Mimivirus*-like sequences have been detected from a BSL viral metagenome (Stedman, K., personal communication). If such NCLDVs are confirmed, this would be the first instance of a eukaryotic virus from a thermoacidic environment, or any extreme environment.

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SUPPORTING INFORMATION

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

Figure S1. DGGE gel image of 18S rRNA PCR, with bands labeled. BSL 16S DGGE band sequences 4-09.docx, BSL 18S DGGE band sequences 11-09.docx: sequences for DGGE analysis (FASTA format) of 16S and 18S rRNA from enrichments and BSL samples.
Videos S1–S5. Time-lapse of *Tetramitus thermacidophilus* strain BSL taken by William Reeder (WR) or Marissa Hirst (MH).
Video S1. Limax ameboid form ingesting *Phialophera* conidia (400X; phase contrast; WR).
Video S2. Limax ameboid form showing pseudopodial extrusion (400X; DIC, MH).
Video S3. Limax ameboid form showing cell division (400X; DIC, MH).
Video S4. Flagellate form showing two polar flagella (400X; DIC, MH).
Video S5. Cyst germinating to ameboid form (400X; DIC, MH).