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Journal

Cell, 189(20)

ISSN

0092-8674

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Publication Date

2026-10-01

DOI

10.1016/j.cell.2026.08.043

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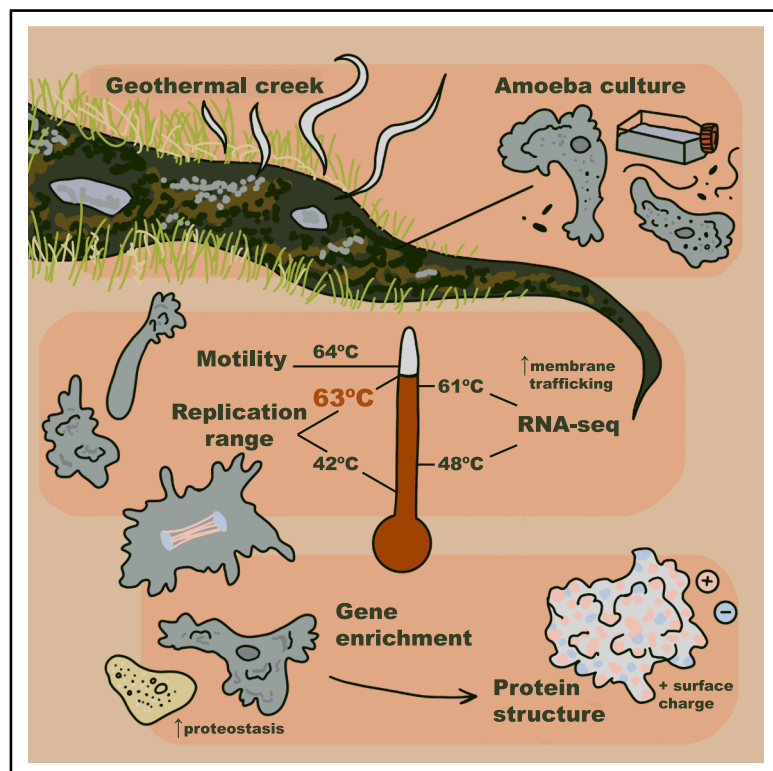
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A geothermal amoeba sets a new upper temperature limit for eukaryotes

Graphical abstract



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In brief

The amoeba *Incandiamoeba cascadiensis* demonstrates that eukaryotic life can withstand temperatures beyond what was thought possible and sheds light on molecular strategies for survival in extreme heat.

Highlights

- *Incandiamoeba cascadiensis* is a novel geothermal amoeba that grows at 63°C
- This organism establishes higher thermal limits for eukaryotes
- Proteostasis, membrane trafficking, and DNA repair are upregulated with temperature
- Protein surface chemistry is convergent with strategies in prokaryotic thermophiles



Short article

A geothermal amoeba sets a new upper temperature limit for eukaryotes

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<https://doi.org/10.1016/j.cell.2026.08.043>

SUMMARY

The study of temperature limits has transformed our understanding of life's boundaries but has focused on bacteria and archaea. For decades after their discovery, no eukaryote was shown to replicate above 60°C. We isolated a geothermal amoeba, *Incendiamoeba cascadiensis*, that divides at 63°C and is motile up to 64°C. We identified an enrichment of genes related to proteostasis and environmental sensing relative to mesophilic amoebae. Comparative RNA sequencing of *I. cascadiensis* revealed upregulation of pathways related to proteostasis, DNA repair, and membrane trafficking at high temperatures. Predicted proteins of *I. cascadiensis* have distinct biophysical properties, including enrichment of positively charged surface residues similar to bacterial and archaeal hyperthermophiles, which suggests a convergent mechanism for protein stability under temperature stress. Together, our findings expand our understanding of where and how life can persist.

INTRODUCTION

The thermal limits of life have long been a source of scientific fascination and discovery. By studying the physical factors at the limits of life, we can broaden our understanding of constraints on cellular biology and of where life can emerge and persist. Temperature regulates life from enzyme stability to cellular functions and metabolic processes, to the distribution of species across ecosystems,^{1,2} and impacts physical parameters such as pH, viscosity, density, and solubility.¹ Characterization of bacteria and archaea that inhabit high-temperature environments such as geothermal springs and hydrothermal vents has uncovered some of Earth's most metabolically and phylogenetically diverse organisms, including most of the candidate archaeal phyla.^{2,3}

Thermophiles are organisms with high thermal limits and optimal growth at or above 45°C.⁴ Thermal limits are defined by three thresholds: completion of the life cycle (e.g., cellular

replication), metabolic activity (e.g., motility and feeding), and survival.¹ The highest known temperature threshold is 122°C, at which only the archaeon *Methanopyrus kandleri* can grow.⁵ Among bacteria, *Geothermobacterium ferrireducens* can grow at temperatures up to 100°C.⁶ These growth temperatures are remarkable given the challenges associated with thermal stress, including increased likelihood of protein and other biomolecule denaturation and impacts on membrane fluidity.^{2,7}

Surprisingly, parallel efforts to characterize eukaryotic thermophiles and define upper temperature thresholds remain extremely limited.^{8–11} The upper temperature limit of growth commonly cited for eukaryotes is 60°C, supported by a few species of fungi and red algae.^{12–17} Other eukaryotes have been documented growing up to 57°C, including the amoeba *Echinamoeba therrum*,¹⁸ but the majority of eukaryotic diversity in high-temperature environments remains unexplored.⁸ Thermophilic red algae and fungi offer insight into adaptations including coordinated signaling pathways for efficient proteostasis and chaperone



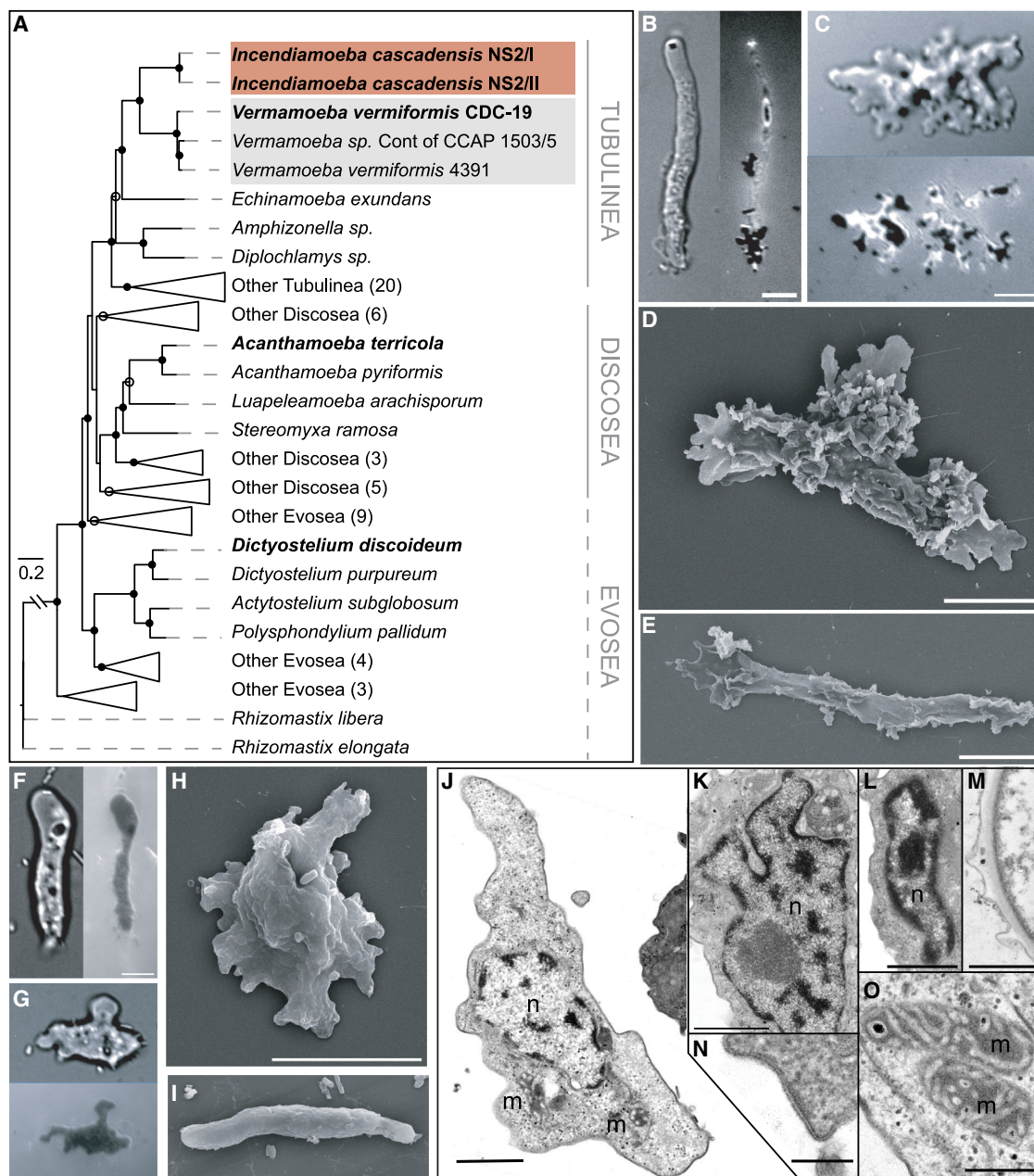


Figure 1. Establishment of *Incandiamoeba cascadiensis*, a novel genus and species within Amoebozoa

(A) Concatenated tree of 102 Amoebozoa genes; filled circles indicate 100% bootstrap support, open circles > 90%. *I. cascadiensis* (orange), *Vermamoeba vermiformis* (gray), bolded species used for genome comparison.

(B and C) Differential interference contrast microscopy (DIC) and corresponding reflection interference contrast microscopy (RIFM) of *I. cascadiensis* in (B) vermiform and (C) amoebiform state.

(D and E) Scanning electron microscopy (SEM) of *I. cascadiensis* in (D) amoebiform and (E) vermiform state.

(F and G) DIC and corresponding RIFM of *V. vermiformis* in (F) vermiform and (G) amoebiform state.

(H and I) SEM of *V. vermiformis* in (H) amoebiform and (I) vermiform state.

(J–O) Transmission electron microscopy (TEM) ultrastructure of *I. cascadiensis*.

(J) Overview of trophozoite nucleus (N) and mitochondria (M).

(K) Lobate nucleus with dispersed chromatin aggregates.

(L) Nucleus with chromatin aggregates beneath the nuclear envelope.

(M) Bilayered cyst wall.

(legend continued on next page)

production.^{19,20} However, both the molecular mechanisms that could limit growth and the true upper temperature limit for eukaryotic cells remain unknown. Although 62°C has been hypothesized as an upper bound based on a presumed lack of organelle membrane thermostability, this threshold was derived from limited samples that did not yield fungal growth above 60°C.¹²

Here, we report the discovery of an amoeba isolated from Lassen Volcanic National Park (LVNP; California, USA) and (1) describe its evolutionary placement, morphology, and biogeography; (2) determine its maximum temperature; and (3) shed light on strategies used to protect structure and function in extreme heat. Our findings establish higher upper temperature limits for cellular replication and motility in eukaryotes.

RESULTS

A novel genus of amoeba cultivated from geothermal springs

We isolated an amoeba from 14 out of 20 geothermal sampling locations over a 3-year period (2023–2025) along a tributary of Hot Springs Creek in LVNP. This tributary is one of the few non-acidic features in LVNP, a vapor-dominated acid-sulfate thermal system.^{21,22} Microbial communities at Boiling Springs Lake, an acid-sulfate geothermal feature 1 km from this study site, have been characterized,^{22,23} but this tributary was not included in previous efforts. Tributary temperatures ranged from 49°C–65°C and pH from 6–7 (Figures S1A–S1C; Table S1), both highly stable over decades.²¹ Samples yielding the amoeba were from water, biofilm, and sediment. To obtain cultures, we enriched samples with a wheatberry to stimulate growth of the *in situ* bacterial community, which in turn enriched growth of the bacterivorous amoeba (Figure S1D). Within 1 to 2 weeks post-enrichment, amoebae were visible from incubations at 57°C and 60°C, and within 3 weeks at 63°C. We recovered the amoeba each year but used strain NS2, isolated in 2023 from site S2, in all experiments and sequencing, and we designate NS2 as the type strain. In 2025, the amoebae were recovered directly at 63°C and have been maintained in a 63°C incubator for months. We also isolated a strain of *Vermamoeba vermiformis* likely present as cysts from site S5, incubated at room temperature (Table S1), and used it for morphological comparison.

To determine the phylogenetic placement of this amoeba, we obtained two replicate near-complete genomes from strain NS2 and used the phylogenomics pipeline EukPhylo.²⁴ We produced a phylogeny from a multi-sequence alignment of 102 concatenated genes. The amoeba falls within Tubulinea (Amoebozoa) (Figure 1A) in the clade Echinamoebida, which notably contains *Echinamoeba*, the genus with the current highest reported amoeba growth temperature. The amoeba is sister to *V. vermiformis*, a thermotolerant amoeba commonly isolated from hot water heaters but capable of growth at room temperature. *Vermamoeba vermiformis* has an upper growth

limit near 40°C–45°C.^{25,26} The 18S rRNA tree supports this placement (Figure S2), and the 18S rRNA sequence of the novel amoeba is 94% identical to *V. vermiformis*, in contrast to less than 1% 18S sequence divergence within the *Vermamoeba* genus.²⁷ The branch length distance between the novel amoeba and *V. vermiformis* fell outside a 95% confidence interval constructed from six other within-genus pairs of species (Table S2). Coupled with the morphological and ecological evidence described below, we propose the amoeba as a new genus, *Incendiamoeba* (Latin *incendarius* meaning igniting fire, “fire amoeba”) and species *cascadensis*, referring to the Cascade mountain range where it was discovered.

To determine whether *Incendiamoeba* has been detected from environmental sequencing efforts, we searched 31,093 publicly available metagenomes from diverse environments globally (JGI IMG). We found an identical 18S rRNA gene sequence (Figure S2) in a geothermal microbial mat in the Taupō Volcanic Zone (Waikite Valley, NZ), which was at 62°C near the source.²⁸ Three metagenomes from Yellowstone National Park (USA; reported temperatures 42°C–90°C) contained sequences in the same clade, two of which form a distinct subclade that may represent additional species-level diversity. *Incendiamoeba* sequences were detected exclusively in geothermal areas, which suggests that their distribution is restricted to high-temperature areas.

We characterized the morphology of *I. cascadensis* (Figures 1B–1E) compared to *V. vermiformis* (Figures 1F–1I) using high-temperature light microscopy and scanning electron microscopy and examined *I. cascadensis* ultrastructure with transmission electron microscopy (Figures 1J–1O). We identified two distinct morphological forms: an elongated, vermiform (worm-like) state similar to that previously described for *V. vermiformis* (Figures 1B and 1E) and a more compact, amoebiform state, characterized by pseudopodia (Figures 1C and 1D). *I. cascadensis* are typically uninucleate and, in rapidly moving vermiform cells, the nucleus appears biased toward the direction of motion (Video S1). *I. cascadensis* are $38.7 \pm 9.4 \mu\text{m}$ in the vermiform state and $23.5 \pm 7.9 \mu\text{m}$ in the amoebiform state (Table S3; see taxonomic appendix in Data S1). Although *V. vermiformis* and *I. cascadensis* cells exhibit comparable motility and switch between similar vermiform and amoebiform morphologies (Videos S2, S3, and S4), the use of reflection interference contrast microscopy (RICM) reveals differences in the extent of surface contact. *I. cascadensis* form smaller and more transient surface attachments than *V. vermiformis* (Figures 1B and 1C compared to Figures 1F and 1G; Videos S2, S3, and S4). Thus, while motility and morphology appear similar between the two species, the molecular and biophysical details may differ.

We observed *I. cascadensis* preferentially feeding on filamentous bacteria (Video S5), during which they employed a protrusive, phagocytic structure that envelops the prey (Figure S3, blue) and exerts forces sufficient to bend and fold the bacterial cells, forming tight loops inside the amoeba (Figure S3, pink;

(N) Cell surface of trophozoite.

(O) Mitochondria with non-branching tubular cristae.

Scale bars in (B)–(I) are 5 μm . Scale bars in (J)–(M) are 1 μm , (N) is 200 nm, and (O) is 500 nm.

See also Figures S1–S3; Tables S1–S3 and S4; and Videos S1, S2, S3, S4, S5, and S6.

Video S6). To characterize the bacterial community in culture with *I. cascadiensis*, we sequenced the strain NS2 metagenome. We recovered 17 bacterial species-level genomes (Table S4), 11 of which represent putatively novel species based on relative evolutionary divergence. Notably, *Meiothermus ruber*, a globally distributed filamentous bacterium found in geothermal environments,²⁹ comprised 41.34% of all reads (Table S4). The growth range of *M. ruber* spans 35°C–70°C,²⁹ which exceeds the thermal range of *I. cascadiensis* and indicates that bacterial food availability is unlikely to restrict the amoeba's thermal limits. The sequence abundance and filamentous morphology of *M. ruber* suggest it is a primary prey of *I. cascadiensis*. Other members include *Pelomicrobium methylotrophicum*, isolated from a mud volcano,³⁰ and additional metabolically diverse thermophilic bacteria. This compositional complexity may partly explain why *I. cascadiensis* was not previously discovered, as culturing efforts for geothermal amoebae typically rely on a single bacterial strain rather than community enrichments.¹⁸

At temperatures above 64°C, *I. cascadiensis* forms compact but irregularly shaped cysts, ranging in diameter from ~5 to ~15 µm (Figure S4); encystment is a common strategy among amoebae to enter dormancy under unfavorable conditions.²⁵ This genus is further distinguished from *Vermamoeba* by its ability to thrive at substantially higher temperatures, as described below.

I. cascadiensis exhibits high-temperature replication and motility

I. cascadiensis grows robustly at the previously defined limit for eukaryotes of 60°C^{12–16} and continues replicating at a maximum temperature of 63°C (Figures 2A and 2B). To determine the upper and lower temperature limits for cellular replication of *I. cascadiensis*, we conducted growth experiments at 17 temperatures from 30°C to 64°C, with four replicate flasks per temperature, monitoring culture density over 7 days, along with a 14-day experiment at 63°C (Table S5). We validated incubation temperatures with multiple independent temperature probes. To determine significant growth, we estimated slopes of linear models fitted to the log-transformed growth curves. The minimum growth temperature is 42°C, with no growth at 40°C or lower; optimal growth is at 55°C–57°C (Figure 2A; Table S6), and maximum growth is at 63°C, with no growth at 64°C or higher. At both 42°C and 63°C, growth was variable across replicates. This temperature range confirms *I. cascadiensis* is an obligate thermophile.

We also visualized cell division, including mitosis, in cells grown in their optimal range of 58°C and incubated for 7 days at their maximum replication temperature of 63°C, using ultra-structure expansion microscopy (U-ExM) following the protocol described in Mikus et al.³¹ With tubulin (anti- α and β) and DNA (Hoechst) staining, we identified cells undergoing mitosis at the point of fixation (Figures 2C and 2D). Metaphase mitotic spindles were wide and acentric, apparently lacking astral microtubules. The direct visualization of mitosis at 63°C demonstrates that *I. cascadiensis* completes cellular replication and not merely hyphal extension or cell elongation at this temperature, which is a distinction that has not yet been established for thermophilic fungi reported near this range.^{12–15,17} The capacity for full life cycle completion at extreme temperatures

also distinguishes *I. cascadiensis* from thermophiles such as the hydrothermal vent worm, *Alvinella pompejana*, which requires substantially cooler temperatures to complete its life cycle.^{32,33}

The temperature dependence of *I. cascadiensis* motility exhibits a broad peak centered at 57°C but extends as high as 64°C. We employed high-temperature live-cell microscopy³⁴ and cell centroid tracking (Figure S5) to characterize motility across a 45°C range (25°C–70°C) (Videos S7, S8, and S9). To discriminate active from passive motion due to fluid flow and random thermal motion, we included 25°C and 70°C conditions in which all cells were encysted. We confirmed that temperature was not correlated with surface bacterial density (Figure S6A).

Consistent with our growth measurements, cell velocity peaked within the optimal growth range at 57°C (14.5 µm/min) (Figure 3A; Table S7). Bacterial density was positively correlated with amoebae velocity ($\rho = 0.40$, $p < 0.0001$) (Figure S6), indicating possible bacteria-driven chemotaxis. Mean squared displacement (MSD) analysis confirmed active motion, with the slope α of log-log MSD versus time peaking between 55°C–64°C ($\alpha > 1$, $p < 0.0001$) (Figure 3B; Table S8), while encysted cells were close to zero. Displacement ratios similarly peaked in the 55°C–64°C range, indicating greatest persistence of motion. To quantify how cell shape relates to movement, we identified cell edges (Figure 3C) and extracted aspect ratios (Figure 3D; Table S3). Cells at 55°C–64°C also displayed the highest aspect ratios (i.e., most elongated), peaking at 57°C, while cysts were most circular (Figure 3D).

I. cascadiensis switches between vermiform and amoebiform modes (Videos S2 and S10), suggestive of two-state motility. Cells switch on average every 91.2 ± 3.2 s at 57°C compared with once every 235.8 ± 2.1 s at 50°C and 2.6 times more frequently than *V. vermiformis* at its optimal temperature (Video S4; Table S9). *I. cascadiensis* spent approximately 30% of its time vermiform, displaying higher velocities (at 57°C: 28.3 µm/min versus 17.1 µm/min amoebiform) (Table S7) and straighter trajectories (Figure 3D; Table S8). In contrast, amoebiform cells explored greater areas (57°C mean convex hull: 58.0 µm² versus 25.0 µm²) (Table S8). Overall, at 57°C, *I. cascadiensis* exhibited lower velocity, explored more space, and changed shape more frequently than *V. vermiformis* at 25°C. This switching may reflect a strategy to alternate between foraging and rapid travel suited to geothermal streams, where temperatures can fluctuate within a few centimeters.³⁵

Similar to other thermotolerant amoebae,^{36,37} *I. cascadiensis* can recover from encystment at temperatures above its cellular replication limit. At 66°C, cells round up but remain partially active, likely initiating encystment (Table S8). When maintained at 70°C for 5 min, cells encyst (Video S11). In response to heat shock, encysting cells display rapidly moving, hair-like structures (Video S12; Figure S4). To our knowledge, this phenomenon is unique to *I. cascadiensis* and may represent a mechanism to sense external temperature.³⁸ Cells can recover from heating to 70°C but not 80°C after the temperature is lowered to 60°C. In contrast, *V. vermiformis* cysts are inactivated at 60°C, which is at least 10°C below the maximum temperature of *I. cascadiensis*.³⁷ As *I. cascadiensis* inhabits a stream with

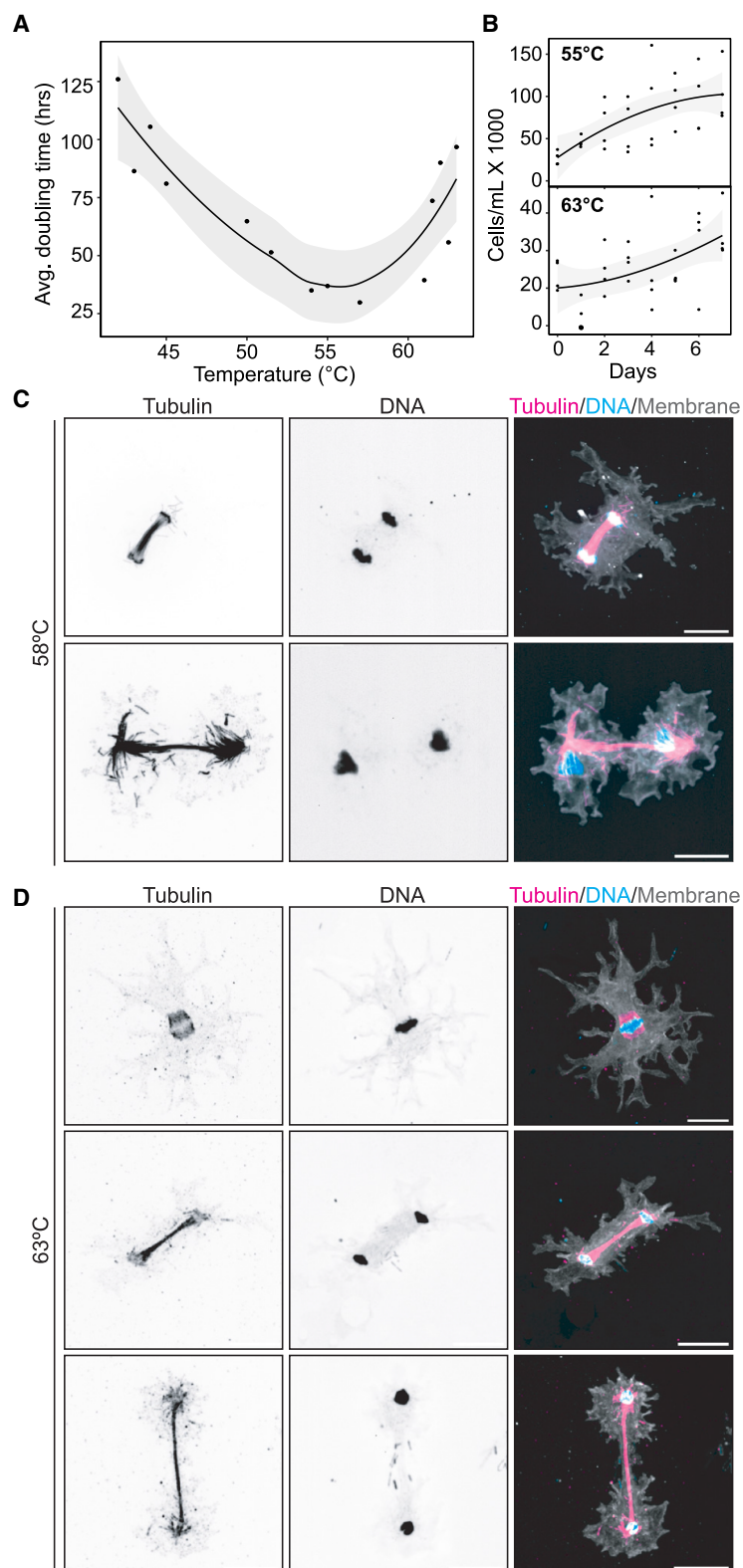


Figure 2. *Incandiamoeba cascadiensis* undergoes cellular replication up to 63°C

(A) Doubling time across temperature range. Points represent mean doubling time from the exponential growth period of a 7-day experiment; gray indicates 95% confidence interval (CI).

(B) Representative growth curves at 55°C and 63°C.

(C and D) Ultrastructure expansion microscopy of mitotic cells fixed at 58°C (C) or 63°C (D), with tubulin (left), DNA (middle), composite (right) with tubulin (pink), DNA (blue), and membrane (gray). Top row in (D) is consistent with metaphase; in (C) and middle row (D), the mitotic spindle array is visible, and cells are arriving at the end of division. The bottom row in (D) displays late telophase. Scale bars, 20 μ m, unadjusted for approximately 4 $\times$ expansion factor.

See also [Figure S4](#) and [Tables S5](#) and [S6](#).

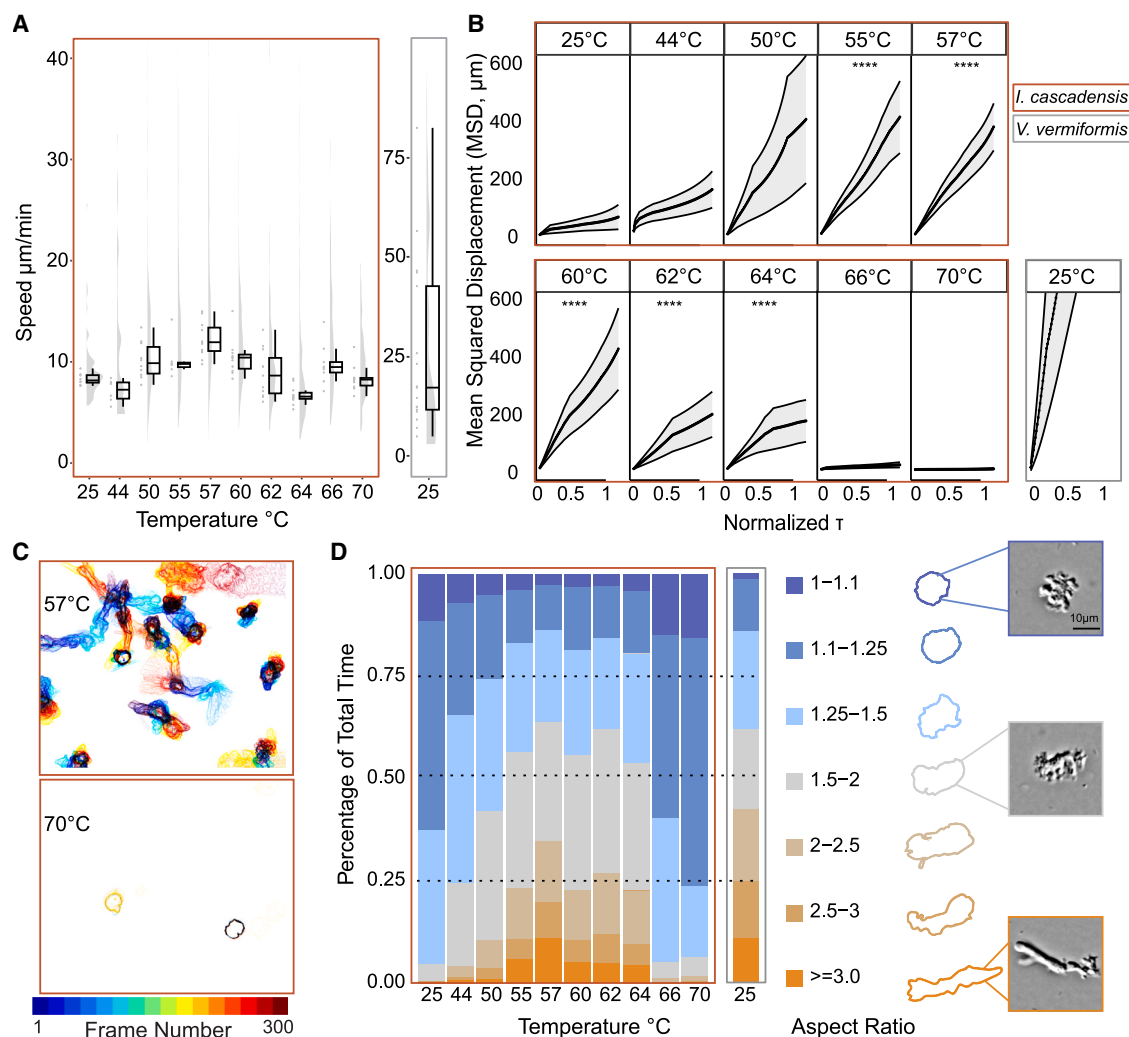


Figure 3. Motility characterization of *I. cascadenensis* over a 45°C temperature range, up to 64°C

(A) Median speed (µm/min) of tracked cell centroids shown as box and whisker plots (75th, median, 25th percentiles; whiskers extend to 1.5 × IQR, outliers excluded). Points represent median values from image sequences; rain clouds represent individual tracks.

(B) MSD over time across temperature; error bars represent standard deviation. Asterisks indicate significance of α comparisons against a reference value of 1 (** $p < 0.0001$); values > 1 indicate directed motion.

(C) Contour traces of cells at 1 s intervals at 57°C and 70°C. Frame number indicated by color.

(D) Percentage of time cells spent at given aspect ratios (vermiform > 2.5 , amoebiform 1.2–2.5, cyst 1.0–1.2) per track, with exemplar cell outlines and corresponding images.

Boxes around (A)–(D) colored by species.

See also [Figures S4–S6](#); [Tables S7, S8, and S9](#); and [Videos S7, S8, S9, S10, S11, and S12](#).

fluctuating temperatures, survival across a broad range of temperatures is likely ecologically relevant.

Comparative analyses shed light on high-temperature adaptation

To better understand how this amoeba survives at high temperatures, we assembled two high-quality genomes using PacBio long-read sequencing ([Figure 4A](#)). The assembly is 48 Mbp with a GC content of 36%, slightly larger (contrary to genome reduction in thermophilic fungi³⁹) and less GC-rich than *V. vermiformis* ([Table S10](#)). Codon usage is distinct from *V. vermiformis* as well

([Figure S7A](#)). Assembly quality metrics were comparable to reference genomes included for comparison: *Acanthamoeba terricola*, *Dictyostelium discoideum*, and *V. vermiformis* ([Table S10](#)). We estimate that the assemblies are 83%–94% complete. We profiled the gene content of *I. cascadenensis* and the three reference Amoebozoa ([Figure 4A](#); [Table S10](#)). *I. cascadenensis* and *V. vermiformis* shared 52% of orthogroups and less than 40% with the other amoebae. Additionally, 6,919 orthogroups were found only in *I. cascadenensis*, comprising $> 50\%$ of the predicted genes. The large portion of potential lineage-specific genes may encode thermotolerance strategies.

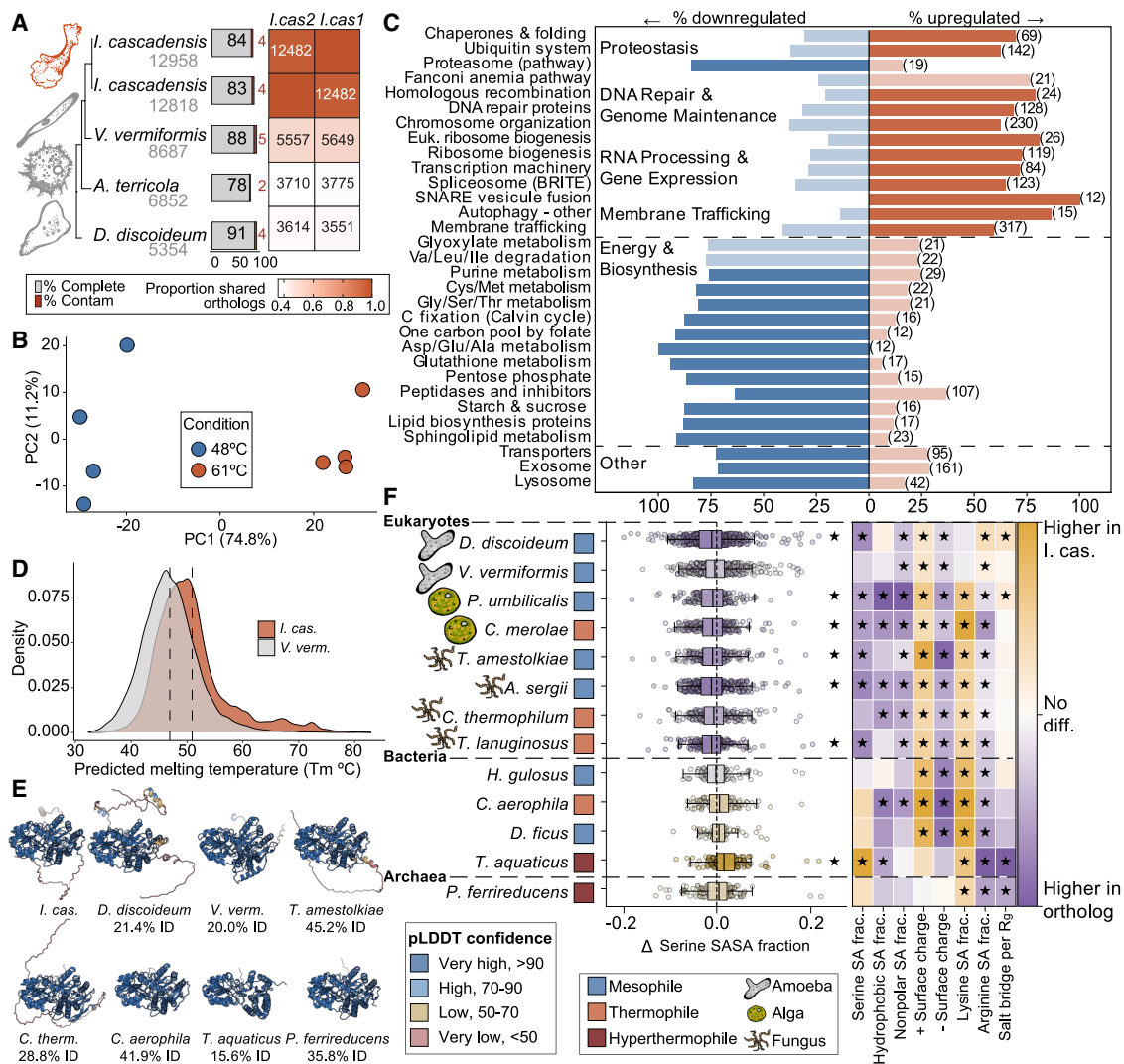


Figure 4. Candidate mechanisms of thermotolerance in *I. cascadenis*

(A) Relationship between *I. cascadenis* genomes and mesophilic amoebae with total predicted orthologs. Bars show completeness and contamination. Heatmap shows shared orthologs.

(B) Principal-component analysis (PCA) of RNA sequencing (RNA-seq) data at 48°C and 61°C.

(C) Pathway bias at 61°C versus 48°C. Solid bars show percentage of upregulated (red) or downregulated (blue) genes for significant pathways (BH $p < 0.05$), semi-transparent bars indicate trending pathways. Numbers in parentheses indicate genes per pathway.

(D) Predicted protein melting temperature distributions.

(E) AlphaFold2 structure predictions of *I. cascadenis* protein and seven low-sequence homology orthologs. Residues colored by predicted local distance difference test (pLDDT) confidence scores.

(F) Distribution of the difference in various protein properties ($\Delta = I. cascadenis$ -ortholog). (Left) Boxplots show the distribution of Δ for different organisms. Each point is a protein pair, box spans median 50% of proteins, center line is the median, and whiskers are the median 90% of the data. (Right) Heatmap shows the median Δ values for parameters. SA, surface area; frac., fraction. Color indicates median difference. Each column is normalized to the maximum absolute Δ for that parameter. Stars indicate significance (FDR-corrected $p < 0.05$).

See also [Figures S7](#) and [S8](#) and [Tables S10](#), [S11](#), and [S12](#).

We identified putative functions and pathways enriched in *I. cascadenis* relative to more mesophilic amoebae ([Figure S7B](#); [Table S11](#)). Signaling networks including calcium, mitogen-activated protein kinase (MAPK), and Ras were all enriched in *I. cascadenis* (false discovery rate [FDR]-adjusted $p < 0.0001$ for all). Calcium signaling pathways can initiate protective responses to thermal fluctuations by altering lipid meta-

bolism,^{40,41} and the enrichment of rapid-response signaling pathways is consistent with the ecology of *I. cascadenis* in a dynamic geothermal stream. *I. cascadenis* may also employ an expanded molecular toolkit to manage proteostasis. Genes encoding molecular chaperones including small heat shock proteins (HSPs), which prevent aggregation and assist in refolding, and HSPA5 which regulates the unfolded protein response,⁴² were enriched

along with ubiquitin-protein ligases⁴³ including RLIM and HERC2 (Figures S7B and S7C; Table S11).

To assess which cellular processes are transcriptionally regulated at near-maximum growth temperatures, we compared gene expression in *I. cascadiensis* grown at 48°C versus 61°C. Samples clustered strongly by temperature condition (74.8% of variance) (Figure 4B). Within proteostasis pathways, there was an upregulation of chaperones, folding catalysts, and the ubiquitin system but a downregulation of the proteasome pathway (Figure 4C). We also observed upregulation of DNA repair and genome maintenance pathways. Membrane trafficking was strongly upregulated, most notably Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) interactions in vesicular transport. SNARE proteins mediate fusion between intracellular membrane compartments,^{44,45} and their upregulation may reflect the sensitivity of membrane-based processes to high temperatures in eukaryotes. In contrast, energy and biosynthesis pathways were downregulated at 61°C (Figure 4C). This may indicate a strategy of metabolic reallocation consistent with other eukaryotes near their upper temperature limits.^{19,46} Notably, 71.5% of the top 200 most differentially expressed genes were unannotated, thus highlighting the extent of uncharacterized temperature responses in *I. cascadiensis*.

We used the complete set of predicted protein sequences from *I. cascadiensis* and *V. vermiformis* to obtain predicted melting temperature (T_m) distributions, using TemBERTure to capture overall trends.^{47,48} The T_m is the temperature at which the population of a protein is 50% unfolded and is used to quantify protein thermostability. The predicted melting temperature of the *I. cascadiensis* proteome is significantly higher, 50.9°C ± 6.6°C, in comparison to 46.9°C ± 5.1°C for *V. vermiformis* ($p < 0.0001$) (Figure 4D). *I. cascadiensis* is also predicted to have five times more proteins with T_m above 60°C than *V. vermiformis*, which may reflect increased protein stability consistent with other studies comparing thermophiles and mesophiles.^{48,49}

We further hypothesized that fingerprints of thermal adaptation would be found in the chemistry of *I. cascadiensis* proteins. Specifically, we reasoned that the most abundant *I. cascadiensis* proteins would exhibit distinct biophysical properties that contribute to stability under heat stress because temperature-induced aggregation and misfolding in abundant proteins would cause widespread dysfunction. Using the 500 most abundant transcripts (49.9% of transcription at 61°C) as a proxy for protein abundance, we aimed to identify orthologs across 13 other mesophiles and thermophiles, including amoebae, fungi, red algae, and prokaryotes. Distant ortholog identification presents a long-standing challenge.⁵⁰ To overcome it, we developed a computational pipeline combining OrthoFinder⁵¹ and the ESM-2 protein language model⁵² to identify ortholog candidates and then validate these using AlphaFold2⁵³ structural predictions and Foldseek⁵⁴ to assess structural similarity (Figure S8A). This identified 3,368 ortholog pairs across the 13 taxa with an average sequence homology of 37% (Figure S8B; Table S12).

We calculated the difference in a range of chemical and structural features between *I. cascadiensis* proteins and their orthologous protein pairs in each of the other taxa, allowing for the systematic identification of properties that may contribute to *I. cascadiensis* thermotolerance (Figure 4E). Our analysis shows

that *I. cascadiensis* is depleted in hydrophobic and non-polar surface residues (Figures 4F and S8C). Surface-exposed hydrophobic residues are inherently “sticky,” especially at high temperatures, which can promote protein aggregation.^{55,56} The minimization of such residues in *I. cascadiensis* can therefore serve as a mechanism to prevent unwanted protein aggregation at high temperatures.

More strikingly, *I. cascadiensis* is enriched in positively charged surface residues and depleted in negatively surface residues, which is similar to only the most thermophilic organisms, *T. aquaticus* (maximum 79°C⁵⁷) and *P. ferrireducens* (maximum 98°C⁵⁸). This suggests a convergent evolutionary mechanism by which increased positive charge can help with protein stability under temperature stress. This enrichment has been previously reported for thermophiles,⁵⁹ and proliferation of charges in general can act to increase protein-solvent interactions that prevent unfolding at high temperatures or reduce heat-driven aggregation through electrostatic repulsion.^{55,56} In prokaryotic thermophiles specifically, the increase in positive charges tends to be driven by arginine proliferation, known to be more thermostable than lysine.⁶⁰ While our data show that amoebae are more enriched in lysine, *I. cascadiensis* is enriched in arginine relative to the other amoebae. This suggests that the enrichment of arginine within the evolutionary constraints in Amoebozoa can be a viable strategy to induce protein thermotolerance.

While *I. cascadiensis* shows a modest enrichment in salt bridges compared to mesophilic *D. discoideum* and *P. umbilicalis*, their presence in *I. cascadiensis* is depleted compared with the most thermophilic prokaryotes. Salt bridges have been previously seen to enhance thermostability through localized ionic contacts⁶¹; however, the overall salt bridge depletion in *I. cascadiensis* indicates this strategy has not evolved similarly to prokaryotic thermophiles. As eukaryotic proteins are often larger and more complex, optimized electrostatic surfaces over the precise ionic contact networks of prokaryotes may serve as a better strategy for thermal stability.^{62,63}

DISCUSSION

Incandiamoeba cascadiensis proliferates at temperatures beyond what was thought possible for any eukaryote. This discovery raises questions about the true maximum temperature a eukaryotic cell can endure. Our findings demonstrate that amoebae, not just fungi and red algae,^{12–16} are capable of occupying the most extreme thermal niches available to eukaryotes, and our biogeographic analysis suggests that additional thermophilic species within this clade await discovery.

Nearly all thermal stress research in eukaryotes has focused on mesophilic organisms pushed to their limits rather than true thermophiles.⁶⁴ Our genomic and transcriptomic analyses point to candidate mechanisms of thermotolerance, including expanded chaperone and degradation machinery, signaling networks, distinct protein biophysical properties, and coordinated regulation of proteostasis, DNA repair, and membrane trafficking, representing leads for future mechanistic investigation. Beyond basic biology, *I. cascadiensis* offers an untapped source of thermostable proteins with promising biotechnological applications.

Limitations of the study

First, *I. cascadiensis* is not yet genetically tractable, meaning candidate genes cannot yet be directly tested. At the genomic level, many lineage-specific genes remain unannotated, and our assembly is not yet chromosome-resolved, limiting the completeness of our comparative analyses. At the protein level, melting temperatures as well as structures for pairwise ortholog comparison are only predictions and require experimental validation. Finally, our transcriptomic data reflect a single pairwise temperature comparison, and experiments across a broader thermal range will improve characterization of the transcriptional response. A clear next step is characterizing how lipid composition and membrane fluidity shift with increasing temperature, and what structural features distinguish candidate thermostable proteins.

I. cascadiensis is straightforward to culture, grows to high densities, and with a sequenced genome is among the most tractable eukaryotic thermophiles available for mechanistic study. We hope this discovery motivates the reassessment of other thermophilic eukaryotes under standardized conditions to inform the true temperature difference between this organism and other eukaryotic thermophiles. Long thought to be impossible, the 4-min mile record was accomplished and then broken again within weeks. Similarly, we hope *I. cascadiensis* generates momentum in the discovery of eukaryotic extremophiles and their capabilities and constraints.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Angela Oliverio (amoliver@syr.edu).

Materials availability

I. cascadiensis strain NS2 has been deposited with the Leibniz Institute DSMZ (Germany) as DSM 122033.

Data and code availability

- Raw reads and genome assemblies generated in this study have been deposited in the NCBI Sequence Read Archive as BioProject: PRJNA1329177. 18S rRNA gene sequences of *I. cascadiensis* and *V. vermiformis* are deposited in NCBI under GenBank: LC894571, GenBank: LC894572, GenBank: LC894573, and GenBank: LC894570.
- All code and analysis datasets have been deposited in GitHub: https://github.com/hbrappaport/incendiamoeba_manuscript, <https://github.com/sukeniklab/DistantOrthologDetection>, and at Figshare: <https://doi.org/10.6084/m9.figshare.c.8546250>.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

We wish to thank Lassen Volcanic National Park for their permits and logistics support. We acknowledge QB3 Genomics, Berkeley, CA, RRID:SCR_022170 for sequencing and thank Eva Myšková for assistance with library preparation. We thank Danielle Jorgens and Reena Zalpuri from the University of California Berkeley Electron Microscope Laboratory for assistance with TEM. We thank Arthur Charles-Orszag for his introduction to the heated microscope setups. We thank Ashley Lasko and Kelly Buch for their feedback on motility analysis. We also wish to thank David Booth and his lab members for the live-cell re-

agents. F.M. thanks the EMBL Advanced Light Microscopy Facility for equipment and expertise. S.S. thanks H. Broder Schmidt for discussion on ortholog assignments. We thank Syracuse University's Research Computing (NSF ACI-1341006) for computational resources. H.B.R. acknowledges an NSF GRFP. A.M.O. was supported by NSF DEB 2439029, NASA Exobiology 24-EXO24-0042, and the APS Lewis and Clark Fund. R.D.M. was supported by NIH NIGMS R35-GM118119 and the HHMI Investigator program. L.A.K. acknowledges NSF DEB 243903 and 2230391. K.M.S. was supported by NSF MCB 2025305 and NIH NIGMS 1R16GM154184. S.S. was supported by NIH NIGMS R35-GM137926, NSF IntBio 2128067, the Water and Life Interface Institute, NSF DBI 2213983, and the Alfred P Sloan Foundation. J.K.N. was supported by Syracuse University Vice President of Research. G.D. acknowledges the European Commission KaryodynEVO 101078291 and the EMBL. O.D. was supported by Swiss NSF TMSGI3_218007, and O.D., G.D., and F.M. were supported by the Gordon and Betty Moore Foundation GBMF13113. The work conducted by the U.S. Department of Energy Joint Genome Institute (<https://ror.org/04xm1d337>), a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy operated under contract no. DE-AC02-05CH11231.

AUTHOR CONTRIBUTIONS

Conceptualization, H.B.R. and A.M.O.; field sampling, H.B.R., A.M.O., K.M.S., G.V.W., I.d.I.H., T.T., and K.S.; culturing, H.B.R., T.T., N.A.P., R.M.S., and A.M.O.; expansion microscopy, F.M., N.A.P., O.D., and G.D.; scanning electron microscopy (SEM), N.A.P.; heated microscopy, H.B.R., N.A.P., K.S., S.J.L., and R.D.M.; motility analyses, K.S. and E.M.; phylogenomics, G.A. and L.A.K.; 18S tree, T.T.; RNA-seq, N.E.G. and A.M.O.; protein analysis, S.S. and J.K.N.; writing, H.B.R. and A.M.O. with contributions from all co-authors; bioinformatics and genomics, H.B.R., K.L., F.S., and A.M.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the authors used Claude (Sonnet v.4.5, Anthropic) to write code to parse the KEGG BRITE hierarchy HTML and organize the RNA-seq analysis scripts. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cell.2026.08.043>.

Received: January 19, 2026

Revised: May 14, 2026

Accepted: August 23, 2026

Published: September 22, 2026

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Alpha Tubulin Antibody	ABCD Antibodies	Cat#ABCD_AA345; RRID: AB_3106979
Anti-Beta Tubulin Antibody	ABCD Antibodies	Cat#ABCD_AA344; RRID: AB_3678982
Anti-HA Tag Antibody	ABCD Antibodies	Cat#ABCD_AF291; RRID:AB_3754698
Anti-mouse AlexaFluor488	ThermoFisher	Cat#A-11001; RRID: AB_2534069
Anti-guineapig AlexaFluor647	ThermoFisher	Cat#A-21450; RRID: AB_2535867
Chemicals, peptides, and recombinant proteins		
Poly-L-Lysine	Sigma	Cat#P8920
NucBlue	ThermoFisher	Cat#R37605
SiR-Actin	Cytoskeleton	Cat#CY-SC001
BODIPY TR Ceramide	ThermoFisher	Cat#D7540
Hoechst 33342	ThermoFisher	Cat#62249
Critical commercial assays		
Monarch Spin gDNA Extraction Kit	New England Biolabs	Cat#T3010S
Monarch DNA Gel Extraction Kit	New England Biolabs	Cat#T1120S
TOPO TA Cloning Kit	ThermoFisher	Cat#K4575J10
Monarch Plasmid Miniprep Kit	New England Biolabs	Cat#T1110S
QuickDNA HMW MagBead Kit	Zymo	Cat#D6060
Monarch Total RNA Miniprep Kit	New England Biolabs	Cat#T2010S
SMRTbell Express Template Prep Kit 2.0	PacBio	PN 100-938-900
Nextera XT Library Prep Kit	Illumina	Cat#FC-131-1024
Monarch Spin RNA Isolation Kit (Mini)	New England Biolabs	Cat#T2110S
Illumina Stranded mRNA Prep	Illumina	Cat#20040534
DNEasy PowerSoil Kit	Qiagen	Cat#47014
Deposited data		
Analysis code and datasets	This study	https://doi.org/10.6084/m9.figshare.c.8546250
Experimental models: Organisms/strains		
<i>Incendiamoeba cascadenensis</i> strain NS2	This study	DSM 122033
<i>Vermamoeba vermiformis</i> strain HSC3	This study	N/A
Oligonucleotides		
18S-EukA: AACCTGGTTGATCCTGCCAGT	Medlin et al. ⁶⁵	N/A
18S-EukB: TGATCCTTCTGCAGGTTACCTAC	Medlin et al. ⁶⁵	N/A
NS2_975F: TCGTTAACGGAATTAACCAGAC	This study	N/A
1350R: CCGTCAATTCCTTTAAGTTTC	Dyková et al. ⁶⁶	N/A
Software and algorithms		
Micro-manager 2.0.3	Edelstein et al. ⁶⁷	https://micro-manager.org/
FIJI 2.16.0/1.54p	Schindelin et al. ⁶⁸	https://imagej.net/software/fiji/
RStudio (R 4.3.2)	Posit Team	http://www.rstudio.com/
MAFFT 7	Katoh et al. ⁶⁹	https://mafft.cbrc.jp/alignment/server/index.html
trimAl 12	Capella-Gutiérrez et al. ⁷⁰	https://vicfero.github.io/trimal/
Jalview 2	Waterhouse et al. ⁷¹	https://www.jalview.org/
IQ-TREE	Trifinopoulos et al. ⁷²	http://iqtree.cibiv.univie.ac.at/
iToL	Letunic and Bork ⁷³	https://itol.embl.de/

(Continued on next page)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
BBTools	Bushnell ⁷⁴	https://github.com/bbushnell/BBTools
Flye v2.9.5-b1801	Kolmogorov et al. ⁷⁵	https://github.com/mikolmogorov/Flye
MetaBAT2 15–5-g1a9bac2	Kang et al. ⁷⁶	https://bitbucket.org/berkeleylab/metabat/src/master/
BUSCO	Simão et al. ⁷⁷	https://busco.ezlab.org/
BRAKER3	Gabriel et al. ⁷⁸	https://github.com/Gaius-Augustus/BRAKER
featureCounts	Liao et al. ⁷⁹	https://subread.sourceforge.net/featureCounts.html
DESeq2 1.5.2	Love et al. ⁸⁰	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
EukPhylo	Katz et al. ²⁴	https://github.com/Katzlab/EukPhylo
OrthoFinder	Emms and Kelly ⁵¹	https://github.com/davideemms/OrthoFinder
TemBERTure	Rodella et al.; Jarzab et al. ^{47,48}	https://github.com/ibmm-unibe-ch/TemBERTure
AlphaFold2 v2.3.2	Jumper et al. ⁵³	https://www.ebi.ac.uk/training/online/courses/alphafold/accessing-and-predicting-protein-structures-with-alphafold/choosing-how-to-access-alphafold/
ESM-2 v2.0.0	Iovino et al. ⁸¹	https://github.com/facebookresearch/esm#esmfold
FoldSeek 10.941cd33	Van Kempen et al. ⁵⁴	https://github.com/steineggerlab/foldseek
MDTraj	McGibbon et al. ⁸²	https://www.mdtraj.org/1.9.8.dev0/index.html
MDAnalysis	Michaud-Agrawal et al. ⁸³	https://www.mdanalysis.org/
OpenMM	Eastman et al. ⁸⁴	https://openmm.org/
KEGG Automatic Annotation Server (KAAS)	Moriya et al. ⁸⁵	https://www.genome.jp/kegg/kaas/
Other		
NanoEntek Neubauer hemocytometer	NanoEntek	Cat#76330-084
EasyLog Temperature Logger	Lascar Electronics	EL-USB-TC-LCD
Traceable Datalogger	Cole-Palmer	Cat#EW-18004-27
Fisherbrand Traceable Type K Thermometer	Fisher	Cat#15-078-187
High temperature incubator	Memmert	IF55plus
Biopetechs Delta-T Culture Dishes	Biopetechs	Cat#04200417B
Objective Heater Controller	Biopetechs	Cat#150803
Heated Lid	Biopetechs	Cat#04200803-1416
Delta T Dish Controller with high heat mod	Biopetechs	Cat#0420-4-03
VaHeat heated stage	Interherence	N/A
Vaheat Smart substrates – Standard Range	Interherence	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

To start a culture of *Incandiamoeba cascadiensis* strain NS2 from a stock, add 5mL of starter *I. cascadiensis* cells and bacterial prey to a vented 250mL tissue culture flask with six autoclaved wheatberries to enrich for the bacteria which *I. cascadiensis* feeds on, and fill the flask to 60mL with autoclaved Page's saline media.⁸⁶ *I. cascadiensis* is cultured for maintenance at 58–60°C in 250mL tissue culture flasks. Weekly, flasks are removed from incubators and media is poured into bleach. To refresh media, 60mL of autoclaved Page's saline media is poured into each flask before returning flasks to incubators. To split cultures, flasks are kept at room temperature for five to ten minutes, gently shaken, and then 30mL of detached cells is poured into a new flask with six autoclaved wheatberries. Both old and new flask are filled to 60mL with Page's saline. *Vermamoeba vermiformis* strain HSC3 is cultured at room temperature (~22°C) in vented, 250mL tissue culture flasks with six autoclaved wheatberries. Weekly, 1–3mL of culture is scraped and pipetted from the bottom of the flask with a p1000 pipette tip and dispensed into a new flask with 60mL of Page's saline. Information on genotype, age, developmental stage, and sex is not applicable to these amoebae.

METHOD DETAILS

Field sampling

We collected sediment, microbial biofilm, and water samples from 11 sites along a tributary of Hot Springs Creek (40° 26.510'N, 121° 24.167'W, ~84m transect), located in the Warner Valley in Lassen Volcanic National Park, CA, USA in August 2023, July 2024, and July 2025 under permits LAVO-2023-SCI-0028, LAVO-2024-SCI-0017, and LAVO-2025-SCI-0016. Although most Lassen geothermal features are acid-sulfate steam-heated, the tributary is a part of Drakesbad, a low chloride, neutral pH hydrothermal system that is likely heated from a shallow source.²¹ Sampling site temperature ranged from 47–64°C and pH averaged 7.55, measured in the lab with a pHenomenal pH meter (VWR; Table S1). We collected and transported samples in sterile 50mL conicals and 1L polycarbonate bottles and kept them at room temperature until cultivation.

Cultivation and isolation

To increase the density of protists from the samples, we established initial enrichment cultures in 50mL plastic tissue culture flasks with 1mL of sample and 19mL of autoclaved Volvic® water within a few days of sampling.¹⁸ We added sterile wheatberries as a nutrient source to grow bacteria sourced from the same environment, which serve as prey for the target protists.⁸⁷ We kept flasks in the dark, so although we observed eukaryotic algae and cyanobacteria in initial cultures, no photosynthetic organisms remained in enrichments. We incubated enrichments across a temperature range from 37°C to 60°C. *Incandiamoeba cascadiensis* became dense and emerged as the sole eukaryotes in cultures incubated at ≥ 55°C. We maintained cultures in 250mL flasks with Page's amoeba saline⁸⁶ and transferred cultures into fresh media weekly.^{66,88} We identified *I. cascadiensis* by morphology across samples ≥ 55°C and confirmed identity by Sanger sequencing of the 18S rRNA gene from sites S2, S13, and S14 (Table S1; Figure S1B).

Growth experiments

To determine the growth range, we conducted growth experiments in a manner similar to Baumgartner et al. (2003).¹⁸ First, we grew amoeba cultures for at least a week to high density and then measured cell density in four replicates with a Neubauer hemocytometer (NanoEntek C-chip 4 chamber). We pipetted dense amoeba stock into 50mL tissue culture flasks with a single autoclaved wheatberry and Page's amoeba saline to reach a final density of 2.50E+04 cells/mL in 10mL. We set up four replicate flasks per temperature and confirmed initial density with a hemocytometer before incubating the flasks at a range of temperatures from 30°C to 64°C. Every 24 hours, we removed flasks from the incubators, imaged them, filled to 10mL with autoclaved DI water to retain a stable salinity after evaporation, and we allowed amoebae to detach from the flask surface at room temperature. After 30 minutes, we gently shook flasks and counted each replicate once with the hemocytometer. After seven days, we calculated the average doubling time for each temperature for the exponential growth period (Table S6). Doubling times were variable, as factors such as initial bacterial input and evaporation likely impacted cell growth in addition to temperature. We cross-checked incubator temperatures with EasyLog Temperature Data Loggers (Lascar Electronics) recording temperature every ten minutes (Table S5) and a NIST-certified Traceable Datalogger. We used temperature experiments to determine the upper and lower boundaries of cellular replication and to estimate the optimal growth temperature.

Temperature-controlled microscopy

We imaged *I. cascadiensis* in a Biopetechs Delta-T dish heated with a Biopetechs Delta-T Dish Controller³⁴ or the Interference VaHeat heated stage from 42°C–70°C. We washed Biopetechs dishes with MilliQ water then primed with sterile-filtered, conditioned growth media. We calibrated dish temperature with a Fisherbrand Traceable Big-Digit Type K Thermometer (0.1°C resolution, NIST certified 1°C precision). We then replaced conditioned media with 2.5mL of dense, freshly detached culture, brought the dish to the appropriate temperature, and then imaged. We plasma cleaned VaHeat substrates, coated them with Poly-L-Lysine (PLL; Simão et al.⁷⁷ (p8920)), and washed with fresh Page's Saline. We then treated substrates with sterile-filtered conditioned growth media for at least 30 minutes prior to exchange with freshly detached cells.

For morphological characterization, we performed live cell imaging at 100X as previously described.³⁴ We performed reflection interference contrast microscopy (RICM) using a Nikon Ti2-LA-FL-3 epi illuminator on a Ti2 microscope, fitted with a liquid light guide illuminated by the Cy3 channel of a Lumencor SpectraX LED source. The dichroic cube held a Chroma 50/50 beamsplitter with a D546/10X exciter filter. We adjusted illumination intensity and the aperture diaphragm on the epi illuminator to achieve the optimal contrast. Dark regions of the RICM image correspond to areas of close contact between the cell membrane and the glass coverslip. We imaged Differential Interference Contrast (DIC) and RICM at 100X using a Photometrics BSI camera. To describe nuclear morphology, we stained nuclei with two drops of NucBlue (ThermoFisher) per dish and excited samples with a 405 laser. We stained actin with 1μM SiR-Actin (Cytoskeleton).

For motility analysis, we imaged cells in the Biopetechs heated dishes by DIC using a 40X Nikon Plan Apo (NA 0.95) objective on a Nikon Ti-E microscope fitted with a Chameleon3 series monochrome camera (Point Grey Research/Teledyne) controlled by Micro-manager 2.0.3.⁶⁷ We acquired 300 frames at one frame/second using one field of view.

SEM and TEM sample preparation

To fix *I. cascadiensis* for SEM, we detached cells from culture flasks grown at 58 or 63°C by pouring off media (~60mL) and resuspending in 5–7mL room temperature Page's saline. We gave cells ten minutes to detach and then plated on plasma cleaned

and PLL-coated 12mm high precision coverslips (Thorlabs CG15NH) in a 24-well plate. We returned plates to the 58°C incubator for 30–60 minutes. We fixed cells by adding an equal volume of prewarmed 2x fixation solution (final concentration 1X PHEM buffer and 4% PFA or 1X PHEM buffer and 2% glutaraldehyde) for 30 minutes, then washed with 0.1M HEPES pH 7.0 and stored at 4°C. To fix *V. vermiformis* for SEM, we transferred plasma cleaned and PLL-coated coverslips to a 6-well plate (multiple coverslips per well). We split cells into the 6-well plate using fresh Page's saline and a wheatberry and allowed cells to grow for one to three days. We fixed cells at the highest density of amoebae by adding 2x fixation solution (final concentration 1X PHEM buffer and 4% PFA). For heat shock, we transferred settled *I. cascadiensis* cells to a 70°C incubator and added 2x fixation buffer (final concentration 1X PHEM and 4% PFA) at the appropriate time points.

For SEM, we performed secondary fixation with 1% osmium tetroxide for one hour at room temperature in the dark. Then, we dehydrated cells in increasing concentrations of ethanol (25%, 50%, 75%, 90%, 100%). The last dehydration step involved solvent-dried ethanol using 4Å, 8–12 mesh molecular sieves that had been rinsed and baked. We then critical-point dried and sputter coated samples with 5nm iridium. We performed SEM imaging on a Phenom Pharos tabletop FEG-SEM.

To prepare samples for TEM, we seeded amoeba cultures grown to high density onto agar plates (2.5% BactoAgar in Page's saline) and incubated at 57°C for one hour. We fixed adhered amoeba cells *in situ* with a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 30 minutes, postfixed with 1% osmium tetroxide and 1.6% potassium ferricyanide for two hours, dehydrated with a series of acetones, and embedded in Epon resin. We examined ultrathin sections stained with 2% aqueous uranyl acetate and 0.3% lead citrate 0.1 N sodium hydroxide using the Tecnai 12 electron microscope at the University of California Berkeley Electron Microscope Laboratory.

Ultrastructure expansion microscopy (U-ExM)

We performed U-ExM as previously described with the following modifications.⁶⁹ We grew cells on plasma cleaned and PLL-coated coverslips at 58 or 63°C and fixed in 1X PHEM buffer with 4% paraformaldehyde for 30 minutes. Next, we carefully washed coverslips with 0.1M HEPES pH 7.0 and shipped them in PBS. For U-ExM, we anchored coverslips overnight at 37°C in 0.7% formaldehyde and 1% acrylamide and embedded them into a swellable hydrogel, which was polymerized for 45 minutes at 37°C in a humid chamber. We denatured samples at 95°C in an SDS containing buffer for 1.5 hours, washed with PBS twice, and expanded in three successive incubations in ddH₂O for 15 minutes each. We measured gel diameters for use as a proxy for cell expansion factors.

We stained after shrinking the gels with three PBS washes. For antibodies, we used ABCD (AntiBodies Chemically Defined; Geneva Antibody Facility), antibodies against alpha- and beta-tubulin (AA345 and AA344) and HA (AF291) at 1:500 in 3% BSA in PBS-T (0.1 % TritonX-100) and incubated at 37°C overnight. We removed unbound antibody by three PBS-T washes for five minutes each before incubating gels in secondary antibodies, anti-mouse AlexaFluor488 (Thermo A-11001) and anti-guineapig AlexaFluor647 (Thermo A-21450), for four hours at 37°C. We again washed gels three times with PBS before staining with Bodipy-TR ceramide (Invitrogen D7540) at 1:2000 and Hoechst 33342 (ThermoFisher 62249) for three hours at 37°C, washing again with PBS and re-expanding them in ddH₂O for imaging.

We acquired images with a Nikon-CSU-W1 SORA 750 spinning disk microscope with a SR P-Apochromat IR AC 60X WI/1.27 objective. We mounted gels onto PLL-coated Ibidi glass bottom chambers for imaging to reduce sample drift. Maximum intensity projections of full volumes are shown (Figure 2). We performed visualization and processing in ImageJ FIJI.⁶⁸ We applied look-up tables to fluorescence channels for display purposes with no pixel intensity values being modified. We adjusted brightness and contrast linearly and applied uniformly across individual channels. Displayed multicolor merge images represent sum composites of the NHS ester, tubulin, and DNA channels, while single channel images are shown as inverted grey scale.

Image analyses

We measured cell size from 2,414 cells across ten temperature conditions (Table S3). We measured nuclear diameter from 29 cells imaged at 60°C. To remove uneven illumination and background artifacts from high temperature, we first processed image sequences using a combined fast Fourier transform and bandpass filter in FIJI (Version 2.16.0/1.54p⁶⁸). We filtered large structures down to 50 pixels, small structures up to five pixels, with no stripe suppression and 5% tolerance of direction. To identify the cell edge for cell shape and tracking, we first duplicated each image sequence. We inverted one image and thresholded both to generate binary masks. We then combined the masks to include both the illuminated cell edges as well as the shadows from differential interference contrast.

Once images were segmented, we conducted particle analysis in FIJI with the following conditions: circularity=0.1–1.00 and size >75µm. We excluded particles on edges to restrict analysis to whole cells and optimize downstream tracking. Size thresholding removes non-filamentous bacteria from the analysis, and circularity thresholding removes the majority of filamentous bacteria. We recorded all thresholded cells as outlines. We extracted major and minor axes, area, and centroids along with frame number using a custom macro and recorded each measurement per frame. In FIJI, we determined centroids by averaging all xy coordinates in the thresholded area and defined major and minor axes by fitting an ellipse to the object and identifying its primary and secondary axes (Fiji Documentation: <https://imagejdocu.list.lu/start>). We defined aspect ratio as the ratio between the major and minor axes. We reported axes and aspect ratio measurements as averages for tracks across frames (Tables S3 and S7) or per frame (Figure 3D).

To track cell positions, we created tracks by assigning particle IDs using a custom script in R (R version 4.3.2). We used the k-nearest neighbors algorithm through the "Fast Nearest Neighbors" R library to link particles across frames. We set maximum allowed

displacement per particle between frames and gap tolerance criteria, or number of frames a particle can disappear and still retain its ID, during each run. Maximum allowed displacement filters completely detached cells, distinguishing passive motion due to fluid flow from active motility. We removed particles appearing in five or fewer frames and assessed performance of the particle-linking process by visual track display (Figure S5A) and by particle ID assignment integrity post hoc. N values for tracks are as follows: N = 25°C:81, 44°C:55; 50°C:181; 55°C:430; 57°C:525; 60°C:337, 62°C:65; 64°C:521; 66°C:185; 70°C:60. We used track length (Figure S5B) as a metric for quality control. As fast-moving particles tended to move out of field of view, we performed a Spearman's rank correlation analysis between track length and measured variables such as velocity to check for bias introduced by tracking quality. We found no significant correlation between measured metrics and track length (Figure S5B).

We calculated the mean squared displacement (MSD) of particles over time. To first calculate displacement, we dynamically set the maximum time lag (τ) based on the median track length for each condition, minimizing bias of short tracks. We calculated squared displacement as $\Delta x^2 + \Delta y^2$ for x,y particle positions. The MSD scales with time lag ($\text{MSD} \propto \tau^\alpha$) where we determined α from the slope of a log-transformed plot of MSD versus τ (Table S8). To classify type of motion, we statistically compared α against a reference value of 1 using two one-sided t-tests. Values greater than 1 indicate directed motion.

As all imaging was performed under xenic conditions, we wanted to understand the influence of bacterial density on motility. We quantified bacterial density with intensity thresholding to measure bacterial cell area and reported it as a fraction of total image area. To distinguish bacterial cells from amoebae, cells were first segmented by size (0.5–10 μm). Values from the first and last frames were computed, averaged per image sequence and then compared to temperature and extracted motility metrics using a Spearman's rank correlation analysis (N = 25°C:8, 44°C:6; 50°C:10; 55°C:7; 57°C:11; 60°C:11, 62°C:11; 64°C:11; 66°C:9; 70°C:10) (Figure S6).

Molecular cloning and phylogenetic analysis

We extracted DNA from established monoeukaryotic cultures of strain NS2 using the Monarch Spin gDNA Extraction Kit (New England Biolabs). We amplified nearly the entire 18S rRNA gene sequence of *I. cascadiensis* (strain NS2, 2023) and *V. vermiformis* (strain HSC3, 2024) by PCR using universal eukaryotic primers EukA and EukB.⁶⁵ PCR conditions followed the Q5 Hot Start Master Mix protocol (New England Biolabs): 98°C for 30 seconds, 34 cycles of 98°C for 10 seconds, 67°C for 30 seconds, 72°C for 1 minute, then 72°C for 2 minutes. We visualized the resulting amplicons by gel electrophoresis and then excised and purified bands of approximately 3 kb (*I. cascadiensis*) and 1.8 kb (*V. vermiformis*) using the Monarch DNA Gel Extraction Kit (New England Biolabs) according to the manufacturer's instructions. Using the TOPO TA Cloning Kit (Invitrogen) and following the manufacturer's protocol for chemical transformation, we ligated purified amplicons into the pCR4-TOPO vector and transformed into One Shot competent cells, plated on LB agar with kanamycin (50 $\mu\text{g}/\text{mL}$), and screened by PCR using M13 primers. We extracted plasmid DNA from overnight cultures using the Monarch Plasmid Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. We sequenced at the UC Berkeley Sequencing Center using M13 primers and internal primers NS2_975F (5'-TCGTTAACGGAATTAACCAGAC-3') and 1350R⁶⁶ for the longer NS2 inserts.

We inspected the 18S rRNA gene sequences for ambiguous base calls and assembled the sequences in Geneious Prime v2025.0.3 (Biomatters) before subjecting them to BLAST⁹⁰ searches against the GenBank (NCBI) and IMG/M⁹¹ databases to identify top hits. To search for previous detection in IMG/M metagenomes, we queried the *I. cascadiensis* 18S rRNA gene sequence against the "18S rRNA public assembled metagenomes 2025-06-16" JGI IMG database (<https://img.jgi.doe.gov>) containing 2,509,354 sequences extracted from 31,093 publicly available genomes. To infer the phylogenetic positions of *I. cascadiensis* and *V. vermiformis* strain HSC3 with 18S gene sequences, we compiled a sequence dataset comprising the top BLAST hits and representatives of major Tubulinea lineages with Centramoebidae as the outgroup. We aligned the dataset using MAFFT v7⁶⁹ with the Q-INS-i algorithm. We trimmed the alignment using trimAl v.12⁷⁰ with trimming thresholds -gt 0.3 -st 0.001, resulting in a final alignment of 1,437 bps, and we inspected the alignment in Jalview v.2.⁷¹ We computed the final tree with the IQ-TREE web server⁷² with TIM3+F+R7 model selected by ModelFinder with Bayesian information criterion.⁹² We tested tree branches by the standard bootstrap analysis⁹³ and the approximate Bayes test.⁹⁴ We visualized the tree in iTOL.⁷³

Genome and transcriptome sequencing

To target amoeba genomes and transcriptomes, we concentrated two sets of four 60mL cultures by centrifuging for ten minutes at 1000g. We added 200 μL DNA/RNA shield (Zymo) to the pellet and extracted DNA with the QuickDNA HMW MagBead Kit (Zymo) according to the protocol for cultured cells. We extracted RNA with the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the protocol for mammalian whole blood. We prepared ultra-low input DNA with the SMRTbell Express Template Prep Kit 2.0 (PacBio) and sequenced in a pooled library with PacBio Revio 25M SMRT Cell at QB3 Genomics, UC Berkeley, resulting in 6.6GB of data and 3,570,574 sequencing reads. We separated the amoeba from bacterial or viral contigs, resulting in a single *I. cascadiensis* bin from each sample (Figure 4A). We deduplicated reads using smrtlink_13.0.0.207600.sif pbmarkdup. For read quality control, we used BBDuk (version 39.19⁷⁴) to remove reads that contained PacBio control sequences and PCR adapters and used IceCreamFinder (version 39.19⁷⁴) to remove reads without SMRTbell sequences. We then assembled reads with Flye (v2.9.5-b1801⁷⁵) and binned with MetaBAT2 (v2.15-5-g1a9bac2⁷⁶). To assess bin completeness and contamination, we assessed presence or duplication of Benchmarking Universal Single-Copy Orthologs (BUSCOs⁷⁷), identifying the single largest bin with highly complete eukaryotic markers as the *Incendiamoeba* draft assembly. We estimated additional completeness in light of shared missing markers across Amoebozoa (as the BUSCO marker set is not specific to Amoebozoa) by adding shared missing markers to our estimate.

To obtain metagenomes for characterization of the enriched bacterial community, we filtered ~60mL of four dense cultures onto 0.2µm filters and extracted DNA with the DNEasy PowerSoil Kit (Qiagen) following manufacturer's instructions. We sent extracted DNA to SeqCoast Genomics (Portsmouth, NH) for Illumina sequencing, generating 13.3 million 150bp paired end reads. We filtered bacterial metagenome assembled genomes (MAGs) through KBase with Trimmomatic,⁹⁵ assembled with metaSPAdes v3.15.3,⁹⁶ and binned with MaxBin2 v2.2.4,⁹⁷ CONCOCT v1.1,⁹⁸ and MetaBAT2 v1.7.⁷⁶ We optimized bins with DasTool v1.1.2.⁹⁹ We assessed the quality of extracted bins with CheckM v1.0.18¹⁰⁰ and assigned taxonomy with GTDB-Tk v2.3.2.¹⁰¹ The 17 genome bins assembled collectively comprise >85% of the total reads, indicating the dominant bacterial community is well recovered at the genome level. We constructed a phylogenetic tree from these bins using KBase SpeciesTree v2.2.0, which relies on a set of 49 core, universal genes defined by COG gene families.

We PolyA selected and prepared transcriptomes with the Nextera XT kit and sequenced 150bp reads with Illumina NovaSeq X 10B. To remove bacterial sequences, we concatenated the 17 bacterial MAGs assembled from metagenomes and mapped transcripts against the resulting database using BBMap (version 39.33⁷⁴). We then mapped the unmapped reads to *I. cascadiensis* assembly. We predicted gene calls with BRAKER3⁷⁸ from RNA reads and draft genomes. We then tabulated paired-end reads assigned to each BRAKER gene (e.g. read counts) with featureCounts.⁷⁹

Phylogenomics and functional analyses

We input BRAKER3 gene calls to EukPhylo²⁴ to produce gene trees for 596 gene families conserved across Amoebozoa (71 Amoebozoa taxa and 253 total taxa). Next, we produced a phylogeny from a multi-sequence alignment using 102 gene families selected from an initial set of 270 based on a minimum of 30 Amoebozoa taxa and both novel amoeba replicates after running gene calls through EukPhylo. To assess the likelihood that the amoeba belonged in the same genus as its nearest neighbor, *Vermamoeba vermiformis*, we computed the average distance separating other species that fall within the same genus in our Amoebozoa phylogeny (Figure 1A). To do this, we calculated the branch length to the nearest shared common ancestor data from six within-genus pairs of species from the following genera: *Nebela*, *Hyalosphenia*, *Diffugia*, *Acanthamoeba*, *Paramoeba*, and *Dictyostelium* (Table S2). Then, we assessed whether the distance between the novel amoeba and *V. vermiformis* fell within a 95% confidence interval from the known within-genus pairs of species across the phylogeny.

To elucidate which gene functions and pathways may be present or enriched in *I. cascadiensis* relative to other amoebae, we obtained publicly available genomes from three additional species of Amoebozoa. We ran these three genomes through BRAKER3 to facilitate comparisons (*Vermamoeba vermiformis* CDC-19 GCA_045837725.1,¹⁰² *Acanthamoeba terricola* Neff GCA_000313135.1 [previously *A. castellanii*],¹⁰³ and *Dictyostelium discoideum* GCA_000004695.1¹⁰⁴). We inferred the orthogroups of the two *I. cascadiensis* draft genomes and three comparative Amoebozoa genomes using OrthoFinder.⁵¹ We calculated the proportions of shared orthogroups between genomes from means of the OrthoFinder output pairwise comparisons of total orthogroups. We confirmed the robustness of independent *de novo* assembly by finding 97% of orthogroups shared between the two *I. cascadiensis* assemblies. We functionally classified predicted genes using the KEGG Automatic Annotation Server⁸⁵ with the bi-directional best-hit method. Following the metric used in Tikhonenkov et al. (2022),¹⁰⁵ we calculated enrichment by adding the number of species that had fewer orthologues than *I. cascadiensis* and calculated depletion by subtracting the number of species that had more orthologues. Then, we used a non-parametric, permutation-based approach to assess whether the number of enriched or depleted gene functions for each pathway/category (BRITE level C) significantly differed from a random sampling. Briefly, we performed 10,000 random permutations of enrichment types (enriched, depleted, neutral; where < -2.5 = enriched, $-2.5 \leq 2.5$ = neutral, and >2.5 = depleted) to simulate a random distribution and then compared observed counts to our permuted counts (two-sided p-values were FDR adjusted for multiple tests).

RNA-seq experiment

We performed an RNA-seq experiment to examine the transcriptional response of *I. cascadiensis* grown in two different temperatures. A parent culture of *I. cascadiensis* was grown at 58°C in sterile AGM media (Key Resources Table) with wheatberries in 75cm² tissue culture flasks. Once dense, 20mL of the parent culture was inoculated into 75cm² tissue culture flasks containing 40mL of fresh media with sterile wheatberries and incubated at either 48°C or 61°C for seven days. Cells were harvested from each flask by pouring off spent media and adding fresh, preheated media (kept at 55°C in a heated bead bath) and gently resuspending cells using sterile cell scrapers. Resuspended cells were pooled across six flasks for each of four biological replicates per temperature and filtered onto a 2µm 47 mm polycarbonate filter. The filters were placed into cryovials and immediately flash frozen in liquid nitrogen and stored at -80°C.

Total RNA was extracted from each filter using the Monarch® Spin RNA Isolation Kit (Mini) following the "Mechanical Lysis of Bacteria or Yeast" lysis protocol. Briefly, filters were placed in MP Biomedicals™ Lysing Matrix E 2mL tubes with 200µL of Monarch StabiLyse DNA/RNA Buffer. The filters were bead-beat using the Vortex-Genie 2 Vortex adaptor for 10 minutes. The supernatant was collected and RNA was extracted following the manufacturer's protocol, using the optional DNase treatment. Resultant RNA was quantified using the Qubit HS RNA quantification kit and quality determined using the NanoDrop. The purified RNA was stored at -80°C until shipped on dry ice.

PolyA-selected RNAseq libraries were prepared and sequenced at SeqCoast Genomics (Portsmouth, NH). Briefly, RNA samples were prepared using the Illumina Stranded mRNA Prep with Illumina Unique Dual Indexes and sequenced on the Illumina

NextSeq2000 platform using a 300-cycle XLEAP-SBS flow cell kit to produce 2x150bp paired reads targeting 50M reads per sample. Reads were demultiplexed, trimmed, and quality-checked using DRAGEN v4.2.7 (NextSeq2000 software). Reads were mapped to the draft *I. cascadiensis* genome using BMap v39.81⁷⁴ and transcript counts were tabulated using featureCounts v2.0.1.⁷⁹ The DESeq2 v1.50.2 R-package⁸⁰ was used to calculate log₂fold changes between 61°C and 48°C, and the Benjamini-Hochberg adaptive false discovery rate (FDR) procedure was used to calculate adjusted p-values. Reads were also normalized using transcripts-per-million (TPM) calculation.¹⁰⁶

To quantify and visualize differences in transcription between the two temperature conditions, we performed a principal components analysis (PCA) on normalized transcription across all genes. For each pathway (Kegg Level C), we quantified whether the number of significantly differentially upregulated and downregulated genes comprised a greater proportion of the total genes in the pathway than expected by chance, using one-sided binomial tests to compare the observed versus expected counts based on the overall dataset proportions. Pathways with fewer than 10 genes were excluded. P-values were corrected for multiple comparisons using Benjamini-Hochberg corrections across all pathways. Pathways meeting significance (BH *p*-value < 0.05) or trending (defined as >75% in one direction and *n* ≥ 20) were included in Figure 4.

Protein melting temperature analyses

To compare predicted melting temperature (*T_m*) distributions of *I. cascadiensis* and *V. vermiformis*, we used the complete set of predicted protein sequences. We obtained *T_m* predictions with TemBERTure, a deep learning framework trained on 48,000 protein melting temperatures.^{47,48} We calculated summary statistics for both species' sets of protein sequences and compared *T_m* means with a one-sided Welch's t-test. We emphasize that these predictions are not accurate for absolute melting temperatures of individual proteins but can capture trends in overall distributions.⁴⁷

Protein ortholog analyses

To compare protein biophysical properties between *I. cascadiensis* to additional thermophiles and mesophilic organisms, we used the top 500 most abundant *I. cascadiensis* mRNA transcripts (as determined by RNAseq at 61°C) and found orthologs for each of these in 13 proteomes from other taxa. A full list of organisms and UniProt proteome accession codes are specified in Table S12. Our ortholog detection pipeline uses a two-stage approach, designed to first reduce the candidate ortholog pool to a tractable size before obtaining structural prediction using AlphaFold2.⁵³ The pipeline is shown schematically in Figure S8A.

In the first stage of this pipeline, we use Orthofinder⁵¹ v2.5.5 on the full *I. cascadiensis* assembled proteome with each target proteome to generate ortholog groups using MSA-based phylogenetic inference with DIAMOND ultra-sensitive search and MAFFT alignment. Second, protein language model embeddings from ESM-2 v2.0.0⁸¹ (esm2_t30_150M_UR50D model⁵²) were generated for all *I. cascadiensis* and target proteomes. Ortholog candidates were identified from these embeddings using a reciprocal best hits approach. For this, each *I. cascadiensis* protein embedding was compared to all protein embeddings of the target taxa by cosine similarity.¹⁰⁷ Candidate ortholog pairs were retained only if both the forward search (*I. cascadiensis* protein to full target proteome) and reverse search (target protein to full *I. cascadiensis* proteome) resulted in high-ranking matches (cosine similarity > 0.85), reducing false positive ortholog identification. Embeddings from multiple internal layers (layers 20 and 25) of the ESM-2 model were evaluated for robust ortholog candidate identification.⁸¹ Orthologs identified through sequence-based clustering and embedding-based identification were combined to create a pool of potential ortholog candidates.

In the second stage of the pipeline, structures for the top 500 *I. cascadiensis* proteins (including all splice variants) and their ortholog candidates (a total of 6,679 proteins) were predicted using AlphaFold2 (version 2.3.2)⁵³ with full genetic databases, and default monomer modeling parameters. The structures predicted for *I. cascadiensis* were compared against all orthologous proteins using FoldSeek (version 10.941cd33),⁵⁴ and ortholog pairs with a high structural similarity score (TM-score > 0.5) were retained for downstream analysis. This structural similarity cutoff was previously established to correspond to proteins that predominantly share the same fold from those with different folds.¹⁰⁸

With this method, we have managed to map 3,368 proteins across 13 organisms, with 63% of the proteins being mapped to exactly one ortholog. The average homology was 37%, but all TM-scores were > 0.5 (See Figure S8B for breakdown). These structures were analyzed using sequence and structural analysis tools (MDTraj,⁸² MDAnalysis,⁸³ and OpenMM⁸⁴). Analyses included radius of gyration (*R_g*), secondary structure composition using DSSP, solvent-accessible surface area with the Shrake-Rupley algorithm, and intramolecular interaction quantification. We performed statistical comparisons between the calculated metrics of *I. cascadiensis* and 13 other taxa using a two-sided paired t-test with Benjamini-Hochberg correction for false discovery.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses are described in the relevant sections of the results, methods, or figure and table legends.