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Research Article

Heterotrophic unicellular eukaryotes feeding on the unicellular red alga *Cyanidiococcus* sp. in moderately hot geothermal sulfuric springs

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Abstract

Sulfuric acidic hot springs (<pH 4.0, >37°C) are found in volcanic regions worldwide, where various bacteria, archaea, and the unicellular red algae Cyanidiophyceae dominate. Regarding heterotrophic eukaryotes, the only known species was the thermophilic amoeboflagellate *Tetramitus thermacidophilus* (class Eutetramitea, phylum Heterolobosea), which feeds on surrounding bacteria and archaea. In this study, we investigated three sulfuric hot springs (34.7°C–50°C, ~pH 2.0) in Japan to determine whether other heterotrophic eukaryotes inhabit these environments. As a result, we isolated and identified cultures of four species capable of surviving at pH 2.0 and 40°C: *Allovahlkampfia* sp. (Eutetramitea, Heterolobosea); *Nuclearia* sp. and *Parvularia* sp. (Nucleariidea, Cristidiscoidea); and *Vannella* sp. (Discosea, Amoebozoa). Phylogenetic analyses suggest that these four species independently evolved from mesophilic and neutrophilic ancestors, separate from each other. Additionally, *Platyophrya* sp. (Colpodea, Ciliophora) and two species of *Neobodo* (Euglenozoa, Kinetoplastea) were also found in the same environment, while their maximum survival temperatures were 35°C and 30°C, respectively. Among these, all species except *Neobodo* were confirmed to grow exclusively by feeding on *Cyanidiococcus* sp., a dominant species of Cyanidiophyceae in the environment. Thus, various lineages of heterotrophic unicellular eukaryotes have independently developed acidophilic and thermotolerant traits, allowing them to colonize sulfuric hot springs.

Keywords: Cyanidiophyceae; Heterolobosea; *Nuclearia*; sulfuric hot spring; *Vannella*

Introduction

Life on Earth is remarkably diverse, thriving in environments ranging from temperate ecosystems to the most extreme habitats. Extremophiles, unique groups of organisms capable of surviving and thriving under extreme physical and chemical conditions, challenge traditional notions of the limits of life. These environments include extreme temperatures, high salinity, intense acidity or alkalinity, elevated pressure, and high levels of radiation (Shu and Huang 2022, Rappaport and Oliverio 2023). The study of extremophiles has revealed remarkable biochemical and physiological adaptations that enable survival under such stresses. This knowledge not only enhances our understanding of evolutionary biology (Shu and Huang 2022, Rappaport and Oliverio 2023) but also offers innovative applications (Littlechild 2015). For instance, extremophiles contribute to environmental remediation by breaking down pollutants or immobilizing heavy metals, and their heat- and salt-stable enzymes, such as DNA polymerases, have become invaluable tools in various industrial and biotechnological processes (Littlechild 2015).

Research on extremophiles has largely focused on prokaryotes (bacteria and archaea), which dominate extreme habitats and are easier to study (Shu and Huang 2022). In contrast, protists (unicellular eukaryotes) have been historically overlooked due to assumptions that their complex structures and high energy demands make them poorly suited for extreme environments (Rappaport and Oliverio 2023). Technical challenges in isolating protists and limited knowledge of their diversity have further contributed to their neglect. However, advances in environmental metagenome sequencing are revealing the presence of diverse protists in extreme ecosystems, challenging earlier assumptions and broadening our understanding of life in such conditions (Rappaport and Oliverio 2023). Still, the detected nucleic acids may originate from organisms that temporarily entered the environment from nearby habitats or from dead cells, necessitating confirmation through other methods, such as cultivation.

Sulfuric hot springs (generally <pH 4.0 and >37°C), found worldwide around volcanic areas, are examples of habitats for extremophiles. The extremely low pH of these waters is due to the dissolution and oxidation of sulfur, sulfur dioxide, and

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hydrogen sulfide exposed to water and oxygen, which produces sulfuric acid. In addition, oxidation of sulfide minerals (e.g. pyrite) in underground rocks and sulfur-oxidizing bacteria also leads to sulfuric acid production (Dopson and Johnson 2012). In addition, the low pH facilitates the solubility of metals in water; therefore, these acidic waters tend to have high concentrations of metals. As a result, organisms thriving in such environments must cope with toxic metals in addition to high (≥40°C) or moderately high (37°C–40°C) temperatures and low pH, all of which are lethal to most eukaryotes. Thus, these organisms are referred to as polyextremophiles (Rappaport and Oliverio 2023). Presumably because of these requirements, the variety of unicellular eukaryotes found in sulfuric hot springs is, as described below, extremely limited. On the other hand, several types of unicellular eukaryotes have been found in highly acidic mesophilic environments or in neutral environments with high temperatures.

So far, analyses of eukaryotic diversity in highly acidic environments have been largely biased toward artificial environments, such as mine-derived streams and lakes, which are highly acidic and generally at moderate or lower temperatures (acid mine drainage; AMD) (Amaral-Zettler et al. 2002, 2011, Hao et al. 2010, Rappaport and Oliverio 2023). Previous studies have shown that species diversity declines sharply below pH 3 (Packroff and Woelfl 2000, Wollmann et al. 2000). In such environments, in addition to various lineages of microalgae, heterotrophic organisms such as some ciliates belonging to families Urotricha and Vorticella, heliozoans *Actinophrys* spp. (class Raphidomonadea, phylum Stramenopila), and amoebae *Vahlkampfia* spp (class Eutetramitea, phylum Heterolobosea) have been identified (Deneke 2000, Packroff 2000, Wollmann et al. 2000). Additionally, multicellular organisms, including fungi (Rappaport and Oliverio 2023), chironomids, and rotifers (Wollmann et al. 2000), have also been found in highly acidic environments.

In high-temperature conditions (≥40°C) with weak acidity or a higher pH (>pH 4.0), amoebae such as *Acanthamoeba* spp. (class Discosea, phylum Amoebozoa) and those belonging to the family *Naegleriidae* (class Eutetramitea, phylum Heterolobosea), including *Naegleria* spp., have been identified. However, these organisms are thermotolerant rather than thermophilic, capable of growing at temperatures above 40°C but exhibiting optimal growth at lower temperatures (Reeder et al. 2015). As thermophiles with an optimal temperature above 40°C, several amoeba strains belonging to the phyla Amoebozoa and Heterolobosea have been found. These include the amoebozoan *Echinamoeba thermarum* (class Tubulinea; Baumgartner et al. 2003) and Heterolobosean amoebae such as *Marinamoeba thermophila* (family Tulamoebidae, class Eutetramitea), found in marine hydrothermal vents (De Jonckheere et al. 2009); *Oramoeba fumarolia* (family unassigned, class Eutetramitea), isolated from marine sediment near a fumarole (De Jonckheere et al. 2011); and *Fumarolamoeba ceborucoi* (family unassigned, class Eutetramitea), found in volcanic fumaroles (De Jonckheere et al. 2011) (classification of Heterolobosea follows Pánek et al. 2025).

On the other hand, most thermoacidophiles thriving in sulfuric hot springs are nonphotosynthetic prokaryotes (archaea or bacteria) and eukaryotic microalgae belonging to the red algae class Cyanidiophyceae, which includes the genera *Galdieria, Cyanidium, Cyanidiococcus*, and *Cyanidioschyzon* (Walker et al. 2005, Cho et al. 2023, Stephens et al. 2024). Regarding eukaryotes, Cyanidiophyceae are usually the only group found in hotter regions (37°C–60°C, with an optimal temperature of 40°C–50°C), forming blue-green mats despite being red algae, as their common ancestor lost the red photosynthetic pigment phycoerythrin (Seckbach 1994). In cooler regions, however, the habitat of Cyanidiophyceae begins to overlap with that of other eukaryotic algae, such as green algae, euglenids, and diatoms (Gross 2000, Ferris et al. 2005). It is well known that cyanobacteria and other phototrophic prokaryotes are absent in environments with a pH below about 4 (Ward et al. 2012). Thus, Cyanidiophyceae had long been recognized as the only phototrophic organisms and eukaryotes found in acidic hot waters. However, later, the photosynthetic euglenid *Euglena* sp. CR-RdV (Sittenfeld et al. 2002) and the unicellular green alga *Chlamydomonas pitschmannii* (Pollio et al. 2005) were found in sulfuric hot springs.

Euglena sp. CRRdV, which is closely related to *E. mutabilis*, another acid-tolerant euglenoid, was found in an acidic hot mud pool (34°C–45°C; pH 2–4) located near the Rincón de la Vieja volcano (northwestern Costa Rica) (Sittenfeld et al. 2002). In the culture, its upper temperature limit for growth was 40°C (Sittenfeld et al. 2002). *Chlamydomonas pitschmannii* was found in the Pisciarelli hot spring located in the hydrothermal system of the Campi Flegrei Caldera (Italy) (Pollio et al. 2005). In the culture, it showed a pH tolerance limit of 1.5, with the best growth occurring at a pH between 2.0 and 2.5. The maximum temperature for growth was 42°C, while the optimal temperature was 37°C. Thus, these two algae are thermotolerant (able to survive at high temperatures but not requiring them for optimal growth) rather than truly thermophilic (thriving and growing best at high temperatures), unlike Cyanidiophyceae.

The only nonphotosynthetic eukaryotic organism ever found in sulfuric hot springs is the amoeboflagellate *Tetramitus thermacidophilus* (family Vahlkampfiidae, class Eutetramitea, phylum Heterolobosea) (Baumgartner et al. 2009, Reeder et al. 2015). This organism was found and isolated from three locations around the world: a sample mainly consisting of filamentous bacteria from the Caldera Uzon in Kamchatka (Russia; strain Cu8) (Baumgartner et al. 2009), a sediment covered with Cyanidiophyceae in Pisciarelli Solfatara near Naples (Italy; strain Pzc6; both thrive at pH 1.2–5 and 28°C–54°C, with an optimum at pH 3 and 45°C) (Baumgartner et al. 2009), and a sample from Boiling Springs Lake (BSL), an acid geothermal feature in Lassen Volcanic National Park in California (strain BSL; optimal growth at 38°C–50°C and pH 2–5), dominated by Cyanidiophyceae (Reeder et al. 2015). As far as it has been investigated, *T. thermacidophilus* feeds on bacteria and archaea present in the environment and does not appear to prey on algae, including Cyanidiophyceae (Baumgartner et al. 2009, Reeder et al. 2015).

Regarding the metagenomic analysis of environmental samples from sulfuric hot springs, the presence of some eukaryotic lineages was suggested at moderately high temperatures around 37°C–40°C. However, no evidence was found for the presence of eukaryotic organisms other than those mentioned above at temperatures of 40°C or higher (Brown and Wolfe 2006, Oliverio et al. 2018, Rappaport and Oliverio 2023, Stephens et al. 2024).

Here, we have investigated whether the scarcity of heterotrophic organisms in sulfuric hot springs, particularly under conditions of ≥40°C and ∼pH 2 (with only *T. thermacidophilus* having been found so far), is due to their phylogenetic rarity, or whether other lineages might also exist at low frequencies. By investigating samples from three sulfuric acidic hot springs in Japan, seven species of heterotrophic unicellular eukaryotes, belonging to a wide variety of eukaryotic lineages, were found in mats of Cyanidiophyceae, with five preying on them. Among these, four species were capable of growth at pH 2 and 40°C. Although the optimal temperature for all of these species was below 40°C, indicating that they are thermotolerant rather than

Figure 1. Photographs of sampling sites and points at sulfuric hot springs for unicellular eukaryotes. (a–b) Tamagawa Hot Spring (a) (Semboku City, Akita Prefecture, Japan) and Sampling Point 1 (b). (c–e) Goshogake Hot Spring (c) (Kazuno City, Akita Prefecture, Japan) and Sampling Points 1 (d) and 2 (e). (f–h) Sai-no-Kawara in Kusatsu Hot Spring (f) (Kusatsu City, Gunma Prefecture, Japan) and Sampling Points 1 (g) and 2 (h). At each point, blue-green mats soaked in the spring water were harvested, except at Tamagawa 1, where blue-green mats on the humid rock above the waterline (arrowhead) were harvested. The pH (except for Tamagawa Sampling Point 1, where the pH could not be determined) and water temperature at the time are indicated in each photo.

thermophilic, unlike *T. thermacidophilus*, these results suggest that several heterotrophic eukaryotic lineages independently acquired resistance to low pH, moderately high temperatures, and probably high metal concentrations in order to colonize niches in sulfuric hot springs.

Materials and methods

Collection, isolation, and cultivation of organisms

The collection of organisms from sulfuric hot springs was conducted on four occasions: twice at Sai-no-Kawara Park in Kusatsu, Gunma, Japan (36°37′26″ N, 138°35′29″ E) on 19 October 2022, and 16 May 2024, and once each at Goshogake Hot Spring in Kazuno, Akita, Japan (39°58′5″ N, 140°48′9″ E) on 13 July 2023, and Tamagawa Hot Spring in Senboku, Akita, Japan (39°57′45″ N, 140°43′43″ E) on 14 July 2023 (Fig. 1).

In the respective hot springs, blue-green mats of Cyanidiophyceae were collected from the surfaces of stones or rocks in hot water streams, or from the surfaces of wet rocks above the waterline, at sites with temperatures of ∼35°C or higher (Fig. 1). The mats were scraped off using a toothbrush and submerged in hot spring water from the same location in 15 ml or 50 ml plastic tubes. The samples obtained during the first collection in Kusatsu, as well as those from Goshogake and Tamagawa, were transported at room temperature and subsequently incubated at room temperature. During the second collection in Kusatsu, two types of samples were prepared: one set was kept at room temperature during transport, and the other at 40°C with a heat storage material (Patthermo, Kaneka, Japan). These were then incubated at room temperature and 40°C, respectively.

Vannella sp. VaKS-1 and *Nuclearia* sp. NuKS-1 were isolated from the samples of Kusatsu Sampling Points 1 and 2 (Fig. 1),

respectively, collected in October 2022 (first collection), which were transported at room temperature. The harvested samples were inoculated into KS medium at pH 2.0, which mimics the water composition of the Kusatsu hot spring (described later; Table 1), and incubated in the dark at room temperature for 1 week. Heterotrophic organisms that appeared to be unicellular eukaryotes and proliferated in the samples were then isolated using a pipette and cultured in KS medium or filter-sterilized Kusatsu hot spring water with *Cyanidiococcus* CcyaKS-1 (an axenic culture of a clone isolated from Kusatsu) or *E. coli* as prey at 24°C. The initial clonal culture was conducted at 24°C instead of 40°C because these organisms had proliferated after incubation at room temperature, and we considered that high-temperature conditions might cause stress to the organisms and slow their growth.

Allovahlkampfia sp. AlKS-1 was isolated from the samples of Kusatsu Sampling Point 1 (Fig. 1), collected in May 2024 (second collection), which were transported while being kept at 40°C. To promote the predominant proliferation of heterotrophic organisms, the samples were incubated in the dark at 40°C for 4 days. To isolate a clone, the sample was serially diluted with filter-sterilized Kusatsu hot spring water supplemented with both *Cyanidiococcus* CcyaKS-1 and *E. coli* as prey, and incubated in 96-well plates at 40°C in the dark. The isolated clone of *Allovahlkampfia* sp. was maintained in KS medium with *E. coli* as prey.

Parvularia sp. PaGS-1 was isolated from the samples of Goshogake Sampling Points 1 (Fig. 1) and *Neobodo* sp. NbGS-1 and *Platyophrya* sp. PlGS-1 were isolated from the samples of Goshogake Sampling Points 2 (Fig. 1), collected in July 2023, which were transported at room temperature. For *Platyophrya* sp. PlGS-1, the harvested sample was inoculated into KS medium at pH 2.0 and incubated at room temperature in the dark for 2 weeks. A *Platyophrya* cell was then isolated using a pipette and cultured in KS medium with *Cyanidiococcus* CcyaKS-1 as prey. For *Neobodo* sp. NbGS-1 and *Parvularia* sp. PaGS-1, the samples were serially diluted in KS medium supplemented with both *Cyanidiococcus* and *E. coli* as prey, and incubated in 96-well plates at 24°C in the dark. The isolated clones of *Neobodo* sp. NbGS-1 and *Parvularia* sp. PaGS-1 were maintained in KS medium with *E. coli* as prey.

Neobodo sp. NbTG-1 was collected from the sample of Tamagawa Sampling Point 1 (Fig. 1), collected in July 2023, which was transported at room temperature. *Neobodo* sp. NbTG-1 was isolated using the same method as for *Neobodo* sp. NbGS-1 described above.

Cyanidiococcus sp. CcyaKS-1, a clone isolated from Kusatsu Sampling Point 1 (Fig. 1; the *rbcL* sequence has been deposited under accession number LC860426), was maintained photoautotrophically in 20 ml of MA medium (an inorganic medium; Minoda et al. 2004) in a 50 ml culture flask at 42°C under continuous illumination (50 µmol photons m^{-2} s^{-1}). This clone was used as prey for the aforementioned organisms. In a previous study, it was found that the natural hot spring water was poor in inorganic nitrogen sources and could not support the batch culture growth of Cyanidiophyceae (Hirooka and Miyagishima 2016). Thus, *Cyanidiococcus* was cultured in an inorganic medium supplemented with a sufficient concentration of NH_4^+ (MA medium; Minoda et al. 2004) under light conditions.

A natural population of *Cyanidiococcus* was prepared from a sample collected at Kusatsu Sampling Point 1 (Fig. 1) in May 2024 (second collection), which had been transported at room temperature. The sample was inoculated into MA medium and incubated at 45°C under continuous light (50 µmol photons m^{-2} s^{-1}) for 3 days. To eliminate heterotrophic organisms, the sample was diluted three times with MA medium in 96-well plates, and a population of *Cyanidiococcus* free from heterotrophic eukaryotes, as confirmed by microscopy, was obtained. The *Cyanidiococcus* population was maintained in the same manner as *Cyanidiococcus* CcyaKS-1 as described above.

Determination of water composition of hot spring waters

The concentrations of NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-} were measured using a Water Analyzer for Total-N and Total-P (AACSIII, BL TEC K.K.). The concentrations of Al, Ca, Co, Cu, Fe, K, Mg, Mn, Na, and Zn were determined using an inductively coupled plasma (ICP) emission spectrometer (ICPE-9000, Shimadzu). Br^- and I^- were quantified using an ICP mass spectrometer (7700X ICP-MS, Agilent). Fe^{2+} and Fe^{3+} were quantified using a spectrophotometric method with 1,10-phenanthroline. F^- was quantified using the lanthanum-alizarin complexone visual colorimetric method. Cl^- was quantified using ion chromatography (LC20AD, Shimadzu). H_2SiO_3 was quantified using the molybdenum yellow visual colorimetric method. The concentration of HBO_2 was determined using the ICP mass spectrometer under the assumption that boron exists as HBO_2 at pH values below 9.2.

A synthetic inorganic medium mimicking the natural hot spring water (KS medium) [15.9 µM $(NH_4)^2SO_4$, 2.34 mM $MgSO_4$, 23.4 µM KH_2PO_4, 2.2 mM $CaCl_2$, 308 µM $FeSO_4$, 4.0 µM $ZnSO_4$, 46.2 µM $MnCl_2$, 1.47 mM $Al_2(SO_4)_3$, 0.48 mM K_2SO_4, 1.4 mM Na_2SO_4, 0.6 mM NaF, 11.6 mM HCl, 31.2 µM NaBr, 18.9 µM NaI, 0.26 mM Na_2SiO_3, 2.84 mM $NaBO_2$, pH 2.0 (adjusted with H_2SO_4); filter-sterilized (the pore size was 0.22 µm)] was prepared based on the results of three water quality surveys conducted in Kusatsu (Table 1).

Determination of growth rate at different pH and temperature ranges

To determine the pH and temperature ranges for the survival or growth of the isolated heterotrophic eukaryotes, the respective cells were cultured statically in 2 ml of KS medium in a well of a 24-well plate (92 424; TPP Techno Plastic Products) in the dark. As prey for the organisms, *E. coli* or *Cyanidiococcus* CcyaKS-1 was added to the culture as indicated for each case, except for *Platyophrya* sp. PlGS-1, for which only *Cyanidiococcus* CcyaKS-1 was added, as it did not grow when fed only *E. coli*.

For the preparation of prey, *E. coli* grown in liquid LB medium and *Cyanidiococcus* CcyaKS-1 grown in MA medium at pH 2.0 were concentrated by centrifugation at 5000× *g* and 1500× *g* for 5 min, respectively. The resulting cell pellets were suspended in KS medium adjusted to the desired pH (pH 1.0–7.0) using H_2SO_4 or NaOH, at 10 times the volume of the original cultures, yielding an OD_{750} of ∼0.1 for *Cyanidiococcus* and an OD_{600} of ∼0.05 for *E. coli*.

Each heterotrophic eukaryote was diluted 20-fold from stock cultures using KS medium supplemented with either *E. coli* or *Cyanidiococcus* CcyaKS-1 as prepared above. For pH tests, the cells were cultured at 25°C (except for *Allovahlkampfia* sp. AlKS-1, which was cultured at 40°C) at various pH levels as indicated. For temperature tests, the cells were cultured in the medium at pH 3.0 (and at pH 5.0 for *Allovahlkampfia* sp. AlKS-1) (except for *Parvularia* sp. PaKS-1, which were cultured at pH 2.0) at various temperatures as indicated. After more than 24 h had passed since the start of cultivation under each condition (allowing each organism to acclimate to its respective condition), cells of heterotrophic eukaryotes

Table 1. Compositions of sulfuric hot spring waters and the medium used in this study (KS medium), designed to mimic the composition of the water at Kusatsu hot spring.

Date Place	2020/7/15 Kusatsu	2021/2/24 Kusatsu	2021/5/13 Kusatsu	2023/7/13 Goshogake	2023/7/14 Tamagawa	KS med.
pH	1.84	1.81	1.99	2.45	1.86	2.00
Water analyzer						
NH_4^+ (μM)	15.0	12.8	18.2	34.8	20.1	15.9
NO_3^- (μM)	0.78	0.46	0.30	2.46	7.79	–
NO_2^- (μM)	0.03	0.20	0.03	2.07	2.07	–
PO_4^{3-} (μM)	25.7	17.0	25.7	5.06	8.06	23.4
ICP-OES						
Al (mM)	1.41	1.42	1.57	1.77	2.32	1.47
Ca (mM)	1.88	2.15	2.56	–	0.27	2.20
Co (μM)	–	0.06	–	1.57	1.62	–
Cu (μM)	–	0.65	1.29	–	–	–
Fe (μM)				658	649	See below
K (mM)	0.90	0.96	1.06	0.01	0.06	0.97
Mg (mM)	2.12	2.35	2.55	–	1.63	2.34
Mn (μM)	40.9	45.4	52.3	0.76	5.15	46.2
Na (mM)	2.70	3.02	2.83	–	0.12	2.85
Zn (μM)	2.81	4.21	4.80	0.94	1.35	4.00
Others	2022/10/18	2024/1/25				
F^- (mM)	0.60	0.60				0.60
Cl^- (mM)	11.7	12.0				11.6
Br^- (μM)	31.2	31.2				31.2
I^- (μM)	18.9	17.3				18.9
HBO_2 (mM)	0.26	0.26				0.26
H_2SiO_3 (mM)	2.84	2.77				2.84
Fe^{2+} (μM)		365				308
Fe^{3+} (μM)		4				

"–" indicates below the detection limit, and a blank space indicates that the measurement was not conducted.

were observed by inverted microscope (CKX-53, Olympus). Photographs were taken with a digital camera (HD Lite 1080P, Relyon), and cell density was determined by counting the number of cells per unit area. After an appropriate interval (ranging from 24 h for fast-growing cultures to 120 h for slow-growing cultures) under conditions with sufficient prey, the cells were counted again, and their growth rates were calculated.

To determine the pH and temperature ranges for the survival or growth of *Cyanidiococcus*, the population of *Cyanidiococcus* harvested from Kusatsu Sampling Point 1, as described above, was grown at 42°C under continuous light (50 μmol photons m^{-2} s^{-1}) in MA medium at pH 2.0 until they reached an OD_{750} of 1.0–2.0. The cells were harvested by centrifugation at 1500× *g* for 5 min and resuspended in 1 ml of MA medium adjusted to the respective pH using H_2SO_4 or NaOH in a well of a 24-well plate to give an OD_{750} of 0.01. Note that *Cyanidiococcus* is an obligate photoautotroph, and a previous study showed that sulfuric hot spring waters contain insufficient concentrations of inorganic nitrogen sources for growth in batch cultures (Hirooka and Miyagishima 2016). Thus, *Cyanidiococcus* cells were grown in MA medium instead of KS medium.

For pH tests, the population of *Cyanidiococcus* was cultured statically at 40°C across seven pH levels ranging from 0.25 to 7.0 under continuous light (50 μmol photons m^{-2} s^{-1}). For temperature tests, the cells were cultured statically in the medium at pH 2.0 across seven temperature levels ranging from 25°C to 55°C under continuous light (50 μmol photons m^{-2} s^{-1}). Both for the pH and temperature tests, after 48 h had passed since the start of cultivation under each condition (allowing *Cyanidiococcus* cells to acclimate to their respective condition), the cells were counted using an inverted microscope. After 48 h, the cells were counted again, and their growth rates were calculated.

To test whether the isolated heterotrophic eukaryotes can grow by feeding only on *Cyanidiococcus* as prey, cells were cultured in KS medium at pH 3.0 (except for *Parvularia* sp. PaGS-1, which was cultured at pH 2.0) with *Cyanidiococcus* CcyaKS-1 in a well of a 24-well plate in the dark. *Vannella* sp. VaKS-1, *Nuclearia* sp. NuKS-1, and *Allovahlkampfia* sp. AlKS-1 were cultured at 30°C, while *Parvularia* sp. PaGS-1, *Neobodo* sp. NbGS-1, and *Platyophrya* sp. PlGS-1 were cultured at 25°C. Growth rates were determined as described above.

Microscopy

Samples from the three sulfuric hot springs or the isolated heterotrophic organisms cultured in 96-well or 24-well plates, as described above, were observed using an inverted phase-contrast microscope (CKX-53, Olympus) with 10×, 20×, or 40× objective lenses.

The details of the cells of the isolated heterotrophic organisms were observed using a microscope (BX51, Olympus) or an inverted phase-contrast microscope (IX71, Olympus; for observing the filose pseudopods of *Nuclearia* sp. NuKS-1 or *Parvularia* sp. PaGS-1 cells) equipped with differential interference optics and 40× or 60× objective lenses.

Phylogenetic analyses

To identify the lineages of the seven heterotrophic unicellular eukaryotes isolated in this study, 18S rDNA genes were amplified by PCR using the primers 5′-AACCTGGTTGATCCTGCCAGT-3′ and 5′-TGATCCTTCTGCAGGTTCACCTAC-3′ (primer

sequences are based on a previous study; Medlin et al. 1988). The *rbcL* gene of *Cyanidiococcus* CcyaKS-1 was amplified by PCR using the primers TTGTTCCGCGTAACTCCTCAAC and TACGTTAGCTGTTGGTGTTTCTACG. The respective amplified DNA sequences were determined and subjected to a BLASTN search against the nonredundant nucleotide sequence database of NCBI.

The obtained 18S rDNA sequences and the corresponding regions from putative related species of each eukaryote, inferred from a BLASTN search, were aligned using ClustalW in MEGA 11 (Tamura et al. 2021) and subjected to maximum likelihood (ML) analysis using MEGA 11 (Tamura et al. 2021). Bayesian inference (BI) was performed with MrBayes 3.2.6 (Ronquist et al. 2012), using 1 000 000 generations of Markov chain Monte Carlo iterations; the first 25% of the generations were discarded as burn-in. The substitution models selected by MEGA 11 were TN93+G+I (ML) and HKY+G+I (BI) for the dataset of *Nuclearia* sp., TN93+G+I (ML) and GTR+G+I (BI) for the dataset of *Parvularia* sp., TN93+G (ML) and HKY+G (BI) for the dataset of *Allovahlkampfia* sp., GTR+G (both ML and BI) for the dataset of *Vannella* sp., T92+G+I (ML) and HKY+G+I (BI) for the dataset of *Platyophrya* sp., and TN93+G+I (ML) and GTR+G+I (BI) for the dataset of *Neobodo* spp.

Results

Isolation of heterotrophic unicellular eukaryotes inhabiting three sulfuric acid hot springs in Japan

To examine whether any heterotrophic unicellular eukaryotes inhabit highly acidic and high or moderately high-temperature environments, we collected blue-green mats, indicative of Cyanidiophyceae, from three sulfuric acid hot springs in Japan: Tamagawa Hot Spring (Fig. 1a and b), Goshogake Hot Spring (Fig. 1c–e), and Sai-no-kawara in Kusatsu Hot Spring (Fig. 1f–h). These mats were observed at locations with temperatures of approximately 35–50°C and pH 2–2.5 (Fig. 1). The mats were collected from the hot spring flow, except at Tamagawa Point 1, where the mat was collected from the surface of a wet rock above the waterline (where pH could not be measured) (Fig. 1).

In the initial sampling series, the samples were brought back to the laboratory at room temperature, without any particular treatment for temperature after sampling, and were incubated in the dark at room temperature for 2 weeks for the Goshogake and Tamagawa samples, and for 4 days for the Kusatsu samples, with the expectation that they would proliferate by consuming surrounding microorganisms. As a result, eukaryotes were observed in the samples, including round cells larger than archaea or bacteria (Fig. 2a and e), ciliates (Fig. 2b), flagellates (Fig. 2c), amoeboid cells (Fig. 2d), which are presumed to be unicellular eukaryotes, and rotifers (Fig. 2f; Supplementary Video S1). Considering the possibility that these eukaryotic organisms might temporarily enter from relatively cooler areas nearby (e.g. the edges of the flow) or survive in dormant states such as cysts and then proliferate at room temperature during transport, we next collected blue-green mats of Cyanidiophyceae from Kusatsu Sampling Point 1 and transported them to the laboratory in an insulated container maintained at 40°C. When the samples were observed under a microscope 6 h after collection, amoeboid organisms were detected (Fig. 2g and h), suggesting the presence of heterotrophic unicellular eukaryotes capable of growing at pH 2 and ≥40°C.

Based on the above results, we decided to isolate and establish cultures of unicellular eukaryotic organisms, excluding the multicellular rotifer, from the collected blue-green mat samples. To set up culture conditions for these organisms, we first examined the primary components of the blue-green mats from the three hot springs and identified *Cyanidiococcus* sp. as the dominant organism. From Kusatsu Sampling Point 1, we established a clonal culture of *Cyanidiococcus* sp. (CcyaKS-1; *rbcL* accession number, LC860426). Additionally, we analyzed the water composition of the three hot springs and prepared a culture medium that mimics Kusatsu hot spring water (KS-1 medium, Table 1). We confirmed that iron (on the order of 0.3 mM) and aluminum (on the order of 1 mM) were highly dissolved due to the strong acidity (Table 1). High concentrations of iron are known to cause oxidative stress within cells and inhibit the absorption of other essential trace elements, such as Mg^{2+} and Zn^{2+} (Harish et al. 2023). Similarly, Al^{3+} is known to be toxic to cells (Sparling and Lowe 1996).

Using *Cyanidiococcus* CcyaKS-1 or *E. coli* as prey, we cultured clones of the unicellular eukaryotic organisms in KS medium (Figs 3–7). Molecular phylogenetic analyses based on 18S rDNA sequences were then conducted to determine their taxonomic affiliations (Figs 3–7). Furthermore, we examined the temperature (Fig. 8) and pH (Fig. 9) ranges at which each organism could grow or survive. The results for each organism are described below. For comparison, we prepared a population of *Cyanidiococcus* isolated from Kusatsu Sampling Point 1 (a population was used instead of a single clone to account for potential genetic variability in the traits of individual clones) and investigated its growth and survival pH and temperature ranges. The results, which were largely consistent with previous reports, showed that *Cyanidiococcus* could grow at pH 0.5–5.0, with an optimum at pH 2.0, and at temperatures between 30°C and 50°C (Figs 8 and 9). At 55°C, the cells did not increase, and when the cells exposed to 55°C for 4 days were subsequently transferred to 40°C, they gradually bleached and died. This indicates that the *Cyanidiococcus* population is unable to survive at 55°C (Fig. 9).

Regarding the rotifer (Fig. 2f; Supplementary Video S1), which was not further examined, it is known that several lineages of rotifers inhabit aquatic environments with moderate or lower temperatures and a pH below 3 (Deneke 2000). However, the rotifer found in the sulfuric hot spring (Fig. 2f) differed from these, as it was able to grow by feeding on Cyanidiococcus (Supplementary Video S1) at 40°C but not at 45°C.

Nuclearia sp. NuKS-1 and *Parvularia* sp. PaGS-1

A spherical unicellular organism, ~20 μm in diameter (morphologically similar to that in Fig. 2e), was isolated from *Cyanidiococcus* mats at Kusatsu Sampling Point 2, which was incubated at room temperature. Another spherical unicellular organism, ~5 μm in diameter (morphologically similar to those in Fig. 2a), was isolated from mats at Goshogake Sampling Point 1, which was also incubated at room temperature. Phylogenetic analysis based on 18S rDNA sequences identified these organisms as belonging to the genera *Nuclearia* and *Parvularia*, respectively (Fig. 3). Both genera are classified under the class Cristidiscoidea of the phylum Cristidiscoidea (Fig. 3). They were named *Nuclearia* sp. NuKS-1 and *Parvularia* sp. PaGS-1, respectively. In accordance with these results, filose pseudopods were evident in both organisms (Fig. 3c and h), consistent with the shared characteristics of this group (Gabaldón et al. 2022).

In their isolated cultures, both *Nuclearia* sp. NuKS-1 and *Parvularia* sp. PaGS-1 grew when supplemented with *Cyanidiococcus* as their sole prey (Fig. 10), ingesting and digesting *Cyanidiococcus* within their cells (Fig. 3b, f, and g). Additionally, both or-

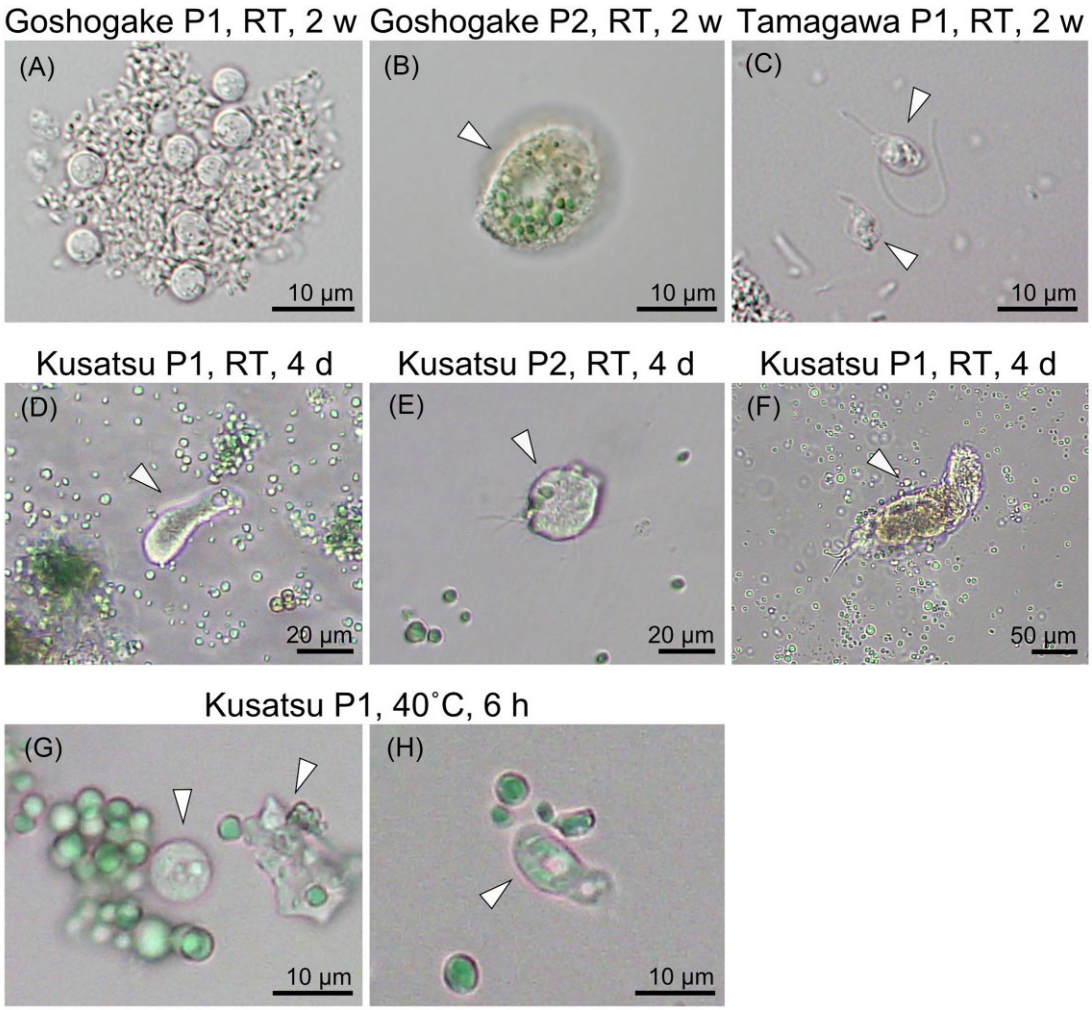

Figure 2. Examples of candidates for eukaryotes, other than the unicellular red algae Cyanidiophyceae, found in samples harvested from sulfuric hot springs. (a–c) A round-shaped organism (a), a ciliate (b), and a flagellate (c) found in samples harvested from Goshogake Sampling Point 1 or Tamagawa Sampling Point 1 after 2 weeks of incubation at room temperature (RT). (d–f) An amoeba (d), a round-shaped unicellular organism (e), and a rotifer (f) found in samples harvested from Kusatsu Sampling Point 1 or 2 after 4 days of incubation at RT. (g and h) Amoeboid organisms found in samples harvested from Kusatsu Sampling Point 1 after 6 h of incubation at 40°C. That harvested samples were kept at 40°C during transportation, between collection and microscopic observation.

ganisms were able to grow by feeding exclusively on *E. coli* (Figs 8 and 9), although it is not present in their natural habitat.

Regarding the pH range, *Nuclearia* sp. NuKS-1 was capable of growing at pH 1.8–7.0, with an optimum at pH 3.0, whereas at pH 1.6 or lower, the cells shrank and eventually died (Fig. 8). Regarding the temperature range, it was capable of growing at 25°C–40°C, with an optimum at 35°C (Fig. 9). At 41°C or higher, the cell number remained unchanged, and the cells retained their shape for 4 days. When these populations were subsequently transferred to 30°C, the cells originally cultured at 41°C proliferated again, but the cells cultured at 42°C or higher did not proliferate at all, indicating that *Nuclearia* sp. NuKS-1 is unable to survive at 42°C or higher (Fig. 9).

Parvularia sp. PaGS-1 was capable of growing at pH 1.2–7.0, with an optimum at pH 2.0, but it could not survive at pH 1.0 (Fig. 8). It was capable of growing at 25°C–40°C, with an optimum at 25°C (Fig. 9). Similar to *Nuclearia* sp. NuKS-1, at 41°C or higher, the cell number of *Parvularia* sp. PaGS-1 remained unchanged, with cells retaining their shape for 4 days; however, when these populations were subsequently transferred to 25°C, they did not proliferate at all, indicating that *Parvularia* sp. PaGS-1 is unable to survive at 41°C or higher (Fig. 9).

Allovahlkampfia sp. AlKS-1

An amoeboid organism (morphologically similar to those in Fig. 2g and h) was isolated from *Cyanidiococcus* mats at Kusatsu Sampling Point 1, which was transferred and incubated at 40°C. Phylogenetic analysis based on 18S rDNA sequences identified it as belonging to the genus *Allovahlkampfia* (class Eutetramitea, phylum Heterolobosea), and it was named *Allovahlkampfia* sp. AlKS-1 (Fig. 4).

The isolated culture of *Allovahlkampfia* sp. AlKS-1 originated from a flagellate cell. During the early stage of the culture, the flagellates proliferated (Fig. 4c); however, as cultivation progressed, the culture began to produce amoeboid cells (Fig. 4a and b) and cysts (Fig. 4d), and eventually, the flagellates disappeared (Fig. 4a). It is known that members of the Heterolobosea generally exhibit these three morphological forms within a single species (Pánek et al. 2025). However, under our culture conditions, flagellates did not reappear even after multiple passages of *Allovahlkampfia* sp. AlKS-1.

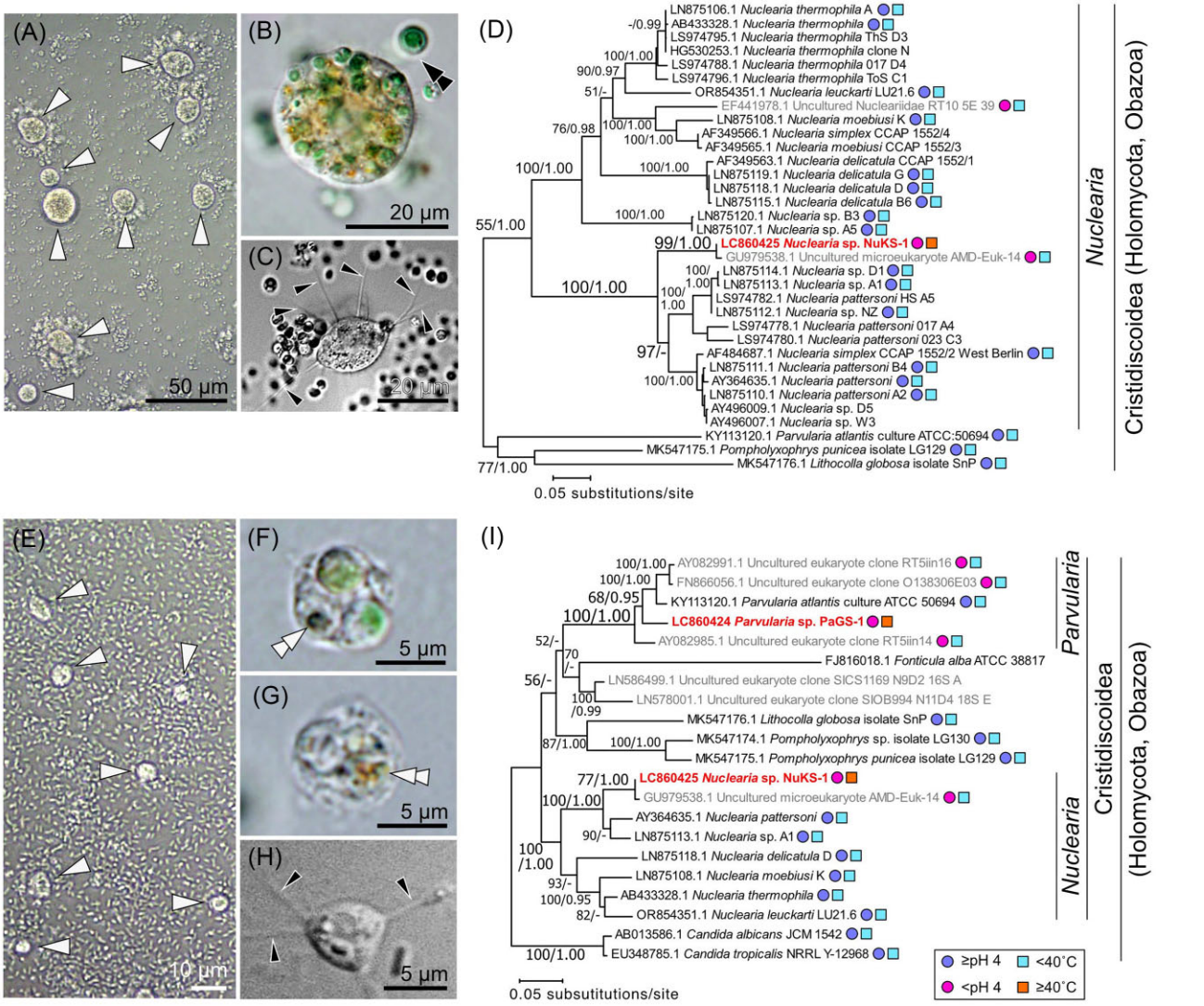

Figure 3. Microscopic images of the culture, cells, and phylogenetic positions of *Nuclearia* sp. NuKS-1, isolated from Kusatsu Sampling Point 2, and *Parvularia* sp. PaGS-1, isolated from Goshogake Sampling Point 1. (a) A phase-contrast image of the *Nuclearia* sp. NuKS-1 culture supplemented with *E. coli* as prey. (b) A differential interference contrast (DIC) image of a *Nuclearia* sp. NuKS-1 cell feeding on *Cyanidiococcus*. (c) A DIC image of a *Nuclearia* sp. NuKS-1 cell showing the filose pseudopods. (d) ML tree of *Nuclearia* sp. NuKS-1 based on 1 992 base pairs of 18S rDNA sequences. The tree was constructed using the ML method based on 18S rDNA sequences. The sequences shown in black text are derived from the DNA of cultured cells, while those shown in gray text are derived from environmental DNA. Bootstrap values (>50%) obtained by ML and posterior probabilities (>0.95) obtained by BI analysis are shown above the selected branches. The branch lengths reflect evolutionary distances, as indicated by the scale bar. The accession numbers of the respective nucleotide sequences are indicated with the species and strain names. When the information is available (with the source listed in Supplementary Table S1), the pH (blue circles represent pH ≥4, magenta circles represent pH <4) and temperature (light blue squares represent temperatures <40°C, orange squares represent temperatures ≥40°C) of the locations where each strain or environmental DNA is collected are also shown. (e) A phase-contrast image of the *Parvularia* sp. PaGS-1 culture supplemented with *E. coli* as prey. (f and g) DIC images of *Parvularia* sp. PaGS-1 cells feeding on *Cyanidiococcus*. (h) A DIC image of a *Parvularia* sp. PaGS-1 cell showing the filose pseudopods. (i) ML tree of *Parvularia* sp. PaGS-1 and *Nuclearia* sp. NuKS-1 based on 1 756 base pairs of 18S rDNA sequences. The rest is the same as in Fig. 3d. The white arrowheads indicate *Nuclearia* sp. NuKS-1 or *Parvularia* sp. PaGS-1 cells. The black and white double arrowheads indicate *Cyanidiococcus* cells located outside the *Nuclearia* sp. NuKS-1 or *Parvularia* sp. PaGS-1 cells (black) and those being digested within them (white), respectively. The black arrowheads indicate the filose pseudopods of *Nuclearia* sp. NuKS-1 or *Parvularia* sp. PaGS-1 cells.

In the isolated culture, *Allovahlkampfia* sp. AlKS-1 grew when supplemented with *Cyanidiococcus* as its sole prey (Fig. 10), ingesting and digesting *Cyanidiococcus* within its cells (Fig. 4b). Additionally, it was able to grow by feeding exclusively on *E. coli* (Figs 8 and 9). *Allovahlkampfia* sp. AlKS-1 was capable of growing at pH 1.6–7.0, with an optimum at pH 5.0, but it could not survive at pH 1.2 or lower (Fig. 8). At pH 1.4, after cultivation for 4 days, the cells were still exhibiting amoeboid locomotion, but the number of cells decreased, indicating that they are unable to survive at pH 1.4 in the long term (Fig. 8).

It was capable of growing at 25°C–40°C, with an optimum at 30°C, but it could not survive at 44°C or higher (Fig. 9). After cultivation for 4 days at 41°C–43°C, the cells were still exhibiting amoeboid locomotion, although the cell number did not increase (Fig. 9). At 41°C and 42°C, almost all cells survived, but at 43°C the cell number decreased, indicating that the upper temperature limit for long-term survival is 42°C (Fig. 9). Under conditions where it could survive, the frequency of cyst formation remained almost constant at each temperature during the cultivation period until the culture became confluent.

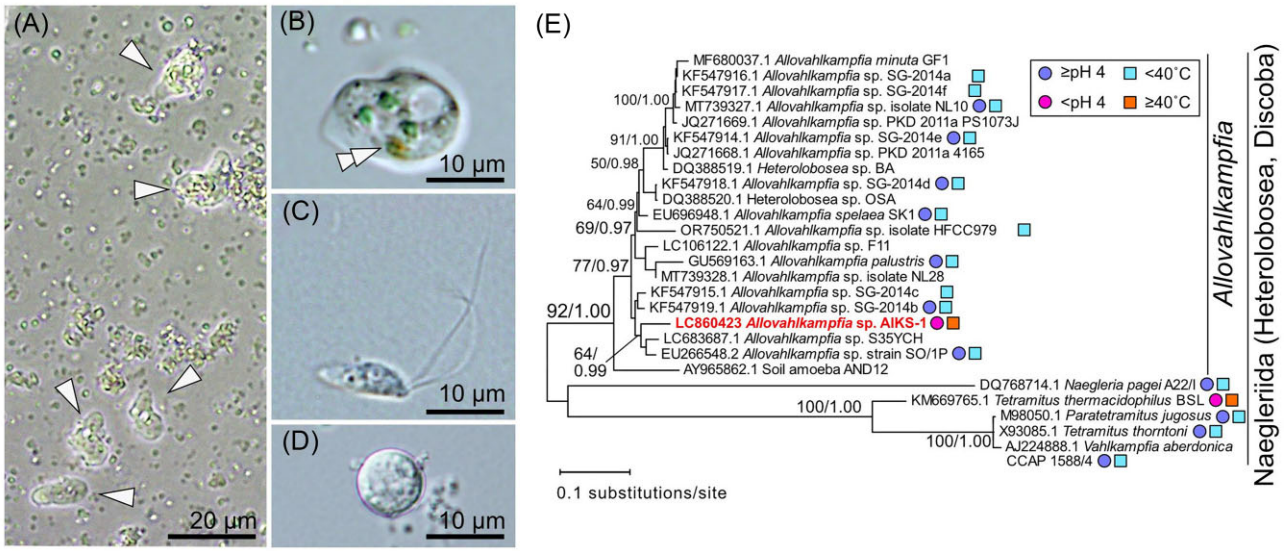

Figure 4. Microscopic images of the culture, cells, and phylogenetic positions of *Allovahlkampfia* sp. AlKS-1, isolated from Kusatsu Sampling Point 1. (a) A phase-contrast image of the *Allovahlkampfia* sp. AlKS-1 culture supplemented with *Cyanidiococcus* as prey. (b–d) DIC images of an *Allovahlkampfia* sp. AlKS-1 amoeboid cell (b), flagellate cell, and cyst (d). (e) ML tree of *Allovahlkampfia* sp. AlKS-1 based on 2 031 base pairs of 18S rDNA sequences. The rest is the same as in Fig. 3d. The white arrowheads indicate *Allovahlkampfia* sp. AlKS-1 cells. The white double arrowhead indicates a *Cyanidiococcus* cell being digested in the *Allovahlkampfia* sp. AlKS-1 cell.

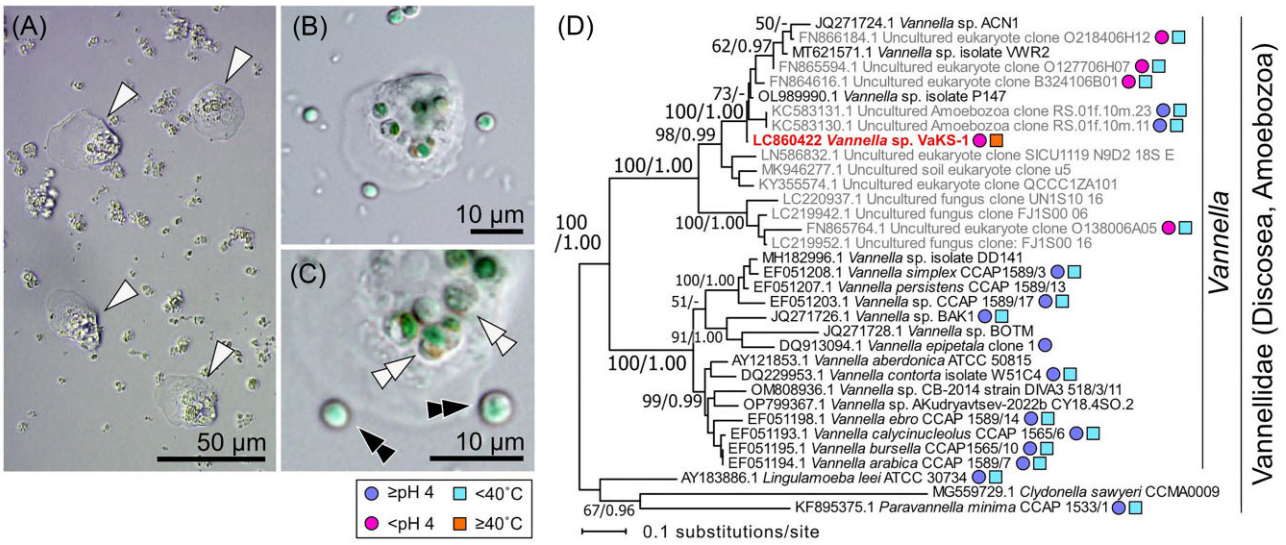

Figure 5. Microscopic images of the culture, cells, and phylogenetic positions of *Vannella* sp. VaKS-1, isolated from Kusatsu Sampling Point 1. (a) A phase-contrast image of the *Vannella* sp. VaKS-1 culture supplemented with *E. coli* as prey. (b and c) DIC images of a cell feeding on *Cyanidiococcus* (b), with an enlarged image showing *Cyanidiococcus* being digested in the cell (c). (d) ML tree of *Vannella* sp. VaKS-1 based on 1 903 base pairs of 18S rDNA sequences. The rest is the same as in Fig. 3d. The white arrowheads indicate *Vannella* sp. VaKS-1 cells. The black and white double arrowheads indicate *Cyanidiococcus* cells located outside the *Vannella* sp. VaKS-1 cell (black) and those being digested within it (white), respectively.

Vannella sp. VaKS-1

An amoeboid organism (morphologically similar to those in Fig. 2g and h) isolated from *Cyanidiococcus* mats at Kusatsu Sampling Point 1, which was transferred and incubated at room temperature, was identified through phylogenetic analysis based on 18S rDNA sequences as belonging to the genus *Vannella* (class Discosea, phylum Amoebozoa) and was named *Vannella* sp. VaKS-1 (Fig. 5).

In the isolated culture, *Vannella* sp. VaKS-1 grew when supplemented with *Cyanidiococcus* as its sole prey (Fig. 10), ingesting and digesting *Cyanidiococcus* within its cells (Fig. 5c). Additionally, it was able to grow by feeding exclusively on *E. coli* (Figs 8 and 9). *Vannella* sp. VaKS-1 was capable of growing at pH 1.4–5.0, with an optimum at pH 3.0, but it could not survive at pH 1.0 or pH 7.0 (Fig. 8). After cultivation for 4 days at pH 1.2, the cells were still exhibiting amoeboid locomotion, but the cell number decreased (Fig. 8), indicating that the lower pH limit for long-term survival is pH 1.4.

It was capable of growing at 25°C–35°C, with an optimum at 35°C (Fig. 9). At 40°C, when fed on *E. coli*, *Vannella* sp. VaKS-1 showed little growth but survived (Fig. 9), exhibiting amoeboid locomotion for at least one week. In addition, at 40°C, it was capable of grow-

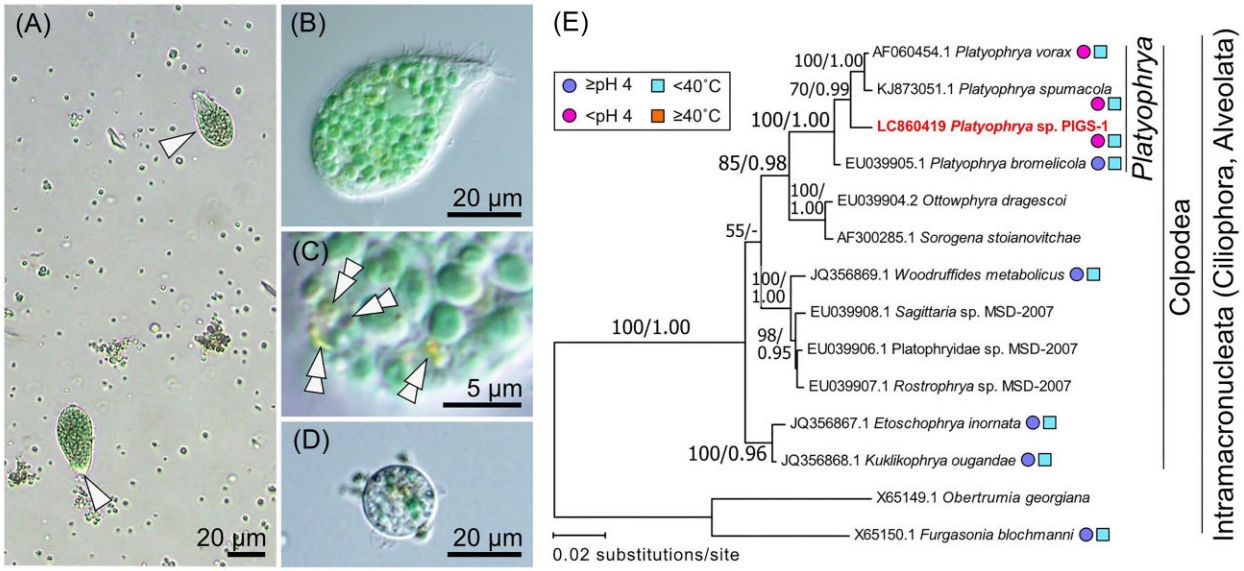

Figure 6. Microscopic images of the culture, cells, and phylogenetic positions of *Platyophrya* sp. PlGS-1, isolated from Goshogake Sampling Point 2. (a) A phase-contrast image of the *Platyophrya* sp. PlGS-1 culture supplemented with *Cyanidiococcus* as prey. (b and c) DIC images of a cell (b), with an enlarged image showing *Cyanidiococcus* being digested in the cell (c). (d) A DIC image of a cyst of *Platyophrya* sp. PlGS-1. (e) ML tree of *Platyophrya* sp. PlGS-1 based on 1 683 base pairs of 18S rDNA sequences. The rest is the same as in Fig. 3d. The white arrowheads indicate *Platyophrya* sp. PlGS-1 cells. The white double arrowheads indicate *Cyanidiococcus* cells being digested in the *Platyophrya* sp. PlGS-1 cell.

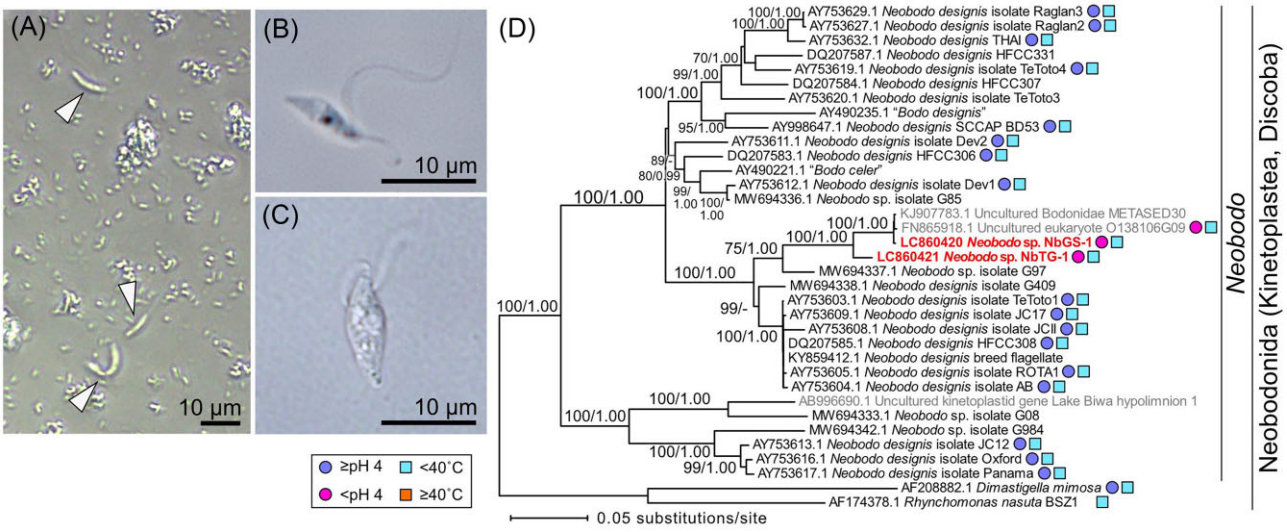

Figure 7. Microscopic images of the cultures, cells, and phylogenetic positions of *Neobodo* sp. NbGS-1, isolated from Goshogake Sampling Point 2, and *Neobodo* sp. NbTG-1, isolated from Tamagawa Sampling Point 1. (a) A phase-contrast image of the *Neobodo* sp. NbGS-1 culture supplemented with *E. coli* as prey. (b and c) DIC images of the cells of *Neobodo* sp. NbGS-1 (b) and *Neobodo* sp. NbTG-1 (c) cultured with *Cyanidiococcus* as the sole prey. (d) ML tree of *Neobodo* sp. NbGS-1 and *Neobodo* sp. NbTG-1 based on 2 116 base pairs of 18S rDNA sequences. The rest is the same as in Fig. 3d. The white arrowheads indicate *Neobodo* sp. NbGS-1 cells.

ing when fed on *Cyanidiococcus* (growth rate $\mu = 0.18$) (Fig. 9). In contrast, it could not survive at 41°C or higher (Fig. 9).

Platyophrya sp. PlGS-1

This organism and the following *Neobodo* spp. were found to be capable of growing at pH 2 but unable to survive at temperatures of 40°C or higher or 35°C or higher, respectively (Fig. 9).

A ciliate (morphologically similar to that in Fig. 2b) isolated from *Cyanidiococcus* mats at Goshogake Sampling Point 2, which was transferred and incubated at room temperature, was identified through phylogenetic analysis based on 18S rDNA sequences as belonging to the genus *Platyophrya* (class Colpodea, phylum Ciliophora) and named *Platyophrya* sp. PlGS-1 (Fig. 6).

In the isolated culture, *Platyophrya* sp. PlGS-1 grew when supplemented with *Cyanidiococcus* as its sole prey (Figs 8–10), ingesting and digesting *Cyanidiococcus* within its cells (Fig. 6c). However, it was not able to grow by feeding exclusively on *E. coli*. *Platyophrya* sp. PlGS-1 formed cysts, as do other Colpodea species (Foissner 1993), after consuming nearly all of the *Cyanidiococcus* prey (Fig. 6d).

In the isolated culture, *Platyophrya* sp. PlGS-1 was capable of growing in a pH range of 2.0–7.0, with an optimum pH of 3.0 (Fig. 8).

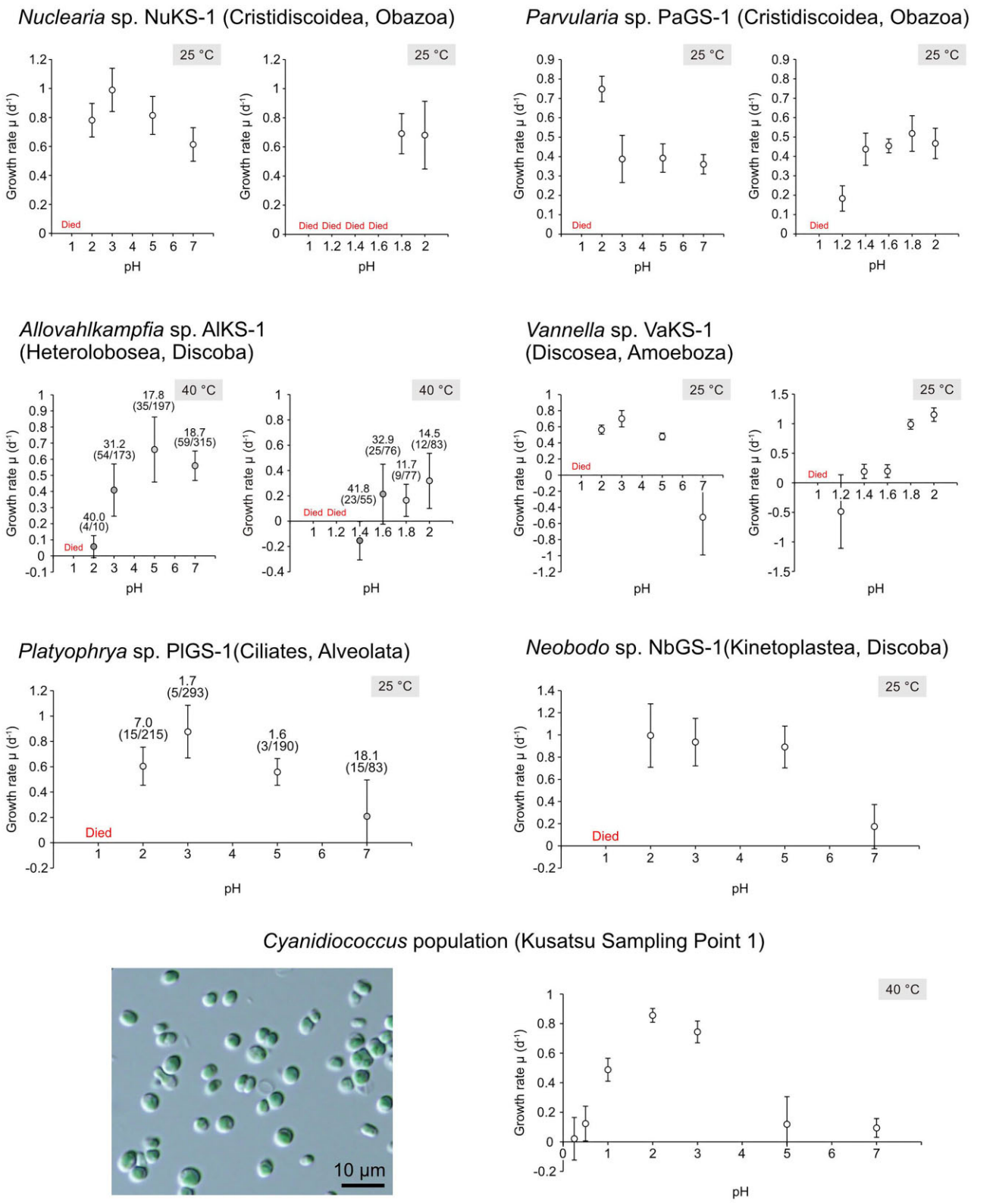

Figure 8. Growth rates of heterotrophic unicellular eukaryotes isolated in this study, along with the unicellular red alga *Cyanidiococcus*, across a range of pH levels. Means ± standard deviations of results from four independent cultures are shown. For *Allovahlkampfia* sp. AlKS-1 and *Platyophrya* sp. PlGS-1, the proportion of cysts (summed across four independent cultures) is shown as a percentage. For the test between pH 1.0 to 7.0 at intervals of 1.0, the heterotrophic organisms were cultivated with *E. coli* as prey, except for *Platyophrya* sp. PlGS-1, which was cultivated with *Cyanidiococcus* as prey, in the dark using an inorganic medium mimicking the composition of Kusatsu hot spring water. For the test between pH 2.0 and 1.0 at intervals of 0.2 (only for *Nuclearia* sp. NuKS-1, *Pavularia* sp. PaGS-1, *Allovahlkampfia* sp. AlKS-1, and *Vannella* sp. VaKS-1), the organisms were cultivated with *Cyanidiococcus* as prey. The cultivation temperature was 25°C, except for *Allovahlkampfia* sp. AlKS-1, which was cultivated at 40°C. A *Cyanidiococcus* population harvested from Kusatsu Sampling Point 1 was cultivated in inorganic MA medium at 40°C. After being transferred to the respective conditions and left for more than 24 h (allowing the cells to acclimate to their respective condition), the growth rate was measured. "Died" indicates that all cells had died where when the cells retained their shape, their numbers were plotted. If "Died" is indicated and no plot is present, this means that the cells disintegrated and were not observed.

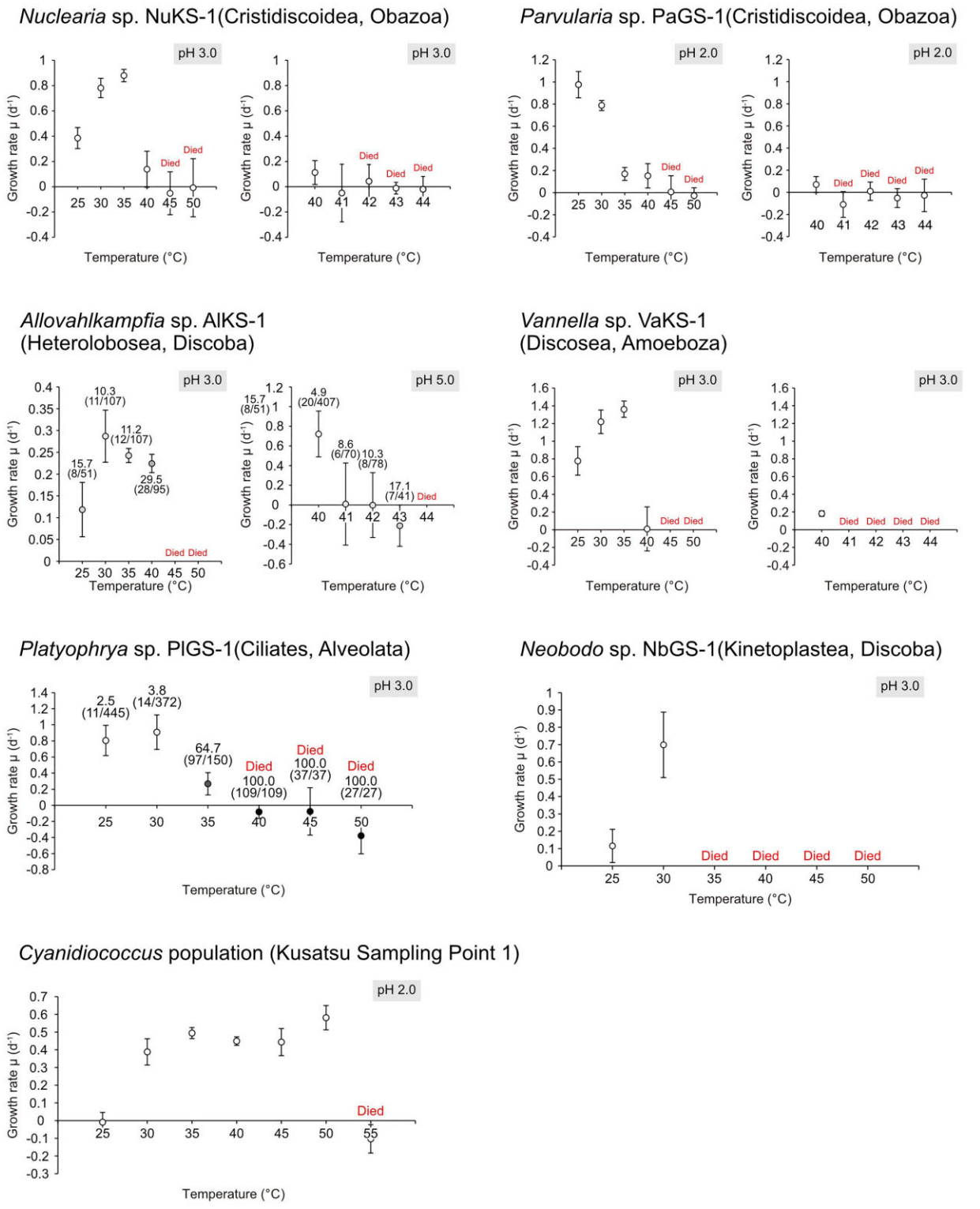

Figure 9. Growth rates of heterotrophic unicellular eukaryotes isolated in this study, along with the unicellular red alga *Cyanidiococcus*, across a range of temperatures. Means ± standard deviations of results from four independent cultures are shown. For *Allovahlkampfia* sp. AlKS-1 and *Platyophrya* sp. PlGS-1, the proportion of cysts (summed across four independent cultures) is shown as a percentage. For the test between 25°C and 50°C at intervals of 5°C, the heterotrophic organisms were cultivated with *E. coli* as prey, except for *Platyophrya* sp. PlGS-1, which was cultivated with *Cyanidiococcus* as prey, in the dark using an inorganic medium mimicking the composition of Kusatsu hot spring water. For the test between 40°C and 44°C at intervals of 1°C (only for *Nuclearia* sp. NuKS-1, *Pavularia* sp. PaGS-1, *Allovahlkampfia* sp. AlKS-1, and *Vannella* sp. VaKS-1), the organisms were cultivated with *Cyanidiococcus* as prey. The medium was set to pH 3.0, except for *Parvularia* sp. PlGS-1, which was cultivated at pH 2.0, and for the test between 40°C and 44 °C of *Allovahlkampfia* sp. AlKS-1, which was cultivated at pH 5.0 (the respective optimal pH for each strain). A *Cyanidiococcus* population harvested from Kusatsu Sampling Point 1 was cultivated in inorganic MA medium at pH 2.0. After being transferred to the respective conditions and left for more than 24 h (allowing the cells to acclimate to their respective condition), the growth rate was measured. "Died" indicates that all cells had died, whereas when the cells retained their shape, their numbers were plotted. If "Died" is indicated and no plot is present, this means that the cells disintegrated and were not observed.

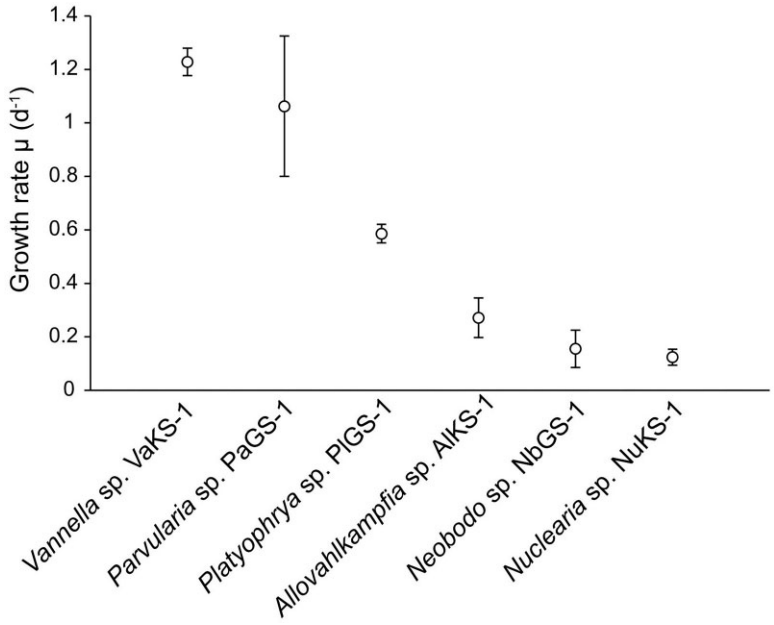

Figure 10. Growth rates of the heterotrophic unicellular eukaryotes isolated in this study while feeding exclusively on the unicellular red alga *Cyanidiococcus*. Means ± standard deviations of results from four independent cultures are shown. The organisms were cultivated with *Cyanidiococcus* as prey in the dark using an inorganic medium mimicking the composition of Kusatsu hot spring water at pH 3.0, except for *Parvularia* sp. PaGS-1, which was cultivated at pH 2.0. The temperature was set to 30°C for *Vannella* sp. VaKS-1, *Allovahlkampfia* sp. AlKS-1, and *Nuclearia* sp. NuKS-1, and 25°C for the others.

It could not survive at pH 1.0; when transferred to the condition, the cells died immediately without forming cysts (Fig. 8). The strain was able to grow at temperatures between 25°C and 35°C, with an optimum at 30°C (Fig. 9). At 35°C, about two-thirds of the cells formed cysts (Fig. 9), but when the culture was transferred to 25°C, most of the cysts germinated within a day (12.9% remained as cysts), indicating that they were alive. At temperatures of 40°C or higher, all cells formed cysts (Fig. 9); however, they did not germinate even when subsequently transferred to 25°C, indicating that they were dead. Under conditions where it could grow, the frequency of cyst formation remained nearly stable at each temperature during the cultivation period until the *Cyanidiococcus* prey was depleted.

Neobodo sp. NbGS-1 and *Neobodo* sp. NbTG-1

Flagellates (morphologically similar to that in Fig. 2c) isolated from *Cyanidiococcus* mats at Goshogake Sampling Point 2 and Tamagawa Sampling Point 1, which were transferred and incubated at room temperature, were identified through phylogenetic analysis based on 18S rDNA sequences as belonging to the genus *Neobodo* (class Kinetoplastea, phylum Euglenozoa) and were named *Neobodo* sp. NbGS-1 and *Neobodo* sp. NbTG-1, respectively (Fig. 7). The analysis also showed that the two strains are very closely related (Fig. 7). Thus, we further examined *Neobodo* sp. NbGS-1 solely in this study.

In the isolated culture, *Neobodo* sp. NbGS-1 was able to grow when supplemented with either *Cyanidiococcus* (Fig. 10) or *E. coli* (Figs 8 and 9; Supplementary Video S2) as its sole prey. However, we never observed *Neobodo* sp. NbGS-1 ingests these prey organisms. Thus, how this organism acquires nutrients remains unclear, whether through phagotrophic digestion or by utilizing organic substances released extracellularly by prey organisms.

Neobodo sp. NbGS-1 was capable of growing at pH 2.0–7.0, with an optimum pH of 2.0–5.0, but it did not survive at pH 1.0 (Fig. 8). It was capable of growing at 25°C–30°C, with an optimum temperature of 30°C, but could not survive at 35°C or higher (Fig. 9).

Discussion

In volcanic sulfuric acidic hot springs, which are characterized by high or moderately high temperatures and extreme acidity, the only known heterotrophic protist had been *T. thermacidophilus* (class Eutetramitea, phylum Heterolobosea), identified from three locations worldwide and exhibiting optimal growth at pH 3 and 45°C (Baumgartner et al. 2009, Reeder et al. 2015). In this study of sulfuric acidic hot springs (34.7°C–50°C, 34.7°C–50°C, ~pH 2.0), we identified four additional heterotrophic protists capable of growing or surviving at 40°C and pH 2.0 (Fig. 11; Table 2): *Nuclearia* sp. NuKS-1 and *Parvularia* sp. PaGS-1 (both in class Nucleariidea, phylum Cristidiscoidea), *Allovahlkampfia* sp. AlKS-1 (class Eutetramitea, phylum Heterolobosea), and *Vannella* sp. VaKS-1 (class Discosea, phylum Amoebozoa) (Fig. 5). Additionally, while their maximum survival temperatures were 35°C and 30°C, respectively (Fig. 9; Table 2), the ciliate *Platyophrya* sp. PlGS-1 (class Colpodea, phylum Ciliophora) (Fig. 6) and the kinetoplastids *Neobodo* sp. NbGS-1 and NbTG-1 (class Kinetoplastea, phylum Euglenozoa) (Fig. 7) were also found in the same environment. In addition, as discussed below, the results of the phylogenetic analyses suggest that the former four species independently evolved from their respective mesophilic, neutrophilic ancestors through the development of acidophilic and thermotolerant traits. Although the four species could grow at 40°C or at slightly higher temperatures (with a maximum of 42°C for *Allovahlkampfia* sp. AlKS-1), these organisms are not thermophilic but thermotolerant, with optimal temperatures below 40°C, unlike the thermophilic *T. thermacidophilus* and the unicellular red algae Cyanidiophyceae (Fig. 9). The rarity of thermophiles, but not thermotolerant eukaryotes, inhabiting moderately high-temperature

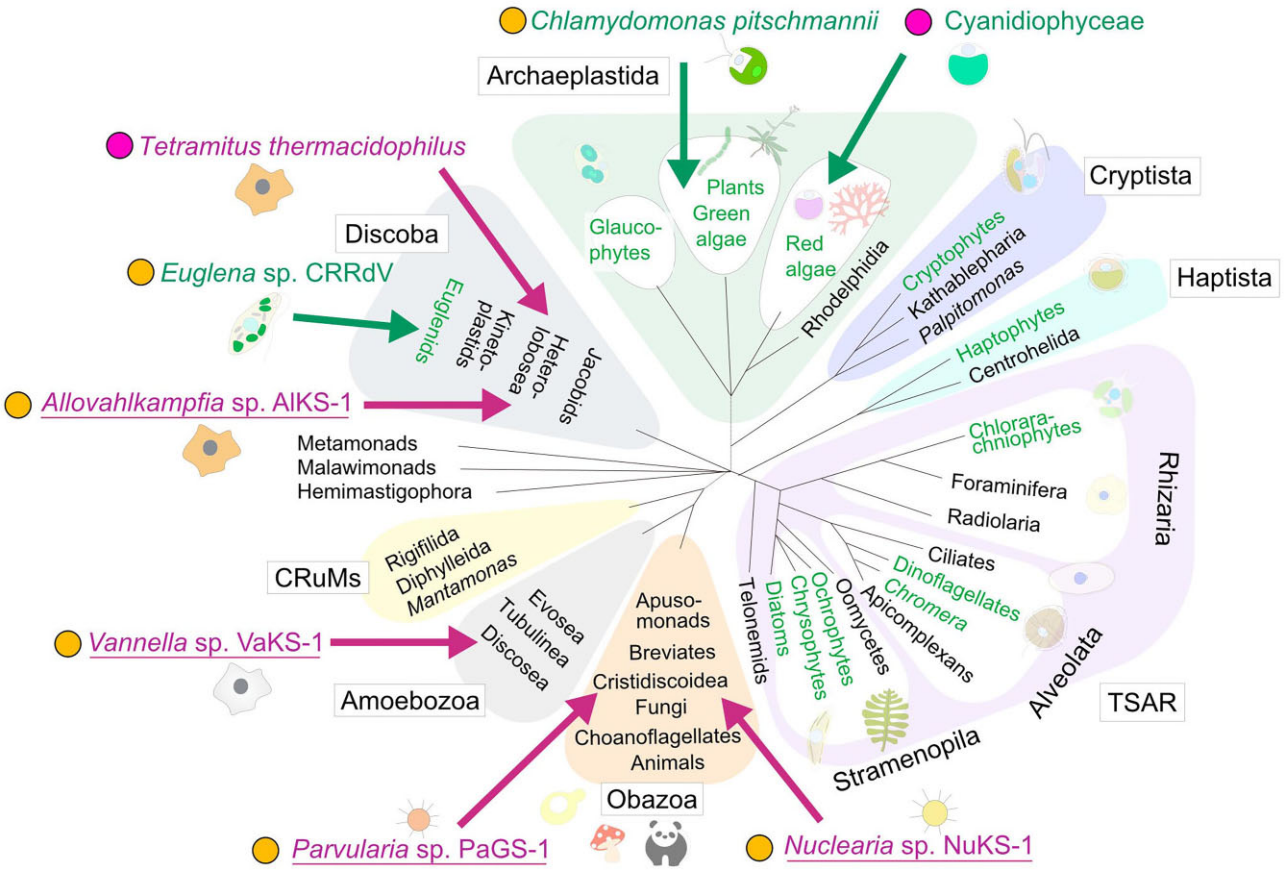

Figure 11. Phylogenetic distribution of thermo-acidophilic and thermotolerant acidophilic organisms in eukaryotes. Photosynthetic and heterotrophic species (capable of surviving at ≥40°C and ≤pH 4.0) are shown in green and magenta text, respectively. Magenta and orange circles indicate thermophilic (optimal temperature ≥40°C) and thermotolerant (optimal temperature <40°C) organisms, respectively. Organisms newly identified in this study are underlined. The tree topology is based on a previous paper (Strassert et al. 2021).

acidic environments is apparently consistent with the results of previous metagenomic analyses (Rappaport and Oliverio 2023).

Thus far, *Nuclearia* species have been isolated from environments with moderate or lower temperatures (<37°C) and neutral to slightly acidic pH levels (≥pH 4) (Fig. 3d and i; e.g. Dirren and Posch 2016). However, environmental metagenomic analyses of the highly acidic, red-colored AMD in Río Tinto (15°C–25°C, pH 2.0; Spain) (RT10 5E 39; Amaral-Zettler et al. 2002, 2011), and AMD in Xiang Mountain (30°C, pH 3.0; China) (AMD-Euk-14; Hao et al. 2010) identified *Nuclearia* spp. from each location. Of these, AMD Euk-14 (Hao et al. 2010) is most closely related to *Nuclearia* sp. NuKS-1 (Fig. 3d). Thus, the result suggests that the common ancestor of *Nuclearia* sp. NuKS-1 and AMD-Euk-14 evolved from a mesophilic, neutrophilic ancestor by acquiring thermotolerant and acidophilic traits.

Regarding the genus *Parvularia*, only one strain, *Parvularia atlantis* ATCC 50694, isolated from a lake in Atlanta, has been described (López-Escardó et al. 2018). However, environmental metagenomic analyses of the AMD in Río Tinto (Amaral-Zettler et al. 2002, 2011) identified three clones of *Parvularia* (RT5iin16; O138306E03; RT5iin14; Fig. 3i). Although it remains unclear when *Parvularia* sp. PaGS-1 acquired acidophilic and thermotolerant capabilities during evolution, the phylogenetic tree pattern and the distribution of organisms across various environments (Fig. 3d and i) suggest that *Nuclearia* sp. NuKS-1 and *Parvularia* sp. PaGS-1 independently evolved acidophilic and thermotolerant traits.

Thus far, Heterolobosean *Allovahlkampfia* species have been isolated from soil, temperate freshwater, or tree bark, and they feed on bacteria. However, all of these species, except for *Allovahlkampfia* sp. AlKS-1, as far as their isolation environments have been documented, have been found in environments with moderate or lower temperatures (<37°C) and neutral to slightly acidic pH levels (≥pH 4) (Fig. 4e; e.g. Geisen et al. 2015). In culture, none of these strains, except for *Allovahlkampfia* sp. AlKS-1, has shown growth at 37°C; some have grown at 30°C, while others have grown only at lower temperatures (Geisen et al. 2015). The thermoacidophilic amoeboflagellate *T. thermacidophilus* (Baumgartner et al. 2009, Reeder et al. 2015) also belongs to class the Eutetramitea but is classified in the family Vahlkampfiidae, unlike *Allovahlkampfia*, which belongs to the family Acrasidae (Pánek et al. 2025) (Fig. 4e). Thus, *Allovahlkampfia* sp. AlKS-1 has evolved acidophilic and thermotolerant traits independently from *T. thermacidophilus*.

Several species of amoeba *Vannella* have been isolated from soil, freshwater, and seawater. To date, except for *Vannella* sp. VaKS-1, there have been no reports of their occurrence in high or moderately high temperatures (>37°C) or highly acidic environments (<pH 4.0) (Smirnov et al. 2007, Kudryavtsev et al. 2021). However, the phylogenetic analysis showed that *Vannella* sp. VaKS-1 is included in a clade to which many environmental metagenomic sequences are assigned (Fig. 5d). Among them, four sequences were obtained from AMD in Río Tinto [15°C–25°C, pH 2.0; O218406H12; O127706H07; B324106B01; O138006A05: (Amaral-Zettler et al. 2011)], while two sequences were from surface

Table 2. Growth temperature and pH, prey organisms used in culture, and phylogeny of unicellular eukaryotes identified in sulfuric acidic hot springs in this study.

Species	Location condition	Culture temp. (°C)	Opt. temp. (°C)	Culture pH	Opt. pH	Prey	Taxonomy
Cyanidiococcus spp.	All	30–50	40–50	0.5–5.0	2.0	–	Cyanidiophyceae Rhodophyta Archaeplastida
Nucleariа sp. NuKS-1	Kusatsu P2 50.0 °C pH 2.3	25–41	35	1.8–7.0	3.0	*Cyanidiococcus*, *E. coli*	Nucleariidea Cristidiscoidea Obazoa
Parvularia sp. PaGS-1	Goshogake P1 38.1 °C pH 2.45	25–40	25	1.2–7.0	2.0	*Cyanidiococcus*, *E. coli*	Nucleariidea Cristidiscoidea Obazoa
Allovahlkampfia sp. AIKS-1	Kusatsu P1 45.7 °C pH 2.07	25–42	30	1.6–7.0	5.0	*Cyanidiococcus*, *E. coli*	Eutetramitea Heterolobosea Discoba
Vannella sp. VaKS-1	Kusatsu P1 45.7 °C pH 2.07	25–40	35	1.4–5.0	3.0	*Cyanidiococcus*, *E. coli*	Discosea Amoebozoa
Platyophyra sp. PIGS-1	Goshogake P2 34.7 °C pH 2.28	25–35	30	2.0–7.0	3.0	*Cyanidiococcus*	Colpodea Ciliophora Alveolata
Neobodo sp. NbGS-1	Goshogake P2 34.7 °C pH 2.28	25–30	30	2.0–7.0	2.0–5.0	*Cyanidiococcus*, *E. coli*	Kinetoplastea Euglenozoa Discoba
Neobodo sp. NbTG-1	Tamagawa P1 42.5 °C ND	ND	ND	ND	ND	ND	Kinetoplastea Euglenozoa Discoba

"–" indicates that no prey was used for cultivation, and "ND" indicates that it was not determined. In the taxonomy column, the class (top), phylum (middle), and, if applicable, supergroup (bottom) are indicated.

waters off the coast of the northeastern Red Sea (25°C at the time of sampling; RS.01f.10 m.23; RS.01f.10 m.11; Acosta et al. 2013). Thus, acid tolerance may have been acquired by the common ancestor of this clade or independently established in each subgroup at a later stage. As for the thermotolerance, *Vannella* sp. VaKS-1 may have acquired it relatively recently after diverging from other members.

The ciliates belonging to the class Colpodea are widespread in various environments including, terrestrial habitats, such as soil, leaf litter, tree holes, freshwater, and seawater (Foissner 1993, Foissner et al. 1999, Dunthorn et al. 2009, Vd'ačný and Foissner 2019). The phylogenetic analysis showed that *Platyophrya* sp. PlGS-1 is closely related to *P. vorax* and *P. spumacola* which have been found in environments ranging from acidic (pH 2.5–4) to neutral soils (Foissner 2000) (Fig. 6e). In addition, *P. bromelicola*, which formed a monophyletic clad with the three species (Fig. 6e), was isolated from tank water of a bromeliad tree (Foissner and Wolf 2009), which is generally acidic, with a pH between 4.0 and 6.5 (North et al. 2023). Thus, it is likely that among Colpodea, *Platyophrya* has adaptively evolved to acidic environments.

The genus *Neobodo*, along with other free-living bodonids and obligate parasitic trypanosomatids, belongs to the class Kinetoplastea of phylum Euglenozoa (Adl et al. 2019). *Neobodo* species are bacterivores that inhabit a wide variety of environments, including freshwater, seawater, and soils (von der Heyden and Cavalier-Smith 2005, Flegontova et al. 2018). The phylogenetic analysis showed that both *Neobodo* sp. NbGS-1 and *Neobodo* sp. NbTG-1 are closely related to an environmental genomic sequence from AMD in Río Tinto (15°C–25°C, pH 2.0; Spain; Uncultured eukaryote O138106G09) (Amaral-Zettler et al. 2011) and to another sequence from thermal and acidic green biofilms in a fumarole (Mexico; Uncultured Bodonidae METASED30; temperature and pH information not provided). These four strains are related to *Neobodo* sp. G97, whose sequence was detected by PCR from the gut of a tsetse fly and is presumed to have originated from environmental water (Votýpka et al. 2021). Other *Neobodo* strains have been isolated from nonacidic environments (Fig. 7; e.g. von der Heyden and Cavalier-Smith 2005). These results suggest that the common ancestor of *Neobodo* spp. inhabiting sulfuric hot springs in Japan and AMD in Río Tinto evolved into an acidophile from a neutrophilic ancestor.

In this study, except for *Neobodo* spp., each organism or its closely related species was found at only one of the three sulfuric acidic hot springs. Furthermore, previous environmental DNA-based metagenomic analyses have not identified any closely related species of these organisms. However, their environmental population density was extremely low compared to Cyanidiophyceae; thus, the survey and sampling were probably far from saturation. Further investigations are needed to determine whether these organisms are widely distributed in sulfuric acidic hot spring environments, similar to Cyanidiophyceae. Nevertheless, all the organisms that morphologically matched those that proliferated in the Cyanidiophyceae blue-green mat when incubated at 40°C or at room temperature (Fig. 2) were successfully cultured and analyzed in this study. In our sampling, after incubation of the algal mats, a greater variety (Fig. 2) and a higher number of presumed heterotrophic eukaryotic cells were observed in the samples that were transported and incubated at room temperature compared to those transported and incubated at 40°C. This is probably because the optimal temperature for these organisms was lower than 40°C, as shown by their isolated cultures (Fig. 9).

For some organisms, especially those isolated from Kusatsu Hot Spring, the reason why the water temperature at the sampling site exceeded the survival limits observed in their isolated

cultures (Table 2) is still unclear. During sampling, we submerged the collected *Cyanidiococcus* mats in nearby spring water. We also collected and observed only the respective spring waters, but we did not find any organisms in the water samples, suggesting that the isolated organisms were in the algal mats. It is likely that some organisms temporarily entered from surrounding cooler areas (e.g. the edges of the flow). However, we cannot rule out the possibility that factors such as the structure of the mat (e.g. biofilms) or coexistence with other organisms may raise the upper temperature limit for survival. In this regard, the isolated *Vannella* sp. VaKS-1 did not proliferate at 40°C when fed with *E. coli*, but it was able to grow when fed with *Cyanidiococcus* under our culturing conditions.

The thermoacidophilic *T. thermacidophilus* feeds on archaea and bacteria but does not appear to prey on Cyanidiophyceae (Baumgartner et al. 2009, Reeder et al. 2015). To date, no predators of Cyanidiophyceae have been reported. However, the heterotrophic protists found in this study were isolated from Cyanidiophyceae mats (Fig. 1), primarily composed of *Cyanidiococcus*, and were found to feed on *Cyanidiococcus* (except for *Neobodo*, whose feeding behavior remains unclear) (Figs 3–7 and 10). These newly identified organisms, other than the ciliate *Platyophrya* sp. PlGS-1, were also able to grow by feeding exclusively on *E. coli* (Figs 8 and 9), suggesting that they likely consume bacteria and archaea in their natural environment as well. Additionally, although not analyzed in detail in this study, a rotifer isolated from the environment (Fig. 2f) was also able to grow by feeding exclusively on *Cyanidiococcus*.

The seven heterotrophic organisms identified in this study are all acidophiles, with optimal pH ranges between 2 and 5 (Fig. 8). However, unlike the unicellular red alga *Cyanidiococcus*, which was found in the same environment and can grow at pH 1.0 and even pH 0.5 (Fig. 8), these heterotrophs are not as highly specialized for extreme acidity and fail to survive at such low pH levels (Fig. 8). In terms of temperature, while *Cyanidiococcus* has a maximum growth temperature of 50°C, the heterotrophic organisms identified in this study have maximum growth temperatures below 40°C (Fig. 9). Among them, four species are thermotolerant, capable of marginal growth or survival at 40°C or at slightly higher temperatures, but they are not thermophiles like *Cyanidiococcus* (Fig. 9; Table 2). Even though their cells are not specifically adapted to conditions such as pH 2.0 and 40°C, Cyanidiophyceae, their primary prey, remains abundant throughout the year. Thus, it is presumed that the population size of these heterotrophic unicellular eukaryotes is limited solely by abiotic environmental factors such as temperature and pH. Additionally, since there are few competing organisms for prey and few predators targeting them, the overall environment may still be favorable for their survival.

This study revealed that various lineages of heterotrophic unicellular eukaryotes have independently developed acidophilic and thermotolerant traits, enabling them to colonize moderately high-temperature, extremely acidic sulfuric hot springs (Fig. 11). To elucidate the mechanisms and evolutionary processes underlying these adaptations, future research involving genomic, physiological, and structural analyses will be necessary. To this end, the information and cultures provided by this study serve as valuable resources for future investigations.

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Author contributions

Yuki Sunada (Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Validation, Writing –original draft, Writing – review & editing), Dai Tsujino (Data curation, Formal analysis, Investigation, Validation, Writing – review & editing), Shota Yamashita (Formal analysis, Investigation, Methodology, Supervision, Validation), Wei-Hsun Hsieh (Formal analysis, Investigation, Validation), Kei Tamashiro (Formal analysis, Investigation, Methodology, Validation), Jin Izumi (Formal analysis, Investigation, Methodology, Validation), Fumi Yagisawa (Formal analysis, Investigation, Methodology, Validation), Baifeng Zhou (Investigation, Resources, Validation), Shunsuke Hirooka (Methodology, Supervision, Validation), Takayuki Fujiwara (Investigation, Methodology, Supervision, Validation), and Shin-ya Miyagishima (Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing)

Supplementary data

Supplementary data are available at *FEMSEC Journal* online.

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Data availability

The 18S rDNA sequences were deposited in the GenBank under accession numbers: LC860419 (*Platyophrya* sp. PlGS-1), LC860420 (*Neobodo* sp. NbGS-1), LC860421 (*Neobodo* sp. NbTG-1), LC860422 (*Vannella* sp. VaKS-1), LC860423 (*Allovahlkampfia* sp. AlKS-1), LC86042 (*Parvularia* sp. PaGS-1), LC860425 (*Nuclearia* sp. NuKS-1).

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