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Meiofaunal Richness in Highly Acidic Hot Springs in Unzen-Amakusa National Park, Japan, Including the First Rediscovery Attempt for Mesotardigrada

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Extreme environments sometimes support surprisingly high meiofaunal diversity. We sampled runoff from the acidic hot springs of Unzen, Japan. This is the type locality of *Thermozodium esakii* Rahm, 1937, the only tardigrade in the class Mesotardigrada, which remains contentious in the absence of corroboration or supporting specimens. Our sampling revealed at least three species of arthropods, four rotifers, and five nematodes living in the hot (ca. 40°C) and acidic (ca. pH 2.5) water, but no tardigrades.

Key words: meiofauna, tardigrade, extreme environments, low pH, nematode, rotifer, arthropod

INTRODUCTION

Sampling for meiofauna in extreme environments has sometimes revealed unexpectedly high diversity and complex ecological interactions. Examples include the fauna of extremely acidic rivers with high concentrations of heavy metals (Amaral Zettler et al., 2002), cryoconite holes on the surfaces of glaciers (Zawierucha et al., 2015), saline lakes (Timms, 2001), and the outflow of hot springs (Wiegert and Mitchell, 1973; Duggan et al., 2007). In particular, the waters of hot springs, often acidic and rich in dissolved minerals, may be particularly challenging to their inhabitants (Uyemura, 1939; Pinder, 1995; Cowan et al., 2012). Animal groups with members that are particularly well-adapted to these extreme environments include platyhelminths, gastrotrichs, annelids, mollusks, rotifers, nematodes, tardigrades, and arthropods (Uyemura, 1939).

Rahm (1937a, b) reported the discovery of a tardigrade, *Thermozodium esakii*, from a runoff of acidic hot springs in the region now known as Unzen–Amakusa National Park in Japan. *Thermozodium esakii* is the only species in the class Mesotardigrada, with characteristics of the classes Eutardigrada and Heterotardigrada of the phylum Tardigrada. Okada and Ito (1938) found no animals other than two dipteran species from Unzen hot springs. No voucher specimens are known for *T. esakii*, and no one has reported the species since Rahm, leading some authorities to doubt the

veracity of Rahm's report.

In May of 2012 and 2013, we sampled the same hot-spring habitat reported by Rahm. Here we report on the richness of the meiofauna from the very acidic hot springs in Unzen–Amakusa National Park, and the results of our search for *T. esakii*.

MATERIALS AND METHODS

Source, collection, and isolation of hot-spring meiofauna

Samples of sediment, moss, lichen, and algae were collected in and around hot water streams from locales within Unzen–Amakusa National Park, 32.44°N, 130.15°E (Fig. 1). Sampling was conducted between 28 and 30 May, 2012 by the current authors, and on 14 and 15 May, 2013 by ACS and HK, the same time of year that Rahm was collecting in the area (Rahm, 1937a, b) (Table 1). The area has many hot springs, and the steam vents and boiling hot pots have led to the common name of the area, 'Jigoku (Hell)'. *Jigoku* names are shown in Table 1, following Yamaguchi et al. (1982).

In an attempt to rediscover *T. esakii*, we sampled the locales described by Rahm (1937a, b). Rahm (1937a) described the environment of Mesotardigrada as "the algae in the little overflowing stream of the hot spring, where minerals of the water have produced some incrustations." In referring to a name of the oldest public bath in the area, he also wrote "Enryaku-yu, which is not very far from the point where we collected the algae" (Rahm, 1937a). Although Rahm provided only "Furu-yu" as the type locality (1937a, b), his described habitat is widely distributed in Furu-yu and Shin-yu, both of which are not very far from Enryaku-yu (Fig. 1). Our sampling efforts focused on Shin-yu, because the flow of water at Furu-yu has diminished. While Enryaku-yu fell out of use at least three years before Rahm's expedition and the amount of hot water

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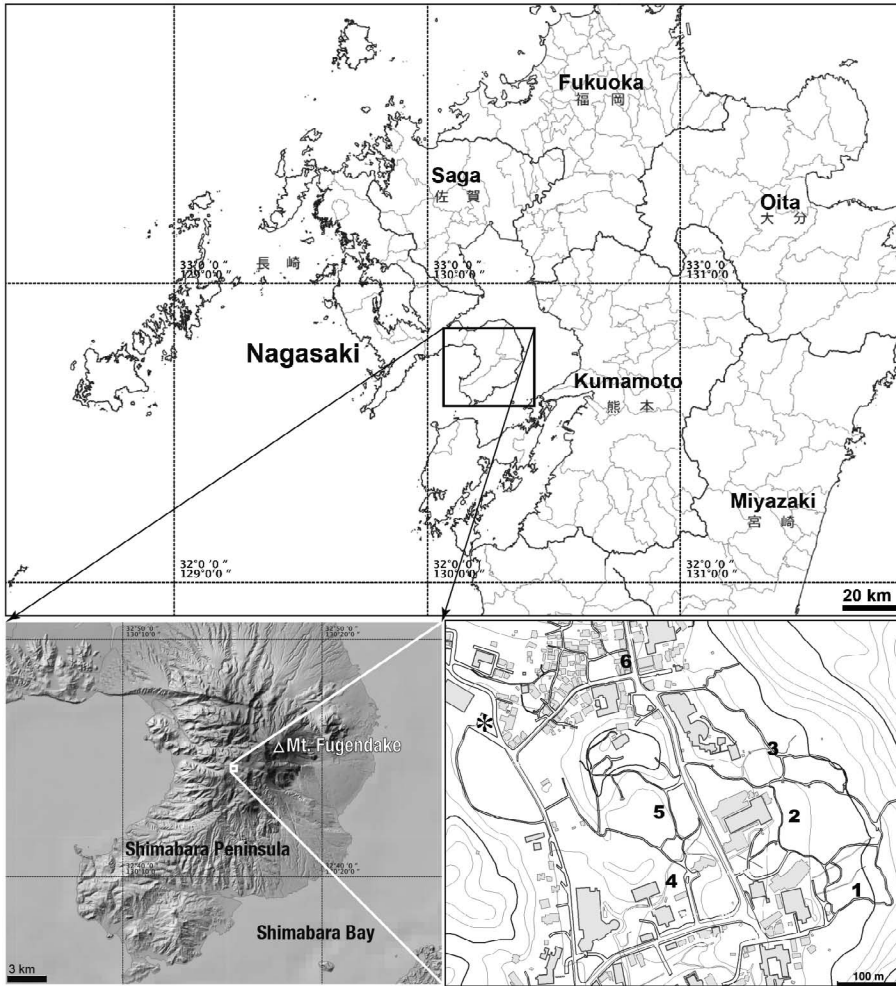


Fig. 1. Sites of the present study in Unzen-Amakusa Park. (1) Kajiya jigoku, (2) Sakaya jigoku, (3) Akkô-Ryôzetsu jigoku, (4) upstream of Sakura Bridge on Yu-River, (5) Old-Hachiman jigoku, (6) Enryaku-yu. Asterisk (*) shows the location of the *ad hoc* field laboratory in the house “Yamabiko Kaikan”.

flow decreased after a construction in 1960 (Yamaguchi et al., 1982), Enryaku-yu was used as a source of hot water until 1992 and was finally exhausted by 2004 (M. Kato, personal communication). Although Rahm did not specify Enryaku-yu as his sampling point, we sampled also from the underground drainage of the remains of Enryaku-yu.

Temperature and pH values at each sampling site were obtained using either a U-10 Water Quality Checker (HORIBA) or a HI991001 pH/Temperature Meter (Hanna Instruments). Samples were processed in an *ad hoc* field laboratory (Fig. 1, asterisk). Large-volume samples were first washed through a 1-mm sieve to remove debris, then decanted over a 32- μ m sieve, and finally transferred to a plastic dish containing tap water. Small-volume samples were submerged in tap water in a plastic dish.

Individual meiofauna were isolated using dissecting microscopes, and were fixed in ethanol or in formalin. The fixed animals were then mounted on glass microscope slides with Hoyer's medium or glycerol, or were mounted alive in water. They were photographed with a Sony NEX-5N digital camera mounted on an Olympus BX50 DIC light microscope. Some specimens from site-1 were rinsed several times in distilled water and fixed in ethanol or DESS solution (20% DMSO, 0.25 M disodium EDTA, and saturated sodium chloride), and preserved at room temperature prior to DNA extraction, as per Yoder et al. (2006).

DNA extraction, amplification, and sequence analysis of 18S rRNA

Specimens were rinsed with distilled water, and transferred to individual 0.2 ml polymerase chain reaction (PCR) tubes containing 20 μ l of 0.25 N NaOH, and kept at room temperature for 12 h, as per Floyd et al. (2002). This lysate was then heated for 3 min at 95°C, and neutralized with 4 μ l of 1 M HCl and 10 μ l of 0.5 M Tris-HCl (pH 8.0), followed by 1 μ l of 2% Triton X-100. The lysate was then heated for an additional 3 min at 95°C, and stored at -20°C until further processing.

PCR amplification was performed on 18S rRNA loci in a 20 μ l reaction volume, containing 1 μ l of lysate as templates, 2 μ l of 10 \times reaction buffer with 2.5 mM MgCl₂, dNTPs at 200 μ M each, 1 μ M of each primer, and 0.5 units of Ex Taq DNA Polymerase (Takara Bio Inc., Japan). The PCR primer sequences used for 18S rRNA were SSU07F (AAA-GATTAAGCCATGCATG) and SSU26R (CATTCTTGGCAAATGCTTTTCG), except for the Bdelloid rotifer samples, for which Bde_SSU26R (CATTCTTGGCGAACGC-CTTCG) was used as per Meldal et al. (2007) and Sands et al. (2008).

Samples were denatured at 94°C for 10 sec, annealed at 52°C for 30 sec, and extended at 72°C for 1 min for 40 cycles, followed by polymerization for 10 min at 72°C as per De Ley et al. (2005), Nadler et al. (2006) and Meldal et al. (2007). PCR products were separated by agarose gel electrophoresis, and purified using a QIAquick Gel Extraction Kit (Qiagen, USA).

(Applied Biosystems, USA).

RESULTS

The water temperature at several Unzen sites was similar to that described by Rahm (1937a, b, c), ca. 40°C (Table 1). Although our sampling efforts at the most extreme environment (49°C; pH 1.9) revealed no meiofauna, larval midges were observed downstream from a hotter spring (60°C; pH 2.5).

Arthropods

Larvae of *Chironomus fusciceps* Yamamoto, 1990 (Insecta: Diptera; Ceratopogonidae) (A1 in Table 1) were abundant and widely distributed in the hot-spring samples. 18S rRNA sequences of 10 larvae from Site 1 were identical (DDBJ/GenBank/EMBL Accession number (Acc#): LC128699). Larvae of a biting midge, *Culicoides* sp. (Insecta: Diptera: Culicoidae) (A2 in Table 1, Fig 2A), were collected at Site 1. Five larvae of this species returned an identical 18S rRNA sequence (Acc#: LC128700).

Tyrphonostrus sp. (Acari: Oribatida: Malaconothridae) (A3 in Table 1, Fig. 2B, C) were widely distributed in the hot

Table 1. Sampling sites and their environments.

Sites ¹⁾	Date	Latitude	Longitude	Temp. ²⁾	pH	Substrate	Meiofauna ³⁾
Sample no.	yyyy/mm/dd	N 32°44'	E 130°15'	(°C)			
1 Kajiya Jigoku							
12.02-1-3	2012/05/29	19.7"	53.5"	32.9	2.41	Algae on rock	A1, A3, R1, R2 , R3, N1, N2
12.02-4	2012/05/28	20.1"	52.0"	39.0	ND	Algae on rock	R2
12.02-6	2012/05/29	19.7"	53.5"	<u>42.0</u>	2.64	Moss (stream 45.9°C)	A2, A3 (fig. 2B,C), R2, N3, N4
12.02-7,8	2012/05/30	19.8"	53.3"	40.1	2.58	Algae on rock	A3, R4, R5
13.01	2013/05/14	20.06"	53.61"	38.8	2.64	Sediments	A1
13.02	2013/05/14	20.11"	53.50"	40.0	2.58	Moss	A1, A3, R1 (fig. 3A), R2, R3, N3, N4
13.03	2013/05/14	20.16"	53.50"	<u>35.9</u>	2.54	Moss (stream 43.2°C)	A2 (fig. 2A), A3, R1, R3
13.041	2013/05/14	20.02"	53.39"	<u>27.0</u>	2.54	Moss (stream 41.2°C)	R1 (fig. 3B), N5
13.042	2013/05/14	20.02"	53.39"	<u>36.0</u>	2.54	Algae on rock (stream 41.2°C)	A1, A3, R1
13.043	2013/05/14	20.02"	53.39"	41.2	2.54	Sediments	A1, R1
13.05	2013/05/14	19.94"	53.34"	41.3	ND	Algae on rock	R2
13.10	2013/05/15	19.84"	52.52"	40.3	2.33	Sediments	A1, R1, R2
13.11(= 13.05)	2013/05/15	19.94"	53.34"	ND	ND	Moss	A2, A3, R1, N4
2 Sakaya Jigoku							
13.06	2013/05/14	24.10"	48.83"	48.7	1.95	Algae on rock	null
3 Akkō-Ryōzetsu Jigoku							
12.04	2012/05/29	27.5"	47.4"	39.3	ND	Algae on rock	R2, R3 (fig. 3D)
4 Yu-river, upstream of Sakura Bridge							
13.12	2012/05/15	20.09"	41.35"	30.0	1.90	Sediments	A1, R1
5 Old-Hachiman Jigoku							
13.071	2013/05/15	23.62"	40.69"	40.8	2.02	Algae on rock	R2 (fig. 3C)
13.072	2013/05/15	23.62"	40.69"	29.2	2.08	Lichen	R1, R2, N6
13.08	2013/05/15	23.99"	39.95"	42.2	2.10	Algae on rock	R2
6 Enryaku-Yu							
12.8	2012/05/30	31.6"	38.6"	31.9	2.55	Algae, sediments	A1, A3, R2

(1) *Jigoku* names are those provided by Yamaguchi et al. (1982).

(2) Underlined values indicate the temperature of the moss or the algae, with the neighboring water temperature noted in parentheses under "Substrate." ND: no datum.

(3) Bold letters indicate those with 18S rRNA data. Abbreviations: **A**, Arthropoda. A1, *Chironomus fusciceps*; A2, *Culicoides* sp.; A3, *Tyrphono-thrus* sp. **R**, Rotifera. R1, bdelloid sp.1 and/or sp.2; R2, *Lecane* sp., R3, *Cephalodella* sp., R4, 'large rotifer'; R5, 'small rotifer.' **N**, Nematoda. N1–N5, nematodes OTU01 - 05 on the basis of their DNA sequences; N6, unsequenced nematode.

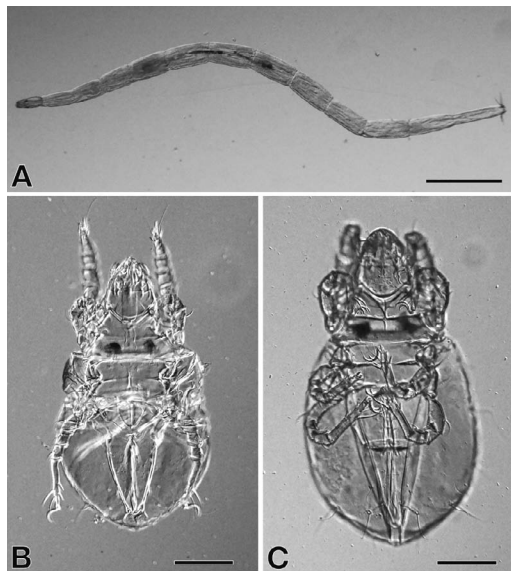


Fig. 2. Arthropods from Unzen hot springs. **(A)** *Culicoides* sp. (Insect: Diptera: Chironomidae), **(B)** *Tyrphono-thrus* sp. (Acari: Oribatida: Malaconothridae), tritonymphal stage **(C)** *Tyrphono-thrus* sp., adult. Scale bars: **(A)** 1.0 mm; **(B)** and **(C)** 100 μ m.

Table 2. Individual numbers of two types of bdelloids.

Sample no.	Bdelloid type A	Bdelloid type B
12.02-3	1	–
13.02	–	1
13.041	9	2
13.042	23	7
13.043	1	2

springs, and were collected in various stages of development. Seven individuals collected from site 1 showed an identical 18S rRNA sequence (Acc#: LC128701), which shared 99.8% (847/849) nucleotide identity with the sequence of *Malaconothrus gracilis* (voucher specimen UMMZ BMOC 08-1119-020 AD1409) (Acc#: JQ000044).

Rotifers

Bdelloid rotifers (R1 in Table 1) were distributed widely among the hot springs. Two types of 18S rRNA sequences were detected from five samples from site 1; 34 individuals of type A and 12 of type B (Acc#: LC128702 and LC128703, respectively). Both types were found in the same samples (Table 2). The rRNA sequences differed at seven points of 832 base pairs. These rotifers also differed morphologically

(Fig. 3A, B). One form (bdelloid sp. 1) had two major uncus teeth in its trophi (inset of Fig. 3A), and the other form (bdelloid sp. 2) had seven (inset of Fig. 3B). We did not obtain 18S rRNA sequences corresponding to those morphology types.

Monogonont rotifers of the genus *Lecane* (R2 in Table 1) (Fig. 3C) were also widely distributed. Seven individuals from three samples from site 1 had identical 18S rRNA sequences (Acc#: LC128704). An additional monogonont rotifer was collected from Sites 1 and 3, and identified as the genus *Cephalodella* (R3 in Table 1) (Fig. 3D) on the basis of morphological characteristics. We also observed unidentified 'small' and 'large' rotifers from Site 1 (R4 and R5 in Table 1). Some of these might be R2 or R1, but they were not observed at high magnification.

Nematodes

We used operational taxonomic units (OTUs) from 18S rRNA sequences for the identification of nematodes. OTUs are a group of sequences sharing high sequence identity. For our purposes, sequences with more than 99.5% shared identity were placed in the same OTU.

We distinguished five nematode OTUs from Site 1. One individual each of OTU01 and OTU02 (N1 and N2 in Table 1) were isolated from a sample (12.01-3). The 18S rRNA

sequence of OTU01 (Acc#: LC128705) shared 99.0% (812/820) nucleotide identity to those of the genera *Acrobeloides*, *Cervidellus*, and *Cephalobus* (Chromadorea: Rhabditida: Cephalobidae). OTU02 (Acc#: LC128706) shared 99.6% (789/792) nucleotide identity to the SB133 strain of *Oscheius guentheri* Sudhaus and Hooper, 1994 (Chromadorea: Rhabditida: Rhabditidae). We collected six nematodes of OTU03 (Acc#, LC128707; N3 in Table 1) and three of OTU04 (LC128708, N4 in Table 1) from multiple samples in Site 1. Although their 18S rRNA sequences shared only 88.4% identity (696/779) with each other, they showed the best BLAST hit to two distinct unidentified species in the genus *Aphelenchoides* (Chromadorea, Aphelenchida: Aphelenchoididae). OTU03 shared 732/782 (93.6%) with *Aphelenchoides* sp. Ap-A (Acc#: AB661626). OTU04 shared 719/792 (90.8%) with *Aphelenchoides* sp. 8 JH-2012 (Acc#: JQ957883). Seven individuals in OTU05 (N5 in Table 1) from a sample (13.041) had identical 18S rRNA sequence (LC128709), shared 99.2% (789/795) with the sequence EU915487, *Eutylenchus excretorius* Ebsary and Eveleigh, 1981 (Chromadorea: Tylenchida: Tylenchidae). We also observed an unsequenced nematode from Site 5 (N6 in Table 1).

Tardigrades

No tardigrades of any species were collected from the Unzen hot springs in either 2012 or 2013.

Other remarks

An unidentified ciliate and a diatom in the genus *Pinnularia* (Bacillariophyceae: Pennales: Raphidineae) were collected at site 5 (40.8°C, pH 2.02). The same type of diatom was also found in the stomach of a *Cephalodella* rotifer from Site 3 (Fig. 3D).

DISCUSSION

Sampling the acidic hot springs in Unzen National Park, Japan (now Unzen–Amakusa National Park), Okada and Ito (1938) found only two dipterans, a shore fly (*Scatella* sp.), and a midge (*Chironomus thummi*, now known as *C. fusciceps*). In contrast, they described greater biodiversity from less-acidic thermal springs around the Mt. Aso volcano, including one annelid, three mollusks, and twelve insects (Okada and Ito, 1938). We sampled the same hot spring environments in Unzen described by Rahm (1937a, b) and Okada and Ito (1938). These environments were hot and highly acidic as previously described. Despite these extreme conditions, we collected midges, mites, bdelloid, and monogonont rotifers, and nematodes, revealing an unexpectedly rich meiofauna. We did not collect *T. esakii*, nor any other tardigrade.

Arthropods

Our sampling revealed larvae of two types of midge. *Chironomus fusciceps* breeds throughout the year in the sulfur-rich, acidic outflow of hot springs in the Unzen area, and adults are reported to cause discomfort to residents of and visitors to Unzen (Yamamoto, 1990; Takagi et al., 1995). We found its larvae to be abundant and widely distributed. *Culicoides* biting midges transmit many diseases of human and veterinary importance in many parts of the

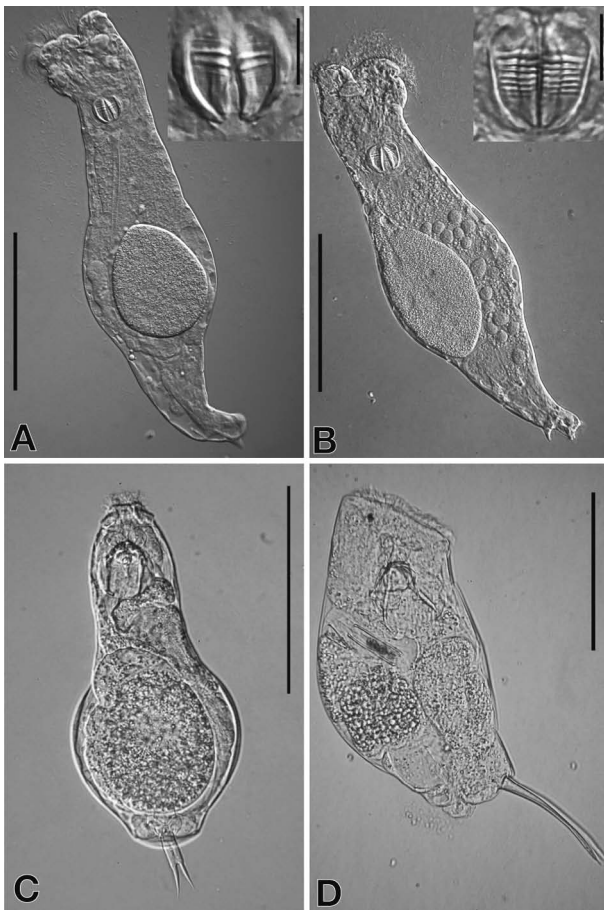


Fig. 3. Rotifers from Unzen hot springs. (A) bdelloid sp. 1, DIC, (B) bdelloid sp. 2, DIC, (C) *Lecane* sp. (Monogononta), DIC, (D) *Cephalodella* sp. (Monogononta), bright field. Scale bars 100 μ m.

world, including Japan (Yanase et al., 2013). Pinder (1995) cited three other examples of midge larvae inhabiting hot springs waters in excess of 40°C in Iceland, New Zealand, and the United States. Although not found in our sampling, Okada and Ito (1938) recorded the fly *Scatella* sp. as a common animal in the acidic, hot springs.

Whereas the majority of oribatid mites inhabit terrestrial habitats, a small proportion of species reside in aquatic conditions (Schatz and Behan-Pelletier, 2008). The speciose family Malaconothridae are known from freshwater habitats including wet moss and sodden organic debris (Kuriki, 2000; Schatz and Behan-Pelletier, 2008; Ermilov et al., 2010). In our samples, we found many individuals of mites at various stages, which showed morphological affinity to *Trimalaconothrus azumaensis* Yamamoto et al., 1993, described from sphagnum in Yachidaira and Ozegahara Marsh, Fukushima Prefecture, Northeastern Japan (Yamamoto et al., 1993; Kuriki and Yoshida, 1999). This species has recently been transferred to the genus *Tyrphonothrus* (see Colloff and Cameron, 2013).

Rotifers

Our sampling revealed two bdelloid and two monogonont rotifers. Individuals of bdelloid types A and B were both collected from hot water ranging from 27.0°C to 41.2°C, *Lecane* sp. from 29.2°C to 42.0°C, and *Cephalodella* sp. from 32.9°C to 40.0°C. Issel (1906) showed twelve rotifers from Italian hot springs, in which the monogononts *Notommata najas* (= *Eosphora ehrenbergi* Weber, 1918) and *Euchlanis plicata* were from temperatures above 40°C, *Metopidia solidus* (= *Lepadella latusinus* (Hisgendorf, 1899)) at 40°C, and a bdelloid *Rotifer vulgaris* (= *Rotaria rotatoria* (Pallas, 1766)) at 38°C. A number of rotifer species are also known from acidic waters (Deneke, 2000; Amaral Zettler et al., 2002), and the genus *Cephalodella* includes species that are particularly tolerant of low pH (Jersabek et al., 2011). In its acidophilic nature, the *Cephalodella* sp. found in Unzen at pH 2.4–2.6 resembles *C. acidophila* Jersabek et al., 2011, reported from acidic (pH 2.4–2.7) mining lakes in Europe. The genus *Lecane* is both speciose and diverse, and includes thermophiles and members tolerant of acidic waters (Segers, 2001).

Nematodes

Some nematode species are resistant to high temperatures and low pH (Issel, 1906; Hoeppli, 1926; Meyl, 1954). Rahm (1937c) documented a nematode, *Aphelenchoides* sp., from a hot spring in New Zealand (61.3°C), and both *Aphelenchoides* sp. and *Plectus* sp. from a hot spring in Chile (57.6°C). Earlier studies of nematodes in hot springs have shown most species to be cosmopolitan in their distribution, more commonly observed in less extreme habitats, although a few species are known only from the outflow of thermal springs (Nicholas, 1984). The habitat of OTU05 was acidic but, in fact, not very hot (27.0°C), therefore this nematode may not be a true resident of the hot spring, in spite of the hot water (41.2°C) in the immediate vicinity. The same circumstance is observed with OTU01 and 02, whereas OTU03 and OTU04 may represent true inhabitants in the hot environment.

Analysis of 18S rRNA sequences of the nematodes that

we collected revealed five OTUs from four families within the class Chromadorea. OTU01 belongs to the family Cephalobidae, one of the most difficult groups of nematodes to identify (Smythe and Nadler, 2006), and its 18S rRNA analysis could not reveal its genus. *Oscheius guentheri*, showing enough similarity to be placed in OTU02, was originally isolated from decaying stem tissue of rice plants from Vietnam (Sudhaus and Hooper, 1994), while closely-related *O. tipulae* is a widespread soil nematode (Beïlle et al., 2008). Sudhaus and Hooper (1994) found that cultures of *O. guentheri* reproduced well at 20–32°C, but died after two days at 34–35°C. OTU02 was found from 32.9°C, the boundary temperature for *O. guentheri* to survive. OTU03 and OTU04 show similarities with genus *Aphelenchoides*, which includes many foliar nematodes and fungivorous species (Rybarczuk-Mydlowska et al., 2012). *Eutyenchus* consists of a small group of migratory ectoparasites of aquatic vascular plants, rarely found in moist sandy soils near streams and rivers (Palomares-Rius et al., 2009).

Tardigrades

In an attempt to rediscover *T. esakii*, the only species in the class Mesotardigrada, we sampled the hot springs of Furu-yu and Shin-yu regions of Unzen-Amakusa National Park. We also sampled the underground drainage of Enryaku-yu. Ours appears to be the first systematic search for *T. esakii* (Grothman et al., 2017, this volume).

Our failure to find mesotardigrades may reflect extreme rarity or extremely cryptic habits, an extirpation of Rahm's population as a result of changes in water flow in the region, or a misinterpretation or misrepresentation by Rahm (see Grothman et al., 2017, this volume). We are aware of only three additional reports of tardigrades, *Macrobotus* sp., in hot springs (Ciofalo, 1927; Okada et al., 1938; Uyemura, 1938), and these were from neutral rather than acidic waters. It seems that tardigrades are infrequent members of the meiofauna of acidic hot springs.

General remarks

Some of the meiofauna that we sampled had a patchy distribution. For example, all seven individuals of the nematode OTU05 were found in a single sample from Kajiya-Jigoku (Site 1), from which twelve samples were collected. Moreover, this nematode was found only in 2013, whereas OTU03 and OTU04 were found in both 2012 and 2013. The heterotardigrade *Carphania fluviatilis* Binda, 1978, has been observed on only a single occasion (Binda and Kristensen, 1986), despite several subsequent attempts to sample it (O. Lisi, personal communication), implying that some members of the phylum are rare and/or elusive. The same is true of the heterotardigrade *Oreella chugachii* Calloway et al., 2011, described from Alaska. In this case a thriving population with 101 individuals was found on a single boulder, but no evidence of that species on any other samples taken in that area (Calloway et al., 2011). If *T. esakii* has the same characteristics, it would be difficult to collect without an even-more extensive sampling effort. If it were to be rediscovered in the future, *T. esakii* might provide important information about tardigrade phylogeny, and the biology of meiofauna in extreme environments.

Many, minute, moss-dwelling animals, particularly rotifers,

nematodes, and tardigrades, exhibit cryptobiosis—an extreme diminution of physiological processes, allowing them to inhabit cold and/or dry conditions. The wide range of extreme habitats occupied by members of these groups, including the very acidic hot springs of Unzen–Amakusa National Park, implies broadly-adaptive anatomical and physiological characteristics. Future study of the meiofauna of extreme habitats, including hot springs, is likely to be rewarding.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

ACS coordinated the research and all authors met at Unzen to collect and examine samples. HK carried out DNA analyses. ACS prepared figures and tables. All authors wrote the paper, read and approved the final manuscript.

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