Rapid Communication

First record of the North American freshwater sponge *Heteromeyenia latitenta* (Potts, 1881) found in Japan (Spongillida: Spongillidae)

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Abstract

A previously unreported freshwater sponge species was discovered from the Tama River in Tokyo Prefecture and the Sagami River in Kanagawa Prefecture, Honshu, Japan, using data and specimens from the National Census on River Environments. The sponge was identified as belonging to *Heteromeyenia latitenta* (Potts, 1881). The lack of reports of this species in previous surveys and the restricted distribution of the gemmules as documented in this report suggest a recent introduction. This is the first record of the species from Japan. We describe the morphological characteristics of this species and compared ITS2 rDNA of sequence data of this species with other freshwater sponges.

Key words: Porifera, Demospongiae, non-native species, gemmules, ITS2

Introduction

Sponges (Porifera) occur at all depths of every ocean and in freshwater bodies (lakes, rivers). Currently, there are 9,372 recognized valid species of which less than 266 (about 3%) are freshwater sponges (Van Soest et al. 2021). In Japan, the taxonomy and distribution of freshwater sponges are well-studied; to date, 25 species in 11 genera have been recorded (Masuda 2006; Funayama et al. 2009). Recently, *Heterorotula multidentata* (Weltner, 1895) and *Trochospongilla pennsylvanica* (Potts, 1882) have conspicuously increased their geographical range in Japan, although they were not recorded before World War II (Sasaki 1934, 1936, 1939, 1941). They may be previously unreported species introduced by human activity (Masuda 2006; Matsuoka 2011).

Most marine sponges do not form gemmules, but many freshwater sponges do through an asexual process. It is thought that inland water bodies are more prone to environmental fluctuations such as freezing and drying out than sea areas. Consequently, the ability to produce gemmules was probably essential for sponges to introduce and establish themselves in freshwater ecosystems (Masuda 2006, 2012). Spongillidae gemmules have a complex design with gemmuloscleres arranged tangentially to radially to create an armor-like coating that protects totipotent cells, and several species have a pneumatic layer that allows for downstream gemmule dispersal (Copeland et al. 2019). This type of gemmule seems to be perfectly adapted for overland aquatic areas (Manconi and Pronzato 2015).

Freshwater sponges have growth form morphologies ranging from encrusting to rounded and fingerlike (Reiswig et al. 2010). Because form can vary substantially within a species, from thin to thick crusts or cushions, be of bulbous or otherwise massive nature, or display branching, subbranching, or pseudobranching projection (Penney and Racek 1968), external morphology is of limited use in the sponge taxonomy. Therefore, the classification of freshwater sponges is based primarily on the structure of gemmule spicules (Penney and Racek 1968; Manconi and Pronzato 2007). However, skeleton and megasclere morphologies and genetic information are also important taxonomic traits.

In this study, we report for the first time in Japan the occurrence of *Heteromeyenia latitenta* (Potts, 1881), a sponge species known, until now, from North America (Reiswig et al. 2010), using a database and specimens from the National Census on River Environments (NCRE). The NCRE has been conducted by the Ministry of Land, Infrastructure, and Transport (MLIT) of Japan since 1991, and is a continuous and ongoing survey of the fauna and flora of 123 rivers (109 river systems) all over Japan (Ikeuchi and Kanao 2003; Sueyoshi et al. 2016). We describe the morphological characteristics of the dormant sponge and gemmules, and we compare the intron 2 of the nuclear ribosomal cistron (ITS2) sequence data of this species with those of some other freshwater sponges.

Materials and methods

Specimens

For morphological study, we used samples from the Tama River system in Tokyo Prefecture and the Sagami River system in Kanagawa Prefecture, Japan. A survey of macroinvertebrates was conducted at 16 sites in the Tama River system in August and November 2017 and February 2018, 10 sites in the Sagami River system in August and December 2018 and February 2019 by NCRE. All collection sites (Figure 1) were freshwater sites and not brackish. Measured minimum and maximum water temperatures throughout the year were 2 °C and 28 °C respectively. Samples were collected using a square frame with a 0.5 mm mesh standard dip net. At each study site, quantitative samples— surface area 25 cm^2, 3 samples per site— and qualitative samples without defined surface area—were collected. After collection, the material was fixed in 5% formaldehyde. In the laboratory, samples were sorted and sponges gemmules were preserved

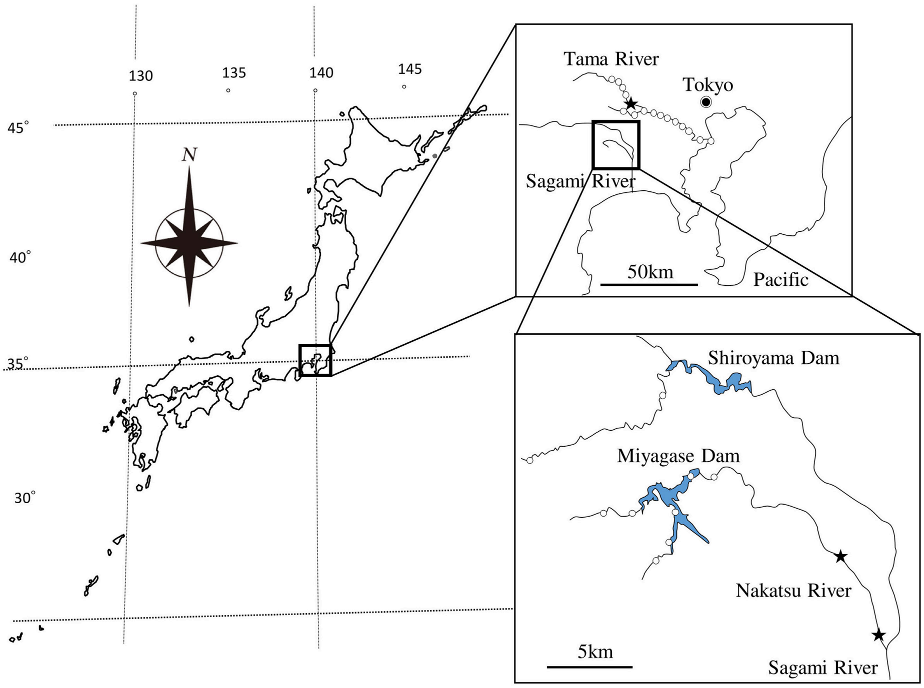

Figure 1. Collection sites. The open circle indicates the sampling site, and the open star indicates the *Heteromeyenia latitenta* was recorded.

in 80% ethanol until further investigation. Bottom sediments from streams and wet soils were repeatedly stirred in water in a tray or bucket, and the gemmules were collected with a 0.5 mm screen. For genetic study, gemmules and dry stage of an encrusting sponge of Japanese *H. latitenta* were collected by the first author from the Sagami River, Kanagawa Prefecture on April 2020, in addition to the aforementioned surveys. And the gemmules of Japanese *H. stepanowii* were collected by the second author in the outflow system of Lake Inba-numa, Chiba Prefecture on 9 November 2020. The gemmules and sponge of American *H. latitenta* were collected by Dr. Copeland (Lincoln Memorial University, Harrogate, Tennessee) from the Pigeon River in eastern Tennessee on 24 July 2015 were used for both morphological and genetic studies. Details of locations are presented in Supplementary material Table S1.

Here we propose a substitute technique for easily observing gemmule spicules using Light Microscopy. The steps are as follows: First, to cut out a hemisphere at the gemmule's terminal regions, using tweezers. Next, the hemisphere gemmules are dehydrated in a graded ethanol series on a slide and cleared in methyl salicylate. Finally, they are mounted whole between slide and coverslip in Canada balsam. As a result, the morphology of the spicules can be observed and studied easily, as is shown in Figure 2C, D, E. This method is effective for identifying freshwater sponges. To determine the range for spicular lengths and rotule diameters 20 of each type of spicules were measured using Image J software. Ten gemmules of each species were measured for diameter.

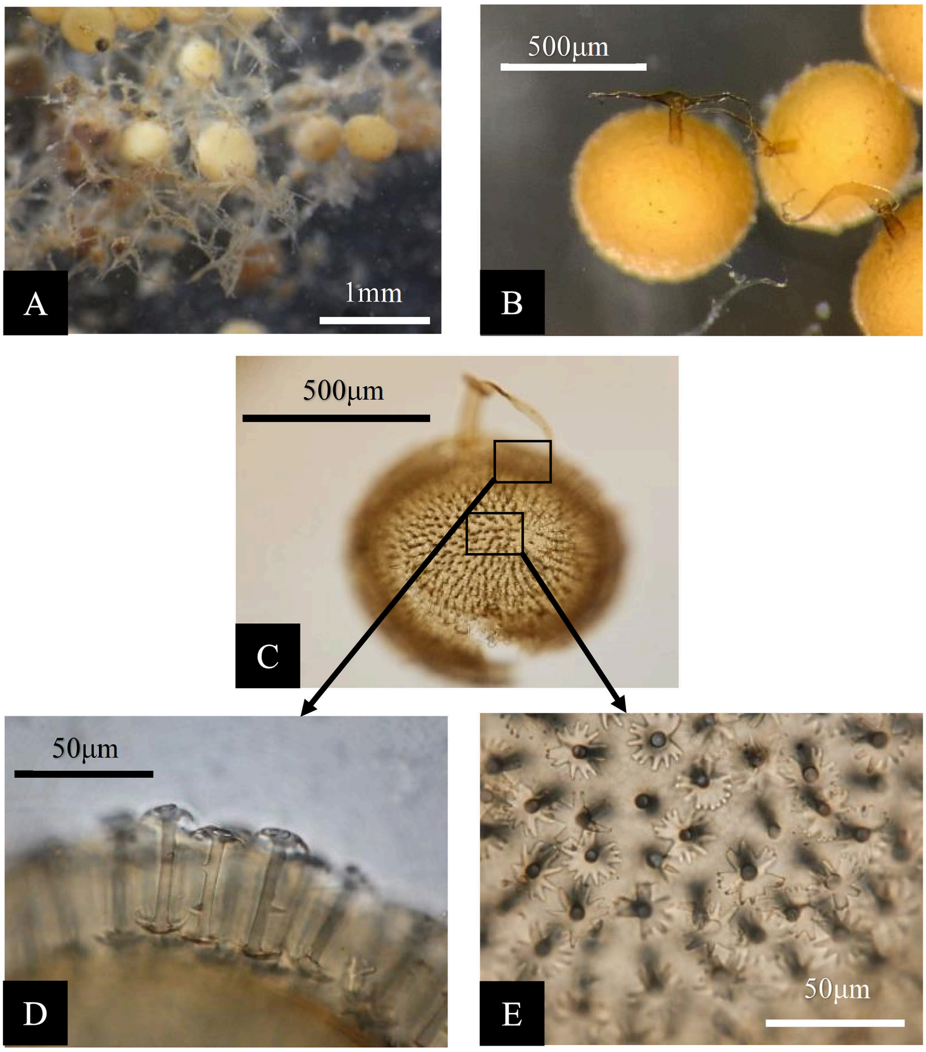

Figure 2. Photos of *Heteromeyenia latitenta* collected from the Sagami River, Japan. (A) skeleton and gemmules (scale bar 1 mm). (B) gemmules (scale bar 500 µm). (C) gemmule cleared in methyl salicylate (scale bar 500 µm). (D) gemmuloscleres cleared in methyl salicylate (scale bar 50 µm). (E) rotules of gemmuloscleres cleared in methyl salicylate (scale bar 50 µm). Photographs by Takaaki Torii.

Morphological identification of sponges was made using the keys of Manconi and Pronzato (2016), Reiswig et al. (2010) and Penney and Racek (1968). Specimens were examined using an Olympus SZX16 stereomicroscope. Details were further studied using an Olympus BX53 compound microscope. The illustrations were made by tracing a photo using Microsoft Excel. Photos were taken with a Wraymer WRAYCAM-NF500 digital camera mounted on an Olympus SZX16 stereomicroscope and Shimadzu Moticam digital camera mounted on an Olympus BX53 compound microscope. Specimens of *H. latitenta* prepared using the proposed techniques described above were deposited in the National Museum of Nature and Science, Tokyo, Japan (NSMT).

DNA sequences

DNA sequences were performed on *H. latitenta* and *H. stepanowii* using *H. latitenta* collected from the Sagami River, Japan and from the Pigeon River, Tennessee, United States and *H. stepanowii* collected from the Lake-Inba in Japan. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) by following the protocol for animal tissue. Tissue was incubated in the extraction buffer and proteinase K mixture (both as provided with the kit) at 56 °C for overnight.

ITS2 regions were amplified with the universal primers ITS-u3 and ITS-u4, producing a 395 bp fragment (Cheng et al. 2016). Since intra-individual polymorphisms may be confirmed in the ITS region, the nucleotide sequence was determined using NGS. When sequencing using NGS, the target region was amplified by 1st PCR and an adapter for Miseq sequencing was added by 2nd PCR. The initial PCRs of ITS2 were performed in reaction mixtures of 12.0 μL with 6.0 μL of KAPA HiFi (Kapa Biosystems, Wilmington, MA, USA), 0.7 μL of 10 μM primer mix for ITS2, 2.0 μL of template DNA, and 3.3 μL of dH2O. The initial PCR of ITS2 was under the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 98 °C for 20 s, annealing at 55 °C for 15 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The initial PCR products of ITS2 were purified with an Agencourt AMPure XP kit (Beckman Coulter, Fullerton, CA, USA). To construct the DNA libraries using the second PCR, the i7 and i5 indexes (Illumina, San Diego, CA, USA) for identifying each sample and P5 and P7 adapters (Illumina) for Miseq sequencing were ligated to the purified initial PCR products. The second PCRs of ITS2 were performed in reaction mixtures of 24.0 μL with 12.0 μL of KAPA HiFi (Kapa Biosystems), 2.8 μL of 10 μM forward and reverse index primers, 2.0 μL of 1/10 1st PCR products, and 4.4 μL of dH2O. The second PCRs of ITS2 were under the following conditions: initial denaturation at 95 °C for 3 min, followed by 12 cycles of denaturation at 98 °C for 20 s and extension at 72 °C for 15 s, with a final extension at 72 °C for 5 min. The DNA libraries of ITS2 purified with an Agencourt AMPure XP kit (Beckman Coulter) were sequenced using a MiSeq Reagent Kit v3 (600-cycle format; Illumina) with the Illumina MiSeq sequencer following the manufacturer's protocol.

ITS2 sequences of *H. latitenta*, *H. stepanowii* and other freshwater sponges obtained from DDBJ were aligned using MEGAX (Kumar et al. 2018). Upon alignment, sequences of other freshwater sponges obtained from DDBJ was trimmed to sequences of *H. latitenta* and *H. stepanowii*. A Maximum Likelihood (ML) analysis was performed in MEGAX with the Tamura 3 model of evolution and 1000 bootstrap replicates. Sequence data of *H. latitenta* and *H. stepanowii* have been deposited in the DNA Data Bank of Japan (DDBJ) and Genbank database under accession numbers LC666919 to LC666921 (Table S2).

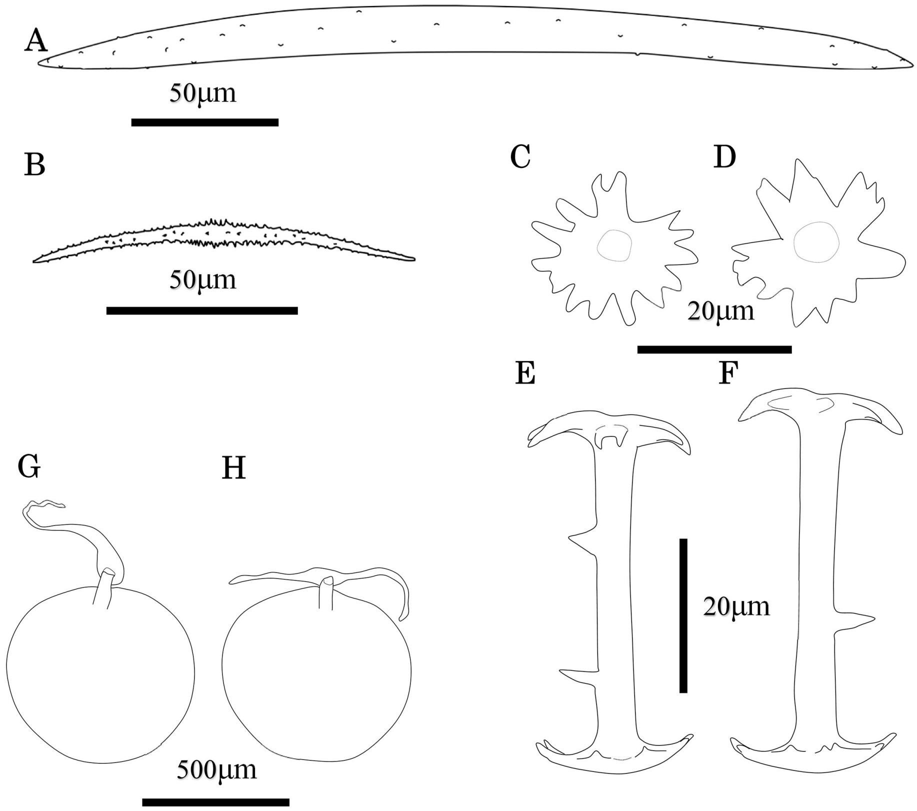

Figure 3. Ultrastructure of *Heteromeyenia latitenta* collected from the Sagami River, Japan. (A) Megascleres (scale bar 50 µm) (B) Microsclere (scale bar 50 µm). (C, D) rotules of gemmuloscleres (scale bar 20 µm). (E, F) gemmuloscleres (scale bar 20 µm). (G, H) gemmules (scale bar 500 µm).

Results

Specimens

In NCRE, the gemmules of *H. latitenta* were collected only at 1 site in the Tama River and the Sagami River respectively in August. Gemmules and dry stage encrusting sponge of *H. latitenta* were collected at an additional site in the Sagami River in April 2020 (Figure 1).

Description of the Japanese species

The consistency of the dormant sponge and gemmules was extremely fragile. Color creamy whitish in the dormant sponge and gemmules. Ectosomal skeleton with scattered microscleres. Choanosomal skeleton is an irregular network of paucispicular parallel fibers. Megascleres and microscleres are isolated and scattered randomly. Gemmules abundant, isolated or in groups, scattered throughout the sponge body. Foramen with irregular shape. Gemmular theca tri-layered with gemmuloscleres radially embedded. Pneumatic layer well developed.

Gemmules: Spherical, size ranging from 490–630 µm in diameter (Figure 2B, C; 3G, H), with long, slender foraminal tubule and cirrous appendages (Figure 2B, D; 3G, H). Foraminal tubules 70–90 µm long. Cirrous projections start from a disk that is initially flat but rounded in its

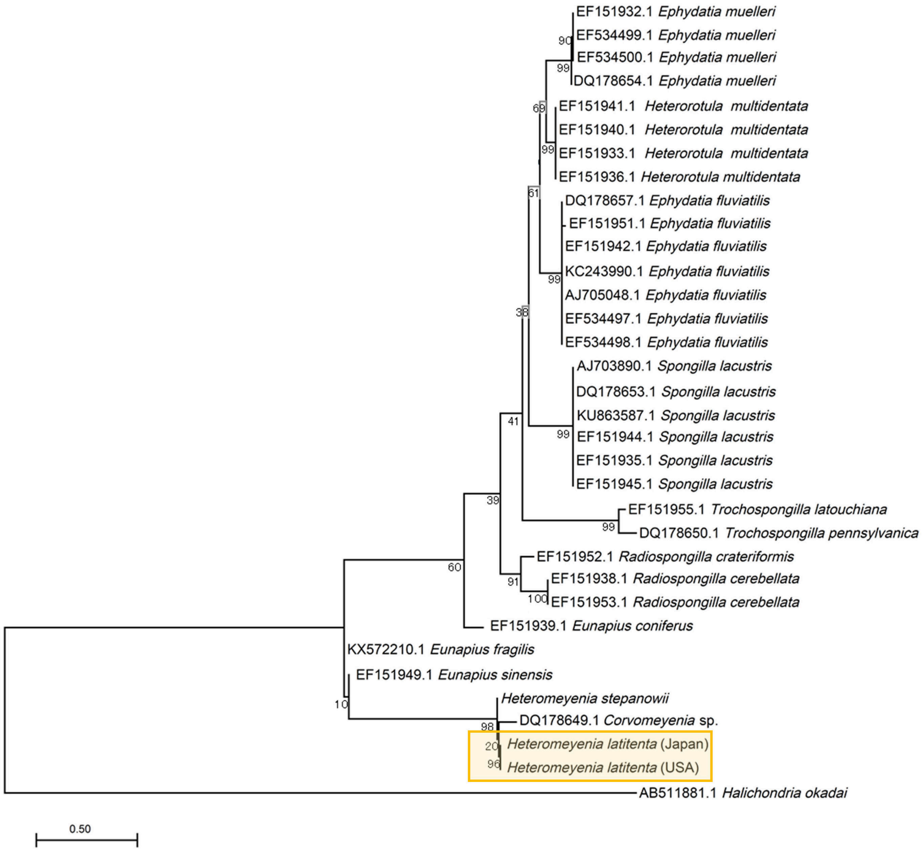

Figure 4. Maximum Likelihood tree of ITS2 sequence divergences for some freshwater sponges. Scale bar indicates Tamura 3-parameter genetic distance of 0.5. Numbers on the internodes represent the bootstrap values with 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches.

terminal regions; number of cirrous appendages between one and three, mainly one or two.

Spicules: Megascleres (Figure 3A) acanthoxeas, straight, sparsely microspined, slender and sharply pointed; length range 255–380 µm, width range13–16 µm. Microscleres (Figure 3B) acanthoxeas, slightly curved, slender and covered with entirely spined with those in the central region only slightly larger than those on the ends; length range 82–127 µm, width 7–8 µm. Gemmuloscleres birotulate amphistromgyla with cylindrical and stout shafts (Figure 3E, F), radially inserted in the theca of gemmules; length range 40–49 µm, width 21–22 µm. Rotules of equal diameter with 9–14 deeply incised and recurved teeth (Figure 3C, D).

Maximum likelihood analysis

The genetic analysis of ITS2 included 34 sequences, comprising 32 Japanese freshwater sponges, seawater sponges as outgroup taxa by referring to the DDBJ database. Due to internal insert/gap, the sequences varied greatly in length from species to species. A maximum likelihood tree of a 333–447 bp fragment of ITS2 was obtained, which is shown in Figure 4. In the ITS2, *H. latitenta* in Japan and the United States were a perfect match. Further, *H. latitenta* formed a cluster together with *H. stepanowii*. However, both had a total of 28 base insert/gap.

Ecology

The specimens were found encrusting on submerged pebbles at the bank of the river where the current was slow.

Remarks

Heteromeyenia latitenta was originally described from the specimens of Chester Creek, Pennsylvania, Western New York as "*Carteius latitenta*" (Potts 1887) and has previously been reported only from the southeastern United States (Reiswig et al. 2010) was recently discovered in the Pigeon River of eastern Tennessee in the southeastern U.S. (Copeland et al. 2019). In this study, gemmules of *H. latitenta* from the Pigeon River were examined in the same way as Japanese specimens. *Heteromeyenia latitenta* is identified from congeners based on the combination of the following morphological characteristics (Frost et al. 2001; Pinheiro et al. 2015): 1) presence of microscleres rod-shaped to needlelike in structure, and gemmuloscleres birotulate. 2) foraminal aperture of gemmules with distinct, terminal cirrous projections. 3) foraminal aperture of gemmules with one to two long cirrous projections starting from a flat disk, flat and ribbonlike at the base and cylindrical at the end. This kind of cirrous projection is a unique characteristic of *H. latitenta* that has never been observed in other congeners (Potts 1887; Pinheiro et al. 2015). These morphological character of traits of gemmules and spicules of Japanese specimens were consistent with the U.S. specimens.

The molecular data indicate that *H. latitenta* is a species different from those Japanese freshwater sponges that have been registered in DDBJ. Furthermore, *H. latitenta* collected in Japan and the United States are considered to be the same species because the sequences of ITS2 were completely the same.

Discussion

The genus *Heteromeyenia* is known from the Palearctic, Oriental, Nearctic, Neotropical, and Australian regions (Batista et al. 2007). The genus comprises ten species worldwide (Pinheiro et al. 2015; van Soest et al. 2021): *H. baileyi* (Bowerbank, 1863), *H. barlettai* Pinheiro, Calheira & Hajdu, 2015, *H. cristalina* Batista Volkmer-Ribeiro & Melão, 2007, *H. horsti* Ezcurra de Drago, 1988, *H. insignis* Weltner, 1895, *H. latitenta* (Potts, 1881), *H. longistylis* Mills, 1884, *H. stepanowii* (Dybowski, 1884), *H. tentasperma* (Potts, 1880), and *H. tubisperma* (Potts, 1881). Among these species, *H. latitenta* is distinguished by one or two, very long, cirrous projections originating from a disk that is initially flat but rounded in the gemmule's terminal regions. This feature can be used to quickly confirm the presence of this species.

This morphological study found that the species collected from Japan corresponded well to the specimens from Tennessee and original description of *H. latitenta* (Potts, 1881) from Pennsylvania and New York, U.S. Results from ITS2 analysis, Japanese and U.S. *H. latitenta* were consistent with the same cluster but separated into different cluster from *Corvomeyenia* and *H. stepanowii* (Figure 4). ITS2 rDNA has a rapid rate of evolution (Hillis et al. 1996) and has been used to resolve closely related sponge genera and species (Wörheide et al. 2004) as well as populations (Lopez et al. 2002; Wörheide et al. 2002; Duran et al. 2004) and the rDNA spacer sequences can be useful in the study of phylogenetic relationships of and the identification of species of freshwater sponges. (Itskovich et al. 2013). *Heteromeyenia latitenta* possesses a different ITS2 haplotype than *H. stepanowii*. These data and morphological differences support the separate taxonomic status of the sponges.

Until now *Heteromeyenia latitenta* had never been found outside the Americas (Reiswig et al. 2010; Copeland et al. 2019). It is currently unknown how it was introduced to Japan. Relatively small alien species now widely distributed throughout Japan, such as *Potamopyrgus antipodarum* (Gray, 1843), *Girardia tigrina* (Girard, 1850), and *Girardia dorotocephala* (Woodworth, 1897), are thought to have been introduced with ornamental plants, fish, and cultured fish (Kawakatsu et al. 2007; Urabe 2007).

The absence of this species in Japan from previous surveys, for instance in the NCRE framework suggest a quite recent introduction of *H. latitenta* to Japan. However, its exact distribution in Japanese inland waters is still unknown. Further studies are needed to understand the exact pathways and vectors of this species.

It is notable that freshwater sponges of the genus *Ephydatia* possess gemmules with birotules that are inserted perpendicularly in a pneumatic layer, their spiny rotules possibly acting as hooks for the adhesion to feathers and scales of migrating birds, such as anatids (ducks), which may transport viable gemmules to remote areas (Pronzato and Manconi 1994). Gemmules of *Heteromeyenia latitenta* have a similar structure and very long cirrous projections. These characters could also act as hooks that can adhere to feathers, which suggests that anatids could be vectors of distribution for *H. latitenta.* If so, it is likely that *H. latitenta* could be distributed throughout Japan in the future by anatids and other waterfowl.

In areas of Japan where *H. latitenta* has been found no negative or positive impacts on indigenous species have been observed at first glance. When sponge overgrowth occurs, unconsolidated sediments can be stabilized and interstitial water flow is reduced, with effects on the interstitial redox regime and water chemistry; and heterogeneity and physical complexity of the substrate can be increased, causing major impacts on local invertebrate communities (Stewart et al. 1998). Therefore, we should pay attention to the distribution dynamics of *H. latitenta*, and its possible environmental and economic impacts in the future. It is important to give a Japanese vernacular name for this species to alert the Japanese people, and we propose the Japanese name "Makige-Kaimen", which means "sponge with curly hair".

The NCRE conducted throughout Japan since 1991 is considered a powerful survey for the assessment of biodiversity in rivers, and it serves as a baseline to develop adequate conservation policies (Nakamura 2012). Additionally, the crucial role of NCRE for early detection of alien species is demonstrated in this report to encourage attention to monitor the expansion to obtain information on the discovery of alien species and hope that a continuous NCRE survey will be conducted.

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Authors' contribution

T.T., study conception and design, acquisition of data, drafting of manuscript; Y.M., study conception and design, critical revision; T.S., performed genetic analyses; T.K., critical revision. The manuscript was written by T.T. and reviewed by all authors.

Ethics and permits

Some of the specimens and data used in this paper were obtained with verbal permission from the Keihin Work Office, Kanto Regional Construction Bureau and Sagami River Wide Area Dam Management Office, Kanto Regional Development Bureau, Ministry of Land, Infrastructure and Transport (MLIT) of Japan.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Details of locations of finds of *Heteromeyenia latitenta*.

Table S2. The accession number registered in this study.

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2023/Supplements/BIR_2023_Torii_etal_SupplementaryMaterial.xlsx