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Latitudinal shift of the associated hosts in *Sagamiscintilla thalassemicola* (Galeommatoidea: Galeommatidae), a rare ectosymbiotic bivalve that lives on the proboscis of echiuran worms

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Abstract

Sagamiscintilla thalassemicola (Bivalvia: Galeommatoidea: Galeommatidae) is a rare ectocommensal bivalve that lives on the proboscis of echiuran worms, *Anelassorhynchus* spp. (Annelida: Thalassematidae: Thalassematinae: Thalassematini), and has been known only from the temperate zones of Japan. In this study, we found *S. thalassemicola* on the proboscis of the large echiuran *Ochetostoma* sp. (Thalassematidae: Thalassematinae: Thalassematini) on intertidal flats of three islands of the Ryukyu Archipelago, southern Japan. These are the first records of *S. thalassemicola* on non-*Anelassorhynchus* hosts and also from the subtropical regions. Additionally, we also collected *S. thalassemicola* from an intertidal flat of Kushimoto, Wakayama, Kii Peninsula, Japan, which is an update of the easternmost record of this species. The genetic differences in the mitochondrial cytochrome *c* oxidase subunit I and nuclear internal transcribed spacer 2 genes among *S. thalassemicola*, including those with *Ochetostoma* sp. from the subtropical region and with *Anelassorhynchus* spp. from the temperate region, can be considered within the intraspecific variation. These suggest that *S. thalassemicola* uses different echiuran hosts in the temperate and subtropical regions, respectively.

Introduction

The family Galeommatidae sensu Ponder (1998) is a group of tiny marine bivalves, with approximately 620 described species and numerous undescribed species (Huber, 2015). Many commensal species within this family live attached to the body surface or burrow walls of benthic invertebrates (Boss, 1965; Morton and Scott, 1989; Goto *et al.*, 2012; Li *et al.*, 2012). *Sagamiscintilla thalassemicola* (Habe, 1962) is a distinctive commensal galeommatid that almost exclusively lives on the proboscis of the echiurans, *Anelassorhynchus* spp. (Annelida: Thalassematidae: Thalassematinae: Thalassematini), and has been known only from the temperate Japan (Habe, 1962; Goto and Ishikawa, 2019). This species was originally described based on the specimens collected from Amakusa, west of Kyushu Island, western Japan (Habe, 1962). The list of the molluscs collected from Wakayama Prefecture, Japan, in Habe (1981) contained *S. thalassemicola* from the middle western part of the Kii Peninsula without detailed information. As there have been no reliable records of this species since the original description, it was once suspected to be extinct (Wada *et al.*, 1996). However, it has recently been rediscovered in southern Kyushu and western Shikoku Islands of western Japan (Goto and Ishikawa, 2019).

In this study, we collected *S. thalassemicola* attached to the proboscis of the large echiuran *Ochetostoma* sp. (Thalassematidae: Thalassematinae: Thalassematini) in subtropical coasts of the Ryukyu Islands, southern Japan, which provides a new host record and significantly updates the southernmost record of this species. We also collected *S. thalassemicola* attached to *Anelassorhynchus* sp. 1 in Kushimoto, Wakayama, Kii Peninsula, middle Japan, which updates the easternmost record of this species. We investigated their morphological characteristics and compared the specimens collected from *Ochetostoma* sp. and *Anelassorhynchus* spp. based on the mitochondrial cytochrome *c* oxidase subunit I (COI) and nuclear internal transcribed spacer 2 (ITS2) sequences to test whether they are conspecific. In addition, morphological and molecular characteristics of the echiuran hosts were provided.

Materials and methods

Sampling

We collected *S. thalassemicola* from echiuran hosts on the intertidal flats of the Ryukyu Archipelago (Kakeroma, Okinawa, and Ikei Islands) in 2010, 2021, and 2022, as well as

Table 1. Sampling information of *Sagamiscintilla thalassemicola*

Sampling date	Sampling locality	Latitude and longitude	Host	No. of bivalves per host
May-14-2010	Chinoura, Kakeroma Island, Kagoshima, Japan	28°10′N, 129°15′E	A: *Ochetostoma* sp.	3
Dec-16-2021	Yakata, Onna, Okinawa Island, Okinawa, Japan	26°29′N, 127°50′E	B: *Ochetostoma* sp.	2
Feb-4-2022	Kamiura, Kushimoto, Wakayama, Japan	33°27′N, 135°46′E	C: *Anelassorhynchus* sp. 1	1
Feb-28-2022	Yakata, Onna, Okinawa Island, Okinawa, Japan	26°29′N, 127°50′E	D: *Ochetostoma* sp.	2
Mar-2-2022	Ikei Island, Uruma, Okinawa, Japan	26°23′N, 127°59′E	E: *Ochetostoma* sp.	1

from the intertidal flat of Kamiura, Kushimoto in the Kii Peninsula, Wakayama, Japan in 2022 (Table 1). Figure 1 shows the sampling localities of this study and the sampling records of the previous studies (Habe, 1962, 1981; Goto and Ishikawa, 2019). The host echiurans in Kakeroma Island and Kamiura were collected from their burrows beneath the rocks embedded in the sediment bottom, whereas those in Okinawa and Ikei Islands were collected from their burrows by using a yabby pump. *Sagamiscintilla thalassemicola* and their host echiurans were photographed in a living state before fixation.

Sagamiscintilla thalassemicola and its host collected from Kakeroma Island were fixed in 70% ethanol except for a small tissue of the echiuran proboscis fixed in 99.5% ethanol, which was already used for molecular phylogenetic analyses of echiurans in Goto *et al.* (2013, 2020) and Goto (2016). Morphological characteristics of the specimen of *Ochetostoma* sp. from Kakeroma Island were described in Goto (2017). *Sagamiscintilla thalassemicola* collected from Okinawa and Ikei Islands, as well as Kushimoto, were fixed in 99.5% ethanol along with small tissues of the proboscis of their echiuran hosts. The trunk and a part of the proboscis of *Anelassorhynchus* sp. 1 collected from Kushimoto were fixed in a 10% formalin solution.

Morphological characteristics of *S. thalassemicola* were observed under a microscope. The following specimens were dissected to observe the hinge structure and internal anatomy: one from Kakeroma (A-1, see Table 2 for sample IDs), two from Okinawa Island (B-1 and B-2), and one from Kushimoto (C-1).

For molecular comparison with *S. thalassemicola* from the Ryukyu Archipelago and Kushimoto, we obtained the sequence data of the COI and ITS2 genes from two individuals of *S. thalassemicola*, which were collected from Ainan, Ehime, Shikoku Island, Japan in the previous study (Goto and Ishikawa, 2019; F-1 and G-1 in Table 2), in addition to the specimens collected in this study. The hosts of these specimens were previously identified as *Anelassorhynchus* sp. (Goto and Ishikawa, 2019). The COI gene of one individual (the host of F-1) was already sequenced in Goto (2016) as '*Anelassorhynchus* sp. 10' (accession number: LC107732). Additionally, we sequenced the COI gene of another individual (the host of G-1) of *Anelassorhynchus* sp. collected in 2015 and used in Goto and Ishikawa (2019).

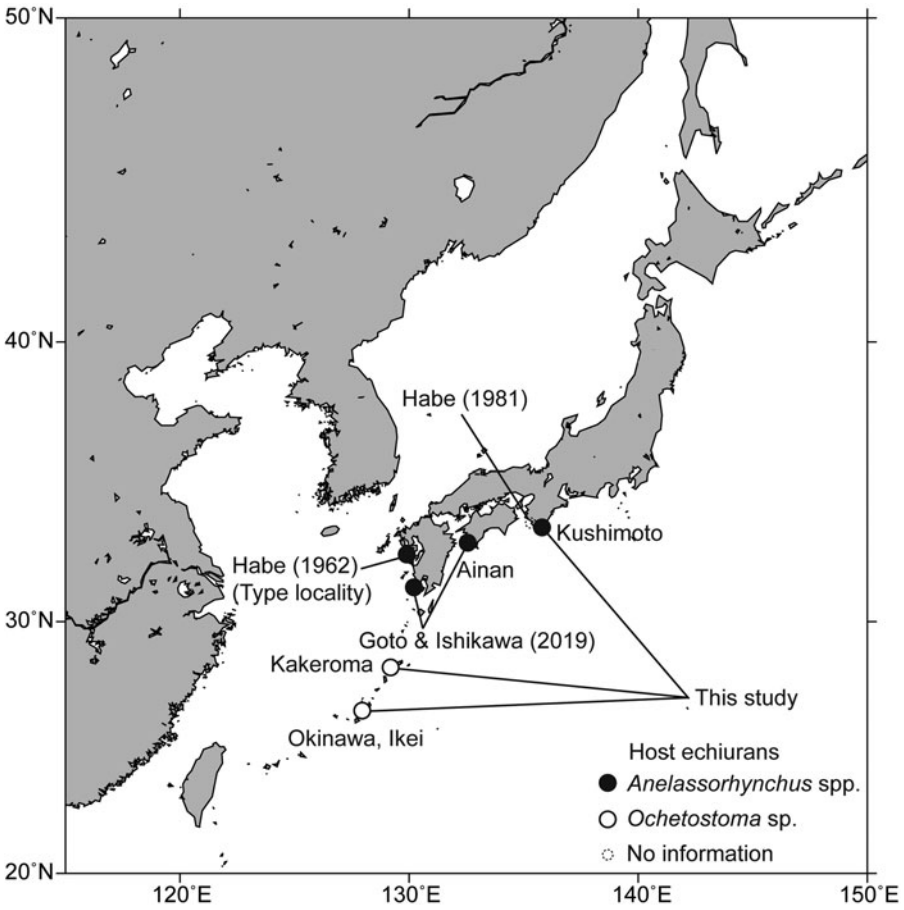

Figure 1. Sampling localities of *Sagamiscintilla thalassemicola* in this study and previous studies (Habe, 1962, 1981; Goto and Ishikawa, 2019) with the host information.

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Table 2. Specimen information of *Sagamiscintilla thalassemicola*

Sample ID	Shell length (mm)	Shell height (mm)	COI	ITS2	Host COI	Host species	Sampling locality	Sampling date
A-1	4.7	3.4	–	–	LC107736	*Ochetostoma* sp.	Chinoura, Kakeroma Island, Kagoshima, Japan	May-14-2010
A-2	–	–	–	–	–	*Ochetostoma* sp.	Chinoura, Kakeroma Island, Kagoshima, Japan	May-14-2010
A-3	–	–	–	–	–	*Ochetostoma* sp.	Chinoura, Kakeroma Island, Kagoshima, Japan	May-14-2010
B-1	3.4	2.8	LC780060	LC780068	LC780076	*Ochetostoma* sp.	Yakata, Onna, Okinawa Island, Okinawa, Japan	Dec-16-2021
B-2	3.0	2.3	LC780061	LC780069	–	*Ochetostoma* sp.	Yakata, Onna, Okinawa Island, Okinawa, Japan	Dec-16-2022
C-1	3.4	2.2	LC780062	LC780070	LC780077	*Anelassorhynchus* sp. 1	Kamiura, Kushimoto, Wakayama, Japan	Feb-4-2022
D-1	5.2	3.7	LC780063	LC780071	–	*Ochetostoma* sp.	Yakata, Onna, Okinawa Island, Okinawa, Japan	Feb-28-2022
D-2	5.0	3.7	LC780064	LC780072	–	*Ochetostoma* sp.	Yakata, Onna, Okinawa Island, Okinawa, Japan	Feb-28-2022
E-1	5.1	3.6	LC780065	LC780073	LC780078	*Ochetostoma* sp.	Ikei Island, Uruma, Okinawa, Okinawa, Japan	Mar-2-2022
F-1*	–	–	LC780066	LC780074	LC780079	*Anelassorhynchus* sp. 2	Ainan, Ehime, Shikoku Island, Japan	Jul-28-2013
G-1*	–	–	LC780067	LC780075	LC780080	*Anelassorhynchus* sp. 3	Ainan, Ehime, Shikoku Island, Japan	Sep-15-2015

Shell length and width of *S. thalassemicola*. Accession numbers of the COI and ITS2 sequences of *S. thalassemicola* and the COI sequence of the host thalassematids. The alphabets and asterisks in the sample IDs of *S. thalassemicola* indicate the host individuals (see Table 1) and the specimens derived from Goto and Ishikawa (2019), respectively.

DNA extraction

We extracted genomic DNA from small tissues of the ethanol-fixed specimens of *S. thalassemicola* and the host echiurans' proboscis using a DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol. Polymerase chain reaction (PCR) was performed using the primers LCO1490/HCO2190 (Folmer *et al.*, 1994) and Echi_cox1L/Echi_cox1H (Tanaka *et al.*, 2014) to amplify the COI genes (~650 bp) of bivalves and echiuran hosts, respectively, and also ITS2F/ITS2R (Xu *et al.*, 2001) for the nuclear ITS2 gene (~522 bp) of bivalves. Thermal cycling for the COI gene was performed with an initial denaturation for 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min 30 s at 42°C, and 1 min at 72°C, with a final 4 min extension at 72°C. As for the ITS2 gene, the annealing temperature was set to 50°C. All PCR products were purified by ExoSAP-IT (Thermo Fisher Scientific K.K., Tokyo, Japan) and then sent to Eurofins Genomics with PCR primers for sequencing. The obtained sequences were deposited in the DDBJ/EMBL/GenBank databases with the following accession numbers: *S. thalassemicola* (LC780060–LC780075), *Ochetosoma* sp. (LC780076 and LC780078), and *Anelassorhynchus* spp. (LC780077, LC780079, and LC780080) (Table 2). The voucher specimens have been retained by the first author in the Seto Marine Biological Laboratory, Kyoto University.

Molecular analysis

Haplotype networks of *S. thalassemicola* were constructed using the Median Joining Network function (Bandelt *et al.*, 1999) implemented in software PopART (Leigh and Bryant, 2015). We compared the Kimura two-parameter (K2P) genetic divergence among the specimens of *S. thalassemicola* and also among *Ochetostoma* sp. using 'distance' function of MEGA11 (Tamura *et al.*, 2021).

Results

Field observation

All the individuals of *S. thalassemicola* collected in three localities of the Ryukyu Archipelago (Kakeroma, Okinawa, and Ikei Islands), southern Japan, were found from the basal part of the proboscis of *Ochetostoma* sp. (Figures 2 and 3), while the individual collected in Kamiura, Kushimoto, Kii Peninsula, middle Japan, were found from the basal part of the proboscis of *Anelassorhynchus* sp. 1 (Figure 4). All the bivalve specimens were found hidden within the gutter of the host's proboscis (Figures 2–4) and were attached around the host's mouth (Figures 3 and 4), except for those from Kakeroma, which were attached inside the host's mouth (Figure 2). Regarding *Ochetostoma* sp., four of five (80%) collected in this study harboured *S. thalassemicola*. Number of individuals per host varied from one to three (Table 1).

Morphological characteristics

General morphological features of the specimens collected in this study were identical to those of *S. thalassemicola* described in Habe (1962) and Goto and Ishikawa (2019): (1) shells are elongated- or rounded-oval in outline and fully covered by a white soft mantle robe with short papillae (Figures 3B–D and 4C, D), (2) the hinge has a single anterior cardinal tooth in front of internal ligament of each valve (Figure 5), and (3) all the specimens dissected possess a single inner demibranch composing both ascending and descending lamellae (Figure 5). The soft mantle of the specimens collected from Okinawa Island formed a keel-like structure from right to left valves through the umbo (Figure 3B–D), which were not described in Habe (1962) or Goto and Ishikawa (2019).

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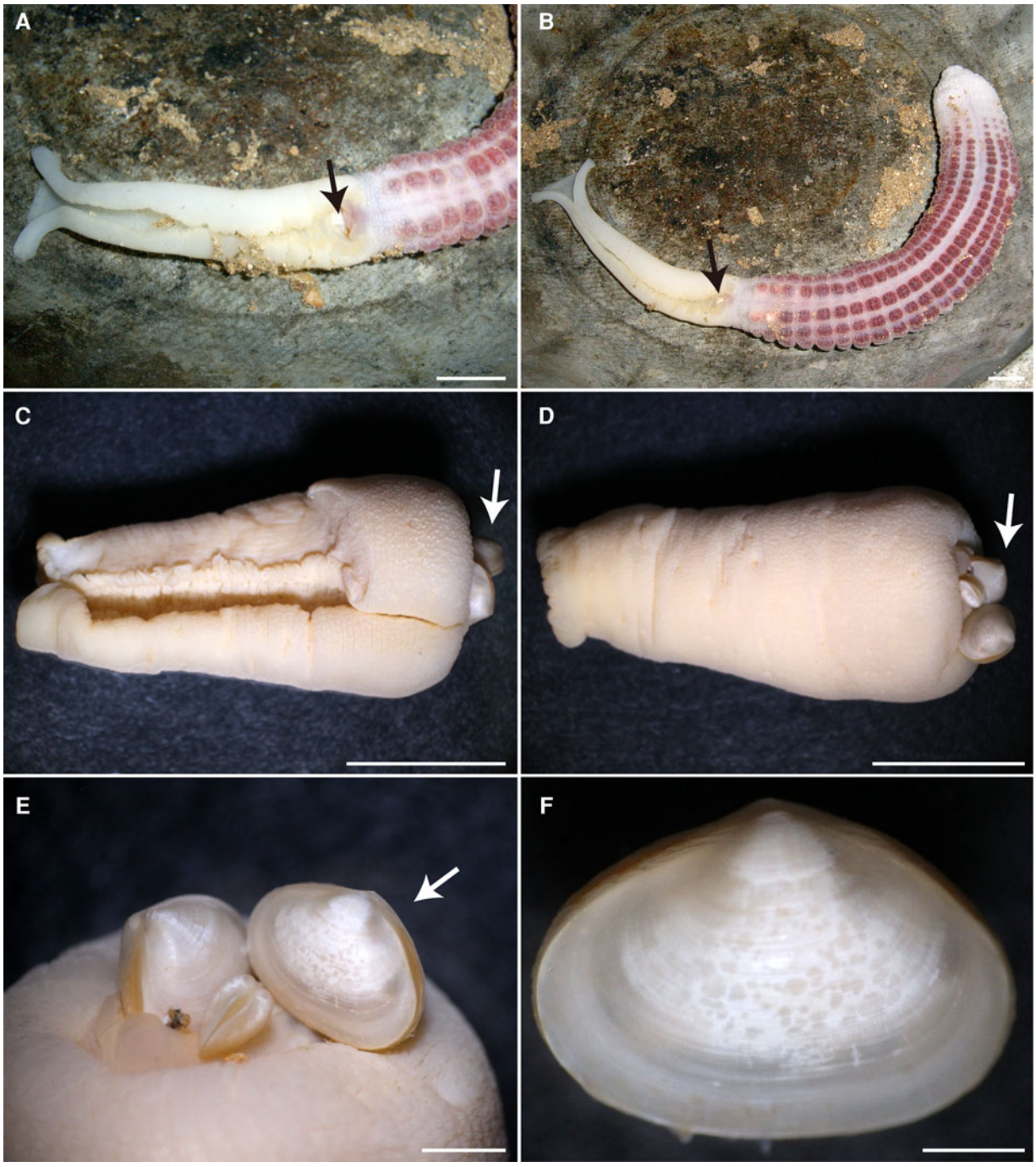

Figure 2. *Sagamiscintilla thalassemicola* (A-1, A-2, and A-3; see Table 2 for sample IDs) with their host *Ochetostoma* sp. collected from Chinoura, Kakeroma Island, Kagoshima, southern Japan. (A, B) *S. thalassemicola* living on the basal part of proboscis (inside the mouth) of *Ochetostoma* sp. (C, D) Ventral and dorsal view of the proboscis of *Ochetostoma* sp., attached by three individuals of *S. thalassemicola*. (E) Close up of the basal part of the proboscis. (F) An individual of *S. thalassemicola* removed from the host's proboscis. Scale bar: (A–D) 1 cm, (E) 2 mm, (F) 1 mm. *Sagamiscintilla thalassemicola* is indicated by white or black arrows.

Shell length and height of the specimens collected from the Ryukyu Archipelago and Kushimoto are shown in Table 2. The shell of *S. thalassemicola* from *Anelassorhynchus* sp. 1 (C-1) is slightly longer than those from *Ochetostoma* sp. even in the same size stage (e.g., B-1) (Figure 5).

Haplotype networks and genetic divergences of S. thalassemicola

Figure 6 shows the haplotype networks of the COI and ITS2 genes of *S. thalassemicol*a collected from the Ryukyu Archipelago (Okinawa and Ikei Islands), Kushimoto, and Shikoku Island. The COI and ITS2 gene haplotype networks contained eight and five haplotypes, respectively. In the COI haplotype network, one haplotype derived from G-1, which was obtained from *Anelassorhynchus* sp. 3 in Ehime, Japan, was separated from the other. No clear genetic structures were detected corresponding to each host genus in both haplotype networks (Figure 6A, B). There were only 0.1–1.5 and 0–1.0% differences in the K2P genetic divergence of the COI and ITS2 genes among the specimens, respectively.

Host echiuran identification

Ochetostoma sp. collected from Kakeroma Island had three pairs of gonoducts and eight longitudinal muscle bands. The host

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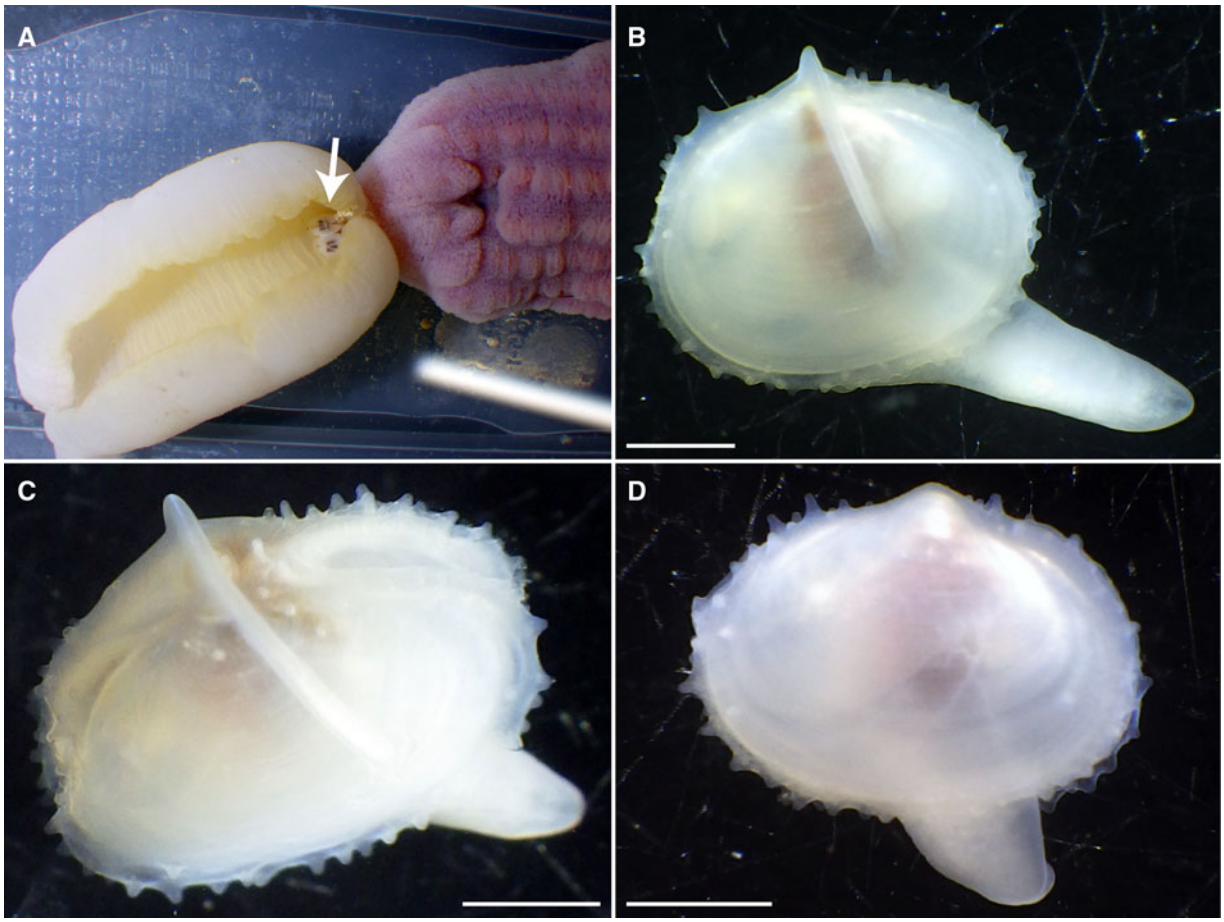

Figure 3. *Sagamiscintilla thalassemicola* (B-1 and B-2; see Table 2 for sample IDs) and their host echiuran *Ochetostoma* sp. collected from Yakata, Okinawa Island, southern Japan. (A) Two individuals of *S. thalassemicola* on the proboscis of *Ochetostoma* sp. (B, C) A larger individual of *S. thalassemicola* (SL 3.4 mm). (D) A smaller individual of *S. thalassemicola* (SL 3.0 mm). Scale bar: 1 mm.

echiuran *Ochetostoma* sp. from Okinawa Island in 2021 had ten longitudinal muscle bands without internal information. There was only 0.2–0.8% K2P genetic divergence in the sequences of COI gene among *Ochetostoma* sp. specimens collected from Kakeroma, Okinawa, and Ikei Islands.

Anelassorhynchus sp. 1 collected from Kushimoto possessed two gonoducts. The COI gene of *Anelassorhynchus* sp. 1 from Kushimoto was identical to that of '*Anelassorhynchus* sp. 9' sequenced in Goto (2016), which was previously collected from Ehime, Japan. Morphological characteristics of this species were described in Goto (2017) as '*Anelassorhynchus* sp. 6'. One of the host specimens of '*Anelassorhynchus* sp.' in Goto and Ishikawa (2019) was previously sequenced in Goto (2016) and corresponds to '*Anelassorhynchus* sp. 10'. In this study, we additionally sequenced the COI gene of another host specimen of '*Anelassorhynchus* sp.' in Goto and Ishikawa (2019) and its sequence corresponded to that of '*Anelassorhynchus* sp. 12' in Goto (2016). Morphology of this species was described as '*Anelassorhynchus* sp. 5' in Goto (2017). Overall, *S. thalassemicola* uses at least three species of *Anelassorhynchus*.

Discussion

The bivalves attaching to the proboscis of *Ochetostoma* sp. in the Ryukyu Archipelago are morphologically identified as *S. thalassemicola*. The genetic divergences among the specimens (0.1–1.5% in COI and 0–1.0% in ITS2), including those from Kushimoto and Shikoku Island with *Anelassorhynchus* spp. and those from the Ryukyu Archipelago with *Ochetostoma* sp., are similar to or less than intraspecific variation reported in other galeommatids [COI: *Koreamya arcuata* (A. Adams, 1856) in Sato *et al.* (2011); *Neaeromya rugifera* (P. P. Carpenter, 1864) in Li and Ó Foighil (2012), ITS2: *Lasaea australis* (Lamarck, 1818) in Li *et al.* (2013)]. Overall, our finding significantly updates the southernmost limit of the distribution of this species and provides a novel host record of this species. *Sagamiscintilla thalassemicola* from *Ochetostoma* sp. tended to be more rounded than that from *Anelassorhynchus* sp. 1 (Table 2). Such host-associated intraspecific morphological differences are also known in other commensal galeommatids (Sato *et al.*, 2011; Li and Ó Foighil, 2012).

Although the number of the longitudinal muscle bands of *Ochetostoma* sp. was different between Kakeroma and Okinawa Islands (eight *vs* ten), there were only 0.8% (5 of 656 bp) genetic differences between them, suggesting that they are conspecific. Longitudinal muscle bands of Thalassematini are known to vary within species (e.g., Tanaka *et al.*, 2014) and thus these can be considered within intraspecific variation. Our results suggest that *Anelassorhynchus* spp. contain at least three species, corresponding to *Anelassorhynchus* spp. 9, 10, and 12 in Goto (2016) and Goto *et al.* (2020). Overall, *S. thalassemicola* uses at least four species of two genera of thalassematids as hosts.

Habe (1981) included *S. thalassemicola* in the list of molluscs collected from Wakayama Prefecture and recorded it from the middle western part of the Kii Peninsula without details. Our collection of *S. thalassemicola* in Kushimoto thus confirms the record from the Kii Peninsula and updates the easternmost record of this species.

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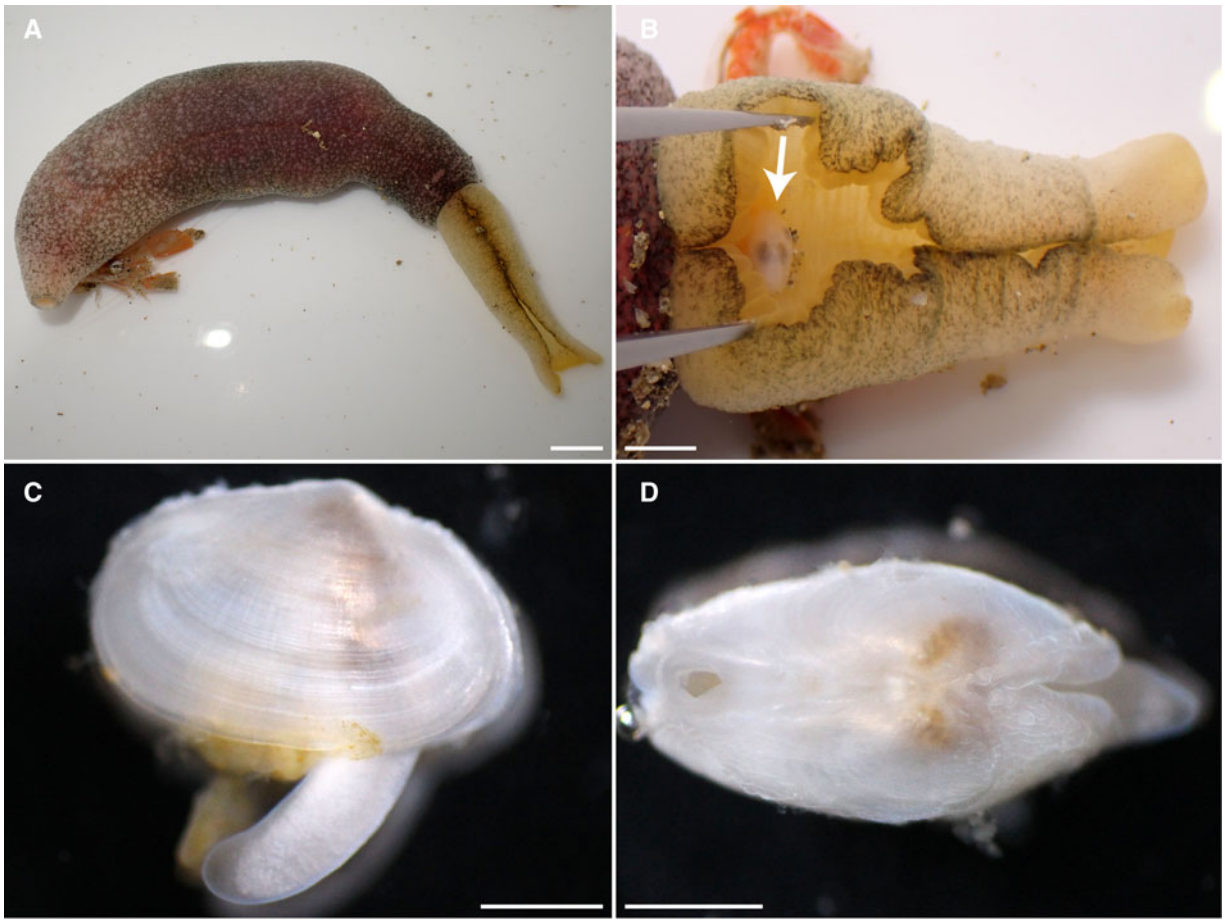

Figure 4. *Sagamiscintilla thalassemicola* (C-1; see Table 2 for sample ID) and its echiuran host *Anelassorhynchus* sp. 1 from Kushimoto, Wakayama, Japan. (A) *Anelassorhynchus* sp. 1 with its commensal shrimp *Alpheus barbatus* Coutère, 1897. (B) *S. thalassemicola* on the host's proboscis. (C, D) Lateral and dorsal view of *S. thalassemicola* removed from the host. Scale bar: 3 mm (A, B), 1 mm (C, D).

About 45 species of commensal galeommatids have been recorded from the Japanese Islands (Goto, 2022). The geographic distribution of each galeommatid species around Japanese Islands is generally limited to either the temperate or subtropical regions (Goto, 2022). The commensal galeommatid species found both in the temperate and subtropical regions are rare, except for a few examples such as *Nipponomontacuta actinariophila* Yamamoto & Habe, 1961 (Miura and Miura, 2015). Our study newly added *S. thalassemicola* to such rare examples.

Most commensal galeommatids use a specific genus or family as host (Sato *et al.*, 2011). Thus, it may not be surprising that *S. thalassemicola* uses host echiurans belonging to multiple genera (i.e., *Anelassorhynchus* and *Ochetostoma*) of the tribe Thalassematini. However, *S. thalassemicola* may not use a broad range of the species of these genera as host. *Ochetostoma* sp. is a very rare echiuran species in the Ryukyu Archipelago. Although the echiuran fauna in the Ryukyu Archipelago has been investigated by us since around 2010, we obtained only five specimens of *Ochetostoma* sp. Despite its rarity, 80% of them harboured *S. thalassemicola* on their proboscis. In contrast, *Ochetostoma erythrogrammon* Rüppel & Leuckart, 1828, which is the most common species of this genus occurring in the Ryukyu Archipelago (Goto and Kato, 2012), has never been found together with *S. thalassemicola*, although we investigated many individuals of this echiuran species for their commensals (Goto *et al.*, 2011; Goto and Kato, 2012). These suggest that *S. thalassemicola* may exhibit a host specificity for a peculiar species of *Ochetostoma*. The species of *Ochetostoma* are common in subtropical and tropical regions, but not in the temperate regions, whereas those of *Anelassorhynchus* are common both in temperate and warmer (subtropical and tropical) regions (Biseswar, 2010; Goto, 2017). We have collected various species of *Anelassorhynchus* in the Ryukyu Archipelago and other Pacific Islands (Goto, 2016, 2017), but never encountered the echiurans of this genus with *S. thalassemicola*. Thus, *S. thalassemicola* also prefers to live symbiotic with the specific species of *Anelassorhynchus* in the temperate zone. Explaining the cause of such a 'picky' host specificity is difficult as the host species of *Anelassorhynchus* and *Ochetostoma* are not so ecologically distinct from other congeneric species: both host echiuran species are burrower in intertidal sediments, which is usual in each of these genera. Interestingly, some galeommatid also shows such a host specificity pattern (Li and Ó Foighil, 2012). However, its cause also remains poorly understood.

Latitudinal shift of the associated hosts is probably common in symbiotic or parasitic marine animals, although it remains not well investigated. If the genetic isolation occurs between the northern and southern populations of such species, it likely leads to the separation of the species associated with different hosts. Some sister species of commensal galeommatids actually show such a distribution pattern: the ectosymbiotic bivalves, *Peregrinamor ohshimai* Shôji, 1938 and its sister *Peregrinamor gastrochaenans* Kato & Itani, 2000, use different upogebiid hosts and are distributed in the temperate and subtropical regions, respectively (Itani, 2004). A further investigation on latitudinal host variation of commensal galeommatids will advance our understanding of the host-associated speciation of symbiotic or parasitic animals in the sea.

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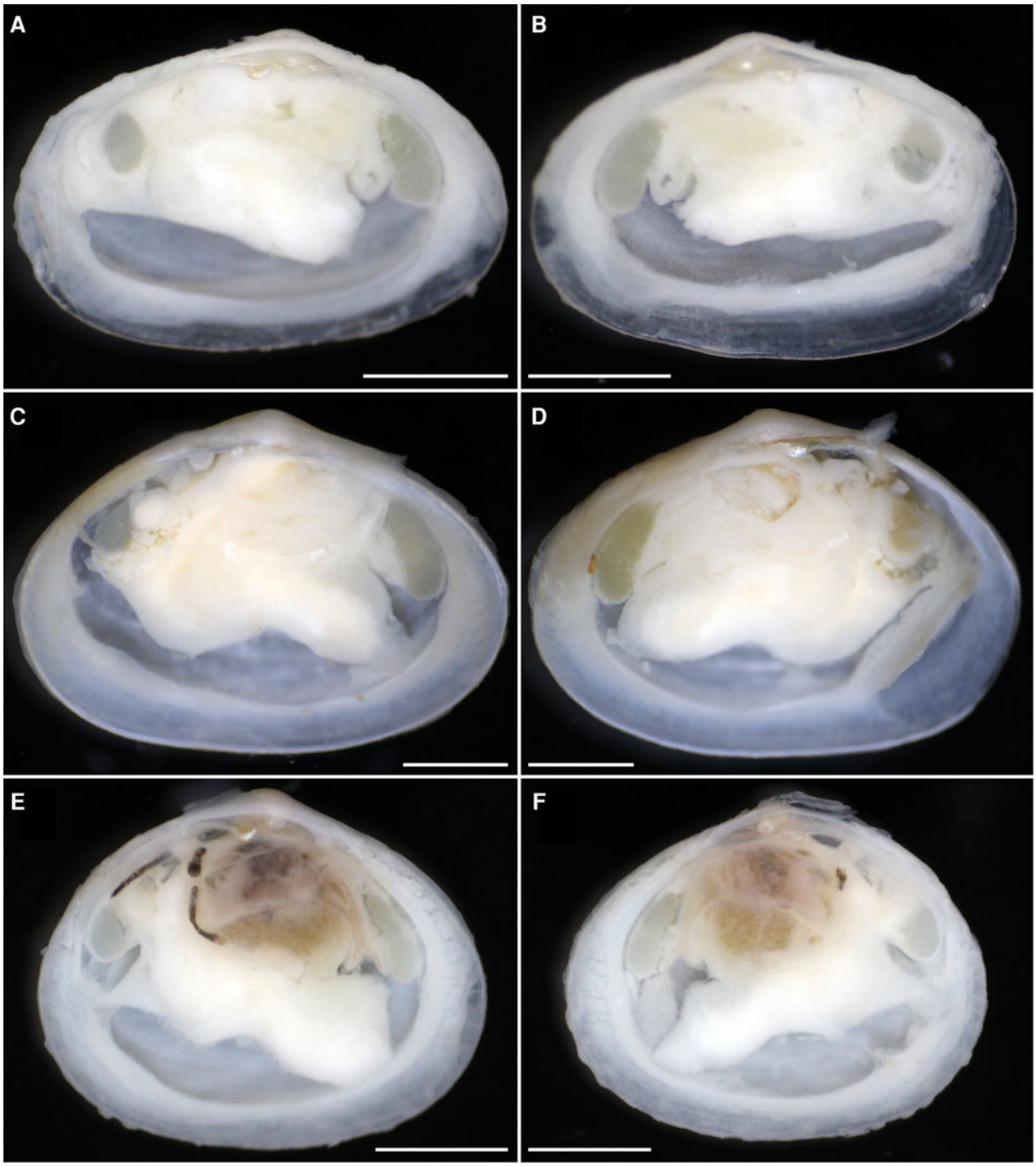

Figure 5. Internal anatomy of left and right valves of *Sagamiscintilla thalassemicola* collected from different localities: (A, B) Kushimoto, Wakayama, (C, D) Kakeroma Island, and (E, F) Okinawa Island. Sample ID: C-1, A-1, and B-1. Scale bar: 1 mm.

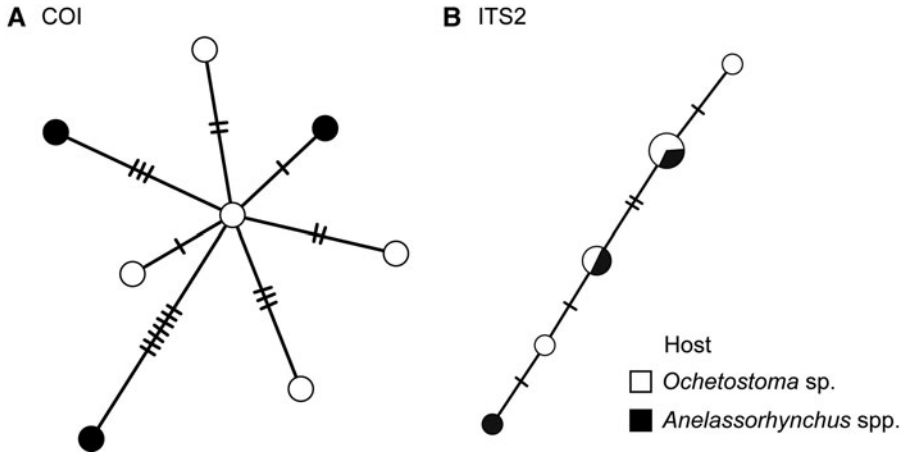

Figure 6. Haplotype networks from COI and ITS2 data for *Sagamiscintilla thalassemicola* from the Ryukyu Archipelago, Kushimoto, and Shikoku Island, Japan. Each circle represents a unique haplotype. Size and colour of the circles represent the haplotype frequency and host echiuran species, respectively. Each connection represents one inferred base-pair change.

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Data availability. The genetic data newly obtained in this study are available on NCBI GenBank at https://www.ncbi.nlm.nih.gov/genbank/ and can be accessed with the following accession numbers LC780060–LC780075 (Table 2).

Author's contribution. R. G. wrote the initial draft of the manuscript, and collected, identified, and sequenced the specimens. T. Sa., H. N., T. Su., and H. I. collected the specimens. All the authors contributed to finalize the manuscript.

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