Secondary metabolites produced by endosymbionts of shipworm woodborers (Bivalvia: Teredinidae) along the coast of Hokkaido

（北海道沿岸の木材穿孔動物フナクイムシ類（二枚貝綱：フナクイムシ科）の共生生物によって生産される二次代謝産物）

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Teredinids, known as shipworms, are unique species known for their wood-boring abilities. They occur in all aquatic ecosystems from cool-temperate to tropical waters, in floating, sunken, or living wood ranging mostly from intertidal zones to a few meters' depths. They are distinct due to several morphological adaptations to the boring and xylophagous (wood-eating) mode of life. They utilize wood as shelter and food source and digest wood with symbiotic cellulolytic and nitrogen-fixing bacteria in their gills.

Shipworm endosymbionts (e.g., *Teredinibacter turnerae*) produce secondary metabolites that can be useful in the production of biofuels and antimicrobial drugs such as tartrolons and turnercyclamycins.

More than twenty shipworm species have been known in Japanese waters mostly from the genera of *Bankia*, *Teredo*, and *Lyrodus*. Despite the recent surge in applied research of teredinid bivalves, fundamental data on the ecology and even distribution in Hokkaido waters are still barely known. Hokkaido, the northernmost part of Japan, has a cold climate. Its eastern coast has the coldest marine environment in Japan because of the strong influence of the cold Oyashio Current. In contrast, other areas are under relatively temperate conditions owing to the effect of the warm Tsushima Current on the Sea of Japan side and its branches, the Tsugaru and Soya currents. Such current settings lead to differences in the distribution of coastal marine animals in Hokkaido.

This study aims to report the interesting and remarkable secondary metabolites produced by the shipworm symbionts collected along the coastal stretches in Hokkaido that showcased a wide variety of biological activities such as antimicrobials and cytotoxic potentials in addition to cellulose-degrading capacity. These promising activities of symbionts will address the ongoing and persistent Antimicrobial Resistance (AMR) as well as chemical scaffolds for anti-cancer drug discovery and sustainable biotechnology. Furthermore, the findings described the recent data on shipworms' occurrence and distribution along the coast of Hokkaido and to continue to monitor their distribution patterns concerning ocean water currents as well as other factors that made them established along the vast coastal stretches.

Teredinids were collected from the drifted wooden debris along the shores from Ishikari Bay to Hakodate (43°N to 41°N), Notsuke Peninsula (43°N), and the northernmost part of Hokkaido, Mombetsu (44°N) between October 2020 and December 2022, some of which were surveyed more than once (e.g., Ishikari Bay and Tomakomai). A total of twelve sampling sites were visited along the vast stretches of Hokkaido waters, with two stations from each area in Ishikari Bay, Tomakomai, Yoichi, Notsuke Peninsula, and Mombetsu. Sampling sites were categorized based on three biogeographical zones: the subarctic zone in eastern Hokkaido (A), the cool temperate zone in central and northern Hokkaido (B), and the intermediate (cool- and warm-mixed) temperate zone in southern Hokkaido (C). Extraction of shipworms from wood involved the use of common tools including a saw, chisels, and hammers. Taxonomic identification of the specimens was done up to the species level based primarily on the morphology of the pallets and identification keys. Pallets and valves removed from the soft parts were preserved in 70% ethanol. The figured voucher specimens of each teredinid were deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, under registration numbers ICHUM 8445–8450.

The gills of the freshly collected shipworms were dissected, rinsed with sterile seawater, and then homogenized with sterile 75% seawater buffered with 20 mM HEPES (pH 8.0). The gill homogenates were streaked on 1.5% Noble agar MSBM plates supplemented with 0.2% Sigmacell as carbon source and incubated at 30 °C until colonies began to appear. Individual colonies were picked and re-streaked more than thrice to ensure the purity of the culture. A 16S rRNA gene sequencing was done on the isolated symbionts for classification and identification. Isolated pure bacterial strains were cultured in large volumes at optimized conditions with constant shaking of 125 rpm and extracted with different solvent polarities. All solvent extracts (fractions and isolates) were subjected to LC/MS chemical profiling and biological activities such as antibacterial against pathogenic bacteria and cytotoxicity assay using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] to MCF-7 cancer cells.

Chapter 1 discussed the occurrence and distribution of five shipworm species namely *Teredo navalis* Linnaeus, 1758, *Lyrodus pedicellatus* (Quatrefages, 1849), *Bankia setacea* (Tryon, 1863), *Bankia bipennata* (Turton, 1819), and *Bankia carinata* (Gray, 1827) collected from the different sites along the coast of Hokkaido. The present new records of two warm-water species, *Bankia bipennata* and *Bankia carinata* represent the first teredinids from temperate (cool to intermediate) waters along the vast coastal stretches of Hokkaido (Fig. I-1). *Bankia bipennata* might have been transported from the Kuroshio region to southern Hokkaido and accidentally brought to the Pacific side by typhoons and other extreme weather events. Likewise, *Bankia carinata* became naturally distributed in the Sea of Japan and transported to Hokkaido by the Tsushima and Tsugaru currents. Furthermore, *Bankia setacea* is restricted to the cold and cool-temperate zone as previously described, whereas *Teredo navalis* has wide occurrence as observed on both sides (western and eastern) of Hokkaido waters with a high tolerance for salinity and temperature fluctuations, while *Lyrodus pedicellatus* favor warmer water and is less likely to survive in subarctic eastern Hokkaido.

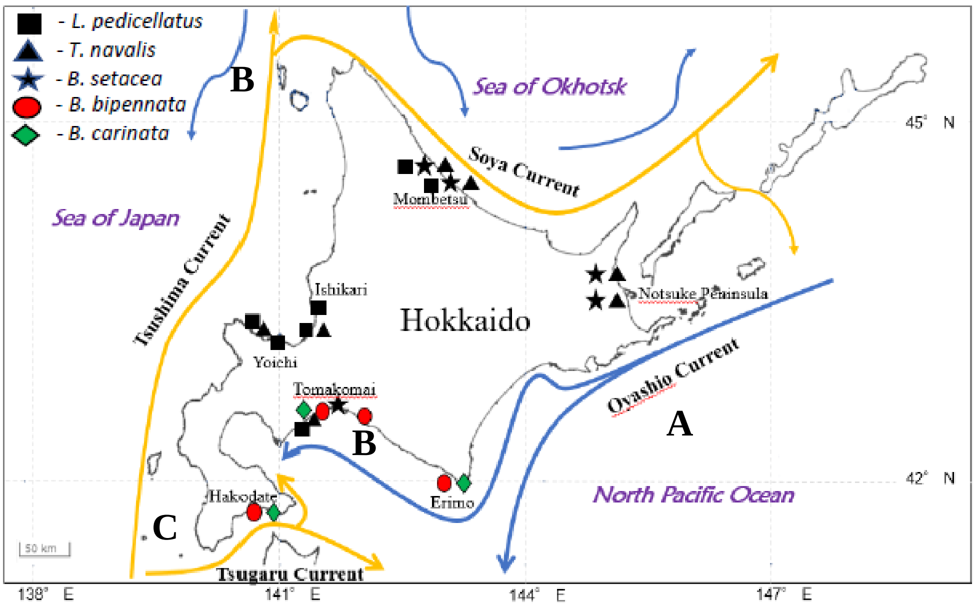
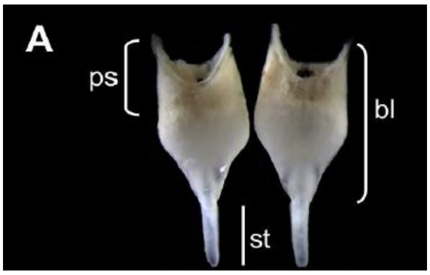


Figure I-1. Collection sites and distribution of shipworms along the coast of Hokkaido, new records from Tomakomai, Hakodate, and Erimo coastlines (red and green).

***Teredo navalis* Linnaeus, 1758**

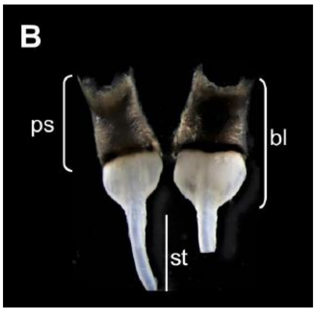


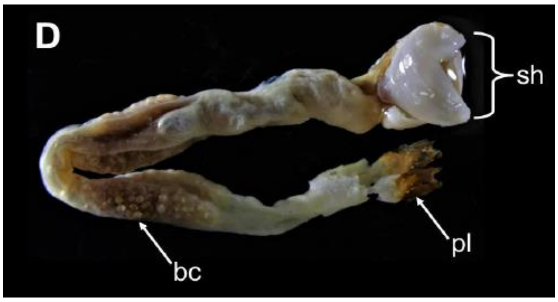
Material examined: ICHUM 8445, a set of pallets; Fig. I-2A.

Description: Pallets short, blade unsegmented with yellow/light brown periostracum and slender stalk. Outer side of blade U or V-shaped large incision. Siphons long, separated. Brooded juveniles observed in the branchial cavity. A 7 mm total length from blade to stalk.

Remarks: This species had the highest frequency of occurrence from ten sampling stations like *L. pedicellatus* and the longest body (5–25 cm) among the teredinids observed. This species is found in temperate and tropical seas and oceans worldwide including the Korean Peninsula, southern Primorye in the Sea of Japan, Sakhalin Islands, and Hokkaido.

***Lyrodus pedicellatus* (Quatrefages, 1849)**



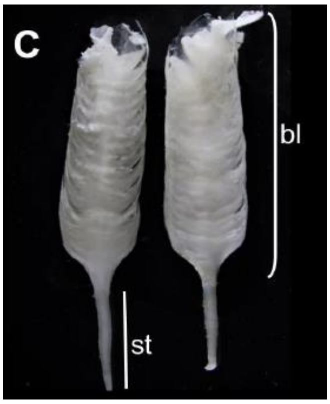


Material examined: ICHUM 8446, a set of pallets; Fig. I-2B; ICHUM 8447, preserved specimen; Fig. I-2D.

Description: Pallet non-segmented, posterior portion of calcareous blade (distal half) lanceolate covered with dark brown/black periostracal cap. Siphons relatively short and separated. Brooded juveniles in the branchial cavity. A 4.5 mm length of pallet (B) and a 4 cm overall length (D).

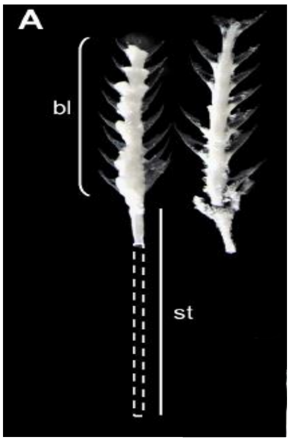
Remarks: Same with *T. navalis*, this species is also abundant and has the highest frequency of occurrence among species collected from driftwood in the present study. They are considered long-term brooder species. Extensively distributed in temperate and tropical zones. Widely distributed along the coasts of Hokkaido, particularly on the side of the Sea of Japan, whereas a small number of individuals occur in the cooler eastern area.

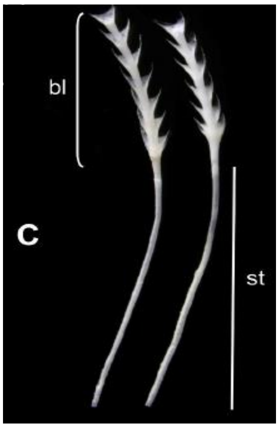
***Bankia setacea* (Tryon, 1863)**



Material examined: ICHUM 8448, a set of pallets; Fig. I-2C.
Description: Blade of pallet elongated, segmented, showing feather-like appearance. Each blade's cone broad, with periostracum whose upper margins without any serrations. Stalk short. *Bankia setacea* is easily distinguished from *B. bipennata* having shorter stalk, broader cone, and periostracum without serrations. No brooding observed in the branchial cavity. A 16 mm in length from blade to stalk.
Remarks: Exclusively distributed in cool temperate and cold-water zones in eastern Hokkaido.

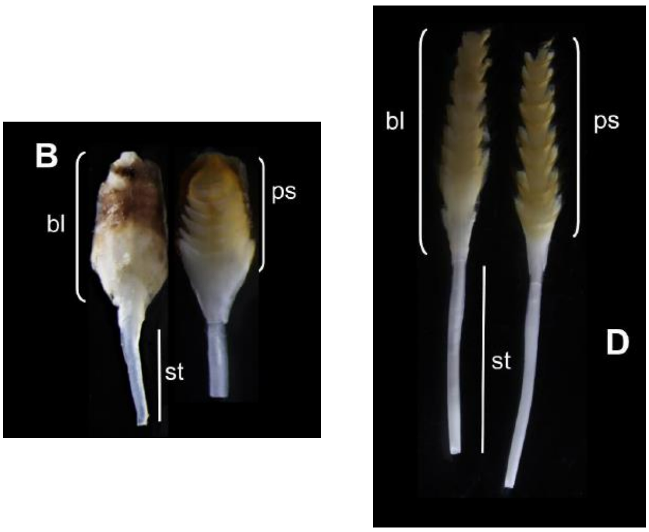
***Bankia bipennata* (Turton, 1819)**





Material examined: ICHUM 8449, a set of pallets; Fig. I-3A & C.
Description: Pallet large, slender, and segmented blade like *B. setacea*. The upper margin of the periostracum covers the blades' cone clearly serrated at the outer side. No brooding in the branchial cavity. A 20 mm total length from blade to stalk (A).
Remarks: They are considered a free-spawning species. It is distributed worldwide from temperate to tropic zones. In Japan, *B. bipennata* is known to occur in warm waters in the Kashima-nada Sea and southwards. So far, seemingly the Sea of Japan does not harbor this species. We confirmed the occurrence of the present species in four collection sites along the Pacific (Hakodate, two areas from Tomakomai, and Erimo), representing the first record of the occurrence in Hokkaido.

Bankia carinata **(Gray, 1827)**



Material examined: ICHUM 8450, a set of pallets; Fig. I-3B & D.
Description: Pallet rather slender, of which oblanceolate calcareous blade segmented and covered with brown, smooth periostracum without distal serrations. No brooding observed in the branchial cavity. A 2 mm pallet length (B).
Remarks: Distributed worldwide in subtropical to tropical zones. In Japan, it is known to occur in warm-temperate waters. The same with *B. bipennata*, our discoveries in this study from Hakodate, Erimo, and Tomakomai represents the first record of the occurrence in Hokkaido.

Chapter 2 described the eight cultivated bacterial strains from shipworms that were successfully isolated and subjected to gram-staining for strain differentiation as Gram-negative or Gram-positive. A partial 16S rRNA gene sequence was conducted on all isolated symbionts. All strains were identified as gram-negative bacteria under Phylum Pseudomonadota with 2 distinct classes—Alphaproteobacteria and Gammaproteobacteria and Flavobacteriia of the Phylum Bacteroidetes. The NCBI Blast results showed that the T2002-1 strain is closely related to the genus *Alteromonas* while the N2104-1 strain is to the genus *Marinomonas* within the class of Gammaproteobacteria that have 98.83% and 95.0% identity, respectively. The phylogenetic analysis indicated that these strains are members of a well-supported clade that also include *Teredinibacter turnerae* — a gamma-proteobacterium. On the other hand, strains T2001-1, I2003-1, YI210-1, H2101-1, and H2101-2 belong to Alphaproteobacteria from the genera of *Thalassospira*, *Tritonibacter*, Cohaesibacter, and *Phaeobacter*, respectively. While symbiont N2103-1 from flavobacteriia belongs to the genus *Tenacibaculum*.

The LC/MS chemical profiling showed the prevalence of tartrolon D from crude extracts of shipworm endosymbionts collected in all study sites from the Sea of Japan to the North Pacific Ocean. Tartrolon D has been isolated from numerous bacteria including marine *Streptomyces* sp., terrestrial rhizobacterium *Gynuella* sp., and shipworm endosymbiont *Teredinibacter turnerae*. In addition, turnercyclamycins A and B were detected in shipworms from three out of twelve locations around Hokkaido which represent cold-water and warm-water currents. As a result, compounds isolated from shipworm symbionts are most likely identical regardless of the source and its geographic location. Based on this, it suggests that antibiotics tartrolons and turnercyclamycins play a role in shipworm chemical symbiosis.

Chapter 3 focuses on two strains: *Alteromonas* sp. T2002-1 and *Marinomonas* sp. N2104-1. *Alteromonas* sp. T2002-1 was isolated from *Lyrodus pedicellatus* shipworm collected in Tomakomai in December 2020 and showed the highest bioactivity among the shipworm symbionts that were tested against pathogenic bacteria and MCF-7 cancer cell line in Chapter 2. Whilst *Marinomonas* sp. N2104-1 strain from *Bankia setacea* shipworm, found in the Notsuke Peninsula in November 2021 shows interesting peaks based on LC/MS data.

Potential bioactive compounds from the marine shipworm symbionts *Alteromonas* sp. T2002-1 and *Marinomonas* sp. N2104-1 were isolated and identified based on HR-MS/MS and NMR data. The isolated compounds were antibiotic macrodiolide tartrolon D and cyclic lipopeptide turnercyclamycin A. Tartrolon D had antibacterial activity against gram-positive bacteria *B. subtilis* and methicillin-resistant *S. aureus* and potential cytotoxic activity against MCF-7 cancer cells with a MIC of 10 μg/mL and IC_{50} of 11 μg/mL, respectively. Conversely, turnercyclamycins have potent activity against the gram-negative bacterium *P. aeruginosa* with a MIC of 10 μg/mL. Turnercyclamycins were only reported from *T. turnerae* shipworm endosymbiont. In addition, new analogues of turnercyclamycins were isolated from *Alteromonas* sp. T2002-1 endosymbiont.

It is worth mentioning that this study adds to the growing list of unexpected features of shipworm-symbiont biology and shows that these curious lifestyles have resulted in the development of secondary metabolites with potential, yet largely untapped medical and biotech applications. Remarkably, T2002-1 and N2104-1, which shows bioactivities are just a few of the strains from a variety of symbionts isolated from shipworms collected in Hokkaido. There are still vast amounts of shipworm symbiont types that are closely related to the *Teredinibacter* with a genome rich in secondary metabolites that need to be uncovered. Indeed, the collective results of this study provide a basis for the continued study of shipworm symbiosis which produces a multitude of diverse and significant compounds that could lead to drug discovery and the development of sustainable biotechnology.