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A novel symbiotic relationship between ascidians and a new tunic-boring polychaete (Annelida: Spionidae: *Polydora*)

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Abstract

Polydora tunicola Abe, Hoshino & Yamada, **sp. nov.**, a new spionid species currently considered an obligate symbiont of styelid ascidians, is described based on materials collected from *Polycarpa* cf. *cryptocarpa kroboja* (Oka, 1906) and *Cnemidocarpa* sp. in Izu-Oshima Island and *Polycarpa* sp. in Wakayama Prefecture, Japan. Polychaete–ascidian symbiotic relationships are known only in two syllid species: *Myrianida pinnigera* (Montagu, 1808) and *Proceraea exoryxae* Martin, Nygren & Cruz-Rivera, 2017. The latter has been the only polychaete known to bore into the tunic of an ascidian. *Polydora tunicola* **sp. nov.** is the second known example of a tunic-boring polychaete, which constructs U-shaped burrows in the tunic of the host ascidians. Worms were often concentrated near the host siphons and assumed to use water currents created by the filter-feeding host for suspension feeding. Although the boring mechanism into ascidian tunica is unknown, the plate assay and zymography results consistently detected cellulase activities, suggesting that cellulose digestion may enable the worms to bore into the cellulose-rich ascidian tunics. *Polydora tunicola* **sp. nov.** is morphologically similar to *P. aura* Sato-Okoshi, 1998, *P. cornuta* Bosc, 1802, *P. fusca* Radashevsky & Hsieh, 2000, *P. glycymerica* Radashevsky, 1993, *P. latispinosa* Blake & Kudenov, 1978, *P. lingulicola* Abe & Sato-Okoshi, 2020, *P. nanomon* Orensky & Williams, 2009, *P. robi* Williams, 2000, and *P. vulgaris* Mohammad, 1972 in having a single median antenna on the caruncle and chaetiger 5 without dorsal superior capillaries but with ventral capillaries. The new species is unique in having a black-rimmed pygidium, distinguishing it from these species. The phylogenetic analyses of the concatenated 18S, 28S, and 16S sequences recovered *P. tunicola* **sp. nov.** as the sister species to *P. aura* within a well-supported clade also including *P. lingulicola* and *P.* cf. *glycymerica*. The bright yellow body color of *P. tunicola* **sp. nov.** in life is similar to that of *P. aura*, however, these two species are distinguished by the former not having modified posterior notochaetae. The symbiotic nature of the association between *P. tunicola* **sp. nov.** and styelid ascidians is discussed.

Key words: cellulase activity, commensalism, *Cnemidocarpa*, *Polycarpa*, *Polydora tunicola* **sp. nov.**

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Introduction

Symbiotic relationships between animals are diversely ubiquitous in the sea. The symbiotic associations among polychaetes, a dominant group of marine benthic invertebrates, have been extensively reported in existing literature (Martin & Britayev 1998, 2018). The family Spionidae Grube, 1850, one of the most abundant polychaete groups in Annelida in terms of species numbers and biomass, is globally found in a wide variety of marine environments (Blake *et al*. 2020). Among them, many members belonging to the *Polydora* complex have symbiotic associations with other marine benthic invertebrates (Martin & Britayev 1998, 2018). The species of the genus *Polydora* have a broad range of hosts including mollusks, barnacles, shells of hermit crabs, corals, sponges, bryozoans, brachiopods, echinoderms (only fossil record), and coralline algae (Martin & Britayev 1998, 2018; Williams & McDermott 2004; Wisshak & Neumann 2006; Rodrigues *et al*. 2008; Abe *et al*. 2019). One remarkable feature is their ability to bore into various hard calcareous substrates (Blake & Evans 1973). Shell borers in mollusk aquaculture are often harmful because a heavy *Polydora* infestation can negatively affect the host mollusk (Sato-Okoshi 1999; Simon & Sato-Okoshi 2015). Although most borers are opportunistically associated with various host species (facultative associates), others are specialists or even obligate symbionts of a particular host or a group of similar host species (e.g., Williams & McDermott 2004; Radashevsky 2012; Martin & Britayev 2018). Recently, a new obligate symbiotic association between a nonboring *Polydora* species and a lingulid brachiopod was discovered (Abe & Sato-Okoshi 2020).

Despite the hundreds of symbiotic polychaete species, polychaete–ascidian symbiotic relationships are only known for two syllid species: *Myrianida pinnigera* (Montagu, 1808) and *Proceraea exoryxae* Martin, Nygren & Cruz-Rivera, 2017 (Okada 1935; Spooner *et al.* 1957; Martin *et al*. 2017). *Proceraea exoryxae* is the only polychaete known to bore into the tunic of an ascidian. In the present study, an undescribed *Polydora* species, which bores into the tunic of solitary ascidians, namely, *Polycarpa* cf. *cryptocarpa kroboja* (Oka, 1906), *Polycarpa* sp., and *Cnemidocarpa* sp., was found on Izu-Oshima Island and in Sakai Port of the Wakayama Prefecture, Japan (Fig. 1). Therefore, this is the second known example of a tunic-boring polychaete and the first spionid known to live in a symbiotic association with ascidians. Here, we describe a new species, *Polydora tunicola* **sp. nov.**, and report on its natural history. We also estimate the phylogenetic position of the new species within the genus based on analyses of nuclear 18S, 28S, and mitochondrial 16S rRNA gene sequences. Furthermore, we examined the presence of cellulase activity of the *Polydora* worms to understand the mechanism of boring into the cellulose-rich ascidian tunics.

Materials and methods

Specimen collection and morphological observation. Host styelid ascidians, *Polycarpa* cf. *cryptocarpa kroboja* and *Cnemidocarpa* sp., were collected from 5 to 40 m depth in the rocky shore environment in Akinohama (34.7868 N, 139.4088 E), Izu-Oshima Island, Japan (Fig. 1), by scuba diving on August 4, 2016 (for morphological and molecular analyses), November 11, 2017 (for micro-CT examination), and February 6, 2018 (for cellulase activity measurements). Both ascidian species had thick leathery tunics and were approximately 5–10 cm long. An additional host specimen, *Polycarpa* sp. was collected from Sakai Port (33.7436 N, 135.3325 E), Minabe Town, Wakayama Prefecture, Japan (Fig. 1) on April 1, 2021. Additionally, field observation has been performed regularly in Akinohama from March 2014 to January 2022 by an author (O. Hoshino), and field photographs were taken using a digital camera (Nikon D80 or D810) with a macrolens (Nikon AF Micro-Nikkor 105 mm). *Polydora* worms were obtained from the tunic by refrigerating the ascidian hosts. When cooled to about 4°C in a refrigerator, many *Polydora* worms emerged from the tunics. The worms remaining in the tunics were flushed out by shooting seawater into one of the bore-hole openings of the U-shaped burrows using a needle attached to a syringe. The *Polydora* and ascidian specimens were fixed in 10% v/v neutral formaldehyde solution in seawater before storing in 70% ethanol for morphological observation or 99% ethanol for molecular analysis.

The morphology of the *Polydora* worms was observed under a stereomicroscope (Olympus SZX9) in both live and preserved conditions. Live specimens were anesthetized in 7% magnesium chloride solution when required. Sections of the fifth chaetiger with major spines were mounted on microscope slides and observed using phase-contrast microscopy (Nikon Eclipse 80i). Light micrographs were taken using a digital camera (α6000; Sony, Tokyo, Japan) attached to the microscope using a c-mount camera adapter (SA20; Wraymer, Osaka, Japan). The type

material is deposited in the National Museum of Nature and Science (NSMT), Tsukuba, Japan, under the museum registration numbers NSMT-Pol H-855 and P-856–857.

X-ray microcomputed tomography (micro-CT) examinations were conducted to examine how and where the *Polydora* worms live in the ascidians. Micro-CT scanning of a host ascidian was performed using the nanofocus CT system (Phoenix Nanotom M, GE Sensing & Inspection Technologies) at 130 kV tube voltage, 110 mA tube current, and 33.3 µm voxel sizes for 500 ms exposure time, and 1,000 images were obtained during the 360° rotation of the sample. For micro-CT examination, formalin-fixed and ethanol-preserved ascidian samples were used without staining and dehydration.

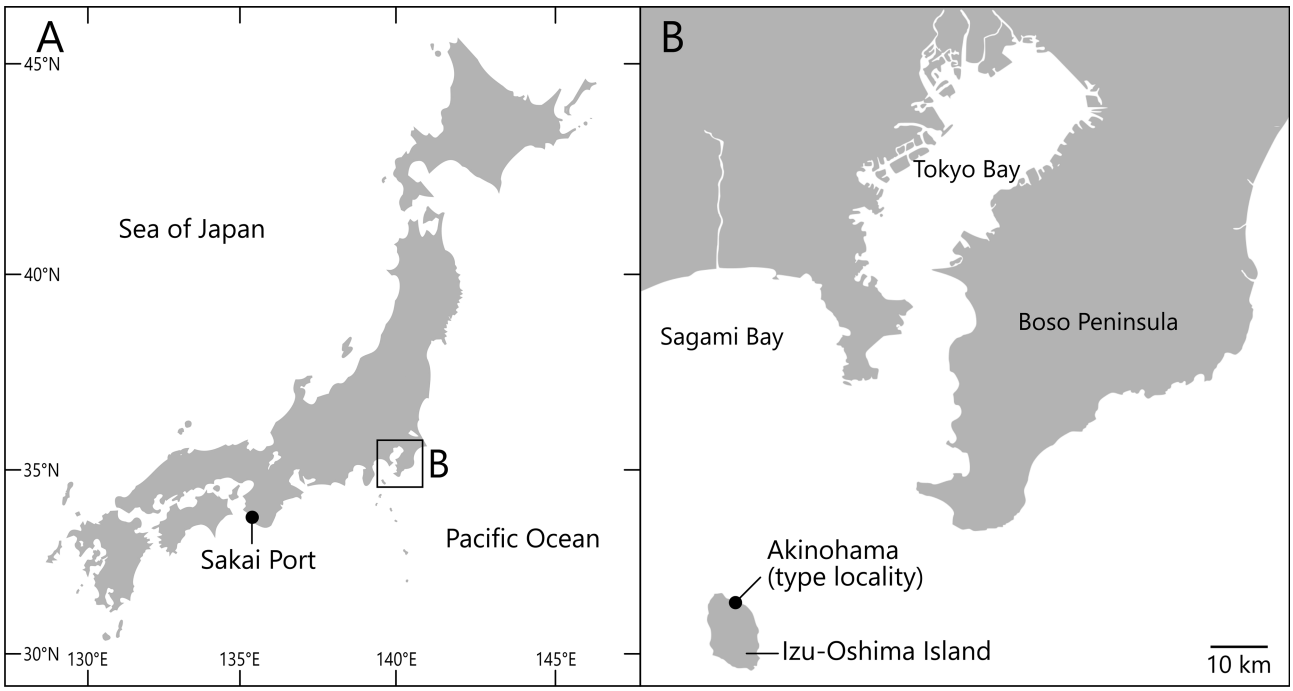

FIGURE 1. Maps showing sampling locations of *Polydora tunicola* **sp. nov.** A: Japan. B: Izu-Oshima Island and its vicinity.

Molecular analysis. We performed nuclear 18S, 28S, and mitochondrial 16S rRNA gene analyses on five *Polydora* specimens. Genomic DNA was extracted from 99% ethanol-preserved tissue by grinding and heating at 95°C for 20 min in 50 µL of TE buffer (pH 8.0) with 10% Chelex 100 (Bio-Rad) according to Richlen & Barber (2005). Tenfold diluted extracted DNA in TE buffer was used as a polymerase chain reaction (PCR) template. Partial sequences of the nuclear 18S, 28S, and mitochondrial 16S rRNA genes were amplified by PCR using the primer pairs 18S-1F1/18S-1R632, 18S-2F576/18S-2R1209, and 18S-3F1129/18S-R1772 for 18S (Nishitani *et al*. 2012); D1R/D2C for 28S (Scholin *et al*. 1994); and 16Sar/16Sbr for 16S (Palumbi *et al.* 1991). PCR was performed in a 25 µL reaction mixture containing 1.0 µL of template DNA, 16.45 µL of sterilized water, 2.5 µL of 2 mM of deoxynucleoside triphosphates (dNTPs), 2.5 µL of 10× PCR buffer, 2.0 µL of 25 mM $MgSO_4$, 0.4 µL of 1 U/µL KOD-Plus-ver. 2 DNA polymerase (Toyobo, Osaka, Japan), and 0.1 µL of 50 µM forward and reverse primers. The PCR cycling conditions were initial denaturation at 94°C for 2 min and 36 cycles of 98°C for 10 s, 58°C (18S and 28S) or 56°C (16S) for 30 s, and 68°C for 45 s. The PCR products were purified using ExoSAP-IT (Affymetrix, Cleveland, OH) and sequenced by Eurofins Genomics (Tokyo, Japan). The forward and reverse complementary sequences and contigs were assembled using GeneStudio ver. 2.2.0.0 (GeneStudio, Inc., Suwanee, GA, USA). All newly generated sequences in this study have been deposited in DDBJ/ENA/GenBank nucleotide sequence database under accession numbers LC677126 to LC677140 (Table 1). PCR using the first primer set of 18S rRNA gene was performed to ensure the future re-verification of the identification of the host ascidians, and the obtained sequences have been deposited in DDBJ/ENA/GenBank nucleotide sequence database under accession numbers LC677141 to LC677143. To reconstruct molecular phylogeny, sequences of the 18S, 28S, and 16S rRNA genes were aligned with the sequences of other *Polydora* species and outgroups obtained from GenBank (Table 1) using MAFFT online service ver. 7 with L-INS-i algorithm (Katoh *et al.* 2017). The gene sequences of *Dipolydora* species obtained from DDBJ/

TABLE 1. DDBJ/ENA/GenBank accession numbers and information on the lifestyle of each *Polydora* species and outgroups (*Dipolydora*) used in molecular phylogenetic analyses in this study. The gene sequences obtained in this study are highlighted in boldface type.

Genus	Species	Country	DDBJ/ENA/GenBank accession number			Reference of accession number	Life style	Reference of life style
			18S	28S	16S			
Polydora	*Polydora tunicola* Abe, Hoshino & Yamada **sp. nov.**	Japan	**LC677126–LC677130**	**LC677131–LC677135**	**LC677136–LC677140**	This study	Tunic-boring	This study
	Polydora aura Sato-Okoshi, 1998	Japan	AB705409	LC500923	LC500931	Sato-Okoshi & Abe (2012); Abe & Sato-Okoshi (2020)	Shell-boring	Sato-Okoshi (1999); Sato-Okoshi & Abe (2012)
	Polydora brevipalpa Zachs, 1933	Japan	AB705407	-	LC595738	Sato-Okoshi & Abe (2012); Abe & Sato-Okoshi (2021)	Shell-boring	Sato-Okoshi (1999); Sato-Okoshi & Abe (2012)
	Polydora calcarea (Templeton, 1836)	Japan	AB705403	-	LC595740	Sato-Okoshi & Abe (2013); Abe & Sato-Okoshi (2021)	Shell-boring	Sato-Okoshi & Abe (2013)
	Polydora cornuta Bosc, 1802	Japan	LC541483	LC541485	LC541484	Abe & Sato-Okoshi (2020)	Free-living (Tube-dwelling) /Sessile	Abe *et al*. (2019)
	Polydora cf. *glycymerica* Radashevsky, 1993	Japan	LC545907	-	LC595742	Abe & Sato-Okoshi (2021)	Shell-boring	Radashevsky (1993)
	Polydora hoplura Claparède, 1868	Japan	LC101841	LC101854	LC101870	Sato-Okoshi *et al*. (2017)	Shell-boring	Sato-Okoshi (1999); Sato-Okoshi & Abe (2012); Sato-Okoshi *et al*. (2017)
	Polydora lingshuiensis Ye, Tang, Wu, Su, Wang, Yu & Wang, 2015	China	KF562240	KF562246	KF562233	Ye *et al*. (2015)	Shell-boring/ Tube-dwelling	Ye *et al*. (2015)
	Polydora lingulicola Abe & Sato-Okoshi, 2020	Japan	LC500909	LC500916	LC500924	Abe & Sato-Okoshi (2020)	Non-boring symbiotic (Tube-dwelling)	Abe & Sato-Okoshi (2020)
	Polydora neocaeca Williams & Radashevsky, 1999	China	KF562241	KF562248	KF562234	Ye *et al*. (2015)	Shell-boring	Sato-Okoshi & Abe (2013); Ye *et al*. (2015); Malan *et al*. (2020)

.....continued on the next page

TABLE 1. (Continued)

Genus	Species	Country	DDBJ/ENA/GenBank accession number			Reference of accession number	Life style	Reference of life style
			18S	28S	16S			
	Polydora cf. *nuchalis* Woodwick, 1953	South Africa	KY677903	-	-	Williams *et al*. (2017)	Tube-dwelling	Williams *et al*. (2017)
	Polydora onagawaensis Teramoto, Sato-Okoshi, Abe, Nishitani & Endo, 2013	Japan	AB691768	LC682719	LC595745	Teramoto *et al*. (2013); Abe & Sato-Okoshi (2021); Sato-Okoshi *et al*. (unpubl.)	Shell-boring	Teramoto *et al*. (2013)
	Polydora cf. *spongicola* Berkeley & Berkeley, 1950	Japan	LC545910	-	LC595747	Abe & Sato-Okoshi (2021)	Sponge-boring	Abe & Sato-Okoshi (2021)
	Polydora triglanda Radashevsky & Hsieh, 2000	Taiwan	JN048718	JN048731	JN048705	Radashevsky & Pankova (2013)	Shell-boring	Radashevsky & Hsieh (2000); Radashevsky & Pankova (2013)
	Polydora websteri Hartman in Loosanoff & Engle, 1943	France	LC682698	LC682720	LC682741	Sato-Okoshi *et al*. (unpubl.)	Shell-boring	Sato-Okoshi (1999); Sato-Okoshi & Abe (2013)
	Polydora sp. 1 sensu Abe & Sato-Okoshi (2021)	Japan	LC545912	-	LC595750	Abe & Sato-Okoshi (2021)	Shell-boring	Abe & Sato-Okoshi (2021)
	Polydora sp. 2 sensu Abe & Sato-Okoshi (2021)	Japan	LC545914	-	LC595752	Abe & Sato-Okoshi (2021)	-	-
	Polydora sp. 3 sensu Abe & Sato-Okoshi (2021)	Japan	LC545915	LC682722	LC595753	Abe & Sato-Okoshi (2021); Sato-Okoshi *et al*. (unpubl.)	-	-
Dipolydora	*Dipolydora bidentata* (Zachs, 1933)	Russia	JX228065	JX228085	JX228103	Radashevsky & Pankova (2013)	Shell-boring	Radashevsky (1993); Radashevsky & Pankova (2013)
	Dipolydora cardalia (Berkeley, 1927)	Russia	JX228073	JX228093	JX228113	Radashevsky & Pankova (2013)	Tube-dwelling	Radashevsky & Pankova (2013)
	Dipolydora carunculata (Radashevsky, 1993)	Russia	JN048711	JN048724	JN048698	Radashevsky & Pankova (2013)	Shell-boring/ Tube-dwelling	Radashevsky (1993); Radashevsky & Pankova (2013)
	Dipolydora giardi (Mesnil, 1893)	France	LC682685	LC682706	LC682728	Sato-Okoshi *et al*. (unpubl.)	Shell-boring	Sato-Okoshi (1999)

ENA/GenBank were used as outgroup taxa according to the molecular phylogeny of the family Spionidae by Abe & Sato-Okoshi (2021). Ambiguously aligned regions were eliminated by employing Gblocks server ver. 0.91b with the least stringent settings (Castresana 2000; Talavera & Castresana 2007). The final lengths of the aligned sequences were 1771, 766, and 469 bp for the 18S, 28S, and 16S rRNA gene sequences, respectively. The alignment has 26 sequences with 3006 columns, 370 distinct patterns 241 parsimony-informative, 112 singleton sites, and 2653 constant sites. A phylogenetic tree was constructed based on the concatenated sequences of 18S, 28S, and 16S rRNA gene regions by maximum likelihood (ML) analyses performed using IQ-TREE (Nguyen *et al*. 2015) implemented in PhyloSuite v.1.2.2 (Zhang *et al*. 2020) under an edge-linked partition model. The TIM2e+I+G4, TIM3+F+I, and GTR+F+I+G4 models were selected as the best substitution models for the 18S, 28S, and 16S rRNA gene regions, respectively, by ModelFinder (Kalyaanamoorthy *et al*. 2017) as implemented in IQ-TREE under the Bayesian information criterion. We evaluated the robustness of the ML trees by the Shimodaira–Hasegawa-like approximate likelihood-ratio test (SH-aLRT) with 5,000 replicates (Guindon *et al*. 2010), approximate Bayes (aBayes) test (Anisimova *et al*. 2011), and ultrafast bootstraps (UFBoot) with 5000 replicates (Hoang *et al*. 2018). SH-aLRT ≥ 80%, aBayes ≥ 0.95, and UFBoot ≥ 95% were defined as robust statistical support. To discuss the biology of the worm to its host, ecological information on each species' lifestyle based on our observations and available literature (Table 1) was mapped onto the phylogenetic tree.

Cellulase activity measurements. For examining the presence of cellulase activity of the *Polydora*, plate assay and zymography using carboxymethylcellulose (CMC) were conducted. A part of the specimens collected on February 6, 2018, was frozen at −20°C for cellulase activity measurements. The whole body of the specimen with 0.039 g wet weight was homogenized with 50 μL of sodium acetate (AcNa) buffer (pH 5.5) using a handheld homogenizer for plate assay. The homogenate was centrifuged at 10,000 × *g* for 10 min, and the supernatant was used as an enzyme solution. A mixture of 1.5% agar (Agarose S, Nippon Gene, Tokyo, Japan) and 0.1% CMC (carboxymethylcellulose sodium salt low viscosity, Sigma-Aldrich, St Louis, MO, USA) in 100 mL of 0.2 M AcNa buffer was heated and poured into a plastic Petri dish to a height of approximately 4 mm. Then, 30 μL of the enzyme solution was applied in a spot with an approximately 4-mm diameter in the gel plate. After incubation at 37°C overnight, the gel was stained with 5 mL of 0.1% Congo red for 1 h and subsequently rinsed with 10 mL of 1 M NaCl solution for 1 h. Endoglucanase activity was indicated by a destained area (halo) around each spot. Crystalline style with 0.005 g wet weight of *Corbicula japonica* purchased in supermarkets and AcNa buffer without homogenate were used as positive and negative controls, respectively.

Zymographic analysis estimated the molecular weight of cellulase according to Béguin (1983) with slight modifications. The whole body of the specimen with 0.016 g wet weight was homogenized with 150 μL of phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 (pH 7.4) using a handheld homogenizer. The homogenate was centrifuged at 10,000 × *g* for 10 min, and the supernatant was collected. The protein concentration of the supernatant was determined using Micro BCA™ Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) and then adjusted to 1 mg/mL with PBS. The adjusted solution was used as an enzyme solution for zymographic analysis. The enzyme solution was mixed with sample buffer (0.35 M Tris-HCl, 10% SDS, 36% glycerol, and 0.1% bromophenol blue) at a ratio of 1:6, followed by SDS-PAGE with 10% acrylamide gel containing 0.1% w/v CMC. Prestained XL-Ladder Broad (APRO Science, Tokushima, Japan) was loaded as a size marker. After electrophoresis, the gels were rinsed twice in 0.2 M AcNa buffer containing 0.1% Triton X-100 for 30 min to remove SDS from the gels and subsequently incubated for 3 h at 37°C. Each gel was then stained with 0.1% Congo red for 30 min and rinsed with 1 M NaCl for 1 day. The molecular size of cellulase was estimated from active bands detected as destained bands in the gel.

Results

Family Spionidae Grube, 1850

Genus *Polydora* Bosc, 1802

***Polydora tunicola* Abe, Hoshino & Yamada, sp. nov.**
[Japanese name: Hoyano-poridora]
(Figs. 2–4)
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Spionidae sp.: Hoshino 2016: page 50–51, 3 figs.
Polydora sp.: Hoshino 2020: page 100–105, 9 figs.

Type material. Holotype: NSMT-Pol H-855, Akinohama, 34.7868 N, 139.4088 E, Izu-Oshima Island, Tokyo Pref., subtidal, 10 m depth, rocky shore, isolated from *Polycarpa* cf. *cryptocarpa kroboja*, 04 August, 2016; 35 **Paratypes**: NSMT-Pol P-856, Akinohama, 34.7868 N, 139.4088 E, Izu-Oshima Island, Tokyo Pref., subtidal, 10 m depth, rocky shore, isolated from *Polycarpa* cf. *cryptocarpa kroboja*, 04 August, 2016; 1 **Paratype**: NSMT-Pol P-857, Sakai Port, 33.7436 N, 135.3325 E, Minabe Town, Wakayama Pref., isolated from *Polycarpa* sp., 01 April, 2021.

Holotype description. A complete 107-chaetiger individual, measuring 27 mm long and 1.1 mm wide at chaetiger 5. Body light tan in alcohol (Fig. 2) and yellow in life (Fig. 3A–C); yellow pigmentation present on prostomium, peristomium, dorsal, ventral, and lateral sides of body in preserved specimen (Fig. 2), but difficult to observe in fresh condition (Fig. 3A–C). Prostomium rounded on anterior margin (Figs. 2B, 3A); eyes absent; caruncle extends posteriorly to middle of chaetiger 3, with small median antenna present on caruncle at level of chaetiger 1. Palps extending posteriorly for about 11 chaetigers, color in alcohol opaque white to light tan, without black pigmentation.

Chaetiger 1 with small noto- and neuropodial postchaetal lamellae compared to subsequent chaetigers (Fig. 3A); notochaetae absent, short capillary neurochaetae present. Postchaetal lamellae well developed on anterior chaetigers except chaetiger 5, gradually reduced on posterior chaetigers. Prechaetal lamellae absent in all parapodia. Neuro- and notochaetae of chaetiger 2–4, 6 winged capillaries, arranged in two rows; capillaries in anterior row more curved and winged than those in posterior row. Number of capillaries per fascicle and wings in capillaries in notopodia of succeeding chaetigers diminished gradually; rows of notochaetae becoming indistinct in posterior chaetigers. Posterior notochaetae only capillaries. Hooded hooks of neuropodia from chaetiger 7 (Fig. 3B, C), not accompanied by capillaries; hooks bidentate, with main fang at right angle to shaft and acute angle with apical tooth; slightly curved shaft having constriction in upper part; curve and constriction of shaft weak in hooks from 7th chaetiger (Fig. 3G) but develop gradually toward posterior chaetigers (Fig. 3H, I)); hooks up to 12 in series.

Chaetiger 5 slightly larger than either chaetiger 4 or 6; not overlapping chaetiger 6 (Figs. 2B, 3A); with curved oblique row of 10 dorsal falcate major spines alternating with pennoned companion chaetae (Fig. 3F); Anterior spines in a row falcate with lateral tooth; posterior spines in a row simple falcate or falcate with a rough-edged transverse shelf (Fig. 3F). Ventral tuft of four short winged capillaries present (Fig. 3C).

Branchiae from chaetiger 7 to end of body, absent only from six posterior-most chaetigers, short, not overlapping mid-dorsal, full-sized from chaetiger 9 or 10 to middle of body, then decreased in size; free from notopodial postchaetal lamellae, with flattened surfaces oriented parallel to body axis.

Pygidium wide disc with mid-dorsal gap; white-colored with black rim (Fig. 2D–F). No gizzard-like structure in digestive tract. Glandular pouches in neuropodia from chaetiger 7 onward, largest in chaetigers 9–12 (Fig. 3D).

Variability. The largest individual 27 mm long, 1.1 mm wide for 107 chaetigers (Holotype). Body pigmentation brown and/or yellow may be present on palp, prostomium, peristomium, dorsal and ventral sides, and pygidium. Bands of black pigment, probably remnants of larval pigmentation, on dorsal side of anterior chaetigers in small individuals. Prostomium rounded or weakly incised on anterior margin. Eyes usually absent in large worms, but occasionally present in small individuals especially in juveniles; four black eyes arranged as trapezoid shape, anterior pair widely spaced, posterior pair closely spaced. Caruncle extending posteriorly to end of chaetiger 2 or 3. Median antenna fingerlike (Fig. 3A) or small and indistinct (Fig. 2B). Palp length varied depending on degree of extension at fixation, extending as long as body length in live individuals especially in juvenile. Tip of anterior most spines in a row in chaetiger 5 occasionally chipped. Tip of companion chaetae often feather-like especially anterior in a row (Fig. 3F). Chaetiger 5 without dorsal superior capillaries but with ventral capillaries. Pygidium cup-shape to a wide flare-disc with mid-dorsal gap; white colored with black or brown rim. Pygidium of several specimens without black or brown rim, possibly due to intraspecific variation, but probably also due to susceptibility to tearing at this region.

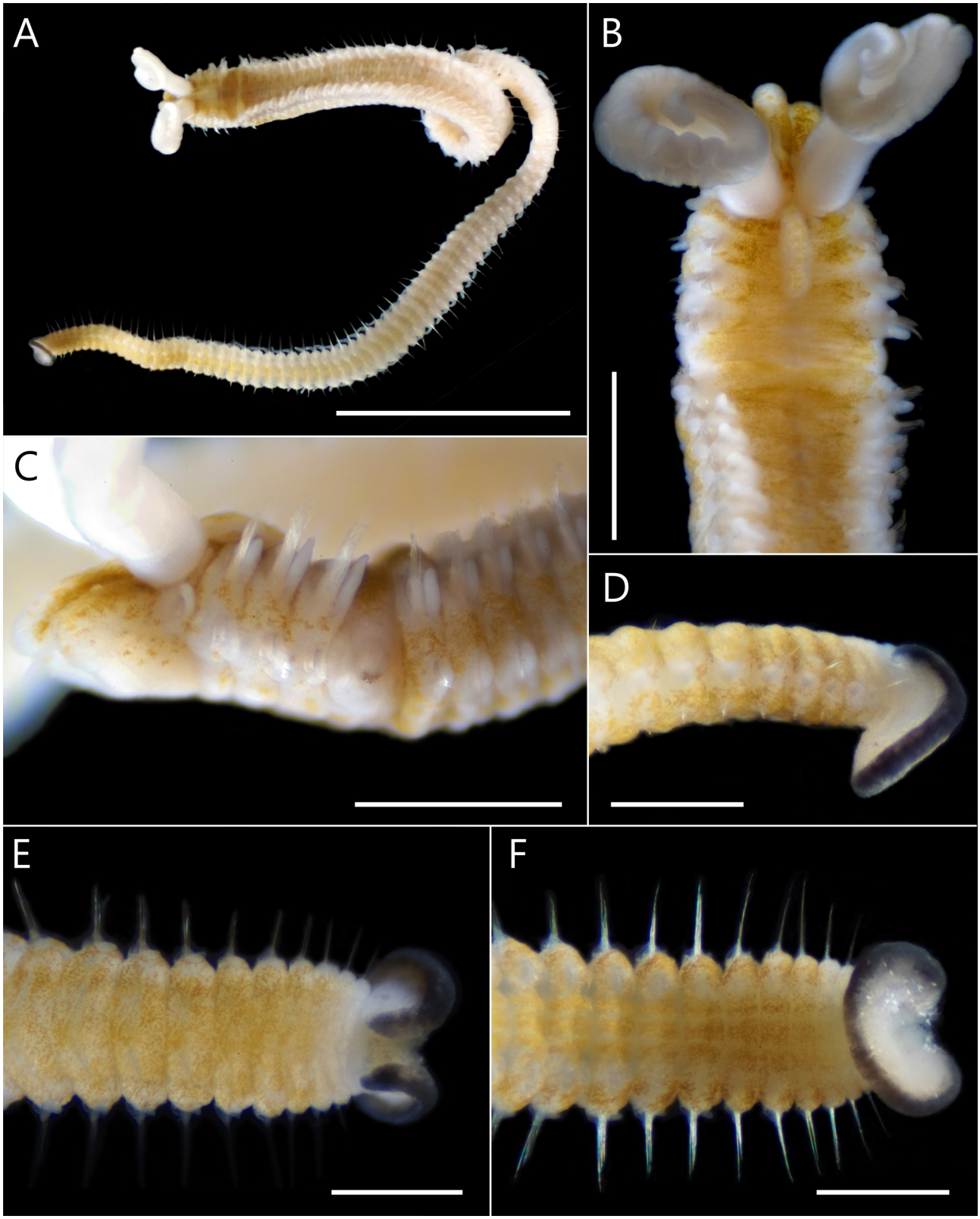

FIGURE 2. *Polydora tunicola* **sp. nov.** Light micrographs showing the morphology of preserved specimens (holotype: NSMT-Pol H-855). A: entire body. B: anterior end, dorsal view. C: anterior end, lateral view. D: posterior end, lateral view. E: posterior end, dorsal view. F: posterior end, ventral view. Scale bars: (A) = 5 mm; (B–C) = 1 mm; (D–F) = 500 μm.

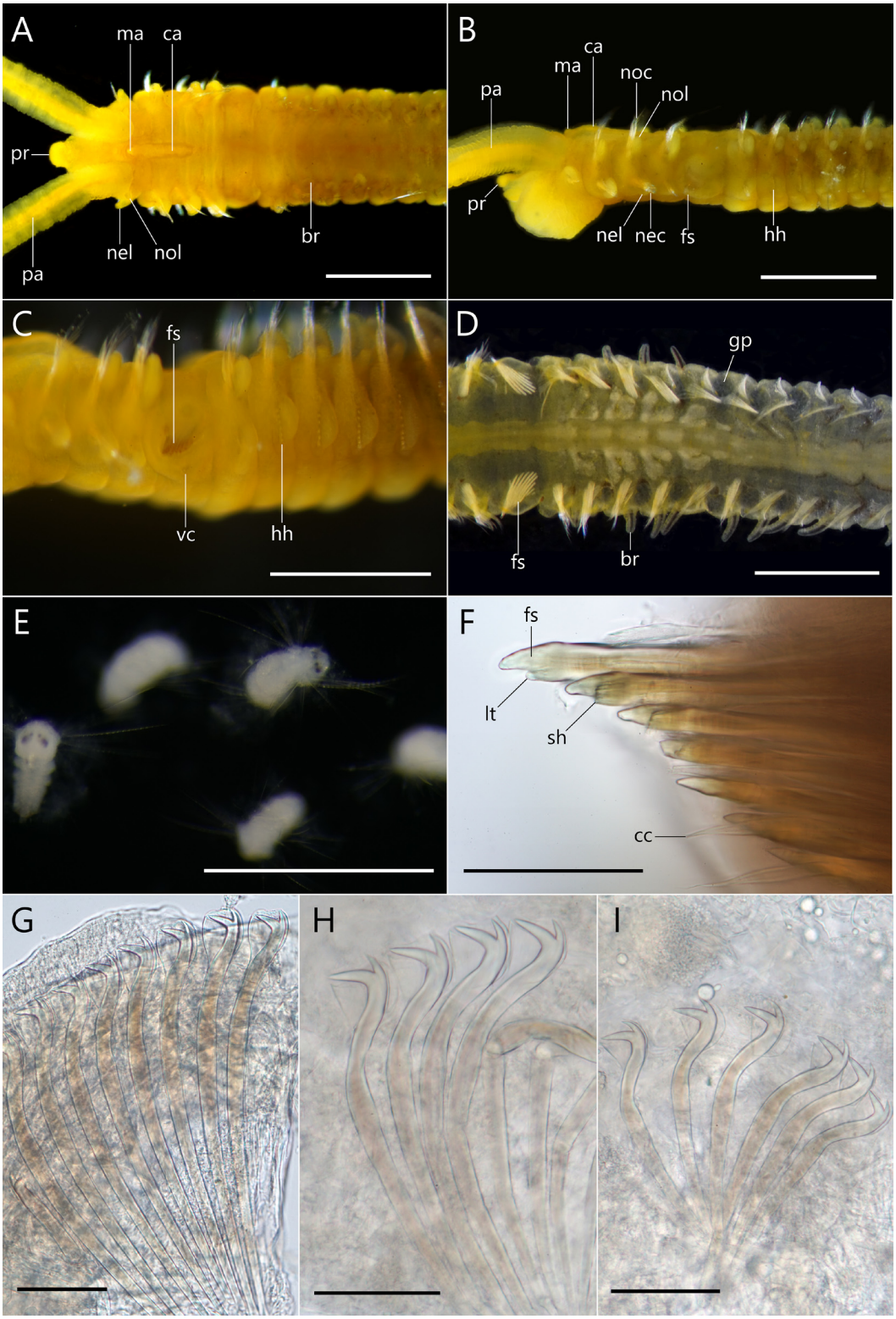

FIGURE 3. *Polydora tunicola* **sp. nov.** Light micrographs showing the morphology of living (A–D) and fixed (E–I) specimens. A–D, F–I: paratypes (NSMT-Pol P-856), E: non-type larvae. A: anterior end, dorsal view. B: anterior end, lateral view. C: 2–11 chaetigers, lateral view. D: glandular pouches in the 7th to 14th chaetigers. E: larvae from a maternal tube. F: notopodial falcate spines and companion chaetae of the 5th chaetiger. G: neuropodial hooded hooks of 7th chaetiger. H: hooded hooks from neurochaetae of 20th chaetiger. I: hooded hooks from neurochaetae of posterior (60th) chaetiger. br—branchia; ca—caruncle; cc—companion chaetae; gp—glandular pouch; hh—hooded hook; lt—lateral tooth; ma—median antenna; fs—falcate spine; nec—neuropodial capillaries; nel—neuropodial lamella; noc—notopodial capillaries; nol—notopodial lamella; pa—palp; pr—prostomium; sh—shelf; vc—ventral capillaries. Scale bars: (A–D) = 1 mm; (E) = 300 µm; (F–I) = 50 µm.

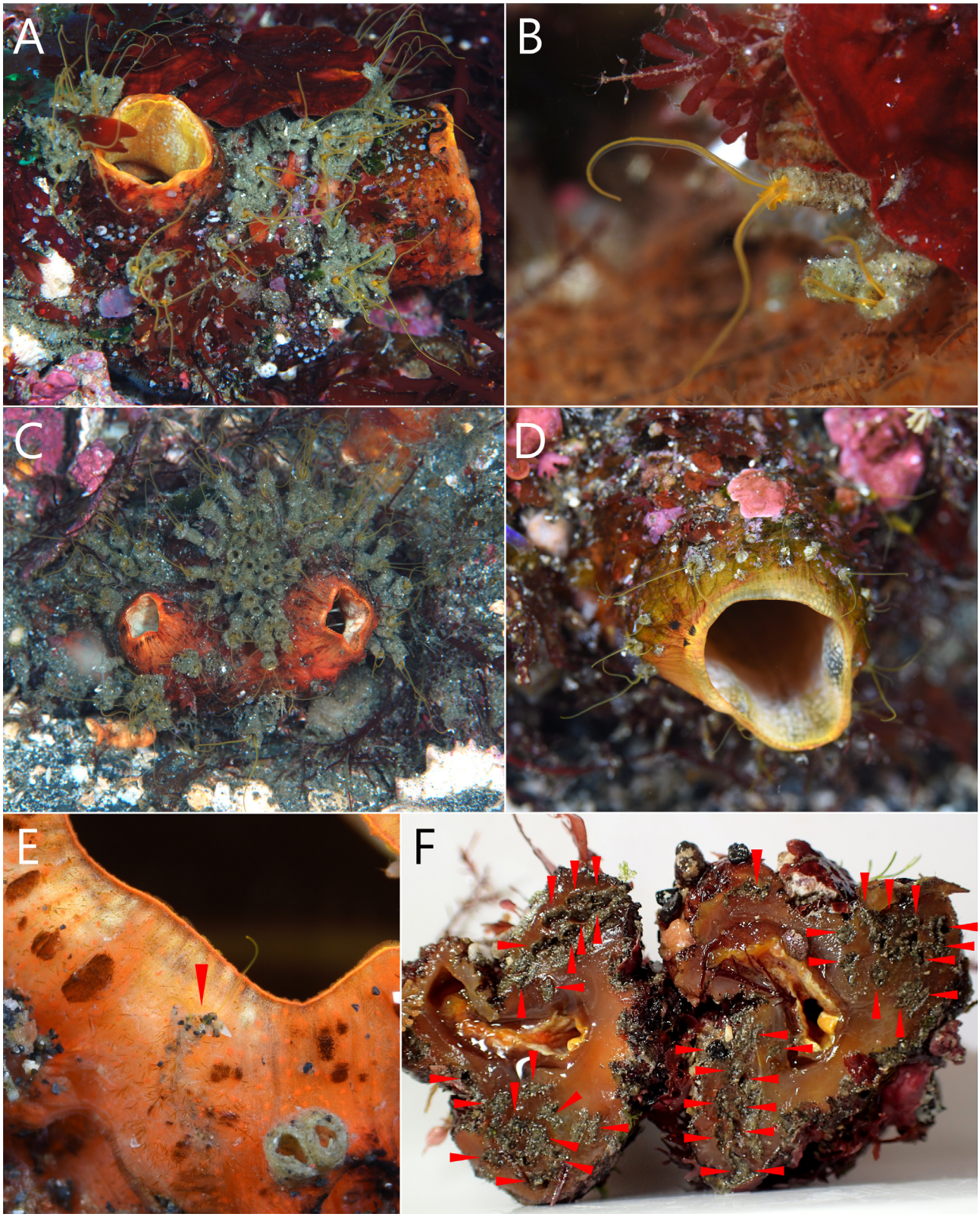

FIGURE 4. *Polydora tunicola* **sp. nov.** A: palps and mud tubes of *Polydora tunicola* **sp. nov.** protruding from their burrows in the tunics of the sessile ascidian *Polycarpa* cf. *cryptocarpa kroboja*. B: closeup of palps and mud tubes of *Polydora tunicola* **sp. nov.** C, D: tubes of *Polydora tunicola* **sp. nov.** concentrated on near siphons of the ascidians. E: juvenile of *Polydora tunicola* **sp. nov.** (arrowhead) constructing a mucous tube on the siphons of the ascidians but not yet bore into the tunic. F: cross-section of a host ascidian showing many pairs of boreholes filled with mud tubes (arrowheads) of *Polydora tunicola* **sp. nov.** in the host tunic. Photo by O. Hoshino (A–E) and H. Abe (F).

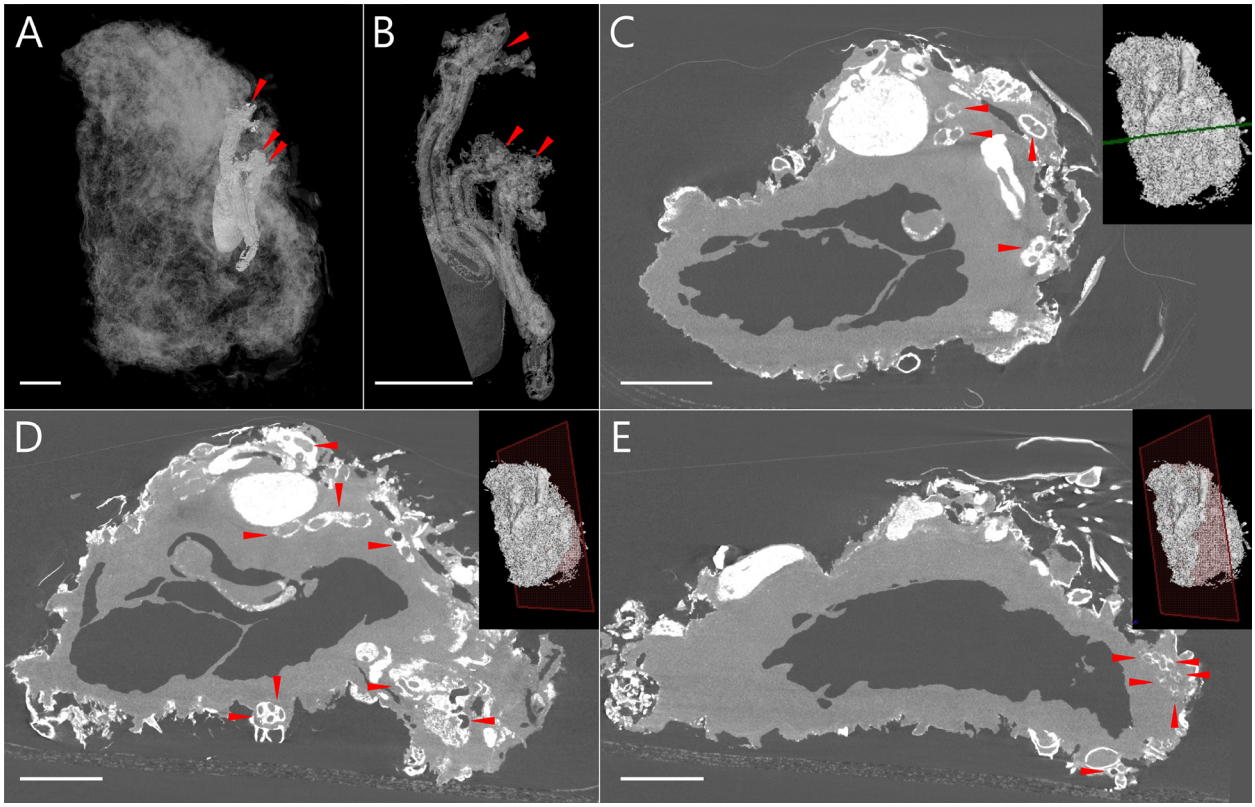

FIGURE 5. Micro-CT images of the host ascidian. A: three-dimensional (3D) reconstructed image of three U-shaped burrows of *Polydora tunicola* **sp. nov.** B: two-dimensional reconstructed images of three U-shaped burrows of *Polydora tunicola* **sp. nov.** extracted from tomographic data. Arrowheads of figures A and B indicate the apertures of the burrows. C–E: Cross-sectional image of the host. Arrowheads indicate the boreholes of *Polydora tunicola* **sp. nov.** in the ascidian tunic. Scale bars = 10 mm.

Remarks. *Polydora tunicola* **sp. nov.** is similar to *P. aura* Sato-Okoshi, 1998, *P. cornuta* Bosc, 1802, *P. fusca* Radashevsky & Hsieh, 2000, *P. glycymerica* Radashevsky, 1993, *P. latispinosa* Blake & Kudenov, 1978, *P. lingulicola* Abe & Sato-Okoshi, 2020, *P. nanomon* Orensky & Williams, 2009, *P. robi* Williams, 2000, and *P. vulgaris* Mohammad, 1972 in having a median antenna on the caruncle and chaetiger 5 without dorsal superior capillaries, but with ventral capillaries. *Polydora tunicola* **sp. nov.** can be distinguished from these similar species by combining morphological characteristics such as maximal caruncle length, number of branchial chaetigers, absence of modified posterior notochaetae, yellow body pigmentation, and unique black-rimmed flaring pygidium (Table 2). *Polydora tunicola* **sp. nov.** closely resembles *P. cornuta* in both having caruncle continued to the end of chaetiger 3, branchiae to near the end of the body, major spines of chaetiger 5 with a lateral tooth, and no modified posterior notochaetae (Table 2). However, these two species are clearly distinguished by the former having a narrow rounded or weakly incised prostomium instead of a broad bilobed prostomium, no eye spots instead of 4 widely spaced eyespots arranged in a square shape, and major spines of chaetiger 5 with lateral shelf instead of a distinct accessory tooth found in the latter species (Blake & Maciolek 1987). The bright yellow body color of *P. tunicola* **sp. nov.** in life condition (Fig. 3A–C) is similar to that of *P. aura* (Sato-Okoshi 1998; Sato-Okoshi & Abe 2012), however, these two species are distinguished by the former not having modified posterior notochaetae. The larger anterior spines are the oldest and usually most worn in *Polydora* species and therefore the lateral tooth is often a worn flange, sheath, or shelf (Blake 1969; Kudenov 1982; Bertasi 2016). The lateral shelf on the unworn newer posterior spines characterizes the major spines of the new species. *Polydora tunicola* **sp. nov.** is unique among the genus *Polydora* and the family Spionidae because they have tunic-boring symbiotic relationships with ascidians.

Etymology. The specific name *tunicola* is a combination of two Latin words, namely, tunica and -cola (= dweller), which refers to the peculiar tunic-boring habit of the species. The Japanese name Hoyano-poridora is a combination of the Japanese words hoya (=ascidian), no (case particle), and poridora (=*Polydora*), meaning "*Polydora* of the ascidian".

TABLE 2. Morphological characteristics of known *Polydora* species which have a single median antenna on the caruncle and chaetiger 5 without dorsal superior capillaries, but with ventral capillaries (based on Abe & Sato-Okoshi 2020).

Species	Prostomium	Caruncle (maximal length)	Branchiae from chaetiger 7 to	Chaetiger 5		Modified posterior notochaetae	Pygidium	Pigmentation	Life style	Reference
				Major spine	Companion chaetae					
Polydora tunicola Abe, Hoshino & Yamada, **sp. nov.**	Rounded or weakly incised	End of chaetiger 3	Near the end	Falcate with lateral tooth or shelf	Present	Absent	Flare-disc with mid-dorsal gap; rimmed with black	Yellowish pigment especially anterior and posterior chaetiger	Tunic-borer	This study
P. aura Sato-Okoshi, 1998	Incised	End of chaetiger 2–3	Near the end	Falcate with lateral flange	Present	Present	Wide flaring disc	Brown and orange pigment on body	Shell-borer	Sato-Okoshi (1998); Sato-Okoshi & Abe (2012)
P. cornuta Bosc, 1802	Bifurcated and flaring laterally	End of chaetiger 3	Near the end	Falcate with lateral tooth and subdistal flange or keel	Present	Absent	Flaring cup to disc with middorsal gap to narrow incision	Usually black spots on lateral sides of chaetigers from 7–10 to 10–19	Tube-dweller	Radashevsky (2005)
P. fusca Radashevsky & Hsieh, 2000	Incised	End of chaetiger 2	About 3/4 of body	Falcate with small lateral tooth	Present	Present	Cup-shaped with dorsal gap	Scattered black pigment on head and body	Tube-dweller	Radashevsky & Hsieh (2000)
P. glycymerica Radashevsky, 1993	Incised	Middle of chaetiger 2	Near the end	Falcate with lateral flange or sheath	Present	Absent	Disc-like with wide dorsal gap	Absent	Shell-borer	Radashevsky (1993)
P. latispinosa Blake & Kudenov, 1978	Incised	End of chaetiger 2	Continuing to posterior end	Falcate with lateral sheath or flange	present	Present	Large flaring disc with wide dorsal gap	Brown pigment along edges of caruncle	Shell-borer	Blake & Kudenov (1978)

.....continued on the next page

TABLE 2. (Continued)

Species	Prostomium	Caruncle (maximal length)	Branchiae from chaetiger 7 to	Chaetiger 5		Modified posterior notochaetae	Pygidium	Pigmentation	Life style	Reference
				Major spine	Companion chaetae					
P. lingulicola Abe & Sato-Okoshi, 2020	Incised	End of chaetiger 2–3	Near the end	Falcate with lateral flange	Present	Absent	Disc-like with middorsal gap	Absent or present (Scattered brown pigment on middle and posterior body)	*Lingula* symbiont	Abe & Sato-Okoshi (2020)
P. nanomon Orensky & Williams, 2009	Incised	End of chaetiger 2	2/3 of body	Falcate with two lateral teeth	Present	Absent	Cup-shaped	Females sometimes exhibit irregular pigmentation patches on anterior and posterior dorsal side	Shell-borer	Orensky & Williams (2009)
P. robi Williams, 2000	Rounded	End of chaetiger 2	End of body	Falcate with small lateral flange	Present	Present	With anal cirri	Absent	Shell-borer	Williams (2000)
P. vulgaris Mohammad, 1972	Incised	Middle of chaetiger 2	Near the end	Falcate with lateral flange	Present	Absent	Saucer-shaped with a dorsal notch	-	Shell-borer	Mohammad (1972); Manchenko & Radashevsky (1994)

Habitat. Five to 40 m depth of subtidal zone of rocky shores.

Distribution. Currently, it is known from Izu-Oshima Island, Sagami Sea, central Japan and Sakai fishing port, Minabe Town, Wakayama Prefecture, Japan. Additionally, although we have not examined the specimen and the locality is unknown, palps of spionids probably attributable to this species were in a photograph of *Polycarpa cryptocarpa kroboja* in a Japanese field guidebook (Nishikawa 2006).

Reproduction and larval morphology. Egg strings and egg capsules were not found. Three-chaetiger larvae were found in the host ascidians during the extraction procedure of adult specimens (Fig. 3E). All observed larvae had eye spots and long larval chaetae.

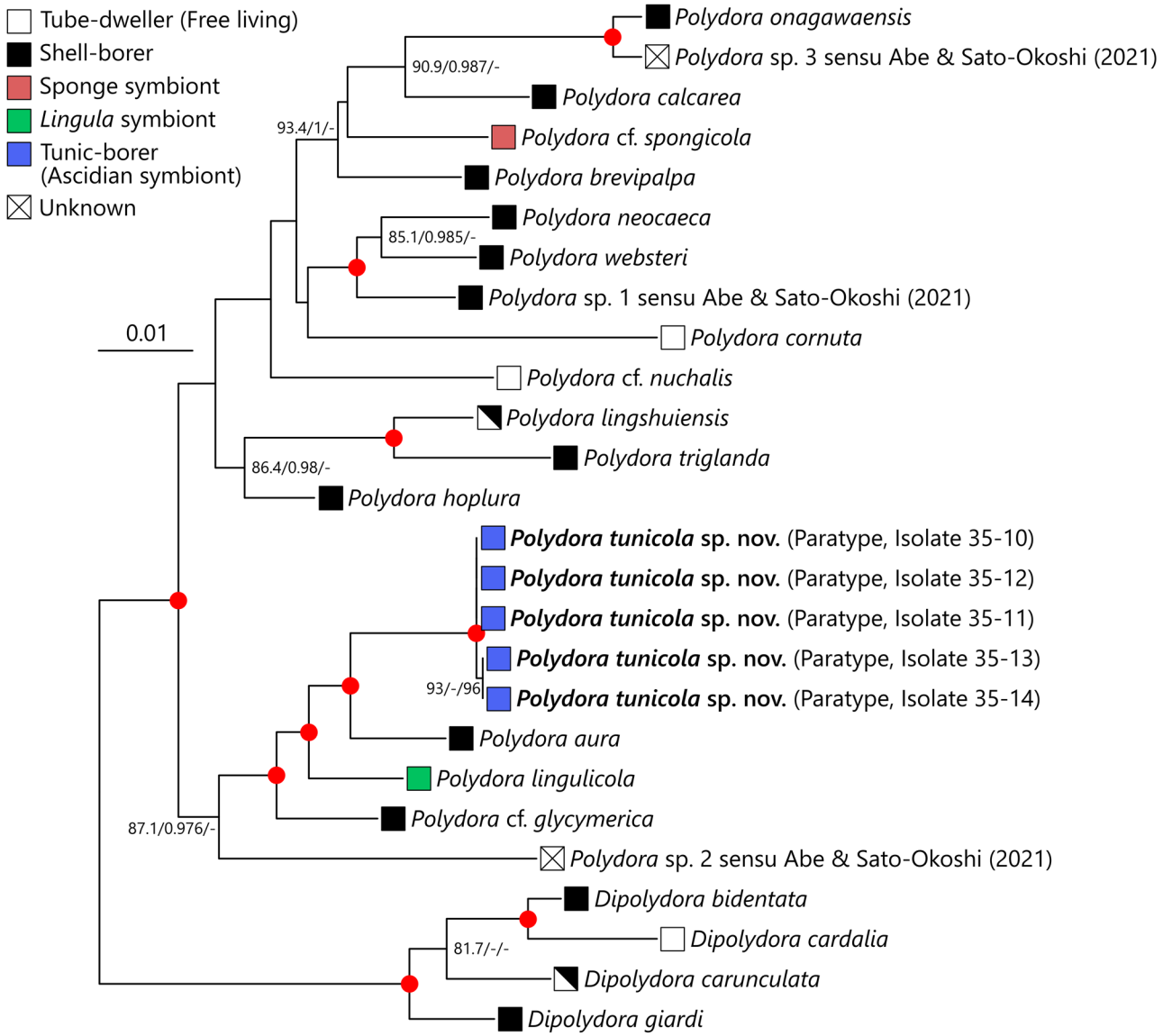

FIGURE 6. Maximum likelihood tree inferred from concatenated sequences of nuclear 18S and 28S and mitochondrial 16S rRNA gene sequences of *Polydora* species obtained in this study and from DDBJ/EMBL/GenBank database (Table 1). The gene sequences obtained in this study are highlighted in boldface. SH-aLRT/approximate Bayes support/ultrafast bootstrap support values of ≥80%/≥0.95/≥95%, respectively, are given beside the respective nodes. Nodes with red circles indicate triple high support values of SH-aLRT ≥ 80, approximate Bayes support ≥ 0.95, and ultrafast bootstrap support ≥ 95. The scale bar represents the number of substitutions per site. Sequences of *Dipolydora* species are used for outgroup rooting. The symbols at the left of the species names indicate the lifestyle based on the available information (Table 1).

Host association. Currently, *Polycarpa* cf. *cryptocarpa kroboja* and *Cnemidocarpa* sp. (Ascidiacea: Styelidae) are known as hosts. All specimens of *P. tunicola* **sp. nov.** burrow into the tunica and construct mud tubes on the host surface (Fig. 4A, B). The positions of the burrows are variable, but most worms construct burrows with the

openings near siphons of host ascidians (Fig. 4C, D). One juvenile worm, which did not bore but constructed a tube at the tip of the host siphon, was observed in December 2017 (Fig. 4E). The number of worms per host was zero to several tens of individuals. The second author (OH) has been observing marine organisms by more than 500 dives per year for more than 20 years in the sea of Izu-Oshima Island, but worms matching the new species of *Polydora* were not observed so far in other substrates including other ascidians (e.g., *Clavelina cyclus* Tokioka & Nishikawa, 1975, *Cnemidocarpa irene* (Hartmeyer, 1906), *Didemnum vexillum* Kott, 2002, *Diplosoma mitsukurii* Oka, 1892, *Polyandrocarpa misakiensis* Watanabe & Tokioka, 1972, *Polycitor proliferus* (Oka, 1933), *Pseudodistoma fragile* Tokioka, 1958, *Pyura mirabilis* (Drasche, 1884), *Rhopalaea circula* Monniot & Monniot, 2001, *Styela clava* Herdman, 1881, and Botryllinae spp.).

Anatomical (Fig. 4F) and micro-CT (Fig. 5) examination of the ascidians showed that *P. tunicola* **sp. nov.** bored into the cellulose-rich ascidian tunics and constructed a U-shaped burrow (Fig. 5A, B). In the cross-sectional images of the host, many pairs of the boreholes of *P. tunicola* **sp. nov.** were observed in the host tunica because the burrows were U-shaped and the two apertures were adjacent to each other (Fig. 5C–E). *Polydora tunicola* **sp. nov.** constructed the external mud tubes on the host surface as extensions of the two openings of the burrow (i.e., approximately twice as many external mud tubes as burrows) and the burrows were lined with mud and silt.

Molecular phylogeny. The intraspecific variation in gene sequences of five *P. tunicola* **sp. nov.** specimens were 0% (0/1749 bp) for 18S, 0%–0.13% (0–1/767 bp) for 28S, and 0%–0.42% (0–2/473 bp) for 16S rRNA. The phylogenic analyses of the concatenated sequences recovered *P. tunicola* **sp. nov.** as the sister species to *P. aura* within a well-supported clade (Fig. 6). *Polydora tunicola* **sp. nov.** and *P. aura* also formed a well-supported clade with *P. lingulicola* and *P.* cf. *glycymerica*.

Cellulase activity. The results of plate assay (Fig. 7A) and zymography (Fig. 7B) consistently detected the cellulase activities in the individuals of *P. tunicola* **sp. nov.** Zymography showed multiple forms of cellulase enzymes with molecular weights near 63 and 50 kDa.

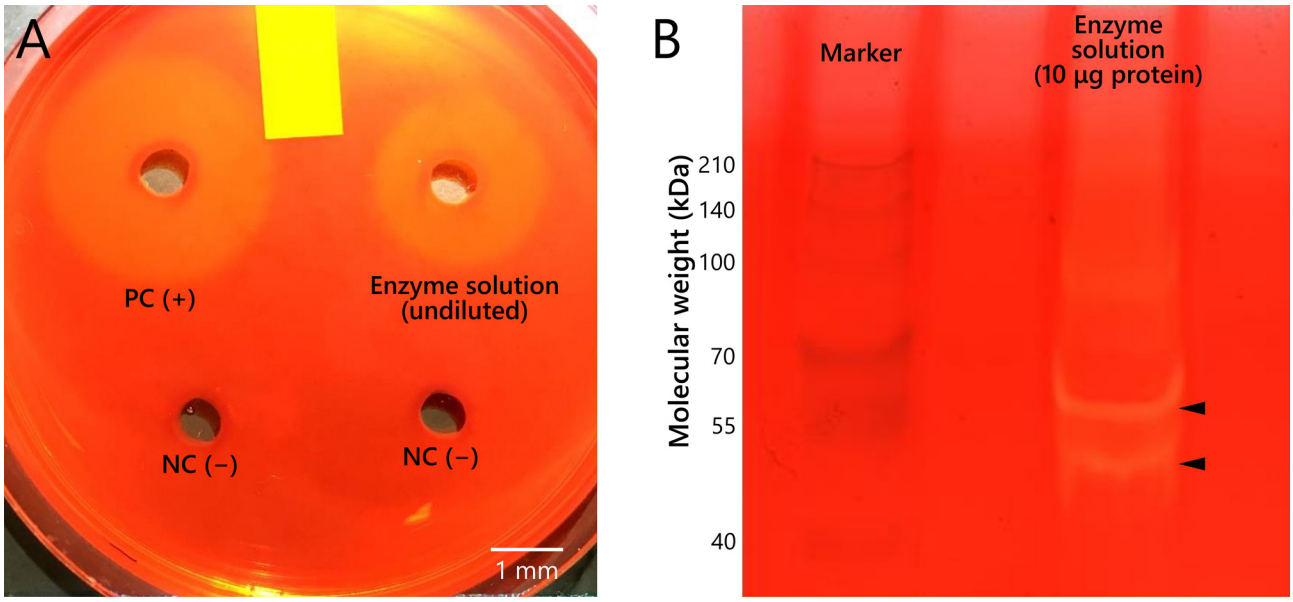

FIGURE 7. Results of plate assay (A) and zymographic analysis (B) for cellulase activity of *Polydora tunicola* **sp. nov.** Haloes (A: destained areas) and destained bands (B: arrowheads) indicate the cellulose decomposition (exhibition of cellulase activity). PC = positive control (crystalline style of a brackish water clam *Corbicula japonica*), NC = negative control.

Discussion

Many invertebrates, including amphipods, shrimps, copepods, pinnotherid crabs, nemerteans, and cnidarians, live in symbiotic associations with ascidians, and most of these animals are commensals that live in the branchial sac or ectoparasites on the respiratory organ of the host (Illg 1958; Stock 1967; Boxshall 2005; Lambert 2005; Monniot 1990; Thiel 2000; Baeza & Díaz-Valdés 2011; White 2011; Kim *et al*. 2016). However, only a few animals inhabit the ascidian tunic: mytilid mussels in the genera *Mytilimeria* and *Musculus* (Say 1822; White 1949; Lambert 2005;

Morton & Dinesen 2011; Cañete & Rocha 2013) and amphipods in the genus *Polycheria* (Skogsberg & Vansell 1928; McClintock *et al*. 2009). In annelids, *Proceraea exoryxae* is the only known polychaete tunic-borer that produces a network of burrows in the tunic of an ascidian species (Martin *et al*. 2017). *Polydora tunicola* **sp. nov.** is the first and second examples among spionids and annelids, respectively, inhabiting excavated galleries in ascidian tunics. In contrast to *Proceraea exoryxae*, which is assumed to feed on host tissues using trepan teeth, *Polydora tunicola* **sp. nov.** does not feed on host tissues because it lacks hard feeding structures. Since we have not assessed whether there is mechanical damage (or other negative impacts) to the host ascidians by the new *Polydora* species, the symbiosis cannot be fully characterized (it is likely commensalism but could be parasitic).

Micro-CT scanning showed that *Polydora tunicola* **sp. nov.** lives in a U-shaped burrow in the host ascidian tunics (Fig. 5). Although various shapes of burrows are known in the shell-boring polychaetes of the genus *Polydora* and the closely related genus *Dipolydora* (Sato-Okoshi & Nomura 1990; Sato-Okoshi 1999), they are basically U-shaped, and mud tubes protrude from the two tube openings, which communicate with the outside of the substrate (Blake & Evans 1973; Sato-Okoshi & Okoshi 1996). The shapes of the burrows of the tunic- and shell-boring *Polydora* are similar, implying a common boring process. Although the mechanism of boring into ascidian tunica is still unknown, the results of plate assay and zymography consistently detected the cellulase activities from the new species (Fig. 7). These results suggest the hypothesis that potential cellulose digestion abilities enable them to burrow into the cellulose-rich ascidian tunics. Cellulase activities were found in some marine annelids, e.g., nereidid *Alitta virens* (Sars, 1835) (Lewis & Whitney 1968, as *Nereis*), *Perinereis mictodonta* (Marenzeller, 1879) (Ito *et al*. 2011, as *P. nuntia brevicirris*), *Hediste atoka* Sato & Nakashima, 2003, *H. diadroma* Sato & Nakashima, 2003, *Tylorrhynchus osawai* (Izuka, 1903), capitellid *Heteromastus* sp. (Kanaya *et al*. 2018, 2019), and sabellid *Sabella spallanzanii* (Gmelin, 1791) (Lewis 1980, as *S. penicillus*). In particular, *P. mictodonta* has an endogenous cellulase gene coding endo-b-1,4-glucanase of 48.6 kDa (Ito *et al*. 2011), with a similar molecular weight as the cellulase of the lower band in this zymography (Fig. 7B). Whether cellulases are used for tunic boring and endogenous or originated from symbiotic microorganisms needs clarification in future studies.

Polydora tunicola **sp. nov.** is often densely concentrated near the ascidian siphons (Fig. 4C, D), suggesting that they gain some benefit from water currents generated by the ascidian filter-feeding activities. Considering that most spionid polychaetes, including *Polydora*, are interface feeders that can switch between suspension and deposit-feeding mode (Jumars *et al*. 2015), *P. tunicola* **sp. nov.** could take advantage of particulate fluxes by suspension feeding of the host ascidians. Some shell-boring polydorid species open their burrows around the siphons of host mollusks and use the water currents created by the host to catch suspended particles using their palps (Radashevsky 1993). Similarly, recently described brachiopod-associated non-boring *Polydora* species construct their tubes near the host lateral pseudosiphons, taking advantage of particulate fluxes in the inhalant current created by the host to collect suspended food particles (Abe & Sato-Okoshi 2020). Ascidians have two siphons: inhalant and exhalant siphons (Petersen 2007). Because *P. tunicola* **sp. nov.** was usually equivalently dense in both siphons (Fig. 4A, C), there seemed to be no tendency to prefer one or the other of these siphons. Therefore, this suggests that the new species is not kleptoparasitic but rather merely uses the water currents created by the filter-feeding host for their feeding activity. The concentration of *P. tunicola* **sp. nov.** near the host's siphons was presumably due to the selective settlement of the larvae. The discovery of an early juvenile of this species just after settlement and before starting boring near siphons of the host supports this assumption (Fig. 4E). Similar to the shell-boring polydorids (Blake & Evans 1973), they seem to first construct silty tubes of the host surface and then start boring into the tunic of ascidians. Three-chaetiger larvae with long larval chaetae found within the burrows of the maternal worms (Fig. 3E) and the absence of nurse eggs suggest that the species has a long-term planktonic larval development because these characteristics are typical for species with such larval development in the *Polydora* complex (Blake & Arnofsky 1999).

Polydora tunicola **sp. nov.** was not observed from other than *Polycarpa* and *Cnemidocarpa*, suggesting it has an obligate symbiotic association with styelid ascidians. Because the substrate must be sufficiently thick for burrowing, it appears that the thick tunics of both host species (c.a. 5–35 mm thick: Fig. 4F) allow *P. tunicola* **sp. nov.** to inhabit them (Fig. 3F). However, as other ascidian species have thicker tunics, further studies on whether *P. tunicola* **sp. nov.** is an exclusive symbiont of *Polycarpa* and *Cnemidocarpa* or also infests other ascidians is necessary. Additionally, because the tunics of both hosts are structurally tough and may contain various antipredatory chemicals typical for ascidian tunic (Stoecker 1980; Hirose *et al.* 2001; Pisut & Pawlik 2002; Joullié *et al*. 2003; Odate & Pawlik 2007; Koplovitz *et al*. 2009), the host ascidian provides a stable and safe habitat for the *Polydora* species. Generally, the life span in ascidians is short, between 6 months to 3 years, but some ascidians have a much

longer life span over four years, especially the cold-water solitary species (Millar 1971; McClintock *et al*. 1991). The life spans of the host ascidians are unknown, but field observations suggest that they might live for more than three years. Since the lifespan of *Polydora* species is usually less than one year (Zajac 1991) and several years at most (Sato-Okoshi et al. 1990; Dualan & Williams 2011; Teramoto et al. 2013), it is likely that the host ascidian has a lifespan long enough for completion of the life cycle of the new *Polydora* species.

Species within the genus *Polydora* have been known to use invertebrates from diverse phyla as hosts, and ascidians are now newly added to the list as hosts. The discovery of the new tunic-boring species in this study leads to questions including how *P. tunicola* **sp. nov.** its lifestyle evolved, in contrast to many members of the genus *Polydora* that are shell-boring species. In general, hosts used by symbiotic organisms are conserved and switching to distantly related hosts among closely related symbiont species is infrequent (e.g., Hoberg & Brooks 2008; Spencer 2009; Gómez *et al*. 2010; Lanterbecq *et al*. 2010). However, marine invertebrate symbionts have been reported to often switch between closely related species to distantly related hosts. In particular, this is predicted for species with inquilinism, i.e., the symbionts are not directly dependent on the host for nutrition and rarely cause harm to the host, allowing the symbionts to switch hosts without major adaptation to host physiology (Goto *et al.* 2012; Kou *et al*. 2015; Horká *et al*. 2016). This may be the case for the new *Polydora* species, which does not feed on the host tissue but indirectly benefits from the water currents created by the host for its interface feeding. However, the cellulase activity of *P. tunicola* **sp. nov.** and its unique position on the host suggests their symbiotic relationship is commensal in nature and indicate a long shared evolutionary history with the host. The results of our molecular phylogenetic analysis showed that the new species forms a clade with the shell-boring species, *P.* cf. *glycymerica* and *P. aura*, and the recently described brachiopod-associated non-boring species *P. lingulicola*. Our study suggests that this clade has an ecological specialization mostly positioned in a certain area of the host with a strong tendency to use the water current created by the host as reported in *P. lingulicola*, *P.* cf. *glycymerica*, and the new species (Radashevsky 1993; Abe & Sato-Okoshi 2020; This study).

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