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Polysaccharide hydrolase of the hadal zone amphipods *Hirondellea gigas*

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ABSTRACT

Hirondellea species are common inhabitants in the hadal region deeper than 7,000 m. We found that *Hirondellea gigas* thrived in the Challenger Deep possessed polysaccharide hydrolases as digestive enzymes. To obtain various enzymes of other *H. gigas*, we captured amphipods from the Japan Trench, and Izu-Ogasawara (Bonin) Trench. A phylogenetic analysis based on the cytochrome oxidase I gene showed close relationships among amphipods, despite the geographic distance between the localities. However, several differences in enzymatic properties were observed in these *H. gigas* specimens. We also carried out RNA sequencing of *H. gigas* from the Izu-Ogasawara Trench. The cellulase gene of *H. gigas* was highly homologous to cellobiohydrolase of Glucosyl Hydrolase family 7 (GH7). On the other hand, enzymatic properties of *H. gigas*'s cellulase were different from those of typical GH7 cellobiohydrolase. Thus, these results indicate that hadal-zone amphipod can be good candidates as the new enzyme resource.

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Amphipods are one of the most common invertebrates in aquatic environments. In marine environments, they widely inhabit from the seashore to the seafloor, and they are scavengers in the ecological community [1–4]. *Hirondellea* species are residents of the hadal zone, which occurs at depths of approximately 7,000–10,000 m [5–11]. *Hirondellea gigas* specimens were collected from the Challenger Deep in the Mariana Trench and Philippine Trench [8,12]. The species name "*gigas*" refers to the organism's distinctively large body size among amphipods. The hadal zone of the deep sea, which is approximately 10,000 m deep, is not hospitable to many life forms because of the high pressure and poor nutrients [6,13–16]. A low number of available organisms can cause serious problems for scavenger species, and even bacteria, which are potential food sources for benthic organisms in the hadal zone, do not easily grow in this region [17–20]. Thus, it remains unclear how the benthic scavenger *H. gigas* can survive under high pressure and grow so large in the oligotrophic environment.

We have reported that *H. gigas* captured from the Challenger Deep has a series of polysaccharide hydrolases as digestive enzyme, and obtained nutrient from plant debris [12]. Especially, the cellulase of *H. gigas* (abbreviated as HGcel) has a novel enzyme to produce glucose and cellobiose from cellulose. This unique property showed the applicability of the enzymes for the saccharification of cellulose. In addition, the low temperature for enzyme reaction was expected to be economical operation of the industrial plant. These polysaccharide hydrolases are mainly used in the food industry, as well as chemical industry for bioethanol production. The deep-sea organisms have a possibility of valuable resource for novel industrial enzymes. In fact, there are a number of examples of animals that have utilized cellulases and hemicellulose hydrolases from symbiotic bacteria, fungi or protozoa as digestive enzymes [21–23]. Plant and wooden debris have been found in deep-sea and hadal trenches [24,25]. Plant debris is an important nutrient for various deep-sea animals living at depths of approximately 3,000 m [26].

With respect to industrial application of the enzyme, the diversity of enzyme properties is very important for appropriate utilization corresponding to the reaction process [27]. Actually, because the bacterial or fungal enzymes have various properties in reaction temperature, pH, substrate, and products, they are widely used in food and medical industries. To expand utilization of enzymes and construct new process in industry, new resource of enzymes is always needed. In this study, we focused the deep-sea animal as a resource for new enzymes. We captured the deep-sea amphipods from

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the Izu-Ogasawara Trench and the Japan Trench, and report industrially applicable polysaccharide hydrolases from the deep-sea amphipods from the Izu-Ogasawara Trench and the Japan Trench.

Materials and methods

Capturing deep-sea amphipods

We lowered an 11,000-m class free-fall lander that was attached to four baited traps containing a slice of mackerel into the hadal zone at a depth of over 9,000 m in the Izu-Ogasawara Trench (IO) (32°12.5766 N, 142°08.0411E, depth: 9,450 m) on 2 July 2013. After 6.5 h, we surfaced the lander, gathered the deep-sea animals (all amphipods) from the baited traps and stored them in an −80 °C deep freezer or liquid nitrogen. We also lowered another deep-sea lander that was attached to five baited traps containing a slice of mackerel into the Japan Trench (J) (34°00.2461 N, 142°00.0697E; depth = 9,255 m) on June 17–18, 2010. We captured more than 7,000 individuals during an approximately overnight period on the seafloor. All of the deep-sea animals captured in the baited traps were amphipods, and they were stored in a -30 °C freezer. We also captured 185 amphipods from the Challenger Deep in the Mariana Trench (M) (11°22.11 N, 142°25.86E, depth of 10,897 m) on September 10, 2009 [12]. The distance between the sampling points in the J and IO was 199.407 km. The Challenger Deep is 2308.07 km south of the sampling point in the IO (Figure 1).

In this study, our sampling points in IO and J were included in Japan's exclusive economic zones. No permission is required in capturing deep-sea animals in the scientific research for non-profit institute.

Preparation of crushed amphipod samples

We crushed amphipods using the same methods as described in a previous report [12] for enzyme experiments and measurement of glucose content. The amphipods were freeze-dried, finely cut with dissection scissors, and crushed with BioMasher II (Nippi Inc., Tokyo, Japan). The enzymes or glucose were extracted with ice-cold deionized distilled water (DDW).

Amphipod glucose content

We measured the glucose content of the amphipods using the methods described in a previous report [12]. Five specimens were crushed, and then extracted three times with 1 mL distilled water. After removing the insoluble particles by centrifugation (20,400 × *g*, at 4 °C for 10 min), the extract was centrifuged using a 10-kDa cut-off Microcon centrifugal filter device (Millipore Co., Billerica, MA) to remove enzymes and other high molecular weight components. The glucose content was then measured using the Glucose CII Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Detection of hydrolytic enzymes in the amphipods

We crushed individual amphipods in 0.5 mL deionized distilled water (DDW) with the same methods as described above. After mixing with a vortex mixer, we plated 50 µL crushed amphipod samples on an agar plate containing a substrate for hydrolytic enzymes. We used 0.5% (w/v) starch azure (Sigma-Aldrich Co., St. Louis, MO, USA) for amylase and 0.1% (w/v) glucomannan (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Azo-CM-Cellulose (Megazyme, Wicklow, Ireland) for cellulase. We observed halo formation around the spots after incubation for 24 h at 30 °C. Crushed *H. gigas* was used as a positive control.

Measurement of hydrolytic enzyme activity in amphipod extracts

The enzymes from the crushed *H. gigas* were extracted three times with 0.5 mL distilled water (4 °C). All of the enzymatic activities were measured at 30 °C. Amylase activity was detected using iodine after incubating the extract with 1% (w/v) soluble starch at pH 5.6. One unit was defined as the amount of extract that hydrolyzed soluble starch to cause a 1% decrease in the absorbance at 620 nm within 1 min. Cellulase activity was measured with a cellulase assay kit with 1% (w/v) Azo-CM-Cellulose (Megazyme, Wicklow, Ireland). One milli-unit was defined with a following equation according to the manufacturer's protocol using *Tricoderma* sp. *endo*-cellulase as standard.

$$1\ \mathrm{mU} = 412.5 \times \delta\mathrm{A590} - 6.0$$

The cellulase activity of the extracts during HGcel purification was measured as the amount of glucose produced from carboxymethyl cellulose (CMC). One unit of cellulase activity was defined as the amount required to hydrolyze enough CMC to produce 1 µmol glucose within one minute. Mannanase activity was measured as the amount of reducing sugar detected by a dinitrosalicylic acid (DNS) assay after reacting with 0.2% (w/v) glucomannan at pH 5.6 [28]. One unit was defined as the amount of extract required to hydrolyze glucomannan to produce 1 µmol reducing sugar within 1minute. Alpha-glycosidase activity was measured as the amount of glucose produced with 1% (w/v) maltose at pH 5.6. One unit was defined as the amount required to hydrolyze maltose to produce 1 µmol glucose within one minute at pH 5.6. β-glucosidase activity was measured as the amount of glucose produced with 0.5% (w/v) cellobiose at pH 5.6. One unit was defined as the amount required to hydrolyze maltose to produce 1 µmol glucose within 1 min at pH 5.6. All of the enzymatic activities were calculated based on the protein content of the sample solutions. The protein content was measured by the Bradford assay using bovine serum albumin as the standard [29].

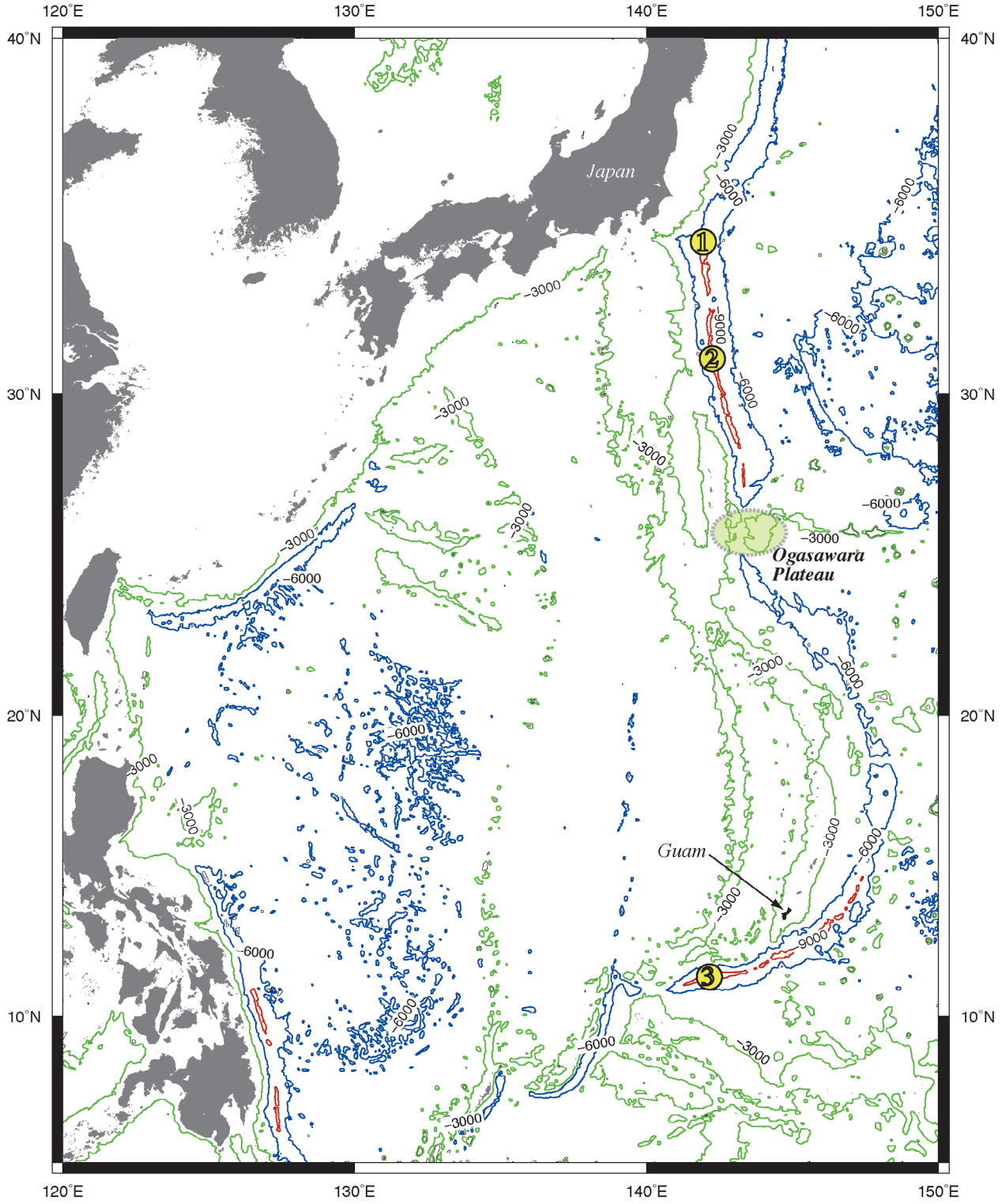

Figure 1. Sampling locations of amphipods from the hadal zone. Light blue, dark blue, and dark red lines represent 3000, 6000, and 9000 m depths, respectively. (1) Japan Trench; (2) Izu-Ogasawara Trench; 3, Challenger Deep in the Mariana Trench [12]. Light green zone indicated the Ogasawara Plateau. We modified the map created by GMT program (https://www.soest.hawaii.edu/gmt/).

Thin-layer chromatography (TLC) analysis of the enzyme reaction products

CMC (1.0% w/v) or cellobiose was incubated with amphipod extracts at 35 °C in a 50 mM sodium acetate buffer (pH 5.6). Samples were collected at intervals and boiled for 5 min, and the products were analyzed using TLC with a butanol/acetic acid/water (2:1:1 v/v/v) solvent. All spots were detected by heating to 105 °C after spraying with 40% H_2SO_4.

Cellulase purification

(1) *The amphipod from the Izu-Ogasawara Trench*
Cellulase was purified from a pool of 20 amphipod specimens, which were crushed on ice and centrifuged (1,000 × *g* for 10 min at 4 °C). The supernatant was collected, and then 10 mL ice-cold DDW was added and the sample was vortexed, with the supernatant collected again after centrifugation (1,000 × *g* for 10 min at 4 °C).

This step was repeated twice. We adjusted the pH of the total extraction to 8.6 by adding 0.5 M Tris-HCl buffer (pH 8.6) and then 5 mL of Toyopearl DEAE-650 M anion exchange resin (TOSOH Co., Tokyo, Japan) for adherence to the cellulase. After the extract was mixed at 4 °C for 30 min, it was centrifuged (8,000 × *g* for 30 min at 4 °C), and the resin was collected and washed with 10 mL 10 mM Tri-HCl buffer (pH 8.6). The resin adhering cellulase was suspended in 4 mL 10 mM Tri-HCl buffer (pH 8.6) containing 0.4 M NaCl, and the supernatant containing cellulase was collected after centrifugation (1,000 × *g* for 10 min at 4 °C). This step was repeated twice. An 8-mL aliquot was desalted and concentrated to 50 µL using Amicon Ultra 50 K columns (Millipore Co.). After precipitating the cellulase by adding ammonium sulfate to a final concentration of 40% (saturation), the precipitate with cellulase was suspended in 20 mM sodium phosphate buffer (pH 6.8) containing 20% (saturation) ammonium sulfate. The cellulase solution was transferred into a butyl-Toyopearl hydrophobic interaction column (TOSOH Co.) (20 mM sodium phosphate buffer (pH 6.8)). The column was eluted with buffer containing a linear gradient of 20–0% (saturation) ammonium sulfate. The cellulase activity was measured based on the amount of glucose produced after reacting with 1% (w/v) CMC (pH 5.6). Then, we collected the cellulase-containing fractions and performed DEAE-Toyopearl column chromatography (TOSOH Co.) (10 mM Tris-HCl (pH 8.6)) with a gradient of 0.1–0.5 M of NaCl after desalting. We collected, desalted and concentrated the cellulase-containing fractions to 200 µL, and cellulase activity was detected in the 0.5 M-NaCl fractions (Figure S1). The buffers used for the measurement of pH dependency of enzyme reaction were 50 mM sodium acetate buffer (pH 4.4, 5.0, 5.6), 50 mM sodium phosphate buffer (pH 6.2, 6.8, 7.4), and 50 mM Tris-HCl buffer (pH 8.0).

(2) *SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)*

We used 5–20% acrylamide gradient gel (Wako, Osaka, Japan) for SDS-PAGE of the proteins. SDS-PAGE was carried out with the molecular marker of Precision Plus Protein Dual color Standard (Bio-Rad, Hercules, CA). Proteins were stained with 2D Silver Stain Reagent II (Cosmo Bio Co. Ltd., Tokyo, Japan)

(3) *The amphipod from the Japan Trench*

Cellulase was purified from a pool of 65 amphipod specimens from the Japan Trench, which were crushed on ice and centrifuged (1,000 × *g* for 10 min at 4 °C). The supernatant was collected, and then 10 mL ice-cold DDW was added and the sample was vortexed, with the supernatant collected again after centrifugation (1,000 × *g* for 10 min at 4 °C). This step was repeated twice. We adjusted the pH of the total extraction to 8.6 by adding 0.5 M Tris-HCl buffer (pH 8.6) and then 5 mL of DEAE-Toyopearl 650 M anion exchange resin (TOSOH Co., Tokyo, Japan) for adherence to the cellulase. After the extract was mixed at 4 °C for 30 min, it was centrifuged (8,000 × *g* for 30 min at 4 °C), and the resin was collected and washed with 10 mL 10 mM Tri-HCl buffer (pH 8.6). The resin adhering cellulase was suspended in 4 mL 10 mM Tri-HCl buffer (pH 8.6) containing 0.4 M NaCl, and the supernatant containing cellulase was collected after centrifugation (1,000 × *g* for 10 min at 4 °C). This step was repeated twice. An 8-mL aliquot was desalted and concentrated to 50 µL using Amicon Ultra 50 K columns (Millipore Co.), and then transferred onto a DEAE-TOYOPEARL anion exchange column (TOSOH Co., Tokyo, Japan) (10 mM Tris-HCl (pH 8.6)). The column was eluted sequentially with buffer containing 0.05 M steps of 0.2–0.5 M NaCl. The cellulase activities were found in 0.4 M NaCl fractions.

DNA and RNA isolation from amphipods

We isolated DNA fraction from liquid N_2 frozen amphipods using the DNAiso solution (TAKARA, Bio Inc., Otsu, Japan) according to prescribed method for animal genome isolation. We isolated total RNA fraction from liquid N_2 frozen amphipods using the RNeasy lipid tissue kit (QIAGEN, Hilden, Germany) according to prescribed method. The isolated DNA and RNA were checked with the agarose gel electrophoresis, and discarded in the case of clearly digested pattern.

PCR amplification and sequencing

The cytochrome oxidase I gene was amplified by PCR using the universal primer pair LCO1490 (5′ – GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCA AAAAATCA-3′)[30]. PCR amplification with a 25 µL reaction volume was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) with Speedstar-HS DNA polymerase (Takara Bio Inc., Otsu, Japan) and the buffer supplied with the enzyme. The PCR conditions were as follows: an initial incubation at 96 °C for 30 s, 30 cycles of 98 °C for 5 s, an incubation at 55 °C for 10 s, another incubation at 72 °C for 15 s, and a final extension at 72 °C for 2 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel, purified using Exo-SAP digestion with Exonuclease I (USB Corp., Cleveland, OH, USA) and shrimp alkaline phosphatase (SAP) (Promega, Fitchburg, WI, USA) at 37 °C for 20 min and then treated at 80 °C for 30 min to inactivate the enzymes. The PCR products were sequenced using the primers described above and DYEnamic ET Dye Terminator reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA) on a MegaBACE 1000 (Amersham Biosciences, Piscataway, NJ, USA) automatic sequencer. The nucleotide sequences were

trimmed, assembled, and translated using Sequencher 3.7 software (Gene Codes Corp., Ann Arbor, MI, USA).

RNA sequencing

To check the quality of purified total RNA, we analyzed RNA size distribution of purified total RNA using Agilent RNA 6000 Nano assay kit (Agilent). We prepared mRNA-seq library DNA with purified 2.4 μg total RNA using a commercial kit (KAPA Stranded mRNA-Seq Kits; KAPA Biosystems) and Y-shaped sequence adaptor (SeqCap Adapter Kit A; Roche) in accordance with the manufacturer's protocol. To prepare long insert DNA, we fragmented purified poly-A selected RNA at 85 °C for 6 min. Before PCR enrichment, we determined optimal number of cycle in PCR enrichment using qPCR system (KAPA Real-Time Library Amplification Kit; KAPA Biosystems). Subsequently, we amplified sequence library DNA with 5 cycles of PCR enrichment. Prepared sequence library DNA was analyzed by using Agilent High Sensitivity DNA Kit (Agilent). The Sequence library DNA was analyzed using a massively parallel sequencer (HiSeq2500; Illumina). By using CLC Genomics Workbench 9.0.1, we trimmed adaptor sequence. Subsequently, we performed a *de novo* assembly of RNA-seq data with Trinity (v. 2.0.6) [31]. Resulted sequences of contigs were used for subsequent analysis.

Phylogenetic and diversity analysis of Hirondellea gigas

The nucleotide sequences of the cytochrome oxidase I (COI) of all amphipod specimens J, IO and M caught in the northwest Pacific Ocean were closely related to known sequences of *Hirondellea gigas* on a preliminary phylogenetic affiliation by the blastn [32]. *H. gigas* and some related species in the genus *Hirondellea* are recently reported to be monophyletic [33]. Therefore, we investigated the phylogenetic placements of amphipod specimens J, IO and M among the genus *Hirondellea*. The COI nucleotide sequences of amphipods J, IO and M were aligned by eyes with those of *H. brevicaudata*, *H. dubia*, *H. gigas*, *Uristes* sp. and *H. wagneri* as outgroup. No ambiguously aligned regions were presented in the alignment. Phylogenetic trees were calculated with the PhyML algorithm based on a selected best substitution model, implemented in TOPALi package ver. 2.5 [34]. The statistical robustness of the analysis was estimated by bootstrapping with 1,000 replicates and Bayesian posterior probabilities by MrBayes method [35] with the following parameters: number of runs: 4, number of generations: 5 million, sample frequency: 10, burn in 25%.

Phylogenetic analysis of polysaccharide hydrolases

We pick out polysaccharide hydrolase genes from RNA sequences of *H. gigas* IO using blastx. We also selected the related polysaccharide hydrolase genes of other species, which showed high homology (E value < 10^{-50}) for phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman model [36]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 [37].

Data accession number

The RNA sequence data obtained from RNAseq of *H. gigas* (IO) has been deposited in the DDBJ (accession numbers IACF01000001-IACF01008777).

Results

Phylogenetic analysis of Hirondellea species

To estimate the evolutionary relationship among the amphipods J, IO and M, and the *Hirondellea* species, we made the maximum-likelihood tree based on COI nucleotide sequences (Figure 2). Topologies of PhyML tree and Bayesian tree were almost identical and did not contradict to those by Ritchie H et al. [33]. As predicted by the high similarities on blastn search, three amphipods J, IO and M were placed into a clade of *Hirondellea gigas*. Their positions strongly suggest the amphipods J, IO and M to be members of *Hirondellea gigas*. The Mariana specimen (M) of *H. gigas* was distant from the other two specimens (IO and J) captured in Izu-Ogasawara Trench and Japan Trench, which were closely related to the specimens of *H. gigas* previously collected near Japan. The phylogenetic variation among *H. gigas* seemed to be associated with their geographical divergence. Morphological variation between *H. gigas* collected in Mariana, Palau, and Philippine Trenches was once reported [38]. In this study, we cannot compare the morphological characters between the J, IO and M due to an insufficient numbers of specimens.

Amphipod polysaccharide hydrolase

Four specimens were crushed and assayed to determine their digestive enzyme activity by observing halo formation on agar plates containing various plant polysaccharides. *H. gigas* from the M contained amylase, cellulase, mannanase, and xylanase, and it was used as a control [12]. We observed halos around all spotted

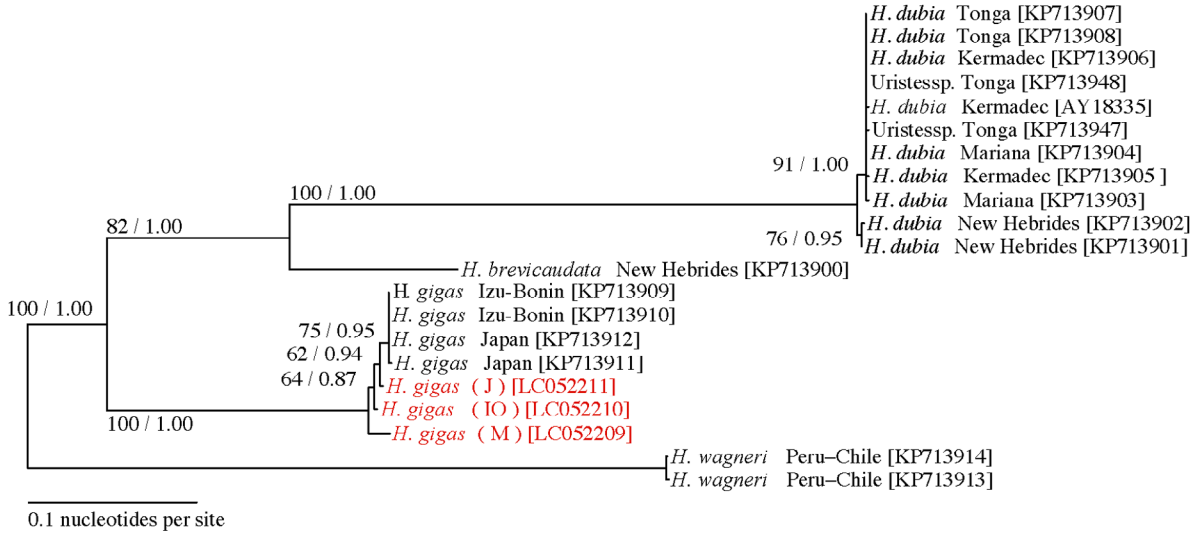

Figure 2. Phylogenetic tree of *Hirodellea gigas* and related amphipods reconstructed using the MTRev + G model from mitochondrial COI protein sequence alignment (223 amino acids). The amino acid sequences of *H. gigas* (M), (J), and (IO) are indicated in red. Bootstrapping values (> 60%) and Posterior probabilities (>95%) as inferred by maximum likelihood and Bayesian inference were shown above branches. Accession number of the gene was followed after the species name. The scale bar for the branch length is given as the estimated number of amino acid substitution per site.

samples on the plates that contained starch, carboxymethyl cellulose (CMC), and glucomannan (Figure 3). We measured enzyme activities of the polysaccharide hydrolase, α-glucosidase, and β-glucosidase in 5 randomly selected individuals. *H. gigas* from the J and IO produced β-glucosidase, which was not detected in *H. gigas* from M. The mannanase and α-glucosidase activity of *H. gigas* from the J and IO were higher than those from M, and the enzyme activity decreased with increased distance from land. However, the cellulase activity of *H. gigas* from the J and IO were approximately 6% of the activity of *H. gigas* from M (Table 1). The average glucose content, which is the digestive product of polysaccharide hydrolase, was around 0.4%(w/w) among the amphipods captured from the three hadal zones (Table 2).

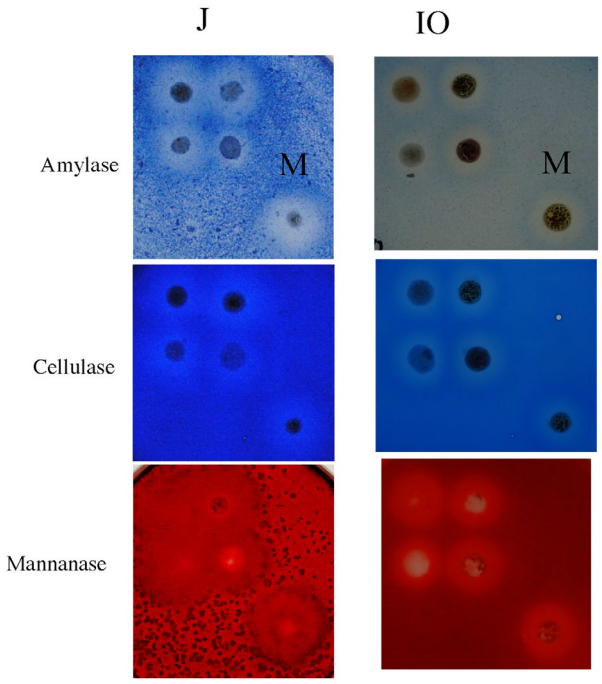

Figure 3. Polysaccharide hydrolase activities of the amphipods captured from the hadal zone. Digestive enzyme activities were assessed by halo formation on agar plates containing starch azure (amylase), Azo-CMC (cellulase), and glucomannan (mannanase). The halos produced by the amylase and cellulase activities were visualized directly, whereas the halos resulting from mannanase were detected after staining with 0.5% Congo red followed by washing with DDW. Four individuals were randomly selected from the Japan Trench and the Izu-Ogasawara Trench. *H. gigas* from the Challenger Deep was used as the positive control.

Table 1. Total digestive enzymatic activities detected in the amphipods' whole-body extracts[1].

Habitat	Amylase (mU)	Cellulase (mU)	Mannanase (μU)	AGL[2] (μU)	BGL[3] (μU)
J	83.4 ± 9.52*	0.15 ± 0.15*	111 ± 58.9**	57.8 ± 22.0*	21.9 ± 14.3*
IO	58.8 ± 3.75*	0.18 ± 0.04*	37.0 ± 18.3**	47.5 ± 17.4*	20.2 ± 8.27*
M[4]	86.2 ± 20.4	2.71 ± 0.56**	22.2 ± 12.5	19.2 ± 5.69	N.D.[5]

Average ± S.D. (n = 5) per individual.
[1]Enzyme activity was measured described in Materials and Method, and total activity was calculated from protein concentration and extract volume per individual.
[2]α-glucosidase.
[3]β-glucosidase.
[4]Calculated from enzyme data of previous report [12].
[5]Not detected.
*No significant ($p > 0.2$); **$p < 0.03$, Sample M was calculated from both J and IO.

Table 2. Glucose content of the amphipods' whole-body.

Trench	J	IO	M[1]
Glucose content (w/w)[2]	0.47± 0.18*	0.39 ± 0.10*	0.43± 0.10

Average ± S.D. (n = 5) per individual.
[1]Data from Ref. [12].
[2]Dry weight.
*No significant ($p > 0.2$), Sample M was calculated from both J and IO.

We focused on cellulase because *H. gigas* from the Challenger Deep had unique cellulase content (HGcel (M)) that can produce glucose and cellobiose from CMC or natural wood [12]. To compare the properties of the cellulase, we examined the cellulolytic activity of *H. gigas* from IO, which was also found to produce glucose and cellobiose from CMC (Figure 4). *H. gigas* from the IO contained β-glucosidase, which produced glucose, and exhibited glucosyl transferase activity, which produced cellobiose isomer [39]. Therefore, two possible enzymatic digestion pathways occurred that can produce glucose and cellobiose. One is the co-reaction of cellobiohydrolase and β-glucosidase, and the other is the reaction of cellulase as HGcel (M). To identify this cellulolytic reaction, we attempted

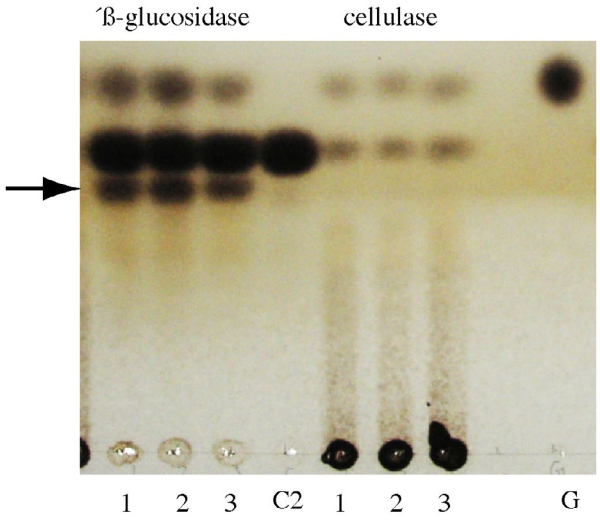

Figure 4. Products of β-glucosidase and cellulase from amphipods from the Izu-Ogasawara Trench. The products of the reactions were determined with TLC as described in the Methods section. Protein fractions of the amphipods were extracted with DDW and precipitated by 60% saturation with ammonium sulfate. Three individuals were crushed and reacted with 0.5% (w/v) cellobiose for β-glucosidase or 1% (w/v) CMC for cellulase for 3 h at 30 °C. The arrow indicates a by-product of the glucotransferase activity of β-glucosidase. G and C2 indicate glucose and cellobiose, respectively.

to purify cellulase from the IO amphipods through hydrophobic interaction chromatography and anion exchange chromatography. As shown in Table 1, the cellulase activity of *H. gigas* from the IO was less than one tenth the activity of *H. gigas* from M. Thus, we succeeded in partially purifying the cellulase of *H. gigas* from IO. The cellulase activity profile and SDS-polyacrylamide gel electrophoresis indicated that cellulase exhibited a 50 kDa band, which was smaller than the 59 kDa exhibited by the HGcel (M) (S1 Figure, Figure 5(A)). Similar to HGcel (M), the cellulase of the amphipods from the IO did not react with cellobiose. The products of the enzyme reaction with CMC at 35 °C showed only glucose and cellobiose (Figure 5(B)). The optimum pH and temperature for the enzyme reaction was 6.4 and 40 °C, respectively (Figure 5(C), (D)). These enzymatic properties were slightly different from those of HGcel (M) (optimum pH and temperature at 5.6 and 25–35 °C, respectively). The HGcel (M) lost its enzyme activity at pH 7.6, whereas the cellulase of *H. gigas* from the IO maintained approximately 75% its activity at pH 7.6. The cellulase of *H. gigas* from the IO showed less than 50% activity at 25 °C, which is the optimum temperature for the reaction of HGcel (M). We also measured cellulase activity at 4 °C, which is almost same temperature as their habitat. The cellulase of *H. gigas* (IO) showed about 65.7% activity of 25 °C. Compared with HGcel (M), the partially purified cellulase fraction did not digest cellobiose. Thus, the β-glucosidase of the collected amphipods has different properties than cellulase.

We also tried purification of cellulase from the 65 amphipods from the Japan Trench. However, the cellulase activity was very low, and we didn't succeed in purification of cellulase. Then, we examined the enzymatic properties of cellulase using partially purified cellulase (Figure S2). The cellulase of the amphipods from the Japan Trench produced glucose and cellobiose from CMC, as same products as the amphipods from the Izu-Ogasawara Trench and the Mariana Trench.

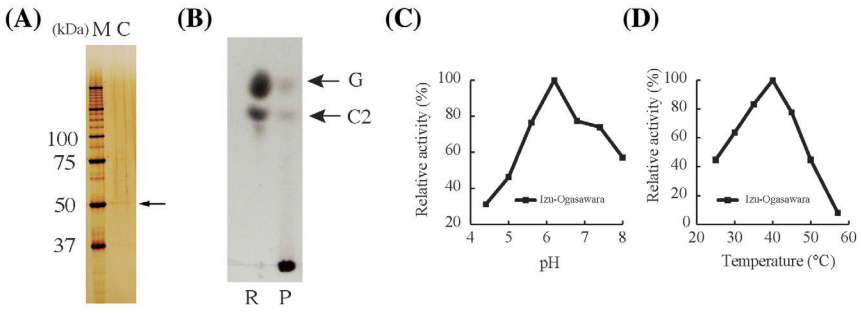

Figure 5. Properties of partially purified cellulase of the amphipods from the Izu-Ogasawara Trench. The SDS-polyacrylamide gel (5–20% gradient) electrophoresis of partially purified cellulase fraction (A) showed a main band of approximately 50 kDa (indicated with an arrow). Amphipod cellulase (Izu-Ogasawara) converted cellulose to glucose and cellobiose (Lane P, indicated with arrows) at pH 5.6. Lane R showed reference of glucose (G) and cellobiose (C2) (B). The effects of pH (C) and temperature (D) on the enzyme's activity and stability are expressed relative to their maximum respective values.

Table 3. Polysaccharide hydrolase genes in mRNA library.

Candidate	Contig	E value	Top hit in Blast search [Species]	Accession No.
Amylase	145202	3.0E-150	amylase [*Eriocheir sinensis*]	ANG56301.1
	154925	3.0E-69	alpha-amylase [*Panulirus argus*]	CDU84835.1
Cellulase	45882	7.0E-71	exoglucanase 1-like [*Hyalella azteca*]	XXP_018027108.1
	83367	2.0E-136	family 7 cellobiohydrolase [*Chelura terebrans*]	AGM37865.1
ß-glucanase	62175	6.0E-97	endoglucanase E-4-like [*Hyalella azteca*]	XXP_018026496.1
	85418	0	endoglucanase A-like [*Hyalella azteca*]	XXP_018016810.1

The optimum temperature and pH of enzymatic reaction was 40 °C and 6.2, which were the same as those of cellulase from Izu-Ogasawara Trench. In addition, the cellulase fractions contained 50 kDa protein, which was the molecular weight of the cellulase of the amphipod from the Izu-Ogasawara Trench. Therefore, the cellulase of amphipod from the Japan Trench would be the same as that of amphipod from the Izu-Ogasawara Trench.

Polysaccharide hydrolase genes of amphipoda IO

In order to obtain transcript sequences of polysaccharide hydrolase, we tried preparation of mRNA from the deep-sea amphipods of the Mariana Trench, the Izu-Ogasawara Trench, and the Japan Trench. We trued isolation of RNA from over 300 individuals of captured *H. gigas*, however; almost all RNAs were degraded. The degradation of RNA would depend on the long time passage after the capture in the deep-sea. We succeeded in the preparation of RNA from only the amphipod of the Izu-Ogasawara Trench. The RNA sequencing was carried out using Hiseq2500 sequencer as described in Materials and Methods. Then, we obtained mRNA library of 162,388 contigs after assembling all reads. There were each 2 contigs showed high homology (E value < 1.0E-50) to amylase, cellulase and ß-glucanase through blast search, respectively.. There was no contig having homology to pectinase or xylanase (Table 3). The amylase candidate genes (contig 145202, 154925) had high homology to amylase of decapods of *Eriocheir sinensis* and *Panulirus argus*, respectively. Both amylase genes were members of the crustacean in the phylogenetic tree based on the amino acid sequences (Figure 6(A), (B)). The top hit of cellulase candidate genes, contig 45882 in blast search was exoglucanase of *Hyalella azteca,* and followed the glycosyl hydrolase family (GH) 7 cellulase genes. The cellulase candidate gene, contig 83367 had homology to cellobiohydrolase of wood boring amphipod *Chelura terebrans* [40]. The phylogenetic analysis based on amino acid sequence showed that the cellulase genes of *H. gigas* were the member of the GH7 and included in the crustacean group (Figure 6(C), (D)). The ß-1. 4 glucanase candidate genes (contig 62175, 85418) had high homology to endoglucanase of *Hyalella azteca*. The phylogenetic analysis of both genes belonged to the GH9, widely distributed in the arthropod (Figure 6(E), (F)). We also analyzed these polysaccharide hydrolases with dbCAN [41] and Hotpep [42], which provide GH family classification based on CAZy database, and obtained the same GH classification as the blastx search.

Discussion

In this study, we found that the polysaccharide hydrolases produced by the hadal-zone *H. gigas* from the J and IO were the same as those produced by the *H. gigas* from M. *H. gigas* from the J and IO contained the same amount of glucose as *H. gigas* from M. Therefore, these hadal-zone amphipods presumably acquire their nutrients from driftwood at the oligotrophic sea bottom. The cellulase activity of the amphipods at both sites was lower than that in *H. gigas* from M at the same reaction conditions. The optimal pH and temperature were used to calculate the total cellulase activity of the amphipods from IO and J, and the values were approximately one tenth the value for *H. gigas* from M. However, the amphipods from the J showed mannanase activity that was five times greater than that of *H. gigas* from M. It has been reported that the digestive enzyme activity in seashore amphipods and crabs reflect food resources [42,43]. Therefore, these differences in digestive enzymes could have been caused by distance from land and ocean currents around the trench, which affect the food influx to the hadal region. The sampling sites in the J and IO are approximately 200 km and 350 km from land, respectively, and both sites are route of the Kuroshio Current, which flows along the southeast coast of Japan [44]. Therefore, the forest in Japan would supply fresh driftwood to both hadal zones. Additionally, high biological production in the Izu-Ogasawara also provides additional carbon sources to the hadal zone [45]. Totally, the deep-sea amphipods can be good candidates for resource of polysaccharide hydrolases.

Some deep-sea amphipods showed genetic diverse corresponding to their habitat [46,47], also a small genetic distance between *H. gigas* from the M and those from IO and J was indicated by the phylogenetic analysis based on the COI gene. Basically, amphipods captured from M, IO, and J would be the same species of *Hirondellea gigas*, as previously reported [10,48]. The habitat of *Hirondellea* species is limited to the bottom of a huge trench, which is one of unique properties differentiating from other common oceanic amphipods. Therefore, the sphere of *Hirondellea* species changes as the trench system is transformed. Approximately 50 Ma, the ancient trench system began as the Japan-South China-Borneo Trench

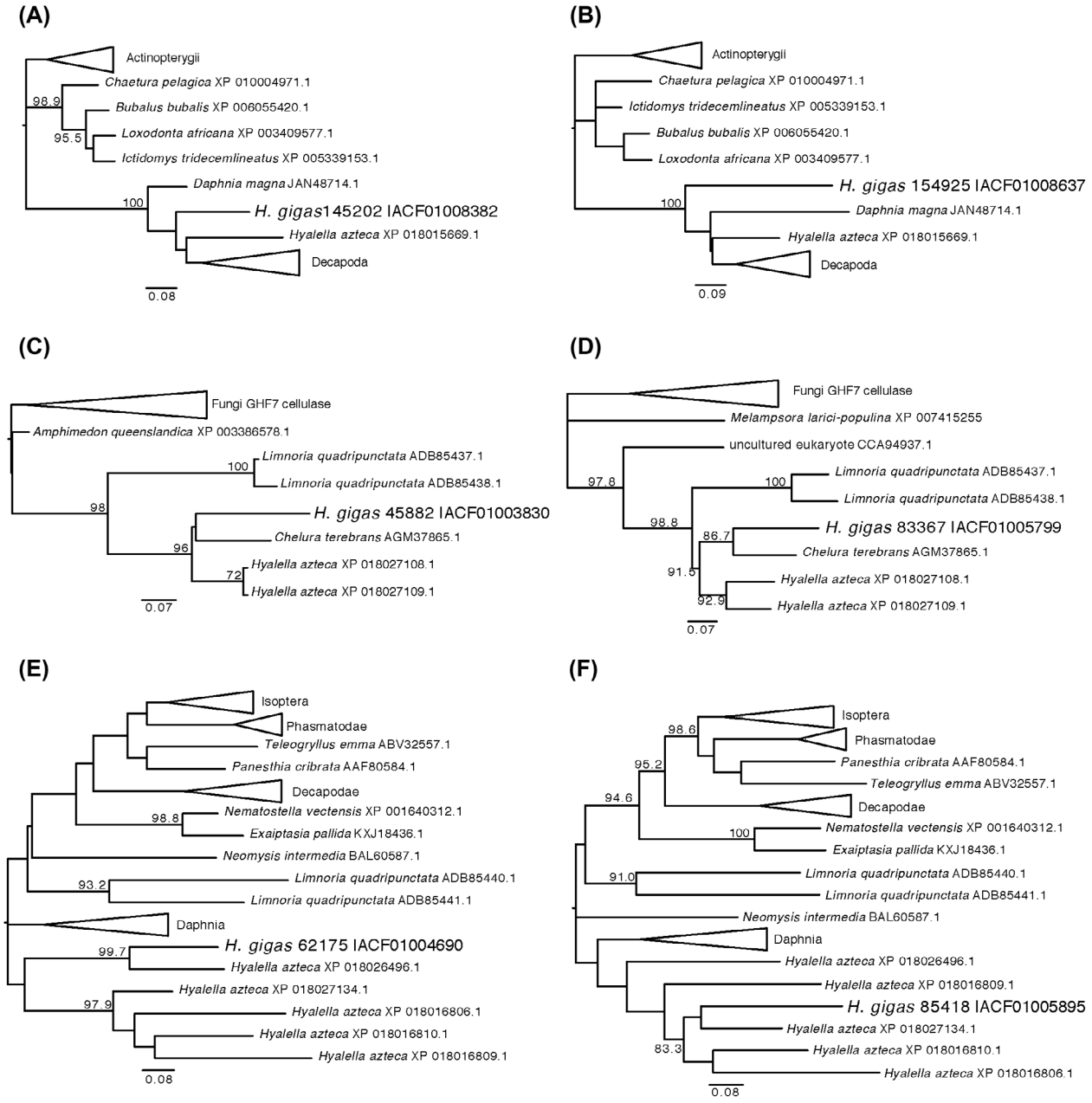

Figure 6. Phylogenetic trees based on polysaccharide hydrolases detected in RNAseq data of *H. gigas* (IO). Tree was constructed from the protein sequence of amylase (contig 145202 (panel A) and contig 154925 (panel B)), cellulase (contig 45882 (panel C) and contig 83367 (panel D)), and ß-glucanase (contig 62175 (panel E) and contig 85418 (panel F)) in Table 3. There were a total of 253 positions (panel A), 152 positions (panel B), 137 positions (panel C), 249 positions (panel D), 196 positions (panel E), and 365 positions (panel F) in the final data-set. Accession number of the gene was followed after the species name. Accession numbers of all genes used in the phylogenetic analysis were listed in Supplemental text. Each phylogenetic tree was reconstructed as described in Materials and Methods.

system near Eurasia, which was then divided into two trenches at approximately 15 Ma: the Philippine Trench and IO-M system [49–52]. Subsequently, the Ogasawara Plateau divided this ancient trench system into the IO and M. The *Hirondellea* species in the three trenches and hadal zone of the Philippine Trench have a distribution that is consistent with geological changes caused by plate tectonics. The partition of the J and the IO occurred at about 17 Ma, due to the opening of the Sikoku Basin with eastward migration of the IO. However, there is no shallow region less than 6,000 m in depth between the J and IO (Figure 1). Therefore, amphipods would be able to move between the J and the IO. This geological isolation of amphipods would cause enzyme genes variation along to nutrient conditions.

The cellulase genes of *H. gigas* IO belonged to the branch of the crustacean cellobiohydrolase included in the GH7 [40]. Other GH7 cellobiohydrolase in the phylogenetic tree were derived from fungi and symbiotic protists. The cellobiohydrolase belonged to GH7 degraded the reducing end of cellulose and produced initially cellobiose, then processed glucose and cellotriose

from cellobiose [53,54]. The GH7 cellobiohydrolase of wood-boring isopod, *Limnoria quadripunctata* was reported to have the same enzymatic property as fungal GH7 cellobiohydrolase except for the salt tolerance [40]. HGcel IO produced glucose and cellobiose from cellulose at initial stage of reaction. And, HGcel M reacted from non-reducing end of cellulose [12]. Therefore, cellulases of *H. gigas* as well as other crustaceans might be new GH family. The contig 45882 and 83367 was around N-terminal and C-terminal of enzymes, respectively. We tried whole cellulase genes from genome by PCR based on contig 45882 and 83367 DNA sequences; however, we cannot obtain cellulase gene fragments. These cellulase gene candidates would occur in genome, independently. The ß-glucanases of *H. gigas* were belonged to GH9 family from domain search. They were widely found in lots of species in the crustaceans. The GH9 family contains various type of glucoside hydrolase including *endo*-cellulase. When extractions of *H. gigas* reacted on cellulose or CMC, we found only glucose or cellobiose. Therefore, the ß-glucanases of *H. gigas* would be other type of glucoside hydrolase. We cannot identify the substrate of these ß-glucanase from sequences. The amylase genes of *H. gigas* were pancreatic amylase found in various animals. Because we could detect only glucose or cellobiose as a product when extracts of *H. gigas* reacted on cellulose or CMC, the ß-glucanases of *H. gigas* would be other type of glucoside hydrolase. Although we cannot identify the substrate of these ß-glucanase from sequences, the amylase genes of *H. gigas* were found to be pancreatic amylase found in various animals.

Author contribution

Conceived and designed the experiments: H.Ko. H.Ko, W.A, H.W, K.O, H.Ki, H.T were performed the experiments. H.Ko, T.N, K.F, Y.K. analyzed phylogenetic data. S. Y, U, M, H. Y, N. performed and analyzed RNA sequencing data. K.F, Y.K analyzed geological data. Contributed reagents/materials/analysis tools: H.Ko, K.O. Wrote the paper: H.Ko. All authors reviewed manuscript.

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Disclosure statement

The authors declare no conflicts of interest associated with this manuscript.

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