A New Find of the Whale-fall Lancelet *Asymmetron inferum* (Cephalochordata) near a Hydrothermal Vent at Oomuro-dashi Submarine Volcano in the Izu Islands, Pacific Coast of Japan, as a Possible Case to Prove the Stepping-stone Hypothesis

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(Received 15 September 2016; Accepted 23 February 2017)

Introduction

In deep-sea reducing bottoms, ephemeral whale-fall communities may provide dispersal stepping stones for a subset of the vent and seep faunas (Smith et al. 1989; Smith and Baco 2003). This dispersal stepping-stone hypothesis has been suggested by finds of whale-fall species of the phyla Annelida and Mollusca also in the reducing vent and/or seep habitats, and *vice versa* (Smith and Baco 2003; Lorion et al. 2013; Sumida et al. 2016). Here will be given the first such example for the phylum (or subphylum) Cephalochordata (for taxonomic rank see Satoh et al. 2014).

The whale-fall lancelet *Asymmetron inferum* Nishikawa, 2004 has so far been known exclusively from reducing bottom sand beneath the bones of the sperm whale *Physeter macrocephalus* Linnaeus, 1758, off Cape Nomamisaki, SW Kyushu, Japan, 219–254 m deep (Nishikawa 2004; Fujiwara et al. 2007; Japan Agency for Marine-Earth Science and Technology 2016). *Asymmetron inferum* has so far been the only lancelet inhabiting whale-fall communities (Smith et al. 2015). The lancelet is not included in Nakajima et al.’s (2014) species list of the deep-sea benthic macrofauna and megafauna from 42 seeps and vents around the Japanese Archipelago. In 2016, however, two lancelet specimens were collected from reducing gravel bottom near a hydrothermal vent, 196 m deep, at Oomuro Hole of Oomuro-dashi submarine volcano in the Izu Islands, Pacific coast of Japan by using the ROV *Hyper-Dolphin* during the RV *Shinsei-maru* KS-16-6 cruise of JAMSTEC. The vent was not referred to by Nakajima et al. (2014). These specimens were safely identified as *A. inferum* in terms of morphology and mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequence data.

The present finding contributes to discussions on the origin of ephemeral populations of the whale-fall lancelet. Further finds of the lancelet from seeps, vents, and whale falls may be highly expected, and will shed new light on the discussions.

Materials and methods

**Sampling.** Two lancelets were collected on 16 May 2016 from pumiceous gravel bottom (34°32.8069’N 139°26.5492’E) near a hydrothermal vent at a depth of 196 m, in Oomuro Hole of Oomuro-dashi submarine volcano in the Izu Islands, Pacific coast of Japan by using the ROV *Hyper-Dolphin* during the RV *Shinsei-maru* KS-16-6 cruise of JAMSTEC; the bottom surface of the sampling site was covered with pale greyish bacterial mat (Fig. 1A). A lancelet was found in the gravel sampled to collect benthic sea anemones by using a scoop (Fig. 1B), while the other in the gravel of a push-core with the diameter of ca. 3 cm. After
taking photographs with an Olympus OM-D E-M1 digital camera on board, the specimens were fixed with 80% ethanol and preserved in 70% ethanol. They are deposited in the National Museum of Nature and Science, Tsukuba (NSMT).

Morphological examination. A stereo microscope was used for morphological observation, measurement of body length, and counting myomeres; specimens were not stained. Body length was measured by using a pair of calipers.

DNA extraction, PCR amplification and sequencing. Total genomic DNA was extracted from muscle pieces on the left side of respective specimens by using DNeasy blood and tissue Kit (Qiagen, Hilden, Germany). A partial region of the COI gene (about 729bp) was amplified by the polymerase chain reaction (PCR) using the following primer pair: AinfCOF214 (5′-CCTGTAATACTGGAGGCTTC-3′) and AinfCOR983 (5′-CCTGCTAGTGACATGCTAATCGAC-3′). The region failed to be amplified by the primer pairs LCO1490 (5′-GGTCAAACATTATAAGATATGGG-3′) and HCO2198 (5′-TAAACTTCAAGGTTAGCCTAACTAC-3′) (Folmer et al. 1994) and AmphL109 (5′-ATTCCGNCGGAAYTNATCNAGC-3′) and AmphH1325 (5′-TCNAGATAYCGNCGWGTTATCNC-3′) (Kon et al. 2006). Amplification conditions were as follows: initial denaturation for 4 min at 94°C; 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C; and final extension for 7 min at 72°C. All reactions were aided by ExTaq HS polymerase (TaKaRa, Otsu, Japan), and the products were purified by ExoSAP-IT (USB). Sequencing reactions were performed according to the manufacturer’s instructions using the BigDye Terminator Cycle Sequencing Reaction Kit ver. 3.1 (Thermo Fisher Scientific, MA, USA). Sequencing reaction products were purified by ethanol precipitation. Labeled fragments were analyzed using an ABI 3500xL Genetic Analyzer (Applied Biosystem). Sequences were obtained from both strands of the gene segments for verification using the same primers. The nucleotide sequences have been submitted to the DNA Data Bank of Japan (DDBJ) under accession numbers LC185086 for a male (NSMT-Pc 1162) and LC185085 for a female (NSMT-Pc 1163).

Phylogenetic analysis. For molecular phylogenetic analysis, obtained from DDBJ were 54 published sequences of *Asymmetron lucayanum* s. str. (accession numbers AB201335–AB201353, AP009354; see Kon et al. 2006, 2007), *A. lucayanum* complex sp. A (AB201315–AB201325, AP009353; see Kon et al. 2006, 2007), *A. lucayanum* complex sp. B (AB201326–AB201334, AB240554–AB240565; see Kon et al. 2006), *A. inferum* (AP009352) (see Kon et al. 2007), and further, as an outgroup, *Branchiostoma japonicum* (AB078191, registered initially as *B. belcheri*; see...
These published sequences and our newly sequenced data from the two Oomuro specimens were analyzed. DNA sequences were aligned using the multiple sequence alignment program CLUSTAL W (Thompson et al. 1994). The sequences from the COI gene were unambiguously aligned without gap. Phylogenetic relationships were reconstructed by the Neighbor-joining method (Saitou and Nei 1987) with MEGA 7.0.14 software (Kumar et al. 2016). In the analysis, Kimura 2-parameter method (Kimura 1980) of nucleotide substitution was used to estimate genetic distances. To estimate statistical support for branching patterns, 1,000 bootstrap replications (Felsenstein 1985) were performed.

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Results

Morphological Examination. The material consists of a 26.5 mm long male (NSMT-Pc 1162; Fig. 1C) and a 25.0 mm long female (NSMT-Pc 1163), each with matured gonads only on the right side; approximate number of myomeres is 85 in the former, while 87 in the latter. Each was provided with a remarkable urostyloid process. Further detailed examination was impossible due to ill shrinkage. However, the asymmetrical arrangement of gonads and the existence of urostyloid process in these specimens may safely justify their affiliation to the genus Asymmetron (Nishikawa 2004). And their myomere numbers (85 and 87) are similar to that of the original description of A. inferum (83) by Nishikawa (2004), quite distinct from the other known congeners (55–72 in A. lucayanum Andrews, 1893...
species complex; Nishikawa 2004, Kon et al. 2006). Thus, the specimen could be safely identified as *A. inferum*.

**DNA barcoding and phylogenetic analysis.** The complete mitochondrial genome sequence of *A. inferum* is registered in the DDBJ (AP009352; Kon et al. 2007), representing so far the only published genome data for this species, based on the specimen from the type locality (off Cape Nomamisaki). In the COI gene, the present sequences differ from the registered one by only 1 (out of 671 nucleotides in the male: LC185086; NSMT-Pc 1162) or 2 (of 668 in the female: LC185085; NSMT-Pc 1163) nucleotides, representing at most 0.3% variation. The male and female sequences differ from each other by only a single nucleotide. On the other hand, the present sequences differ from the registered ones of congeneric three species of tropical shallow-water *A. lucayanum* species complex by 16–18% (AB201315–201353, AB240554–240565; see Kon et al. 2006). Further, our Oomuro specimens are markedly included in a distinct clade with that of *A. inferum* collected from the type locality (Fig. 2). Therefore, the molecular analyses also support the present Oomuro specimens to be identified as *A. inferum*.

**Discussion**

The two specimens were found in a very limited volume of bottom gravel, and they are matured. Therefore, the present Oomuro population of *Asymmetron inferum* can be supposed stable, possibly with a rather high density. Therefore, it may be said that the newly found vent population probably represents a new stable habitat for the whale-fall lancelet, and that the present find suggests the dispersal stepping-stone hypothesis. Future detailed comparisons must be highly expected between the Kagoshima and Oomuro populations of this species in terms of molecular population genetics, which will reveal their history of colonization. Further finds of this lancelet may be highly expected from seeps and vents, as well as whale falls.

**Acknowledgments**

The authors sincerely thank the captain and crew of the RV *Shinsei-maru* and the operating team of the ROV *Hyper-Dolphin* of JAMSTEC for their help in collecting specimens on board, and also the two anonymous referees for their good advices.

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