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Molecular Phylogenetic Analysis of Chemosymbiotic Solemyidae and Thyasiridae

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Abstract

In order to invade and adapt to deep-sea environments, shallow-water organisms have to acquire tolerance to high hydrostatic pressure, low water temperature, toxic methane and hydrogen sulfide, and feeding strategies not relying on photosynthetic products. Our previous study showed that the "evolutionary stepping stone hypothesis", which assumes that organic falls can act as stepping-stones to connect shallow sea with deep sea, was supported in Mytilidae. However, it is not known whether other bivalves constituting chemosynthetic communities experienced the same evolutionary process or different processes from mytilid mussels. Therefore, here, we performed phylogenetic analyses by sequencing the nuclear 18S rRNA and mitochondrial COI genes of solemyid and thyasirid bivalves. In Solemyidae, the two genera *Solemya* and *Acharax* formed each clade, the latter of which was divided into three subgroups. The *Solemya* clade and one of the *Acharax* subgroups diverged in the order of shallow-sea residents, whale-bone residents, and deep-sea vent/seep residents, which supported the "evolutionary stepping stone hypothesis". Furthermore, in Thyasiridae, the two genera *Thyasira* and *Maorithyas* formed a paraphyletic group and the other genera, *Adontorhina*, *Axinopsis*, *Axinulus*, *Leptaxinus*, and *Mendicula*, formed a clade. The "evolutionary stepping stone hypothesis" was not seemingly supported in the other lineages of Solemyidae and Thyasiridae.

Keywords

Whale Bone, Deep Sea, Nuclear DNA, Mitochondrial DNA, Stepping Stone Hypothesis

1. Introduction

In 1977, a community whose primary production is dependent on chemosynthetic bac-

teria was found at a hydrothermal vent along the Galapagos Rift [1] [2]. In the 1980s, similar communities were discovered on a seep in the Gulf of Mexico [3] [4] and on whale bones in the Santa Catarina Basin [5]. Vents and seeps emit methane and hydrogen sulfide that are oxidized by chemosynthetic bacteria to produce energy for the maintenance of these communities. Shallow-water organisms encountered difficulties in adapting to severe deep-sea circumstances when they invaded and settled in deep sea. The organisms must cope with high hydrostatic pressure and low water temperature; they must also circumvent toxic methane and hydrogen sulfide emitting from vents and seeps. Moreover, the organisms have to refine their feeding strategies or develop novel techniques to acquire energy under deep-sea conditions which are nutritionally poor due to a lack of photosynthetic products. It is unclear how the organisms have adapted to deep-sea conditions and overcome the difficult environment.

Distel *et al*. [6] proposed the "evolutionary stepping stone hypothesis", which assumes that organic falls can act as stepping-stones to connect shallow sea with deep sea. According to this hypothesis, shallow-water organisms utilized organic falls to colonize deep-sea vents and seeps. Organic falls, which are sporadically available from shallow to deep waters, provide the animals with an opportunity to acquire tolerance to toxic methane and hydrogen sulfide, high hydrostatic pressure, low water temperature, and feeding strategies against oligotrophy. We assumed that high dispersal ability can also be acquired in organic falls owing to the organisms requiring the ability to exploit patchy and ephemeral habitats. The "evolutionary stepping stone hypothesis" has been supported by previous studies of mytilid mussels [7] [8]. We also showed that mytilid mussels in organic falls and deep-sea vents and seeps had high dispersal ability [9] [10]. However, other deep-sea organisms may have experienced different processes to adapt to deep-sea environments. In the present study, we focus on bivalves belonging to Solemyidae and Thyasiridae to elucidate whether the "evolutionary stepping stone hypothesis" can be supported or other hypotheses are needed by these bivalves.

Solemyidae is an ancient group of bivalves whose fossil records date back to the Ordovician [11] [12] and includes, as modern solemyids, two major genera, *Solemya* and *Acharax*. Solemyid bivalves are distributed at various depths, reside in environments such as anaerobic sandy and muddy bottoms and organic falls [13] [14] [15], and nutritionally depend on intracellular chemosynthetic symbionts, which are harbored in their gills [16]-[22].

The known fossil records of Thyasiridae date back to the Cretaceous [23] [24] or Jurassic [25], and modern thyasirids comprise 11 genera [26]. Thyasiridae predominantly live in fine sediments of the boreal coastal area by burrowing [27]. It is known that a part of the thyasirid species harbor extracellular chemosynthetic symbionts on their gills [28] [29] [30]. However, *Maorithyas hadalis* from the hadal zone in the Japan Trench has exceptionally two types of intracellular symbionts [29], and *Thyasira kaireiae* is intermediate between extracellular and intracellular symbioses. Chemosynthetic bacteria of *T. kaireiae* are enclosed with the cuticle that does not have the membrane structure clearly, whereas the chemosynthetic endosymbionts are generally enclosed in membrane-bound vacuoles [20].

In solemyid and thyasirid bivalves, symbiosis does not depend on depth and existence of organic falls, although only mytilid mussels that inhabit organic falls and deep-sea vents/seeps represent bacterial symbiosis, but not shallow-sea mytilids. Therefore, it is conceivable that solemyids and thyasirids did not require organic falls to acquire tolerance to toxic hydrogen sulfide and methane and to develop symbiosis, which suggest that they might adapt to deep-sea environments in a way(s) that is different from that by mytilid mussels. In other words, it is possible that the "evolutionary stepping stone hypothesis" cannot be supported by solemyid and thyasirid bivalves.

In the present study, we determine the nucleotide sequences of the nuclear 18S ribosomal RNA (18S rRNA) gene and the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and deduce the phylogenetic relationships in solemyids and thyasirids to give an insight into their deep-sea adaptation.

2. Materials and Methods

2.1. Materials

The specimens, of which DNA sequences were determined in the present study, are listed in Table 1, and their collection sites are mapped in Figure 1. Most solemyid and thyasirid bivalves were collected by submersibles such as "Kaiko 7000 II", "Hyper-Dolphin 3000", and "Shinkai 6500" operated by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). *Acharax japonica* from Nabeta Bay, *Axinopsis*

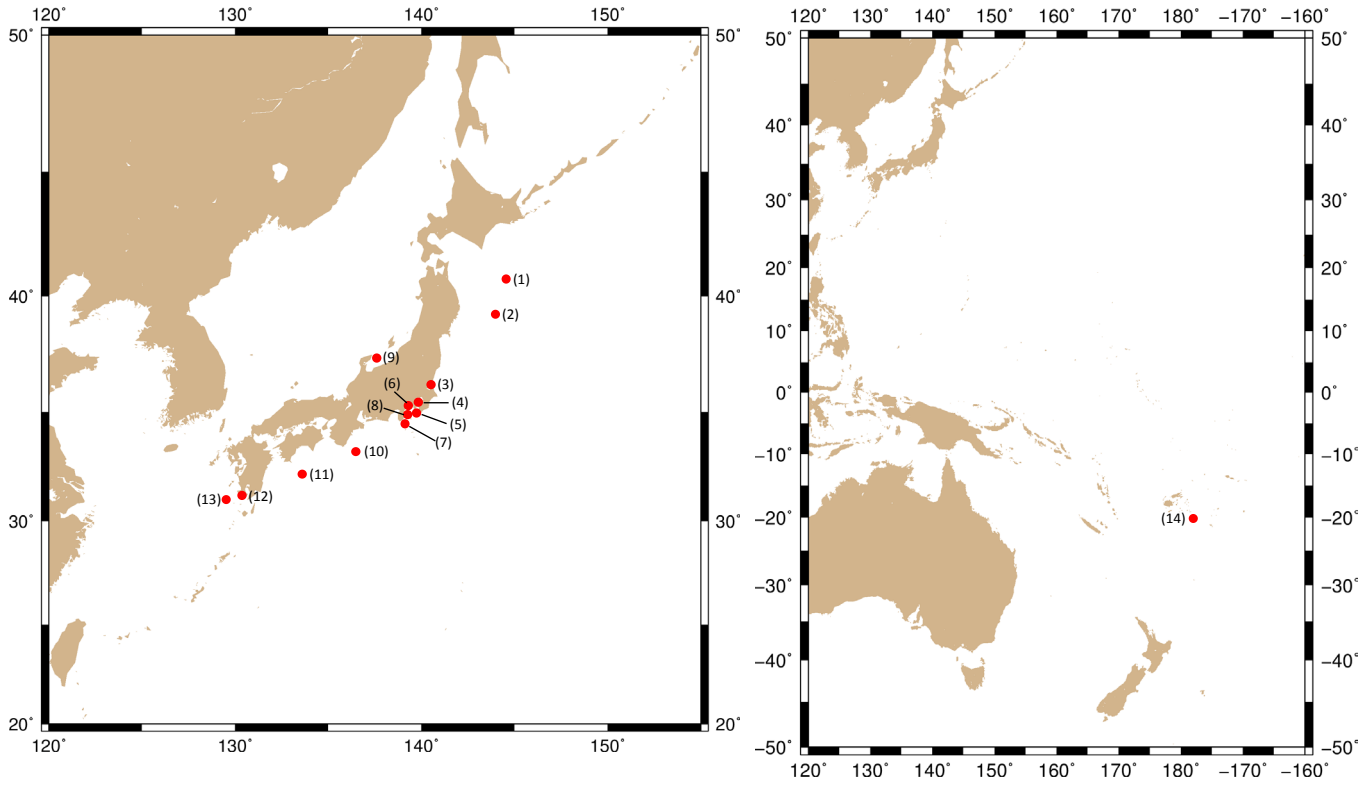

Figure 1. Localities where the samples were collected. (1) Chishima Trench; (2) Japan Trench; (3) Off Kawarago; (4) Koajiro Bay; (5) Okinoyama Bank Site, Sagami Bay; (6) Off Hatsushima, Sagami Bay; (7) Nabeta Bay; (8) Off Inatori; (9) Joetsu Knoll, Toyama Trough; (10) Nankai Trough; (11) Off Ashizuri Cape; (12) Wakamiko Caldera, Kagoshima Bay; (13) Off Noma Cape, Kagoshima Bay; (14) Hine Hina, Lau Basin.

Table 1. Sample list.

Species	Abbreviation	Site (locality number in Figure 1)	Depth (m)	Habitat type	18S	COI
Solemyidae						
Acharax japonica	SHI4-1	Nabeta Bay, Shimoda City (7)	Unknown	Shallow water	LC186952	
Acharax japonica	SHI4-2	Nabeta Bay, Shimoda City (7)	Unknown	Shallow water	LC186953	
Acharax japonica	SHI4-5	Nabeta Bay, Shimoda City (7)	Unknown	Shallow water	LC186954	LC186990
Acharax japonica	SHI5-6B	Nabeta Bay, Shimoda City (7)	Unknown	Shallow water	LC186955	
Acharax johnsoni	AJK1-1	Nankai Trough (10)	2049	Seep	LC186956	LC186991
Acharax johnsoni	AJK1-2	Nankai Trough (10)	2049	Seep	LC186957	LC186992
Acharax johnsoni	AJH1-2	Off Hatsushima, Sagami Bay (6)	1176	Seep	LC186958	LC186993
Acharax sp.	AJH1-1	Off Hatsushima, Sagami Bay (6)	1176	Seep	LC186959	LC186994
Acharax sp.	HS2-1	Off Hatsushima, Sagami Bay (6)	1153	Seep	LC186960	LC186995
Acharax sp.	AJO1-1	Okinoyama Bank Site, Sagami Bay (5)	Unknown	Seep	LC186961	LC186996
Acharax sp.	CST1	Chishima Trench (1)	4970	Seep	LC186962	LC186997
Acharax sp.	AJJ1-1	Japan Trench (2)	5315	Seep	LC186963	LC186998
Acharax sp.	AJJ2-2	Japan Trench (2)	5300	Seep	LC186964	LC186999
Acharax sp.	AJJ02	Japan Trench (2)	5300	Seep	LC186965	
Acharax sp.	JT1A	Japan Trench (2)	5300	Seep	LC186966	
Acharax sp.	JT1B	Japan Trench (2)	5300	Seep	LC186967	LC187000
Acharax sp.	JT4	Japan Trench (2)	5300	Seep	LC186968	
Acharax sp.	W2-1	Off Noma Cape, Kagoshima (13)	217	Whale bone	LC186969	LC187001
Acharax sp.	Lau1	Hine Hina, Lau Basin (14)	1817	Vent	LC186970	LC187002
Solemya pervernicosa	SW3-1	Off Noma Cape, Kagoshima Bay (13)	Unknown	Whale bone	LC186971	
Solemya pervernicosa	SW4-1	Off Noma Cape, Kagoshima Bay (13)	236	Whale bone	LC186972	LC187003
Solemya pervernicosa	SW5-1	Off Noma Cape, Kagoshima Bay (13)	226	Whale bone	LC186973	LC187004
Solemya pusilla	SPM	Koajiro Bay, Miura City (4)	7.1 - 8.1	Shallow water	LC186974	LC187005
Solemya tagiri	KGS1-1	Wakamiko Caldera, Kagoshima Bay (12)	94 - 98	Vent	LC186975	LC187006
Solemya tagiri	KGS1-2	Wakamiko Caldera, Kagoshima Bay (12)	94 - 98	Vent	LC186976	LC187007
Solemya tagiri	KGS2-1	Wakamiko Caldera, Kagoshima Bay (12)	102	Vent	LC186977	LC187008
Solemya tagiri	KGS2-3	Wakamiko Caldera, Kagoshima Bay (12)	102	Vent	LC186978	
Solemya tagiri	KGS3-1	Wakamiko Caldera, Kagoshima Bay (12)	185	Vent	LC186979	
Solemya tagiri	KGS4-1	Wakamiko Caldera, Kagoshima Bay (12)	198	Vent	LC186980	
Solemya tagiri	KGS4-2	Wakamiko Caldera, Kagoshima Bay (12)	198	Vent	LC186981	
Solemya tagiri	KGS5-1	Off Noma Cape, Kagoshima (13)	249	Whale bone	LC186982	LC187009
Solemya tagiri	KGS6-1	Off Noma Cape, Kagoshima (13)	226	Whale bone	LC186983	LC187010
Solemya sp.	SWC1	Wakamiko Caldera, Kagoshima Bay (12)	100	Vent	LC186984	
Solemya sp.	SWC2	Wakamiko Caldera, Kagoshima Bay (12)	196	Vent	LC186985	LC187011
Solemya sp.	AC1-1	Off Ashizuri Cape (11)	575	Seep	LC186986	LC187012
Solemya sp.	AC1-2	Off Ashizuri Cape (11)	575	Seep	LC186987	LC187013
Solemya sp.	W6-1	Off Noma Cape, Kagoshima Bay (13)	227	Whale bone	LC186988	LC187014
Solemya sp.	W12-1	Off Noma Cape, Kagoshima Bay (13)	252	Whale bone	LC186989	LC187015

Continued

Thyasiridae						
Axinopsis rubiginosa	RA-K1	Off Kawarago, Hitachi City (3)	350	Fine sand	LC187016	LC187041
Maorithyas hadalis	TH-JT1	Japan Trench (2)	7333	Seep	LC187017	LC187042
Thyasira kaireiae	PK3	Japan Trench (2)	5345	Seep	LC187018	LC187043
Thyasira kaireiae	PK6	Japan Trench (2)	5345	Seep	LC187019	LC187044
Thyasira kaireiae	PK7	Japan Trench (2)	5345	Seep	LC187020	LC187045
Thyasira sp.	INT-1	Off Inatori (8)	ca. 400	Mud	LC187021	
Thyasira sp.	INT-2	Off Inatori (8)	ca. 200	Mud	LC187022	LC187046
Thyasira sp.	IO1	Off Hatsushima, Sagami Bay (6)	1158	Vent	LC187023	LC187047
Thyasira sp.	TH1	Off Hatsushima, Sagami Bay (6)	1158	Seep	LC187024	
Thyasira sp.	TH2	Off Hatsushima, Sagami Bay (6)	927	Seep	LC187025	
Thyasira sp.	TH3	Off Hatsushima, Sagami Bay (6)	927	Seep	LC187026	
Thyasira sp.	TH4	Off Hatsushima, Sagami Bay (6)	927	Seep	LC187027	LC187048
Thyasira sp.	TH5	Off Hatsushima, Sagami Bay (6)	855	Seep	LC187028	LC187049
Thyasira sp.	TH6	Off Hatsushima, Sagami Bay (6)	855	Seep	LC187029	LC187050
Thyasira sp.	TH7	Off Hatsushima, Sagami Bay (6)	855	Seep	LC187030	LC187051
Thyasira sp.	TH8	Off Hatsushima, Sagami Bay (6)	855	Seep	LC187031	LC187052
Thyasira sp.	TH11	Off Hatsushima, Sagami Bay (6)	1173	Seep	LC187032	LC187053
Thyasira sp.	TH12	Off Hatsushima, Sagami Bay (6)	1173	Seep	LC187033	LC187054
Thyasira sp.	TH19	Off Hatsushima, Sagami Bay (6)	927	Seep	LC187034	LC187055
Thyasira sp.	TH21	Off Hatsushima, Sagami Bay (6)	1170	Seep	LC187035	LC187056
Thyasira sp.	TH32	Joetsu Knoll, Toyama Trough (9)	986	Seep		LC187057
Thyasira sp.	TH33	Off Hatsushima, Sagami Bay (6)	855	Seep		LC187058
Thyasira sp.	TH34	Off Hatsushima, Sagami Bay (6)	856	Seep	LC187036	LC187059
Thyasira sp.	TH35	Off Hatsushima, Sagami Bay (6)	927	Seep	LC187037	LC187060
Thyasira sp.	TH37	Off Hatsushima, Sagami Bay (6)	927	Seep	LC187038	LC187061
Thyasira sp.	TT1	Joetsu Knoll, Toyama Trough (9)	986	Seep	LC187039	LC187062
Thyasira sp.	TN1	Off Noma Cape, Kagoshima Bay (13)	226	Whale bone	LC187040	LC187063

rubiginosa from off Kawarago, solemyid bivalves from off Ashizuri Cape, and *Thyasira* sp. from off Inatori were collected by dredging. All samples were frozen and preserved at −80˚C or in 100% ethanol and deposited at JAMSTEC. The specimens, of which DNA sequences were quoted from the DNA Data Bank of Japan (DDBJ), are listed in Table 2. Almost all thyasirid specimens were very small and often damaged during collection. Thus, we gave priority to molecular analyses using whole bodies than to morphological identification and measurements such as counting the number of ctenidial demibranchs.

2.2. Sequencing of the Nuclear 18S rRNA Gene and the Mitochondrial COI Gene

Total DNA was prepared from the soft tissue using a DNeasy® Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Table 2. Sample list (quoted from DDBJ).

Species	Abbreviation	Site	Depth (m)	Habitat type	18S	COI
Solemyidae						
Acharax sp.	A1-Makran	Makran	2200	Unknown	AJ563759	
Acharax sp.	A1-Java	Java	2940	Unknown	AJ563756	
Acharax sp.	A2-Java	Java	2940	Unknown	AJ563757	
Acharax sp.	A5-Aleutian	Aleutian Trench	4810	Unknown	AJ563760	
Acharax sp.	A3-Costa Rica	Costa Rica	763	Unknown	AJ563763	
Acharax sp.	A1-Oregon	Oregon	780	Unknown	AJ563751	
Acharax sp.	A2-Oregon	Oregon	910	Unknown	AJ563753	
Acharax sp.	A8-Oregon	Oregon	780	Unknown	AJ563752	
Acharax sp.	A10-Oregon	Oregon	910	Unknown	AJ563754	
Acharax sp.	A13-Oregon	Oregon	910	Unknown	AJ563755	
Acharax sp.	A1-Peru	Peru	ca.3500	Unknown	AJ563762	
Solemya elarraichensis	*Solemya elarraichensis*	Unknown	Unknown	Unknown		KC984719
Solemya reidi	*Solemya reidi*	Santa Monica Bay, sewage outfall	Unknown	Shallow water	AF117737	
Solemya velesiana	*Solemya velesiana*	Unknown	Unknown	Shallow water		KC984744
Solemya velum	*Solemya velum*	Unknown	Unknown	Shallow water	AF120524	
Solemya velum	*Solemya velum*	Unknown	Unknown	Shallow water		KC984745
Thyasiridae						
Adontorhina cyclia	*Adontorhina cyclia*	San Diego, California, USA	Unknown	Shallow water	AM392455	
Axinulus eumyaria	*Axinulus eumyaria*	Fanafjord, Norway	Unknown	Shallow water		AM706494
Axinulus sp.	*Axinulus* sp.	Scotia Ridge, Antarctica	285	Unknown	AM392441	
Leptaxinus indusarium	*Leptaxinus indusarium*	Arabian Sea, Pakistan	775	Mud	AM392454	
Mendicula ferruginosa	*Mendicula ferruginosa*	Northern North Sea	125	Shallow water	AM392456	
Mendicula ferruginosa	*Mendicula ferruginosa*	Flesland, Norway	Unknown	Shallow water		AM706496
Thyasira cf *subovata*	*Thyasira* cf *subovata*	Scotia Ridge, Antarctica	3894	Vent	AM392451	
Thyasira equalis	*Thyasira equalis*	Gullmarsfjorden, Sweden	100	Shallow water	AM392453	
Thyasira equalis	*Thyasira equalis*	Korsfjord, Norway	Unknown	Shallow water		AM706521
Thyasira flexuosa	*Thyasira flexuosa*	Plymouth, UK	Unknown	Shallow water	AJ581870	
Thyasira gouldi	*Thyasira gouldi*	Firth of Forth, UK	Unknown	Shallow water	AJ581871	
Thyasira granulosa	*Thyasira granulosa*	Fanafjord, Norway	Unknown	Unknown		AM706503
Thyasira methanophila	*Thyasira methanophila*	Concepcion, Chile	780	Seep	AM392447	
Thyasira obsoleta	*Thyasira obsoleta*	Korsfjord, Norway	Unknown	Unknown		AM706505
Thyasira perplicata	*Thyasira perplicata*	Angola	1950	Seep	AM392448	
Thyasira polygona	*Thyasira polygona*	Northern North Sea	125	Shallow water	AM392449	
Thyasira sarsi	*Thyasira sarsi*	Northern North Sea	139	Shallow water	AM392450	
Thyasira sarsi	*Thyasira sarsi*	Korsfjord, Norway	Unknown	Shallow water		AM706508
Thyasira sp.	*Thyasira* sp. Fiji	Fiji Back Arc Basin	1977	Vent	AM392452	
Nuculidae						
Acila castrensis	*Acila castrensis*	Unknown	Unknown	Unknown	KC429319	KC429087
Lucinidae						
Myrtea spinifera	*Myrtea spinifera*	Banyuls, France	Unknown	Unknown	AJ581861	
Myrtea spinifera	*Myrtea spinifera*	Unknown	Unknown	Unknown		AY070139

To amplify the partial fragments of the 18S ribosomal RNA (18S rRNA) and cytochrome *c* oxidase subunit I (COI) genes, PCR was performed in reaction solutions containing template DNA and KOD Dash (Toyobo Co., Ltd., Osaka, Japan) under the following condition: 1) 30 cycles of denaturation at 94˚C for 30 s, annealing at 45˚C for 5 s, and extension at 74˚C for 10 s. The primers used in the present study are described in Table 3. When PCR amplification under this condition was not successful, PCR was

Table 3. Primers used in the present study.

Specimen	Gene	Primer	Sequence	Orientation	Reference
C	18S rRNA	1F	TACCTGGTTGATCCTGCCAGTAG	Forward	[40]
C	18S rRNA	3R	AGGCTCCCTCTCCGGAATCGAAAC	Reverse	[40]
C	18S rRNA	3F	GTTCGATTCCGGAGAGGGA	Forward	[40]
C	18S rRNA	5R	CTTGGCAAATGCTTTCGC	Reverse	[40]
C	18S rRNA	5F	GCGAAAGCATTTGCCAAGAA	Forward	[40]
C	18S rRNA	9R	GATCCTTCCGCAGGTTCACCTAC	Reverse	[40]
C	18S rRNA	18Sbi	GAGTCTCGTTCGTTATCGGA	Reverse	[41]
C	18S rRNA	1Fn	CGCGAATGGCTCATTAAATC	Forward	This study
C	18S rRNA	9Rn	GTACAAAGGGCAGGGACGTA	Reverse	This study
S	18S rRNA	Sol1S	TTACCTGGTTGATCCTGCCAGTAG	Forward	This study
S	18S rRNA	Sol1A	ATTCCAATTACGGGGCCTCGAAC	Reverse	This study
S	18S rRNA	Sol2S	CTGCCCTATCAACTGTCGATGGTAG	Forward	This study
S	18S rRNA	Sol2A	GAACTACGACGGTATCTGATCGTC	Reverse	This study
S	18S rRNA	Sol3S	CGGTGTTAGAGGTGAAATTCTTGG	Forward	This study
S	18S rRNA	Sol3A	CGACTTTTACTTCCTCTAAGCGATC	Reverse	This study
T	18S rRNA	Th1F	GATCCTGCCAGTAGTCATATGC	Forward	This study
T	18S rRNA	Th1R	AGACTTGCCCTCCAATGGATC	Reverse	This study
T	18S rRNA	Th2F	GTCTGCCCTATCAACTTTCGATGG	Forward	This study
T	18S rRNA	Th2R	GGCATCGTTTATGGTCAGAACTACG	Reverse	This study
T	18S rRNA	Th3F	GCATTCGTATTGCGGTGTTAGAGG	Forward	This study
T	18S rRNA	Th3R	CGACTTTTACTCCCTCTAGTCC	Reverse	This study
C	COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Forward	[42]
C	COI	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Reverse	[42]
S	COI	ACS1	GGGCTTTGTTAGGGGATGAT	Forward	This study
S	COI	ACA1	TCCGGTTAAAACAGGTAAGGA	Reverse	This study
S	COI	ACS2	TTTAAGATTATTAATTCGGGCTGAAC	Forward	This study
S	COI	ACA2	CCGGTTAAAACAGGTAAGGATAATAA	Reverse	This study
S	COI	So1F	TTGGTCAACCTGGAGCATT	Forward	This study
S	COI	So1R	AATTGCTCCGGCTAAAACT	Reverse	This study
S	COI	So2F	GCTATTTGAGCCGGAATAGTAGG	Forward	This study
S	COI	So2R	CTGCGGGATCGAAGAATGATGTA	Reverse	This study
S	COI	So3R	TAGAATAGGATCTCCACCTCCTG	Reverse	This study
T	COI	CA1	GGTATACGGGGTAACCC	Reverse	This study
T	COI	CS2	CGCTTAGAACTTAGCCAGCC	Forward	This study
T	COI	CA2	ATAGGATCCCCCCCTCCAC	Reverse	This study
T	COI	CS3	ATTTACTGGGTTAGCTGGGAC	Forward	This study
T	COI	CA3	CACGAGGATCAAAAAACCTAC	Reverse	This study

C, for Solemyidae and Thyasiridae; S, for Solemyidae; T, for Thyasiridae.

performed under the following modified conditions: 2) initial denaturation at 94˚C for 2 min, 5 cycles of denaturation at 94˚C for 30 s, annealing at 48˚C for 1.5 min, and extension at 72˚C for 1 min, followed by 35 cycles of denaturation at 93˚C for 30 s, annealing at 51˚C for 1.5 min, and extension at 72˚C for 1 min, and final extension at 72˚C for 7 min. Alternatively, 3) first PCR was performed under the 1) or 2) condition, and the second PCR was performed under the 1) or 2) condition using primers different from those used in the first PCR. Only for two thyasirid bivalves from off Inatori (INT-1 and INT-2), the first PCR was performed with 1F and 9R primers for 18S rRNA and with LCO1490 and HCO2198 primers for COI under the following condition: initial denaturation at 94˚C for3 min, 35 cycles of denaturation at 95˚C for 45 s, annealing at 55˚C for 3 min, and extension at 72˚C for 1.5 min, and final extension at 72˚C for 7 min; the second PCR was performed with Th1F and Th1R, Th2F and Th2R, and 5F and 9Rn primers for 18S rRNA and with CS2 and CA2 primers for COI under the 2) condition. PCR products were purified using a QIAquick® PCR purification Kit (Qiagen GmbH, Hilden, Germany).

Direct sequencing of the double-stranded PCR product was performed using an ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA, USA) and the primers used for PCR on Model 377 and 377XL DNA sequencers (Applied Biosystems Inc., CA, USA) according to the manufacturer's directions. Alternatively, direct sequencing was performed using a GenomeLab™DTCSQuick Start Kit on a CEQ™ 2000XL DNA Analysis System (Beckman Coulter Inc., CA, USA) according to the manufacturer's directions. DNA sequences were aligned with DNASIS (Hitachi Software Engineering) and MEGA 6.0 [31]. All sequences obtained in the present study were registered in DDBJ (accession numbers LC186952-LC187063).

2.3. Phylogenetic Analysis

We constructed three trees based on only 18S rRNA sequences, only COI sequences, and concatenated 18S rRNA + COI sequences for Solemyidae and Thyasiridae, respectively. Trees were constructed by the neighbor-joining (NJ) and maximum parsimony (MP) methods using MEGA 6.0 [31] and the PAUP*4.0 beta 10 software [32], respectively. Genetic distances were computed using the Kimura's two-parameter method [33]. The reliability of trees was evaluated by producing 1000 bootstrap replicates. The majority-rule consensus MP tree was constructed by conducting a heuristic search based on the 1000 bootstrap replicates with an unweighted ts/tv ratio. The Bayesian tree was constructed using the MrBayes version 3.1 software [34] based on the model evaluated by the MrModel test 2.2 [35]. The best models were SYM + G for 18S rRNA, GTR + G for COI, and GTR + I + G for 18S rRNA + COI in Solemyidae. On the other hand, the best models were SYM + I + G for 18S rRNA, GTR + G for COI, and GTR + I + G for 18S rRNA + COI in Thyasiridae. The Monte Carlo Markov chain (MCMC) length was 5 million generations, and we sampled the chain after every 100 generations. MCMC convergence was assessed by calculating the potential scale reduction factor, and the first 25,000 generations were discarded. The outgroup species *Acila castrensis* (Bivalvia, Nuculidae) for Solemyidae and *Myrtea spinifera* (Bivalvia, Lucinidae) for Thyasiridae were used.

3. Results

3.1. Phylogenetic Relationships of Solemyidae

In the NJ tree based on 18S rRNA sequences (1300 bp, 253 variable sites, and 119 informative sites), *Acharax* formed a paraphyletic group composed of three clades, *Acharax* 1, *Acharax* 2, and *Acharax* 3. Moreover, *Solemya* formed a clade (Figure 2). In the NJ tree based on COI sequences (400 bp, 256 variable sites, and 178 informative sites), *Acharax* and *Solemya* formed clades, respectively, with an only exception of *Acharax* sp. Lau 1 (Figure 3). In the NJ tree based on concatenated 18S rRNA + COI sequences (1700 bp, 464 variable sites, and 284 informative sites), *Acharax* and *Solemya* formed clades, respectively, and the *Acharax* genera were divided into three clades: Subgroups 1, 2, and 3 (Figure 4). The tree showed that the *Solemya* clade and Subgroup 3 in the *Acharax* clade diverged in the order of shallow-sea residents, whale-bone residents, and deep-sea vent/seep residents, although this was not well supported by MP bootstrap values and Bayesian posterior probabilities. Subgroups 1 and 2 were constituted

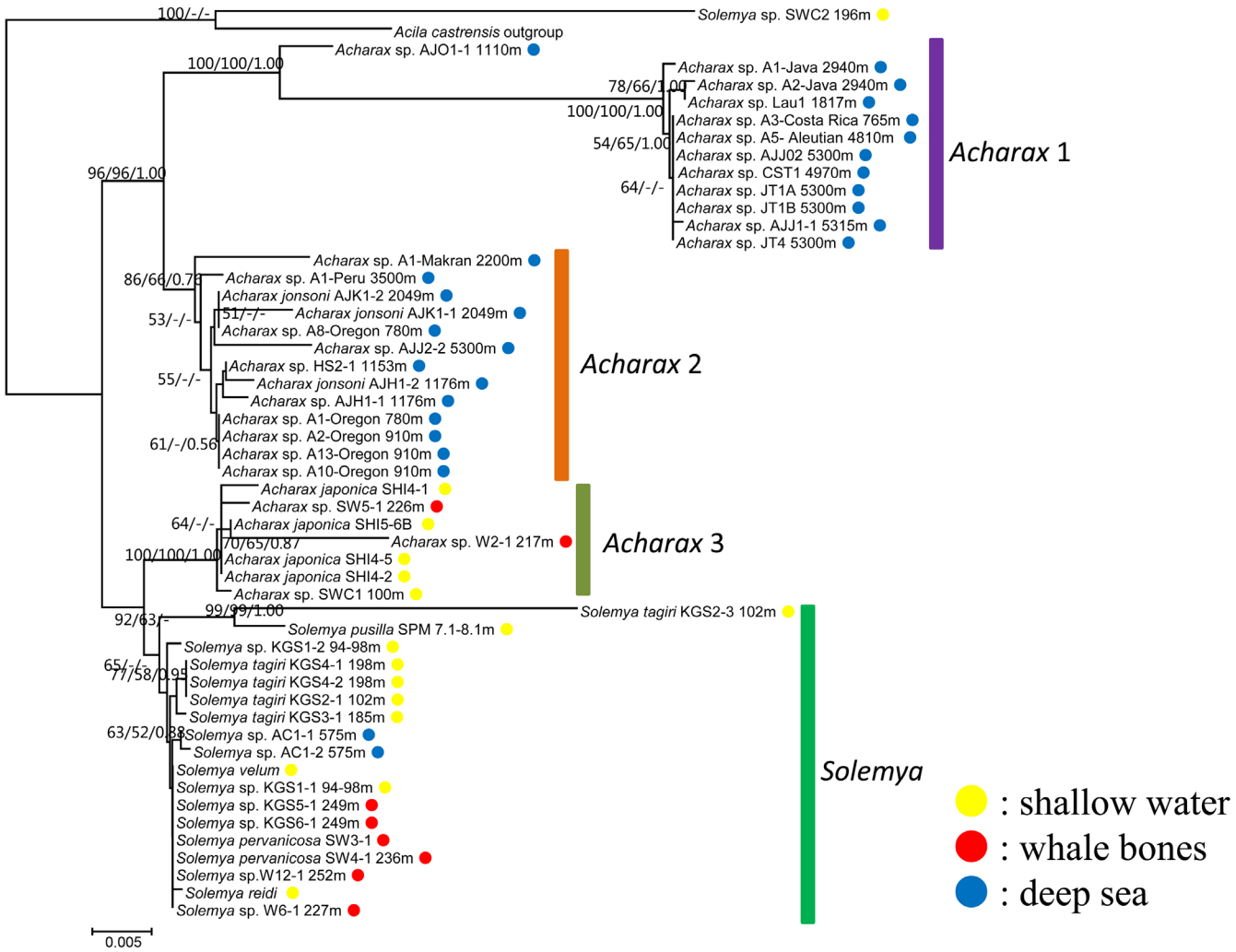

Figure 2. Phylogenetic relationships of solemyid bivalves based on 18S rRNA sequences (1300 bp). The NJ, MP, and Bayesian trees were constructed using *Acila castrensis* as an outgroup species. Only the NJ (left) and MP (middle) bootstrap values ≥ 50 and the Bayesian (right) posterior probabilities ≥ 0.50 are specified. The scale bar indicates 0.005 substitutions per site.

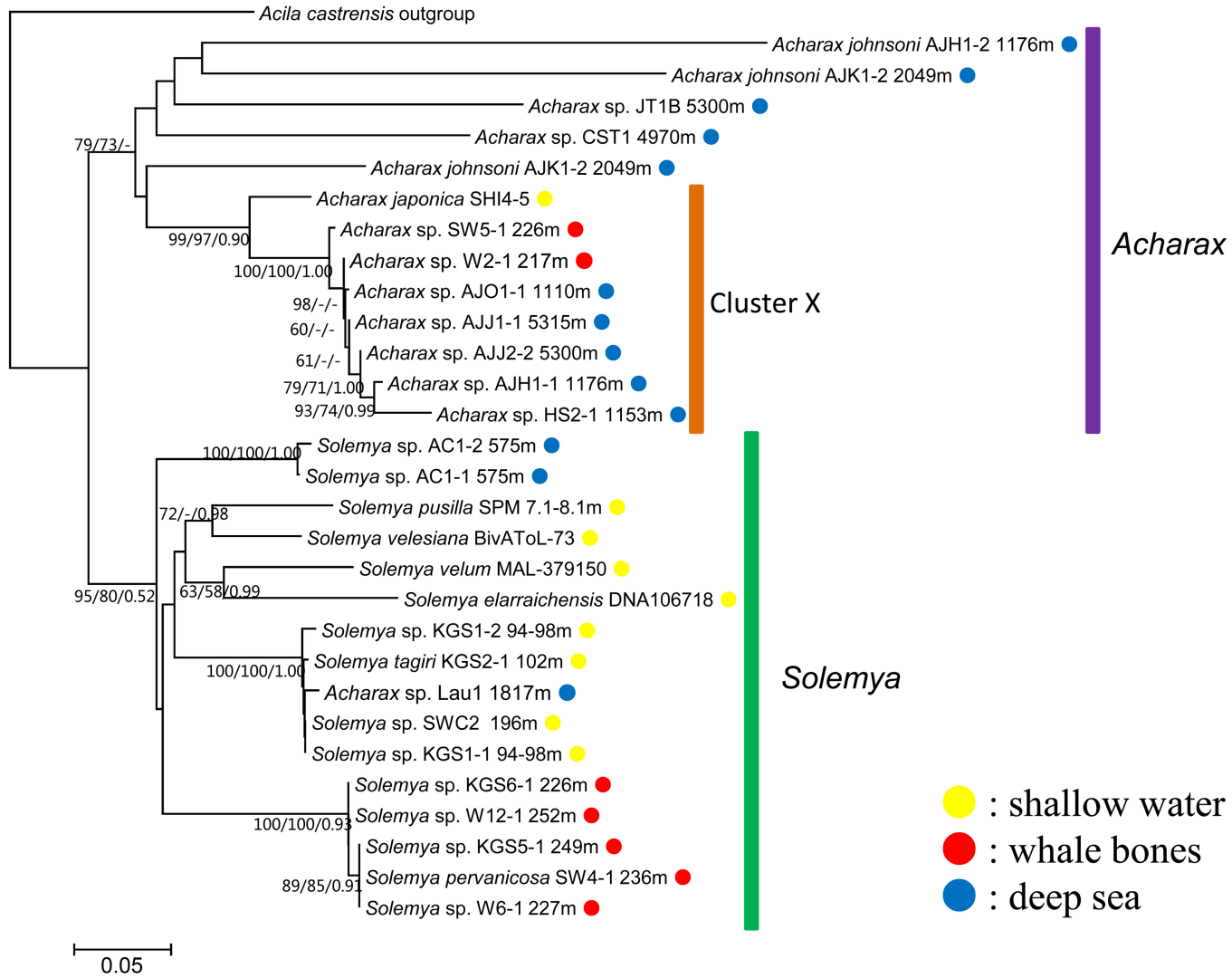

Figure 3. Phylogenetic relationships of solemyid bivalves based on COI sequences (400 bp). The NJ, MP, and Bayesian trees were constructed using *Acila castrensis* as an outgroup species. Only the NJ (left) and MP (middle) bootstrap values ≥ 50 and the Bayesian (right) posterior probabilities ≥ 0.50 are specified. The scale bar indicates 0.05 substitutions per site.

by only deep-sea specimens. *Acharax* sp. Lau 1 was included in *Acharax* 1 of the 18S rRNA tree (**Figure 2**) and in *Acharax* Subgroup 1 of the 18S rRNA + COI trees (**Figure 4**), but in the *Solemya* clade of the COI tree (**Figure 3**). *Solemya* sp. SWC2 was included in the *Solemya* clade of the COI tree (**Figure 3**), but diverged basally from other solemyid mussels in the 18S rRNA and 18S rRNA + COI trees (**Figure 2** and **Figure 4**). We constructed the three trees, 18S rRNA, COI, and 18S rRNA + COI. The 18S rRNA tree had more taxa than the two other trees, because more data have been registered in DDBJ. The 18S rRNA + COI tree seems the most reliable, because it was shown that larger sequence data provided more reliable tree [36]. The COI tree was consistent with the 18S rRNA + COI tree in that *Solemya* and *Acharax* formed each clades, whereas the 18S rRNA tree was consistent with the 18S rRNA + COI tree in division of *Acharax* into the three subgroups and phylogenetic positions of *Acharax* sp. Lau 1 and *Solemya* sp. SWC2.

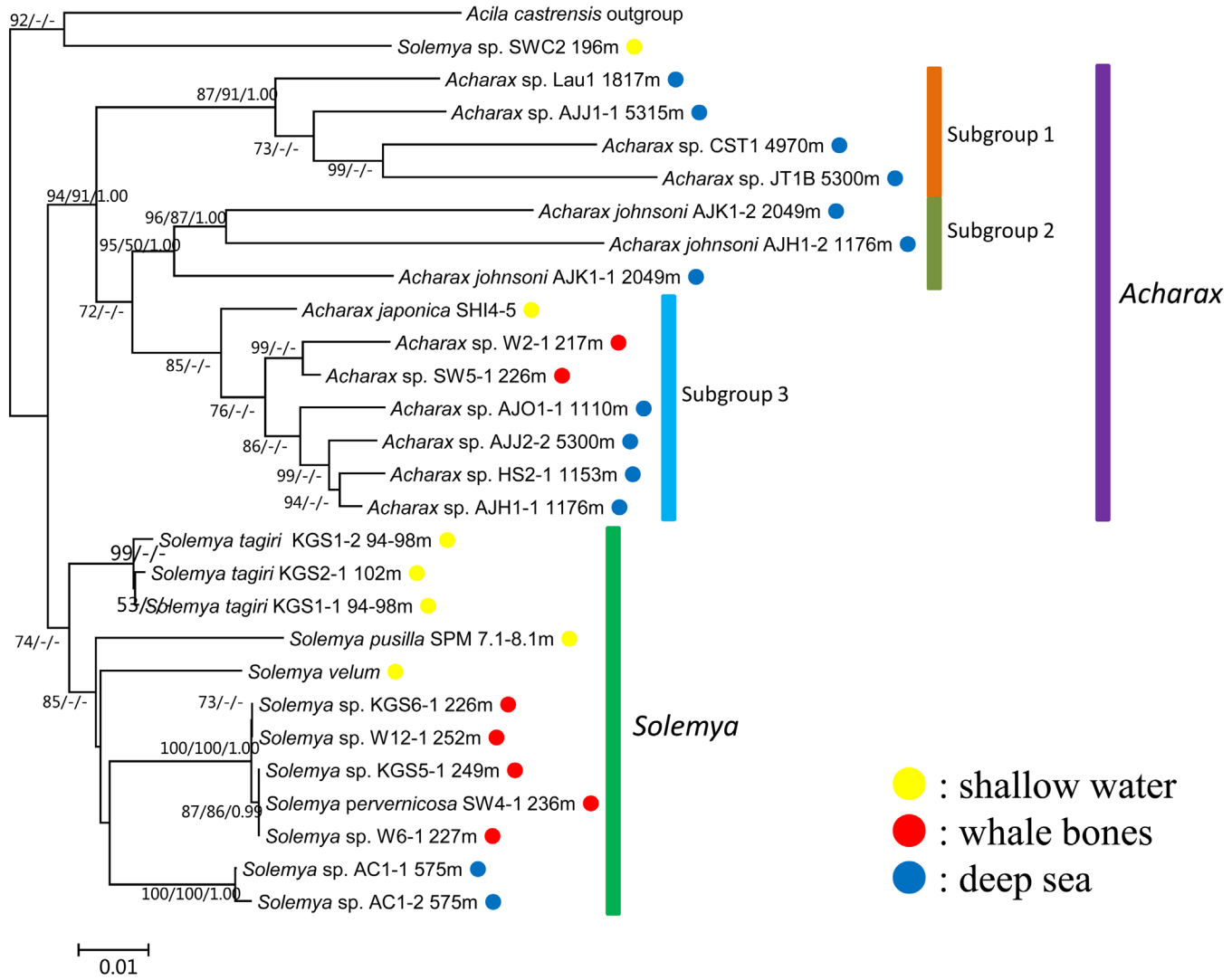

Figure 4. Phylogenetic relationships of solemyid bivalves based on concatenated 18S rRNA + COI sequences (1700 bp). The NJ, MP, and Bayesian trees were constructed using *Acila castrensis* as an outgroup species. Only the NJ (left) and MP (middle) bootstrap values ≥ 50 and the Bayesian (right) posterior probabilities ≥ 0.50 are specified. The scale bar indicates 0.01 substitutions per site.

3.2. Phylogenetic Relationships of Thyasiridae

In NJ trees based on 18S rRNA (793 bp, 231 variable sites, and 118 informative sites), COI (317 bp, 200 variable sites, and 145 informative sites), and concatenated 18S rRNA + COI (1110 bp, 400 variable sites, and 237 informative sites) sequences, *Thyasira* formed a paraphyletic group (Figures 5-7). The genera, *Thyasira* and *Maorithyas*, included specimens that have two demibranchs and symbiotic bacteria. The other genera, *Adontorhina*, *Axinopsis*, *Axinulus*, *Leptaxinus*, and *Mendicula*, formed a clade including specimens that have one demibranch and no symbiotic bacteria. Thyasirid bivalves did not diverge in the order of shallow-sea residents, whale-bone residents, and deep-sea vent/seep residents. *Thyasira kaireiae* in the Japan Trench and *Thyasira* sp. off Hatsushima formed a clade as shown by Cluster A in Figure 5, Cluster B in Figure 6, and Cluster C in Figure 7. *Thyasira sarsi* was included in the *Thyasira* cluster of the 18S rRNA tree, but diverged basally from other thyasirid bivalves in the COI tree and 18S rRNA + COI

Figure 5. Phylogenetic relationships of thyasirid bivalves based on 18S rRNA sequences (793 bp). The NJ, MP, and Bayesian trees were constructed using *Myrtea spinifera* as an outgroup species. Only the NJ (left) and MP (middle) bootstrap values ≥ 50 and the Bayesian (right) posterior probabilities ≥ 0.50 are specified. The scale bar indicates 0.01 substitutions per site. Numbers following species names denote the number of ctenidial demibranchs. A and S following the numbers or species names denote the absence and presence of symbiotic bacteria.

trees. Similarly to the above Solemyidae, the 18S rRNA tree included more data from DDBJ. In Thyasiridae, the three trees, 18S rRNA, COI, and 18S rRNA + COI, were generally consistent, although the phylogenetic position of *Thyasira sarsi* in the 18S rRNA tree was different from that in the COI and 18S rRNA + COI trees.

4. Discussion

4.1. Mytilidae

Miyazaki *et al.* [7] indicated that the "evolutionary stepping stone hypothesis" was supported in mytilid mussels, because they diverged in the order of shallow-sea residents, whale-bone residents, and deep-sea vent/seep residents. Moreover, the transition of symbiotic systems in mytilid mussels also supported the hypothesis [7]. Furthermore, Lorion *et al.* [8] revealed, by investigating many mytilids obtained from organic falls, that the evolutionary process as indicated by the "evolutionary stepping stone hypothesis" had occurred not only once in Mytilidae, but also several times in parallel.

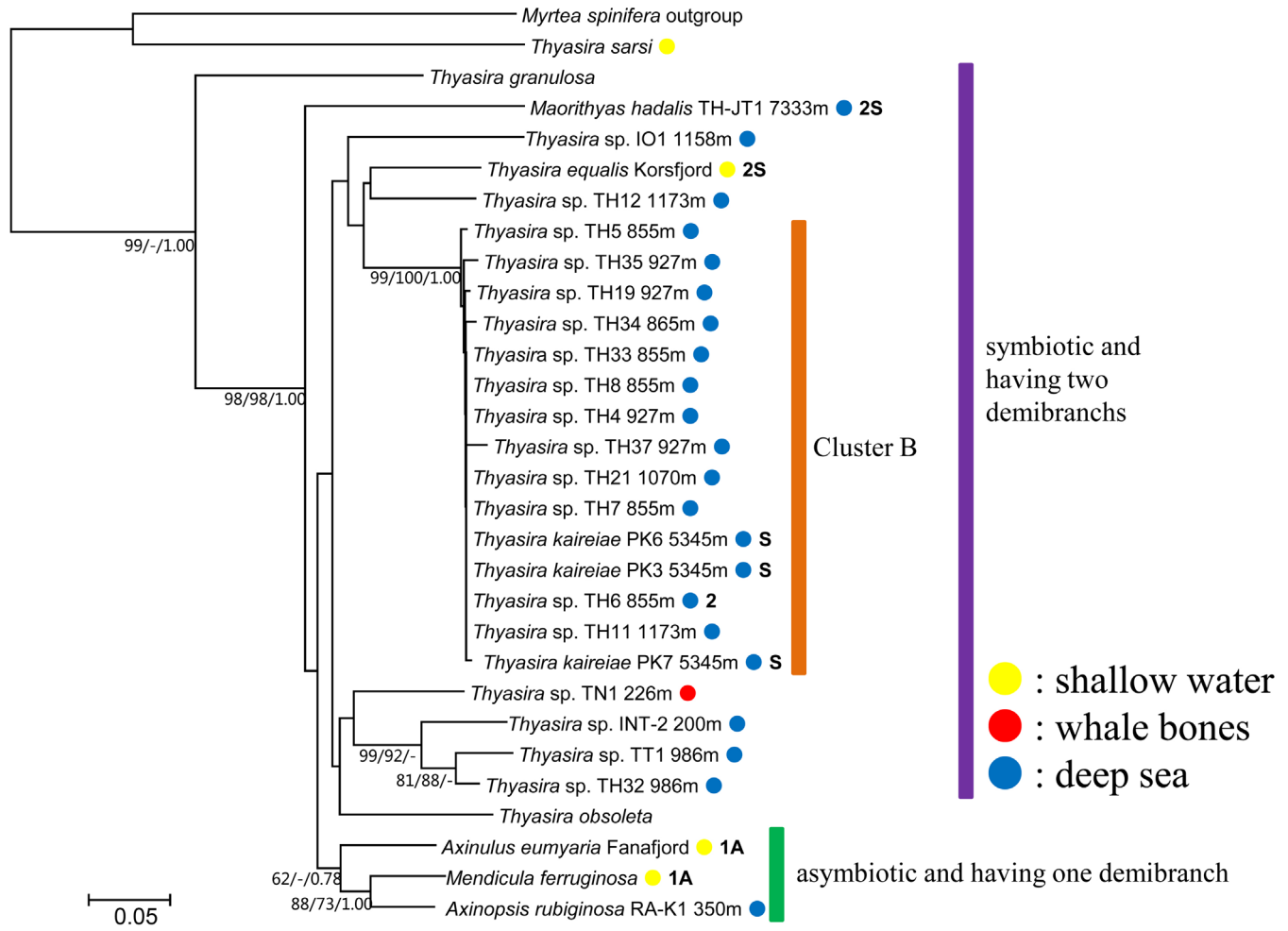

Figure 6. Phylogenetic relationships of thyasirid bivalves based on COI sequences (317 bp). The NJ, MP, and Bayesian trees were constructed using *Myrtea spinifera* as an outgroup species. Only the NJ (left) and MP (middle) bootstrap values ≥ 50 and the Bayesian (right) posterior probabilities ≥ 0.50 are specified. The scale bar indicates 0.01 substitutions per site. The numbers following the species names denote the number of ctenidial demibranchs. A and S following the numbers or species names denote the absence and presence of symbiotic bacteria.

4.2. Solemyidae

As in the mytilid mussels, splitting in the order of shallow-sea residents, whale-bone residents, and deep-sea vent/seep residents was shown in the Cluster X of the COI tree (Figure 3) and the Subgroup 3 and the *Solemya* clade of the 18S rRNA + COI tree (Figure 4). This suggested that a part of *Acharax* and *Solemya* adapted in parallel to deep-sea environments in the process indicated by the "evolutionary stepping stone hypothesis."

Acharax sp. Lau 1 and *Solemya* sp. SWC2 presented markedly divergent phylogenetic positions between the 18S rRNA and COI trees, although we used the same specimen in each taxon for sequencing. The present study cannot explain the discrepancies of their phylogenetic positions between the trees.

4.3. Thyasiridae

Thyasira bivalves did not diverge in the order of shallow-sea residents, whale-bone

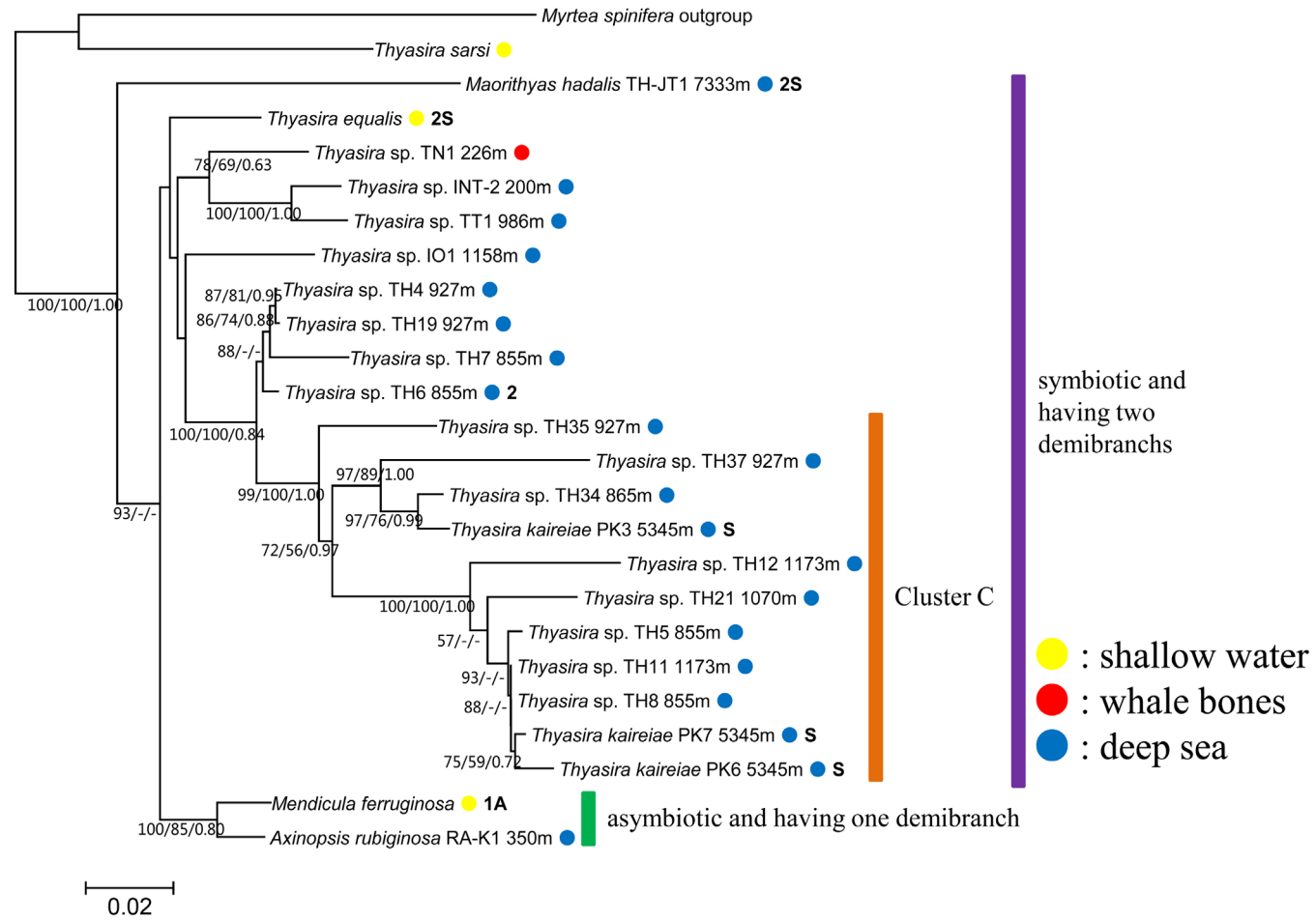

Figure 7. Phylogenetic relationships of thyasirid bivalves based on concatenated 18 S rRNA + COI gene sequences (1110 bp). The NJ, MP, and Bayesian trees were constructed using *Myrtea spinifera* as an outgroup species. Only the NJ (left) and MP (middle) bootstrap values ≥ 50 and the Bayesian (right) posterior probabilities ≥ 0.50 are specified. The scale bar indicates 0.01 substitutions per site. The numbers following the species names denote the number of ctenidial demibranchs. A and S following the numbers or species names denote the absence and presence of symbiotic bacteria.

residents, and deep-sea vent/seep residents (Figures 5-7) and it suggested that the "evolutionary stepping stone hypothesis" was not supported in this group. However, to evaluate the "evolutionary stepping stone hypothesis" in Thyasiridae, more whale-bone thyasirids have to be investigated, because we used only one whale-bone specimen.

The paraphyletic group composed of *Thyasira* and *Maorithyas* included specimens which have two demibranchs and symbiotic bacteria, whereas the clade composed of other genera, *Adontorhina*, *Axinopsis*, *Axinulus*, *Leptaxinus*, and *Mendicula*, included specimens which have one demibranch and no symbiotic bacteria. Taking the tree topologies, we assume parsimoniously that the ancestor of Thyasiridae has two demibranchs and symbiotic bacteria, and that the latter genera derived from the former genera. Two demibranchs may be advantageous for symbiosis by increasing the gill surface area where chemoautotrophic bacteria dwell and absorb hydrogen sulfide.

Our phylogenetic analysis showed that *T. kaireiae* in the Japan Trench (5345 m depth) and *Thyasira* sp. off Hatsushima (855 - 1173 m depth) were very closely related with each other and might be the same species. If that is the case, this species can be

only bivalves that inhabit deep sea with a range of over 4000 m depth.

Thyasira sarsi also indicated a discrepancy in phylogenetic positions between the 18S rRNA and COI trees. *Thyasira* sp. Fiji was closely related to *M. hadalis* in the 18S rRNA tree (**Figure 5**). We cannot determine whether this specimen was misidentified as *Thyasira*, because we did not have this specimen, and only the 18S rRNA sequence was available in the database.

4.4. Antarctica-Origin Hypothesis

The "evolutionary stepping stone hypothesis" was supported by two lineages of Solemyidae. However, we could not draw explicit conclusions whether this hypothesis was refuted in the other lineages of Solemyidae and Thyasiridae owing to the lack of whale-bone specimens, especially in Thyasiridae. If the "evolutionary stepping stone hypothesis" is not supported, a new hypothesis is needed to explain their invasion and settlement in deep sea. Therefore, we propose the "Antarctica-origin hypothesis". In this hypothesis, we assume that benthoses on the narrow continental shelf of the Antarctica are ejected from there and sunk into deep sea by expansion of the ice shelf, and survivors in the deep-sea environments expand their habitats from the Antarctic to worldwide deep sea. Shallow-sea residents around the Antarctica have been tolerable to low water temperature and all Solemyidae and some shallow-water Thyasiridae have already acquired symbiosis. Thus, symbiotic Solemyidae and Thyasiridae around the Antarctica need to acquire only tolerance to high hydrostatic pressure to invade deep-sea environments. The expansion of deep-sea organisms from the Antarctic deep sea to worldwide deep sea is supported by some studies. Held [37] showed that Serolidae (Isopoda) invaded deep-sea environments from the Antarctic region. Embryos of shallow-water urchins around the Antarctica had tolerance to high hydrostatic pressure [38]. Bivalvia, Gastropoda, Amphipoda, and Decapoda around the Antarctica had an ability to live in broader depth than those in the Atlantic [39].

5. Conclusion

To dissolve the strategies of the organisms for invasion and adaptation to deep sea, we analyzed the nuclear 18S rRNA and mitochondrial COI genes of thyasirid and solemyid bivalves, which constitute chemosynthetic communities. In the most reliable 18S rRNA + COI tree of Solemyidae, *Solemya* formed a clade. *Acharax* formed a clade composed of three subgroups, two of which consisted of only deep-sea taxa. In the most reliable 18S rRNA + COI tree of Thyasiridae, *Axinopsis* and *Mendicula* (and probably *Adontorhina*, *Axinulus*, and *Leptaxinus*) formed a clade, whereas *Thyasira* and *Maorithyas* formed a paraphyletic group to the clade. The "evolutionary stepping stone hypothesis" was supported by the *Solemya* clade and one of the *Acharax* subgroups of Solemyidae, but seemingly was not in the other lineages of Solemyidae and Thyasiridae. Nevertheless, we have to be careful in drawing a conclusion (refutation against the hypothesis), because whale-bone specimens were not enough, especially in Thyasiridae. In the present study, we represented an outline in evolutionary relationships in the two families. However, the reliabilities of the trees were partly not high, the topologies were sometimes inconsistent between trees constructed by different methods, and some taxa pre-

sented highly divergent phylogenetic positions between the trees. These warrants further molecular phylogenetic analyses using more specimens, especially those obtained from organic falls, and using other genes to elucidate phylogenetic relationships and evolutionary history in Solemyidae and Thyasiridae. In addition, morphological investigations such as counting the number of ctenidial demibranchs, which could not be done in this study because of tininess and damages of thyasirid specimens, are necessary to know adaptive changes in the evolutionary process.

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