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Authors: Hikosaka-Katayama, Tomoe, Koike, Kanae, Yamashita, Hiroshi, Hikosaka, Akira, and Koike, Kazuhiko

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Mechanisms of Maternal Inheritance of Dinoflagellate Symbionts in the Acoelomorph Worm *Waminoa litus*

Tomoe Hikosaka-Katayama[1]*, Kanae Koike[1], Hiroshi Yamashita[2], Akira Hikosaka[3], and Kazuhiko Koike[4]

[1]*Center for Gene Science, Hiroshima University, 1-4-2 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8527, Japan*
[2]*Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Fisheries Research Agency, Fukai-Ohta, Ishigaki, Okinawa 907-0451, Japan*
[3]*Graduate School of Integrated Arts and Sciences, Hiroshima University, 1-7-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8521, Japan*
[4]*Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan*

Waminoa litus **is a zooxanthella-bearing acoel worm that infests corals. It is unique to Bilateria in that it transmits its algal symbionts vertically via eggs irrespective of the heterogeneity of the symbionts. It simultaneously harbors two dinoflagellate genera: *Symbiodinium* and *Amphidinium*. In this study, we examined the timing and vertical transmission pathway of algal symbionts in *W. litus* using light and electron microscopy. The oogenesis of the worm can be divided into three stages: stage I, in which the ovary is absent; stage II, the early vitellogenic zone containing immature oocytes formed in the ovary; and stage III, with both early and late vitellogenic zones in the body. In the early vitellogenic zone at stage II, oocytes are surrounded by accessory-follicle cells (AFCs). Both *Symbiodinium* and *Amphidinium* symbionts are not initially observed in the oocytes, but are observed in the AFCs. In the late vitellogenic zone at stage III, oocytes are enveloped by a complete sheath of AFCs; the algal symbionts are taken up by the late vitellogenic oocytes. These observations suggest that AFCs mediate the transfer of the algae from the parent to the oocytes. Ribotype analyses of the *Symbiodinium* symbionts revealed that they differ from those harbored by coral in the same experimental aquarium. These results indicate that *W. litus* has an active algal transport pathway and maintains a specific lineage of *Symbiodinium* via vertical transmission.**

Key words: algal symbiosis, Acoela, dinoflagellate, oogenesis, vertical transmission, *Symbiodinium*, *Amphidinium*

INTRODUCTION

Coral reef ecosystems are among the most biodiverse habitats in marine environments. In such systems, photosynthetic dinoflagellates, which are often called zooxanthellae, establish mutualistic symbioses with numerous invertebrates, including sponges, cnidarians, molluscans, and acoels. They play important roles in the biodiversity of such oligotrophic waters by translocating their photosynthetic products to the host animals. In the initial stage of such symbiosis, the host animal must acquire zooxanthellae in one of two ways: vertically from the parent host to offspring, or horizontally from the environment. Symbiont acquisition fails less frequently in the former, providing an advantage in terms of probability of establishing symbiosis; however, vertical transmission is uncommon in most host animals and is described in less than 15% of cnidarian species (Schwarz et al., 2002). This may be attributable to mechanistic difficulties in the transmission process during oogenesis. The anthozoan oocyte is enveloped by a thin mesogleal layer and an outer layer of endodermal follicular cells. In anthozoan species that transmit zooxanthellae vertically, the follicular cells mediate the transfer of the symbionts from the outer cells of the parent to the oocyte during oogenesis in the breeding season (Benayahu et al., 1992; Hirose et al., 2001). These observations suggest that follicular cells play important roles in this transmission pathway.

Acoel species living in coral reefs are interesting because of their unique symbiotic relationships with various algal symbionts. For example, *Convolutriloba longifissura* harbors the prasinophyte alga *Tetraselmis* sp. (Hirose and Hirose, 2007); *Waminoa* acoels, which are epizoic on living corals, harbor two types of dinoflagellate symbionts: *Symbiodinium* sp. and *Amphidinium* sp. (Winsor, 1990; Ogunlana et al., 2005), an exceptional case in which two different genera of algae coexist in a single host. *Waminoa* worms can take up coral mucus as a food source from the coral

* Corresponding author. Tel. : +81-82-424-4630;
Fax : +81-82-424-3498;
E-mail: tomkata@hiroshima-u.ac.jp
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surface (Naumann et al., 2010), and species of this genus have been found in the Red Sea (Barneah et al., 2007a), Australia (Winsor, 1990), and Indonesia (Haapkylä et al., 2009). *Waminoa* infestations sometimes completely cover the host coral in the field. Interestingly, coral reef hobbyists often report infestations by *Waminoa* acoels in their aquarium. Thus, the worms can be easily maintained in the laboratory with a host coral (Hikosaka-Katayama and Hikosaka, 2010). *Waminoa*'s host coral also contains *Symbiodinium* but not *Amphidinium*.

Acoels are mostly small free-living animals that lack a gut lumen. They have traditionally been classified in the phylum Platyhelminthes (Brusca and Brusca, 1990). However, early phylogenetic studies based on the 18S rRNA gene indicate that Acoela is the earliest bilaterian (Katayama et al., 1993; Ruiz-Trillo et al., 1999). Since that report, the position of Acoela as basal bilaterians has been tested using many phylogenetic analyses. In 2004, the group was placed in a new phylum, Acoelomorpha (Baguñà and Riutort, 2004). However, Philippe et al. (2011) recently proposed a different phylogenetic relationship, in which Acoelomorph flatworms are deuterostomes related to Xenoturbella, and hence their phylogenetic position remains controversial.

Acoels are hermaphroditic, possessing both female and male gonads, and typically copulate with mutual cross-insemination (Hyman, 1951). Acoels produce endolecithal eggs covered with individual eggshells. Oogenesis comprises two phases: previtellogenic and vitellogenic. Developing oocytes of acoels are surrounded by accessory-follicle cells (AFCs) (Falleni and Gremigni, 1990; Falleni et al., 1995; Raikova et al., 1995).

Algal symbionts can be inherited by newborn acoel worms derived from both asexual and sexual reproduction. In asexually produced (i.e., fission or budding) worms, symbiotic algae are directly transmitted in buds or fragments that form new worms (Åkesson et al., 2001). In sexually produced acoels (e.g., *Symsagittifera roscoffensis*) in general, a new generation of larvae acquire algal symbionts from their environment (i.e., horizontal transmission) (Douglas, 1983). In contrast, *W. brickneri* larvae inherit their symbionts directly from their parents (i.e., vertical transmission) (Barneah et al., 2007b). This is the first example of vertical transmission of algal symbionts via sexual reproduction in Acoela. However, to our knowledge, there are no published reports on the process and mechanism of algal symbiont transfer from maternal tissues to oocytes in *Waminoa* acoels.

In this study, we investigated the process of oogenesis from the early to late vitellogenic stages, as well as the location of the algal symbionts in *W. litus* ovaries during oogenesis, to elucidate the timing and pathway of vertical transmission of algal symbionts. In addition, we report the molecular phylogeny of symbionts in *W. litus* and the symbiont of the host coral infested by the acoels.

MATERIALS AND METHODS

Animals

A *W. litus*-infested stony coral, *Symphyllia valenciennesii*, was purchased from a pet shop (Ocean Life, Hiroshima, Japan) in February 2009 and maintained in laboratory cultures following the methods described by Hikosaka-Katayama and Hikosaka (2010). The worms were collected from the coral surface using a Komagome pipette. *Waminoa* sp. 1 was collected from *Trachyphyllia geoffroyi* purchased from another pet shop (Takayama, Hiroshima, Japan) and maintained in another aquarium in the laboratory. *Waminoa* sp. 2 was collected from *Acropora vaughani* maintained in a private aquarium (Higashi-Hiroshima, Hiroshima, Japan). The original localities of the corals are unknown.

Microscopy

Sexually immature and mature *W. litus* specimens were transferred to finger bowls containing filtered culturing seawater. Whole adult worms were narcotized with 10% magnesium chloride and compressd with a slide and cover glass. To determine the maturation stage of the ovary, we observed slides using a stereomicroscope (Leica, MZFLIII; Leica microscopy systems Ltd., Heerbrugg, Switzerland). For light and electron microscopic observations, early- and late-stage specimens were fixed with 2.5% glutaraldehyde in seawater for several days at room temperature. The materials were postfixed with 1.5% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) for 1.5 h at 4°C followed by several washes in the buffer without fixative. After dehydration in an ethanol series, the samples were embedded in EPON 812 (TAAB, Berkshire, UK) and subsequently polymerized at 35°C, 45°C, and 60°C for four days. Sections were cut using an Ultracut E ultramicrotome (Reichert-Jung, Austria) with a diamond knife. For light microscopic observations, 0.5–1-μm-thick sections were stained with 0.05% toluidine blue. Ultrathin sections were stained with 3% (w/v) uranyl acetate for 15 min and lead citrate for 5 min, and observed under a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan) operating at an accelerating voltage of 80 kV.

DNA extraction, amplification, cloning, and sequencing of the *Symbiodinium* ITS-rDNA region

Total DNAs of *Waminoa* species and their symbionts were extracted from 10–20 worms using a NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany). Total DNA of the host coral *S. valenciennesii* and its symbiont algae was extracted from 3–5 tentacles in the same manner. Nuclear internal transcribed spacers (ITS-1-2) and the 5.8S regions of the rRNA gene (i.e., the ITS-rDNA regions) of *Symbiodinium* symbionts were amplified as described by Santos et al. (2001). The purified PCR amplicons were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Several clones were then sequenced using an ABI Genetic Analyzer 3130 × l (PE Applied Biosystems, Foster City, CA, USA) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The sequences determined in this study were deposited in GenBank/EMBL/DDBJ under the accession numbers AB610858–AB610872.

Sequence alignment and phylogenetic analysis of *Symbiodinium*

The obtained sequences were aligned with the homologous sequences sampled from GenBank/EMBL/DDBJ by Clustal X version 2.0 (Larkin, 2007). The alignment was then visually inspected and manually edited. All ambiguous sites observed at the 5′ and 3′ ends of alignments were removed from the dataset for phylogenetic analyses. The edited alignment included 661 sites of 35 taxa, including the clade-F *Symbiodinium* isolated from foraminifera species as an outgroup, and was used for phylogenetic analyses. Maximum likelihood (ML), neighbor joining (NJ), maximum parsimony (MP), and Bayesian analyses were conducted for the aligned sequence. ML analysis was performed using PhyML (Guindon and Gascuel, 2003) with the Hasegawa, Kishino, and Yano model (HKY85; Hasegawa et al., 1985) of nucleotide substitution, and the parameters were estimated from the dataset. ML bootstrap trees (100 replicates) were constructed using the same parameters described above. NJ and MP analyses were performed using the MEGA 4.0 program (Tamura et al., 2007). The sites containing gaps

were completely deleted from the dataset, and the remaining 622 sites were used for NJ and MP analyses. NJ analysis was carried out with Kimura's two-parameter model (Kimura, 1980). The close-neighbor-interchange method on random trees was chosen for the MP tree search. NJ bootstrap trees (1,000 replicates) and MP bootstrap trees (100 replicates) were also constructed. In addition, Bayesian trees were constructed using MrBayes 3.12 (Ronquist and Huelsenbeck, 2003) with the HKY model, which was the best model selected by MrModeltest 2.3 (Nylander, 2004). One cold and three heated Markov chain Monte Carlo (MCMC) chains at default temperatures were run for 5,000,000 generations while sampling log likelihoods and trees at 100-generation intervals. The first 1,250,000 generations were set as "burn-in," and Bayesian posterior probabilities were estimated from the remaining 37,500 trees.

Molecular identification of *Amphidinium*

To assess *Amphidinium* within *W. litus* isolated from *S. valenciennesii*, a nearly full-length nuclear small subunit ribosomal DNA (SSU-rDNA) sequence was determined by direct sequencing using a primer set of TimA and TimB (Noren and Jondelius, 1999). In addition, a partial nuclear large subunit ribosomal DNA (LSU-rDNA) sequence (regions D1–D6) was amplified using a primer set of 1F and 11R (Iwataki et al., 2008). The purified PCR amplicons were cloned and sequenced as described above. Sequences similar to the obtained sequences were searched in the database, and an ML tree was constructed with other *Amphidinium* species using MEGA version 5 (Tamura et al., 2011).

RESULTS

Staging of ovarian maturation during oogenesis

We examined the sexual maturity of adult *W. litus* individuals in our experimental aquarium and defined three stages of ovarian maturation using the unaided eye (Fig. 1A–C) and stereomicroscopic observations (Fig. 1D–F). Fig. 1A–C shows *W. litus* worms on the coral *S. valenciennesii*. Their cinnamon-brown body color is derived from abundant algal symbionts. At the no-ovary (previtellogenic) stage (stage I), the androgenic copulatory apparatus was visible as a white spot, and no ovaries were observed in the bodies of premature individuals (Fig. 1A, D). At the early vitellogenic stage (stage II), vitellogenic oocytes were recognizable as whitish dots to the unaided eye (Fig. 1B). A pair of ovaries was located across the midline in the anterior half of the body (Fig. 1E); each ovary contained about 50–70 oocytes in this zone (early vitellogenic zone). At the late vitellogenic stage (stage III), the ovaries extended to the posterior of the body (late vitellogenic zone) in fully mature adults (Fig. 1C). Mature worms had two clear whitish areas (mature ovaries) that cast a shadow in the center of the posterior body with transmitted light under the stereomicroscope (Fig. 1F, inset). At this stage, the ovaries contained both early and late vitellogenic oocytes in the anterior and posterior zones, respectively (Fig. 1F). Many elongated oocytes were observed in the posterior zone (Fig. 1F).

Localization of algal cells in the *Waminoa* ovary at the early vitellogenic stage

Cells of the two types of algae were distributed in the parenchyma of adult *W. litus* worms (Fig. 2A), as observed in *W. brickneri* (Ogunlana et al., 2005). The more abundant and smaller type (7–11 μm) was identified as a species of *Symbiodinium* due to its characteristic pyrenoid covered by a starch layer and a thick cell-wall structure (Fig. 2B). The larger type (18–25 μm) was tentatively identified as a species of *Amphidinium* due to its characteristic epicone projec-

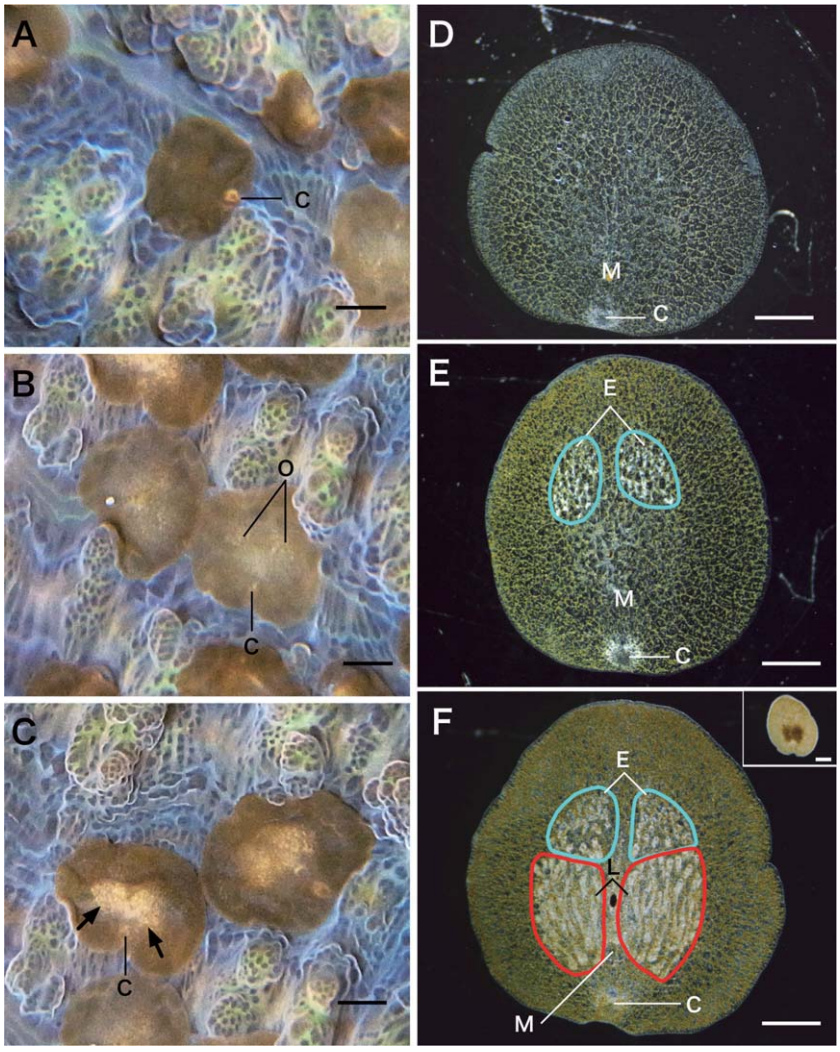

Fig. 1. **(A–C)** Stages of sexual maturity in *W. litus* on the body surface of the coral *Symphyllia valenciennesii*. **(A)** Stage I: no-ovary stage; **(B)** stage II: early vitellogenic stage; **(C)** stage III: late vitellogenic stage. Arrows indicate mature ovaries. **(D–F)** Stereomicroscopic views of compressed specimens of sexually maturing *W. litus*. The worms are larger than the originals because of the compressing preparation. **(D)** Stage I: no-ovary stage; **(E)** stage II: early vitellogenic stage; **(F)** stage III: late vitellogenic stage. Inset: transmitted light view of narcotized specimen. C, male copulatory apparatus; E, zone of the early vitellogenic oocytes; L, zone of the late vitellogenic oocytes; M, mouth; O, ovary. All scale bars are 1 mm.

tion (Fig. 2C). *Symbiodinium* is also a symbiont of a coral in our aquarium (*S. valenciennesii*), whereas *Amphidinium* is not.

The oocytes in the early vitellogenic zone (early vitellogenic oocytes) were 50–100 μm in diameter. At this stage, each oocyte had a large rounded nucleus with a prominent nucleolus (Fig. 2D, E). Numerous mitochondria were observed gathered along the nuclear envelope (Fig. 2E). The surfaces of the oocytes were not covered with microvilli unlike those of anthozoans (Benayahu et al., 1992; Hirose et al., 2001) and other animals (Huebner and Anderson, 1976).

Localization of the algal cells in the early vitellogenic zone was examined using light and electron microscopy. In this zone, the oocytes were surrounded by AFCs (Fig. 2D). The algal cells were not found within the oocytes, but were distributed intracellularly within the AFCs surrounding the oocytes (Fig. 2D, E). Both the symbiotic species were observed in the AFCs (Fig. 2D) and were contacted by thin cytoplasmic projections of the AFCs (Fig. 2D). In the AFCs, algal cells were not covered by a perialgal membrane, which is commonly observed in cnidarian–*Symbiodinium* systems (e.g., the symbiosome membrane reported in Hinde and Trauman (2002)), and were directly distributed in the cytoplasm (Fig. 2E). *Symbiodinium* cells in the cytokinesis phase were occasionally observed, indicating that they proliferate in the cytoplasm of the AFCs (data not shown).

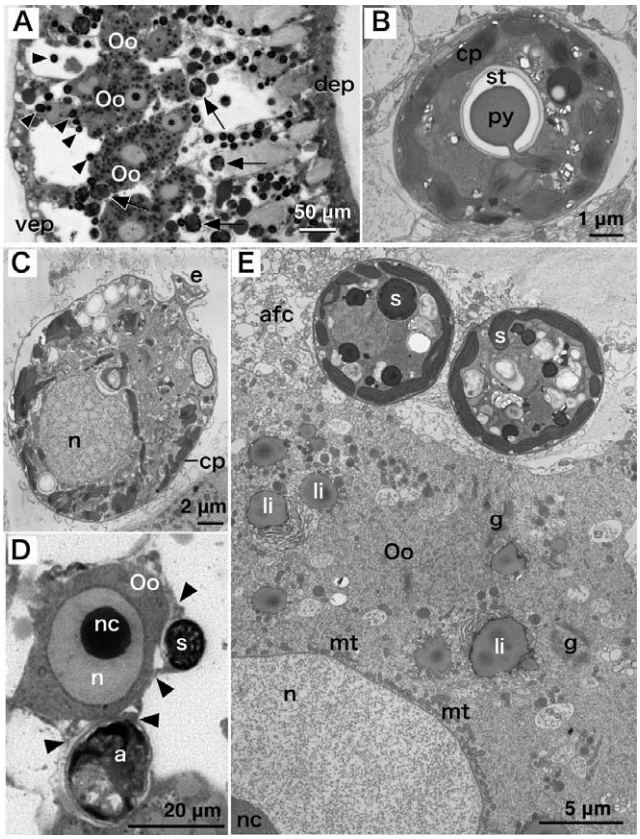

Fig. 2. Symbionts of *W. litus* at the early vitellogenic stage. **(A)** Light micrograph of the early vitellogenic zone in a longitudinal section of *W. litus*. The larger algal cells (black arrows) are *Amphidinium* symbionts, and the smaller ones (black arrowheads) are *Symbiodinium* symbionts. dep, dorsal epidermis; vep, ventral epidermis; Oo, oocyte. **(B)** Transmission electron micrograph of a *Symbiodinium* cell within the parenchyma of *W. litus*. cp, chloroplast; py, pyrenoid; st, starch. **(C)** Transmission electron micrograph of an *Amphidinium* cell within the parenchyma of *W. litus*. cp, chloroplast; e, epicone; n, nucleus. **(D)** Light micrograph of an early vitellogenic oocyte in a horizontal section of *W. litus*. An oocyte (Oo) surrounded by the accessory-follicle cell (AFC) cytoplasm (arrowheads) containing the algal cells (i.e., *Symbiodinium* (s) and *Amphidinium* (a) cells). n, nucleus; nc, nucleolus. **(E)** Electron micrograph of an early vitellogenic oocyte and AFC. An early vitellogenic oocyte (Oo) surrounded by an AFC (afc) containing *Symbiodinium* cells (s). g, Golgi complex; li, lipid; mt, mitochondria; n, nucleus; nc, nucleolus.

Localization of algal cells in *Waminoa* ovary at the late vitellogenic stage

At the late vitellogenic stage, the oocytes in the late vitellogenic zone (late vitellogenic oocytes) were larger than those in the early vitellogenic zone (Fig. 3A). The large nuclei became distorted, and the ooplasm invaginated into the nuclear location (Fig. 3A). At this stage, *Amphidinium* and *Symbiodinium* cells were found not only in the AFCs but also in the oocytes (Fig. 3A). The oocytes were entirely enclosed by a thin cytoplasmic layer of the AFCs containing abundant long cisternae of rough endoplasmic reticulum (RER) (Fig. 3B). In the AFCs, some algae were occasionally encased within symbiosome membranes (Fig. 3B), while the others were not enveloped by a membrane (Fig. 3C). Closely aligned vesicles (50–500 nm) were observed at regular intervals (300–700 nm) on the inside of the symbiosome membrane (Fig. 3D, E), and membranous structures, which appeared to have detached from *Symbiodinium* cells, were often observed in this space.

The cell membranes at the interface between an oocyte and AFC were not apparent in places (i.e., the "membrane-devoid region," MDR) (Fig. 4A–D). In the AFCs, some algal cells and lipid droplets were observed near the openings of the MDRs (Fig. 4B, C), and a vesicle was detected on the MDR (Fig. 4D).

No microvilli were observed on the surface of the oocytes at this stage, as in the previous stage (Fig. 3B). The cell membrane of the AFC was in close contact with the oolemma (Fig. 3E). The oocytes and the AFCs were interdigitated in places (Fig. 4A, E). An electron-dense eggshell was observed in the space between the cytoplasmic membranes of the AFC and oolemma (Fig. 4A).

In the oocytes, algal cells were distributed evenly throughout the ooplasm, in a similar manner to yolk granules, mitochondria, the Golgi complex, and lipid droplets. Symbiosome membranes of the algal cells were not observed any more in the oocytes; rather, randomly deformed vesicles appeared around the algal cells (Fig. 4F, G). Furthermore, some dividing *Symbiodinium* cells were observed within the oocytes (Fig. 4G).

Molecular phylogenetic analysis of *Symbiodinium* ITS-rDNA sequences derived from *W. litus* and the host coral

In our experimental aquarium, *W. litus* worms were maintained for more than two years with a host coral that also harbored *Symbiodinium*. The worms can proliferate by asexual reproduction, as worm fragments have the capacity

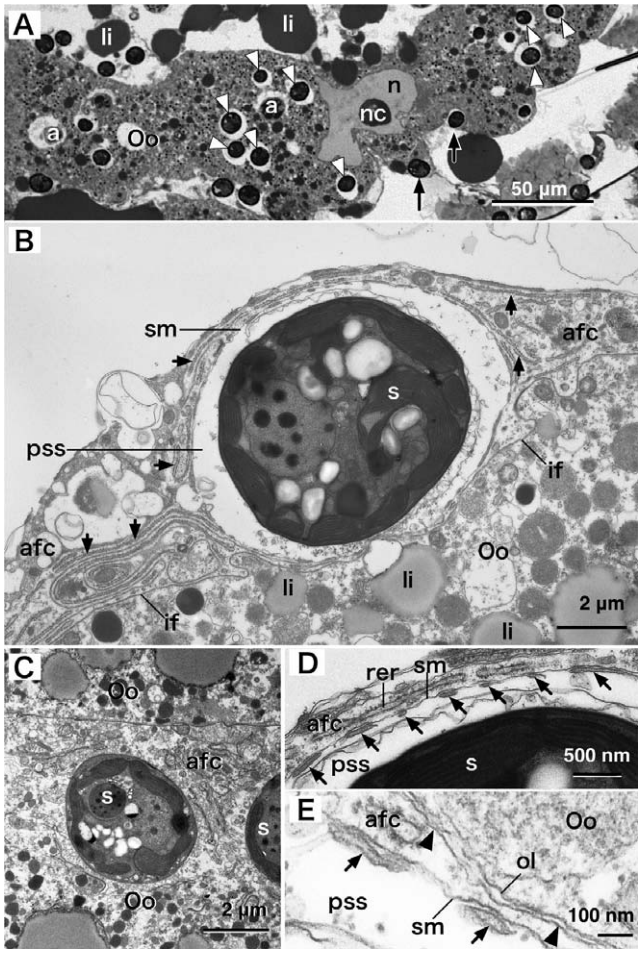

Fig. 3. **(A–C)** Symbionts of *W. litus* in the late vitellogenic stage. **(A)** Light micrograph of a late vitellogenic oocyte. *Symbiodinium* cells (white arrowheads) and *Amphidinium* cells (a) are taken up by the oocyte (Oo). Arrows indicate *Symbiodinium* cells present in AFCs. li, lipid; n, nucleus; nc, nucleolus. **(B)** Electron micrograph of a *Symbiodinium* cell (s) enclosed within an AFC (afc) that encloses an oocyte (Oo) at the late vitellogenic stage. Arrows, rough endoplasmic reticulum; if, AFC–oocyte interface; li, lipid; sm, symbiosome membrane; pss, peri-symbiont space. **(C)** Electron micrograph of two *Symbiodinium* cells (s) in an AFC (afc) located between two oocytes (Oo) at the late vitellogenic stage. Neither a symbiosome membrane nor peri-symbiont space is present around the algae. **(D–E)** Electron micrographs of symbiosome membrane (sm) and the surrounding narrow AFC (afc) cytoplasm. **(D)** Enlarged view of symbiosome membrane. **(E)** High-magnification view of an oocyte–AFC interface. Arrows, vesicles; arrowheads, cytoplasmic membrane of the AFC; Oo, oocyte; ol, oolemma; rer, rough endoplasmic reticulum; sm, symbiosome membrane; pss, peri-symbiont space; s, *Symbiodinium* cells.

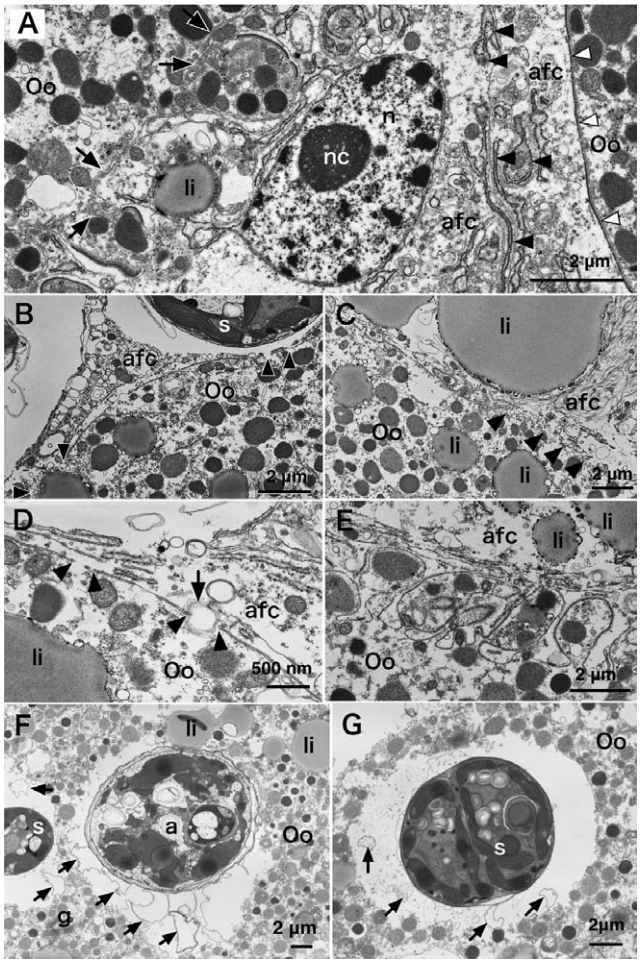

Fig. 4. **(A–E)** Electron micrographs of the oocyte–AFC interface in the late vitellogenic stage. **(A)** Two AFCs (afc) lie between two late vitellogenic oocytes (Oo). Black arrowheads indicate RER. The oocytes and left AFC are interdigitated, and the membrane between them disappears in patches (arrows: "membrane-devoid region," MDR). Eggshell formation has already begun at the surface of the oocyte lying on the right side (white arrowheads). **(B)** A *Symbiodinium* cell (s) enclosed by an AFC located adjacent to the interface. Arrowheads indicate interruptions in the membranes. **(C)** Lipid droplets adjacent to the interface. The membranes are almost absent on the right side of the interface (arrowheads). **(D)** Magnified view of MDR (arrowheads) at the interface. A vacuole (arrow) located at an opening in the membranes. **(E)** Interdigitation of the interface. li, lipid droplet; n, nucleus; nc, nucleolus. **(F–G)** Electron micrographs of symbionts within late vitellogenic oocytes. **(F)** A *Symbiodinium* cell (s) and an *Amphidinium* cell (a) in an oocyte (Oo). No symbiosome membrane is present around either of the symbionts. **(G)** A dividing *Symbiodinium* cell. Arrows indicate randomly deformed vesicles. li, lipid droplet; g, Golgi complex.

to regenerate the whole body (unpublished observation). Furthermore, the worms appeared to continue undergoing sexual reproduction in the aquarium as we occasionally observed *Waminoa* larvae (data not shown). To investigate whether *W. litus* retained a specific lineage of *Symbiodinium* regardless of this co-cultivating condition, we investigated an ITS-rDNA region of *Symbiodinium* within *W. litus* and its host coral, and also within other *Waminoa* species (*Waminoa* sp. 1 and sp. 2).

The sequences of the *Symbiodinium* ITS-rDNA region obtained from *W. litus* in our experimental aquarium included four different sequences. A BLAST search of the DNA databases retrieved the highest hit for the sequence as clade-C *Symbiodinium* harbored by a bivalve *Corculum cardissa* (99% [636/639], AB294632). The sequences obtained from several tentacles of the aquarium coral *S. valenciennesii* included three different *Symbiodinium*

sequences; the highest similarity was observed with clade-C *Symbiodinium* of type 152 clone C1_1179 from an anthozoan *Rhodactis osculifera* (former *Discosoma sanctithomae*) (99% [633/637], EU074885).

In the ML tree (Fig. 5), *Symbiodinium* from *Waminoa* species were robustly clustered with symbionts of *C. cardissa* (bootstrap probabilities ML = 95%, NJ = 88%, MP = 88%, and 1.00 posterior probability). Although the bootstrap probability was relatively low for monophyly of the symbionts of *Waminoa* species (ML = 70%, NJ = 74%, MP = 62%, 0.97 posterior probability), this lineage was distinct from the lineage comprising *Symbiodinium* harbored by *S. valenciennesii*, the host coral of *W. litus*.

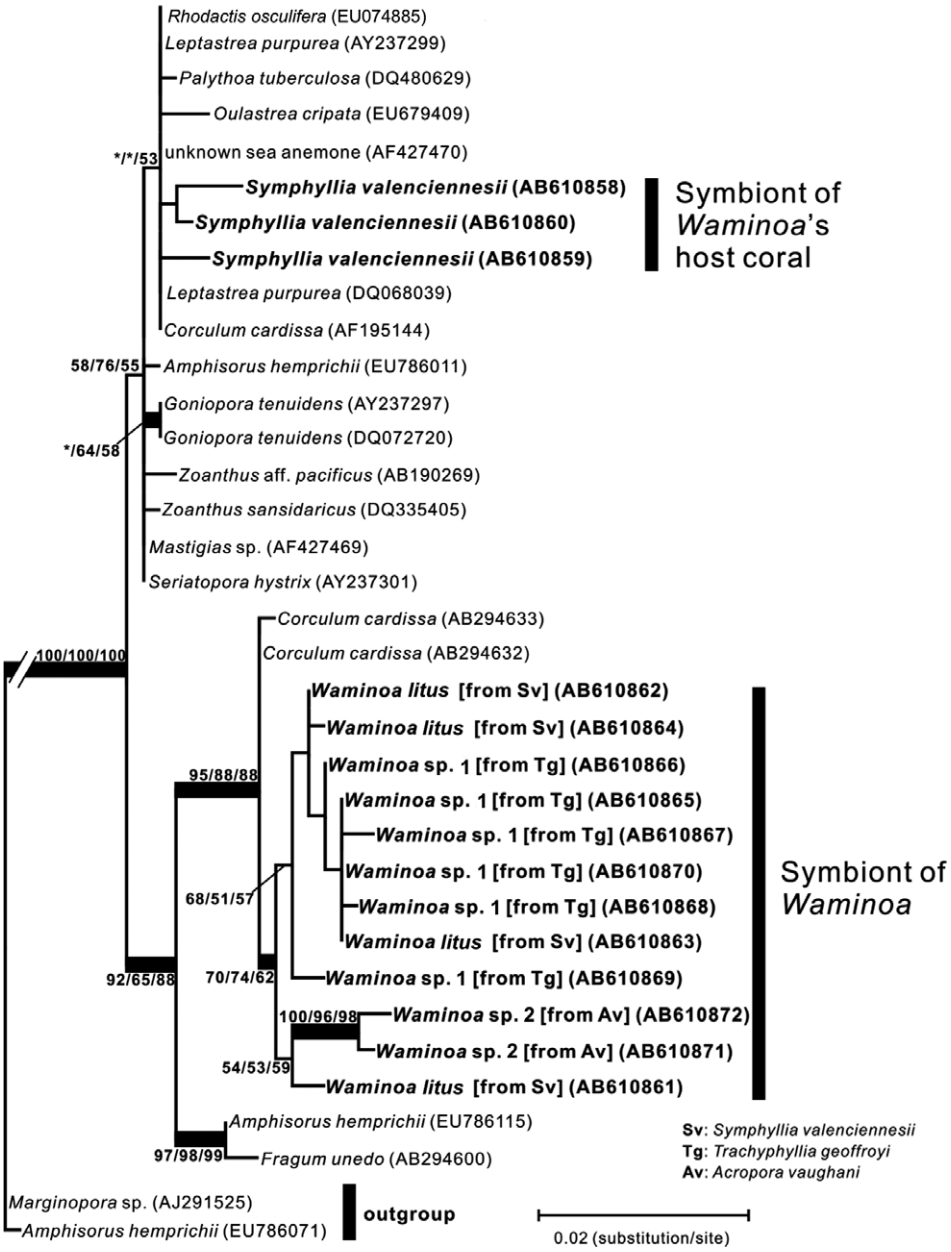

Fig. 5. Maximum likelihood phylogeny of the ITS-1, ITS-2, and 5.8S rRNA gene sequences from clade-C *Symbiodinium*. Two clade-F *Symbiodinium* were used to root the tree. Bootstrap probabilities are shown for nodes with support over 50% (ML/NJ/MP). The thick branches represent branches with Bayesian posterior probabilities greater than 0.80. Asterisks indicate support values less than 50%; sequences from *Waminoa* symbionts and symbionts of *Waminoa*'s host coral are indicated by bold font; *Waminoa* hosts are in brackets; sequence accession numbers are in parentheses.

Phylogenetic affinity of *Amphidinium* SSU/LSU ribosomal DNA sequence

A nearly full-length SSU-rDNA sequence (AB626895) was determined for the *Amphidinium* sp. within *W. litus*. A BLAST search revealed similarities with *A. belauense* (99% [1704/1707], L13719), *Amphidinium* sp. strain Y42 (99% [1704/1707], AB107845), and *A. klebsii* strain CMSTAC018 (99% [1703/1708], EU046335).

We further investigated nuclear LSU-rDNA sequences to more precisely determine the phylogenetic positions of the *Amphidinium* symbionts. Partial sequences obtained from *W. litus* (AB626894) were nested within an *A. klebsii-gibbosum* clade (Fig. 6) and produced the highest identities to those of the *A. gibbosum* strain SI-36-50 (99% [1323/1333], AY460587), *A. gibbosum* strain CCMP120 (99% [1284/1291], AY455672), and *A. klebsii* strain CMSTAC018 (98% [1277/1290], EU046328).

DISCUSSION

Waminoa spp. inherit their symbionts vertically. In this study, we divided the ovarian development of *W. litus* into three stages. Further, we investigated the timing and pathway of the entry of algae into oocytes by examining the localization of algal cells during oogenesis. A schematic view of the symbiont transfer is shown in Fig. 7. Our observations suggest that the oocytes originate in the anterior zone (early vitellogenic zone), and migrate and elongate toward the posterior zone (late vitellogenic zone) as oocyte maturation proceeds (Fig. 1D–F). Algal cells were located outside the oocytes at the early vitellogenic stage (Fig. 2D, E), indicating that algae are not continuously retained in the germline cells, but are taken up into oocytes during oogenesis. Our results also reveal that algal cells are initially present in AFCs (Fig. 2D, E) and subsequently enter the oocytes at the late vitellogenic stage (Figs. 3A, 4F, G). Furthermore, the plasma membrane of an AFC is attached to that of an oocyte, and inclusions

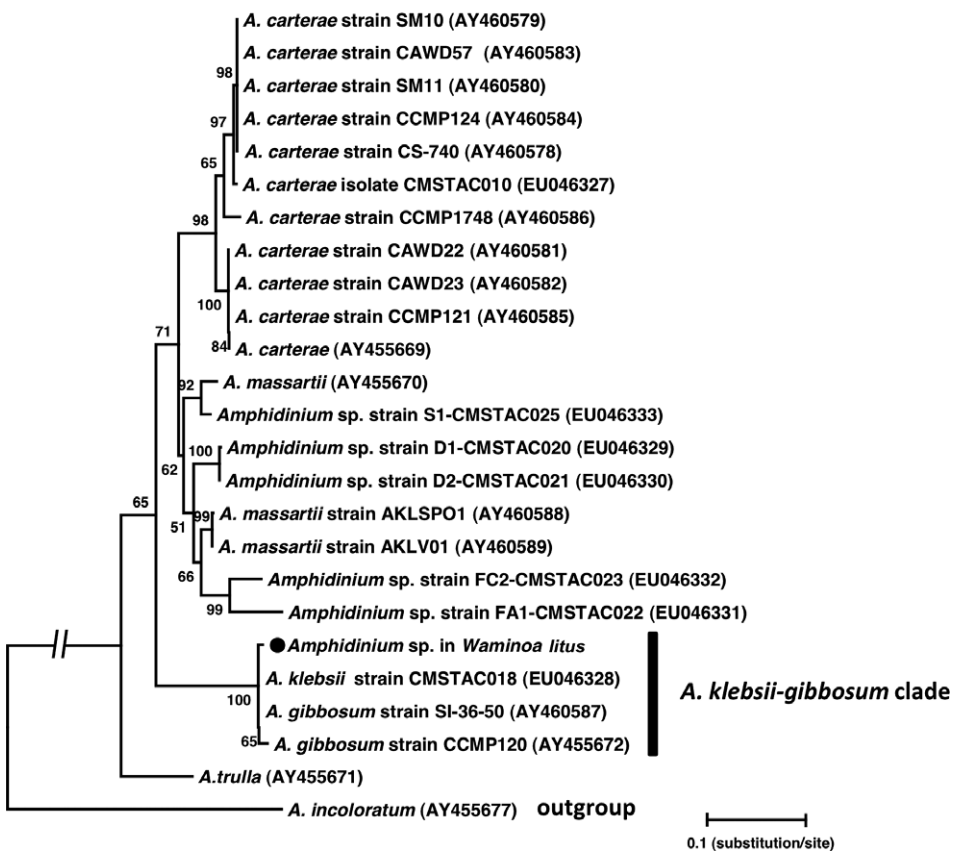

Fig. 6. Maximum likelihood phylogeny of the partial large subunit (LSU) rDNA gene sequence (regions D1–D6) from *Amphidinium* sp. within *W. litus* (solid circle). *Amphidinium* sp. within *W. litus* is nested within a *A. klebsii-gibbosum* clade and with a strain isolated from an acoel *Amphiscolops langerhansi* (CCMP120). The *A. gibbosum* strain SI-36-50 is free-living. The habitat of the *A. klebsii* strain is unkown.

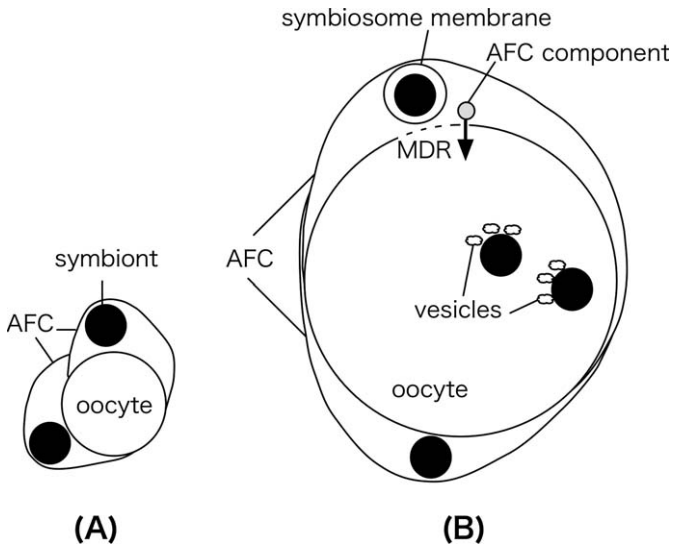

Fig. 7. Schematic diagram showing localization of symbionts in *Waminoa* ovary at the **(A)** early and **(B)** late vitellogenic stages (see Discussion for detail).

of the AFC appear to flow into the oocyte via the MDR (Fig. 4A–D). These observations suggest that *Waminoa* spp. may divert the function of AFCs in oogenesis into the algal transport pathway. As eggshell formation began in the later vitellogenic stage (Fig. 4A), the algae could not be taken up after this stage.

It is unclear how AFCs obtain symbionts and whether these symbionts localize intracellularly or intercellularly in the parenchyma. One possibility is that symbionts are intracellular throughout the *Waminoa* life cycle, and that parenchymal cells containing symbionts differentiate into AFCs. Alternatively, symbionts may temporarily enter the cytoplasm during oogenesis and ontogenesis. It is also unclear how subcellular localization of the symbionts changes during their transfer. It appears that symbionts are, in most cases, directly present in the cytosol of AFCs and oocytes. In our study, no symbiosome membrane was detected around the algae in AFCs in the early vitellogenic zone. In the late vitellogenic stage, however, a *Symbiodinium* cell was observed within a symbiosome membrane (Fig. 3B) in the AFC which contains abundant RER. The membrane appeared to be newly formed in the AFC cytoplasm and transiently enclosed the dinoflagellate cell; however, its function is unclear. It is uncertain whether the algae retain the symbiosome membrane at the time of traversing the AFC–oocyte interface. In the late vitellogenic oocytes (Fig. 4F, G), other vesicles were present around the algae instead of the symbiosome membrane; the origin and function of these vesicles are unknown at present. Further studies are needed to elucidate the details of the symbiosomes.

Some previous studies on the vertical transmission of maternal algal symbionts to oocytes in cnidarians suggest that the algae are phagocytized by follicle cells and subsequently pass into the space between the mesoglea and oocyte microvilli through temporary gaps in the mesoglea (Benayahu et al., 1992; Hirose et al., 2001). The surface structures of the oocytes and surrounding AFCs in *W. litus* were quite distinct from those of cnidarians. First, oocytes and AFCs do not have any microvilli on their surfaces. Second, acoels have no extracellular matrix (Rieger et al., 1991) such as mesoglea in cnidarians, and AFCs attach directly to the surface of an oocyte. Therefore, the plasma membrane of an AFC is in close contact with that of an oocyte in *W. litus*. Third, MDRs were observed at the

oocyte-AFC interface. Furthermore, in cnidarians algae are enclosed in symbiosome membranes within oocytes (Campbell, 1990; Benayahu, 1992; Hirose et al., 2001), whereas no symbiosome membrane was observed around the algae within the *Waminoa* oocytes (Fig. 4F, G). These distinctions suggest that the unique algae-transferring mechanism of *Waminoa* spp. may have arisen during evolution in the acoela lineage.

In our experimental aquarium, the worms were maintained for over two years with the host coral isolated from an open environment; the worms also underwent sexual and presumably asexual multiplication. Even in this situation, the *Symbiodinium* symbionts of *W. litus* were definitely different from that of the coral. Although both the worms and its host coral harbored a member of clade-C *Symbiodinium*, our phylogenic analysis based on ITS-rDNA sequences revealed that the worm-borne *Symbiodinium* were monophyletically clustered and distinct from the symbionts of the coexisting coral. This observation suggests that worm-borne *Symbiodinium* are exclusively vertically transmitted without uptake from the host coral. These results are concordant with those of Barneah et al. (2007a) who used PCR-denaturing-gradient gel electrophoresis (DGGE) analysis and revealed that wild epizoic worms do not acquire their symbionts from the host coral. These results also suggest that *Waminoa* spp. have evolved a specific engagement with a certain *Symbiodinium* type without acquiring exogenous *Symbiodinium* from the environment, including their host corals.

The fact that the coexisting coral did not harbor *Amphidinium* sp. suggests that *Amphidinium* sp. is also exclusively inherited by the worms. A BLAST search of the SSU and LSU-rDNA sequences revealed high similarity with the group of *Amphidinium* species. *Amphidinium* spp. are commonly found in benthic communities, such as sand and macroalgal surfaces, and are also known as endosymbionts in acoels (Taylor, 1971; Trench and Winsor, 1987; Kobayashi and Tsuda, 2004). In the phylogenetic tree based on LSU-rDNA sequences, our *Amphidinium* sequence was nested within *A. klebsii* and *gibbosum* clade. An *Amphidinium* strain in the clade (CCMP120) was originally isolated from the acoel *Amphiscolops langerhansi* (Murray et al., 2004), whereas the others appeared to be derived from the environment. This clade could be considered a composite of both “symbiont” and “free-living” *Amphidinium* spp. that are able to adapt to both lifestyles.

The present results suggest that *Waminoa* spp. have evolved an intimate symbiotic association with particular lineages of the algal symbionts *Symbiodinium* and *Amphidinium* via unique vertical transmission mechanisms. However, because the *Symbiodinium* populations in the worms are heterogeneous and the *Amphidinium* spp. are not resolved as symbiont-specific, we cannot eliminate the possibility that the worms are able to acquire symbionts from the environment. To clarify whether *Waminoa* possess such a capacity for symbiont acquisition, further investigation involving symbiont infection tests is needed.

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