Vol. 592: 119–128, 2018
https://doi.org/10.3354/meps12474

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published March 29

Symbiosis process between *Acropora* larvae and *Symbiodinium* differs even among closely related *Symbiodinium* types

Hiroshi Yamashita[1,*], Go Suzuki[1], Chuya Shinzato[2,3], Mitsuru Jimbo[4], Kazuhiko Koike[5]

[1]Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Japan Fisheries Research and Education Agency, Ishigaki, Okinawa 907-0451, Japan
[2]Marine Genomics Unit, Okinawa Institute of Science and Technology Graduate University, Onna, Okinawa 904-0495, Japan
[3]Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Chiba 277-8564, Japan
[4]School of Marine Biosciences, Kitasato University, Sagamihara, Kanagawa 252-0373, Japan
[5]Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8528, Japan

ABSTRACT: The symbiosis between corals and the dinoflagellate genus *Symbiodinium* is a fundamental pillar of coral reef ecosystems. Many of the coral species, including *Acropora*, acquire *Symbiodinium* from the environment during early ontogeny for symbiosis. Genetically diverse members of *Symbiodinium* have been identified from the environment, but naturally settled *Acropora* recruits (i.e. settlers or spats) usually harbor specific members of *Symbiodinium*. Thus, symbiosis between *Acropora* and *Symbiodinium* is not established haphazardly in the wild. Conversely, under laboratory conditions, *Acropora* larvae can acquire other *Symbiodinium* varieties. However, it remains unclear whether a stable symbiosis between these *Symbiodinium* that are never detected in natural *Acropora* recruits and corals is successfully established. Here, we artificially supplied *A. tenuis* larvae with 3 closely related *Symbiodinium* culture strains, namely, type A1 (common within natural *Acropora* recruits), type A2-relative (never detected within corals), and type A3 (often found within natural *Acropora* recruits). We then determined the percentage of *Symbiodinium*-infected larvae and the density of *Symbiodinium* cells infecting the larvae. For types A1 and A3, the percentage of *Symbiodinium*-infected larvae and the density of *Symbiodinium* cells did not decrease even after stopping the *Symbiodinium* supply. This result indicated that stable symbiosis was successfully established, even in the laboratory. However, the percentage of *Symbiodinium*-infected larvae and *Symbiodinium* cell density of type A2-relative declined after stopping the *Symbiodinium* supply. Thus, under laboratory conditions, *A. tenuis* larvae can acquire *Symbiodinium* that is never detected within natural corals; however, stable symbiosis between these *Symbiodinium* and corals was not successfully established.

KEY WORDS: Early symbiosis · *Symbiodinium* · Zooxanthellae · *Acropora* · Coral larvae

INTRODUCTION

Corals harbor symbiotic dinoflagellates of the genus *Symbiodinium*, commonly referred to as zooxanthellae. The host corals use *Symbiodinium* photosynthetic products for their own growth (e.g. Edmunds & Davies 1986). This relationship is essential for coral survival. For example, coral bleaching, which often results in mass mortality of corals, can occur as a result of *Symbiodinium* loss (Brown 1997). However, some *Symbiodinium* can be isolated from host animals and maintained in culture vessels (Schoenberg & Trench 1980). The genus *Symbiodinium* has been divided into 9 phylogenetically dis-

*Corresponding author: hyamashita@fra.affrc.go.jp

tinct groups (clades A–I; Pochon & Gates 2010), and each clade consists of numerous small groups described as types (LaJeunesse 2005). The physiological response to environmental stresses such as irradiance or high or low temperature may differ among clades, and even among types (e.g. Rowan et al. 1997, Kinzie et al. 2001, Tchernov et al. 2004, Frade et al. 2008, Sampayo et al. 2008, Hennige et al. 2009); therefore, the characteristics of symbionts often affect the physiological response of their host animals (Rowan 2004).

New generations of corals must acquire their own *Symbiodinium* via 2 possible mechanisms: vertical transmission (acquisition by maternal inheritance via the egg) or horizontal transmission (acquisition from the environment) (Trench 1987). It is possible to acquire *Symbiodinium* cells reliably via vertical transmission, but about 80% of spawn-gamete-type corals acquire the symbionts from the environment (Baird et al. 2009). For example, *Acropora* corals, the most common and widespread genus in the Indo-Pacific coral reef environment, employ horizontal transmission to acquire *Symbiodinium*. In the wild, *Symbiodinium* clades A–H have been detected in the reef water column or sediments, although it is unclear whether all of the *Symbiodinium* cells were alive (Hirose et al. 2008, Manning & Gates 2008, Pochon et al. 2010, Reimer et al. 2010, Takabayashi et al. 2012, Yamashita & Koike 2013, Yamashita et al. 2013, Cunning et al. 2015, Granados-Cifuentes et al. 2015). Thus, new generations of corals appear to have a highly diverse range of potential symbionts from among which they can select. However, *Acropora* coral recruits (i.e. settlers or spats) in the wild often harbor certain *Symbiodinium* types, usually those belonging to clade A or D (Abrego et al. 2009, Yamashita et al. 2013, 2014). Recent laboratory experiments have revealed that the specific association of *A. tenuis* and *Symbiodinium* comprises 2 selection steps: an initial attraction step and a subsequent step of selective uptake of certain *Symbiodinium* types by coral (Yamashita et al. 2014); findings have also suggested that the coral lectins are involved in this selective acquisition step (Kuniya et al. 2015). Nonetheless, based on the results of previous laboratory infection tests, it is evident that these processes to ensure specific associations are less than perfect. For example, some laboratory experiments have demonstrated that coral larvae can acquire a variety of *Symbiodinium* types; thus, coral larvae are also thought to be promiscuous (see Cumbo et al. 2013). Furthermore, under laboratory conditions, *A. tenuis* larvae also take up a 'free-living' *Symbiodinium* type, which is not harbored by natural host animals. Namely, Yamashita et al. (2014) demonstrated that when *A. tenuis* larvae were exposed to a high density of free-living-type *Symbiodinium* cells (14 000 cells l^{-1}), larvae could acquire these cells, but this was not achieved under a low cell density (140 cells l^{-1}). It should be noted that *Symbiodinium* cell densities in the reef water column are usually low (several 100 cells l^{-1}) throughout the year off Ishigaki Island, Okinawa, Japan (Yamashita et al. 2013), and thus such high cell densities as created in the laboratory are intrinsically unusual in the wild (but see Littman et al. 2008). However, corals have the ability to acquire *Symbiodinium* types that are never detected within natural corals if a sufficient number of *Symbiodinium* cells were to accumulate around corals. In such a situation, it remains unclear whether stable symbiosis between these types of *Symbiodinium* and *Acropora* coral would be successfully established. The *Symbiodinium* clade A lineage consists of rather broad phylotypes and includes both symbiotic and free-living types. *S. microadriaticum* (type A1) is commonly found in natural *Acropora* recruits, and *S. tridacnidorum* (type A3) is often found within natural *Acropora* recruits. In contrast, *S. pilosum* (type A2) and the closely related species *S. natans* (type A2-relative) have never been detected within natural corals. In this study, we artificially introduced types A1, A2-relative, and A3 *Symbiodinium* culture strains to *A. tenuis* larvae and observed their retention within the larvae. We found that type A1 and A3 *Symbiodinium* successfully established symbiosis with the larvae, even under the laboratory conditions, whereas it was not established between corals and type A2-relative *Symbiodinium*, although these *Symbiodinium* cells were acquired by the larvae.

MATERIALS AND METHODS

Symbiodinium culture strains

Culture strains AJIS2-C2 (type A1; *Symbiodinium microadriaticum*) and ISS-C2-Sy (type A2-relative; *S. natans*) were originally isolated by Yamashita & Koike (2013). The internal transcribed spacer (ITS) rDNA sequences of AJIS2-C2 and ISS-C2-Sy have already been determined by Yamashita & Koike (2013), and their GenBank accession numbers are AB704031 and AB704008, respectively. The culture strain CS-161 (type A3; *S. tridacnidorum*) was purchased from the Commonwealth Scientific and

Industrial Research Organization (Black Mountain, ACT, Australia). The GenBank accession number for the ITS rDNA region of CS-161 is AF186055 (Baillie et al. 2000). Types A1 and A3 *Symbiodinium* have been found in naturally settled *Acropora* recruits off Ishigaki Island, Okinawa, Japan, and types A1 and A3 *Symbiodinium* culture strains (AJIS2-C2 and CS-161 respectively) were acquired by *A. tenuis* larvae even under low cell density conditions in laboratory experiments (Yamashita et al. 2014). In contrast, type A2 *Symbiodinium* and the closely related species type A2-relative *Symbiodinium* have never been found in *Acropora* corals in the field. Under laboratory conditions, however, a few cells of type A2-relative *Symbiodinium* culture strain (ISS-C2-Sy) were acquired by *A. tenuis* larvae when a high density of ISS-C2-Sy cells was supplied to the larvae (Yamashita et al. 2014). These 3 cultures were maintained in a 27°C incubator under a light regime of 80–120 µmol photons m^{-2} s^{-1} (12 h light:12 h dark cycle) in IMK medium (Sanko Jyunyaku).

Preparation of apo-symbiotic planula larvae

Parental *A. tenuis* colonies were collected from Sekisei Lagoon between Ishigaki and Iriomote islands in the southern part of Okinawa, Japan, on 8 May 2014. The colonies were immediately transferred into outdoor overflow tanks in our institute, and maintained in running sand-filtered natural seawater. After 1 wk, spawning was artificially induced by adding H_2O_2 (Hayashibara et al. 2004), and the fertilized eggs were washed to remove any remaining sperm and unexpected *Symbiodinium* contaminants using ultrafiltrated (UF) seawater (Membrane Technology). The eggs were then transferred to UF seawater in 100 l polycarbonate tanks (egg or planula densities of approximately 500 ind. l^{-1}) placed indoors (water temperature of 25.8°C). The water was changed once a day until used for the infection test.

Infection test

Groups of 2000 individuals of 2 d old larvae were allocated from the abovementioned polycarbonate tanks to each of 12 cylindrical bottles filled with 2 l of UF seawater; then, 100 000 cells of type A1, A2-relative, and A3 *Symbiodinium* strains were added to 3 bottles each (triplicate experiments). No *Symbiodinium* cells were added to the remaining 3 bottles (control bottles: apo-sym.). The bottles were put into the abovementioned incubator (27°C; 80–120 µmol photons m^{-2} s^{-1}; 12 h light:12 h dark cycle). After 24 h, 10 individual larvae were randomly retrieved from each bottle and observed under an epifluorescent microscope (BX50; Olympus; 400–410 nm excitation) to count the *Symbiodinium* cells within the larval body. The larval observations were performed in the same manner as described by Yamashita et al. (2014). The percentage of *Symbiodinium*-infected larvae was calculated as the number of infected larvae/number of observed larvae (i.e. 10 ind.) × 100. The density of *Symbiodinium* cells infecting individual larvae (cells per individual larva) was calculated as the average number of *Symbiodinium* cells infecting *Symbiodinium*-infected larvae. These calculations were performed for each bottle. After observation, all larvae remaining in the bottles were retrieved using a 20 µm sieve and the seawater was thrown away. The empty bottles were washed with UF seawater, and refilled with 2 l of fresh UF seawater. The retrieved larvae were returned to the bottles, and then the same number of cells (100 000 cells) of each *Symbiodinium* strain were again added to the bottles. These steps were repeated until the larvae were 5 d old. After an age of 6 d, only exchange of the UF seawater was performed, without the addition of any *Symbiodinium* cells. Observations of the percentage of *Symbiodinium*-infected larvae and *Symbiodinium* cell densities within the larvae were in principle performed once every 2 d, until the larvae were 17 d old. In addition, throughout the observation period, micrographs of the *Symbiodinium* cells infecting the larvae were taken using the abovementioned epifluorescent microscope with an ultrasensitive charge-coupled device camera (DP-73, Olympus).

Statistical analysis

One-way ANOVA was performed to analyze the differences among the treatment groups (i.e. added *Symbiodinium* types), and among the observation days within each treatment group. We compared the data for percentage of *Symbiodinium*-infected larvae and *Symbiodinium* cell density within the larvae. It should be noted that the data from control (apo-sym.) bottles were excluded from all analyses, because *Symbiodinium* cells were not added to the control bottles, and thus there were no *Symbiodinium*-infected larvae. The percentage of *Symbiodinium*-

infected larvae and *Symbiodinium* cell numbers within the larvae at the age of 6 d (just after the last *Symbiodinium* addition) and 17 d (the end of observations) were used as variables for the analyses among the treatment groups. In all analyses, the null hypothesis was that there were no differences among the *Symbiodinium* types. To analyze the differences in the percentage of *Symbiodinium*-infected larvae and *Symbiodinium* cell density within larvae after the addition of *Symbiodinium* cells were stopped, we separately compared these data for each treatment group. Analyses within treatment groups were performed using data at larval ages of 6, 8, 10, 12, 14, and 17 d. In all analyses, the null hypothesis was that there were no differences based on the day of observation (larval age). If the null hypothesis was rejected in each analysis, we performed Tukey's honestly significant difference test for multiple comparisons. In the present study, p-values less than 0.05 were considered statistically significant. All of the statistical tests were performed using R version 3.1.2 (R Core Team 2014).

RESULTS

Percentages of *Symbiodinium*-infected larvae

Percentages of *Symbiodinium*-infected larvae for each *Symbiodinium* type are shown in Fig. 1. Infected larvae were never observed in the control bottles (without the addition of *Symbiodinium* cells: aposym). Thus, it was assumed that there was no unexpected *Symbiodinium* contamination during the experimental period. In the bottles with type A1 (culture strain AJIS2-C2) *Symbiodinium* cells, the percentage of *Symbiodinium*-infected larvae gradually increased from 23.3 ± 11.5% when they were 3 d old to 93.3 ± 5.8% when 6 d old. Even after stopping the addition of *Symbiodinium*, the percentage of infected larvae remained over 90%. In the bottles with type A2-relative (culture strain ISS-C2-Sy) *Symbiodinium* cells, the percentage of *Symbiodinium*-infected larvae increased from 6.7 ± 5.8% at an age of 3 d to 76.7 ± 5.8% at 6 d. The maximum percentage (83.3 ± 20.8%) was observed when larvae were 10 d; this percentage

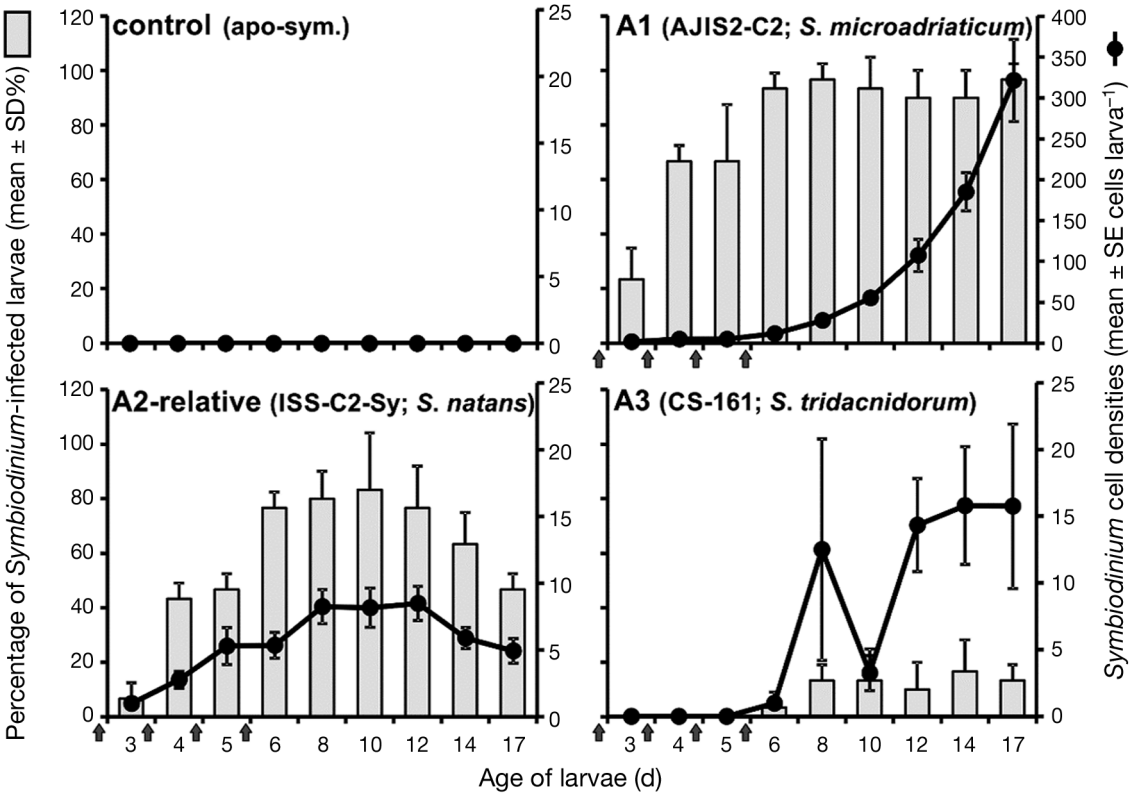

Fig. 1. Percentage of *Symbiodinium*-infected *Acropora tenuis* larvae and density of *Symbiodinium* cells infecting *A. tenuis* larvae during the infection test for 3 *Symbiodinium* culture strains. Bars indicate percentage of *Symbiodinium*-infected larvae (mean ± SD of triplicate experiments; left axis) and lines indicate *Symbiodinium* cell densities (mean ± SE cells larva^{-1}; right axis) for each larval age. Applied *Symbiodinium* type, culture strain name, and *Symbiodinium* species name are shown at the top left of each graph. The timings of *Symbiodinium* cell supply are indicated by arrows under the *x*-axis

gradually decreased to 46.7 ± 5.8% at 17 d. In the bottles with type A3 (culture strain CS-161) *Symbiodinium* cells, *Symbiodinium*-infected larvae were only observed after the age of 6 d. The infected larvae were very few in number, and the maximum percentage was achieved at the age of 14 d, but the percentage was low (16.7 ± 11.5%). However, in contrast to type A2-relative, the percentage of type A3-infected larvae did not decrease during the observation period. The percentage of *Symbiodinium*-infected larvae at the age of 6 d was significantly different among the *Symbiodinium* types (1-way ANOVA, $p < 0.001$). Subsequent multiple comparisons found differences between all of the comparisons, i.e. A1 vs. A2-relative ($p = 0.0285$), A1 vs. A3 ($p < 0.001$), and A2-relative vs. A3 ($p < 0.001$). Percentages of *Symbiodinium*-infected larvae aged 17 d were also significantly different among the *Symbiodinium* types ($p < 0.001$), and significant differences were found between all of the comparisons (A1 vs. A2-relative, $p = 0.001$; A1 vs. A3, $p < 0.001$; A2-relative vs. A3, $p = 0.0009$).

After stopping the addition of *Symbiodinium* cells, the percentages of type A1 and A3 *Symbiodinium*-infected larvae did not significantly change ($p = 0.860$ for A1, $p = 0.562$ for A3). However, the percentages of type A2-relative *Symbiodinium*-infected larvae changed after the *Symbiodinium* cell supply was stopped ($p = 0.0341$). Subsequent multiple comparisons found differences between 10 d old vs. 17 d old larvae ($p = 0.0370$). None of the other comparisons yielded significant differences ($p \geq 0.0634$; all p-values of subsequent multiple comparisons are listed in Table A1 in the Appendix).

Symbiodinium cell densities within individual larva

The number of *Symbiodinium* cells infecting individual larvae is also shown in Fig. 1. In the bottles with type A1 addition, the *Symbiodinium* cell density rapidly increased even after stopping *Symbiodinium* addition. At the end of the observation period (17 d old larvae), the *Symbiodinium* cell density reached 321.4 ± 50.2 cells per larva. Type A2-relative *Symbiodinium* cell densities within larvae gradually increased until the age of 8 d. The maximum *Symbiodinium* cell density (8.5 ± 1.3 cells ind.$^{-1}$) was recorded when the larvae were 12 d old, and then gradually decreased to 4.9 ± 0.9 cells ind.$^{-1}$ when 17 d old. In the bottles to which type A3 was added, the maximum *Symbiodinium* cell density was 15.8 ± 4.4 cells ind.$^{-1}$ at age 14 d. Although the number of type A3 *Symbiodinium* cells infecting individual larvae was quite variable, the *Symbiodinium* cell numbers did not decrease, in contrast to the bottles to which type A2-relative had been added. The number of *Symbiodinium* cells infecting 6 d old larvae was significantly different among the supplied *Symbiodinium* types ($p < 0.001$). Subsequent multiple comparison found differences for A1 vs. A2-relative ($p = 0.0032$) and A1 vs. A3 ($p < 0.001$), whereas A2-relative vs. A3 ($p = 0.115$) was not significantly different. When larvae were 17 d old, the *Symbiodinium* cell numbers among treatments were also significantly different ($p < 0.001$), and differences were found for A1 vs. A2-relative and A3 ($p < 0.001$), whereas in the comparisons of A2-relative vs. A3, significant differences were not found ($p = 1.00$).

After the addition of *Symbiodinium* cells was stopped, type A3 *Symbiodinium* cell densities within individual larva did not significantly change ($p = 0.383$). However, for type A1 and type A2-relative *Symbiodinium*, cell densities within larvae did change significantly ($p < 0.001$ for A1, $p = 0.0058$ for type A2-relative). Multiple comparisons of type A1 *Symbiodinium* cell densities found differences at larval ages of 6 vs. 14, 6 vs. 17, 8 vs. 14, 8 vs. 17, 10 vs. 14, 10 vs. 17, 12 vs. 17, and 14 vs. 17 d ($p \leq 0.0107$ for summary of these comparisons), whereas significant differences were not found for other comparisons ($p \geq 0.119$). All p-values of subsequent multiple comparisons using type A1 *Symbiodinium* cell densities are listed in Table A2 in the Appendix. Multiple comparisons of type A2-relative *Symbiodinium* cell densities found significant differences at larval ages of 8 vs. 17, 10 vs. 17, and 12 vs. 17 d ($p \leq 0.0463$ for summary of these comparisons), whereas significant differences were not found for other comparisons ($p \geq 0.279$). All p-values of subsequent multiple comparisons using type A2-relative cell densities are also listed in Table A2 in the Appendix.

Symbiodinium cells within the larvae

Fig. 2 shows fluorescent micrographs of *Symbiodinium* cells infecting 6 d old larvae (right after the last *Symbiodinium* supply), followed by those of 10 and 17 d old larvae (the end of observations). The infected cells within the larvae were recognized to be healthy because all cells emitted bright autofluorescence from their chloroplasts. Dividing or divided doublet cells, which were undergoing or had undergone cell division, were often found within the larvae in all bottles.

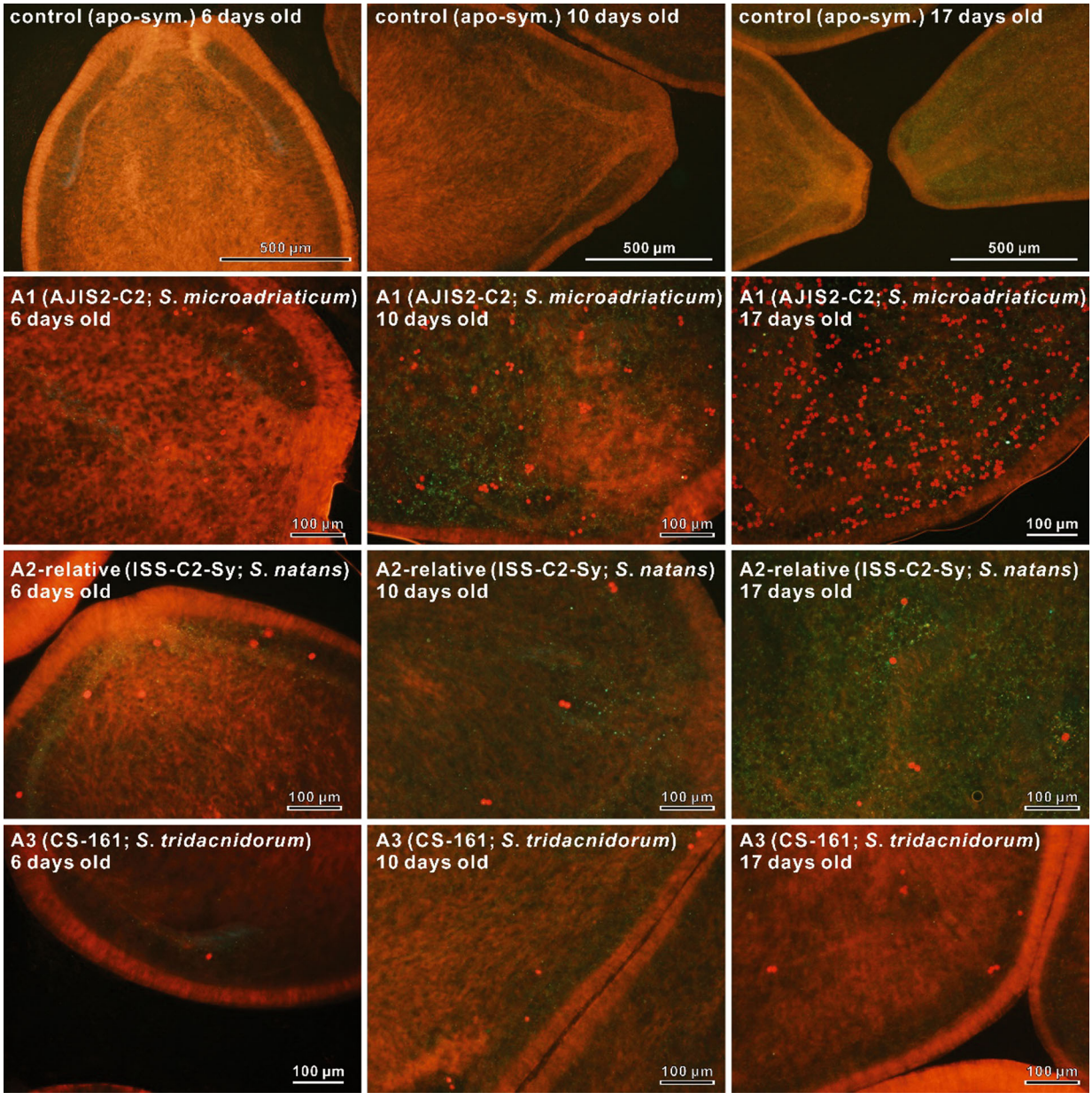

Fig. 2. Fluorescent micrographs of the tested larvae at the age of 6, 10, and 17 d. Applied *Symbiodinium* type, culture strain name, *Symbiodinium* species name, and larval age are shown at the top of each micrograph. *Symbiodinium* cells are circular shapes with a bright red color due to the autofluorescence of chlorophyll. *Symbiodinium* cells were within the larval body; hence, the cells appear slightly blurry. Dividing or divided (doublet) cells can be found within all treatments. This indicated that the 3 tested types of *Symbiodinium* can multiply within the larval body

DISCUSSION

Natural *Acropora* recruits harbor specific *Symbiodinium* clades and types, even when diverse *Symbiodinium* clades and types occur in their surroundings (Yamashita et al. 2013, 2014). However, under laboratory conditions, corals often promiscuously acquire a variety of *Symbiodinium* types (see Cumbo et al. 2013). Furthermore, even the free-living-type *Symbiodinium* (type A2-relative) was acquired by *A. tenuis* larvae under conditions of high *Symbiodinium* cell density (14 000 cells l^{-1}), which is usually unlikely in natural reef environments (Yamashita et al. 2014). This may indicate that *Acropora* corals non-selectively acquire *Symbiodinium* cells when surrounded by abundant *Symbiodinium* cells. However, it re-

mains unclear whether a stable symbiotic relationship is subsequently successfully established.

In this study, we artificially introduced free-living type A2-relative as well as symbiotic type A1 and A3 *Symbiodinium* to *A. tenuis* larvae under conditions of extremely high *Symbiodinium* cell density compared with levels in the natural environment. At a cell density of 50 000 cells l^{-1}, even type A2-relative *Symbiodinium* was acquired by the larvae. Interestingly, the number of type A2-relative *Symbiodinium* cells infecting individual larvae were slightly increased within the period from 6 to 8 d old. The supply of *Symbiodinium* cells had already been stopped at 6 d old; thus, this increase in cell number was considered to be attributable to *Symbiodinium* cell division within the larvae. In fact, cells undergoing and/or those that underwent the division process were often found within the larvae (Fig. 2), which indicated that not only the symbiotic types (A1 and A3) but also the free-living type (A2-relative) can grow within the larval body. However, cell densities of type A2-relative and percentages of type A2-relative-infected larvae gradually decreased after a larval age of 10–12 d. The type A2-relative *Symbiodinium* is intrinsically a free-living type; thus, it is plausible that the environment within the larval body may not be suitable for these *Symbiodinium* cells. To account for this, Hill & Hill (2012) proposed 2 hypotheses (magnesium inhibition hypothesis and arrested phagosome hypothesis) to explain *Symbiodinium*-based interactions in the symbiosis, rather than viewing the host as having complete control over *Symbiodinium* cells. In the present study, however, type A2-relative *Symbiodinium* cells infecting the larvae seemed to be healthy, emitted brilliant chloroplast fluorescence, and propagated within the larval body because cells undergoing and/or those that underwent division were frequently observed, like types A1 and A3. Thus, it is difficult to simply conclude that the environment within the larval body might be unsuitable for type A2-relative *Symbiodinium*. Another possibility to explain the decrease of type A2-relative *Symbiodinium*-infected larvae and cell densities within the larvae is the elimination of *Symbiodinium* cells by the larvae.

The observation period was short at only 24 h; however, an interesting finding in this context was made by Dunn & Weis (2009), who demonstrated that inappropriate symbionts were eliminated from *Fungia scutaria* larvae by host-cell apoptotic activity. Furthermore, Bay et al. (2011) also observed the rejection of some *Symbiodinium* types within *A. tenuis* larvae in early ontogeny. Although data were obtained from adult corals, some corals may alternatively discharge certain clades of *Symbiodinium* from their body (Yamashita et al. 2011). Considering these results, it is plausible that *A. tenuis* larvae also have the ability to eliminate or reject certain *Symbiodinium*. In fact, the repertoire of innate immunity-related genes of *Acropora* corals is more complex than that of other non-symbiotic cnidarians (Shinzato et al. 2011, Hamada et al. 2013). Thus, coral innate immunity is thought to involve a post-phagocytic winnowing process (Dunn & Weis 2009).

Under culture conditions (in the test tube), the growth rate of type A2-relative *Symbiodinium* was higher than that of type A1 or A3 (Yamashita & Koike 2016). Unfortunately, we could not determine growth rates of these *Symbiodinium* cells within the larvae; however, the type A2-relative *Symbiodinium* cell densities within the larval body increased from 6 to 8 d larval age. Although type A2-relative *Symbiodinium* cell densities within the larvae did not change from the age of 8 to 12 d, after 12 d the cell density as well as percentage of type A2-relative *Symbiodinium*-infected larvae gradually decreased. Considering these results, it is plausible that the elimination of type A2-relative *Symbiodinium* cells by larvae eventually exceeded the growth rate of this type of *Symbiodinium* after a larval age of 12 d.

Under laboratory conditions, even the free-living type A2-relative *Symbiodinium* can be acquired by *A. tenuis* larvae, and the cells can multiply within the larvae; however, stable symbiosis is not established with this combination. In contrast, the percentages of type A1 *Symbiodinium*-infected larvae remained high, and type A1 cell densities within the larvae constantly increased during the experimental period. These results indicate that *A. tenuis* larvae and type A1 *Symbiodinium* successfully established a stable symbiosis. In the case of type A3, the percentage of *Symbiodinium*-infected larvae and *Symbiodinium* cell densities were low. The same tendency was also observed in previous laboratory experiments that supplied *A. tenuis* larvae with a low cell density (1000 cells l^{-1}) of A3 *Symbiodinium* (Yamashita et al. 2014). Furthermore, *Symbiodinium* clade A-type compositions within naturally settled *Acropora* recruits in our study area comprised mainly type A1 and few A3 (Yamashita et al. 2014). Thus, it is plausible that *Acropora* corals in our study area mainly acquire type A1 *Symbiodinium*, but a few individuals also have the ability to acquire type A3 *Symbiodinium*. Although only a few larvae have the ability to acquire type A3 *Symbiodinium*, the percentage of A3 *Symbiodinium*-infected larvae and A3 *Symbiodinium* cell densities within these larvae never

decreased during our observation period. Thus, symbiosis appeared to have also been successfully established between this combination.

During the initial acquisition process, *A. tenuis* larvae can selectively uptake certain *Symbiodinium* cells by a 2-step selection (Yamashita et al. 2014). The different *Symbiodinium* strains have been reported to be differentially bound to lectins, which are sugar-binding proteins (Logan et al. 2010), and Wood-Charlson et al. (2006) also demonstrated that glycan ligands of the *Symbiodinium* cell surface play a role in the recognition process during initial contact at the onset of symbiosis with *F. scutaria* larvae. Furthermore, a galactose-binding lectin in the octocoral *Sinularia lochmodes* was also shown to recognize specific *Symbiodinium* (Koike et al. 2004). Thus, coral lectins are probably involved in the selective acquisition of certain *Symbiodinium* cells (Kuniya et al. 2015). These processes are considered to occur as the preliminary stage and pre-stage of the winnowing process mentioned by Nyholm & McFall-Ngai (2004). It is thought that the lectin–glycan interactions are also involved in the winnowing process after *Symbiodinium* cells are acquired by corals. Bay et al. (2011) demonstrated that these interactions play a role in controlling the post-infection growth of some *Symbiodinium* types. This mechanism may also be involved in the decrease of type A2-relative *Symbiodinium* cells infecting larvae in our observations.

Genetically diverse *Symbiodinium* has been found in natural reef environments, but the specificity of the relationship between *Acropora* recruits and *Symbiodinium* with certain genotypes appears to be successfully established. However, under laboratory conditions in which an extremely high *Symbiodinium* cell density is established compared with that in the wild, the *Acropora* corals often acquire even free-living type *Symbiodinium* cells. However, it is plausible that these *Symbiodinium* cells are subsequently excluded by corals. If this is true, in some laboratory experiments in which *Symbiodinium* with a variety of genotypes are artificially introduced into larvae, stable symbiosis might not be achieved.

Symbiodinium pilosum (type A2) and its closely related species *Symbiodinium natans* (type A2-relative) are considered to be free-living species (e.g. LaJeunesse 2002, Yamashita & Koike 2013) and have never been detected within coral hosts in the wild. *Symbiodinium* belonging to these groups basically does not infect cnidarian hosts (LaJeunesse 2001); however, *A. tenuis* larvae can acquire type A2-relative *Symbiodinium* cells when exposed to high cell densities that are unlikely to occur in the wild. Nonetheless, our results clearly showed that these cells are not maintained within the corals for a long time. There are various possibilities to explain the reduction of *Symbiodinium* cells from the coral body, such as expulsion and/or digestion of *Symbiodinium* cells by corals, and/or simple cell death of *Symbiodinium* within the coral body. In the present study, cell division of type A2-relative *Symbiodinium* could be observed, and also, we did not find damaged *Symbiodinium* cells even within 17 d old larvae. Considering these results, type A2-relative *Symbiodinium* cells might be expelled by *A. tenuis* larvae. In contrast, *S. microadriaticum* (type A1) grows rapidly within *A. tenuis* larvae, reflecting the successful establishment of symbiosis, in the laboratory as well as in the wild. This indicates that the combination of *A. tenuis* and type A1 *Symbiodinium* can be used as a model symbiosis system in the laboratory.

Acknowledgements. The authors thank the staff at the Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Japan Fisheries Research and Education Agency for their support during this investigation. The authors thank Enago (www.enago.jp) for the English language review. This work was supported by JSPS KAKENHI Grant Number 26291094 to H.Y. Sampling of corals for research purposes was permitted by Okinawa Prefectural Government (No. 25-49).

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Appendix

Table A1. P-values from subsequent multiple comparisons of percentage of type A2-relative *Symbiodinium*-infected larvae after supply of *Symbiodinium* cells was stopped

Comparisons (d old)	p-value
6 vs. 8	0.9994
6 vs. 10	0.9850
6 vs. 12	1.0000
6 vs. 14	0.7868
6 vs. 17	0.1074
8 vs. 10	0.9994
8 vs. 12	0.9994
8 vs. 14	0.6087
8 vs. 17	0.0634
10 vs. 12	0.9850
10 vs. 14	0.4308
10 vs. 17	0.0370
12 vs. 14	0.7868
12 vs. 17	0.1074
14 vs. 17	0.6087

Table A2. P-values from subsequent multiple comparisons of type A1 and A2-relative *Symbiodinium* cell densities after supply of *Symbiodinium* cells was stopped

Comparisons (d old)	p-value	
	Type A1	Type A2-relative
6 vs. 8	0.9971	0.5125
6 vs. 10	0.8322	0.4236
6 vs. 12	0.1185	0.5581
6 vs. 14	0.0001	0.9998
6 vs. 17	<0.0001	0.8128
8 vs. 10	0.9768	0.9999
8 vs. 12	0.3080	0.9999
8 vs. 14	0.0008	0.3544
8 vs. 17	<0.0001	0.0382
10 vs. 12	0.7675	0.9999
10 vs. 14	0.0107	0.2794
10 vs. 17	<0.0001	0.0256
12 vs. 14	0.3091	0.3953
12 vs. 17	<0.0001	0.0463
14 vs. 17	0.0005	0.9196

Editorial responsibility: Karen Miller, Hobart, Tasmania, Australia

Submitted: March 3, 2017; Accepted: January 3, 2018
Proofs received from author(s): March 14, 2018