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Chromera velia is Endosymbiotic in Larvae of the Reef Corals *Acropora digitifera* and *A. tenuis*

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Scleractinian corals occur in symbiosis with a range of organisms including the dinoflagellate alga, *Symbiodinium*, an association that is mutualistic. However, not all symbionts benefit the host. In particular, many organisms within the microbial mucus layer that covers the coral epithelium can cause disease and death. Other organisms in symbiosis with corals include the recently described *Chromera velia*, a photosynthetic relative of the apicomplexan parasites that shares a common ancestor with *Symbiodinium*. To explore the nature of the association between *C. velia* and corals we first isolated *C. velia* from the coral *Montipora digitata* and then exposed aposymbiotic *Acropora digitifera* and *A. tenuis* larvae to these cultures. Three *C. velia* cultures were isolated, and symbiosis was established in coral larvae of both these species exposed to all three clones. Histology verified that *C. velia* was located in the larval endoderm and ectoderm. These results indicate that *C. velia* has the potential to be endosymbiotic with coral larvae.

Key words: Coral reefs; Apicomplexa; symbiosis; parasitism; *Chromera velia*.

Introduction

Symbiosis is defined as the co-existence of different species, with either one or both species benefiting from the association. Symbiotic interactions are classified into three categories; mutualistic, commensal or parasitic (Douglas 1994). The symbioses between scleractinian corals and the dinoflagellate, *Symbiodinium* spp., is one of the best studied in nature, having first been identified by Brandt (1881). The symbiosis is mutualistic, with endosymbiotic algae providing the coral host with up to 90% of their energy requirements in the form of translocated carbon derived from photosynthesis (Muscatine 1990). In return, *Symbiodinium* cells benefit from a relatively stable environment and a supply of nutrients from the host (Trench 1979).

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However, corals form symbioses with many organisms, including both prokaryotes and eukaryotes (Ainsworth et al. 2010; Knowlton and Rohwer 2003; Rosenberg et al. 2007), and not all the associations are beneficial to both partners (Bourne et al. 2008).

Symbionts of corals include organisms that live within the coral cells, the skeleton, or the coral's surface mucus layer (Knowlton and Rohwer 2003). Some of these associations are mutualistic, such as the cyanobacteria that co-exist alongside *Symbiodinium* in the endodermal cells of *Montastraea cavernosa* and are a potential source of nitrogen for the host (Lesser et al. 2004). However, other symbioses can result in disease and death of the host. For example, black-band disease, white pox and white plague are all caused by bacteria that reside in coral surface mucus layer, which, when the host is under stress, can proliferate and result in coral tissue degradation and death (Patterson et al. 2002; Richardson et al. 1998; Richardson 2004; but see Lesser et al. 2007). Parasitic microorganisms include the ciliate *Halofolliculina corallasia*, which causes skeletal eroding disease (Willis et al. 2004) and *Helicostoma nonatum*, which is thought to be the causative agent for brown band disease (Bourne et al. 2008).

Another group of organisms, recently identified in association with corals are the Apicomplexa (Moore et al. 2008; Toller et al. 2002). Apicomplexans are a group of mostly parasitic protists. Many apicomplexans contain a non-photosynthetic plastid called the apicoplast (e.g. Moore et al. 2008). Apicomplexan gene sequences have been detected in tissue extracts of the octocoral *Plexaura kuna* (Goulet and Coffroth 2003) and *Montastraea annularis* (Toller et al. 2002). Recently, Moore et al. (2008) described a new apicomplexan-like organism, *Chromera velia*, which was isolated from two coral species: *Plesiastrea versipora* from Sydney Harbour and *Leptastrea purpurea* from One Tree Island (Moore et al. 2008). *C. velia* is the closest known photosynthetic relative of the apicomplexan parasites, and it is also related to dinoflagellates (Moore et al. 2008).

The photosynthetic plastid of *C. velia* is related both to the non-photosynthetic chloroplast remnant (termed the 'apicoplast') of apicomplexan parasites and the photosynthetic chloroplast of the dinoflagellate *Symbiodinium* (Moore et al. 2008). The discovery and characterisation of *C. velia* provided support for the hypothesis that dinoflagellates and apicomplexans share a common ancestor (Gajadhar et al. 1991), and confirmed that dinoflagellates and apicomplexans share a common ancestral chloroplast lineage (Fast et al. 2001; Ralph et al. 2004). In terms of lifestyle evolution, it is interesting to ask whether *C. velia* may represent an ancestral symbiosis-ready lineage, whose own ancestors developed into the dinoflagellate and apicomplexan lineages respectively. Additionally, *C. velia* is a potentially valuable research tool in studying how organisms evolve from symbiosis to parasitism, because unlike parasitic apicomplexans it can live without a host and grows readily in culture (Cumbo 2005; Moore et al. 2008).

Chromera velia was isolated during research into the chemical ecology of the scleractinian coral, *Montipora digitata* (Cumbo 2005). *M. digitata* was chosen for culturing studies because of 11 scleractinian species studied, it was the only one with antimicrobial activity in the eggs. *M. digitata* was also the only species with *Symbiodinium* in the eggs, suggesting the symbionts may be the source of the bioactive compounds (Marquis et al. 2005). During attempts to produce monoclonal *Symbiodinium* cultures from the eggs and tissue of *M. digitata*, *C. velia* was isolated from tissue. The aim of this study was to explore the symbiosis between *C. velia* and a coral host. In particular, we aimed to test whether or not *C. velia* could establish symbioses with coral larvae.

Results and Discussion

The algal cultures isolated from the nubbins of *Montipora digitata* were identified as *Chromera velia*. The shape of the chloroplast was similar to that previously described for *C. velia* and very different to those of *Symbiodinium* (Fig. 1). Similarly, each of the DNA sequences from the cultures were analysed by BLASTn (blast.ncbi.nlm.nih.gov) and were found to be almost identical to that of *C. velia* CMS22 (Moore et al. 2008). The fact that *C. velia* was repeatedly isolated from coral tissue, suggests it is symbiotic with the coral host.

Successful, and repeatable, infection of *Acropora* larvae with all three *C. velia* cultures assayed (Figs 2, 3), and the presence of *C. velia* within the larval endoderm and ectoderm (Fig. 2B) suggests the symbiosis is endosymbiotic. *C. velia* was taken up by both species of *Acropora* larvae within 24 h of exposure to the algal cultures. When exposed to *A. digitifera* larvae, *C. velia* Mdig3 was the most infective culture. After one day of exposure the mean proportion of larvae infected with *C. velia* Mdig3 was 0.63 ± 0.14 (mean $\pm$ SE), which was ~31.5% and 54.2% higher than larvae infected with *C. velia* Mdig1 and *C. velia* Mdig2 respectively

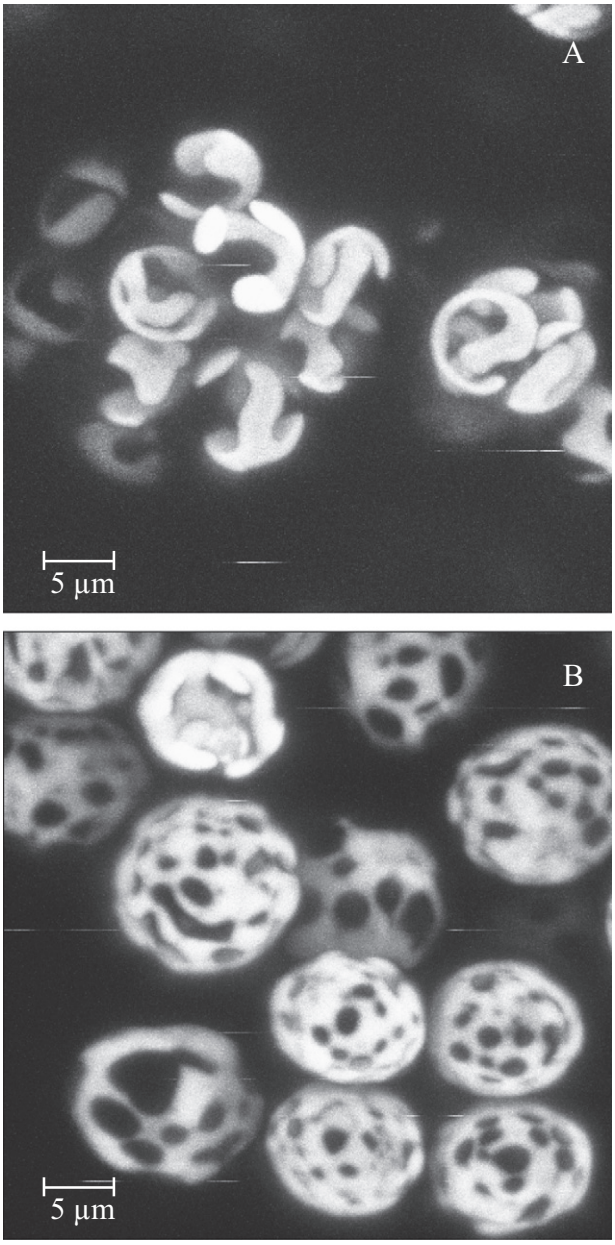

Figure 1. Confocal image of the chloroplast of the algae *Chromera velia* Mdig1 (**A**) and *Symbiodinium* (**B**) by detection of chlorophyll autofluorescence (for details see Methods).

(Fig. 3A). However, the rate of increase in the proportion of larvae infected with each *C. velia* culture between day 1 and 2 was similar, ranging from 0.17 – 0.21 (Fig 3A). By day two, the mean proportion of infected *A. digitifera* larvae ranged from 0.29 ± 0.11 when exposed to *C. velia* Mdig2, to 0.79 ± 0.04 when exposed to *C. velia* Mdig3 (Fig. 3A). *Acropora tenuis* larvae were also successfully infected with *C. velia* with the mean proportion of infected larvae ranging from 0.23 ± 0.03 (SE) when exposed to *C. velia* Mdig2, to 0.33 ± 0.15 when exposed to *C. velia* Mdig1. On day three it was evident that *C. velia* Mdig3 was again more infective than the other two culture, with three to five- fold more *A. tenuis* larvae infected by *C. velia* Mdig3 compared to *C. velia* Mdig2 and *C. velia* Mdig1 (Fig. 3B). The DNA sequence of *C. velia* Mdig3 differed by only one base when compared to the other two *C. velia* cultures. Similar differences in infectivity are common among closely related *Symbiodinium* types (Schoenberg and Trench 1980).

Histology revealed that *C. velia* was located within both the endoderm and ectoderm of *A. digitifera* larvae (Fig. 2B), demonstrating that it can be endosymbiotic. In contrast, *Symbiodinium* was only found in the endoderm, the typical location of this symbiont in planula larvae and adults (Harii et al. 2009). *C. velia* could have entered the larval tissue either directly through the ectoderm, as occurs with *Symbiodinium* in developing larvae of the octocoral, *Anthelia glauca* (Benayahu and Schleyer, 1998), and the scleractinian coral, *Fungia scutaria* (Marlow and Martindale 2007) or via the oral pore, which is the more typical method of uptake of *Symbiodinium* in coral larvae (Harii et al. 2009; Schwarz et al. 1999).

Patterns of uptake and development of *C. velia* by *A. digitifera* larvae suggests that the symbiosis might persist because both the proportion of *A. digitifera* larvae infected (Fig. 3A) and the density of *C. velia* cells within larvae generally increased through time (Fig 4A) although not to the same extent as for *Symbiodinium* C1, where there was a 40-fold increase in the mean number of *Symbiodinium* C1 in larvae between day 1 and day 3 (Fig. 4B). In contrast, in *A. tenuis*, the proportion of larvae infected and the density of cells within larvae generally deceased through time (Figs 3B, 4B).

Apicomplexans produce many unique metabolites (Lim and McFadden 2010; Obornik et al. 2011) and therefore *C. velia* could benefit the host by producing antimicrobial compounds. However, extracts from the cultures of *C. velia* were inactive against numerous microorganisms in disc diffusion assays in marked contrast to extracts from crude pellets of the tissue and eggs of *Montipora digitata* that were active against a range of bacteria (Cumbo 2005). In addition, because *C. velia* is photosynthetic, it could provide nutrition to the coral host. Finally, the decline in density of *C. velia* through time within *A. tenuis* larvae may indicate it is digested by the host, and is therefore a direct source of nutrition.

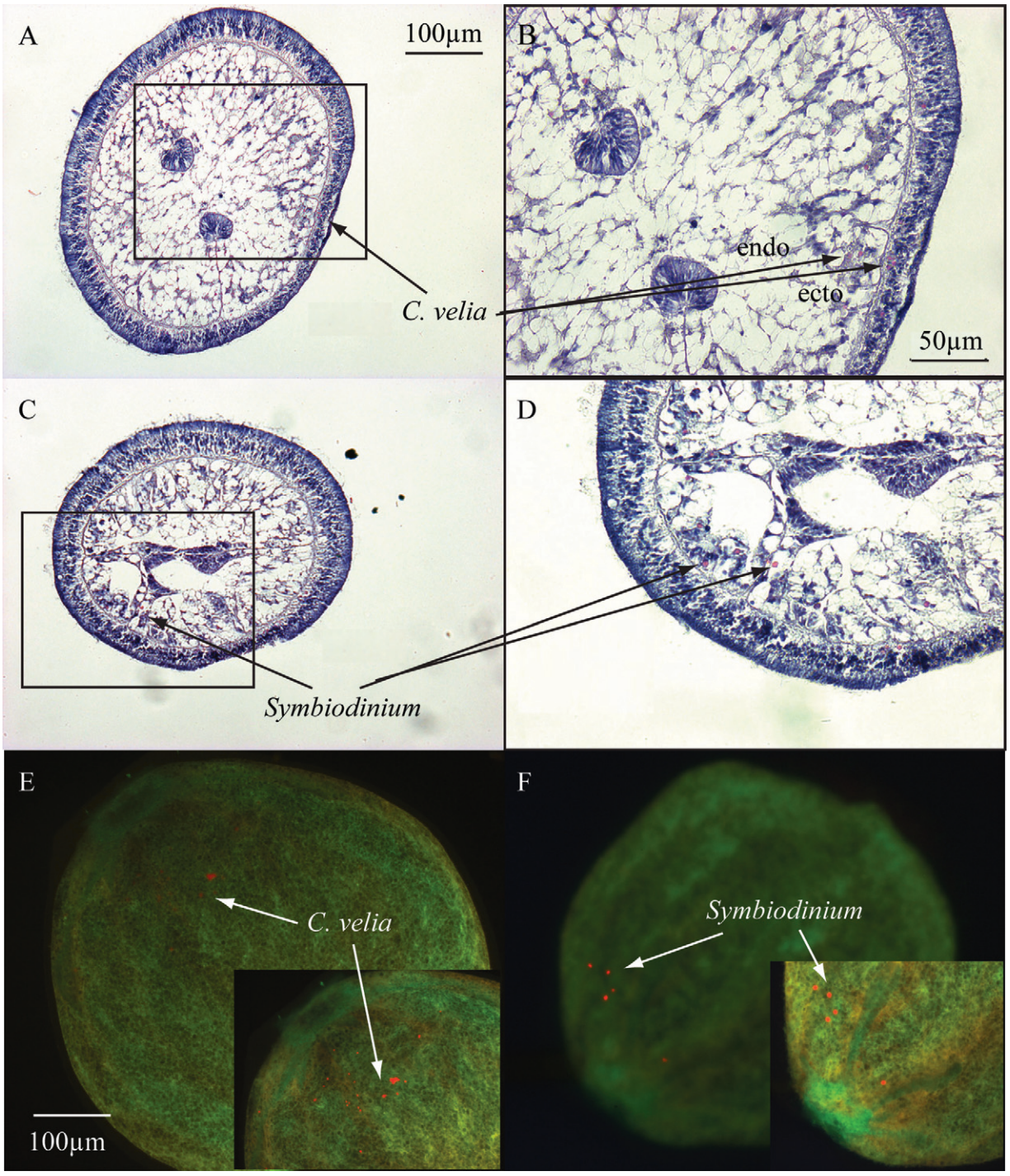

Figure 2. Images of successful uptake of *Chromera velia* and *Symbiodinium* in *Acropora digitifera* and *A. tenuis* larvae. Histological section of *A. digitifera* larvae infected with *C. velia* under 20x (**A**), and 40x objective (**B**) showing algal cells inside the endoderm (endo) and ectoderm (ecto). Section showing larvae infected with *Symbiodinium* C1 control inside the endoderm under 20x (**C**) and 40x (**D**). The larval section is purple while the symbiont cells are pink. Confocal images of uptake of *C. velia* (**E**) and *Symbioidnium* (**F**) in *A. tenuis* larvae. The larvae fluoresce green while the symbiont cells are red.

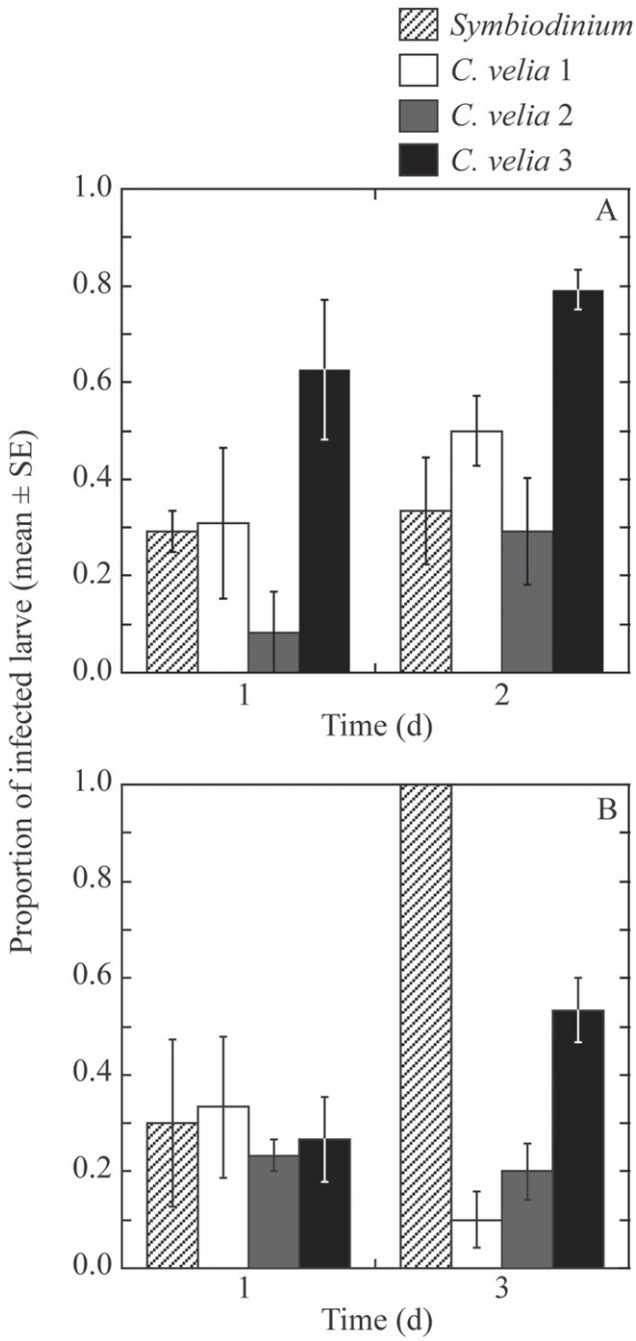

Figure 3. The mean (± SE) proportion of *Acropora digitifera* (**A**) and *A. tenuis* (**B**) larvae infected with different cultures of *Chromera velia* and the *Symbiodinium* control.

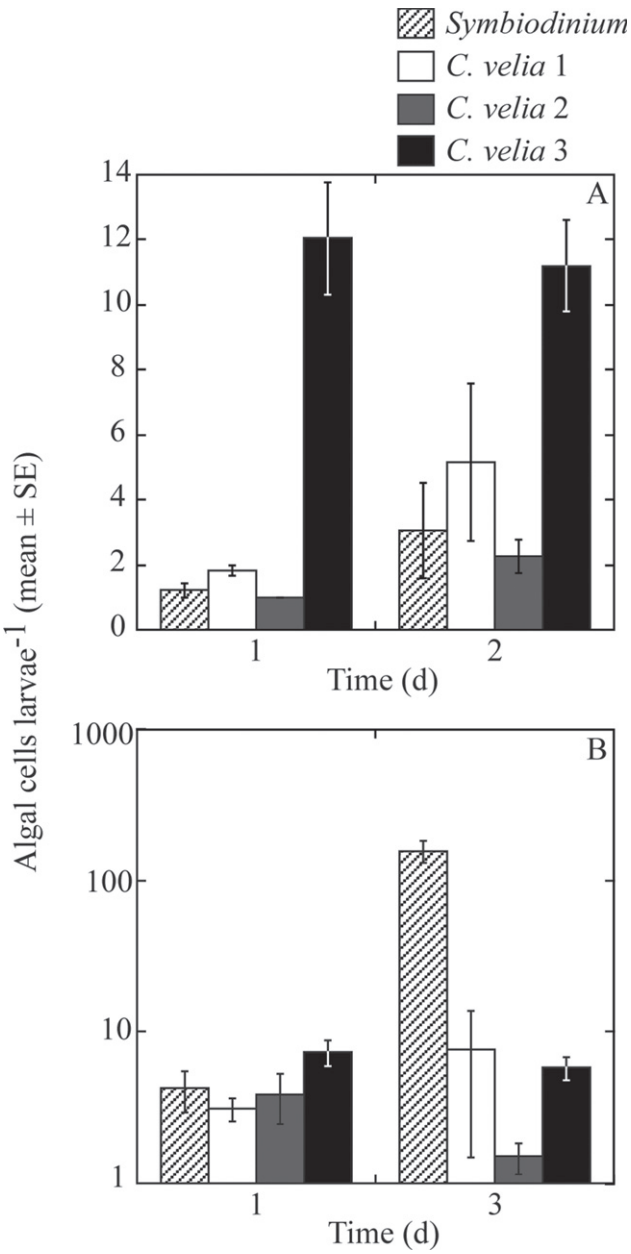

Figure 4. The mean (± SE) numbers of *Chromera velia* and *Symbiodinium* cells within *Acropora digitifera* (**A**) and *A. tenuis* (**B**) larvae.

The association between coral and *C. velia* appears to be quite common. *C. velia* associates with at least three coral genera at sites separated by over 3000 km on the east coast of Australia, and an apicomplexan gene sequence has been isolated from the Caribbean coral *Montastrea annularis* (Toller et al. 2002).

In conclusion, *C. velia* is commonly associated with adult scleractinian corals over a broad geographic scale. It can also establish an endosymbiotic relationship with coral larvae from at least two species of *Acropora* and is likely to be an important component of the coral holobiont.

Methods

Isolation of *Chromera velia*: *Montipora digitata* nubbins were collected from Nelly Bay, Magnetic Island (Lat 19°09′44.39″S, Long 146°51′14.90″E) on the Great Barrier Reef (GBR) and washed in filtered seawater (FSW). Epi- and endophytic algae, along with coral tissue were removed using a high-pressure air gun (Air-Pik). The resulting algal slurry was centrifuged and washed to remove residual coral tissue after which approximately 30 μl of each algal pellet was transferred into 15 mL Falcon tubes with 8 mL of modified f/2 (germanium dioxide was used in place of silicate) medium (Guillard and Ryther 1962) and placed in indirect sunlight to promote algal growth.

The f/2 medium was changed three times over a period of 10 days by centrifuging the samples at 2000 *g* for 5 min, decanting the medium and adding fresh f/2. After transportation to the University of New South Wales (UNSW) the cultured algae were transferred into 100 mL Erlenmeyer flasks containing 50 mL of f/2 medium, which was changed every 2-3 weeks. Cultures were placed in a growth cabinet at 25 °C–27 °C with a light/dark cycle of 12/12 h and a light intensity of approximately 200 $\mu Em^{-2} s^{-1}$. These preliminary cultures were viewed under a fluorescence microscope (Leica DM LB) which revealed the presence of two distinct cell types, one resembling *Symbiodinium*, which are easily identifiable by their single large and distinctive pyrenoid and cell size (7-12 μm) and a second type quite unlike *Symbiodinium*.

Developing monoclonal cultures: Fluorescence-activated cell sorting (FACS) (Sensen et al. 1993) was undertaken to develop monoclonal cultures from the algal cultures isolated from coral nubbins. Monoclonal cultures were achieved by separating cells based on their size and emission fluorescence. Aliquots (~2 ml) of the algal cell suspensions were centrifuged at 1500 *g* for 10 min and more than half of the supernatant was decanted to concentrate the cell samples. Samples were filtered through 40 μm sterile nylon mesh (Sefar) into FACS sample tubes to reduce clumping of the cells. FACS was undertaken using a MoFlo MLS (Dakocytomation). The chlorophyll autofluorescence (FL4) within the cells was excited with a 200 mW argon ion laser (Innova 90 ion laser, Coherent) at 488 nm and measured through a 590 nm long-pass filter. The forward scatter (FSC) and the side scatter (SSC) of the laser beam were also measured. The samples were sorted under sterile conditions at 16 psi using a 70 μm ceramic nozzle with a drop drive frequency of 97.4 kHz, a drop drive amplitude of 14.61 V and a flow rate of between 300 and 500 cells per second. The sheath fluid contained 1 x PBS. The cells were sorted into 96-well microtitre plates (1 cell per well) that contained 100 μL of f/2 medium. After 5 weeks the cultures were observed under a light microscope to determine which wells contained monoclonal cell cultures. Twelve monoclonal cultures from each 96-well microtitre plate were randomly chosen and transferred into 24-well microtitre plates containing 2 ml of f/2 medium (1 monoclonal culture per well). After approximately 3 weeks the cultures were observed again under a light microscope and 6 of the 12 monoclonal cultures were randomly selected and transferred into 100 mL Erlenmeyer flasks containing 50 mL of f/2 medium. Subsequently, three of these cultures were used in the infection study. The cultures are maintained at UNSW and will be made available upon request.

Visualisation of algal chloroplast using confocal microscopy: The chloroplast structure of the monoclonal algal cells was visualised with confocal laser scanning microscopy and compared to the chloroplast morphology of *Symbiodinium*. Imaging was by a Nikon Eclipse E800 microscope fitted with a Bio-Rad Radiance Plus Confocal Scanning System (Bio-Rad Microscience Ltd.) and a 100x oil immersion objective. The algal chloroplasts were imaged by chlorophyll autofluorescence excited by a 488 nm laser line of the Kryron/Argon laser (blue laser) and the chlorophyll emitted a red light above 680 nm using the technique developed by Salih et al. (1998). Serial optical sections at 0.15 μm incremental depths were made of randomly selected cells to a depth of approximately 8 μm (cell diameter), which resulted in approximately 53 sections scanned. The serial scans were reconstructed into a three dimensional (3D) image of the cell's chloroplast using the software VoxelView Ultra 2.1.2 (Vital Images) on an Indigo computer workstation (Silicon Graphics).

DNA extractions, PCR and sequencing of algal isolates: Genomic DNAs from the three cultures that were used in this infection study (ultimately named *C. velia* Mdig1, *C. velia* Mdig2, *C. velia* Mdig3) were obtained for 18S rDNA sequence analysis. A Qiagen DNeasy Tissue Kit was used to extract the DNA following the DNA extraction of animal tissue protocol. DNA was eluted twice from the spin column, first with 100 μl and second with 50 μl of elution buffer into a fresh microcentifuge tube. DNA concentrations were determined using a Nanodrop ® ND-100 Spectrophotometer.

PCR amplification of the 18S rDNA was performed using universal eukaryotic Forward (ss5 – 5′- GGTTGATCCTGCCAGTAGTCATATGCCTTG - 3′) and Reverse (ss3 – 5′-GATCCTTCCGCAGGTTCACCTACGGAAACC - 3′) primers (Rowan and Powers 1992) (Sigma-Aldrich) to obtain a PCR product of ~1800 bp in size. PCR was performed using 100 ng genomic DNA as template with 25 μl master mix (2.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM of each dNTP (Invitrogen), 1.25 units GoTaq® DNA Polymerase (Promega) and a buffer supplied by the manufacturer (Promega)). The PCR conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min, and then a final extension at 72 °C for 10 min.

The PCR products were electrophoresed in an agarose gel and were purified by gel extraction using the Wizard® SV Gel and PCR Clean-Up System (Promega). Sequencing PCR reactions were performed using the ABI Big Dye terminator 3.1 System (ABI) with 100 ng of purified PCR product as templates. Sequencing was performed at the UNSW Ramaciotti Centre for Gene Function Analysis.

Sequences were edited and combined using the BioEdit sequence alignment editor version 7.0.0. Culture identities were analysed at blast.ncbi.nlm.nih.gov, using the sequences individually as BLASTn queries. Sequences for the three cultures were submitted to Genbank (*C. velia* Mdig1 (JN986788.1), *C. velia* Mdig2 (JN986789.1) and *C. velia* Mdig3 (JN986790.1).

***Chromera velia* acquisition experiment:** Acquisition experiments were run using larvae from *Acropora digitifera* in Japan 2007, and *A. tenuis* in Australia 2008. The same three *C. velia* cultures (*C. velia* Mdig1, *C. velia* Mdig2, *C. velia* Mdig3) were used for both sets of experiments.

Acropora digitifera colonies were collected from Oku, Okinawa, Japan (26°50′48″N 128°17′21″E) and spawned on 29th June 2007. The resulting larvae were exposed to each of three *C. velia* cultures, cultured *Symbiodinium* ITS1 C1 obtained as the positive control, and no algal as the negative control. The cultured *Symbiodinium* C1 was isolated from *A. tenuis* from Magnetic Island in 2005. A total of 40 larvae were transfer to one of fifteen 200 mL containers containing 150 mL of 0.2 μm filtered seawater (FSW). Algal densities of ~5 x 10^4 cells mL^{-1} were added, resulting in three replicate containers per algal treatment. To detect acquisition of algal cells, 8 larvae from each replicate were sampled after one and two days of exposure to the algal cultures and visualised under a fluorescent

microscope. Sampled larvae were not returned to the experiment containers.

Acropora tenuis larvae cultured from adults that spawned on 20th of October 2008 at Magnetic Island, GBR were also exposed to each of three *C. velia* cultures, a positive *Symbiodinium* ITS1 C1 control, or a negative control (no algae). The positive *Symbiodinium* control differed from the previous positive control because instead of being cultured, it was freshly isolated from *A. tenuis* nubbins following the protocol in Bay et al. (2011). A total of 100 *A. tenuis* larvae were transferred into one of fifteen 350 mL containers containing 200 mL of 0.2 μm FSW. Algal densities of $\sim 1 \times 10^5$ cells mL^{-1} were added to each container, and there were three replicates $treatment^{-1}$. Ten larvae were sub-sampled from each of the replicates after one and three days.

To determine the successful uptake of algae in *A. digitifera* and *A. tenuis* larvae, data on the proportion of larvae infected, and the density of cells within the larvae were obtained by visually inspecting the larvae under a fluorescent microscope at 20x magnification. Prior to examination, larvae were washed by rapidly pipetting individual larva in FSW to ensure no algae were attached to their surface. Larvae were placed on microscope slides in a small droplet of FSW and cover slips were gently placed over the larvae to immobilise them. Cells were excited in the green excitation range with a bandpass 515-560 nm excitation filter, a 580 nm dichromatic mirror and a longpass 590 nm suppression filter. Upon excitation, the chlorophyll within the symbiotic cells fluoresced red, thus assisting cell counts within the larvae. For each larva, the number of symbiont cells was counted. Larvae of both species did not initially contain any algae, and larvae from the negative control (no algae) container did not acquire symbionts, indicating that all symbionts in the treatment containers were acquired from the algal cultures.

Histological analysis was performed on *A. digitifera* larvae to further verify successful uptake of *C. velia* and *Symbiodinium*. After experiment day 2, 5 larvae from each replicate were removed, washed and placed in 2.5% glutaraldehyde with FSW for 5 h. The larvae were removed from the glutaraldehyde solutions, washed 3 times in phosphate buffer and stored in 0.1 M phosphate buffer (pH 7.2) until processing. Histological analysis of the larvae were performed following the method outlined Miura et al. (2008), however cross-sections were cut every 5 μm instead of every 7 μm. Larval sections were visualized under light microscope at 20x and 40x magnification.

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