Doctoral Dissertation

博士論文

GROWTH, CELL DIVISION AND DYSFUNCTION OF CORAL TISSUES AND SYMBIOTIC ZOOXANTHELLAE IN THE SCLERACTINIAN *Pocillopora damicornis* (Linnaeus) REVEALED BY LIGHT AND ELECTRON MICROSCOPY

ハナヤサイサンゴ（イシサンゴ目）におけるサンゴ組織と共生褐虫藻の成長、細胞分裂および機能不全に関する光学および電子顕微鏡による研究

2017 年 02 月

Graduate School of Kuroshio Science

Kochi University

高知大学大学院総合人間自然科学研究科

黒潮圏総合科学専攻

ALEX PULVINAR CAMAYA

アレックス プルビナール カマヤ

Abstract

Pocillopora damicornis Linnaeus belongs to the order Scleractinia and is one of hermatypic coral species that is responsible for the deposition of hard calcareous structures in reef ecosystem. Since the coral bodies of *P. damicornis* exhibit polymorphism with less verrucae branches in the wide range of coastal habitat, the Japanese name for this species is 'hana-yasai sango' (meaning 'cauliflower corals'). The coral species is distributed widely from warm tropical to sub-tropic waters including regions influenced by the 'Kuroshio Current'. In this research, colonies of *P. damicornis* were collected off Yokonami Peninsula facing Tosa Bay in Kochi Prefecture, Japan and used as model imperforate species to perform coral fragmentation *in vitro*. The primary intention is to provide clone samples for live growth experiments and histological examination dealing with growth, reproduction (cell division) and dysfunction of coral and its symbiotic zooxanthellae. Considering the importance of coral tissue regeneration as the key output of the present culture method, this served as the baseline product in order to demonstrate the aims of this dissertation.

The profound linkages among studies come from the association of the physiological properties of symbioses *vis-a-vis* growth–reproduction–dysfunction, in which the sustainability of the relationship of host animal to its co-existing algae were being regulated. The vital findings obtained from researches in this dissertation are as follows (1) tissue fragment of imperforate corals were capable of growing from typical *in vitro* system where distinct modes of tissue regenerations were first observed from controlled environment; (2) the changes in the growth of coral tissues along with densities of *in situ* zooxanthellae cells were evident upon receiving radiations from monochromatic light spectra, as blue light further promote growth whereas ultraviolet-A and far-red rays significantly suppressed their proliferation; (3) the cell division of

the *in situ* zooxanthellae was followed by the division of coral cells where the successive invaginations of the symbiont membrane structures pose dynamics to cell shape regulation, while the novel role of coral nucleus in pre-cytokinetic mechanism in host animal was first reported among cnidarians; (4) pathomorphologies such as autophagosomes and electron-dense lysosomes were the plausible causes of the collapse host-zooxanthellae organization in the coral ultrastructure as they induced necrosis and autolytic digestions of the cytoplasm leading to release of various gastrodermal entities. This dissertation aims to contribute knowledge to further understand the underlying mechanisms towards the persistence of coral-algal symbioses. Our results could be a useful tool to address coral resiliency concern, mainly to provide inputs for future studies *i.e.* mass coral production to restore damaged reef due to unprecedented effect of natural and human perturbations.

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Main papers used in creating the dissertation

Peer-reviewed paper

1. Alex Pulvinar Camaya, Satoko Sekida and Kazuo Okuda (2016), Changes in the ultrastructure of the coral Pocillopora damicornis after exposure to high temperature, ultraviolet and far-red radiation, Cytologia, 81(4): 465-470.

Conference presentations

1. *Alex Pulvinar Camaya, Satoko Sekida and Kazuo Okuda, Ultrastructure of cell division of symbiotic zooxanthellae in the scleractinian coral *Pocillopora damicornis*, 9th International Kuroshio Science Symposium - Kaohsiung, Taiwan, November 17–20, 2015

2. *Alex Pulvinar Camaya, Satoko Sekida and Kazuo Okuda, Changes in the ultrastructure of the coral *Pocillopora damicornis* exposed to high temperature, ultraviolet and far-red rays, 13th International Coral Reef Symposium – Honolulu, Hawaii USA, June 19–24, 2016

* *speaker during conferences*

1. General Introduction and Thesis Outline

1.1. Early symbiosis establishment and symbionts localization in host cnidarians

The establishment of symbiosis is one of the most exiting study fields in cell biology on animal-algal symbiosis, where symbiotic zooxanthellae (unicellular dinoflagellates) can be acquired by cnidarians through maternal inheritance or, more commonly, anew with each generation from the surrounding seawater (as reviewed Davy *et al*. 2012). In most species, the infection of symbiotic zooxanthellae to host corals is performed by horizontal transfer and proceeds after the settlement of planulae (Hirose *et al*. 2008). The acquisition of symbionts during larval stages occurs after the early development of mouths and coelenterons, which facilitate the intake of symbionts for the onset of symbiosis (Harii *et al*. 2009). However, it has been reported that in some scleractinians, embryos may acquire new symbionts from external environment and restrict them into the gastrodermis during a gastrulation period (Marlow & Martindale 2007). On the other hand, there are three processes in the maternal entry of zooxanthellae into oocytes newly produced. According to Hirose *et al*. (2000; 2001), zooxanthellae are first observed in the follicle of female gonads. They are expelled from the follicle cells, passed through temporary gaps produced in mesoglea and finally entered oocytes through phagocytosis. Used as model species in this research, the scleractinian coral *P. damicornis* is hermaphroditic species that broadcast spawn both spermatozoan and oocytes in the water which gametogenesis and planulation per head commonly performed during spring and summer following a lunar cycle (Stimson 1978; Richmond & Jokiel 1984; Stoddart & Black 1985; Steiner & Cortés 1996; Permata *et al*. 2000). Pocilloporids such as *P. damicornis* and *P. meandrina* were vertical transmitter where they acquired symbiotic zooxanthellae by maternal seeding especially during gastrulation (Marlow &

Martindale 2007; Haryanti *et al*. 2015). Further, this species was revealed also demonstrating with asexual mode of planulation where the brooded planulae had showed exact inheritance of parental genotypes as revealed (Stoddart 1983).

Having a diploblastic body pattern, corals are simply arranged with two tissue layers: the epidermis (also referred to as ectodermis) and the gastrodermis (endodermis), which are separated by a thin, connective tissue layer, the mesoglea (Muller-Parker and D'Elia 1996; Venn *et al*. 2008; Fournier 2013) (see Fig. 1.1a). The population of zooxanthellae resides within the membrane-bound vacuoles in the gastrodermis (Trench 1987). Zooxanthellae are distributed mainly in the tentacles and column wall much denser than in other regions of polyps (Sekida & Okuda 2012). Symbiotic algae also reside in various intracellular localities in other invertebrates. The clam *Corculum cardissa* that accommodates its algal symbionts predominantly in the mantle and gills (Farmer *et al*. 2001), while in nudibranchs zooxanthellae are situated within host-derived 'carrier' cells associated with the digestive gland (Kempf 1984). In all of these localities, the intercellular communication between them is likely having molecular 'cross-talk' involving host-symbiont signalling and signal transduction such as carbon, nitrogen and phosphate uptake and fixation (Trench 1993; Davy *et al*. 2012; Fournier 2013) (see also Fig. 1.1b). Corals acquire the majority of their energetic and nutrient requirements in several dynamic mechanisms such as photosynthetic metabolism of the symbiotic algae, heterotrophy, and feeding of planktonic organisms from the water column (Lesser 2004).

In general, there are various processes in which cnidarians/coral-dinoflagellate symbioses could be established and persisted (as reviewed Davy *et al*. 2012). This includes recognition and phagocytosis, regulation of host-symbiont biomass, metabolic exchange and nutrient trafficking, and calcification.

1.2. Scleractinia and the host-algal symbioses in *Pocillopora damicornis*

1.2.1. Brief history, habitat, distribution and diversity

Scleractinians characterized primarily by corallite structure (Lesser 2004), were first reported to appear during Triassic era in tropical shallow water environment where they evolutionarily coexist with symbiotic algae (Stanley & Swart 1995). Due to the lack of appropriate fossil records, our knowledge of the origin of those symbiotic dinoflagellates is highly inadequate (Trench 1987). In terms of diversity and affinity, there is a high degree of uniformity in the scleractinian corals within the main body of the Coral Triangle (CT; global heart of coral distribution extending from the Philippines, Sunda Shelf to Solomon Is.), in which the highest richness resides in the Birds Head Peninsula of Indonesian Papua that hosts 574 species from the total of 627 in the entire CT (Veron *et al*. 2009; 2015). With attenuation to the north, the Kuroshio as one of the world's strongest boundary currents clearly creates the pattern of coral diversity in this region (Fig. 1.2). The graph also showed that the corals in Japanese locations are peripheral to the core of Kuroshio region.

Among the scleractinians, *P. damicornis* was considered a common species with abundant occurrence in the Indo-West Pacific region (Hoeksema *et al*. 2014) as depicted in its geograhic distribution in Fig. 1.3. The species can be found in the Philippines (various sites) and Japan (recorded from Tosashimizu, Kochi and Southwest Shikoku) and has the same range of variation from the Great Barrier Reef in Australia (Veron & Hodgson 1989; Veron 1992). This species occurs in all shallow water habitats from exposed reef fronts to mangrove swamps and wharf piles and are found in mono-specific stands or multi-species reefs throughout its range from near the surface to a maximum depth of 20 m (Hoeksema *et al*. 2014). Due to wide range of habitats where this species occurred, polymorphism in their morphological structures was

evident as illustrated in Fig. 1.4 (Veron 2013). In this study, the skeletal morphology of *P. damicornis* collected at Tosa Bay, Kochi, Japan resembled those found at the upper reef slope.

1.2.2. *P. damicornis*: transition from uniform recruits to polymorphic adult

The coral *P. damicornis* Linnaeus is one of the scleractinian coral well described at the early recruitment stage in controlled environment (Vandermeulen & Watabe 1973; Babcock *et al*. 2003). Immediate after the larval settlement, skeleton formation has been performed where two primary calcareous elements *i.e.* flattened spherulitic platelets and small rod-like granules have been produced (Vandermeulen & Watabe 1973). The species had exhibited similar patterns in skeleton formation to those in *Seriatopora hystrix* and *Stylophora pistillata* on *i.e.* septa, columella, and corallite wall (Babcock *et al*. 2003). The first apparent structures such as basal plate and three differentiated cycles of basal ridges are revealed as the distinguishing characteristic of young pocilloporid against acroporids. The corallite wall forms through the growth and fusion of synapticulae of basal ridge. After 1 week, a solid coenosteum, prominent septa and columnella become evident. Despite similarity in the pattern of development, the significant difference in the morphology of juvenile corallum allows this species to be recognized.

Upon reaching adult stage, its branching colonies usually reach less than 30 cm tall (Hoeksema 2015). The species is distinguished from other species by having thinner branches and less regular verrucae. As a result, *P. damicornis* exhibits greater branching than *P. verrucosa*. It commonly forms compact clumps on upper reef slopes exposed to strong wave action while pigment highly varies from pale to dark brownish-purple or green, sometimes pink, then dark yellow-grey to brownish-purple (Veron 1992). Coral *P. damicornis* can be used to

illustrate progress and the present state of knowledge in molecular taxonomy (reviewed Veron 2013). The species forms a species complex, characterized by high levels of plasticity within clades and cryptic points of differentiation between clades; thus considered as a highly polymorphic species.

1.2.3. Dinoflagellates: their distinguishing structures and life cycle

The evolutionary history of dinoflagellates is somewhat obscure due to lack of appropriately preserved materials in the fossil record (Trench 1987). It was in the early 1970's when the earliest literatures in the morphological and cellular structures of dinoflagellates (Phylum Dinoflagellata Bütschli 1885) was published by Dodge (1971, 1973) distinguishing them as unicellular flagellates. As the name implied, dinoflagellates have two flagella, one of which is the *longitudinal* flagellum with conventional type while the other is the *transverse* flagellum which is helical in construction. Dinoflagellates at free-living or motile stages revealed unique cell covering termed as *theca* (armor) which in several genera and orders showed variations in developments and ornamentations (Dodge 1965, 1971; Loeblich & Sherley 1979; Chapman *et al*. 1981; Netzel and Dürr 1984; Bricheux *et al*. 1992; Sekida *et al*. 2001). The detailed overview of the cortical plate pattern or tabulations in the diverse number dinoflagellates was concised by Netzel and Dürr (1984) where corticotypes where separated into five organizations namely prorocentroids, dinophysoids, gonyaulacoids, peridinoids and gymnodinioids. Some species were described *naked* or *unarmored* (did not contain cortical plates) where cell cortex revealed either liquid or flocculent materials which gives empty appearances to the vesicles. Other unusual if not unique structures of dinoflagellates were the trichocysts and pusule which the latter can only be found in dinoflagellates but not in all species (Dodge 1971).

When *in hospite*, the symbiotic dinoflagellate (commonly gymnodinioid in type, Freudenthal 1962), remained with highly evident thecal plates enclosed within thecal/amphiesmal vesicles (Loeblich & Sherley 1979; Wakefield *et al*. 2000; see Chapter 4). These structures were found in between the broad membrane layers containing the periplasts (Taylor 1968; Kevin *et al*. 1969) to more detailed cortical fine structures (Wakefield *et al*. 2000; see Chapter 4), of which complex membranes overlying algal plasma membrane were technically pronounced of algal origin (Wakefield *et al*. 2000). In the cytoplasm, the most striking structure of dinoflagellates is the nucleus where chromosomes lacks histone and permanently condensed (evident in all stages), and nuclear envelope persisting throughout its life cycle (Dodge 1973; Triemer & Fritz 1984). Ultrastructural information on the chloroplasts, pyrenoids, eyespots and associated organelles were provided from the early works of Dodge (1968, 1975) and Dodge & Crawford (1969).

The detailed life cycle of the symbiotic dinoflagellate (*Symbiodinium microadriaticum* Freudenthal) had been reconstructed by Freudenthal (1962) in Fig. 1.5 with reference from early descriptions of McLaughlin and Zahl in the late 1950's (as cited). At the early vegetative phase, an spherical cell exhibited single and thin cell wall, ochraceous color and homogenous cytoplasm due to lack of food products. As it grew, the vegetative cells lost some of its chloroplasts and turned into brownish-orange pigmentation. When the cell divides, they undergo binary fission producing two vegetative daughter cells with cellular inclusions equally distributed them. Equatorial zone of constriction occurred after nuclear division then the two cells separate due to deepened furrow. In some cases, 4-celled configurations with paired axes appeared. When cell developed into cyst, the cell wall thickened and may follow one of the three courses namely (1) zoospore or aplanospore; (2) mitotic cell with two autospores or (3) gamete production. Cysts

which contain gymnodinioid cell yield motile cell while zoospore was quiescent within parent wall.

1.2.4. Symbiotic zooxanthellae and the identification of *Symbiodinium* spp.

In recognition of the early proposed generic index for the symbiotic algae by Brandt in 1881 who first identified *Zooxanthella nutricula* from the radiolarian *Collozoum inerme* Haeck (Loeblich & Sherley 1979), the term 'zooxanthellae' [(Greek *zoon* (animal) + *xanthellos* (diminutive form of yellow)] was coined. During this period for the coral pioneers Peyssonel and Ellis, intriguing questions arises whether these tiny single-celled organisms discovered in their fleshy tissues regarded at first as infestation could be possibly an animal origin (Bowen and Bowen 2003). Until then, the earlier studies of the *in vitro* culture of gymnodinioid zooxanthellae from *Cassiopeia* sp. by McLaughlin and Zahl in 1957 had inspired Freudenthal (1962) and further proposed *Symbiodinium microadriaticum* to be assigned as new species name. The latter claimed that genus *Symbiodinium* was tenable classification derived (Greek *symbion* living together + *dinos* whirling) from the family Blastodiniaceae and species (=*microadriaticum*) resembling *Gymnodinii adriaticum*, described with formal generic diagnosis. To date, *S. microadriticum* Freudenthal remains highly recognized as single pandemic species despite the repeatedly amended ultrastructural descriptions (Kevin *et al*. 1969; Trench & Blank 1987) and augmenting number of divergent molecular clades (Stat *et al*. 2006).

1.2.5. Dysfunction of coral-algae symbioses and the treat from climate change

The context of symbiosis breakdown between coral and its symbiotic algae has been one of the highlights of physiological studies in the recent times. This offered a number of opportunities for

reef scientists worldwide to investigate mass mortality in the last decade (Brown 1996). The phenomenon of extensive coral reef bleaching (whitening of corals as a result of dysfunction of coral-algae symbioses) in the Indo-Pacific region from 1979 to early 1980's was the earliest documented events associated with El Niño-Southern Oscillation (ENSO) (see Glynn 1993; Brown 1997; Hoegh-Guldberg 1999; Fitt *et al*. 2001; Baker *et al.* 2008 for reviews). Since then, episodes of natural coral mortality occurred including in Caribbean due primarily to climate change-induced ocean warming which prompted much concern among scientists to focus on the mechanism of bleaching. Gates and co-workers (1992) have represented five potential mechanisms of symbiont loss from cnidarian host tissue such as in *P. damicornis* that includes exocytosis, apoptosis, necrosis, pinching and host cell detachment concomitant with thermal bleaching under experimental stress conditions (see Fig. 1.6). Others have considered the degrading potential of autophagy (Dunn *et al*. 2007; Downs *et al*. 2002; Hanes & Kempf 2013), along with grastrodermal autolytic digestion that weakened cell-to-cell adhesion causing the expulsion of zooxanthellae (Camaya *et al*. 2016).

1.3. Aims and Hypotheses, Significance and Framework of Thesis

1.3.1. Aims and hypotheses of studies

The ultimate aim of this thesis is to observe and describe using light and electron microscopy the growth, cell division and the dysfunction of coral host and the symbiotic zooxanthellae in scleractinian *P. damicornis*. In each study chapter, the hypothesis and specific objectives were sought as follows;

In **Chapter 2**, this study aims to (a) demonstrate the method used for the *in vitro* culture system of coral tissue, and (b) to characterize the two modes of the induced tissue regenerations

of corals. As hypotheses, the scleractinian corals could be cultured in a closed system similar in principle to those in aquaria exhibitions; however setting up an ideal culture system for coral growth remains difficult (Borneman 2008) that depends mainly on the nature of study. Likewise investigating the regenerative mechanism of corals by microscopy could be a challenging option as most of the available literatures have reported such event from the natural reef environment.

For **Chapter 3**, the objectives were to (a) determine the effect of different monochromatic light spectra on coral tissue growth and density of *in situ* symbiotic zooxanthellae, and (b) identify the relationship of growth conditions between host tissue and the population of symbiotic zooxanthellae from different light treatments. The hypotheses drawn are that coral growth response will have significant changes under varying light regimes, as reported in some spectral regime such as blue, red, green and UV. Examining the regenerated tissues from live coral specimen through microscopy is a novel approach in which results relies most from the validity of sampling procedure.

Chapter 4 is mainly directed to (a) describe the process of cell division of the symbiotic zooxanthellae in host coral, and to (b) identify the roles of the cortical fine structure and other cytoplasmic components during cell division. It is hypothesized that zooxanthellae division either precedes or followed by the division of the host cell as mitotic index of symbionts had reported to peaked earlier than its host animal. At ultrastructural level, cell division remains were in fact poorly demonstrated in which the detail of the process were highly considerable.

Then in **Chapter 5**, this study aims to (a) compare the changes in the ultrastructure of host coral tissue and its symbiotic zooxanthellae in *P. damicornis* when exposed under these stressors, and to (b) determine the underlying cellular mechanism involved and implication in the early stage of bleaching in coral. Further it is hypothesized that despite the overwhelming studies

on coral bleaching, yet no sole mechanism is established so far. This indicates that the phenomenon of coral bleaching is highly variable, in which the mechanism involved depend on the various aspects including stress conditions, species used, environment and others.

1.3.2. Significant contributions to knowledge

This section discusses insights to which this thesis might contribute to knowledge dealing with;

1. The aspect of coral reproduction that demonstrate how corals proliferate asexually by fragmentation in closed system and characterize in details the coral tissue regeneration observed from light microscope, considering that most of the available literatures mainly reported this aspect from the natural reef settings.

2. Contribute knowledge on the dynamics of coral photobiology to which coral growth responses depend mainly on the form and intensity of light received. Physiological growth conditions of coral tissue along with the densities of symbiotic zooxanthellae changed dramatically under monochromatic form of light spectra, which is highly attributed in this experimental study.

3. Contribute to the field of ultrastructural examination in cnidarian symbioses where studies on scleractinian corals remain limited to date due to complication in the fixation procedure. This thesis first to revealed the detailed stages of cell division of zooxanthellae *in hospite* emphasizing the physiological process of cytokinesis by describing the behavior of the cortical fine structures and the novel role of coral nucleus in the division process.

4. The ultrastructural examination dealing with the early stage of bleaching further contribute knowledge on the mechanisms involved in the cellular dysfunction of coral-algae symbioses where findings from far-red light radiations were first reported. As the main study of

this dissertation, this chapter demonstrates a comprehensive comparison of the effect of three distinct stressors in coral.

1.3.3. Research framework and structure of thesis

Figure 1.7 and 1.8 illustrated the framework of the research studies conducted for this thesis and the linkages of every study chapter to one another. The linkages between studies are mainly based in the methodologies used. From these diagrams, the study on the coral *in vitro* culture (Chapter 2) served as the baseline or the source of specimen used for various cellular analyses done for growth experimental (Chapter 3) and histological examinations (Chapters 4 & 5). Chapters 3 and 5 were linked due to the methods used for inducing light treatment (Fig. 1.8) as stressor to corals *i.e.* UV-A and far-red radiations, where these spectral rays were initially found to inhibit coral growth in Chapter 3 then continuously examined at ultrastructural level in Chapter 5. To demonstrate the results of the micrographs in each study chapter, specimens were primarily observed through light microscopy (Chapter 3) and transmission electron microscopy (Chapters 4 & 5). In general, these studies may pose potential implication for establishing future set-up to produce mass clone fragments for various cellular examinations or for coral transplantation project as a current plausible restoration scheme for the damaged reef.

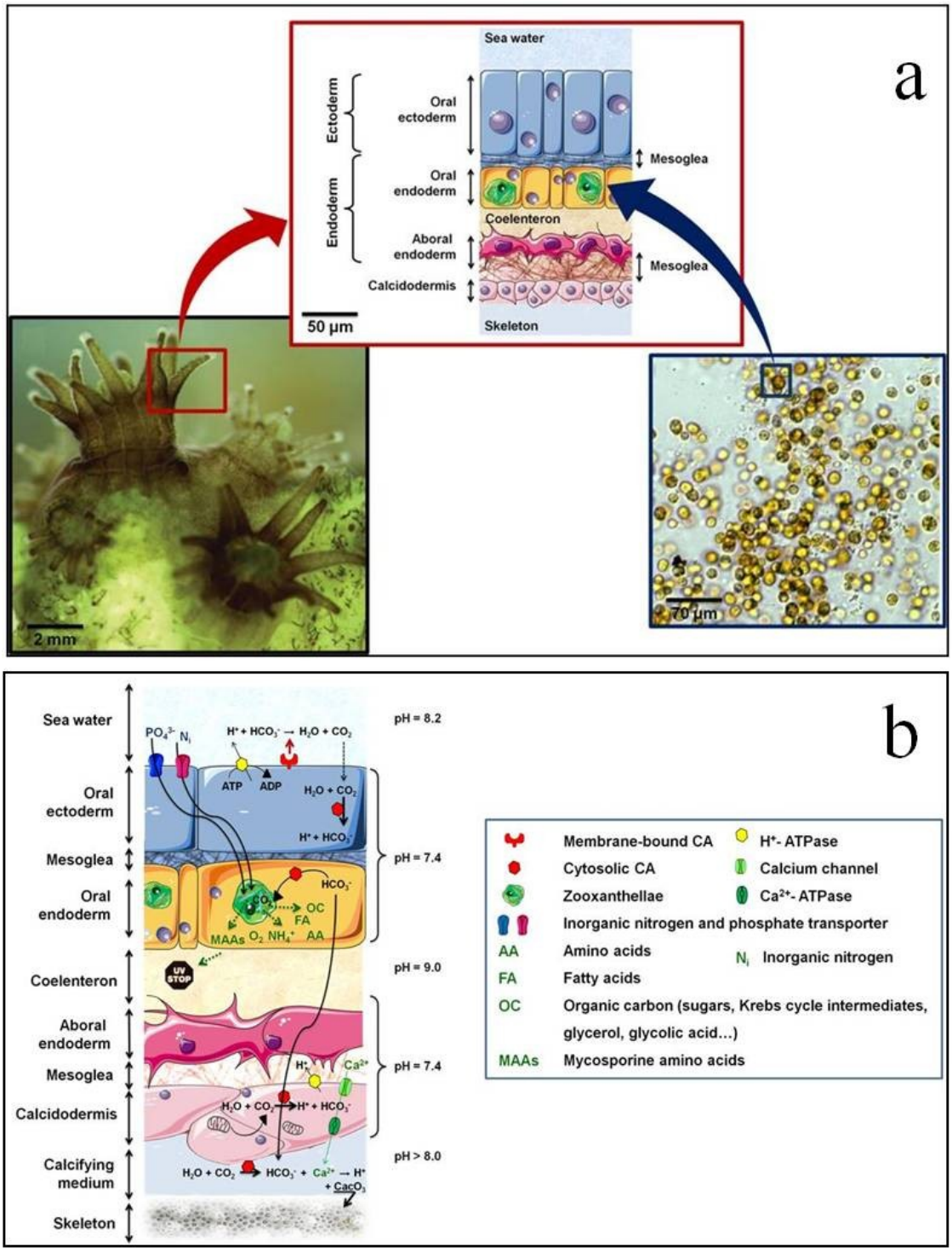

Fig. 1.1. (**a**) Localization of the symbiotic algae in the coral gastrodermis and (**b**) processes of metabolic interaction and calcification. (Illustrations and texts adopted from Fournier 2013)

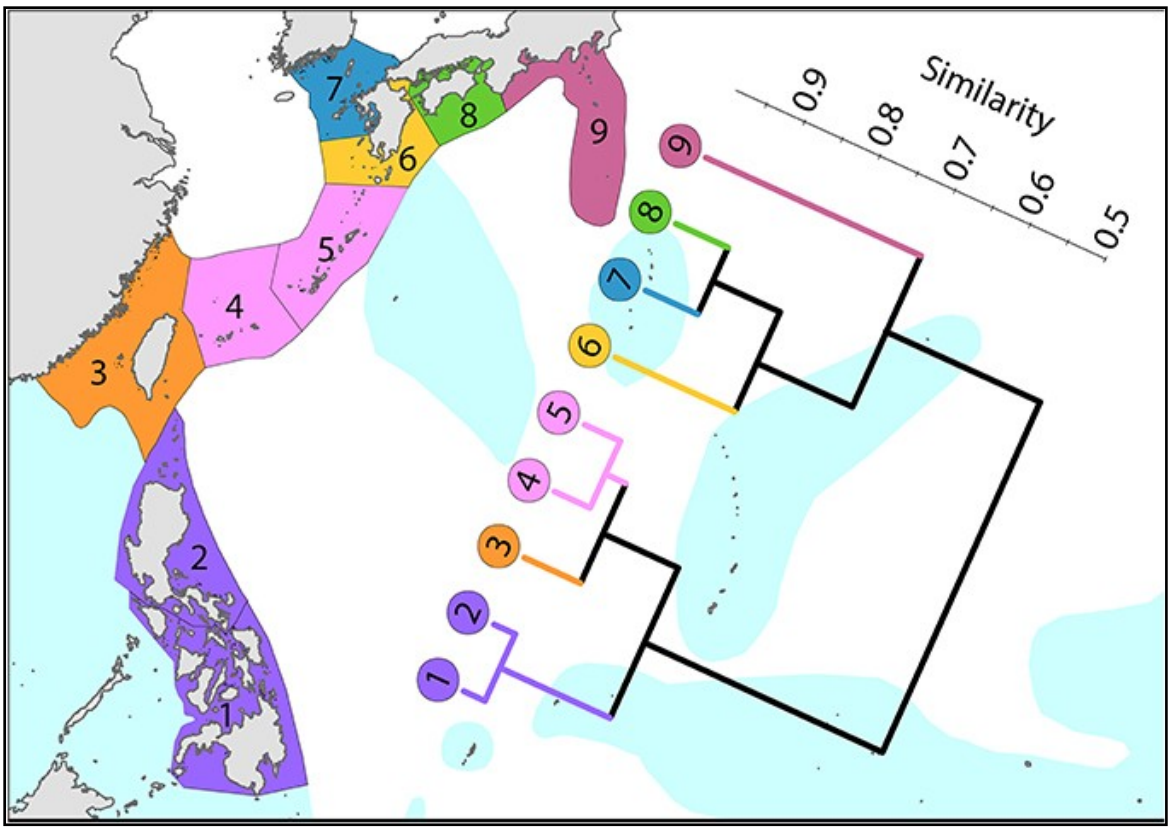

Fig. 1.2. Attenuation patterns of scleractinian coral affinity and diversity along the boundary current of Kuroshio. (Map adopted from Veron *et al*. 2015)

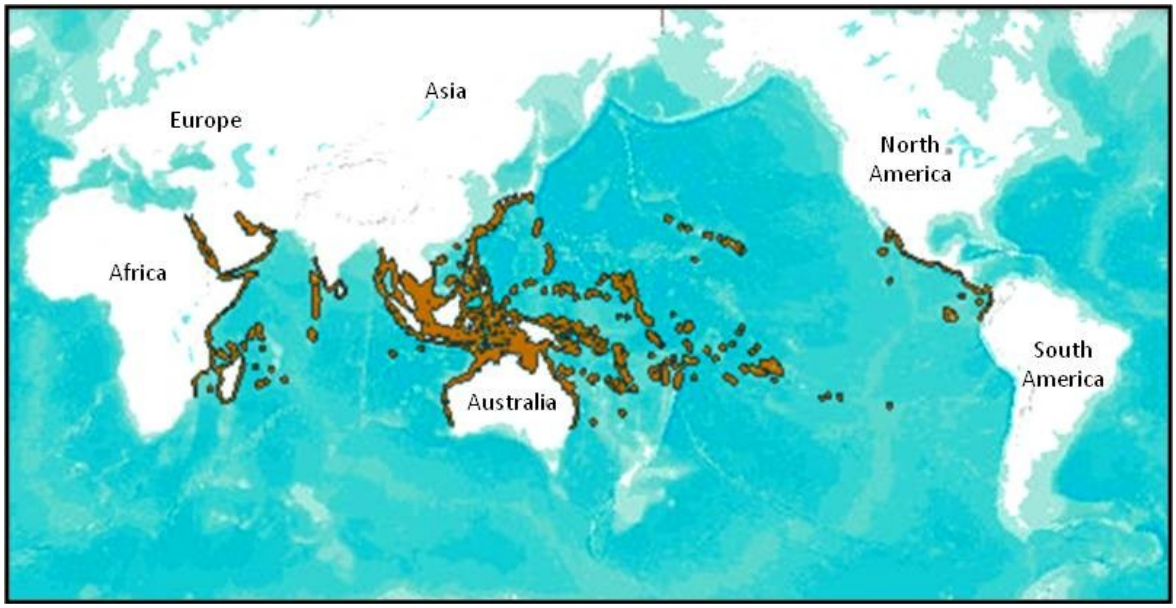

Fig. 1.3. Worldwide geographic distribution of *Pocillopora damicornis* (*brown marks*). The species is considered as common and least concern (LC) The IUCN Red List of Threatened Species (Map adopted from IUCN Red List, Hoeksema *et al*. 2008).

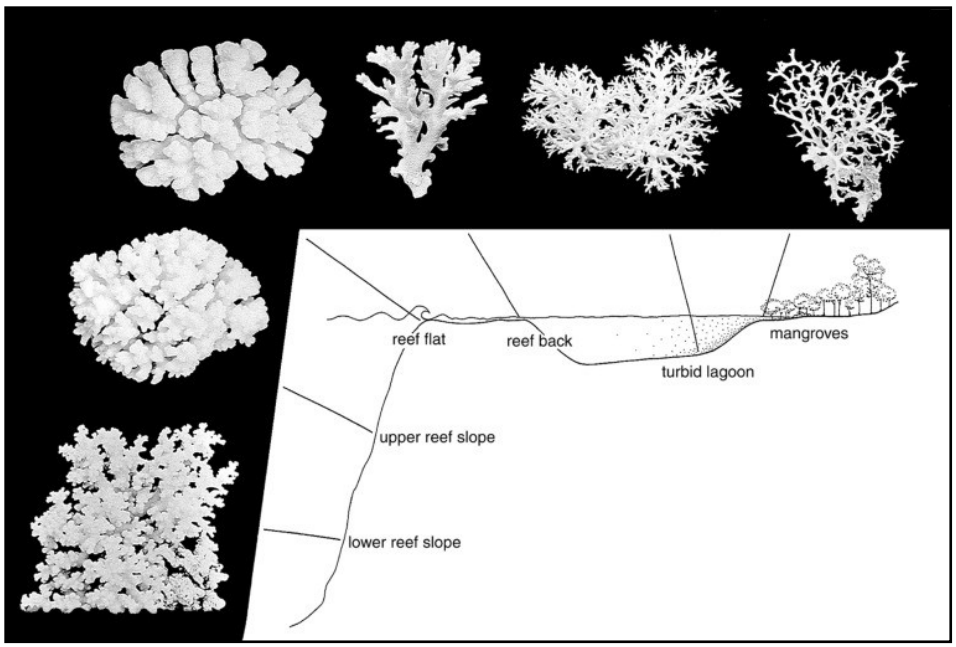

Fig. 1.4. Polymorphism of the skeletal structure of *Pocillopora damicornis* from a wide range of habitats (Illustration adopted from Veron 2013).

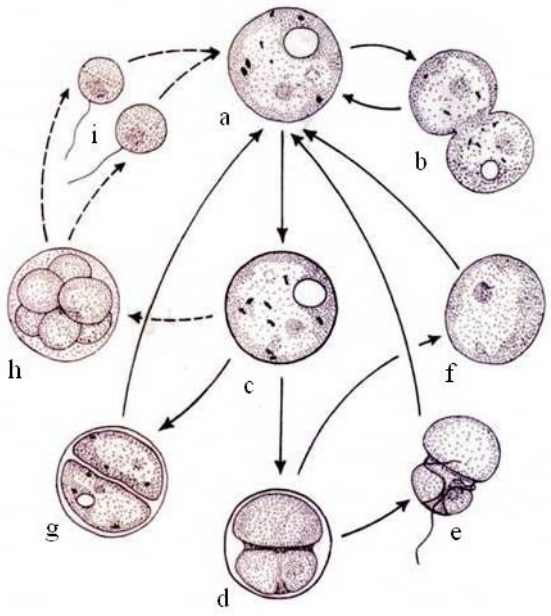

Fig. 1.5. The life cycle of the symbiotic dinoflagellate *S. microadriaticum* Freudenthal isolated from the host *Cassiopeia* sp. (**a**) Vegetative cell with thin cell wall. (**b**) Cell during binary fission producing two daughter cells. (**c**) Vegetative cyst with thick cell wall. (**d**) Mature zoosporangium containing a gymnodinioid zoospore. (**e**) Gymnodinioid zoospore. (**f**) Aplanospore. (**g**) Cyst containing two autospores. (**h**) Cyst containing developing isogametes. (**i**) Liberated isogametes. (Illustrations and texts adopted from Freudenthal 1962)

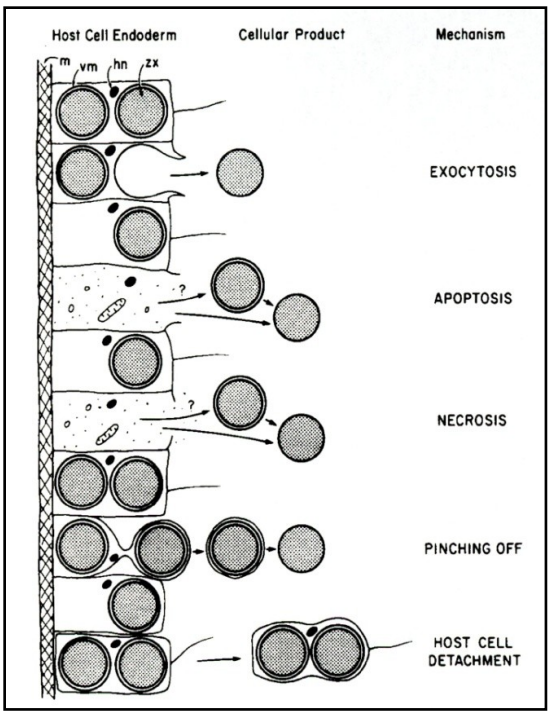

Fig. 1.6. Five potential mechanisms of zooxanthellae release from the host endoderm. (Illustration and texts adopted from Gates *et al*. 1992)

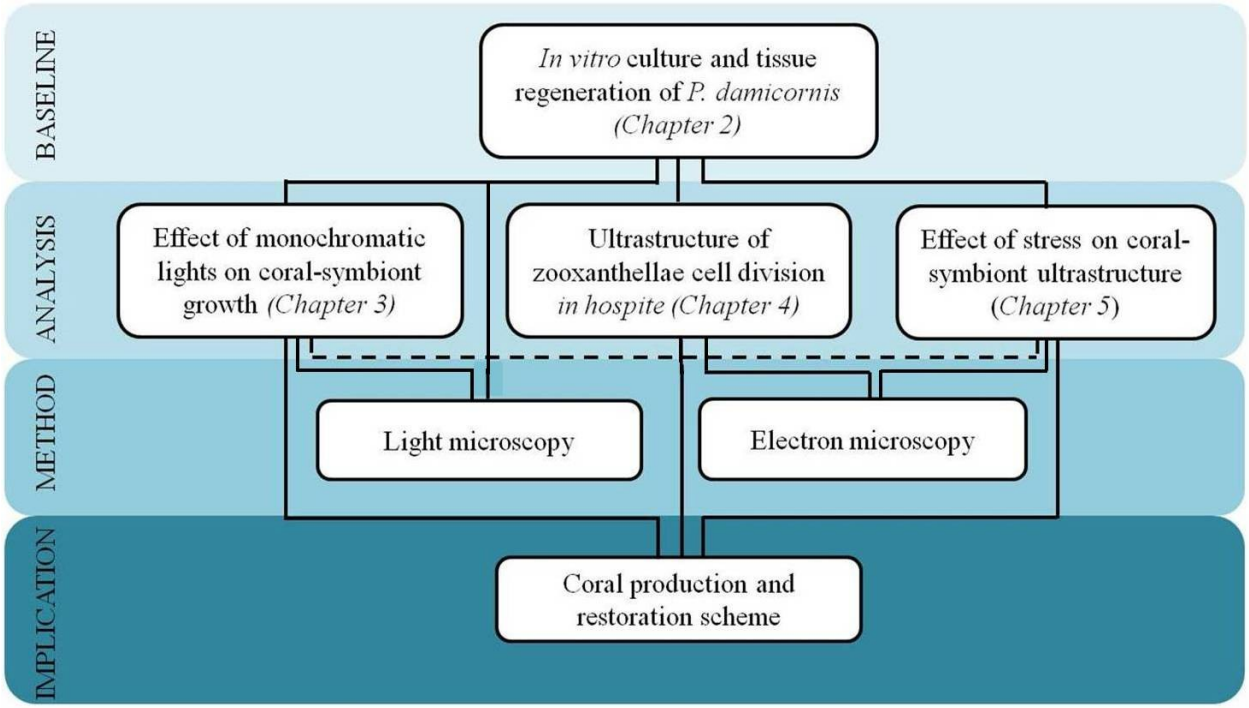

Fig. 1.7. Research framework showing the whole context of dissertation and linkages of study chapters to one another.

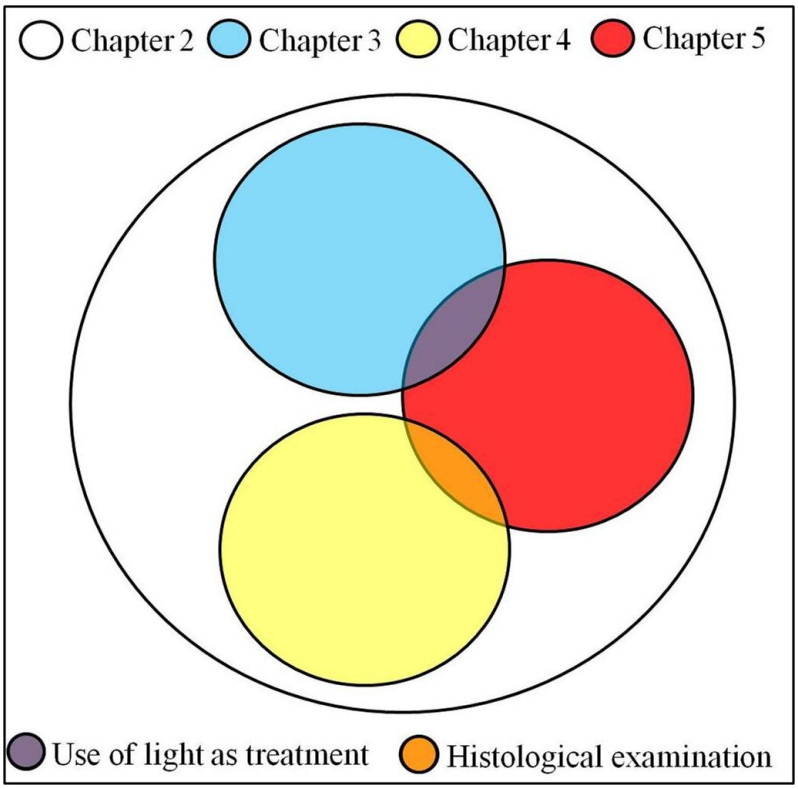

Fig. 1.8. Simplified structure of linkages of the methodologies used in all study chapter of thesis.

2. Regeneration and tissue culture from small coral fragments

2.1. Introduction

To better examine coral growth, one has to understand how corals may survive and reproduce under controlled conditions. For maintaining corals in captivity, it is important to note that seawater quality used should follow those found in nature where corals originally live (Borneman 2008). High degree of success in maintaining corals can be obtained from the establishment of culture conditions with optimal water parameters for coral growth (Borneman 2008; Osinga *et al*. 2011). These parameters include seawater salinity and temperature and may critically affect coral health. Changes in the parameters often result in rapid bleaching or mortality of corals in nature and laboratories (Borneman 2008). Although it is usually difficult to establish an ideal culture system for coral growth, Borneman (2008) claimed that maintenance of coral cultivation could be performed by setting adequate methodologies, equipment and techniques.

Corals reproduce asexually by means of fragmentation of colonies. This was evident that after the predominant breakage or fragmentation of corals during natural disturbances, reef recovery has been reported elsewhere (Highsmith 1982). Understanding the mechanisms related to asexual reproduction holds a key for new and better ways of coral culture (Kramarsky-Winter *et al*. 2011). Through asexual growth mechanisms, the widespread concept to transplant corals has been carried out for species with high growth rates (Yap *et al*. 1992; van Treeck & Schuhmacher 1997; Soong & Chen 2003). However at some points, fragmentation could either altered the growth morphology or reduced the sexual reproduction especially for scleractinian coral *P. damicornis* (Permata & Hidaka, 2005; Zakai *et al*. 2000) or inevitably lead to mortality (Yap *et al*. 1992; Zakai *et al*. 2000). Yet despite these findings, the species has been the subject

of various reproductive examinations, in captivity (Stimson 1978; Zakai *et al*. 2000; Permata & Hidaka, 2005). Experimentally induced injuries or lesions in this species further led to tissue regeneration (Hall 1997; Permata & Hidaka 2005).

Most corals have its innate capacity to regenerate or to extend new tissue over damaged area (Meesters *et al*. 1997; Kramarsky-Winter *et al*. 2011). Bak & Steward-Van Es (1980) found that the surrounding tissue on the lesion enlarged shortly due to the retraction which then followed by successful regeneration until the regenerative capability decreases upon reaching stabilization period. The process generally occurred from the outside to the inside, towards the center of the injury/lesion (Sabine *et al*. 2015). The closure of lesion was assumed to be assisted by polyp development or integration of colonies (Bak and Steward-Van Es 1980; Oren *et al*. 2001). Most studies that we have referred mainly suggested that the capacity of corals to regenerate was strongly influenced by the size of the lesion (Bak & Steward-Van Es, 1980; Wahle 1983; Hall 1997; Meesters *et al*. 1997; Oren *et al*. 1997, 2001; van Woesik 1998; Kramarsky-Winter & Loya, 2000; Cróquer *et al*. 2002; Titlyanov *et al*. 2005; Fishers *et al*. 2007; Sabine *et al*. 2015). This indicates that experimental infliction of injury is the prominent approach to investigate coral regeneration. Most of these compelling studies were performed prominently in the natural setting, suggesting that the examination of coral regenerations were well restricted from the controlled environment. Its diverse regenerative capability remains poorly documented as attempts of characterizing regenerated tissues from the coral fragments *in vitro* had been hardly done so far.

There has been a novel finding that demonstrated the coral re-morphogenesis from the cultured tissue fragment *in vitro* (Kramarsky-Winter *et al*. 2011). Kramarsky-Winter *et al*. (2011) found that soft tissue devoid of skeleton can be cultured in seawater-based medium under

relatively low temperature resulting in the formation of spheroids which may be kept viable in the culture for several months and may be induced to form a developed coral polyp upon increasing the temperature.

In this study, the concept of *in vitro* system is likewise presented with the method of culturing small coral tissue (with intact skeleton) in the glass dish maintained under ambient room condition. One of the highlights of this study had involved the characterization of the tissue regeneration that remains rarely examined *in vitro*. Scleractinian coral such as *P. damicornis* possessed an imperforate tissue (Stimson 1997; Kinzie *et al*. 1984) that induces diverse mode of regeneration in closed system. The survival rates of the cultured fragments and the potential factors that affect them were also discussed. Further, the timing of cell division of the *in situ* symbiotic zooxanthellae was also examined.

2.2. Materials and methods

2.2.1. Collection of coral samples

Coral samples used in this study were collected from shallow water (1–3 m in depth) off the Yokonami Peninsula facing Tosa Bay, Susaki City, Kochi Prefecture in Japan, using SCUBA gears. Two to three small and healthy colonies (ca. 5-8 cm in length) of *P. damicornis* and *Acropora* sp. were collected monthly by bare hands and placed into a mesh bag. After collection, the coral were immediately transferred into re-sealable buckets with seawater taken at the collection site then aerated. Samples were then transported to the laboratory at Kochi University (Asakura Campus) by car for 45 minutes. The preliminary samples were collected in April 2014 (intended for studies in Chapter 3). After 4 months, year-round samples were collected until July 2015 to produce sufficient stock cultures. However, due to high mortality of corals at the first

three months, the collection was extended until December 2015. During the extension period *Acropora* sp. sample was not continuously collected as a result of high mortality.

In Table 2.1, the dates of coral collections and monthly survival rates of two coral species were shown, however in several periods information on the number of isolates produced and survival rates were absent (no data recorded) due to failure of transcribing such information. Further, the mortality in *Acropora sp*. fragments was severe where they it bleached or died eventually from isolation due to its fragile structure as compared from *P. damicornis* with sturdy skeleton. During fragmentation, branches excised from the apical region of the colony easily broke which may affect its tissue integrity. From the series of monthly collection with careful handling and fragmentation procedure, this species remains vulnerable to isolation. For this reason, *P. damicornis* became the focal species solely cultured for this research studies.

2.2.2. Coral fragmentation and culture technique

After arriving in the laboratory, coral fragmentation was immediately followed (Fig. 2.1). To avoid stress, corals were hand-held on the proximal edge of the colonies above the basin with seawater. Small fragments (ca. 3-5 mm) were excised from apical region of the branches using pliers. Fragmentation was done as quickly as possible. The excised fragments were remained in the seawater at all times. Using forceps, fragments were transferred separately in individual Petri dish containing approx. 150 mL of raw seawater medium collected from Kochi University Marine Biological Station (Fig. 2.8). Fragments were then gently mounted on the small drop of silica gel (Dow Corning Toray, Japan) placed on the cover glass (18 x 18 mm) in the Petri dish. Cultures were maintained in room conditions exposed to room fluorescent light and temperatures range of 25-27°C. The number of isolates produced monthly depends on the condition of the

colony collected (Fig.2.6). Exchanging medium and removing debris and growing algae were done twice. The truncated colonies were maintained in aerated aquaria containing raw seawater (approx. 12 L) for future use.

2.2.3. Identifying the ideal site for coral culture survival

The survival of the coral fragment in the culture dishes is the essential output of this study. In the early attempts of cultivation, fragments were placed in the various sites in the laboratory to determine the ideal place for corals to survive. Extremely fast and high mortality of samples occurred from the window areas especially after exposure to elevated day-light. During event of coral mortality, indiscriminate expulsion of symbiotic zooxanthellae occurred as reported in Camaya *et al*. (2016), where considerable amounts of pellets were released then shedding off the entire tissue from its skeleton. From the shaded areas with low exposure to day light and room fluorescent light, mortality of the samples was rather delay but remains high. However from the common desk with direct exposure to room fluorescent light (irradiance level of 8-10 μmol $m^{-2}s^{-1}$), the survival was significantly high among *P. damicornis*.

In the year-round sampling, the monthly survival rate of the coral isolates was computed (formula shown below). From the total number of samples produced monthly, all samples that survived for the first month of isolation were counted. This was based from the present observation that the initial 30 day period is critical for coral to survive in isolation.

$$\text{Survival rate} = \frac{\text{No.of alive samples}}{\text{Total no.of prepared isolates}} \times 100$$

2.2.4. Time lapse micrography of coral tissue growth and zooxanthellae division

When coral isolates of *P. damicornis* survived after 8-10 days, subsequent tissue regeneration was observed however the induction of tissue growth varied among samples. To monitor the tissue regeneration, time-lapse micrography (Fig. 2.2.) was performed for the coral fragments with exposed lesion and for the lesion directly mounted on silica gel. Micrographs were taken automatically every 1 h from 10 to 30 days using dissecting microscope (SZX7; Olympus Optical Co., Ltd, Tokyo, Japan) fitted with digital camera (Coolpix P6000; Nikon Co., Ltd, Tokyo, Japan) and a digital monitor (Sharp Aquos LC-13SX7A, Japan).

To measure the growth rates of tissue regeneration, low magnification micrographs were analyzed. Using image processing software ImageJ (Rasband, 2003), the length of the tissue regenerated was measured in five different locations. Length was measured at fixed scale of 373.33 pixels/1000 μm. The marginal growth rate (MGR) formula modified after Ichiki *et al*. (2001) was used, expressed as;

$$\text{MGR} = \frac{\bar{x}(l_2 - l_1)}{\bar{x}(t_2 - t_1)}$$

where l = length and t = time at *1* (final) and *2* (initial), respectively. On the other hand, the timing of cell division of the *in situ* symbiotic zooxanthellae was determined using automatic time-lapse microscopy (Fig. 2.4). High magnification micrographs obtained for 24-48 h in were analyzed. All symbiotic zooxanthellae cells that appeared contiguous or with doublet formations were thoroughly compared from its previous configuration. This strategy helps to verify whether the cell(s) likely to divide or simply overlapping to one another.

2.2.5. Assessment of field and laboratory water parameters

After the coral samples have been collected, water parameters such as the temperature and salinity were measured from the collection site using hand-held thermometer and refractometer (AS ONE, IS/Mill-E, Japan), respectively. This was measured to determine fluctuations of these factors during the year-round culture period. This aspect was found useful especially in determining the factor that might affect coral survival.

2.3. Results

2.3.1. Two distinct modes of tissue regenerations

Small apical fragments excised from the coral colonies of *P. damicornis* were cultured in ambient room temperature and light conditions. Coral fragments were mounted on cover slips and placed in Petri dishes containing filtered seawater medium. They regenerated new tissues and continued to survive for several weeks. The maximum thickness of new tissues regenerated was around 0.21 mm. Two distinctive modes were found in tissue regeneration. After coral fragments were excised from colonies, the injured side of the fragments was situated at the upward direction, exposing directly to seawater. New tissues regenerated centripetally from surviving tissues on the injured side 8 days after fragmentation. This mode was termed centripetal regeneration. Time-lapse micrographs (Fig. 2.2A1–3) show that the regenerating tissues covered superficially on the naked skeleton surfaces of the cut ends of coral fragments and grew towards the mid region of the naked skeleton.

When the lesion portion of coral fragments was situated at the downward direction and mounted directly on a cover slip, new tissues grew radially from the edges of surviving tissues and extended over the surfaces of a cover slip (Fig. 2.2B1–3). This was termed a radial

regeneration mode. However in the apically fragmented samples that were placed erect on the glass cover (Fig. 2.3A), the portion of the lesion hanging from the glass substrate exhibited centripetal regeneration while the portions leaning on substrate tended to outgrew. Here, outgrowing was referred to the 'radial' mode of tissue regeneration which initiated 10 days after fragmentation. This was also the typical regeneration mode observed in the lesion mounted on the silica gel where the tissues overgrew towards the smooth glass substrate (Figs. 2.2B and 2.3B). The surrounding lip of the lesion was estimated to have average width of ±41.7 mm (non-contracting). The latter was observed colorless due to the absence of the *in situ* zooxanthellae cells, a region closely characterized as the epidermis. During tissue regeneration, developing polyps were evident. Juvenile polyps developed on the regenerated tissues (Fig. 2.2B3), distributed at an mean distance of ±1.3 mm between polyps or polyp density of ±3.0 individuals/mm^2. The regenerated coral tissues were also noted to overgrow the debris such as shedded endoskeleton attached on the glass substrate. During the formation of polyps, localized assemblages of symbiotic zooxanthellae were evident. As they increased in number, the coral tissue bulge then subsequent juvenile tentacles and oral cavity were developed. As polyps matured, spots of calcified materials formed behind its loci were deposited on the glass substrate. In the event where the regenerated corals retracted (Fig. 3.1, Chapter 3) or subsequently died, remnants of these spots and rings of calcification showing the increments on the substratum were observed. Corals that exhibit regeneration apparently became pale than its original pigmentation. Loosening of spaces between the *in situ* symbiotic zooxanthellae was apparent as the host tissue regenerate.

2.3.2. Coral tissue growth rates

In the time lapse monitoring, the centripetal tissue regeneration took 6 days to completely close or heal the surrounding lesion. This gives a mean growth rate of 67.8 μm/day for the tissues that regenerate on its skeletal surface. The result was obtained from two samples where tissues tended to close within 6 and 7 days, respectively. On the other hand, the coral cultures with lesions mounted on the silica gel initiated tissue regeneration at the earliest period of 7 days. Depending on the health condition (possibly amount of stress obtained), some coral cultures exhibited delayed growth after isolation into the Petri dishes. When the tissue regeneration persisted, it extended radially on the cover glass at the mean growth rate of 55.2 μm/day. This was the result from the three coral cultures measured with continuous tissue growth for 10 days. To compare these two modes, the growth rate by centripetal regeneration where the tissue traverses rough surfaces was relatively faster than the radial regeneration growing on the smooth surface such as a glass substrate.

2.3.3. Timing of cell division of *in situ* zooxanthellae

The cell division of the *in situ* symbiotic zooxanthellae in the regenerated tissue had exhibited no specific timing based on the 24-h daily pattern. In the time-lapse monitoring (Fig. 2.4), the apparent doublet cell formation which was the characteristic of the cytokinesis (as discussed in Chapter 3) occurred at 06:10 (morning); 18:10 (afternoon) and 00:10 (midnight) (Fig. 2.4). This suggested that diel pattern is absent in the division process of the symbiotic zooxanthellae *in hospite*. Time-lapse micrographs also showed that zooxanthellae cells used to develop spherical doublet cell formation within 4 h and 6 h, respectively. If this is the case, symbiont cells may possibly took at least 4 h to completely develop the entire division cycle.

2.3.4. Survival of the coral cultures

The monthly survival rate of *P. damicornis* cultured *in vitro* for one year period vary monthly as shown in Table 2.1 and Fig.2.5. At the initial period, high mortality of this species occurred probably due to inevitable stress from acclimatization factors of the coral. The data had showed that sample collected in November 2014 had low survival rate. There were low increase in the succeeding months but tended to decline in the final month of winter season (March). In the onset of spring, survival rate increased significantly until May when no mortality of the samples was noted. In June relatively more mortality was observed however on the following months in summer until autumn season, survivals apparently improved at stable rate.

2.3.5. Variation of field and laboratory seawater conditions

Due to the variation in the survival pattern of the coral isolates, it is speculated that this phenomenon might be attributed to the acclimation of the coral being cultured *in vitro*. For this reason, the temperature and salinity of the seawater in Tosa Bay and medium used in the laboratory were analysed.

In both parameters, the seawater from Tosa Bay showed fluctuations during one year sampling period from November 2014 until September 2015, which is a normal phenomenon brought by the changing season in a sub-tropical region. The temperature began to decline in the onset of winter (between November and December) and reached the lowest of 15°C in January. Before spring ended, temperature increased between April and May. It had peaked in summer at 30°C in July then gradually decreased on way to autumn season. In terms of water salinity used to exhibit an inverse pattern against temperature. In general it is understood that seawater cools

during winter or warm during summer when it used reached an extreme temperature limit, however in salinity this could be affected by some factors for instance severe precipitation in summer. From November 2014, salinity slightly increased until the entire winter season with a peak rate of 37 occurring from January to February of the succeeding year. It gradually decreased during spring season followed by abrupt decline to 21 in the mid summer (July). After reaching the lowest rate, it suddenly elevated until end of summer and maintained the increase through autumn.

This study noted that the ambient temperature and salinity of the seawater medium used for coral cultures were 25–27°C and 27–29, respectively. At these levels, coral *in vitro* survive at high rate and regenerate its tissue. As mentioned earlier, this temperature level was first established due to incidental increase of growing cultures where they maintained in the normal temperature with common white fluorescent light irradiance. In the case of the salinity of seawater medium, such range was obtained because of prevailing temperature in the room where medium were being stored for several months, considering that medium used in cultures were not enriched, nor sterilized.

When the measurement for the medium used and the seawater in Tosa Bay was compared, the temperature and salinity have showed variation pattern that were associated with season (see Fig. 2.6). In both parameters, high variations were evident during winter and summer seasons. Likewise moderate to low variations have been observed in spring and autumn seasons. As shown in the graph, the seawater temperature in Tosa Bay used to intercept with the medium-based level in May to June and in September, while for salinity in early June and late July. This showed that the water parameters from the coral collection site have matched with the ambient

laboratory level during the transition seasons such as spring and autumn, when the survival rates of the coral cultures were also high.

2.4. Discussion

This study had demonstrated that scleractinian coral *P. damicornis* experimentally induced regeneration of the tissue fragments cultured in Petri dishes containing raw seawater medium for more than a week at favorable condition. This further supports the study of Kramarsky-Winter *et al*. (2011) that had revealed the potentials of corals to propagate using *in vitro* technique. Unlike the experiments where corals were being transplanted in the recirculating tanks, aquaria or natural reef to monitor growth (Kinzie *et al*. 1984; Yap *et al*. 1992; Stimson 1997; van Treeck & Schuhmacher 1997; Soong & Chen 2003; Borneman 2008), this procedure became a very useful to innovation that produces 'coral cultures in glasses' readily available for various growth or tissue regeneration examination use. In addition, the advantages of this system were considered to be (1) amenable - where tissue culture is acquiescent to simple laboratory set-up within the ambient room condition; (2) cost efficient – which minimally required typical laboratory materials without further aeration or continuous water-flow, medium enrichment, feeding, complicated lighting fixtures and other growth enhancer; and (3) resource-wise – where small apical fragments are viable culture size requirement that could ecologically help minimize coral extraction from the reef. This highly suggests that the ability of coral to grow is not selective as to the nature, form and size of its environment.

The aspects of coral tissue regeneration have been widely reported from the natural reef, as cited earlier. These reports mainly discussed the closure mechanism of the inflicted lesion on the coral, which was termed as 'centripetal' regeneration in the present study. Further, most

studies have emphasized that the regenerative ability of the corals were highly dependent on the size of the lesion/injury inflicted upon. It has been suggested that the bigger the surface area or perimeter of the lesion, the lower the probability to completely close or heal the injured area. For instance in Meester *et al*. (1997), the massive coral *M. annularis* could regenerate a maximum of 4.7 mm^2 per mm of perimeter length, only 67% of the lesion could be closed by the lesion size 83 mm^2 but not capable of recovering larger than 130 mm^2. Similar with *P. astreoides* that regenerated about 50% of the 1 cm^2 and <10% for *A. agaricites* but both species could not perform in the larger injury of 5 cm^2 (Bak & Steward-Van Es 1980). In such phenomenon, various reasons were explained. Coral tissue regeneration may depend on other intrinsic factors such as the decrease in the capacity of corals to regenerate in time, shape, type and position of lesion and coral morphology (Bak & Steward-Van Es 1980; Bak 1983; Wahle 1983; Hall 1997; Meester *et al*. 1997; Oren *et al*. 1997, 2001; van Woesik 1998; Cróquer *et al*. 2002; Titlyanov *et al*. 2005). It was also attributed to extrinsic factors like algal or animal colonization, sedimentation, depth and temperature range, and diseases that possibly impede the healing process (Wahle 1983; Hall 1997; Kramarsky-Winter & Loya, 2000; Cróquer *et al*. 2002; Titlyanov *et al*. 2005; Fishers *et al*. 2007; Sabine *et al*. 2015). The unrecovered area may lead to permanent dead lesion (Bak 1983; Bak & Steward-Van Es 1980) functionally referred to as 'asymptote' (Meesters *et al*. 1997).

Comparing such conditions with the present result although the centripetal regeneration of *P. damicornis* was entirely (100%) completed, it shows that the growth rate of coral regeneration from *in vitro* condition was relatively slower than in natural setting. In terms with the induction of growing tissue, the centripetal regeneration in present study had commenced 8 days after the fragmentation similar in Meester *et al.* (1997) and Sabine *et al*. (2015) when the

regeneration were also reported to initiate after the injuries were inflicted. However in this study the mean growth rate of 0.068 mm/day (centripetal mode) is too far to compare with the mean rate of 6.71-15.99 mm/day (Meester *et al.* 1997) and with 5.0-17.5 mm/day (Sabine *et al*. 2015) even though the tissue growth rates in these studies were reported to decline in the succeeding days. Further it must be considered that extrinsic factors such as those mentioned earlier were absent from *in vitro* system while the recovery of corals inflicted lesion in the natural reef remains dynamic. If this is the case, it was assumed that the state of the coral environment together with its physiological state affects tissue regeneration processes, synergistically (Wahle 1983; Kramarsky-Winter & Loya, 2000; Titlyanov *et al*. 2005).

Dealing with two modes of regeneration in the present study, the growth by radial regeneration is apparently driven by similar mechanism with centripetal mode except for the growth rates. In centripetal mode, the rough surface of the exposed skeleton may assisted the tissue to grow rapidly than smooth surfaces in radial mode, although this is mere speculation in time. In *Acropora*, rapid tissue regeneration on the skeletal surfaces was observed to be assisted by the equivalent production of skeleton that helps seal the lesion in its fastest way (Hall 1997).

The coral *P. damicornis* having an imperforate tissue (Stimson 1997; Kinzie *et al*. 1984), possessed a tissue that could overgrow on any surface. In this study, the surrounding lesion mounted on the silica gel regenerates until the cover glass, both representing soft and hard substrata with smooth texture. Likewise in the present observations coral tissues used to grow even on the attached debris such as shedded calcareous materials; algal settlers (Meesters *et al*. 1997; Titlyanov *et al*. 2005) and over the accumulated debris and sediments (van Woesik 1998). This further supports the fact that the attachment of coral tissue into the substrate is not selective (Highsmith 1982; van Treeck & Schuhmacher 1997; Lirman 2000). Conclusively, tissue

regeneration is a dynamic behaviour, which Fishers *et al*. (2007) reported that coral tissue regeneration persisted for a year or more in the reef setting.

The survivorship of the coral fragments *in vitro* probably was the most essential aspect of this study. In this experiment, it was assumed that the survival and tissue regeneration were assisted by other factors including the fragment size, light irradiance, and water temperature. Dealing with fragment size, tissue fragment from the apical branches used in this study measured about 3-5 mm which was noted to thrive in a Petri dish culture containing 150 mL seawater medium. The survival of fragments by sizes has been investigated from the transplanted coral experiments in natural reef (Bruno 1998; Lirman 2000), where they indicated that survival of fragment has no significant relationship with the size of fragments. This suggested that coral survival does not depend on its size in the reef setting while in closed system this condition is unknown so far.

In terms of room light, the white fluorescent irradiance of 8-9 μmol $m^{-2}s^{-1}$ used in the present study can be pronounced an ambient light intensity due to survival and growth of coral tissue fragment by *in vitro* system. Verifying from that of Borneman (2008), the irradiance level used in this study was within the SSI (sea surface irradiance) limit of 30-40% for the 'reef' range or equivalent to 6-8 μmol $m^{-2}s^{-1}$, considering that the normal SSI is 20 μmol $m^{-2}s^{-1}$.

As to coral survival, it was found that in the late spring and early autumn the temperature of the seawater in Tosa Bay was nearly closed to the standard temperature of 26°C the medium used in the laboratory (refer to Fig. 2.6). In these seasons, coral cultures have higher survival rates than winter season where seawater in Tosa Bay was extremely low (refer to Table 2.1 or Fig. 2.5). Considering the association of temperature and survival rate of cultures, in this case such condition could be attributed to acclimatization ability of corals where coral samples

collected during warmer season used to survive and grow well than those collected during winter. This phenomenon remains a speculation as this present method used is poorly demonstrated in the early studies. In an outdoor tank experiment, spring season was also reported with high growth rate for *P. lutea* found Okinawa reef (Titlyanov *et al*. 2005). In this season also, it was reported that five coral species from the Caribbean Sea exhibited its peak tissue biomass (Fitt *et al*. 2000). On the other hand, during autumn season the lesion inflicted on *F. granulosa* from Red Sea had completely recovered (Kramarsky-Winter & Loya, 2000).

Although it is indicated that adaptive mechanism was inherent among corals to survive in closed system (Borneman 2008), it remains plausible that the temperature must be highly considered when shifting environment for coral cultures. Further this study suggests that when conducting similar procedure, spring and autumn season are the suitable season to propagate corals from Petri dishes. However if year round cultures are necessary, medium used must either thermally parallel or directly obtained from similar site where coral samples were gathered.

In conclusion, this successful attempt of culturing scleractinian coral *in vitro* is a considerable significance of this study. It is likewise pronounced that the capability of coral to grow was not definite as to nature of environment, while the culture system could be done not as complicated as previously thought. The diverse mode of tissue regeneration exhibited by imperforate coral such as *P. damicornis* suggested that other species having similar tissue characteristic could also perform similar process. Likewise the possibility of introducing various coral species into this form of culture system can be possible. This study may provide inputs that could assists coral biologists in establishing future experimental set-up and produce mass coral clones for use in various histological and ecological investigations.

Collection Dates	*Acropora* sp.		*P. damicornis*	
	No. of fragments produced	Survival rate (%)	No. of fragments produced	Survival rate (%)
August 17, 2014	–	–	20	–
September 8, 2014	–	–	20	–
October, 2014	–	–	20	–
November 26, 2014	10	–	25	80
December 15, 2014	10	–	10	90
January 21, 2015	12	40	25	88
February 24, 2015	12	60	20	90
March 25, 2015	15	53	20	85
April 9, 2015	15	50	21	90
May 15, 2015	5	10	20	100
June 12, 2015	5	40	15	93
July 29, 2015	12	80	28	96
August 17, 2015	12	75	20	95
September 29, 2015	10	–	20	95
October 26, 2015	10	–	20	95

Table 2.1. Coral collection dates and survival rates of *Acropora* sp. and *P. damicornis* after 30 days of *in vitro* culture. The number of fragments produced depends on the availability of healthy colonies. (–) sign indicates no data recorded.

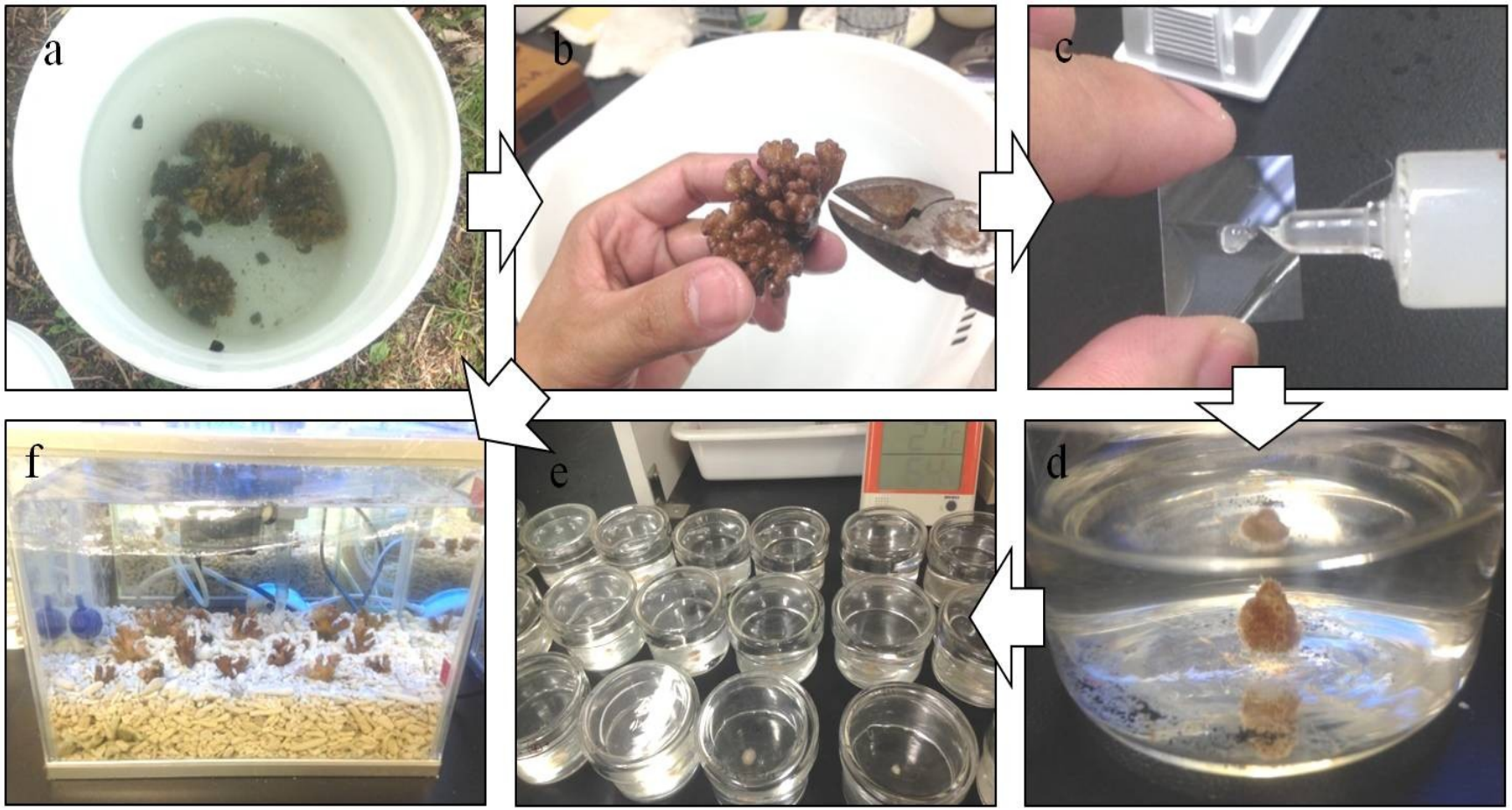

Fig. 2.1. (A–F) Procedure for the *in vitro* culture of tissue fragments of *P. damicornis*. (**a**) Small colonies were collected at Tosa Bay, Kochi, Japan then sealed in the bucket before being transported to the laboratory. (**b**) Apical branches were gently excised using pliers. (**c**) Cover glass was placed with silica gel then placed in individual Petri dish with raw seawater. (**d**) Small fragment was gently mounted on the silica gel to stand erect. (**e**) Isolates were maintained on the laboratory desk exposed to room fluorescent light and temperature from 25 to 27°C. (**f**) Truncated colonies were maintained in the aerated aquarium for future use.

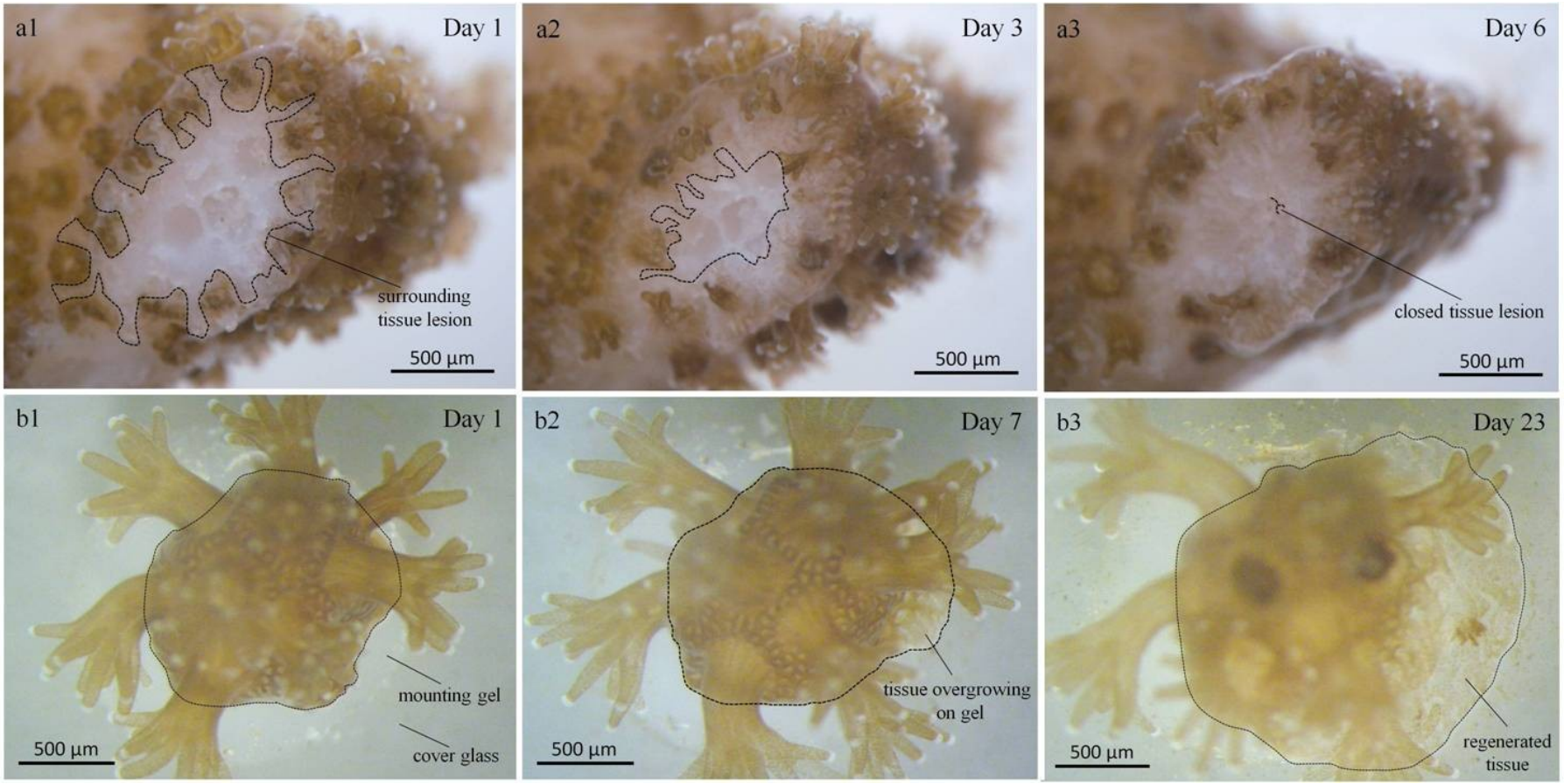

Fig. 2.2. Time-lapse micrographs of the two modes of tissue regenerations in *P. damicornis*. (**a1-3)** Note the closure of the surrounding lesions (*indicated by trace lines*) on the coral fragment for 6 days that exhibited centripetal regeneration. (**b1-3)** While the coral fragment with lesion directly mounted on the silica gel exhibited radial regeneration extending towards the cover glass for 23 days.

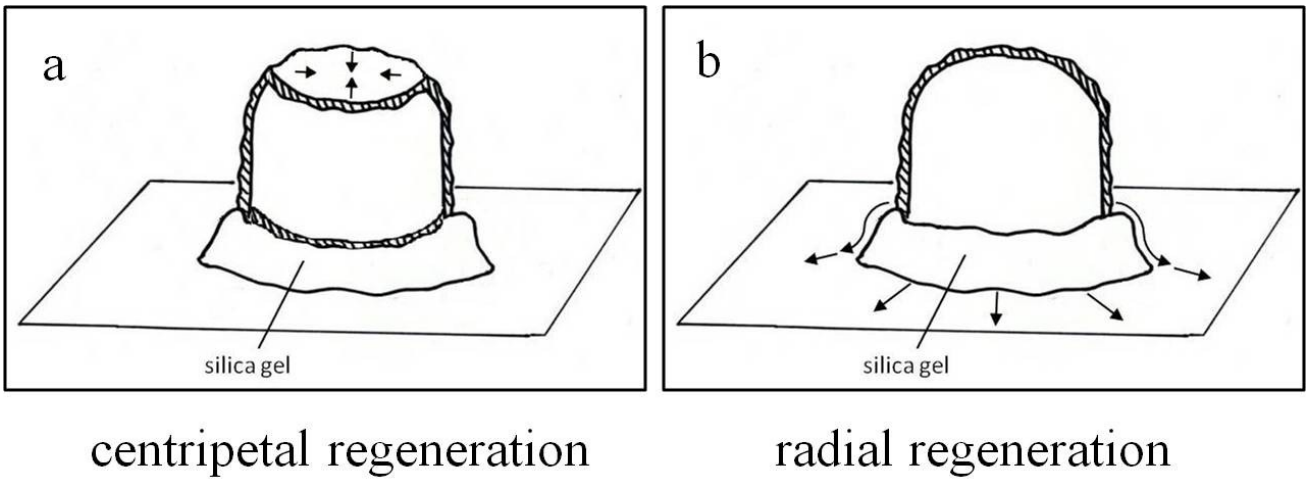

Fig. 2.3. Schematic diagram of the two modes of tissue regenerations in *P. damicornis*. (**a**) Centripetal regeneration of coral tissue or the typical closure/healing of lesion (*arrows*). (**b**) Radial regeneration of coral tissues or the outgrowing mechanism to disperse extensively (*arrows*).

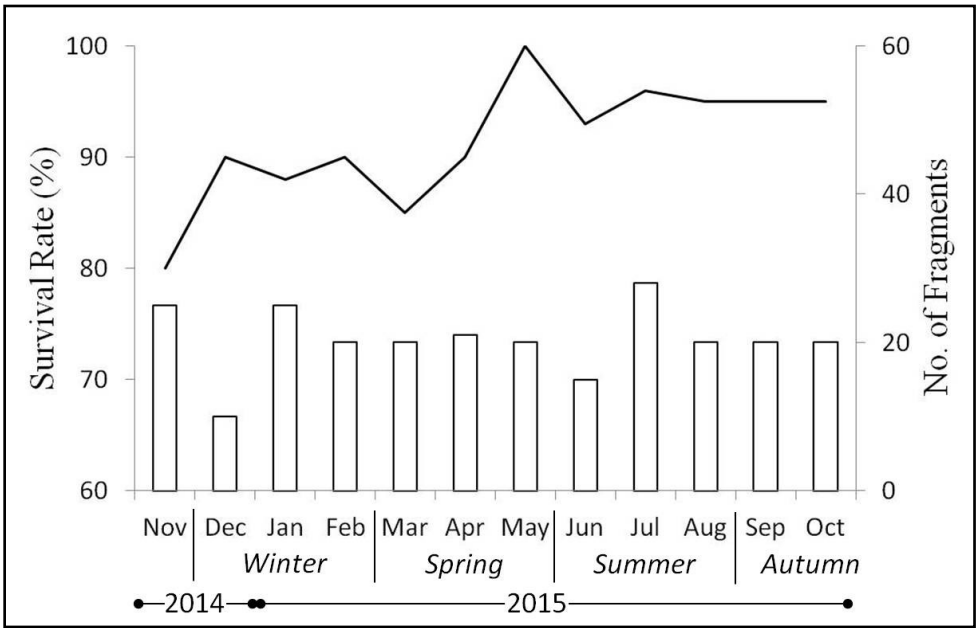

Fig. 2.4. Survival rates of *P. damicornis* from *in vitro* culture. Line graph represents the survival rate (%) and bar graph for the number of coral fragments. The number of samples produced varied due to availability of healthy colonies.

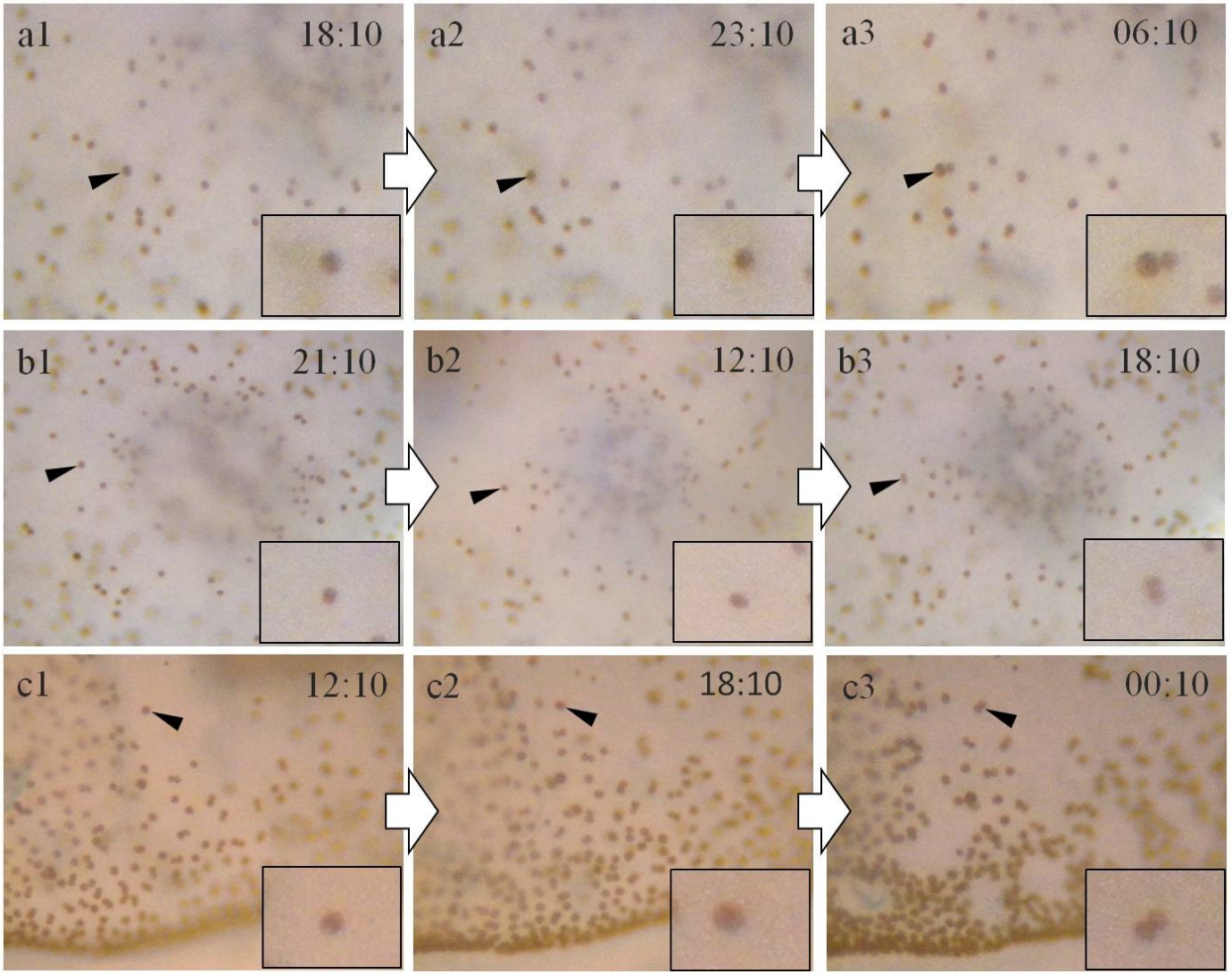

Fig. 2.5. Timing of cell divisions of *in situ* zooxanthellae in the regenerated tissue of *P. damicornis*. Division of zooxanthellae (*arrowheads*) developing into doublet cells (*inlet*) in (**a3**) morning, (**b3**) afternoon and (**c3**) midnight.

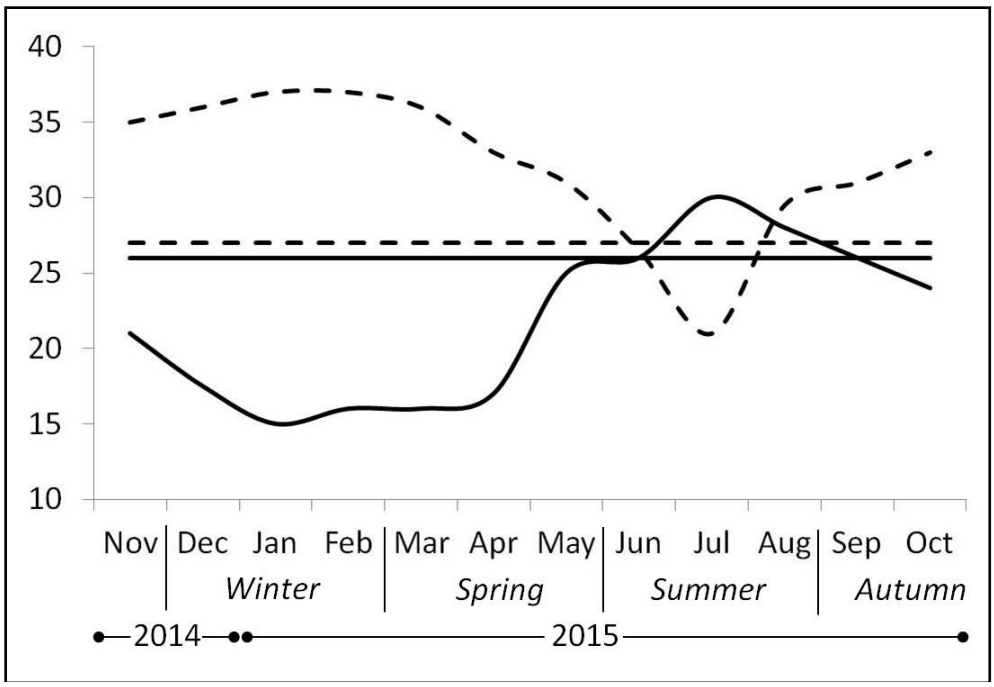

Fig. 2.6. Variation of water parameters from Tosa Bay (*curve*) from standard medium level (*vertical*). Salinity (‰) is indicated by dotted lines and temperature (°C) by continuous lines.

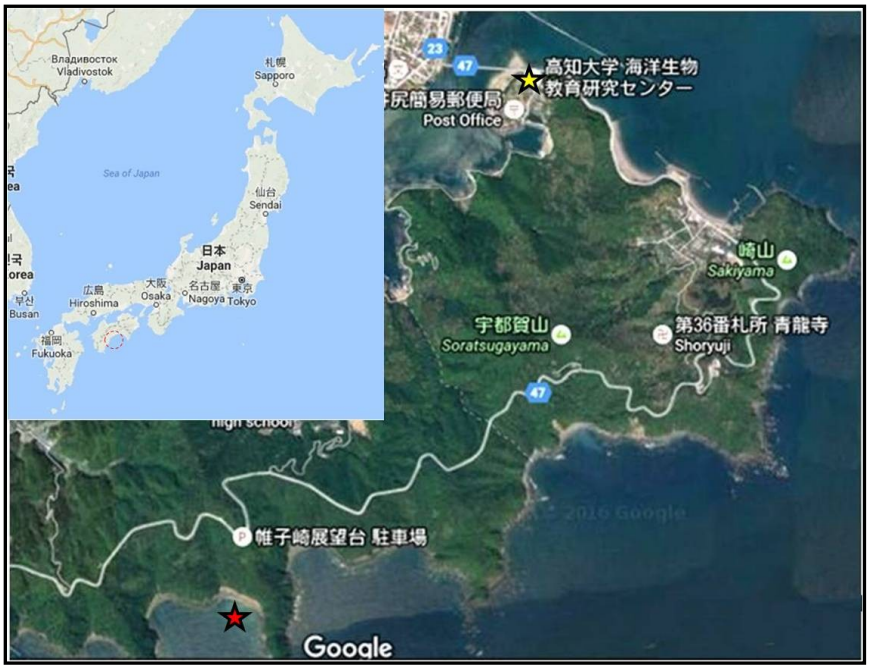

Fig. 2.7. Map showing the source of seawater medium at Kochi University - Usa Marine Biological Station (*yellow star*) and coral collection site at Tosa Bay (*red star*). (maps courtesy of Google)

3. The effect of monochromatic lights on the growth of coral regenerated tissues and the density of *in situ* symbiotic zooxanthellae

3.1. Introduction

Most reef-building corals such as *P. damicornis* are typical photo-symbiotic organisms, because they live in symbiosis together with photosynthetic zooxanthellae, which utilize light to produce organic substances. Many coral species are supplied with organic substances from symbiotic zooxanthellae. Therefore, corals cannot grow at the places deeper than the extension of their photic zone (Beer *et al*. 2014).

In the sea, water columns behave such a monochromator or filter that absorbs certain wavelengths in sunlight where violet and blue have minimal light extinction than red consisting of longer wavelengths when the depth of water increases (Mass *et al*. 2010). The monochromatic adaptability or the effect of single type of light/spectrum exposure on the corals has been studied in various settings (Kinzie *et al*. 1984; Kinzie & Hunter, 1987; D'Angelo *et al*. 2008, Mass *et al*. 2010; Wijgerde *et al*. 2014). In their studies, growth conditions of the corals that live within ambient photosynthetically active radiation (PAR) vary significantly when the corals were exposed to light with different spectral signature. While coral acclimation to light intensity had pronounced effect, it has been suggested that the light color may influence corals in several growth indicators such as photosynthetic, pigment rate, skeletal growth and zooxanthellae density (Kinzie *et al*. 1984; Kinzie & Hunter, 1987; D'Angelo *et al*. 2008, Mass *et al*. 2010; Wijgerde *et al*. 2014). Under normal reef condition, corals inflicted with lesion have tissue growth rate reached between 5.0 -15.5 mm^2 day^{-1} (Meester *et al.* 1997; Sabine *et al*. 2015). For the *in situ* zooxanthellae, its established density in many hermatypic corals ranges from 0.5 to 5 x

10^6 cells cm^{-2} (as cited by Hoegh-Guldberg & Smith 1989; Jones & Yellowlees 1997; Stimson 1997). Exposure from blue light promoted more coral growth and associated increase in the density of zooxanthellae (Kinzie *et al*. 1984; Wijgerde *et al*. 2014), that further enhanced coral pigmentation (D'Angelo *et al*. 2008). However, exposures to green and red lights have resulted in the lowest growth rate (Kinzie *et al*. 1984; Wijgerde *et al*. 2014). Increased irradiance levels of UV radiation such as at peak wavelength of 320-400 nm (UV-A) potentially mediates coral bleaching (Gleason & Wellington 1993). Information on the experimental exposure to corals *in vitro* under extensive varieties of light resembling the PAR spectra is far lacking.

In this study, the effect of monochromatic light spectra on the tissue development and density of its *in situ* zooxanthellae cell were investigated. Coral growth is highly affected by the intensity of light and depends much on the spectral composition of light, however this physiological condition remains unknown among coral cultures *in vitro* system.

3.2. Materials and Methods

3.2.1. Coral exposure to various monochromatic light treatments

The procedure of the coral fragmentation and *in vitro* culture technique were similar as described in Chapter 2. Coral cultures exposed under monochromatic light treatments have used those with lesions mounted on the silica gel which regenerate tissues on the flat surfaces (glass substrate), appropriate for measuring tissue length. LED (light emitting diode) (IS-Mini Series; CCS Inc., Tokyo, Japan) was used for monochromatic light sources, each of which had a peak wavelength (see Table 3.1). Three coral fragments were cultured separately in each Petri dish containing seawater for some weeks to induce regenerated tissues and exposed to each monochromatic light in a dark box for 10 days at the irradiance levels of 1.0 W/m^2, 1.5 W/m^2

and 2.0 W/m^2. LED light illumination was set with intermittent treatment of 14h light and 10h dark under irradiance levels 1.0 W/m^{-2}, 1.5 W/m^{-2} and 2.0 W/m^{-2}, respectively. Corals were examined using light microscopy with similar procedure for monitoring tissue regeneration by time-lapse microscopy in Chapter 2 except that micrographs were obtained manually. Micrographs were captured consistently at a specific site of regenerating tissue magnified 40x and 100x, respectively. Micrographs was obtained prior to exposure (day 0) then every 48h for 10 days. Pen marking were place at the bottom of the glass dishes, while the images showing the location in the coral samples were printed to immediately locate the focal sites during microscopy. Two control samples were considered, the ambient condition as standard for the developing tissues and dark condition (total darkness) for the degenerating tissues. The entire procedure was performed at ambient room temperature from 25°C to 27°C.

3.2.2. Measurement of coral tissue length

Using the processing software ImageJ (Rasband 2003), low magnification images were analyzed to measure the distance of extending or retracting tissues. At one stable point such as visible calcification mark located at the base of the regenerating tissue, the distance towards the tissue edge was measured at five locations (Fig. 3.1). Length scale was set to 373.33 pixels/mm with standard measurement based from an objective micrometer. Data such as average length and standard deviation were derived from the computed values by the software database.

3.2.3. Counting the density of in situ zooxanthellae

The density of the zooxanthellae *in situ* was determined by manual counting where high magnification micrographs were analyzed. Nine quadrats (three each in outer, middle and inner

zones) with standard area of 32 x 32 μm (1000 μm^2) were plotted randomly on the micrographs (Fig. 3.2). All zooxanthellae cells found inside the quadrats were counted. Dividing cells were counted as two individuals (Guillard & Sieracki, 2005), those that showed doublet cells. In case of overlapping cells, the number was estimated by analyzing micrographs from various focuses.

3.2.4. Growth data analysis

Data analyses were done using spreadsheet. To determine the increment/decrement in coral tissues, similar growth rate formula in Chapter 2 was used. To compute for the density of the *in situ* zooxanthellae (% cells per unit mm^2), the formula was expressed as;

$$\text{Relative density rate} = 100 \bullet (d_2 - d_1) / (t_2 - t_1)$$

where d = density and t = time at *1* (final) and *2* (initial), respectively. Using the mean values, the correlation coefficient (r value) between the coral tissues and zooxanthellae density in time was computed. The over-all growth conditions of corals from the different light treatments was then determined. Using growth rate data, coral samples with tissue increment higher than standard rates of ambient control were categorized as 'good', values within or equivalent to standard rates as 'average', then lower than standard rate as 'poor'.

3.3. Results

3.3.1. Growth rates of coral tissue under monochromatic lights

The growth rate of coral tissue exposed under different monochromatic spectra was shown in Table 3.2 and in Fig. 3.4. In control ambient conditions, the normal growth rate of regenerated tissues of coral cultures in 10 days was 55.2 μm/day. However under continuous dark environment, the tissue growth in all corals declined at mean rate of -29.2 μm/day. This absolute

type of growth response was also observed in corals exposed from monochromatic BB and FR light spectra. Under BB light, all samples from three irradiance levels have significant increase in tissue length. The mean growth rate at 1.0 W/m^2 was 14.9 µm/day, while at 2.0 W/m^2 the tissue growth increased to 20.3 µm/day. Both rates were lower than control ambient conditions. However, at the irradiance 1.5W/m^2 tissue growth was highly significant to 159.7 µm/day, obtaining three times higher than the standard control rate. Upon exposure to FR, all coral samples completely degenerated in time. At 1.0W/m^2 coral tissues tended to retract by -14.6 µm/day followed by 2.0W/m^2 with -20.5µm/day. Corals at 1.5W/m^2 degenerate significantly with -60.5µm/day twice higher than control dark condition. During continuous tissue retraction, widespread expulsions of zooxanthellae cells were observed by which pellets were deposit pellets on the substrate. Some corals subsequently died after the 10-day experiment.

In other light spectra, varying irradiance levels *per se* promoted inconsistent coral growth responses. Under VV, GG and YY light spectra, most corals did not grow from irradiance level 1.0W/m^2 where they retract at the rates of -10 µm/day (VV), -2.0 µm/day (GG) and -8.2 µm/day (YY). These decrement rates were relatively higher than control dark condition. However at irradiance 1.5W/m^2 tissue growth persisted with 8.9 µm/day (VV), 1.9 µm/day (GG) and 3.6 µm/day (YY). Similarly at 2.0 W/m^2, further increases were observed 17.3 µm/day (VV), 29.2 µm/day (GG) and 17.8 µm/day (YY), respectively. However, these rates remained lower than normal growth from ambient conditions. Corals exposed from R62 and R68 lights also tended coral tissues to retract both at irradiance levels 1.0 and 1.5W/m^2. Under R62, decrement rates in both irradiances were -4.4 µm/day and -1.9 µm/day, while in R68 with -6.4 µm/day and -3.9 µm/day. These rates were relatively high as compared from control dark condition. Exposure to UV-A spectrum, corals tended to retract tissue by -30.5 µm/day at irradiance 2.0 W/m^2, while at

1.0 and 1.5W/m^2 coral tissue grow with 9.9 µm/day and significant rate of 136.2 µm/day, respectively. Comparing rate from ambient condition, only corals from irradiance 1.5W/m^2 had enhanced growth beyond the standard rate of ambient condition.

3.3.2. Density of the *in situ* zooxanthellae cells

Table 3.2 and Fig. 3.5 have shown the mean density rate of the *in situ* zooxanthellae cells in corals exposed to different light spectra. In ambient control conditions was 23.3 cells/mm^2/day. In contrast to dark control condition where the number of symbionts decreased due to degeneration of host tissue, the mean decrement rate was -123.4 cell/mm^2/day. Similarly in all irradiance levels of UV-A and FR rays, significant decline in symbiont density were evident from -15.5 to -143.6 cell/mm^2/day (UV-A) and -52.4 to -130.7 cell/mm^2/day (FR). The decrement of symbiont density at irradiance 1.5W/m^2 was lower as compared from 1.0W/m^2, while the density rate at irradiance 2.0W/m^2 were higher than the standard rate of dark control condition. Likewise the expulsion of zooxanthellae was noted.

In other light treatments, zooxanthellae density varied significantly with the irradiance levels. In VV ray, the zooxanthellae population found *in situ* used to increase in all light intensities with 18.7 cells/ mm^2/day at 1.0W/m^2 that was higher than 1.5W/m^2 with 16.2 cells/ mm^2/day. Although compared from the ambient control samples these rates are lower, except in irradiance 2.0W/m^2 with mean density of 46.7 cells/mm^2/day. Under BB ray, the zooxanthellae cells density at irradiances 1.0 W/m^2 and 2.0W/m^2 were 35.8 cells/mm^2/day and 35.3 cells/$mm^{2/}$day were relatively higher than ambient control condition. Remarkably at irradiance 1.5W/m^2 the population decreased to -32.7cells/mm^2/day, however no associated expulsion of zooxanthellae cells was observed unlike in growth-inhibiting lights where population decline of

the symbionts were evident. This trend likewise occurred in R68 light. The density of symbionts at irradiance 1.5W/m^2 was -54.5 cells/mm^2/day, lower than 1.0W/m^2 with 13.5 cells/ mm^2/day. At 2.0W/m^2 the zooxanthellae density was 37.8 cells/mm^2/day, which appeared higher than the rate from ambient control condition.

Contrary to the earlier results, the population of zooxanthellae exposed to GG, YY and R62 lights was apparently inclined with light irradiance levels. Its density at irradiance 1.0W/m^2 decreased significantly at the rates of -7.7 cells/mm^2/day (GG), -61.2 cells/mm^2/day (YY) and -1.5 cells/mm^2/day (R62) but remains higher compared from control dark condition. Rate increments were further observed at irradiance 1.5W/m^2 and 2.0W/m^2. Density rates at these irradiance levels from YY and R62 rays were relatively higher than ambient control condition.

3.3.3. Correlation between coral tissue length and zooxanthellae density

The correlation between the coral tissue growth and the density of the *in situ* zooxanthellae cell were analyzed in each monochromatic light treatment. In ambient control condition, the correlation coefficient (*r*) value was -0.61, showing that both factors have moderate indirect relationship to each other. This might be the result of coral samples that exhibited significant growth of tissue against low increase in the density of *in situ* zooxanthellae. In control dark condition, *r* value was moderate to 0.52 which indicated rather that the retraction of coral tissue was accompanied by loss of zooxanthellae population in host cells.

Regardless of light irradiance levels, the correlation coefficients of corals exposed to monochromatic spectra varied significantly. Several light treatments have exhibited strong to modest positive correlation that includes VV ray with 0.70, UV-A = 0.60, GG =0.50 and R68 =0.58, respectively. Others have showed very weak positive correlation such as FR with 0.16 and

YY light having 0.07. On the other hand, the coefficient from R62 showed negatively weak correlation = -0.25. In terms of BB light, *r* value was -0.66 with almost similar case as in ambient control condition. Most corals at irradiance 1.5 W/m^2 exhibited low reproduction of zooxanthellae cells that appeared inversely proportional with the extensive growth of host tissue.

3.3.4. Over-all coral growth condition under monochromatic lights

Corals cultured at ambient environment significantly changed its growth responses when exposed under the different monochromatic light spectra. In Fig. 3.6, the graph showed that corals exposed to UV-A ray appeared with highest number of corals with 'good' growth condition *i.e.* 33%. However, UV-A had a least number (11%) of 'moderately' grown corals while 'poor' growth were abundant to 60%. This was followed by VV and BB rays, both of which had 22% 'good' coral growth condition. The remaining 78% of corals in VV light grew with 'poor' condition. Under BB light, 44% of corals exhibited 'moderate' growth while only 33% were poorly developed. The BB ray remains a significant growth-enhancing monochromatic light for coral and its symbiotic zooxanthellae. Corals exposed to GG, YY and R68 lights exhibited the same result where 11% of corals developed with 'moderate' condition while 89% led to 'poor' growth. Lights such as R62 and FR have common effect to all of its corals where entirely (100%) had subsequent 'poor' condition. This showed that no corals in these light qualities developed within the standard growth condition. The effects of R62 and FR to corals were congruent with the dark regime where both host tissue and symbionts failed to proliferate.

3.4. Discussion

This study had significantly demonstrated a novel and simple method by examining the effect of monochromatic light spectra in coral through microscopy using *in vitro* cultures with induced regenerated tissues. In Chapter 1, it was indicated that the tissue regenerations in both modes revealed a dynamic behaviour, but how far would a tissue grow radially from *in vitro* system remains unknown.

To further solve the issue, corals that induced radial regenerations were experimentally cultured under light treatment. Upon exposure, corals dramatically showed varying growth responses of regenerated tissues when they received irradiance from different monochromatic light spectra. This study had revealed that both light quality at peak wavelength and irradiance levels influenced the growth of coral regenerating tissues and the density of *in situ* symbiotic zooxanthellae. Blue spectra (470 nm) enhanced the growth of coral regenerating tissues best of all monochromatic lights. Some coral exposed to blue light at an intermediate irradiance level of 1.5 W/m^2, significantly enhanced the coral regenerated tissue beyond the growth rate of the corals cultured under ambient 'white' light environment. In contrast, red spectra such as R62 (620 nm) and far red (737 nm) light entirely affected the corals in all irradiance levels, while UV-A (470 nm) affected poorly corals specifically from the strongest irradiance of 2.0 W/m^2. Under growth-inhibiting spectra, corals were mainly characterized with discriminate tissue retraction, and 'wiggling-to-expulsion' reactions of the *in situ* symbiotic zooxanthellae (Camaya *et al*. 2016). The present result further supports the earlier findings of Kinzie *et al*. (1984) and Wijgerde *et al*. (2014), whose suggested that the spectral composition of light *per se* such as blue ray promoted the coral health and skeletal growth while the red lights represses its physiological development. Further in this present study, the types of red spectra that negatively affected the

coral development were significantly distinguished as those having the shortest wavelength of 620 nm and the longest wavelength of 737 nm, respectively. In the case of other light colors used in this experiment, they induced diverse growth patterns dissociated with the irradiance level. Whether the coral growth were blue enhanced or red-repressed (Kinzie *et al*. 1984; Wijgerde *et al*. 2014), it is now relatively clear that monochromatic light spectra along with intensity level were committed on the growth response towards maintaining coral-zooxanthellae symbioses.

This study had observed the enormous increase in tissue regeneration of the corals exposed from blue rays at irradiance 1.5 W/m^2 and yet the density of zooxanthellae tended to decline despite the absence of symbiont expulsion. This appeared that only the host cell growth was enhanced, although it is not known how blue spectra directly affect coral tissue growth. Kinzie *et al*. (1984) indicated that blue light act through photosynthetic efficiency by stimulating the chlorophyll *a* content of the symbiotic cells. As to why photsynthetic zooxanthellae cells apparently did not proliferate well under such condition remains an unknown fact.

In the case of growth inhibition such as in red spectra where retraction of regenerated tissue were evident, such event was further explained by Brown *et al*. (1994) whose indicated that the retraction is a physiological response of corals to unfavourable change in environment such as intense light irradiance and a further response to bleaching. Similarly, corals in captivity exposed to low irradiance tended the tissue recession to occur (Borneman 2008). In Wijgerde *et al*. (2014), deteriorating corals exhibit necrosis until mortality. The recent study of Camaya *et al*. (2016) had demonstrated the effect of distinct light stressors such as UV-A and far red on the ultrastructure of the coral species and suggested that a common mechanism might be involved in the early stage of bleaching such as autophagy and gastrodermal cell necrosis. In this regards, it is assumed that similar process of cell degradation also occurred during indiscriminate retraction

of tissue from other growth-inhibiting spectra in this experiment *i.e.* red 620 as relative coral strain and monochromatic form of light also have been used.

With the variation in the growth responses between coral tissue and symbiotic zooxanthellae in each light treatment along with irradiance levels, this study assumed that their growth patterns might be direct or indirectly associated to one another (see Fig. 7). A directly associated pattern is either 'enhanced' where the growth of host tissue is coupled with the increase in density of symbionts such as in blue and some samples from violet and green spectra, or 'inhibited' where both degenerate in time *i.e.* far red, red 620 and several corals in UV-A. The present result further supports the fact that correlation between host and symbiont growth exist (Davy *et al*. 2012). As host size increases, zooxanthellae abundance increases proportionately (Muscatine *et al*. 1986). In fact in our recent ultrastructural examination of coral under ambient condition (see Chapter 4), had confirmed the assumption of Davy *et al*. (2012) that symbiont cell division was followed by the division of coral host cell. In this case, it is plausible that the normal growth rate between host and symbiotic cells must be equal.

Dealing with the 'inhibited' growth pattern, the present study also suggests that the decline in growth of host tissue was equivalent to loss of symbiont population. The subsequent death of some corals under far red light were due to massive release of zooxanthellae, which supports the earlier reports that continuous loss of zooxanthellae led to bleaching (Lesser *et al*. 1990; Gleason & Wellington, 1993) or slowed down the recovery of coral from bleaching (Jones & Yellowlees, 1997). The intense exposure to UV radiation is one of the profound driving factors of zooxanthellae depletion leading to experimental and natural bleaching in corals (Lesser & Shick 1990; Lesser *et al*. 1990; Gleason & Wellington 1993).

This study further demonstrated that the growth response of host tissue from light spectra was indirectly associated with symbiont population. The good examples for this event are the coral growth pattern from blue and UV-A rays profoundly at 1.5W/m^2, and those from red 62 and red 68, as discussed earlier. If this is the case, it is suggested that a paradoxical pattern of zooxanthellae population appeared when coral tissue tended to retracts or increase. We speculate that the population of zooxanthellae may increase due to the congestion from retracting coral tissue, or may decongest when host tissues grow extensively. In this study, no analysis of mitotic index has been done, however it may be suggested that cellular control and regulation of symbiotic algae by the coral host may exist. The control and synchrony of cnidarians host and symbiont division are not fully understood however some potential mechanism was might be involved in the inhibition of symbiont growth and division (Davy *et al*. 2012). Asynchronous division may occur, as the division pattern of host and symbiont cells used to exhibit separate phasing (Fitt *et al*. 2000). The regulation of zooxanthellae population density likely occurred by facultative increase and expulsion of zooxanthellae, but factors involved is unknown (Muscatine *et al*. 1986). The fact that this event became evident due to light manipulation regime, pertinent inconsistency in growth pattern between host and symbionts remains feasible (Jones & Yellowlees, 1997; Fitt *et al*. 2000).

While it is now understood that the growth condition between the host and its symbiotic zooxanthellae changes dramatically when corals were exposed to monochromatic light treatment, the underlying mechanism involved on the varying growth responses was highly complicated. Some light colors induced direct effect on either enhancement or inhibition of growth between host tissue and symbionts while others stimulate reverse growth pattern depending on the irradiance levels. With these variations revealed in the correlation coefficients, it is important to

note that the declining rate of zooxanthellae density does not primarily mean degeneration or expulsion. Hence it remains necessary to look after the nature of responses whether host coral commits implication to bleaching or may potentially undergo regulation of symbiont proliferation.

Light Spectra	Wavelength (nm)	Irradiance levels		
		1.0 W/m^2	1.5 W/m^2	2.0 W/m^2
Ultraviolet (UV-A)	375	0.9 V	1.4 V	1.8 V
Violet (VV)	405	0.9 V	9.5 V	1.8 V
Blue (BB)	470	1.5 V	2.3 V	3.0 V
Green (GG)	525	2.8 V	4.2 V	5.6 V
Yellow (YY)	570	2.0 V	3.0 V	4.0 V
Red (R62)	620	2.2 V	3.3 V	4.4 V
Red (R68)	680	2.5 V	3.8 V	5.0 V
Far Red (FR)	737	2.5 V	3.8 V	5.0 V

Table 3.1. Monochromatic spectra used as light treatment coral culture. Arranged from shortest to longest wavelengths, light intensities (Voltage) per irradiance level vary in each spectrum.

Light Treatments	1.0 W/m^2						1.5 W/m^2						2.0 W/m^2					
	S1		S2		S3		S1		S2		S3		S1		S2		S3	
	Cor	Zoo	Cor	Zoo	Cor	Zoo	Cor	Zoo	Cor	Zoo	Cor	Zoo	Cor	Zoo	Cor	Zoo	Cor	Zoo
UV-A	-15.4	23.4	14.3	-105.8	30.8	-66.9	148.7	-9.4	108.6	-17.1	151.3	-20.2	-44.4	-126.0	-9.4	-77.8	-37.6	-227.1
VV	15.8	143.1	-11.7	-59.1	-7.2	-28.0	2.4	17.1	27.3	11.5	-3.1	20.2	2.1	42.0	44.7	122.9	5.0	-24.9
BB	6.7	9.4	32.1	57.5	6.0	40.5	242.3	-87.1	199.9	20.2	36.8	-31.1	11.5	38.9	21.2	26.5	28.1	40.5
GG	-3.7	14.0	-4.5	7.8	2.2	-45.1	-7.7	-15.5	-1.5	57.5	14.9	6.0	18.1	23.4	74.8	48.2	-5.4	-18.6
YY	-14.9	18.6	-4.8	-52.9	-4.9	-149.4	7.7	-17.1	1.7	21.8	1.4	70.0	39.2	-12.5	16.0	28.0	-1.7	77.8
R62	-21.7	17.1	10.3	-15.5	-1.7	-6.2	2.4	18.2	-13.7	143.1	5.6	-10.9	13.1	-84.0	8.8	164.9	5.5	101.1
R68	-12.0	20.2	-4.0	7.8	-3.2	12.5	10.1	74.6	-33.6	-132.2	11.8	-105.8	3.1	-42.0	0.0	62.2	47.5	93.4
FR	-24.5	-21.8	0.0	-14.0	-19.4	-150.9	-36.8	-37.4	-119.7	-99.5	-25.0	-20.2	-14.1	-108.9	-9.7	-121.4	-37.6	-161.8
Dark	-37.5	-133.8	-27.1	-150.9	-23.0	-85.5												
Ambient	21.4	45.1	58.2	3.1	86.1	21.8												

Table 3.2. Summary of coral tissue growth rates (*cor*) (µm/day) and the relative densities of *in situ* zooxanthellae (*zoo*) (cell/mm^2/day) in individual coral sample *S1*, *S2*, *S3* at three irradiance levels.

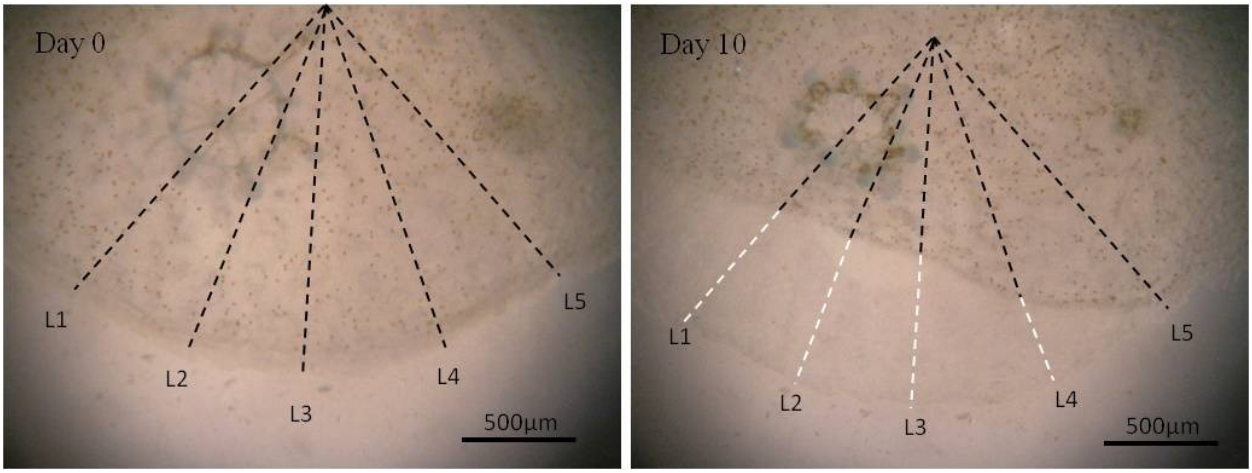

Fig. 3.1. Measurement of coral tissue length observed under low magnification micrographs. Using Image J software, the length of the tissue was randomly measured in five sites. Visible mark such as permanent calcification was used as point of origin of line plots. Note the extent of the regenerated tissue before exposure to far red rays (a) and after 10 days (b) where retraction was evident (white lines).

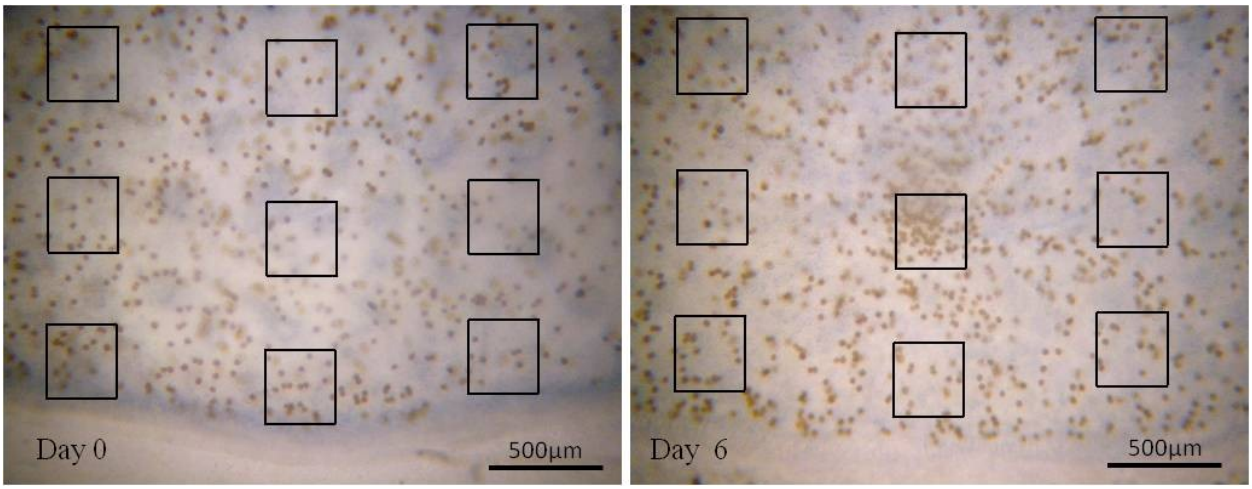

Fig. 3.2. Density of the *in situ* symbiotic zooxanthellae cells observed under high magnification micrographs. All cells were counted inside the nine fixed quadrats (1x1 mm) randomly plotted on the coral regenerated tissue. Note the density of symbiont cells prior to exposure from blue light (a) and after 6 days exposure (b).

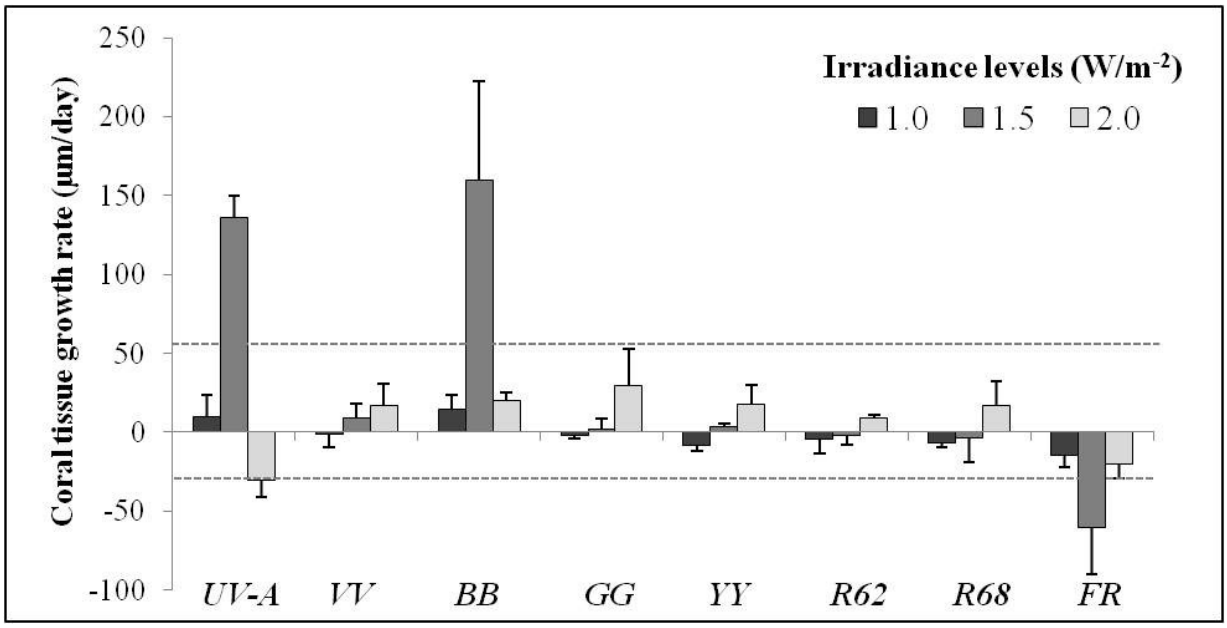

Fig. 3.3. Growth rates of *P. damicornis* tissue fragments experimentally exposed to various monochromatic light spectra with three irradiance levels. Vertical dotted lines indicates the normal rates of ambient control (+) and dark control (-). Values are means + s.e. (*n = 3*).

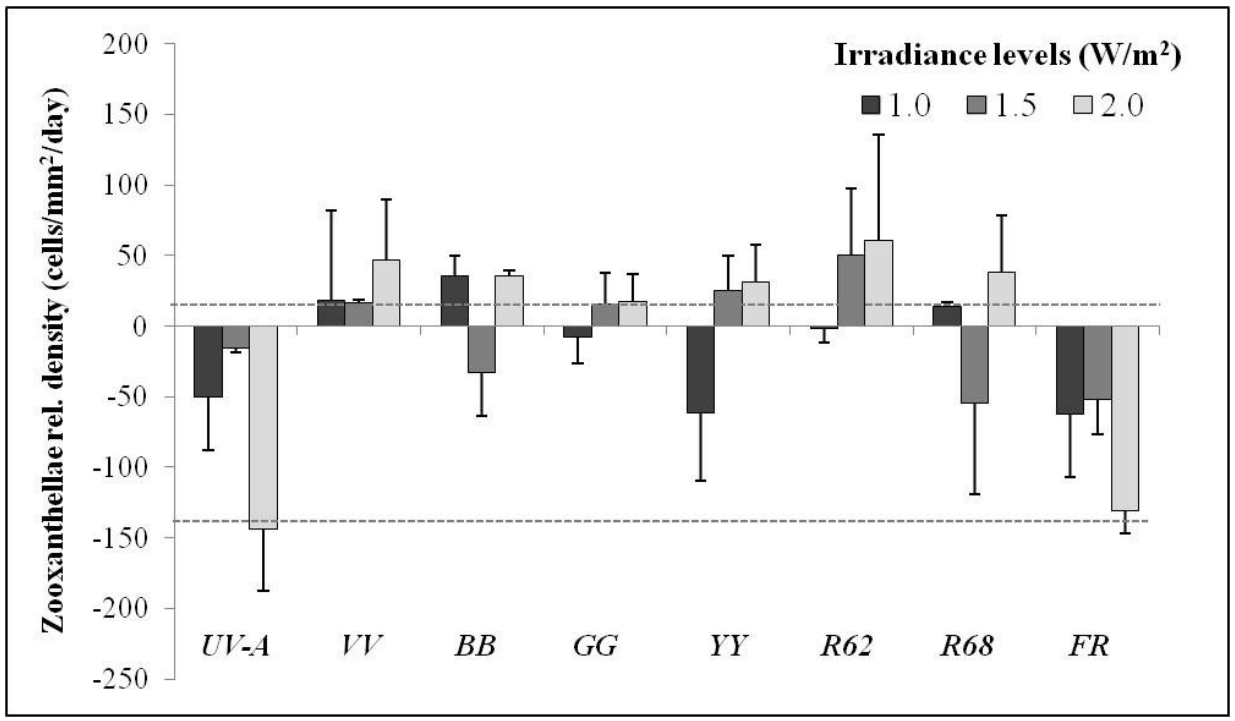

Fig. 3.4. Relative density of the *in situ* symbiotic zooxanthella in *P. damicornis* tissue fragments experimentally exposed to various monochromatic light spectra with three irradiance levels. Vertical dotted lines indicate the normal rates of ambient control (+) and dark control (-). Values are means + s.e. (*n = 3*).

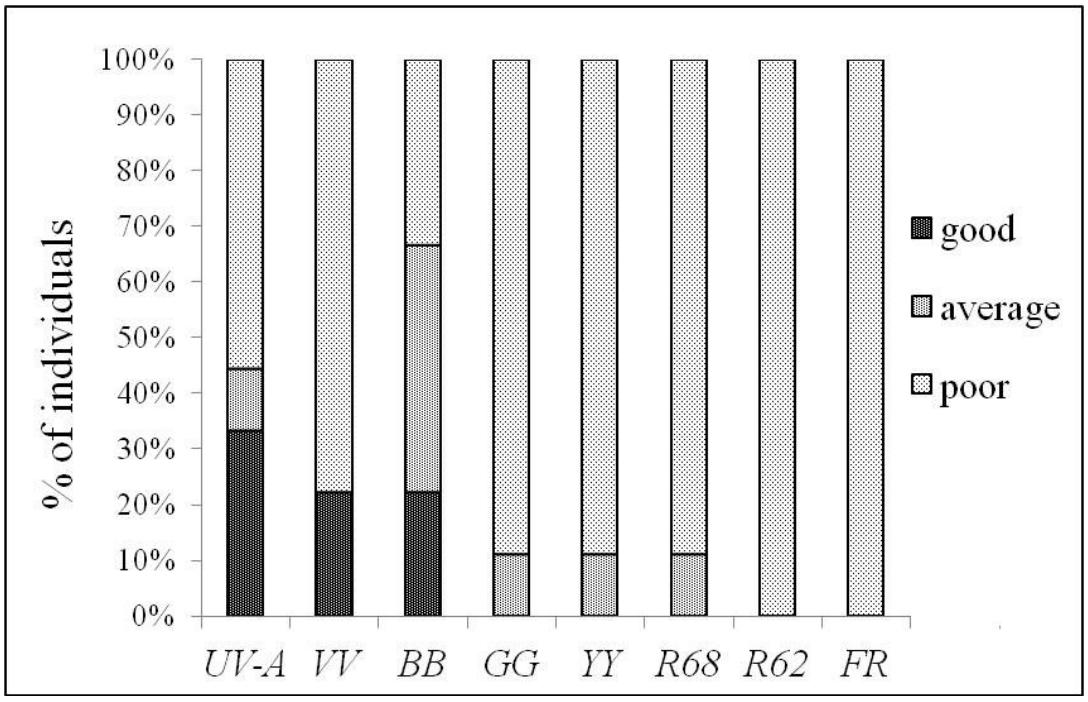

Fig. 3.5. Over-all growth conditions of *P. damicornis* under monochromatic light spectra.

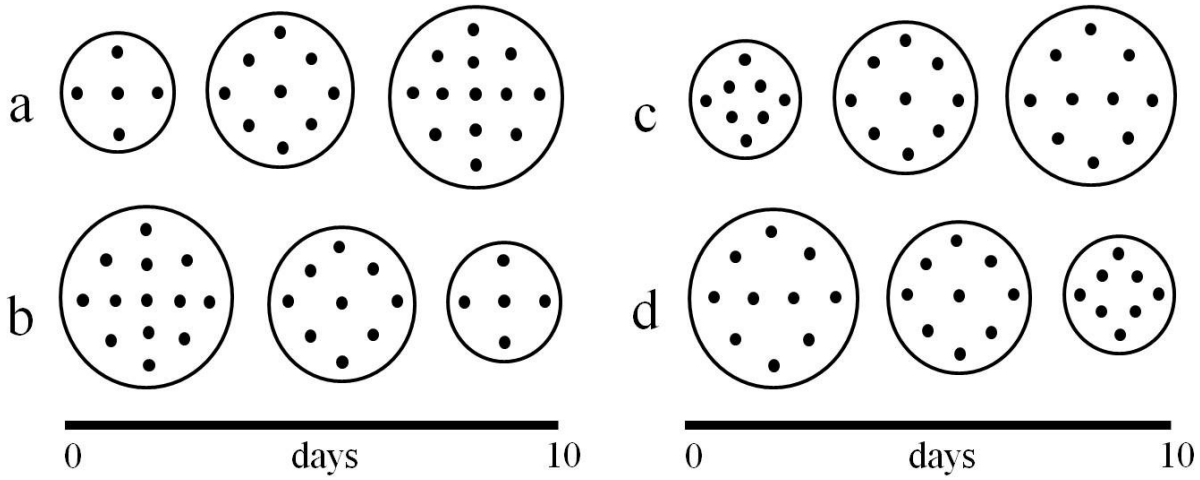

Fig. 3.6. Schematic representation of growth relationship between coral tissues (*circles*) *in situ* symbiotic zooxanthellae (*dots*) under control conditions and monochromatic light spectra. (**a-b**) This pattern indicates direct association of host-symbiont growth directed towards (**a**) growth-enhanced or (**b**)growth-inhibited. (**c-d**) Indirect association of host-symbiont growth pattern either by (**c**) 'promoted coral tissue-growth only' or (**d**) 'promoted symbiont-growth only'.

4. Ultrastructure of cell division of symbiotic zooxanthellae and their host coral cells

4.1. Introduction

Cellular division is a dynamic process and one of the fundamental properties in all living cells. In cnidarians, the proliferation of the symbiotic or host cell by means of cell division must be kept in pace to one another, for the symbiosis to persist in a steady state (Davy *et al*. 2012). There are diverse studies that associate the proliferation of symbiotic species and host animal with the rhythmic mechanism, diel mitotic index, growth rates, seasonal population density and pigment content (Sweeney & Hastings 1958; Gladfelter 1983; Fitt & Trench 1983; Wilkerson *et al*. 1983, 1988; Muscatine *et al*. 1986; Hoegh-Guldberg 1994; Jones & Yellowlees 1997; Fitt 2000; Fitt *et al*. 2000). Although phased division pattern has been found to occur in symbionts or with its host cell (Gladfelter 1983; Wilkerson *et al.* 1988; Hoegh-Guldberg 1994; Fitt 2000), it has been reported that zooxanthellae did not showed periodicity in some cnidarian (Wilkerson *et al.* 1988; see Chapter 1). The more precise about the aspect of cell division in cnidarian symbioses is that, the division of the *in situ* zooxanthellae must be followed by a similar response of the host cell, although the mechanism involved is far understood (Davy *et al.* 2012).

The most common mode of reproduction in dinoflagellates is asexual reproduction that involves the process of mitosis (karyokinesis) and subsequent cytokiesis (Spector 1984). In the case of the *Symbiodinium*, they divide in coccoid state bybinary fission*in hospite* and from isolates (Freudenthal 1962; Trench 1993). Cell biologists distinctly characterize dinoflagellates due to the presence of unusual nucleus exhibiting no traces of DNA histone, permanently condensed chromosomes and a nuclear envelope persisting throughout its life cycle (Dodge 1973; Triemer & Fritz 1984). Ultrastructurally, the mitosis of dinoflagellates in various genera

has been extensively examined in the past (Triemer & Fritz 1984). Investigations include the mitotic process or other aspect primarily from free-living dinoflagellates such as in *Gonyaulax tamarensis* (Dodge 1964) and *G. polyedra* (Schmitter 1971); *Woloszynskia micra* (Leadbeater & Dodge 1967); *Gyrodinium cohnii* (Kubai & Ris 1969); and *Heterocapsa niei* (Morill & Loeblich 1984).

Among the symbiotic zooxanthellae, Freudenthal (1962) originally described the histological morphology and life cycle of gymnodinioid dinoflagellates that were isolated from scyphozoans and proposed the species as *Symbiodinium microadriaticum* Freudenthal. Six years later, Taylor (1968) reported the formal ultrastructural description of *in situ* symbiotic zooxanthellae 'akin' to *S. microadriaticum* Freudenthal isolated from actinians and briefly suggested a probable origin of membrane structures of symbiotic cells. The process of the cell division of the symbiotic zooxanthellae was not clearly discussed. Later, Kevin *et al*. (1969) proposed the revised ultrastructure and the amendment of *S. microadriaticum* Freudenthal that derived from two strains isolated from cnidarian hosts however the details on the cell division of *S. microadriaticum* Freudenthal remained lacking. In the study of zooxanthellae isolated from mollusks, *Tridacna* species, no description of dividing cells has been reported (Taylor 1969). Another modified ultrastructural description of the zooxanthellae was proposed together with other gymnodinioid symbionts in other cnidarians (Trench & Blank 1987), but likewise information regarding cell division is absent. Further, similar natures of reports were done on various symbiotic species (Trench *et al* 1981; Kempf 1984; Trench & Thinh 1995; Banaszak *et al.* 1993), which taxonomic approaches are highly emphasized. Recently, the detailed fine structure of the *Symbiodinium* spp. from cnidarians was elucidated from Wakefield *et al*. (2000) but once again was confined to the revision of the ultrastructural description of the

zooxanthellae. Despite these considerable contributions, most studies conventionally deals with non-dividing features of the symbiotic zooxanthellae. Our understanding on the process of their proliferation through which symbiotic relationship with the host animal persist is far lacking. At present, little has been known on how coccoid zooxanthellae are maintained through observation of its dividing life cycle in host cells.

We speculate that the current absence of formal ultrastructural examination of the dividing zooxanthellae from host cell may either overlooked or merely due to complication in the fixation of endosymbionts. To address the poor accessibility of corals to histological microscopy due to calcification (Weis *et al*. 2008), this study examined the dividing zooxanthellae in the gastrodermal cells of scleractinian coral *P. damicornis*. This emphasizes the behaviour of the cortical membranes and fine structures by freeze-substitution methods, an ideal fixation procedure for anthozoans (Wakefield *et al*. 2000). Our results have showed the first detailed ultrastructural description of the *in situ* cytokinesis process in zooxanthellae and coral host and demonstrated how the symbiont regulates its coccoid cell shape in light of cytomorphogenesis. For the first time, we have revealed the novel role of host nucleus prior to the establishment of its own mitosis which is undescribed before in the aspect of cnidarian cell division.

4.2. Materials and methods

4.2.1. Extraction of live coral tissues

Using the *in vitro* cultures of *P. damicornis* that induced regenerated tissues (described in Chapter 2), three healthy-looking coral samples were placed under the light of table lamp to let polyps extend fully. To avoid the retraction of polyps, colonies were anesthetized by gently adding about 1/3 of the medium volume with 3.6% $MgCl_2 \cdot 6H_2O$ solution (Janes 2008). After 15

min, the polyps became dormant (inactive) then were they were cut off using dissecting scissors. Excised polyp fragments were remained in the sea water to prevent from desiccation and tissue death.

4.2.2. Freeze-substitution fixation

Specimen was fixed using freeze-substitution methods, but unlike in Wakefield *et al.* (2000), in this study a high-pressure freezer (HPM100; Leica Microsystems, Wetzlar, Germany) was used. Using fine forceps, excised polyps were transferred carefully into gold rivets, add with optimum amount of cryoprotectant (fungi extract solution) then rapidly frozen. The frozen specimens were transferred to acetone containing 1% osmium tetraoxide at –80°C and stored in a deep freezer at –80°C for 72 h. Subsequently, samples were placed in –20°C for 24 h, held at 4°C overnight, and then warmed to room temperature for 2 h. They were rinsed with dry acetone five times for 3 minutes then fragmented into small pieces using an improvised fine razor. Samples were infiltrated with dry acetone and Spurr's resin solution series and embedded in molder with pure Spurr's resin before finally polymerized at 70°C for 48 h.

4.2.3. Electron microscopy

Embedded samples were fragmented using razor then fixed into resin capsule. Serial ultrathin sections were made using a diamond knife (Diatome 45°, Nisshin EM Co., Ltd. Japan) on a Leica Utracut UCT ultramicrotome (Leica Microsystems). Using fine forceps, thin sections were mounted on the copper grid (100 mesh) with thin vinyl coating then stained with 1% uranyl acetate for 1 to 2h followed by lead citrate for 10 to 15min. Specimens were carbon-coated using

vacuum evaporator (JEE 4B, JEOL Co., Ltd. Japan). They were then examined with a JEOL JEM-1400 electron microscope (JEOL Co., Ltd., Tokyo, Japan).

4.3. Results

4.3.1. Non-dividing zooxanthellae *in hospite*

Typically, a spherical non-dividing zooxanthella was individually harbored by a host gastrodermal cell. Several prominent cytoplasmic components were observed (Fig. 4.1a). The zooxanthella nucleus was irregularly shaped and commonly located almost at the center of the cell. It consisted of several dark condensed chromosomes and was enclosed by the double-membrane nuclear envelope. A single pyrenoid was observed in elliptical shape. Since it was found to be continuous from the inner surface of a chloroplast by a single stalk, the profile was enclosed with thick starch coverings. The accumulation bodies were distinct from other organelles due to huge and dark structure. They appeared spherical in shape, from one to two profiles occurring in each cell. Several mitochondria were observed with elongated shapes. They had short and bulbous cristae bounded in double membrane compartments. The globose-shaped lipid vacuoles could be found separately or in groups. However, not all cells contained lipid vacuoles. The chloroplasts mostly found surrounding the cell periphery occurred in series of lobate structure. They contained thylakoid lamellae that were often stacked in parallel arrangement and were enclosed by thick layers of roughly three membranes.

At the cell cortex, fine structures showed intact orientation (Fig. 4.1b-c). In details from the inside, series of cortical microtubules *cmi* were found with diameter ranging approximately from 20 nm to 25 nm. Each microtubule was composed of approximately 18 sub-spherical protein tubulin arranged in circular formation. Overlying them were discontinues layers of

flattened amphiesmal vesicles *amv*. They exhibited varying thickness (ca. ±19 nm to 80 nm in diameter) and length (may reach 1400 nm or longer). Inside the *amv* the presence of thin and continuous thecal plate *tpl* was evident. Enclosing these vesicles were stacks of membranous structures. The zooxanthellae plasma membrane z*pm* having approx. 6-7 nm thickness was the outermost membrane that enclosed the entire cytoplasm. It curved normally and showed even distances from the adjacent intermediate membrane *inm*. Thus the intermembrane space *isp* was found in between them during this phase and showed comparative thickness. Bordering the space lied the *inm* termed here as *im1* and *im2*. Localized multi-layered membranes *mm* were also noted above the *inm*. This was observed only in few sectioned cells. Then the outer membrane referred as symbiosome membrane *sym* appeared with thick undulated structure.

4.3.2. Initiation of zooxanthellae cell division

When cell division initiated, the disorientation of the peripheral *ch* at the indefinite site became evident (Fig. 4.2). The underneath lobes of chloroplasts commonly split apart and bend towards the central portion of the cell. When viewed under high magnification, the *zpm* at the site began to furrow forming a shallow 'U-shaped' cleavage. Its distance from the adjacent membrane increased as *im1* remained intact. Within *cw* an inner layer with electron-dense composition was exposed. Likewise the underlying *amv*'s and *cmi*'s tended to protrude inwardly.

4.3.3. Invagination of the zooxanthellae plasma membrane

Later, the zooxanthellae moderately turned its shape into slight elliptic (Fig. 4.3a). This was because the *zpm* from the opposite poles began to invaginate forming a 'Y-shaped' cleavage (Fig. 4.3b). Its distance from *im1* further increased as it remained intact. The inward protrusion

of *amv* was relatively sharp while some *cmi* appeared next to the leading edge. As it persisted, the cleavage of the invaginating *zpm* deepened towards the center of the cytoplasm. The preceding *zpm* had leading edges noted to appear blurred (Fig. 4.3c). This condition was similarly to *amv*'s and *cmi*'s traversing the center of the cytoplasm. The peripheral *ch*'s from the opposite poles tend to follow the path of the division furrows. Concomitant to invagination the nuclear division of the symbiont progressively occurred. Its elongated body was found constricting transversely against the invaginating *zpm* (Fig. 4.3a). The *cr* remained at condensed state and randomly dispersed in the entire nucleus. Meanwhile, groups of spindle fibers *sp* were found at the opposite distal sides (Fig. 4.3c inlet). The *cnu* migrated to one of the cleavage site which was rarely observed. In other sections, the condition was different where there were two of separate *znu* emerged while the cytokinesis was just under way.

4.3.4. Zooxanthellae Cytokinesis

When the nuclear division was completed, two separate *znu*'s surrounded by peripheral *ch* emerged (Fig. 4.4a). The coccoid zooxanthella transformed into semi-circular doublet cells. At the division furrow, the cleavage led to a broad 'V-shaped' structure where *cw* exposure dramatically increased due to intact position of the *im1* (Fig. 4.4b). At the center, two *zpm* exhibited a parallel structure contiguous to one another with clearly developed layers of *amv*'s and *cmi*'s beneath them (Fig. 4.4c). These indicated that cytokinesis was significantly completed where two daughter cells were developed.

4.3.5. Invagination of the intermediate and symbiosome membranes

When the other membrane structures *i.e.* intermediate and symbiosome membranes began to constrict, the daughter zooxanthellae gradually reshaped again into the typical coccoid cells (Fig. 4.5a). The division furrow farther became widened. The coral nucleus *cnu* migrates to one of the cleavage sites which was noted because the coral plasma membrane *cpm* tended to bulge as it cut through the cleavage area. The successive invagination was led by *im1* and followed by *im2* as observed with their leading edges furrowing in-progress (Fig. 4.5b). As they traversed the center of the cytoplasm, the parallel *zpm* were separated from one another. Beneath it, clearly developed *amv*'s and *cmi*'s were evident. After the constriction of the *inm* was completed the *sym* then invaginated. Compared from the previous membranes, the latter had broad leading edge leading the contiguous *inm* to split apart (Fig. 4.5c).

4.3.6. Intervention of the host nucleus

Shortly, *cnu* began to intrude between the doublet daughter zooxanthella (Fig. 4.6a-b). As it migrated toward the narrow intercellular space, the cortical regions of the zooxanthellae cells tended to bend (Fig. 4.6b). However, no displacement on the cortical fine structures was observed. Coral nuclei exhibited a flexible body, where it elongates to fit into the narrow space resulting to the expansion of the distance between the daughter zooxanthellae (Fig. 4.6c). When the *cnu* occupied entirely the intercellular space, mitosis subsequently followed. Coral mitosis was not described in details due to limited sections observed undergoing this process.

4.3.7. Host nuclear division between daughter zooxanthellae

When the distance between the daughter zooxanthellae increased, the host cell was constricted and resembled a 'dumb bell-shaped' configuration (Fig. 4.7a). Likewise the division of *cnu* occurred *in situ* (Fig. 4.7a-b). Coral nuclei exhibited a transverse division pattern where the continuous coral nuclear envelope *cne* was invaginated towards the center to divide into two profiles. Unlike the symbiotic cell, the *cpm* did not invaginate. Each daughter *cnu* was intended to be developed facing to the zooxanthellae, to which it will be associated with the completion of cytokinesis. Several coral *cr* appeared condensed, were dispersed evenly and extended up to *cnu* periphery. Series of host perinuclear microtubules *pmi* (approx. ±12nm in diameter) were evident at the distal sides of each *cnu* near the coral nuclear envelope *cne* (Fig. 4.7c). After this process was completed, coral cell subsequently undergo cytokinesis (may refer to Fig. 4.8). Coral cytokinesis will finally distribute individual zooxanthella and nucleus to each of the daughter coral cells. All other stages during the cellular division were schematically illustrated in Fig. 4.8 to better understand the entire process.

4.4. Discussion

Dealing with the general morphology, the fine structures of the symbiotic zooxanthellae cells observed in *P. damicornis* showed correspondence with the progressing descriptions of the typical *Symbiodinium* spp. in cnidarian hosts (Freudenthal 1962; Taylor 1968; Kevin *et al*. 1969; Dodge 1973; Triemer & Fritz 1984; Trench & Blank, 1987; Wakefield *et al*. 2000). These structures typically found in this species which were also observed in the present study includes the distinguishing characteristics of the cytoplasmic inclusions such as the nucleus with permanently condensed chromosomes and persistent nuclear membrane, multi-lobed peripheral

chloroplasts, huge stalked pyrenoid protruding from the lamellae and other organelles, and complex cortical membrane structures. At the molecular level, *P. damicornis* had roughly identified hosting 2 to 3 phylogenetic clades of zooxanthellae belonging to *Symbiodinium* spp. (Rowan & Powers, 1991; Magalon *et al*. 2007), which could highly supports the identification of the *in situ* zooxanthellae in this study. Similarly, these ultrastructural characteristics were also extensively reported from free-living dinoflagellates (Leadbeater & Dodge 1966, 1967; Dodge 1964, 1973, 1975; Dodge & Crawford 1971; Schmitter 1971; Horiguchi & Pienaar 1994). The cortical features of free-living (motile) dinoflagellates are complex, and it has been reported that they developed and proceeded an overwhelming cytomorphological processes (Leadbeater & Dodge 1967; Morrill & Loeblich 1981, 1984; Chapman *et al*. 1981; Morrill 1984; Bricheux *et al*. 1992; Sekida *et al*. 2001, 2004). For instance in *Scrippsiella hexapraecingula*, the species at motile stage was observed with outermost plasma membrane overlying the series of amphiesmal vesicles and groups of cortical microtubules where thecal plates were enclosed in these vesicles (Sekida *et al*. 2001). When settling during non-motile phase, the species ecdysed (shedding of coverings) resulting in the formation of first pellicle layers, fusion of inner amphiesmal vesicles membrane to form new plasma membrane, deposition of second pellicle layer under the first one, and the appearance and fusion of juvenile amphiesmal vesicle. In *Glenodinium foliaceum*, the development of the complex pellicle underlying the thecal plates occurred after ecdysis (motile phase) that formed four distinct layers of outer layer of randomly oriented fibril, a 50 nm of fibril oriented to the dense layer, dense trilaminate structure and wide homologous layer (Bricheux *et al*. 1992). In this case, the cortical features of a certain dinoflagellates became more complex due to variation in the life cycle.

The present study examine the zooxanthellae cell division *in situ* host cell and showed a unique process where nuclear division occurred while invagination of the plasma membrane is in progress. This is rather congruent with the findings in *Woloszynskia micra* where the constriction in the nucleus during nuclear division was the result of cytoplasmic inpushing brought by membrane invagination (Leadbeater & Dodge, 1967). In several sections observed in this study, two separate nuclei emerged while the invagination of the plasma membrane remained in progress. This implied that many have exhibited the usual process where nuclear division occurred prior to cytokinesis. This event was profoundly exhibited by the symbiotic zooxanthellae (*S. microadriaticum*) isolated from the host *Cassiopeia* sp., where the formation of equatorial zone by the constriction of the cytoplasm occurred after the division of nucleus (Freudenthal 1962).

On the other hand, the parallelism in the alignment of the chloroplasts along with plasma membrane was evident in this study during the invagination. When the process of cytokinesis terminated, the daughter zooxanthellae cells were completely bounded by the peripheral chloroplasts. Such condition was also presented by Kevin *et al*. (1969) as shown on their representation in Fig. 8. In the present study, we did not find the doubling chloroplasts that had kept in pace with the cytoplasmic partitioning. However it is a fact that the event of chloroplast division used to occur among algal species primarily in the endosymbiontic microalgae (see reviews from Dodge 1973; Kuroiwa 2000; Okazaki *et al*. 2010). Performed by the rapid constriction of the ring protein complex at the center of the chloroplast, both the inside and the outside of its two membranes span before the algal cytokinesis occurs (Dodge 1973; Kuroiwa 2000; Okazaki *et al*. 2010). In this case, this may explain why chloroplasts were consistently

appeared with branching or multi-lobed structure (Taylor 1968; Wakefield *et al*. 2000), possibly due to some event, such as constriction that took place anytime (Dodge 1973).

This study had found the constriction of the intact nuclear envelope undergoing mitosis with condensed chromosomes in the *in situ* zooxanthellae. To date, this fact remains highly recognizable with the plausible findings during karyokinesis in dinoflagellates (Leadbeater & Dodge 1967; Kubai & Ris 1969), with some exception in *G. tamarensis* that was reported without defined nuclear membrane (Dodge 1964). Leadbeater & Dodge (1967) further claimed that the microtubule-containing cytoplasmic channels traverse the nucleus in order to divide although the chromosomes had no evident association with the spindle fibers observed. These observations are rather analogous to our study. The cortical microtubules used to traverse the cytoplasm along with the plasma membrane that did not showed any link with chromosomes dispersed all over the dividing nucleus. Likewise the concomitance of mitosis with the progressing cytokinesis was evident (as discussed earlier). However, other sectioned cells followed the general process, where cytokinesis occurred after the nuclear division. Here, it is speculated that there might be some discrepancy in the timing of cytokinesis against nuclear division. The reason for this is unclear. So far there is no obvious tight temporal coupling between the completion of mitosis and the beginning of mitosis (as reviewed by Scholey *et al*. 2003), that have been extensively demonstrated. To date, little is known about the synchrony of host and symbiont division in cnidarians (Davy *et al*. 2012).

Detailed examination of the cortical fine structures of the dividing zooxanthellae *in hospite* helps provides the detailed information to reconstruct the process of zooxanthellae cell division in Fig. 4.8. Our observation showed that the zooxanthellae plasma membrane was the first to reveal the early manifestation of the cytokinesis through the formation of the division

furrow. However it is not clear where and what was the probable precursor at the cortical region that initiated the furrowing. This is rather similar to *Heterocapsa niei* which exhibited the first sign of cytokinesis from the widened hypocone though the process, by which its cortical structures expand, was unclear (Morrill & Loeblich 1984). In *Gonyaulax,* the division furrow emerged from the centripetal out-grows within the division vesicles beneath the margin of the overlapping plate (Netzel & Dürr 1984 for Fig. 4.10). With this common problem in determining the site of cytokinesis in many cells, many have claimed of the responsibility of whole spindle microtubules such as astral MTs, ipMTs and kMTs that mediates the furrow induction (as reviewed by Scholey *et al*. 2003), during the cytokinesis in cell. If this is the case, perhaps there must be the presence of microtubular structures that facilitates the division furrow of the zooxanthellae plasma membrane which was unveiled in this study by ultrastructural examination. In fact, there could be one thing that makes cytokinesis congruent among dinoflagellates – the division of the cell by bipartition that arises fundamentally in the cortex (Netzel & Dürr 1984). In the phenomenon such as *Peridinium*-type cytokinesis, the plasmalemma (plasma membrane) indents centripetally developing division sutures thus forming division furrow in both region (see Fig. 4.9).

During zooxanthellae cytokinesis, this study had found only one membrane structure *i.e.* zooxanthellae plasma membrane surrounding the cytoplasm that invaginated, similar to the more precise findings of Wakefield *et al*. (2000). For this reason, the present observation did not complement with the earlier reports of Taylor (1968) who described that periplast surrounding the symbiont were comprised of two membranes that borders each daughter symbiont cell after the binary fission was completed. In Kevin *et al*. (1969), two membranes layers were described that sandwiched the thick amorphous layer. Both Taylor (1968) and Kevin and co-workers

(1969) might not clearly observe these fine structures under highly magnified micrographs as chemical fixative was used. Using such method rather produced the presence of unusual artefacts often occurred in chemically-fixed sample (Wakefield *et al*. 2000).

On the other hand, during the similar event in the present study, the furrowing zooxanthellae plasma membrane rather exposed a space at the division furrows called here as 'intermembrane space'revealing an electron-dense layer at the inner portion. However, when cleavage furrow became wider (Fig. 4.4b), the said layer was unrecognizable. Referring to study of Wakefield *et al.* (2000), which upon reviewing their micrographs presented in Fig. 10, apparently the electron-dense layer was considered a part of what they termed as 'cell wall'. It seems this contradicted to our observation which we assumed that the dense layers were merely remnants of the invaginating plasma membrane, although both this study and of Wakefield have used freeze-substitution methods. Considering such inconsistency, whether the dense layers is present or not, it would be feasible therefore to describe it as amorphous layer.

Once the cytokinesis of zooxanthellae was done, successive invaginations of the other distinct membrane structures surrounding the cell wall occurred. This event had assisted in the significant cell shape transformation from semi-circular to spherical daughter cells, a stage when doublet cell emerged in one host gastrodermal cell as described by Camaya *et al*. (2016). This also contradicted with the observation of Taylor (1968) suggesting that the outer three membranes of parent cells (referred as the two intermediate membranes and the symbiosome membrane in this study) did not participate in the cell division that tends to break and resynthesize. This study finds no basis to this earlier hypothesis of Taylor (1968), considering that the present examination had demonstrated that dynamic constrictions of these membranes have led to the formation of multi-membrane layers around the newly developed daughter

zooxanthellae. The observation of Taylor (1968) gives no clue on how and when these membranes originated, as he claimed.

Further dealing with these membranes, the findings from the present study is rather different from that of Wakefield *et al*. (2000) who described the presence of thick 'multiple layers of symbiosome membrane' (ml) between membranous outer layer (ol) and outer symbiosome membrane (sm) in their study. Although Wakefield *et al*. (2000) recognized that this disproportionate number of membranes was observed in one side of the symbiont in some specimen, but still considered as part of the boundary between host and symbiont. In the early work of Taylor (1968), he also described the 'scroll-like' structure in the outer layer of cell wall that occurred periodically. We also observed an equivalent structure to ML (termed here as multi-layered membranes) (Fig. 4.2c) from a few sections but was not entirely present in the cell. Considering the aspect of cell division, this study finds no relevance of considering the multi-layered membranes as innate part of the cortical structure of the *in situ* symbiotic zooxanthellae. The basis is that, this study had showed that after cytokinesis only three well-defined membranes have successively invaginates to completely develop the entire cortical structure of the symbiont cells. No evidence on how multiple membranes were developed as only those distinct membrane structures exhibit dynamic reformations during the course of the symbiont reproductive cycle. Wakefield *et al.* (2000) further hypothesized its formation mainly from the zooxanthellae ecdysis cycle, unrecognizing the aspect of its cell division.

In the nuclear divisions of host coral cell, the dynamic mechanism of coral nucleus intervention that assisted in the separation of the daughter zooxanthellae and its karyokinesis in the site was one of the important findings of this study, which remains undescribed in the past. This is the first report in cnidarian symbioses which revealed the novel role of a host nucleus

during the division of both cells. This study revealed that coral nucleus played a disruptive behavior to provide the daughter zooxanthellae and its own mitotic division with distinctive places prior to the cytokinesis of coral cytoplasm. In searching for any comparative studies to this phenomenon, apparently no related cellular event in the endosymbioses has been reported so far. In the past, there has been just one report as cited by Dodge (1973) that illustrated the migration of nuclei in *Closterium* (see Fig. 4.10). However, the nuclei used to intervene the pre-formed breaks that were found in the chloroplasts, and not associated with the reproductive process. Due to limitation of information, the physiology behind the host nuclear intervention remains unknown. This finding deserves further examinations such as tracking the migration of host nucleus through fluorescence imaging, more importantly to elucidate its course of action during cell division.

The nuclear division in host coral cells was relatively different from its symbiotic cells where the coral plasma membrane did not traverse its nucleus to divide into two profiles. Although the coral nuclear envelope remains persistent throughout the invagination process, the cytoplasmic in-pushing apparently did not exist. We assume that in this case, there was the presence of contractile rings that mediate the constriction (Scholey *et al*. 2003) in the nuclear envelope.

In conclusion, we understand how zooxanthellae used these cortical fine structures from the onset of mitosis towards the cytokinesis to regulate its coccoidal shape during its symbiotic life. Cytokinesis comes along with cell reshaping in which all the membrane complexes consisting of membrane series working together, successively. The entire process was probably always directed, if not brought about by the microtubules (Dodge, 1973). This investigation clearly demonstrated to address the postulation, and what symbiotic cells reproduce preceding its

host cells (Davy *et al*. 2012) is indeed plausible. It is not known which gives signal to symbiont to undergo division. The fact that host cell through its nucleus provides assistance during the reproduction of symbiotic zooxanthellae and subsequently performed its division, this implied that both cells have physiological commitment in maintaining their symbiotic relationship through cellular proliferation.

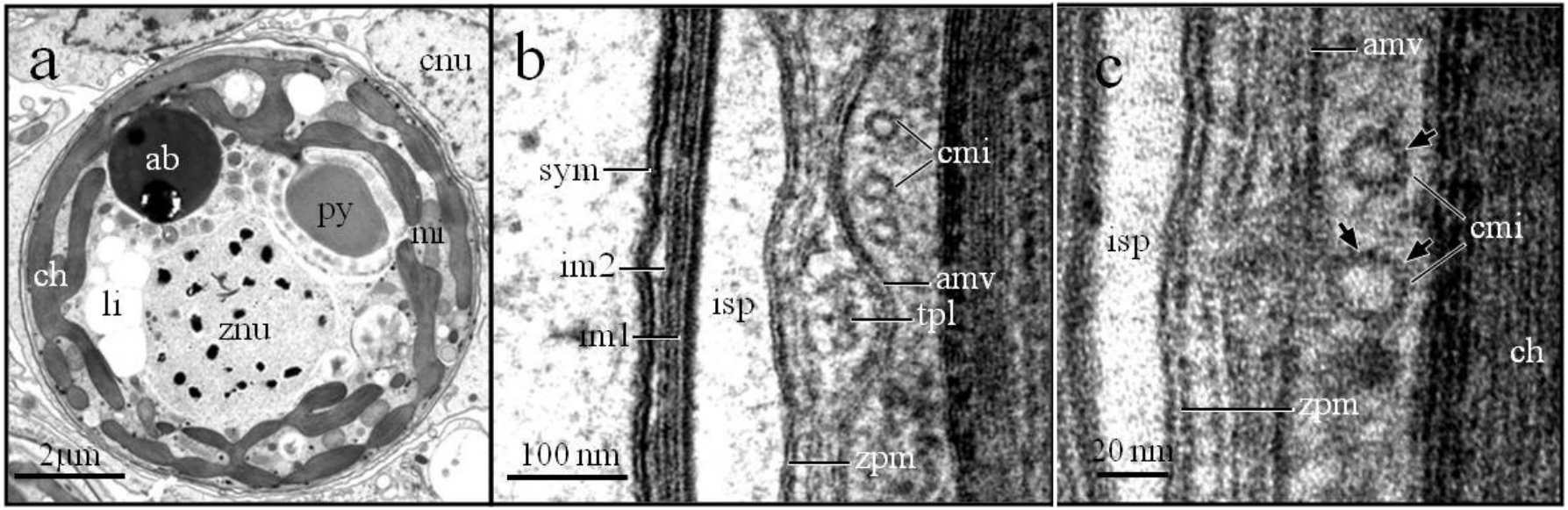

Fig. 4.1. Ultrastructure of symbiotic zooxanthellae *in hospite* at non-dividing stage. (**a**) An spherical-shaped zooxanthella and its intracellular components such zooxanthellae nucleus (znu) with multi-condensed chromosomes (cr), accumulation bodies (ab), pyrenoid (py), mitochondrion (mi), lipid vacuoles (li) and peripheral chloroplasts (ch) while the host coral nucleus (cnu) appeared adjacent to the symbiotic cell. (**b**) The cortical fine structures consisting (*in order from inside*) the series of cortical microtubules (cmi), amphiesmal vesicles (amv) with thin thecal plate (tpl) inside, zooxanthellae plasma membrane (zpm), intermembrane space (isp), intermediate membrane1 (im1) and intermediate membrane 2 (im2) and the symbiosome membrane (sym). (**c**) At high magnification, micrograph showing a pair of *cmi* each having approx. 18 sub-spherical protein tubulin (*arrows*).

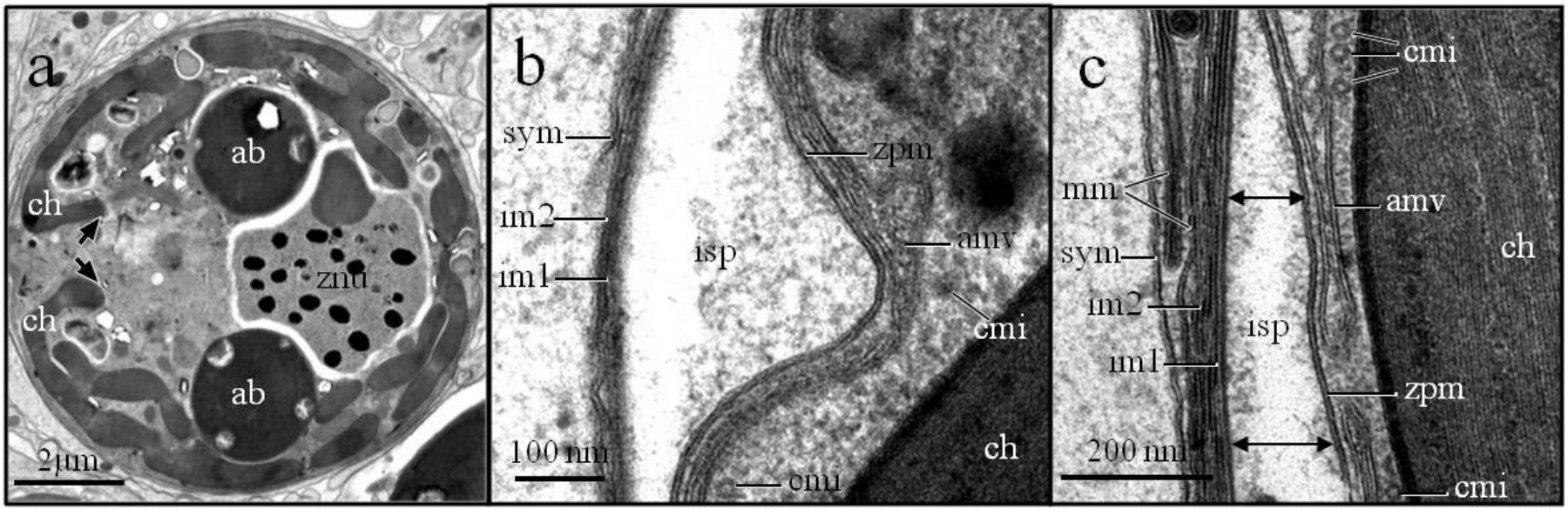

Fig. 4.2. Ultrastructure of symbiotic zooxanthellae *in hospite* during onset of mitosis. (**a**) The peripheral chloroplasts (ch) at an indefinite site is apparently disoriented as they bend towards the center (*indicated by arrows*). (**b**) At the cortical region, the zooxanthellae plasma membrane (zpm) developing a shallow 'U-shaped' furrow while the amphiesmal vesicles (amv) beneath protruding inwardly. The intermembrane space (isp) tended to widen. (**c**) The thickness of the latter varies from areas near (*bottom arrow*) and far (*top arrow*) from the cleavage furrow. Partial inclusion of multi-layered membrane (mm) was evident between intermediate and symbiosome membranes.

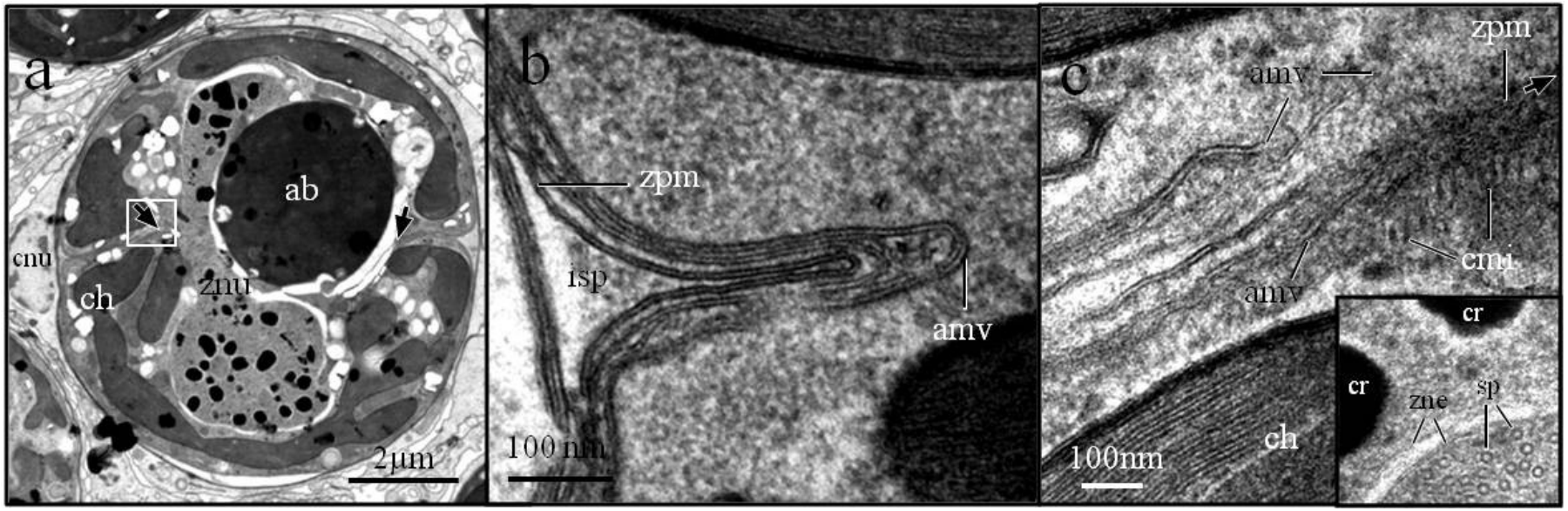

Fig. 4.3. Ultrastructure of symbiotic zooxanthellae *in hospite* during invagination of plasma membrane. (**a**) The zooxanthellae apparently turn into slightly elliptical shape as the zooxanthellae plasma membrane (zpm) from the opposite poles invaginates (*leading edges indicated by arrows*). Simultaneously the zooxanthellae nucleus (znu) divides showing transversely constricted body against the invaginating plasma membrane. The chromosomes (cr) are randomly dispersed in entire profile. The coral nucleus (cnu) appeared adjacent to one of the cleavage sites. (**b**) An earlier invagination of zooxanthellae plasma membrane (zpm) forming 'Y-shape' configuration while sharp protrusion of amphiesmal vesicle (amv) was evident. Some cortical microtubules (cmi) appeared adjacent at the leading edge. (**c**) An enlarged image from Fig. 1A showing thin sheet of invaginating zooxanthellae plasma membrane (zpm). Its leading edges along with developing amphiesmal vesicles (amv) and cortical microtubules (cmi) were apparently blurred. The inlet showed the group of spindle fibers (sp) at the distal side of the dividing zooxanthellae nucleus (znu) located adjacent the nuclear envelope (zne). The chromosomes (cr) remains at condensed state.

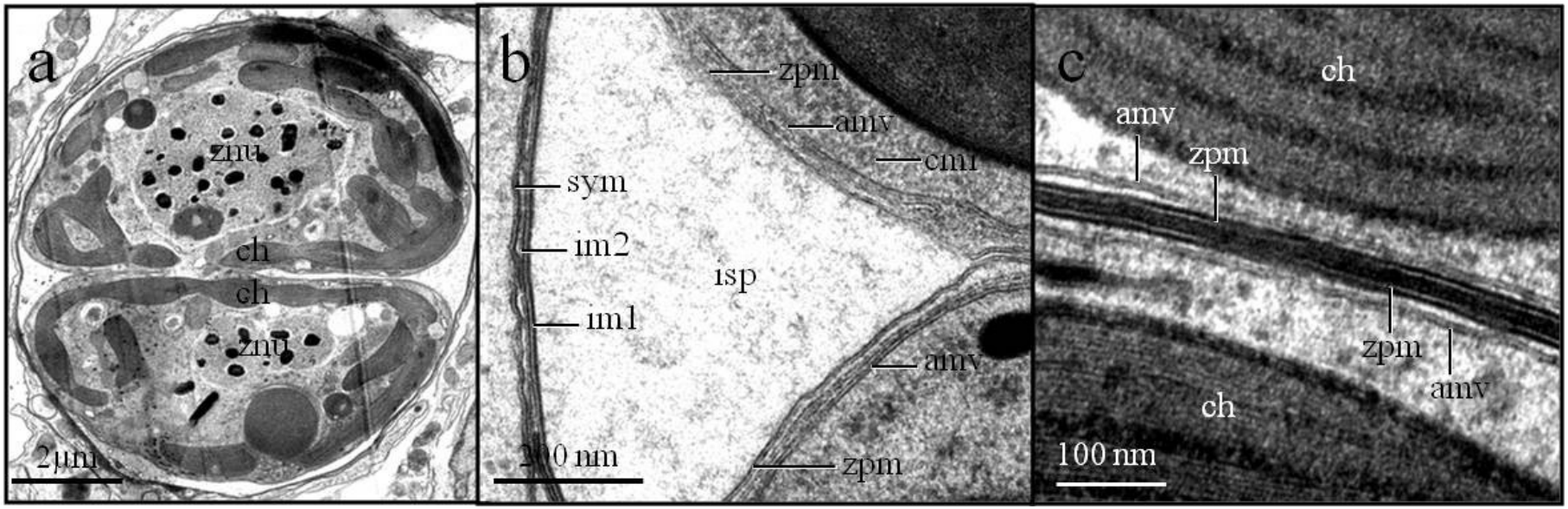

Fig. 4.4. Ultrastructure of symbiotic zooxanthellae *in hospite* during cytokinesis. (**a**) Zooxanthellae formed into semi-circular doublet cell during the cytokinesis where zooxanthellae nucleus (znu) and peripheral chloroplasts (ch) were distributed to each daughter cell. (**b**) 'V-shaped' division furrows tends to widen the intermembrane space (isp) while the intermediate membranes 1 (im1) and 2 (im2) and the symbiosome membrane (sym) remains intact. (**c**) At the division site, the two fully invaginated zooxanthellae plasma membrane (zpm) remained parallel and contiguous to each other. Beneath them were new layers of amphiesmal vesicles (amv).

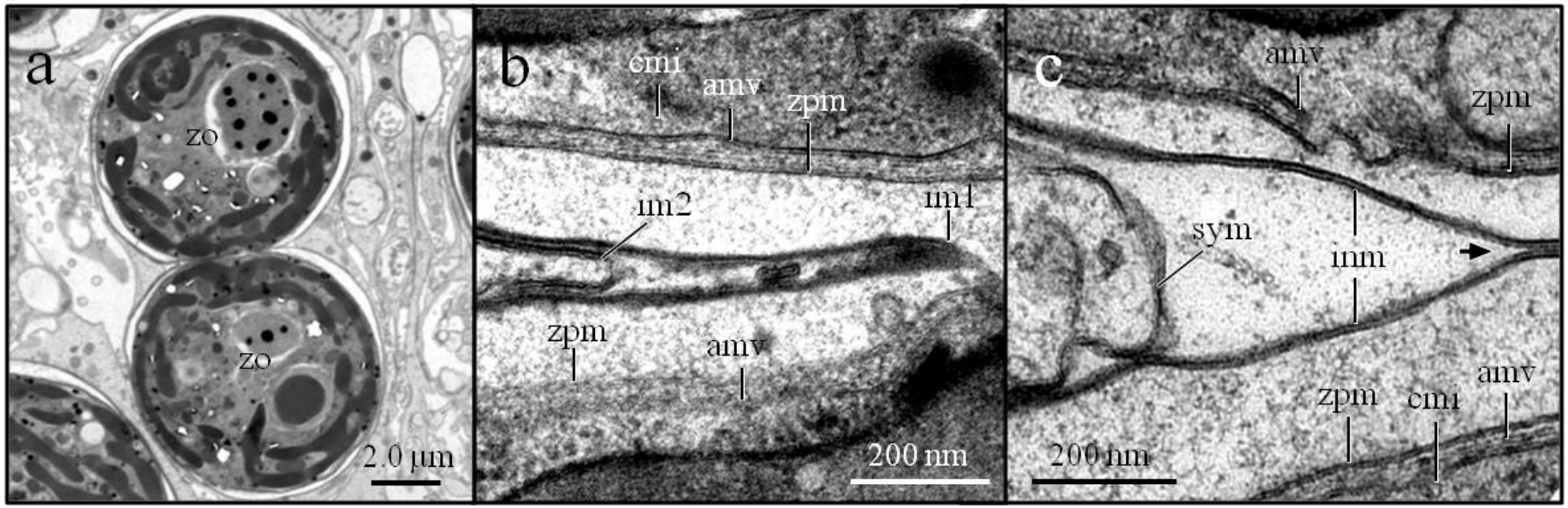

Fig. 4.5. Ultrastructure of daughter zooxanthella *in hospite* during invagination of intermediate and symbiosome membranes. (**a**) The daughter zooxanthellae (zo) transformed into spherical-shaped cells while still contiguous to each other. (**b**) The leading edges of the invaginating intermediate membranes 1 (im1) and 2 (im2) were successively observed in-progress. Wide space between the parallel zooxanthellae plasma membrane (zpm) was evident with the developed fine structures beneath them. (**c**) Later the symbiosome membrane (sym) invaginates with its broad leading edge. While it traversed towards the central region, those intermediate membranes (inm) begin to splits apart (*indicated by arrow*).

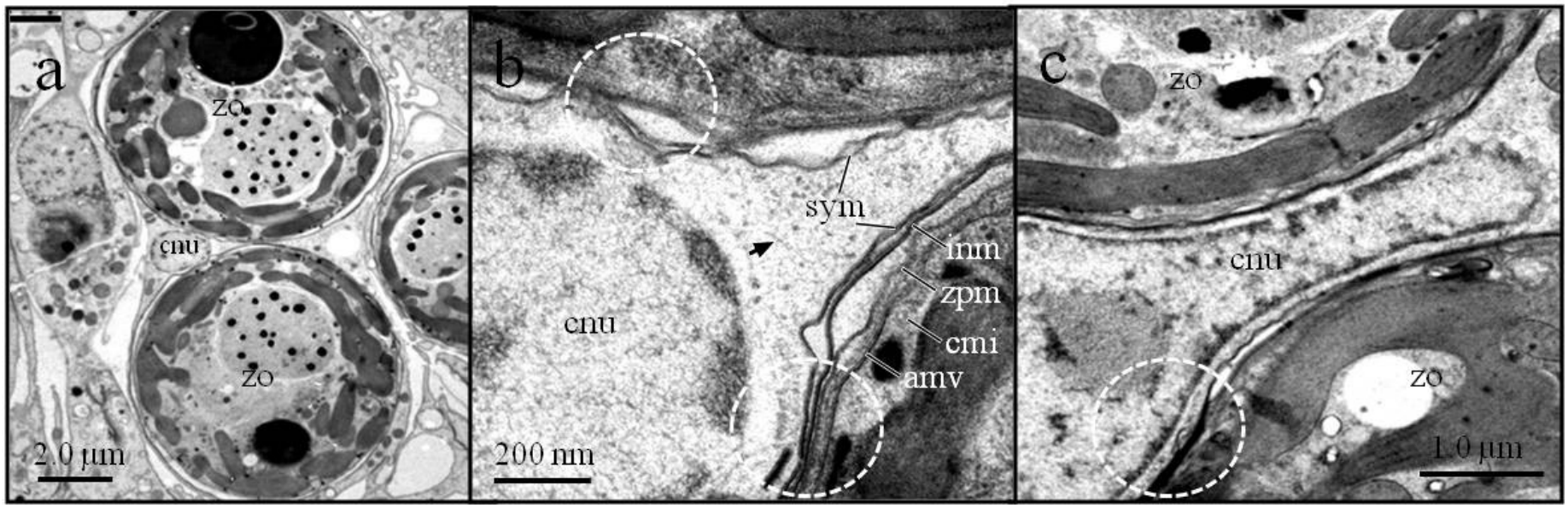

Fig. 4.6. Ultrastructure of daughter zooxanthellae *in hospite* during intervention of coral nucleus. (**a**) The daughter zooxanthellae (zo) with the coral nucleus (cnu) appeared in one of the cleavage sites. (**b**) At high magnification, micrograph from other section show the zooxanthellae cortical region tends to protrude inwardly (*dotted circle*) as the coral nucleus (cnu) intervene the narrow intercellular space. No disorientation on the cortical fine structures was evident. (**c**) An elastic body of coral nucleus (cnu) that fits through the intercellular space. Note the increase in distance between the daughter zooxanthellae (zo) with the inward protrusion of its cortex.

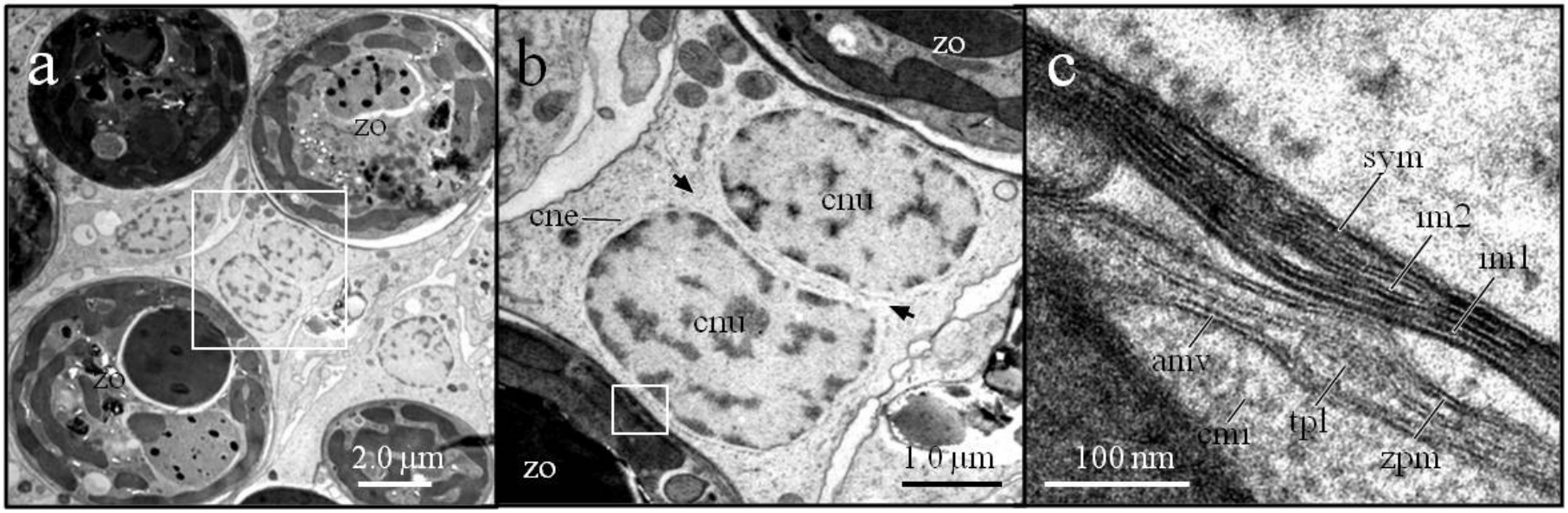

Fig. 4.7. Ultrastructure of daughter zooxanthella *in hospite* during coral nuclear division. (**a**) The coral nuclear membrane (cne) begin to constrict as daughter zooxanthellae (zo) dispersed far apart that resembles 'dumb bell-shaped' configuration. Simultaneously coral nucleus (cnu) divides between them. (**b**) Enlarged micrograph from Fig. 4.7A shows that the nuclear division just recently completed with two continuous coral nuclear envelope (cne) appearing transversely between daughter coral nucleus (cnu) (*indicated by arrows*). The coral plasma membrane (cpm) remains intact. The division pattern intends each coral nucleus (cnu*)* contains condensed chromosomes (cr) dispersed evenly that extend up to the periphery. (**c**) Enlarged image from Fig. 4.7b had showed clearly the cortical region in one of the daughter zooxanthellae.

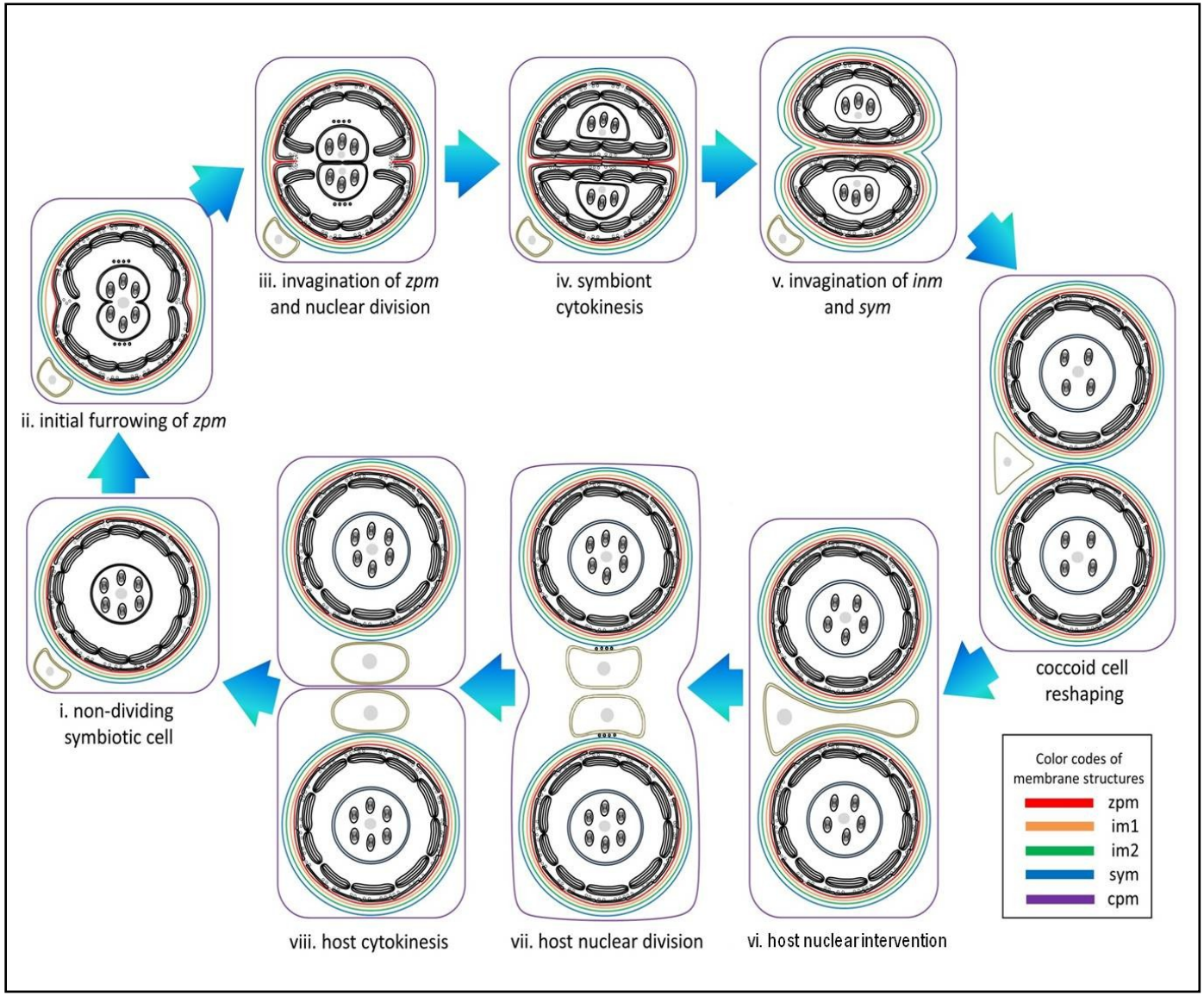

Fig. 4.8. Schematic diagram of the stages of symbiotic zooxanthellae cell division *in hospite*. As cortical structures of symbiont successively invaginate, physiological transformations of its cell shape occurred. Coral nucleus performed dynamic intervention and division between daughter symbionts as pre-cytokinetic mechanism of the host coral cells.

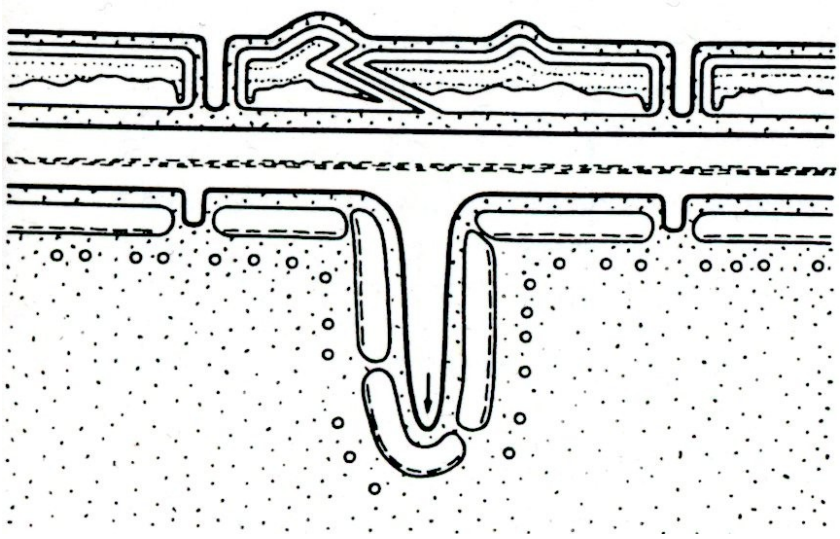

Fig. 4.9. Schematic diagram of the cortex development in *Peridinium*-type of cytokinesis. Note the indention (down-pointed arrow) of plasmalemma centripetally along the division suture thus forming the division furrow. (Illustration adapted from Netzel & Dürr, 1984)

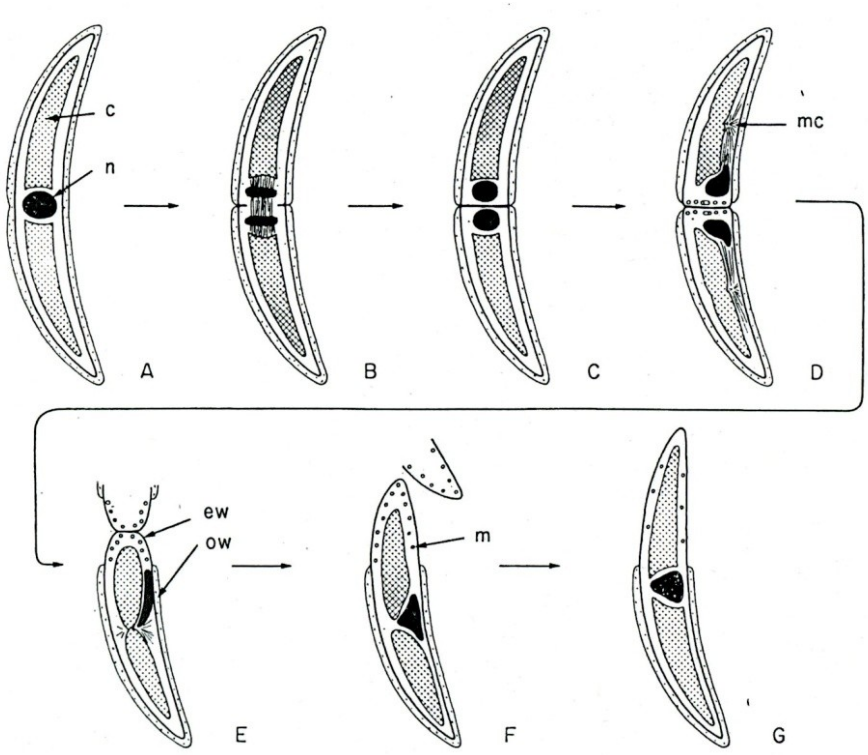

Fig. 4.10. Schematic diagram of cytokinesis in the desmid *Closterium*. Note that migration of the nucleus (shown in D-G) from the division site towards the pre-formed break in the chloroplasts. The two chloroplasts move apart as the nucleus intrudes between them. (Illustration adopted from Dodge 1973)

5. Changes in the ultrastructures of the coral *Pocillopora damicornis* after exposure to high temperature, and ultraviolet and far-red radiation

5.1. Introduction

Reef-building corals contain large populations of symbiotic dinoflagellate algae of the genus *Symbiodinium,* commonly referred to as zooxanthellae, and are fragile marine organisms which are vulnerable to natural disturbances (Birkeland 1997). Coccoid zooxanthellae *in situ* are located within the cells of the host gastrodermis (Trench 1993), and are bound by a membrane complex consisting of a series of membranes of algal origin and an outermost host-derived membrane. This entire entity is referred to as the symbiosome (Wakefield *et al*. 2000).

The loss of symbiotic algae from coral host tissues, which eventually leads to indiscriminate coral bleaching, is driven by various stress factors such as an increase in temperature and irradiance level, lowered pH, and salinity changes (Goreau 1964; Glynn 1993; Hoegh-Guldberg 1999; Fitt *et al*. 2001; Anthony *et al*. 2008; Delgadillo-Nuño *et al.* 2014). High temperature conditions damage various intracellular components of zooxanthellae (Brown 1997; Hoegh-Guldberg 1999), and the effects of high temperature are promoted synergistically by intense light (Weis 2008). Chloroplasts are one organelle that is remarkably affected by these stresses (Downs *et al.* 2002; Weis *et al.* 2008). When corals are exposed to high temperature or intense light irradiance, stress-induced products with oxidative activities can be formed in the chloroplasts of zooxanthellae and may trigger the expulsion of zooxanthellae from the coral host (Lesser 1997; Nii & Muscatine 1997; Downs *et al*. 2002, 2009, 2013; Franklin *et al.* 2004; Lesser & Farell 2004; Smith *et al.* 2005). However, the mechanisms by which coral bleaching occurs in tissues and at the cellular level remain unclear (Downs *et al.* 2002; Weis *et al.* 2008). In addition, high irradiance to red light (Kinzie *et al.* 1984; Kinzie & Hunter 1987; Wijgerde *et*

al. 2014) and exposure to ultraviolet radiation (Lesser & Shick 1990; Gleason & Wellington 1993; Shick *et al.* 1996) significantly decrease both coral growth and zooxanthellae density. It had been poorly understood how these stressors induce coral degradation, especially in the early stage of exposure.

The loss of symbiotic algae is associated with cellular degradation. Cell necrosis is known to be a considerable deleterious stress response of corals and causes the widespread detachment and rupturing of many cells throughout coral tissues (Dunn *et al.* 2000). Coral necrosis involves autolysis, where digestive enzymes may be produced to degrade cells (Müller *et al.* 1984). It has been reported that the degradation of host cells is caused by autophagy, which plays a role in digesting the engulfed cytoplasmic constituents and other materials (Dunn *et al.* 2007; Dunn & Weis 2009; Hanes & Kempf 2013). Other factors that bring about host cell degradation include programmed cell death (PCD), or apoptosis (Dunn *et al*. 2000, 2002; Dunn & Weis 2009; David *et al.* 2005; Paxton *et al.* 2013), pathogenic bacteria (Ben-Haim *et al.* 2003), or viral infection (Lohr *et al.* 2007). It is unknown if these distinct factors mutually influence coral host cells or how various environmental stresses promote digestive activities in coral cells (Fitt *et al.* 2001; Downs *et al.* 2009).

In this study, the hermatypic coral *P. damicornis* was experimentally exposed to three distinct stressors: high temperature (HT), ultraviolet (UV), and far-red (FR) radiation. We examined the changes in the ultrastructure of the host cells and symbiotic zooxanthellae under these stress conditions.

5.2. Materials and methods

5.2.1. Treatment of coral isolates with stressors

Coral isolates were exposed to the three distinct stressors, UV-A, far red and high temperature. Two coral isolates were placed in the black boxes, on which LED monochromatic light units (CCS Inc., Japan) were installed. Light intensities of UV-A (405 nm) and far red (735 nm) was 2.0 W/m^2 and 4.5 W/m^2, respectively. UV-A has hazardous effects to photosynthetic plants (Hollósy, 2002), so the irradiance level increased two and half folds as much as in far red to ensure the expulsive reaction of symbiotic zooxanthellae. For treatment with high temperature (HT), isolates were placed into water bath with temperature of 32°C maintained using heater (Yamato Thermo-mate BF-21, Japan). Samples exposed to UV-A or far red were observed under a light microscope every 3 h (until 24 h after the initiation of experiments) or every 6 h (from 24 h to 48 h after the initiation of experiments). Samples exposed to high temperature were observed every 10 min (until 1 h after the initiation of experiments) or every 1 h (during 1 h to 4 h after the initiation of experiments).

Prior to stress induction, healthy isolates with intact regenerated tissues (no indication of retraction) and stationary zooxanthellae *in situ* were observed. When exposed to FR for 12 h, a few symbionts were noted to move by 'wiggling'. Later, the agitating cells increased while some rigorously moved from one place to another. Moving cells increased in time until the expulsions of symbionts and cellular debris from the hollow cavity became evident after 24 h. Under UV, a little debris was expelled as compared with FR after 24 h. effect of HT appeared faster than that of UV or FR, and the contraction of polyps and the clumping of zooxanthellae at the lower tentacles were noted 20 min after the stress treatments. Wiggling of symbiotic cells was obvious

90 min after the stress treatments, while massive expulsions of zooxanthellae occurred 120 min after the stress treatments.

After these pre-fixation trials to determine stress treatment period and the behavior of the *in situ* zooxanthellae, two new coral samples were exposed in similar irradiance light level for 6h, 12h, 30h, and 48h in both FR and UV lights. Samples treated under high temperature (32°C) were exposed for 15, 30, 60 and 120 minutes, respectively. All procedures were performed in ambient room temperature, as mentioned above.

5.2.2. Chemical fixation

The specimen used for control condition was obtained from the corals fixed by freeze-substitution methods as described in Chapter 4. Since colony tentacles retracted immediately after they were exposed to HT, UV, and FR, it was difficult to fix them by freeze-substitution procedures. Therefore, chemical fixation was adopted for the samples exposed to these stressors. Whole colony segments were immersed in a fixative containing 4% paraformaldehyde, 2% glutaraldehyde and 0.1 M sucrose dissolved in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature and stored for 48 h at 5°C. Fixed colonies were decalcified in 5% ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (pH 7.2) containing 0.2 M sucrose (Kurahashi 1965) for 4–5 days. Decalcified colony samples were rinsed with 0.1 M phosphate buffer containing 0.1 M sucrose five times, cut into several pieces with a fine razor and then post-fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% $KMnO_4$ and 0.1 M sucrose for 30 min at 5°C. Post-fixed specimens were rinsed with the phosphate buffer, dehydrated in an acetone series for 2.5 h and embedded in Spurr's resin before finally polymerized also at 70°C for 48h.

5.2.3. Transmission electron microscopy

The observed specimens under electron microscope, similar procedure were done in preparing the ultrathin section, staining and carbon coating method used in Chapter 4. Micrographs were obtained in each fixation periods of every stress factors used.

5.2.4. Quantitative analysis of autophagy

The number of autophagosomes and lysosomes found in the gastrodermal cells were counted on thin sections. One hundred sectioned cells were observed for both the control treatment and the samples that were exposed to HT, UV, and FR for each different exposure interval for a total of 1,300 coral cells. Both structures found either in non-hosting or hosting-symbionts gastrodermal cells were counted separately then the average density were determined. The test of significant difference in-between control and stress variables in each treatment period were determined using ANOVA ($P < 0.05$) derived from Tukey's HSD *post hoc* test using a public domain programming language for statistical computing *R* software (R Version 3.2.5, 2016).

5.3. Results

5.3.1. Control condition

Coral tissues in the control treatment showed a body pattern typical of diploblastic organisms and consisted of two layers, the epidermis and gastrodermis, interconnected by an acellular matrix mesoglea (Fig. 5.1a). Some cells of the gastrodermis harbored 1–2 symbiotic zooxanthellae, whereas the others contained no zooxanthellae. Each symbiotic zooxanthella had peripheral chloroplasts with a pyrenoid, a mesokaryotic nucleus and other organelles (Fig. 5.1b). The cortical region of the cell was composed of the outermost plasma membrane and a layer of

flattened amphiesmal vesicles with underlying cortical microtubules (Fig. 5.1c). The entire symbiotic zooxanthella cell was enclosed by a symbiosome membrane in the host gastrodermal cell. There were stacks of membranous fragments between the plasma membrane of the zooxanthella and the symbiosome membrane. In some gastrodermal cells, small, dark-stained lysosomes and another type of membrane structure were observed (Fig. 5.1d). The latter was initially a cup-shaped double-membrane sac, which later expanded in size, and its open end eventually closed to form a double-membrane vesicle. This is referred to as an autophagosome in this study. Autophagosomes contained several distinct inclusions (Fig. 5.1d). The average density of autophagosomes observed on a sectioned gastrodermal cell was 0.66 (Fig. 5.6).

5.3.2. Effect of high temperature

When corals were exposed to HT for 15 min, several electron-dense granules appeared in the cytoplasm of the cells without zooxanthellae (Fig. 5.2a-b). These dense granules were regarded as lysosomes. The average number of autophagosomes contained in a sectioned gastrodermal cell was 2.3 for that exposure interval (Fig. 5.6). Exposure to HT for 30–60 min caused severe vacuolization of the gastrodermal cells without zooxanthellae (Fig. 5.2 c-d). This disintegrated the gastrodermis, and thus the cells containing zooxanthellae detached from the gastrodermis and were released into gastrovascular cavities (Fig. 5.2e), followed by the dispersal of their various cellular contents (Fig. 5.2f). Under the dissecting microscope, particles of zooxanthellae *in situ* began to wiggle within the coral tissues and were suspended in gastrovascular cavities 90 min and 2 h after the initial HT exposure, respectively (data not shown).

5.3.3. Effect of ultraviolet-A radiation

After corals were exposed to UV for 6 h to 48 h, autophagosomes increased in size and number and contained distinct inclusions (Fig. 5.3a and f). The average number of autophagosomes per sectioned gastrodermal cells was 1.6 and 2.7 after exposure to UV for 12 h and 48 h, respectively (Fig. 5.6). The cells without zooxanthellae became vacuolated and disconnected from the adjacent cells 12 h after the initial UV exposure (Fig. 5.3b). Such disorganization of the gastrodermis (Fig. 5.3c) led the cells containing zooxanthellae to detach from the gastrodermis after exposure to UV for 30 h and 48 h. Under *in situ* observation by light microscopy, zooxanthella particles wiggled in the coral coenenchyme and floated in gastrovascular cavities 12 h and 30 h after the initial UV exposure, respectively (Fig. 5.5). Disruptions of chloroplasts (Fig. 5.3e) and degradation of intracellular components occurred not only in the gastrodermis, but also in symbiotic zooxanthellae (Fig. 5.3c). The cytoplasm of zooxanthellae was vacuolated in places, and the chloroplasts were often destroyed completely, leaving dark amorphous materials. Numerous electron dense granules appeared in host cytoplasm along with widespread vacuolization (Fig. 5.3f)

5.3.4. Effect of far-red radiation

When corals were exposed to FR for 6 h, a number of small lysosomes appeared in the cytoplasm of the gastrodermal cells (Fig. 5.4a). The average number of autophagosomes per sectioned gastrodermal cell became 1.5 and 2.6 after exposure to FR for 12 h and 48 h, respectively (Fig. 5.6). The cells without zooxanthellae became amorphous and began to degenerate, after 12 h exposure (Fig. 5.4b). Such degenerated cells increased in number concomitantly with an increase in the duration of FR exposure. Exposure to FR for 12 h induced

the wiggling movement of zooxanthella particles *in situ* (data not shown). When zooxanthella particles *in situ* were expelled from the coenenchyme and floated in gastrovascular cavities 30 and 48 h after the initial FR exposure, we observed via electron microscopy that the cells containing zooxanthellae were detached from the gastrodermis (Fig. 5.4e-f). Exposure to FR also affected the structure of symbiotic zooxanthellae, including the disruption of the zooxanthella cytoplasmic contents and thylakoid membranes of the chloroplasts (Fig. 5.4d).

5.4. Discussion

Through the use of transmission electron microscopy, this study has demonstrated that in the coral *P. damicornis*, exposure to HT, UV, and FR destroys the organization of the coral gastrodermis. Early ultrastructural changes (refer to Table 5.1 for the summary) were noticed in the gastrodermal cells without symbiotic zooxanthellae after the initial exposure to these stressors. The number of lysosomes and autophagosomes increased in the cells. Longer exposure to the stressors led to the vacuolation and fragmentation of the cytoplasm, resulting in the breakdown of the cells. Patterns of cellular degradation occurring in the gastrodermal cells when the coral was exposed to these stressors may be characterized as necrosis. Dunn *et al.* (2000, 2002) have reported that autolytic digestion of cytoplasm in coral host cells occurs through necrosis under stressful conditions. In addition to necrosis, it has been reported that autophagic activities degrade coral host cells in response to stress conditions (Hanes & Kempf 2013). The degradation of host cells is triggered by the appearance of autophagosomes or autophagolysosomes (Hanes & Kempf 2013), where cytoplasmic components are engulfed and digested by autophagosomes (Shibutani & Yoshimori 2014). These reports suggest that the vacuolation and fragmentation of the gastrodermal cells of *P. damicornis* under stress conditions

are due to necrosis and autophagy. This study showed that in even control conditions without any stresses, gastrodermal cells had autophagosomes in *P. damicornis*. In this case, autophagosomes seemed to selectively degrade cytoplasmic components as normal metabolism in the cells. Once the coral was placed in stress conditions, autophagosomes in the gastrodermis increased and digested the cells they were present in. These facts suggest that in *P. damicornis,* stress factors such as HT, UV, and FR might induce autophagosomes to lose the specificity where targets to degrade are selective.

In this study, prolonged exposure to HT, UV, and FR caused the cells containing zooxanthellae to be discharged from the gastrodermis. In the control treatment, adjacent cells in the gastrodermis were tightly connected with each other. Since the gastrodermal cells without zooxanthellae collapsed under stress conditions, it is plausible that cell-to-cell adhesion in the gastrodermis weakened, the gastrodermis became disorganized, and eventually the cells containing zooxanthellae were expelled from the gastrodermis.

Symbiotic zooxanthellae are known to generate reactive oxygen species (ROS) under UV radiation and high temperature conditions (Lesser *et al.* 1990; Lesser, 1997; Nii & Muscatine 1997). ROS oxidize membrane lipids and make proteins and nucleic acids to denature and damage thylakoids in chloroplasts (Smith *et al.* 2005). However, Downs *et al.* (2009, 2013) have reported that oxidative stress substances may be produced mainly by coral host cells. In this study, chloroplasts and other cellular components in some zooxanthellae were disrupted by exposure to UV and FR. In this study, chloroplasts and other cellular components in some zooxanthellae were disrupted by exposure to UV and FR. Symbiotic zooxanthellae in *P. damicornis* might be susceptible to UV and FR more than HT. However, even short exposure to HT damaged the coral gastrodermis more severely than in UV or FR. Three different stressors,

HT, UV, and FR, brought about the degradation of gastrodermal cells without zooxanthellae, so that the gastrodermal cells containing zooxanthellae were released from the gastrodermis. This suggests that the coral may receive distinct stress factors, but has a common mechanism by which the gastrodermal cells without zooxanthellae are selectively digested.

Summary of Ultrastructural Changes	Control	High temperature	Ultraviolet-A radiation	Far-red radiation
Occurrences of electron dense granules and autophagosomes	—	○	○	○
Increase in density of autophagosomes	—	○	○	○
Vacuolization and fragmentation of coral cells containing no symbiotic zooxanthellae	—	○	○	○
Release of coral cells containing zooxanthellae and other cellular entities	—	○	○	○
Degeneration of chloroplast and other cytoplasmic components of zooxanthellae	—	○	○	○

Note: (—) denotes no occurrence and (○) with occurrence.

Table 5.1. Summary of the changes in the ultrastructure of coral host and symbiotic zooxanthellae cells in control and stressed conditions. Marks with (—) denotes no occurrence while (O) with occurrence.

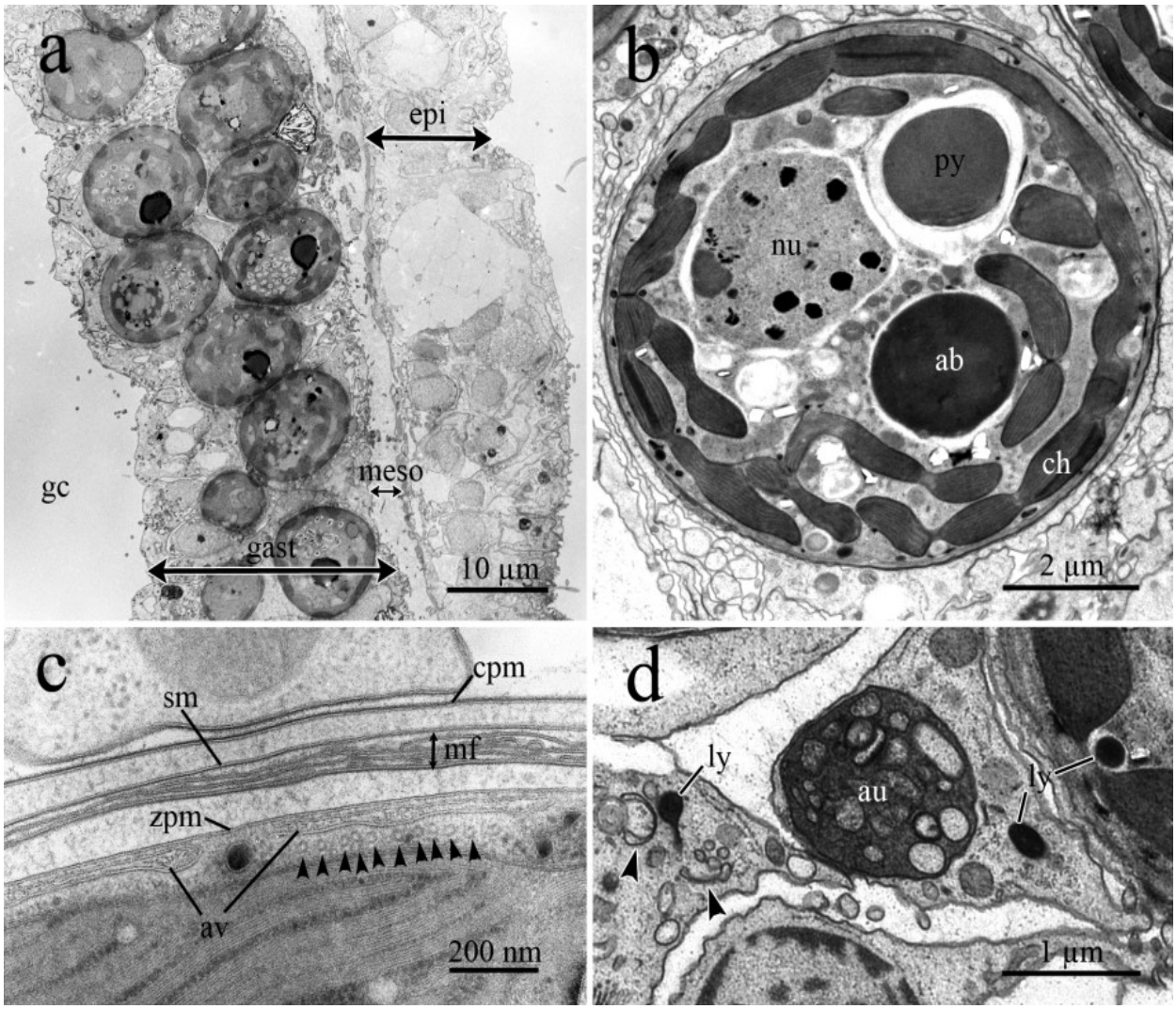

Fig. 5.1. Electron micrographs of coral in control condition. Samples were made through chemical fixation (a) and freeze-substitution (b-d) (**a)** Longitudinal section of a tentacle showing intact two layers of epidermis (epi) and gastrodermis (gast) interconnected by mesoglea (meso); gastrovascular cavity (gc) (**b**) Symbiotic zooxanthella occupying the large space of a gastrodermal host cell. The intracellular components were intact such as nucleus (nu), pyrenoid (py), accumulation bodies (ab), and peripheral chloroplasts (ch). (**c**) Cortical structure of symbiotic zooxanthella were also intact consisting of amphiesmal vesicle (av), zooxanthellae plasma membrane (zpm), , membranous fragments (mf), symbiosome membrane (sm), and coral plasma membrane (cpm),. (**d)** Autophagosome (au) and lysosomes (ly) found in gastrodermis. Arrows showing cup-shaped double membrane sacs.

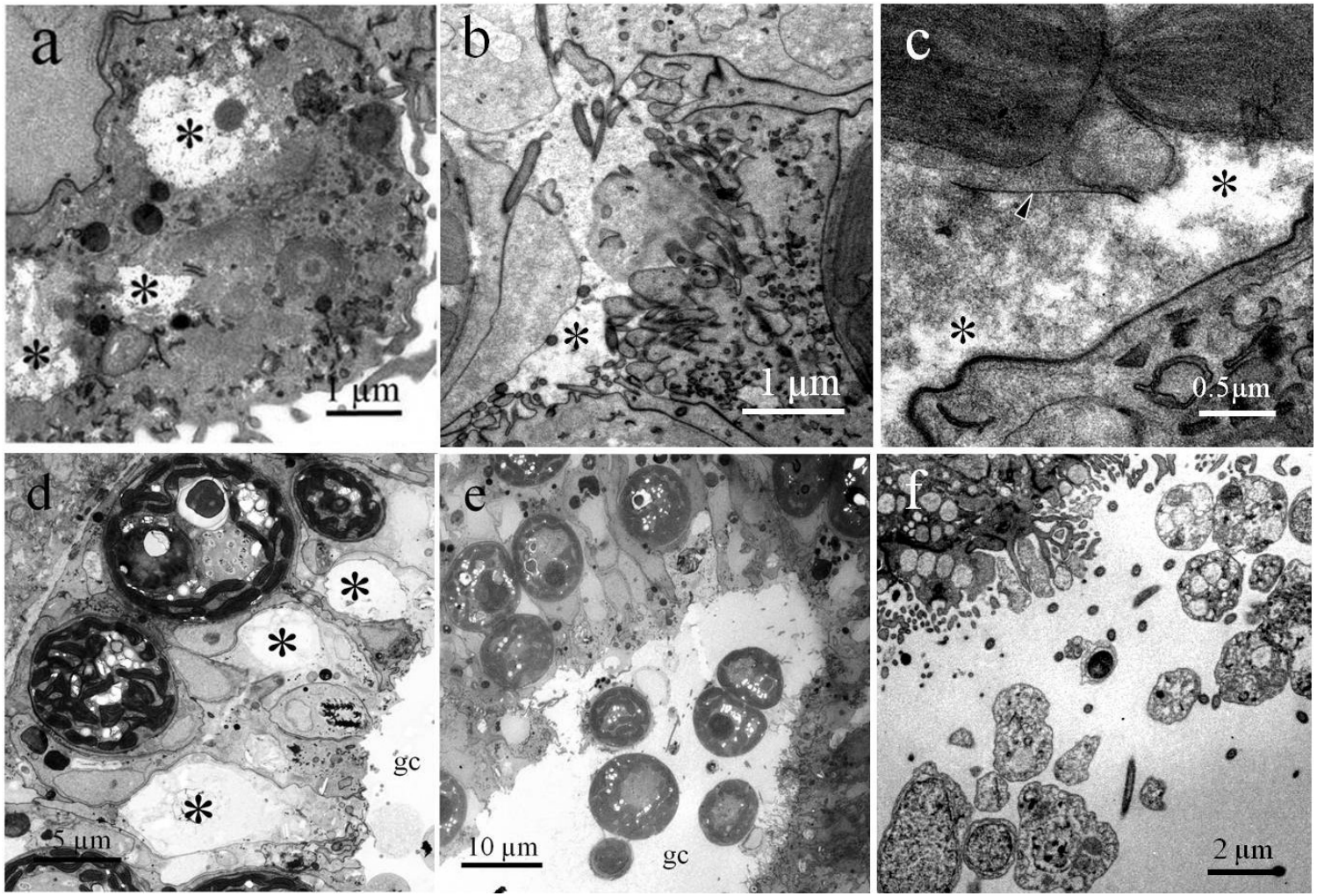

Fig. 5.2. Electron micrographs of coral exposed to high temperature. (**a-b**) Electron dense granules and vacuolated areas (*asterisks*) in gastrodermal cells without zooxanthellae for 15 to 30 min. (**c**) Remaining structure of the disrupted cortical membrane of zooxanthellae due to vacuolizations (*asterisks*) after exposure to heat for 1 h. (**d**) Cells without zooxanthellae that were vacuolated and almost lost their cytoplasm (*asterisks*) after 1 h, (gc) refers to gastrovascular cavity. (**e-f**) Gastrodermal cells with zooxanthellae and other entities released from gastrodermis to gastrovascular cavity (gc) after 1-2 h thermal stress.

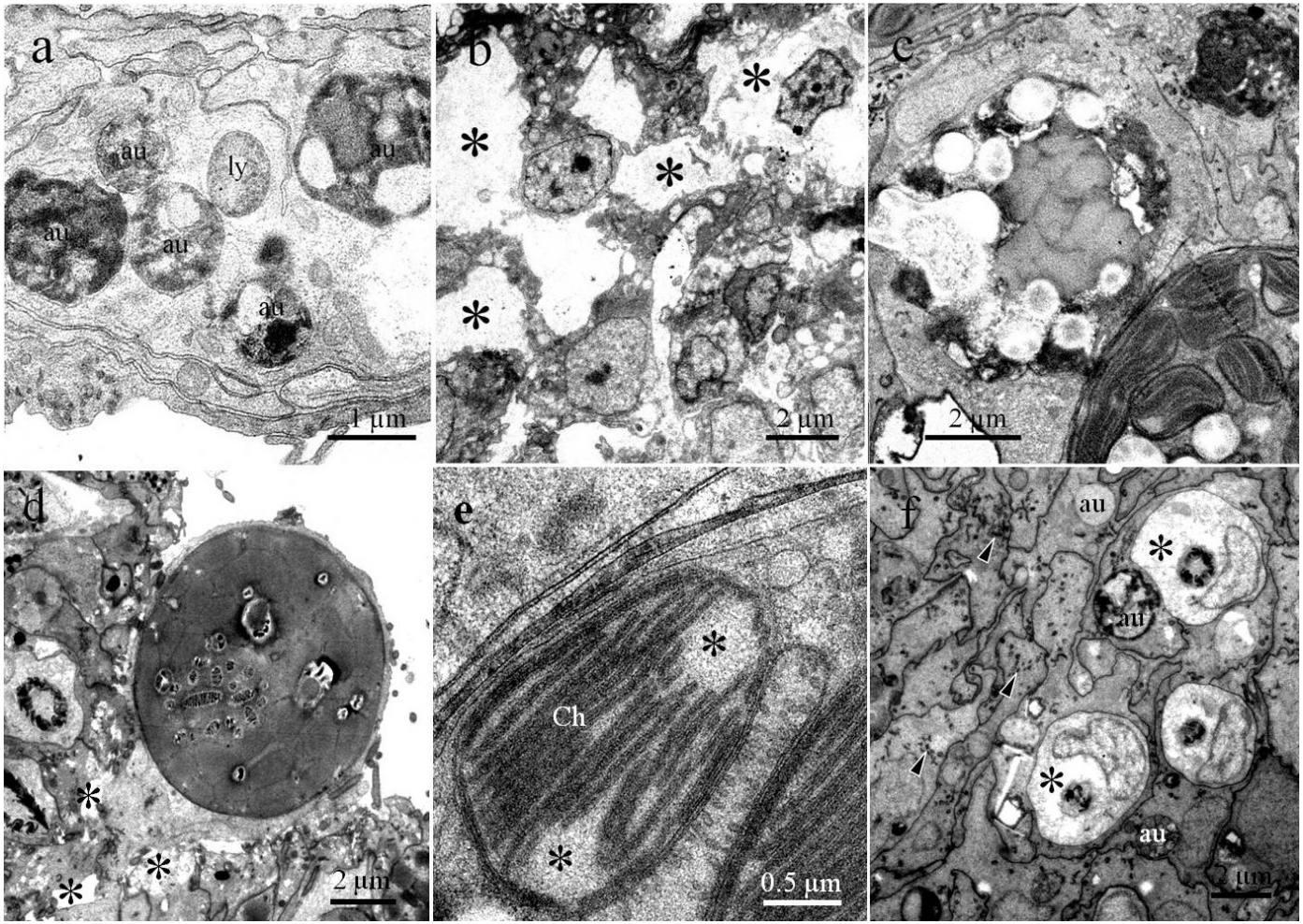

Fig. 5.3. Electron micrographs of coral exposed to ultraviolet-A radiation. (**a**) Several autophagosomes (au) and lysosomes (ly) in a gastrodermal cell without zooxanthellae appeared after 6h. (**b**) Degenerating cells without zooxanthellae showing the occurrence of vacuolated areas (*asterisks*) after 12 h. (**c**) A zooxanthella whose cytoplasmic components were destroyed after exposure to UV for 12 h. (**d**) A host cell containing zooxanthella about to detach due to surrounding vacuolation that occurred after 30 h. (**e**) Disruptions (asterisks) in the lamellae of the zooxanthella chloroplast (ch) after 30 h exposure. (**f**) Widespread appearances of autophagosomes (au), electron-dense granules (*arrowheads*) and vacuolization (*asterisks*) in the gastroderm after 48 h exposure.

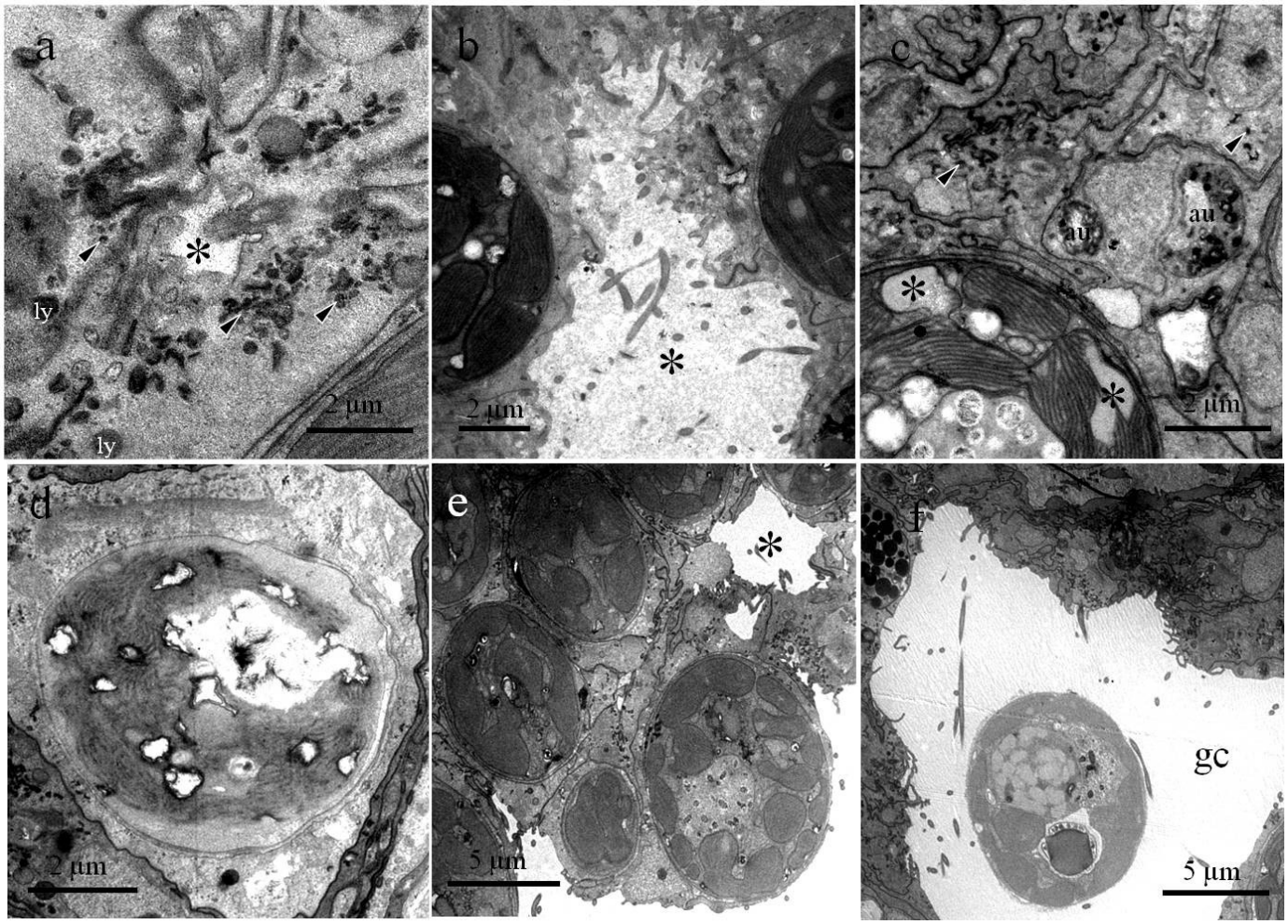

Fig. 5.4. Electron micrographs of coral exposed to far-red radiation. (**a**) Several lysosomes (ly) and electron dense granules (*arrowheads*) surrounding the vacuolated host cell (*asterisks*) without zooxanthellae after exposure for 6 h. (**b**) Vacuolization (asterisks) became evident in host cells between zooxanthellae after 12 h. (**c**) Widespread occurrence of autophagosomes (au), granules (arrowheads) adjacent to zooxanthellae with disrupted chloroplast (asterisks) after 12 h exposure. (**d**) Degenerated zooxanthella after 30 h. (**e**) Coral cells containing zooxanthellae just before detaching from gastrodermis due to vacuolization (*asterisks*) after 30 h. (**f**). Expelled zooxanthellae into gastric cavity (gc) after 48 h.

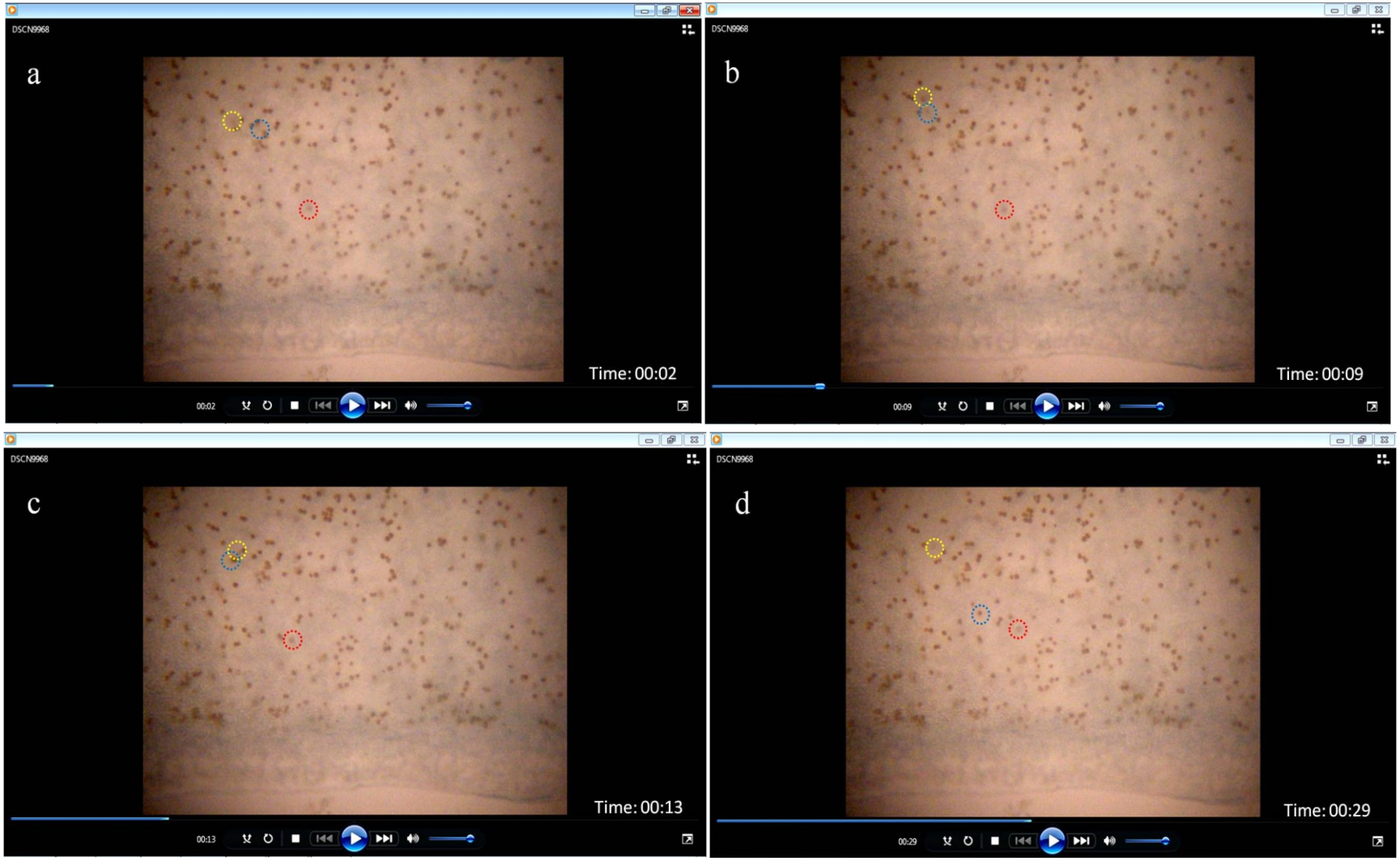

Fig. 5.5. Movement of the *in situ* zooxanthellae cells after exposure to stressors. (A-D) Printed images of the actual time lapse video of the zooxanthella particles that wiggle and float in the coral coenenchyme 30 h after the initial exposure to UV-A. Note the movements of three cells marked with red, blue and yellow circles at elapsed times 2 sec (a), 9 sec (b), 13 sec (c), and 29 sec (d).

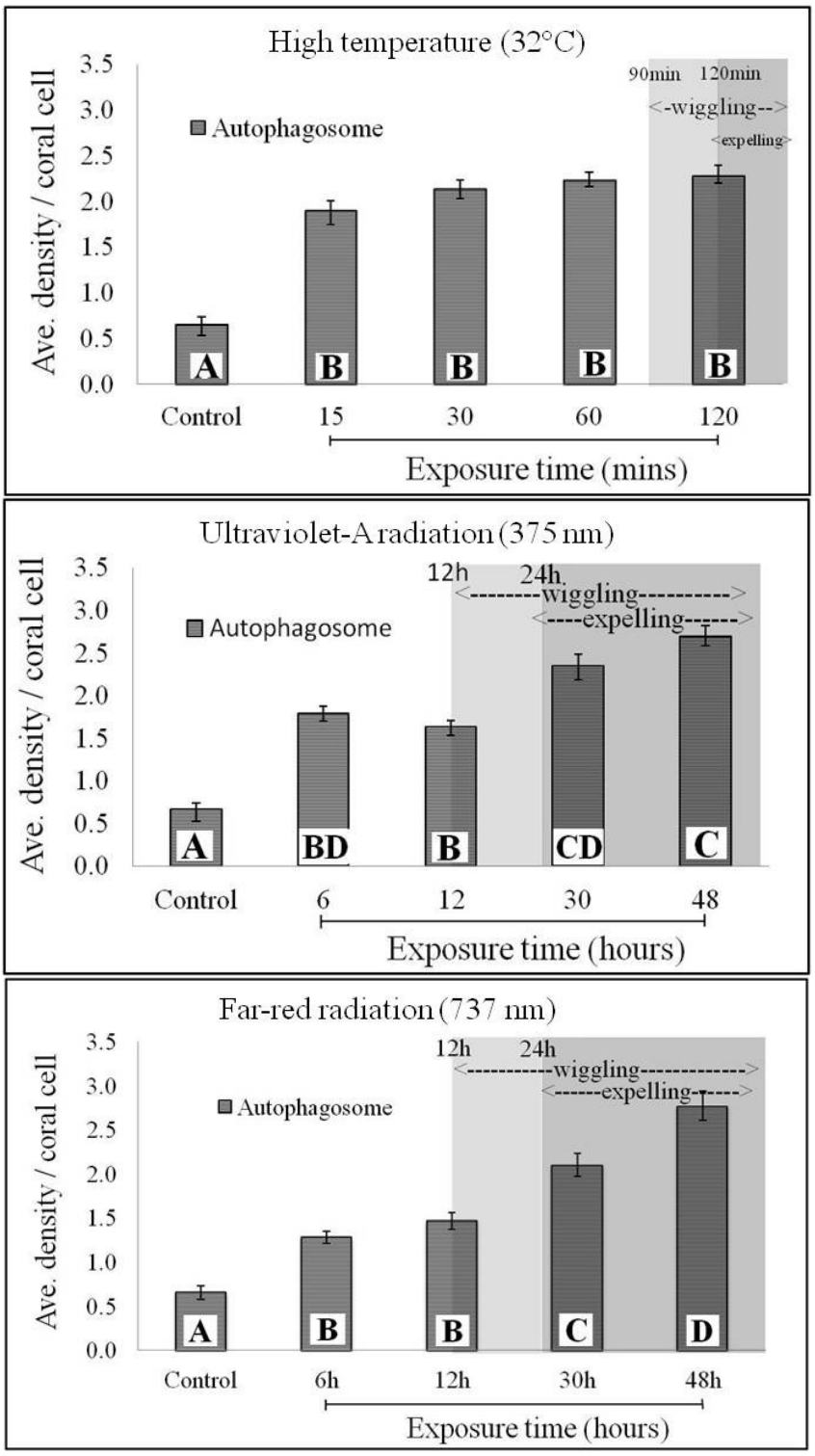

Fig. 5.6. Density of autophagosomes in the host gastrodermal cells from control and stressed conditions. Background indicates timing of zooxanthellae *in situ* movement responses observed under light microscope. Bars with same letter and numbers are not significantly different per Tukey's HSD Test ($P < 0.05$), Values are mean, ± s.e. n=100.

6. General Discussion and Conclusion

6.1. Scleractinian corals and the search of model systems for understanding cnidarian symbioses

Reef-building corals are in fact generally more prominent among cnidarian species with high ecological relevance (Birkeland 1996; Dubinsky & Stambler 2011). However various aspects relating the disadvantages of using these organisms in understanding cnidarians symbioses with symbiotic zooxanthellae have been evaluated (Weis *et al*. 2008). Hence shifting to model species like the sea anemone *Aiptasia* was emphasized, stating its favorable edge against the use of hermatypic corals in cell biological studies with particular notes such as its ability to be voluntarily re-infected with symbiotic algal strains, living temporarily at symbiont-free state and calcification (Weis *et al*. 2008). This was further supported with relevant studies at the molecular level (Baumgarten *et al*. 2015; Bucher *et al*. 2015), revealing the accessibility of the latter species for genomic rearrangement in taxonomy, host dependence on alga-derived nutrients and lineage-specific gene transfer (Baumgarten *et al*. 2015).

However in the broader concept, studies with coral-algal association remain ultimately investigated ever since (Birkeland 1996; Lesser 2004; Dubinsky & Stambler 2011). One proof of prime interest of scientists to corals is the establishments of various coral research institutes and marine laboratories in the different parts of the world undertaking various disciplines of symbioses studies (Richmond & Wolanski, 2011). To further understand cnidarian symbioses, the concept dealing with CZAR (contribution of zooxanthellae to animal respiration) was further established (Richmond & Wolanski, 2011), where scleractinian corals as model species were highly utilized. The general concept is to investigate coral host-algal symbiotic relationship via

metabolic interaction with frontiers aimed to understand biochemical processes behind coral growth and dysfunction (bleaching).

In this research, the ubiquitous scleractinian coral *P. damicornis* had been used as a model species to examine the growth, cell division and dysfunction as major aspects involved in investigating cnidarians symbioses. The use of raw seawater medium and extremely minimal maintenance procedure in the *in vitro* fragment cultureindicates that corals have the significant attributions to simple set-up of *in vitro* 'host cell culture'. This attributes remains complicated among anemones (Weis *et al*. 2008). Thus addressing the vigorous utilization of 'host anemone-model system' approach proposed by Weis *et al*. (2008), may only be optional as the use of sample may be highly dependent on the nature of investigation and the ability of cnidarian species to stimulate response required for the type of research conducted.

6.1.1. Cell division and the role of light in the host-symbiont proliferation

In the earlier attempt to compare cnidarian and symbiotic cell division *in hospite*, host mitotic pattern appeared to peak at night prior to zooxanthellae division with peak rate at dawn (approx. 12 hours apart), following the host animal feeding (Fitt 2000). This indicated that periodicity in host cell division against its symbiont species vary significantly mainly under certain circumstances. In the present study (Chapter 2), the timing of division of the *in situ* symbiotic zooxanthellae was investigated and found that doublet cells (cytokinetic configuration) appeared at all time by 24h daily cycle, thus exhibit no diel pattern. Along with this, extensive coral tissue regenerations were induced, suggesting that both host and zooxanthellae cells reproduce evenly but the phasing pattern was unknown. In Chapter 4, this was somewhat justified as ultrastructural examination had revealed that coral cell division subsequently occurred after symbiont division.

If this is the case, this further supports the assumption of Davy *et al*. (2012) that the reproduction of coral and symbiont cells were kept in pace with each other where proliferation must persisted in a steady state. Further when exposed under varying light spectra and intensities as discussed in Chapter 3 of this study, the growth pattern varied essentially between host tissue and zooxanthellae density. Reproductive-stimulating effect was highly observed from blue light radiation in contrast to the degenerating effect exhibited by UV-A and far red radiation.

The result in Chapter 3 showed that cell divisions in both cells are highly regulated by light. As reviewed by Davy *et al*. (2012), light influences cell division of the symbiotic algae presumably because of photosynthetically-fixed carbon needed for the assimilation of dissolved inorganic nutrients and supports the metabolic demands of cell cycle between host and its symbionts. Radiotracer experiments with ^{14}C-bicarbonate demonstrated that the algae release up to underestimate of 50% of photosynthetically-fixed carbon to the animal host (Venn *et al*. 2008). On the other hand, continuous darkness significantly repressed the symbiont cell division (Fitt 2000), thus the susceptibility of host division and growth is much inclined with the photosynthetic capacity of the zooxanthellae to maintain the symbioses with host cnidarian.

6.1.2. Host-symbiont cell degradation and death

The dysfunction of the symbiotic relationship between coral and zooxanthellae is widely assessed from ecological (Glynn 1993; Hoegh-Guldberg 1999), physiological (Fitt *et al*. 2001; Douglas 2003; Smith *et al*. 2005), molecular and cellular aspects (Brown 1997; Weis 2008; Weis *et al*. 2008; Davy *et al*. 2012). With the extensive examinations done stressors act one thing in common in corals – which is to promote deleterious response (Douglas 2003) or death of corals. In this research, the early stage of coral bleaching observed was attributed to the collapse of the

host cell-to-cell adhesion due to necrosis (Camaya *et al*. 2016). The autolytic digestion of the cytoplasm was associated with the occurrences of multiple electron-dense granules and autophagosomes. Symbiotic zooxanthellae *in hospite* were likewise affected where thylakoid membranes and other cytoplasmic inclusions deteriorates.

6.1.2.1. Symbiont 'chloroplast': the etiology of oxidative damage

One of the leading causes of coral bleaching is oxidative stress (Lesser 2006; Weis 2008). Thermal and high irradiation stresses or their synergistic action could reduce the threshold light intensity of the symbiotic zooxanthellae and trigger the process of photoinhibition, (Weis 2008). During stresses, the etiology of damage comes from the chloroplasts potentially occurring at the three sites: D1 protein in PSII, Calvin cycle and the thylakoid membranes (Lesser 2006; Venn *et al*. 2008; Weis 2008). Photoinhibition due to damaged PSII suppressed the chloroplasts' repair machinery that affects the photosynthetic efficiency (Takahashi *et al*. 2004). This promotes high photosynthetic rates absorptions that produce reactive oxygen species ROS in the form of singlet oxygen ($^1O_2$) and superoxide (O_2^-) then converting into reactive hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2) (Lesser 1997, 2006; Franklin *et al*. 2004; Venn *et al*. 2008; Weis 2008). The increase in ROS production makes the symbiont antioxidant enzymes SOD and ascorbate peroxidase decrease and inhibit its detoxifying function (Lesser *et al*. 1990; Downs *et al*. 2002; Franklin *et al*. 2004). Dealing on its effect to host tissue, controversies arise on how ROS trigger host cell to bleach (Weis 2008). ROS may have leaked into host cell via diffusion (Venn *et al*. 2008), or host cells directly produced them via impairment of mitochondria (Nii & Muscatine 1997) while other proposed the production evolved from both cells (Lesser & Farell 2004), that plays a critical role in the bleaching cascade of corals. This highly implied that coral

bleaching is a host innate immune response to a compromised symbiont where ROS played a central role in both injuries to the partners and to the inter-partner communication of a stress response (Weis 2008).

In the present research, the finding on the early stage of bleaching in *P. damicornis* due to oxidative stress was not confirmed. However as the result on the distinctive damaged observed from the symbiont' thylakoid membranes by high temperature and from UV-A and far red radiation, it is likely that ROS may potentially developed. Further, the dissolution of the zooxanthellae cortical structures may suggest an implication to ROS leakage into the adjacent host gastrodermis leading. These are highly speculative at present, in which cytochemical analysis is required to confirm such conclusion.

6.1.2.2. Cell degradation *via* autophagy and autolysis

Bleaching in corals due to hyperthermic and light irradiance stressors weres highly associated with host and symbiont cell degradation (Gates *et al*. 1992; Brown *et al*. 1995; Dunn *et al*. 2002; Franklin *et al*. 2004; Hanes and Kempf 2013). At these occurrences, cell necrosis showed cytoplasmic vacuolization or dilation, cellular structure disruption and condensation, prominent lysosomes and electron-dense bodies and detachment of various entities. Some of the probable causes are derived from autophagy and autolytic digestion (Müller *et al*. 1984; Glynn *et al*. 1985; Dunn *et al*. 2007; Weis 2008; Hanes & Kempf 2013; Franklin *et al*. 2004). Autophagy is known to physiologically develop by engulfing any cytoplasmic constituents, long-lived proteins and intracellular organelles. The crescent-shaped phagophore closes to form double-membrane autophagosome (Samara *et al*. 2008; Hanes and Kempf, 2013). As they uphold, cytoplasmic digestion occurs after they fuse with lysosomes. Similarly, autolysis mainly involves lysosomes

as ‘self-digesting machinery’, via its enzyme carbonic anhydrase although it degenerating effect in scleractinia used to be evident from non-living calcareous matrix (Müller *et al*. 1984).

As presented in Chapter 5, autophagic structures appeared even in intact coral gastrodermis from the control specimen. However under stress conditions, the multiplication of the autophagosomes and the diminutive lysosomes occurred along with the time exposures. Their association with the autolytic degradation of host and zooxanthellae cytoplasm suggested that the stimulation of their density and degenerating effect are triggered by stress factors, profoundly associated with the increasing intensities.

6.2. Future perspective of studies

6.2.1. Mitotic index and *P-I* curve for growth analysis

With the treatment of light spectra, the further effects on growth of host tissue and symbiont cell number were examined in Chapter 3. However there are some aspects of studies that are important to be considered in the future, *i.e.* analysis of mitotic index for corals exposed under various monochromatic light radiations. . The condition of coral tissue regeneration and the density of symbiont had deduced three distinct growth patterns, which deserve this form of analysis. For instance, by applying fluorescent nuclear stain 4′,6′-diamidino-2-phenylindole (DAPI), the mitotic figures of host digestive cells could be elucidated through epifluorescent microscope while symbiont densities can be analyzed by maceration of coral tissue, as previously demonstrated by Gladfelter (1983) and Fitt (2000). On the other hand, considering the measurement of photosynthesis (photosynthetic-irradiance light saturation, *P-I* curve) is another option. This method was proven sound for massive and branching types of corals as performed with basic spectral regimes (Kinzie & Hunter 1987; Wijgerde *et al*. 2014). By conducting such

investigation, this could determine the photosynthetic efficiency of the corals when experimentally exposed to different forms of monochromatic lights. In this perspective, it is expected that the acclimation intensity and the color of light treatment will have pronounced effect on the photosynthetic response, as observed in the green, red and blue light spectra (Kinzie & Hunter 1987; Wijgerde *et al*. 2014). Comparative photosynthetic responses from violet, yellow and various wavelengths of red (R62, R68 and far red) light treatments used in the present study (Camaya *et al*. 2016), further deserves to be examined.

6.2.2. Cytochemical analysis for coral bleaching pathomorphologies

In Chapter 5, the changes in the ultrastructure of the host gastrodermis and the *in situ* symbiotic zooxanthellae upon exposure to thermal and UV-A and FR spectral stressors were described using an electron microscopy. Some of the vital procedures to test the presence of pathomorphologies in host gastroderm such as autophagy and electron-dense granules is through cytochemical analysis (Dunn *et al*. 2007; Hanes & Kempf 2013). In their studies, to induce autophagy in a cnidarian specimen, they used rapamycin in anemone *Aiptasia* spp. Among hard corals, the detection of autophagic activities utilizing such chemical remains poorly done so far from the available literatures. On the other hand, photo-oxidative stress test is another potential analysis by subjecting host tissue to protein carbonyl as biomarker of protein oxidation (Downs *et al*. 2013) and algal cells to antioxidant levels for enzymes SOD and ascorbate peroxidase (Lesser *et al*. 1990; Downs *et al*. 2002, 2013; Franklin *et al*. 2004). In the proposed oxidative theory of coral bleaching by Downs *et al*. (2002), it was indicated that coral bleaching was tightly coupled to the antioxidant and cellular stress capacity of the symbiotic corals. Considering the ultrastructural changes in *P. damicornis* observed due to the effect of distinct

stressors (Camaya *et al*. 2016), such condition could be subjected for these form of tests to further verify the underlying pathways of the cellular degradation during early stage of coral bleaching.

6.3. General conclusions and implications of studies

This dissertation has demonstrated the regenerative growth, and the process of cellular division and dysfunction of host-algal symbioses in the scleractinian coral *Pocillopora damicornis* revealed through light and electron microscopy. There are four major conclusions that were drawn from each study chapter: (1) tissues fragments of coral had the physiological ability to grow in the glass dishes where imperforate tissue regenerate by diverse modes depending on the position of the lesion – an observation first reported from *in vitro* settings; (2) the growth of coral regenerated tissues and the density of the *in situ* symbiotic zooxanthellae changed dramatically when exposed from the radiation of different monochromatic lights where growth were either promoted under blue rays or inhibited under UV-A and far red rays; (3) the ultrastructure of cell division of symbiotic zooxanthellae *in hospite* had revealed the dynamic behavior of cortical fine structures in maintaining its coccoid cell shape, while coral nuclei performed a novel role in the distribution of daughter symbionts in each dividing host cells; (4) changes in the ultrastructure of coral and zooxanthellae cells occurred when stressed to high temperature, and ultraviolet-A and far red radiations, where pathomorphologies such as autophagy and electron-dense lysosomes were pandemic that induced cell necrosis and autolytic digestion of gastrodermal cells leading to the release of symbionts and other intracellular entities.

By analyzing the key linkages of these findings, the vital associations between studies are indicated considering aspects involving (a) the dynamics of photomorphogenesis of coral species

in which the induced tissue regeneration from ambient white light irradiance (Chapter 2) used to exhibit diverse growth responses upon receiving various monochromatic light spectra (Chapter 3); (b) the doublet cell formation of the *in situ* zooxanthellae along with evident growth of coral tissue by regeneration discussed in Chapter 2 was conclusively revealed at ultrastructural level in Chapter 4 that symbiont cell division was followed by equivalent division of host cell; (c) the degenerating effect of UV-A and far-red rays leading to indiscriminate retraction of coral tissues and expulsion of zooxanthellae (Chapter 3) was in fact attributed to necrosis and autolysis of host gastroderm and its zooxanthellae causing the loss of cell-to-cell adhesion that subsequent led to release of the algal symbionts (Chapter 5).

Further these studies have significant implications in the continuing effort to understand coral-zooxanthellae relationship dealing the two major aspects of cnidarian symbioses such as the reproduction and dysfunction. Indeed, the persistence of this unique relationship poses profound importance to the success of the corals either to thrive in a natural reef ecosystem or in a controlled environment. To address the alarming issue on the unprecedented coral bleaching brought about by increasing intense of natural calamities, sea surface warming, acidifications due to changing climate and human perturbations, this research may contribute an input for the restoration of the natural population of reef-building corals. With the success of *in vitro* fragmentation system and understanding of the effects of spectral radiation as well as stress in coral, this may provide inputs for considering future studies on establishing mass production of resilient clones to implement coral transplantation project in the damaged reef along disaster-prone area *i.e.* Kuroshio region. Cellular biology of coral-algae symbiosis is essential if we are to fully understand the underlying mechanisms of its growth and dysfunction and to overview the current perspectives on how coral could survive climate change in the coming decades.

Acknowledgements

I am deeply indebted to several persons and institutions that made my life as graduate student in Kochi University worth a meaningful success, whom I named some as recognition.

To **Dr. Kazuo Okuda**, who foremost introduced me to the world of microscopy since my first advent to this field in 2007 and greatly improved my perceptions in cell structure and function. As my profound mentor, he unselfishly shared his supervision, knowledge and techniques in making this dissertation a realization.

To **Dr. Satoko Sekida**, who is all-time open for guidance and support to this research. Her willingness to share her expertise had much improved my skill in microtechnique.

To **Dr. Takahiro Taguchi**, as my second supervisor who had shared his knowledge to further improved this dissertation.

To **Dr. Yoshinori Morooka**, as dedicated external examiner and for all sorts of 'fatherly' advices and support, ever since the KU-BU collaboration was established.

To **Dr. Yohei Nakamura**, for his helpful advices and unwavering assistance to provide my coral samples until the very last time I needed it most.

To **Dr. Ichiro Mine**, for his technical assistance which aided much in performing my lab works.

To **Dr. Akira Tominaga**, **Dr. Teruyuki Shinbo**, **Dr. Yoshiaki Iiguni, Dr. Satoshi Kubota, Dr. Mina Hori**, **Dr. Sota Tanaka** and the entire **Graduate School of Kuroshio Science** and **International Student Center** for their warm assistance.

To the **Japanese Government - Monbukagakusho** (**MEXT**) for my scholarship grant.

To **Dr. Fay Lea Patria M. Lauraya**, **Dr. Plutomeo M. Nieves**, **Dr. Victor S. Soliman**, colleagues and staff of **Bicol University** for the kind support to make it possible to pursue my PhD study. Especially mentioned, **Dr. Soliman** served as my former mentor who opened my mind in the field of marine research.

To my fellow graduate students in GSKS especially **Jayvee**, **Emma**, **Renan**, **Jonel**, **Allyn**, **Alvin**, **Janice**, **Yumi**, Japanese and Filipino friends and many others for the momentous time shared in Kochi for more than three years.

To my wife **Naty** and children **Al John** and **Alexie Carl** as my 'inspirations to this goal', dearest mother **Nellia**, brothers, sister and our entire family for the love and support.

To my late father ***Tony*** and brother ***Al***, whom I dedicated my success for PhD.

'To GOD be the Glory'

Literatures Cited

Anthony, K.R.N., Kline, D.I., Diaz-Pulido, G., Dove, S., Hoegh-Guldberg, O. 2008. Ocean acidification causes bleaching and productivity loss in coral reef builders. Proceeding of the National Academy of Science 105(45): 17442–17446. doi:10.1073/pnas.0804478105.

Babcock R.C., Baird, A.H., Piromvaragorn, S., Thomson, D.P., Willis, B.T. 2003. Identification of scleractinian coral recruits from Indo-Pacific Reefs. Zoological Studies 42(1): 211-226.

Bak, R.P.M. 1983. Neoplasia, regeneration and growth in the reef-building coral *Acropora palmata*. Marine Biology 77: 221-227.

Bak, R.P.M., Steward-Van Es, Y. 1980. Regeneration of superficial damage in the Scleractinian corals *Agaricia agaricites f. purpurea* and *Porites astreoides*. Bulletin of Marine Science 30(4): 883-887.

Baker, A.C., Glynn, P.W., Riegl, B. 2008. Climate change and coral reef bleaching: An ecological assessment of long-term impacts, recovery trends and future outlook. Estuarine, Coastal and Shelf Science 80: 435-471. doi: 10.1016/j.ecss.2008.09.003.

Banaszack, A.T., Iglesias-Prieto, R., Trench, R.K. 1993. *Scrippsiella velellae* sp. nov. (Peridiniales) and *Gloeodinium viscum* sp. nov. (Phytodiniales), dinoflagellate symbionts of two hydrozoans (Cnidaria). Journal of Phycology 29: 517-528.

Baumgarten, S., Simakov, O., Esherick L.Y., Liew, Y.J., Lehnert, E.M., Michell, C.T., Li, Y., Hambleton, E.A., Guse, A., Oates, M.E., Gough, J., Weis, V.M., Aranda, M., Pringle, J.R., Voolstra, C.R. 2015. The genome of *Aiptasia*, a sea anemone model for coral-symbiosis. Proceedings of the National Academy of Sciences 112(38): 11893-11898. doi/10.1073/pnas.1513318112.

Ben-Haim, Y., Zicherman-Keren, M., Rosenberg, E. 2003. Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. Applied Environmental Microbiology 69(7): 4236–4242. doi: 10.1128/AEM.69.7.4236-4242.2003.

Beer, S., Björk, M., Beardall, J. 2014. Photosynthesis in the marine environment. USA: John Wiley & Sons and Blackwell Publishing, pp. 208.

Birkeland, C. 1997. Life and death of coral reefs. New York: USA. Chapman and Hall, 536p.

Borneman, E. 2008. Introduction to the husbandry of corals in aquariums: a review. *In* Advances in Coral Husbandry in Public Aquariums. Public Aquarium Husbandry Series, Vol. 2. (Ed: Leewis, R.L., Janse, M.). Arnhem, The Netherlands: Burgers' Zoo, p. 3.

Bowen, J., Bowen, M. 2003. The Great Barrier Reef: History, Science, Heritage. London, UK: Cambridge University Press, pp. 211.

Bricheux, G., Mahoney, D.G., Gibbs, S.P. 1992. Development of the pellicle and thecal plates following ecdysis in the dinoflagellate *Glenodinium foliaceum*. Protoplasma 168: 159-171.

Brown, B.E. 1996. Disturbances to reefs in recent times. *In* Life and Deaths of Coral Reefs (Ed. Birkeland, C.). New York, USA: Chapman and Hall, pp. 354.

Brown, B.E. 1997. Coral bleaching: causes and consequences. Coral Reefs 16: 129-138.

Brown, B.E., Le Tissier, M.D.A., Bythell, J.C. 1995. Mechanisms of bleaching deduced from histological studies of reef corals sampled during a natural bleaching event. Marine Biology 122: 655-663.

Brown, B.E., Le Tissier, M.D.A., Dunne, R.P. 1994. Tissue retraction in the scleratinian coral *Coeloseris mayeri*, its effect upon coral pigmentation, and preliminary implication for heat balance. Marine Ecology Progress Series 105: 209-218.

Brunell, S.A., Hazard, E.S., Sotka, E.E., Van Dolah, F.M. 2007. Characterization of a dinoflagellate cryptochrome blue light receptor with a possible role in circadian control of the cell cycle. Journal of Phycology 43: 509-518.

Bruno, J.F. 1998. Fragmentation in *Madracis mirabilis* (Duchassaing and Michelotti): how common is size-specific fragment survivorship in corals? Journal of Experimental Marine Biology and Ecology 230:169-181.

Bucher, M., Wolfowics, I., Voss, P.A., Hambleton, E.A., Guse, A. 2015. Development and symbiosis establishment in the cnidarian endosymbiosis model *Aiptasia* sp. Scientific Report 6: 19867. doi: 10.1038/srep19867.

Camaya, A.P., Sekida, S., Okuda, K. 2016. Changes in the ultrastructures of the coral *Pocillopora damicornis* after exposure to high temperature, and ultraviolet and far-red radiation. Cytologia 81(4): 465-470.

Chapman, D.V., Livingstone, D., Dodge, J.D. 1981. An electron microscope study of the excystment and early development of the dinoflagellate *Ceratium hirundinella*. British Phycological Journal 16: 183-194.

Cróquer, A., Villamizar, E., Noriega, y N. 2002. Environmental factors affecting tissue regeneration of the reef-building coral *Montastraea annularis* (Faviidae) at Los Roques National Park, Venezuela. Revista de Biologia Tropical 50(3): 1055-1065.

D'Angelo, C., Denzel, A., Vogt, A., Matz, M.V., Oswald, F., Salih, A., Nienhaus, G.U., Wiedenmann, J. 2008. Blue light regulation of host pigment in reef-building corals. Marine Ecology Progress Series 364: 97-106.

David, C.N., Schmidt, N., Schade, M., Pauly, B., Alexandrova, O., Böttger, A. 2005. Hydra and the evolution of apoptosis. Integrative and Comparative Biology 45: 631–638.

Davy, S.K., Allemand, D., Weis, V.M. 2012. Cell biology of cnidarians-dinoflagellate symbiosis. Microbiology and Molecular Biology Reviews 76(2): 229-261. doi:10.1128/MMBR.05014-11.

Delgadillo-Nuño, M.A., Liñam-Cabello, M.A., Reyes-Gómez, J., Soriano-Santiago, O. 2014. Response to pH in the reef-building coral *Pocillopora capitata* (Anthozoa-Scleractinia). Revista de Biologia Marina y Oceanografia 49(3) 449-459.

Dodge, J.D. 1964. Nuclear division in the dinoflagellate *Gonyaulax tamarensis*. Journal of General Microbiology 36: 269-276.

Dodge, J.D. 1965. Thecal fine-structure in the dinoflagellate genera *Prorocentrum* and *Exuviaela*. Journal of the Marine Biological Association of the United Kingdom 45: 607-614.

Dodge, J.D. 1968. The fine structure of chloroplasts and pyrenoids in some marine dinoflagellates. Journal of Cell Science 3: 41-48.

Dodge, J.D. 1971. Fine structure of the Pyrrophyta. The Botanical Review 37(4): 481-507.

Dodge, J.D. 1973. The fine structure of algal cells. London: Academic Press Inc., 261 pp.

Dodge, J.D. 1975. A survey of chloroplast ultrastructure in the Dinophyceae. Phycologia 14(4): 253-263.

Dodge, J.D., Crawford, R.M. 1969. Observations on the fine structure of the eyespot and associated organelles in the dinoflagellate *Glenodinium foliaceum* Journal of Cell Science 5: 479-493.

Dodge, J.D., Crawford, R.M. 1971. A fine structural survey of dinoflagellate pyrenoid and food reserves. Botanical Journal of Linnean Society 64(2): 105-115.

Douglas, A.E. 2003. Coral bleaching – how and why? Marine Pollution Bulletin 46: 385-392. doi:10.1016/S0025-326X(03)00037-7.

Downs, C.A., Fauth, J.E., Halas, J.C., Dustan, P., Bemiss, J., Woodley, C.M. 2002. Oxidative stress and seasonal coral bleaching. Free Radical Biology and Medicine 33: 533–543.

Downs, C.A., Kramarsky-Winter, E., Martinez, J., Kushmaro, A., Woodley, C.M., Loya, Y., Ostrander, G.K. 2009. Symbiophagy as a cellular mechanism for coral bleaching. Autophagy 5(2): 211-216.

Downs, C.A., McDougall, K.E., Woodley, C.M., Fauth, J.E., Richmond, R.H., Kushmaro, A., Gibb, S.W., Loya, Y., Ostrander, G.K., Kramarsky-Winter, E. 2013. Heat-stress and light-stress induce different cellular pathologies in the symbiotic dinoflagellate during coral bleaching. Public Library of Science 8(12):e77173.doi: 10.1371/journal.pone.0077173.

Dubinsky, Z., Stambler, N. 2011. Coral reefs: an ecosystem in transition. The Netherlands: Springer, 552 pp.

Dunn, S.R. and Weis, V.M. 2009. Apoptosis as a post-phagocytic winnowing mechanism in a coral-dinoflagellate mutualism. Environmental Microbiology 11(1): 268–276.

Dunn, S.R., Schnitzler, C.E. and Weis, V.M. 2007. Apoptosis and autophagy as mechanisms of dinoflagellate symbiont release during cnidarians bleaching: every which way you lose. Proceedings of the Royal Society B 274: 3079–3085.

Dunn, S.R., Bythell, J.C., Le Tissier, M.D.A., Burnett, W.J., Thomason, J.C. 2002. Programmed cell death and cell necrosis activity during hyperthermic stress-induced bleaching of the symbiotic sea anemone *Aiptasia* sp. Journal of Experimental Marine Biology and Ecology 272: 29–53.

Dunn, S.R., Thomason, J.C., Le Tissier, M.D.A., Bythell, J.C. 2000. Detection of cell death activity during experimentally induced bleaching of the symbiotic sea anemone *Aiptasia* sp. *In*: Proc. 9th International Coral Reef Symposium Bali, pp. 145–155.

Farmer, M.A., Fitt, W.K., Trench, R.K. 2001. Morphology of the symbiosis between *Corculum cardissa* (Mollusca: Bivalvia) and *Symbiodinium corculum* (Dinophyceae). Biological Bulletin 200: 336-343.

Fisher, E.M., Fauth, J.E., Hallock, P., Woodley, C.M. 2007. Lesion regeneration rates in reef-building corals *Montastrea* spp. as indicators of colony condition. Marine Ecology Progress Series 339: 61-71.

Fitt, W.K. 2000. Cellular growth of host and symbiont in a cnidarians-zooxanthellar symbiosis. Biological Bulletin 198: 110-120.

Fitt, W.K., Brown, B.E., Warner, M.E., Dunne, R.P. 2001. Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. Coral Reefs 20: 51–65. doi: 10.1007/s003380100146.

Fitt,W.K., McFarland, F.K., Warner, M.E., Chilcoat, G.C. 2000. Seasonal patterns of tissue biomass and densities of symbiotic dinoflagellates in reef corals and relation to coral bleaching. Limnological Oceanography 45(3): 677-685.

Fitt, W.K., Trench, R.K. 1983. Endocytosis of the symbiotic dinoflagellate *Symbiodinium microadriaticum* Freudenthal by endodermal cells of the scyphistomae of *Cassiopeia xamachana* and resistance of the algae to host digestion. Journal of Cell Science 64: 195-212.

Fournier, A. 2013. The story of symbiosis with zooxanthellae, or how they enable their host to thrive in a nutrient poor environment. BioSciences Master Reviews 1-8.

Franklin, D.J., Hoegh-Guldberg, O., Jones, R.J., Berges, J.A. 2004. Cell death and degeneration in the symbiotic dinoflagellates of the coral *Stylophora pistillata* during bleaching. Marine Ecology Progress Series 272: 117–130.

Freudenthal, H.D. 1962. *Symbiodinium* gen. nov. and *Symbiodinium microadriaticum* sp. nov., a zooxanthella: taxonomy, life cycle, and morphology. Journal of Protozoology 9(1): 45-52.

Gates, R.D., Baghdasarian, G., Muscatine, L. 1992. Temperature stress causes host cell detachment in symbiotic cnidarian: implications for coral bleaching. Biological Bulletin 182: 324-332.

Gladfelter, E.H. 1983. Spatial and temporal patterns of mitosis in the cells of the axial polyp of the reef coral *Acropora cervicornis*. Biological Bulletin 165: 811-815.

Gleason, D.F., Wellington, G.W. 1993. Ultraviolet radiation and coral bleaching. Nature 365: 836–838.

Glynn, P.W. 1993. Coral reef bleaching: ecological perspectives. Coral Reefs 12: 1–17.

Glynn, P.W., Peters, E.C. Muscatine, L. 1985. Coral tissue microstructure and necrosis: relation to catastrophic coral mortality in Panamá. Diseases of Aquatic Organisms 1: 29-37.

Goreau, T.F. 1964. Mass expulsion of zooxanthellae from Jamaican reef communities after hurricane Flora. Science 145: 383–386.

Guillard, R.R.L., Sieracki, M.S. 2005. Counting cells in cultures with the light microscope. *In* Algal Culturing Techniques. (Ed. Andersen, R.A.) USA: Elsevier Academic Press, pp. 239.

Hall, V.R. 1997. Interspecific differences in the regeneration of artificial injuries on scleractinian corals. Journal of Experimental Marine Biology and Ecology 212: 9-23.

Hanes, S.D., Kempf, S.C. 2013. Host autophagic degradation and associated symbiont loss in response to heat stress in the symbiotic anemone, *Aiptasis pallida*. Invertebrate Biology 132: 95–107.

Harii, S., Yasuda, N., Rodriguez-Lanetty, M., Irie, T., Hidaka, M. 2009. Onset of symbiosis and distribution patterns of symbiotic dinoflagellates in the larvae of scleractinian corals. Marine Biology 156: 1203-1212. doi:10.1007/s00227-009-1162-9.

Haryanti, D., Yasuda, N., Harii, S., Hidaka, M. 2015. High tolerance of symbiotic larvae of *Pocillopora damicornis* to thermal stress. Zoological Studies 54:52 doi:10.1186/s40555-015-0134-7.

Highsmith, R.C. 1982. Reproduction by fragmentation in corals. Marine Ecology Progress Series 7: 207-226.

Hirose, M., Kinzie III, R.A., Hidaka, M. 2000. Early development of zooxanthellae-containing eggs of the corals *Pocillopora verrucosa* and *P. eydouxi* with special reference to the distribution of zooxanthellae. Biological Bulletin 199: 68-75.

Hirose, M., Kinzie III, R.A., Hidaka, M. 2001. Timing and process of entry of zooxanthellae into oocytes of hermatypic corals. Coral Reefs 20: 273-280. doi:10.1007/s003380100171.

Hirose, M., Yamamoto, H., Nonaka, M. 2008. Metamorphosis and acquisition of symbiotic algae in planula larvae and primary polyps of *Acropora* spp. Coral Reefs 27: 247-254. doi:10.1007/s00338-007-0330-y.

Hoegh-Guldberg, O. 1994. Population dynamics of symbiotic zooxanthellae in the coral *Pocillopora damicornis* exposed to elevated ammonium [$(NH_4)_2SO_4$] concentrations. Pacific Science 48(3): 263-272.

Hoegh-Guldberg, O. 1999. Climage change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research 50: 839–866.

Hoegh-Guldberg, O., Smith, G.J. 1989. The effect of sudden changes in temperature, light and sainity on the density and export of zooxanthellae from the reef corals *Stylophora pistillata* Esper and *Seriatopora hystrix* Dana. Journal of Experimental Marine Biology and Ecology 129: 279-303.

Hoeksema, B. 2015. *Pocillopora damicornis* (Linnaeus, 1758). accessed through: World Register of Marine Species @ http://www.marinespecies.org.

Hoeksema, B.W., Rogers, A. & Quibilan, M.C. 2014. *Pocillopora damicornis*. The IUCN Red List of Threatened Species 2014.

Hollósy, F. 2002. Effects of ultraviolet radiation on plant cells. Micron 33: 179-197.

Horiguchi, T., Pienaar, R.N. 1994. Ultrastructure of a new marine sand-dwelling dinoflagellate, *Gymnodinium quadrilobatum* sp. nov. (Dinophyceae) with special reference to its endosymbiotic alga. European Journal of Phycology 29: 237-245.

Ichiki, S., Mizuta, H., Yasui, H., Yamamoto, H. 2001. Effects of irradiance and water temperature on the photosynthesis and growth of the crustose coralline alga *Lithophyllum yessoense* Foslie (Corralinales, Rhodophyceae). Bulletin of the Faculty of Fisheries Hokkaido University 52(2): 103-109.

Janes, M.P. 2008. Laboratory methods for the identification of soft corals (Octocorallia: Alcyonacea). *In* Advances in Coral Husbandry in Public Aquariums. Public Aquarium Husbandry Series, Vol. 2. (Ed: Leewis, R.L., Janse, M.). Arnhem, The Netherlands: Burgers' Zoo, p. 413.

Jones, R.J., Yellowlees, D. 1997. Regulation and control of intracellular algae (= zooxanthellae) in hard corals. Philosophical Transactions of the Royal Society B 352: 457-468.

Kempf, S.C. 1984. Symbiosis between the zooxanthella *Symbiodinium* (= *Gymnodinium*) *microadriaticum* (Freudenthal) and four species of nudibranchs. Biological Bulletin 166: 110-126.

Kevin, M.J., Hall, W.T., McLaughlin, J.J.A., Zahl P.A. 1969. *Symbiodinium microadriaticum* Freudenthal, A revised taxonomic description, ultrastructure. Journal of Phycology 5: 341-350.

Kinzie III, R.A., Hunter, T. 1987. Effect of light quality on photosynthesis of the reef coral *Montipora verrucosa*. Marine Biology 94: 95–109.

Kinzie III, R.A., Jokiel, P.L., York, R. 1984. Effect of light of altered spectral composition on coral zooxanthellae associations and on zooxanthellae *in vitro*. Marine Biology 78: 239–248.

Kramarsky-Winter, E., Loya, Y. 2000. Tissue regeneration in the coral *Fungia granulosa*: the effect of extrinsic and intrinsic factors. Marine Biology 137: 867-873.

Kramarsky-Winter, E., Loya, Y., Vizel, M., Downs, C.A. 2011. Method for coral tissue cultivation and propagation. United States Patent Application Publication. US 2011/0179504 A1.

Kubai, D.F., Ris, H. 1969. Division in the dinoflagellate *Gyrodinium cohnii* (Schiller): a new type of nuclear reproduction. The Journal of Cell Biology 40: 508-528

Kurahashi, Y. 1965. Perfusion fixation with glutaraldehyde and its application for cytochemistry of developing teeth. Journal of Electron Microscopy 14: 337–338.

Kuroiwa, T. 2000. The discovery of the division apparatus of plastids and mitochondria. Journal of Electron Microscopy 49(1): 123-134.

Leadbeater, B., Dodge, J.D. 1966. The fine structure of *Woloszynskia micra* sp. nov., a new marine dinoflagellate. British Phycological Bulletin 3(1): 1-17.

Leadbeater, B., Dodge, J.D. 1967. An electron microscope study of nuclear and cell division in a dinoflagellate. Archiv für Mikrobiologie 57: 239-254.

Lesser, M.P. 1997. Oxidative stress causes coral bleaching during exposure to elevated temperatures. Coral Reefs 16: 187–192.

Lesser, M.P. 2004. Experimental biology of coral reef ecosystems. Journal of Experimental Marine Biology and Ecology 300: 217-252. doi:10.1016/j.jembe.2003.12.027.

Lesser, M.P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annual Review of Physiology 68: 253-78. doi:10.1146/annurev.physiol.68.040 104.110001.

Lesser, M.P., Shick, J.M. 1990. Effects of visible and ultraviolet radiation on the ultrastructure of zooxanthellae (*Symbiodinium* sp.) in culture and in situ. Cell Tissue Research 261: 501–508.

Lesser, M.P., Stochaj, W.R., Tapley, D.W., Shick, J.M. 1990. Bleaching in coral reef anthozoans: effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. Coral Reefs 8: 225–232.

Lesser, M.P., Farell, J. H. 2004. Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. Coral Reefs 23: 367–377.

Lirman, D. 2000. Fragmentation in the branching coral *Acropora palmata* (Lamarck): growth, survivorship, and reproduction of colonies and fragments. Journal of Experimental Marine Biology and Ecology 251: 41-57.

Loeblich, A.R, Sherley, J.L. 1979. Observation on the theca of the motile phase of free-living and symbiotic isolates of *Zooxanthellae microadriatica* (Freudenthal) comb. nov. Journal of the Marine Biological Association of the United Kingdom 59: 195-205.

Lohr, J., Munn, C.B., Wilson, W.H. 2007. Characterization of a latent virus-like infection of symbiotic zooxanthellae. Applied Environmental Microbiology 73(9): 2976–2981. doi:10.1128/AEM.02449-06.

Magalon, H., Flot, J.-F., Baudry, E. 2007. Molecular identification of symbiotic dinoflagellates in Pacific corals in the genus *Pocillopora*. Coral Reefs. 26: 551-558.

Marlow, H.Q., Martindale, M.Q. 2007. Embryonic development in two species of scleractinian coral embryos: *Symbiodinium* localization and mode of gastrulation. Evolution & Development 9(4): 355-367.

Mass, T., Kline, D.I., Roopin, M., Veal, C.J., Cohen, S., Iluz, D., Levy, O. 2010. The spectral quality of light is a key driver of photosynthesis and photoadaptation in *Stylophora pistillata* colonies from different depths in the Red Sea. Journal of Experimental Biology 213: 4084-4091. doi:10.1242/jeb.039891.

Meesters, E.H., Pauchli, W., Bak, R.P.M. 1997. Predicting regeneration of physical damage on a reef-building coral by regeneration capacity and lesion shape. Marine Ecology Progress Series 146:91-96.

Morill, L.C., Loeblich III, A.R. 1984. Cell division and reformation of the amphiesma in the pelliculate dinoflagellate, *Heterocapsa niei*. Journal of Marine Biological Association of United Kingdom 64: 939-953.

Muller-Parker, G., D'Elia, C.F. 1997. Interactions between corals and their symbiotic algae. *In* Life and death of coral reefs (Ed. Birkeland, C.) New York, USA: Chapman and Hall, pp. 96.

Müller, W.E.G., Müller, I., Zahn, R.D., Maidhof, A. 1984. Intraspecific recognition system in scleractinian corals: morphological and cytochemical description of the autolysis mechanism. The Journal of Histochemistry and Cytochemistry 32(3): 285–288.

Muscatine, L., Wilkerson, F.P., McCloskey, L.R. 1986. Regulation of population density of symbiotic algae in a tropical marine jellyfish (*Mastigias* sp.). Marine Ecology Progress Series 32: 279-290.

Netzel, H., Dürr, G. 1984. Dinoflagellate cell cortex. *In* Dinoflagellates (Ed. Spector, D.L.) USA: Academic Press Inc, pp. 143.

Nii, C.M., Muscatine, L. 1997. Oxidative stress in the symbiotic sea anemone *Aiptasia pulchella* (Carlgren, 1943): contribution of the animal to superoxide ion production at elevated temperature. Biological Bulletin 192: 444–456.

Okazaki, K., Kabeya, Y., Miyagishima, S. 2010. The evolution of the regulatory mechanism of chloroplast division. Plant Signaling and Behavior 5(2): 164-167.

Oren, U., Benayahu, Y., Loya, Y. 1997. Effect of lesion size and shape on regeneration of the Red Sea coral *Favia favus*. Marine Ecology Progress Series 146: 101-107.

Oren, U., Benayahu, Y., Lubinevsky, H., Loya, Y. 2001. Colony integration during regeneration in the stony coral *Favus favus*. Ecology 82(3): 802-813.

Osinga, R., Schutter, M., Griffioen, B., Wijffels, R.H., Verreth, J.A.J., Shafir, S., Henard, S., Taruffi, M., Gili, C., Lavorano, S. 2011. The biology and economics of coral growth. Marine Biotechnology 13: 658-671. doi: 10.1007/s10126-011-9382-7.

Paxton, C.W., Davy, S.K., Weis, V.M. 2013. Stress and death of cnidarians play a role in cnidarian bleaching. The Journal of Experimental Biology 216: 2813–2820. doi: 10.1242/jeb.087858.

Permata, W.D., Kinzie III, R.A., Hidaka, M. 2000. Histological studies of planulae of the coral *Pocillopora damicornis.* Marine Ecology Progress Series 200: 191-200.

R version. 3.2.5. 2016. The R foundation for statistical computing platform. www.r-project.org.

Rasband, W.S. 2003. Image J. National Institute of Health, Bethesda, Maryland, USA. Available @ https://imagej.nih.gov/ij/. accessed 2 April 2015.

Richmond, R.H., Jokiel, P.L. 1984. Lunar periodicity in larva release in the reef coral *Pocillopora damicornis* at Enewetak and Hawaii. Bulletin of Marine Science 34(2): 280-287.

Richmond, R.H., Wolanski, E. 2011. Coral research: past research and future horizons. *In* Coral reefs: an ecosystem in transition (Ed. Dubinsky, Z., Stambler, N). The Netherlands: Springer, p. 3.

Rowan, R., Powers, D.A. 1991. Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). Marine Ecology Progress Series 71: 65-73.

Sabine, A.M., Smith, T.B., Williams, D.E., Brandt, M.E. 2015. Environmental conditions influence tissue regeneration rates in scleractinian corals. Maine Pollution Bulletin 95: 253-264.

Samara, C., Syntichaki, P., Tavernakis, N. 2008. Autophagy is required for necrotic cell death in *Caenorhabditis elegans*. Cell Death and Differentiation 15:105-112.

Schmitter, R.E. 1971. The fine structure of *Gonyaulax polyedra*, a bioluminescent marine dinoflagellate. Journal of Cell Science 9: 147-173.

Scholey, J.M., Brust-Mascher, I., Mogilner, A. 2003. Cell division. Nature. 422: 746-752.

Sekida, S., Okuda, K. 2012. Ultrastructure and physiological behaviors of symbiotic zooxanthellae in corals. Aquabiology 34(4): 338-342.

Sekida, S., Horiguchi, T., Okuda, K. 2001. Development of the cell covering in the dinoflagellate *Scrippsiella hexapraecingula* (Peridiniales, Dinophyceae). Phycological Research 49: 163-176.

Sekida, S., Horiguchi, T., Okuda, K. 2004. Development of thecal plates and pellicle in the dinoflagellate *Scrippsiella hexapraecingula* (Peridiniales, Dinophyceae) elucidated by changes in stainability of the associated membranes. European Journal of Phycology 39(1): 105-114.

Shick, J.M., Lesser, M.P., Jokiel, P.L. 1996. Effects of ultraviolet radiation on corals and other coral reef organisms. Global Change Biology 2: 527–545.

Shibutani, S.T., Yoshimori, T. 2014. A current perspective of autophagosome biogenesis. Cell Research 24: 58–68.

Smith, D.J., Sugett, D.J., Baker, N.R. 2005. Is photoinhibition of zooxanthellae photosynthesis the primary cause of thermal bleaching in corals? Global Change Biology 11: 1–11. doi:10:1111/j.1365-2486.2004.00895.x.

Soong, K., Chen, T. 2003. Coral transplantation: regeneration and growth of *Acropora* fragments in nursery. Restoration Ecology 11 (1): 62-71.

Spector, D.L. 1984. Dinoflagellates: an introduction. *In* Dinoflagellates (Ed: Spector, D.L.). USA: Academic Press Inc., pp. 1.

Stanley, Jr., G.D., Swart, P.K. 1995. Evolution of the coral-zooxanthellae symbiosis during Triassic: a geochemical approach. Paleobiology 21(2): 179-199.

Stat, M., Carter, D., Hoegh-Guldberg, O. 2006. The evolutionary history of *Symbiodinium* and scleractinian hosts-symbiosis, diversity, and the effect of climate change. Perspectives in Plant Ecology, Evolution and Systematics 8: 23–43. doi:10.1016/j.ppees.2006.04.001

Steiner, S.C.C., Cortés, J. 1996. Spermatozoan ultrastructure of scleractinian corals from the eastern Pacific: *Pocilloporidae* and *Agariciidae*. Coral Reefs 15: 143-147.

Stimson, J.S. 1978. Mode and timing of reproduction in some common hermatypic corals of Hawaii and Enewetak. Marine Biology 48: 173-184.

Stimson, J.S. 1997. The annual cycle of density of zooxanthellae in the tissues of field and laboratory-held *Pocillopora damicornis* (Linnaeus). Journal of Experimental Marine Biology and Ecology 214: 35-48.

Stoddart, J.A. 1983. Asexual reproduction of planulae in the coral *Pocillopora damicornis*. Marine Biology 76: 279-284.

Stoddart, J.A., Black, R. 1985. Cycles of gametogenesis and planulation in the coral *Pocillopora damicornis*. Marine Ecology Progress Series 23: 153-164.

Sweeney, B.M., Hastings, J.W. 1958. Rythmic cell division in populations of *Gonyaulax polyedra*. Journal of Protozoology 5(3): 217-224.

Takahashi, S., Nakamura, T., Sakamizu, M., van Woesik, R., Yamasaki., H. 2004. Repair machinery of symbiotic photosynthesis as the primary target of heat stress for reef-building corals. Plant Cell Physiology 45(2): 251-255.

Taylor, D.L. 1968. *In situ* studies on the cytochemistry and ultrastructure of a symbiotic marine dinoflagellate. Journal of Marine Biological Association of United Kingdom 48: 349-366.

Taylor, D.L. 1969. Identity of zooxanthellae isolated from some Pacific Tridacnidae. Journal of Phycology 5: 336-340.

Titlyanov, E.A., Titlyanova, T.V., Yakovleva, I.M., Nakano, Y., Bhagooli, R. 2005. Regeneration of artificial injuries on scleractinian corals and coral/algal competition for newly formed substrate. Journal of Experimental Marine Biology and Ecology 323: 27-42.

Trench, R.K. 1987. Dinoflagellates in non-parasitic symbiosis. *In* Biology of Dinoflagellates (Ed. Taylor, F.J.R.). Blackwell Oxford, pp. 530.

Trench, R.K. 1993. Microalgal-invertebrate symbioses: a review. Endocytobiosis & Cell Research 9: 135–175.

Trench, R.K., Blank, R.J. 1987. *Symbiodinium microadriaticum* Freudenthal, *S. goreauii* sp. nov, *S. kawagutii* sp. nov. and *S. pilosum* sp. nov.: Gymnodinioid dinoflagellate symbionts of marine invertebrates. Journal of Phycology 23: 469-481.

Trench, R.K., Thinh, L.V. 1995. *Gymnodinium linucheae* sp. nov.: the dinoflagellate symbiont of the jellyfish *Linuche unguiculata*. European Journal of Phycology 30: 149-154.

Trench, R.K., Wethey, D.S., Porter, J.W. 1981. Observations on the symbiosis with zooxanthellae among the Tridacnidae (Mollusca, Bivalvia). Biological Bulletin 161: 180-198.

Triemer, R.E., Fritz, L. 1984. Cell cycle and mitosis. *In* Dinoflagellates (Ed: Spector, D.L.). USA: Academic Press Inc., pp. 149.

van Treeck, P., Schuhmacher, H. 1997. Initial survival of coral nubbins transplanted by a new coral transplantation technology – options for reef rehabilitation. Marine Ecology Progress Series 150: 287-292.

van Woesik, R. 1998. Lesion healing on massive *Porites* spp. corals. Marine Ecology Progress Series 164: 213-220.

Vandermeulen, J.H., Watabe, N. 1973. Studies on reef corals. I. skeleton formation by newly settled planula larva of *Pocillopora damicornis.* Marine Biology 23: 47-57.

Venn, A.A., Loram, J.E., Douglas, A.E., 2008. Photosynthetic symbioses in animals. Journal of Experimental Botany 59(5) 1069-1080. doi:10.1093/jxb/erm328.

Veron, J.E.N. 1992. Hermatypic corals of Japan. Australian Institution of Marine Science Monograph Series 9, pp. 37.

Veron, J.E.N. 2013. Overview of the taxonomy of zooxanthellate Scleractinia. Zoological Journal of the Linnean Society 169: 485-508.

Veron, J.E.N., DeVantier, L.M., Turak, E., Green, A.L., Kininmonth, S., Stafford-Smith, M., Peterson, N. 2009. Delineating the Coral Triangle. Galaxea 11: 91-100.

Veron, J.E.N., Hodgson, G. 1989. Annotated checklist of the hermatypic corals of the Philippines. Pacific Science 43(3): 234-287.

Veron, J.E.N., Stafford-Smith, M., DeVantier, L.M., Turak, E. 2015. Overview of distribution of zooxanthellate Scleractinia. Frontiers in Marine Science 1(81): 1-19. doi:10.3389/fmars.2014.00081.

Wahle, C.M. 1983. Regeneration of injuries among Jamaican gorgonians: the roles of colony physiology and environment. Biological Bulletin 165: 778-790.

Wakefield, T.S., Farmer, M.A. and Kempf, S.C. 2000. Revised description of the fine structure of in situ "zooxanthellae" Genus *Symbiodinium*. Biological Bulletin 199: 76–84.

Weis, V.M. 2008. Cellular mechanisms of cnidarian bleaching: stress causes the collapse of symbiosis. The Journal of Experimental Biology 211: 3059–3066. doi:10.1242/jeb.009597.

Weis, V.M., Davy, S.K., Hoegh-Guldberg, O., Rodriguez-Lanetty M., Pringle, J.R. 2008. Cell biology in model systems as the key to understand corals. Trends in Ecology and Evolution 23(7): 369–376. doi:10.1016/j.tree.2008.03.004.

Wijgerde, T., van Melis, A., Silva, C.I.F., Leal, M.C., Vogels, L., Mutter, C., Osinga, R. 2014. Red light represses the photophysiology of the scleractinian coral *Stylophora pistillata*. Public Library of Science 9(3): e92781. doi: 10.1371/journal.pone.0092781.

Wilkerson, F.P., Kobayashi, D., Muscatine, L. 1988. Mitotic index and size of symbiotic algae in Caribbean reef corals. Coral Reefs 7: 29-36.

Wilkerson, F.P., Muller-Parker, G., Muscatine, L. 1983. Temporal patterns of cell division in natural populations of endosymbiotic algae. Limnological Oceanography 28(5): 1009-1014.

Yap, H.T., Aliño, P.M., Gomez, E.D. 1992. Trends in growth and mortality of three coral species (Anthozoa: Scleractinia), including effects of transplantation. Marine Ecology Progress Series 83: 91-101.

Zakai, D., Levy, O., Chadwick-Furman, N.E. 2000. Experimental fragmentation reduces sexual reproductive output by the reef-building coral *Pocillopora damicornis*. Coral Reefs 19: 185-188.