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Compositional information on the calcification medium in reef-building corals derived from identification of mineral phases

造礁性サンゴ骨格を形成する鉱物相が示す石灰化母液の組成

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Abstract

Reef-building corals are animals inhabiting the shallow sea in the tropical or sub-tropical regions and have the external skeletons formed by calcium carbonate. It is worthy noted that coral skeletons are formed by mineral “aragonite”, although calcite is the thermodynamically stable mineral phase of calcium carbonates in the coral living environment. It has been suggested that aragonite crystallizes in the so-called “calcification medium” filling the small calcification space less than a few micron meters between skeletons and living organisms, however, there is a little knowledge about mechanisms and process of coral biomineralization. Compositional information on the calcification medium could be an important factor controlling crystallization of aragonite. However, the information about the calcification medium is little, because the calcification space is too small to be measured directly. Thus, in chapter 2, I observed skeletal textures and crystal morphology of the massive reef-building corals from the micro- to nano scale in order to obtain some information about the growth mechanism of aragonite. Furthermore, identification of mineral phases could be important to estimate chemical composition of the calcification medium, because the mineral phases forming the skeletons must be crystallized in it. Thus, I performed two kinds of experimental approaches based on mineral identification in the skeletons to obtain some compositional information on the calcification medium in chapter 3 and 4.

The skeletal texture and crystal morphology of the massive reef-building coral *Porites lobata* were observed from the micro- to nanometer scale using an analytical transmission electron microscope (ATEM). The skeletal texture consists of centers of calcification (COCs) and fiber areas. Fiber areas contain bundles of needle-like aragonite crystals that are elongated along the crystallographic *c*-axis and are several hundred nanometers to one micrometer in width and several micrometers in length. The size distribution of aragonite crystals is relatively homogeneous in the fiber area. Growth lines are observed sub-perpendicular to the direction of aragonite growth. These growth lines occur in 1–2 μm intervals and reflect a periodic contrast in the thickness of an ion-spattered sample and pass through the interior of some aragonite crystals. These observations suggest that aragonite crystals precipitate inorganically in the calcification medium and the medium maintains a $CaCO_3$-supersaturated state during fiber growth. It is also

suggested that growth lines are possibly formed due to changes in the chemical and physical conditions of the medium during fiber growth.

Identification of mineral phases of massive reef-building coral skeleton were performed from the micro- to nanometer scale using an ATEM and a Fourier transform infrared spectrometer (FT–IR). The most notable result was the discovery of halite (NaCl) in the coral skeleton. From the TEM observation, average size of halite crystals is about 60-80 nm and their crystal shape exhibits the square cross-sectional form that is typical euhedral shape of halite. In addition, some special relations of crystallographic orientation can be found between halite and host aragonite. These microstructural and crystallographic features of this halite suggest that it precipitated almost simultaneously with aragonite during coral calcification. The existence of such halite not only represents new evidence for the validity of extracellular calcification, but also provides chemical information about the calcification medium. Moreover, the absence of gypsum ($CaSO_4 \cdot 2H_2O$) and anhydrite ($CaSO_4$) in association with the halite indicates that a selective ion transport mechanism operates from seawater to the calcification space in coral. The primary deposition of a mineral phase other than aragonite in coral skeleton has been confirmed for the first time.

Acropora digitifera was cultured in artificial seawater formulated at 5.0, 2.5 and 1.2 of Mg/Ca ratios in order to investigate the effect of seawater Mg/Ca ratios on mineral phases occurred in the coral skeleton. In this study, larval coral polyp was grown from egg to remove influence of already formed aragonite and symbiotic algae. X-ray diffraction analysis, Raman scattering analysis and TEM observation showed that only aragonite could be identified in all skeletons. Because this result is consistent with the evidence that only aragonite precipitates inorganically in model seawaters with more than 1.0 of Mg/Ca ratio at 25°C (Morse et al., 1997), it is suggested that the calcification medium has a similar value to the Mg/Ca ratio in the surrounding seawater. Ries et al. (2006) showed that calcite can precipitate in the skeletons of some coral specimens with symbiotic algae, cultured in artificial seawater with less than 3.5 of the Mg/Ca ratio. These evidences indicate that symbiotic algae play an important role for decreasing the Mg/Ca ratio in the calcification medium against seawater.

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1. General Introduction

Reef-building Coral Biomineralization

The coral reefs made of coral skeletons provide some of the most diverse ecosystems on earth. It is concerned about how coral growth is influenced by recent changing of the surrounding seawater, such as global warming and ocean acidification. In order to estimate the influence of changing environment on coral calcification precisely, it is important to know how corals make their skeleton. The minerals produced by living organisms are called as biominerals. These biominerals sometimes show unique mineralogical characteristics which are different from those created by inorganic process. For example, the exoskeletons of reef-building corals with zooxanthellae consist of calcium carbonate ($CaCO_3$) in the form of aragonite, although aragonite is a thermodynamically metastable phase of $CaCO_3$ in coral growth environments and calcite is the stable phase there (e. g., Redfern 2000). Coral skeletons are formed through biogenic processes, thus people believe that some biogenic processes contribute to the aragonite formation. However, the detailed contribution on the formation process of coral skeletons has been still unknown.

Biology of Reef-Building Corals

Reef-building corals exist in oligotrophic seawater in the tropical and subtropical regions. The optimum temperature for most coral reefs is 26–27 °C, and few reefs exist in waters below 18 °C. Reef-building corals live in only shallow seawater. This is because corals want to establish a symbiotic relationship with algae (zooxanthellea) that live inside of coral tissues. The symbiotic relationship is advantageous to survive in such regions.

Corals can reproduce both sexually and asexually. Sexual reproduction mainly occurs once a year (Fig. 1-1). Symbiotic algae do not exist in these eggs. Larval planulae hatched from eggs are usually planktonic and dispersive to some degree (Harrison and Wallace 1990). Planulae often get symbiotic algae from sweater to inside of their tissue. After the planulae successfully attach and settle permanently on hard substratum, it metamorphoses from larval form into a juvenile polyp that initiates the formation of the aragonite exoskeleton. Coral polyp phase is characterized by growth of tissue and skeleton. Coral polyp can propagate by asexual budding. Asexual budding of polyps leads the formation of coral colony and allow their life-span to be more than 100 years.

Site of Coral Calcification

The anatomy of corals was described in Johnston (1980) and Fautin and Marical (1991). The skeleton is located outside the animal with an oral tissue facing the seawater and an aboral tissue facing the skeleton (Fig. 1-2). Each tissue is composed of two epithelial cell layers, the external and internal layers generally called as ectoderm and endoderm respectively. These cell layers are separated by the mesoglea, a thin extracelluar matrix of collage (Schmid et al. 1999). The symbiotic algae are inside of oral endoderm cell.

Skeletal aragonite is considered to be precipitated in calcification space located between skeleton and aboral ectoderm cell called calicoblastic cell-layers (Johnston 1980; Clode and Marshall 2003; Allemand et al. 2004; Tambutte et al. 2007). Dimension of the calcification space between skeletons and calicoblastic cell-layers was reported as about 1 μm in width (Johnston 1980; Isa 1986). Some researchers claim that the space with 1μm in width may be overestimate, because of expanding during the microscopic observation time under vacuum (Clode and

Marshall 2003; Tambutte et al. 2007). The calcification space is filled with calcification medium. It is important to know chemical and physical conditions of the calcification medium for understanding coral calcification. However, such information about the calcification medium is little, because the calcification space is so small that physico-chemical states in the space are quite difficult to be measured directly. The calcification medium is considered to come from seawater. However, the composition of calcification medium may be different from that of seawater, because seawater has to pass through coral cell layers to the calcification space and the activity of coral tissue including symbiotic algae may select elements. In fact, a pioneer work of direct measurements of calcification medium revealed that Ca^{2+} concentration and pH value are higher than those of seawater (Al-Horani et al. 2003).

Investigation of Coral Skeleton for Understanding Coral Calcification

The micro-structure and chemistry of coral skeletons have been studied to elucidate the mechanism of biomineralization (e.g., Ogilvie 1896; Bryan and Hill 1941; Nothdurft and Webb 2007). Especially, it has been one of major debate how living organization controls the coral mineralization. The variety of crystallographic morphologies and textures should reflect chemical and physical conditions in calcification medium during the formation of skeletons. For example, nacreous layer of seashell is known to be consisted of aragonite and the aragonite formation should be controlled organic matrix strongly. The crystal shape of aragonite is well-organized and almost equal-size hexagonal prism plate. And the crystallographic *c*-axis is parallel to the axis of prism. This crystallographic feature is different from inorganic aragonite crystals which usually show needle-like shape elongating along the crystallographic *c*-axis, because inserted organic matrix layers in shell control and limit these crystal growth strongly

(e.g., Suzuki et al. 2009).

Ogilvie (1896) observed coral skeleton under an optical microscope. She described fibrous aragonite bundles and dark points. The dark points are encompassed by fibrous aragonite crystals. It is believed that these dark points form first and then crystals in fibrous bundles start to grow (Bryan and Hill 1941; Cohen et al. 2001). Thus, the fibrous bundles and dark points are called the fiber area (Partz 1882) and centers of calcification (COCs) (Ogilvie 1896), respectively. In fiber areas, concentric micrometer-thick layers are recognized around a COC, which are called as growth lines (Barnes 1970). Coral skeleton are consisted of aggregation of such structural unit of fiber and COC (Fig. 1-3).

Barnes (1970) pointed out that the texture of the structural unit is similar to the colloform texture. The collform texture is formed by a simple geometrical selection. In Geometrical selection, at first numerous crystals are formed in inorganic condition at random orientation, however crystals inclined to the substrate surface will make contact with crystals growing perpendicularly and stop their growth. Thus, only the crystals having extended direction perpendicular to the substrate can grow. Cohen et al. (2001) performed more detailed observations of *Porites lutea* by a scanning electron microscope (SEM). They found that the fiber area is built of needle-like crystals longer than 1 μm and submicron-sized granular crystals consist of COCs. Benzerara et al. (2011) also observed such granular crystals in COC of *Porites* sp. by using a synchrotron-based scanning transmission X-ray microscopy and a transmission electron microscopy (TEM). The skeletal texture of coral skeleton seems to be similar to collform texture. Holcomb et al. (2009) synthesized abiogenic aragonite precipitated experimentally from seawater. They suggested that the aggregate of synthetic aragonite have similar texture to the structural unit consisting of coral skeletons. These evidences observed in

coral suggest that coral skeletons could be formed thought inorganic process.

On the other hand, some researchers advocated that organic matrix should control the formation of coral skeletons actively. Isa (1986) observed the fusiform form of accumulated aragonite crystals with hollow in *Acropora* sp. and insisted that organic matrix would fill in the hollow and control the mineralization. Cuif and Dauphin (2005b; 2005a) carried out chemical analyses with nano scale and suggested that organic compounds are inserted into coral skeleton corresponding with COCs and growth lines. Because of these periodically distributions of organic compounds, they considered that organic compounds involve in mineralization and expected that the crystallographic difference should appear at location of existence of organic compounds. However, there were a few observations of crystallographic texture on coral skeleton at the same scale.

Chemical Information in Coral Skeleton

Many elements other than Ca, such as Mg, Sr, Na, K and so on, were reported in coral skeletons by chemical analysis (e. g., Okai et al. 2002). Those elements (Me) are considered to be contained in calcification medium and incorporated into coral skeleton during calcification. The fluctuation of Me/Ca ratio in coral skeleton along the growth direction can provide a wealth of high-resolution information relating to physical and chemical marine conditions (e. g., Smith et al. 1979; Weil et al. 1981). Then, coral skeleton has been shown to be particularly useful in paleoclimatology. In general, it is confirmed by using bulk X-ray diffraction pattern (XRD) that coral skeletons are consisted of aragonite phase. Then, these Me are considered to be incorporated in aragonite and/or absorbed on surface of aragonite crystals. On the other hand, the existence of strontianite ($SrCO_3$) and brucite ($Mg(OH)_2$) were also reported as independent

phases by an extended X-Ray absorption fine structure (EXAFS) and SEM respectively (Greegor et al. 1997; Nothdurft et al. 2005). It was suggested that these phases are too minor to detect by XRD (Allison et al. 2001; Nothdurft et al. 2005). Although some of these mineral phases were concluded to be secondary precipitation or precipitated under unusual situation, it is important to know if there are some mineral phases other than aragonite in coral skeletons. The form of Me in coral skeleton can provide chemical information on calcification medium.

I report the results from three experiments for revealing the compositional information about coral calcification medium. This thesis is divided into this general introduction, following three chapters and conclusions.

In chapter 2, skeletal texture and crystal morphology of the massive reef-building coral, *Porites lobata*

In chapter 3, precipitation of halite during calcification in massive reef-building coral, *Porites lobata*

In chapter 4, mineral phase of skeleton in reef-building coral polyp cultured in artificial seawater with Mg/Ca ratio

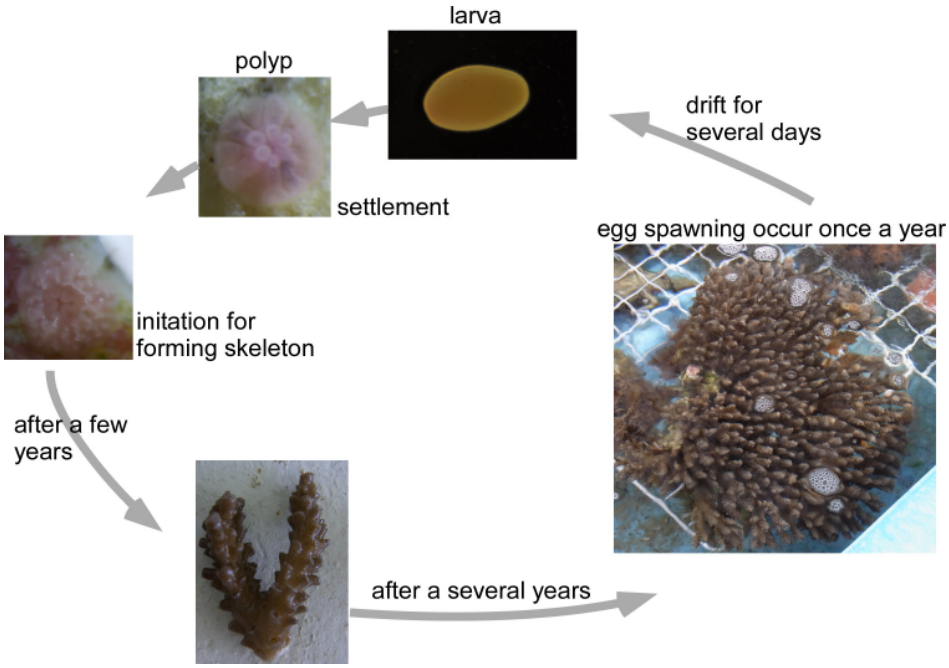

Figure 1-1. Life cycles of corals. The species of all photos is *Acropora tenuis*.

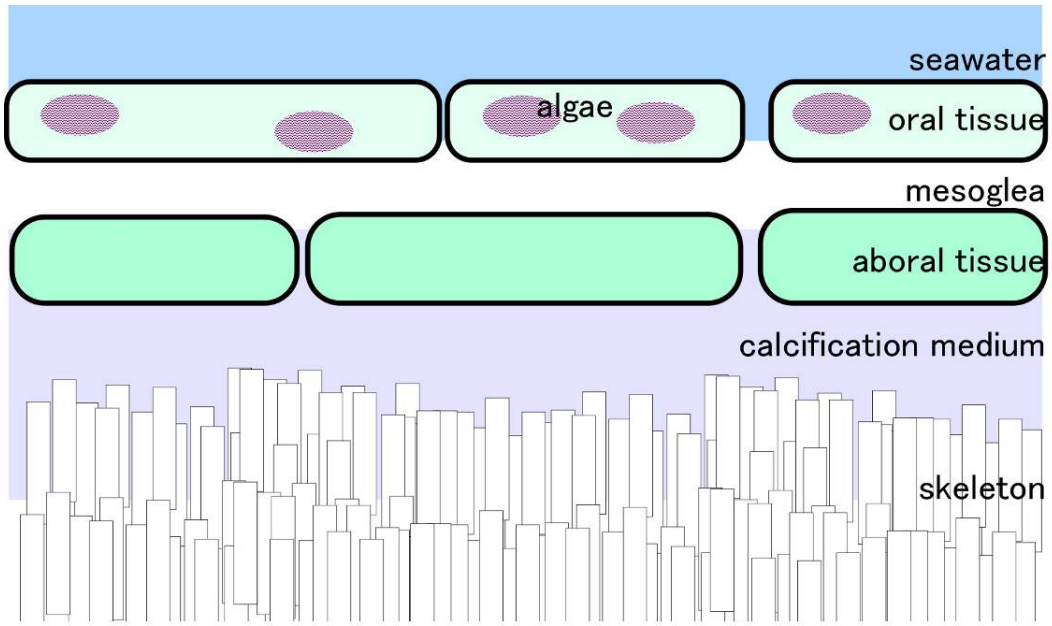

Figure 1-2. Schematic section of the histology of a coral across is coenenchyme.

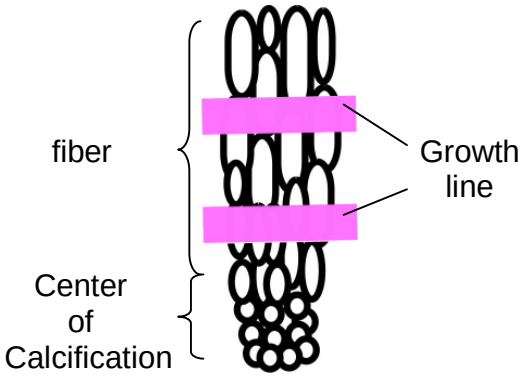

Figure 1-3. Schematic section of the structural units of coral skeleton, center of calcification (COC) and fiber.

2. Skeletal texture and crystal morphology of the massive reef-building coral, *Porites lobata*

2-1. Purpose

To elucidate the biomineralization mechanism, the microskeletal structure and chemical analysis of coral skeletons have been studied over a century (e.g., Cohen and McConnaughey 2003; Meibom et al. 2007; Nothdurft and Webb 2007). These previous studies show that coral skeletons were built with the fundamental units that consisted of “center of calcification” (COC) (Ogilvie 1896) and “fibers” emerging from a COC (Partz 1882). Corals are assumed to make their skeleton by precipitating aragonite crystals in the calcification medium filled with calcification space located between the skeleton and the calicoblastic cell-layer (Allemand et al. 2004). Traditionally, COC is considered a place where the first nucleation of crystalline starts (Bryan and Hill 1941). After COC formation, fibers are formed, with fibrous aragonite in turn constructed by sequential growth of micrometer-thick layers and the boundaries are arranged concentrically around the COC. To understand coral biomineralization, it is important to reveal the mineralization process of fibers, which compose approximately 97 % of coral skeleton structures (Allison 1996).

The boundary of layers is called the “growth line” on the fiber area. The growth lines were considered to reflect a day cycle (Barnes 1972), and it was pointed out that calcification processes of COCs and fibers related to the diurnal cycle with photosynthesis by symbiotic algae in coral tissue (Gladfelter 1983; Cohen and McConnaughey 2003). The forming mechanism of growth lines seems to have strong connection with the life rhythm of coral skeleton. It is

important for establishing the mechanism to become clear the relevance of the biological activity of coral tissue and the forming mechanism of coral skeletal.

The growth line is thought to be resulting from periodic different crystallographic texture, such as crystal morphology and grain size. Previous observations by scanning electron microscope (SEM) and atomic force microscope (AFM) showed crystallographic difference related with growth lines in fiber area (e.g., Cuif and Dauphin 2005a). However, in SEM observation, it is difficult for crystals on a coral skeleton to retain the original skeletal texture and crystal morphology, because acid etching is usually performed during sample preparation for SEM in order to distinguish the layered structure clearly. Particle size determined by AFM analysis does not always correspond directly to grain size, because of the high sensitivity to surface features. These observations may not characterize accurate crystallographic texture.

Here, this chapter will report the mineralogical skeletal texture of growth lines on fiber in coral skeleton of *Porites lobata* observed by an analytical transmission electron microscope (ATEM) from the nano- to micrometer scale. *Porites* sp. is a popular massive coral as reef-building coral. It is possible to distinguish growth lines on massive coral skeletons easier than dendritical coral skeletons under optical microscope observation without chemical etching. The skeletons have been commonly used for reconstructing paleo climate, then chemical analysis on their skeleton were reported a lot. It is possible to observe crystallographic texture in coral skeleton and identify the mineral phase in-situ by ATEM. This observations show a distinct crystallographic texture and comparing the crystallographic texture with reported chemical data will provide new insight into coral calcification.

2-2. Materials and Methods

Sample Preparation

The examined skeletons of the massive coral *Porites lobata* were collected from Ishigaki Island, Okinawa prefecture, Japan, in August 2007. *Porites lobata* was fist-form, 6 cm in diameter and cut off horizontally to the growth axis. To remove coral tissue, detrital materials and salt from the surface of the skeletons, the following treatments were performed. The samples were cleaned ultrasonically, first, in distilled water; second, in ethanol at room temperature several times; then, in 30 % H_2O_2 for removing organic materials from surface of coral skeleton at 40 °C for one hour; and finally, in distilled water at room temperature several times. After the cleaning, the samples were dried at room temperature for a few days. The dried samples were imbedded in epoxy and polished into petrological thin sections for optical microscopic observation.

ATEM observation

Nanoscale observations were performed using a ATEM (JEOL JEM-2010, Fig. 2-1) operated at 200 kV. Element distribution mapping was obtained using scanning transmission electron microscope STEM (HD-2000) with EDX (Genesis) operated at 200 kV and 30 nA (Fig. 2-2). For ATEM and STEM sample preparation, the samples were cut from petrological thin sections and mounted on molybdenum grids. A Gatan Duomil 600 (Fig. 2-3) Ar ion milling apparatus was used to thin the central region of these pieces to a thickness less than 100 nm. These samples were coated with carbon for the elimination of sample charge-up. It is noted that chemical etching was not carried for the samples.

2-3. Results

Thin section images of coral skeleton under an optical microscope are shown in Fig. 2-4. A characteristic fan-shaped texture is observed. The fan areas have relatively higher interference colors under cross-polarized light and correspond to so-called “fibers” described in Pratz (1882). Center of a fun with lower-interference colors is the “center of calcifications (COC)” (Ogilvie 1896). Layered patterns are observed on the fiber area under the plane-polarized light. The layered patterns have 1-2 μm intervals and are arranged in a wavelike formation concentrically to the COCs. These features correspond with the “growth lines” (Cuif et al. 2003).

TEM observation for the fiber area including several growth lines in Fig. 2-4 provides additional detailed mineralogical information about the coral skeleton (Fig. 2-5). The fiber area shows bundles of needle-like crystal shapes a few hundred nm in width and several μm in length. Electron diffraction patterns indicate that the mineral phase of the crystals is only aragonite elongated along the crystallographic *c*-axis (Fig. 2-5a). The dark-field image in Fig. 2-5b reveals that the crystal orientation next to each other does not seem to have any other preferred orientation except *c*-axis of aragonite extension. If crystal *a*-axis and *b*-axis have any preferred orientation, the contrast should show regular pattern in the dark field image reflected the crystallographic orientation.

Characteristic periodic bright and dark contrast with 1-2 μm intervals can be observed in the fiber area. The contrast is oriented almost perpendicularly to the growth direction of aragonite and seems to match the growth lines observed by an optical microscope.

Elemental mapping images are conducted in the same area as the ATEM observation. Periodical contrast is observed in the Ca mapping image (Fig. 2-6a), although the S and Mg contents are too small to detect the periodical contrast. SEM image, which reflects surface

features of materials, also show periodical contrast corresponding to the Ca mapping image (Fig. 2-6b). Then the contrast of Ca mapping relates to the sample thickness.

Fig. 2-7a shows the crystals across growth lines. The area including holes is corresponding to bright area in growth line. The electron diffraction patterns from three different areas (Fig. 2-7b) indicate that these are from a same crystal, because these patterns have same crystallographic zone axis and same crystallographic orientation. This indicates that some growth lines are across the interior of a crystal grain.

2-4. Discussion

Two interesting main models were proposed for the fiber mineralization process involving inorganic or organic model. Both models considered that the growth lines in the fiber area reflected different grain size distributions. Holcomb et al. (2009) performed precipitation experiments of crystalline aragonite by the stepped addition of Na_2CO_3 solution which changes the $CaCO_3$ saturation state of seawater solution. They confirmed that the precipitated assemble had a texture similar to the growth line in coral skeleton, and the layered structure was characterized by fibrous grains with micron size and granular grains with sub-micron size. The size of crystal grains in a same mineral phase is determined by the rate of nucleation relative to the rate of crystal growth in crystallization process. In general, the grain size is smaller when nucleation rate predominant over crystal growth rate. Changing the $CaCO_3$ saturation degree by addition and stop of Na_2CO_3 solution provide favorable environment for nucleation and crystal growth alternately. Thus, the periodic size distribution should appear as a result of chemical changing the $CaCO_3$ concentration in the calcification medium filled calcification space repeatedly caused by the activity of daily cycles in coral tissue. On the other hands, Cuif and Dauphin (2005b; 2005a) suggested that the difference in aragonite grain size was caused by organic matrix sheets. Nanograins of aragonite observed by AFM may precipitate when the matrix sheets are inserted into the coral skeleton in connection with the biogenetic cycle because they considered that the organic material promotes the nucleation of aragonite.

Nevertheless previous works (Cuif and Dauphin 2005a; Holcomb et al. 2009) expected periodically appearance of nanosize aragonite corresponding to growth lines in the fibers area, there is no evidence of assembly with nanosize aragonite grains related to growth lines in this study. Crystalline aragonite in the fiber area shows relatively equigranular needle-like grains.

The lack of a specific size distribution of aragonite crystals in the fiber area implies that crystals continue growing as they form growth lines. Thus, coral maintains $CaCO_3$ supersaturated environment where growth rate of aragonite crystal is predominant over nucleation in the calcification space. It is necessary to reexamine the biomineralization model of the forming of growth lines.

How are the growth lines made on fibers without different crystal sizes? This study shows that the growth line is relatively weak for ion spattering. Some crystals across growth lines imply the thickness is different in the same aragonite crystal. Such contrast due to the sample thickness is not common, because TEM samples are usually expected to be made wedge-shaped by the ion-spattering process in the case of the homogeneous sample preparation. It is well known that the hardness of metals for ion spattering strongly depends on the amount of incorporated elements (Collings and Collings 1975). This dependency may also occur for the case of aragonite assemblies in coral skeletons because a clear correlation between growth lines and some element, such as S and Mg which might be as organic compounds, have been reported (Cuif et al. 2003; Meibom et al. 2007). It can be observed in a chemically etched SEM sample reported (e.g., Nothdurft and Webb 2007) that as the aragonite solubility in acid solution changes, periodical chemical heterogeneity in the aragonite crystal occurs. It is supported by that a $CaCO_3$ crystal, at least calcite, could continue to grow while the crystal incorporate the organic molecule into themselves (Heinz 1996).

As described above, growth lines are not characterized by periodical distribution of grain size resulted from daily activity of coral tissue in our observation. Moreover, it is possible to form the crystallographic texture in this study under physico-chemical environment. The resemblance between the texture in coral skeleton and collform texture formed by geometric selection, as

pointed out also in Barnes (1970), supports this possibility. When organic components control the crystallization, the crystallographic texture and morphology may be characterized specifically. The aragonite crystals of nacre shells show unique crystal morphology of hexagonal platelets and harmonically oriented along a crystallographic direction by organic matrix-inserted (Watabe 1965; Yoshimi et al. 2004). On the other hand, the present observation shows that crystalline aragonite of fiber area in coral skeleton are needle-like grains aligned in the crystallographic *c*-axis of aragonite. Crystallographic alignment of the *a* and *b*-axis seem to be random orientation. It is well known that the crystalline growth direction of aragonite is along the *c*-axis in general and that they have the crystal morphology of needle-like shapes without special force.

2-5. Summary

The observations for fiber in coral skeleton reveal that the fibers consist of relatively equigranular needle-like aragonite crystal. The growth lines characterized as the layered texture of fibers in coral skeletons are caused by different hardness in the same crystal. These suggest that coral holds $CaCO_3$ supersaturated fluid in the calcification space during fiber forming. The activity of coral tissue can change the elemental composition of calcification liquid. However, it does not influent strongly for changing crystallographic morphology of crystals in fiber.

The results in this chapter were published as an article in the Journal of Structural Biology 180, 389-393 (2012).

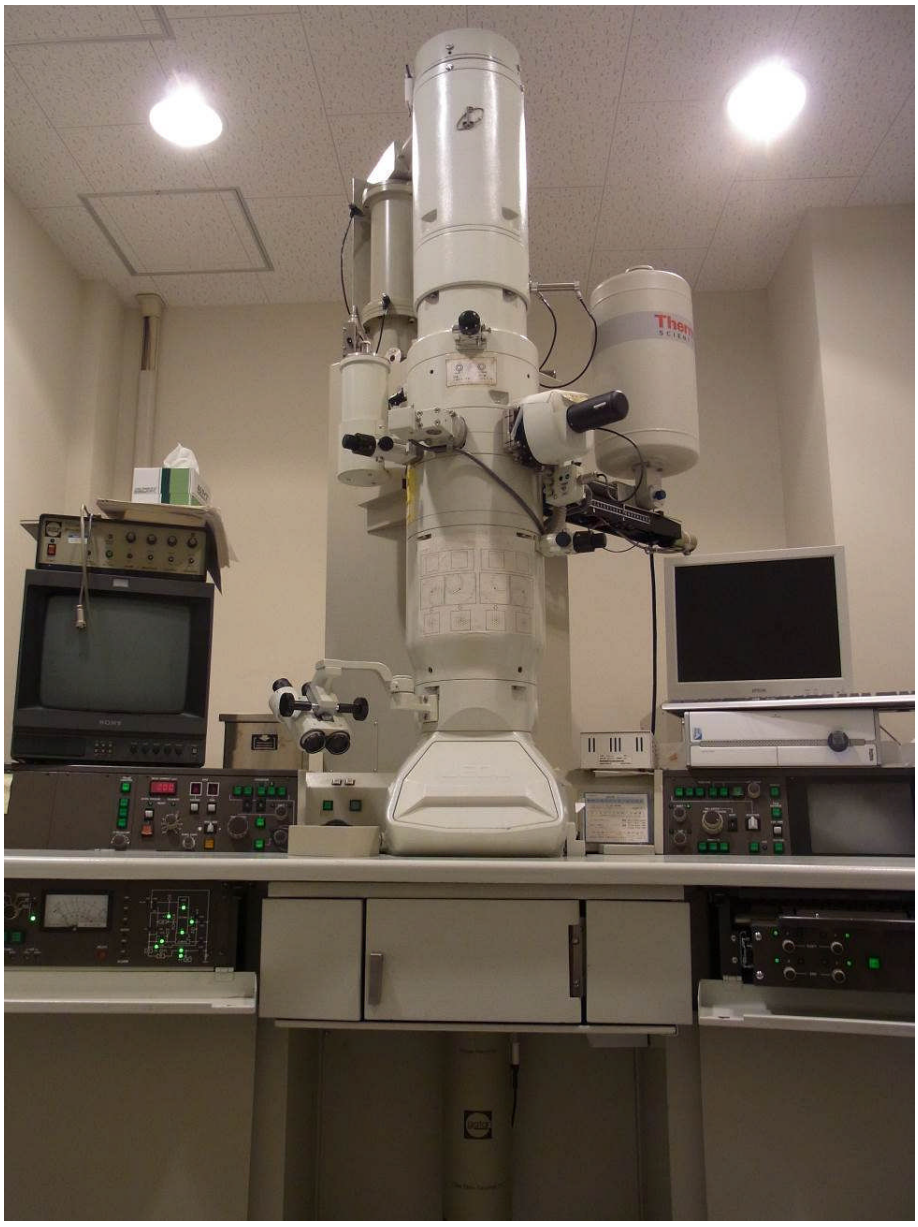

Figure 2-1. Photo of analytical transmission electron microscopy (ATEM) installed in our laboratory.

Figure 2-2. Photo of scanning transmission electron microscope STEM (HD-2000) with EDX (Genesis) in Creative Research Institution, Hokkaido University.

Figure 2-3. Photo of A Gatan Duomil 600 Ar ion milling apparatus in our laboratory.

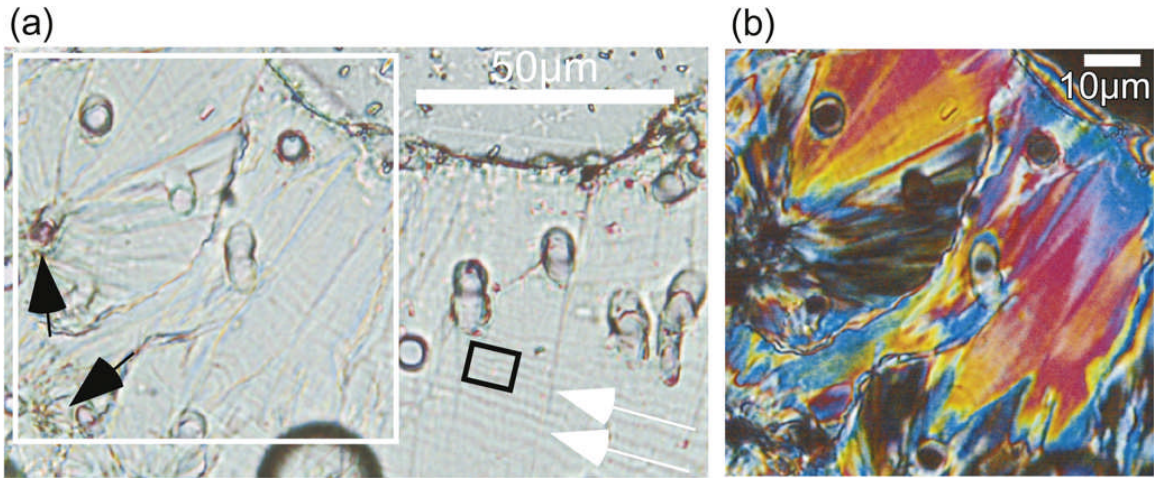

Figure 2-4. Thin section of *Porites lobata* skeleton under a microscope: (a) transmitted light and (b) polarized light of white frame area in (a). The growth direction of coral skeleton is from the bottom to the top of the picture. The skeleton consisted of a center of calcification (COC; black arrow) and fibers. The crystals radiated from the COC. There are striped growth lines (white arrow) a few μm in width and parallel to the COC on fibers.

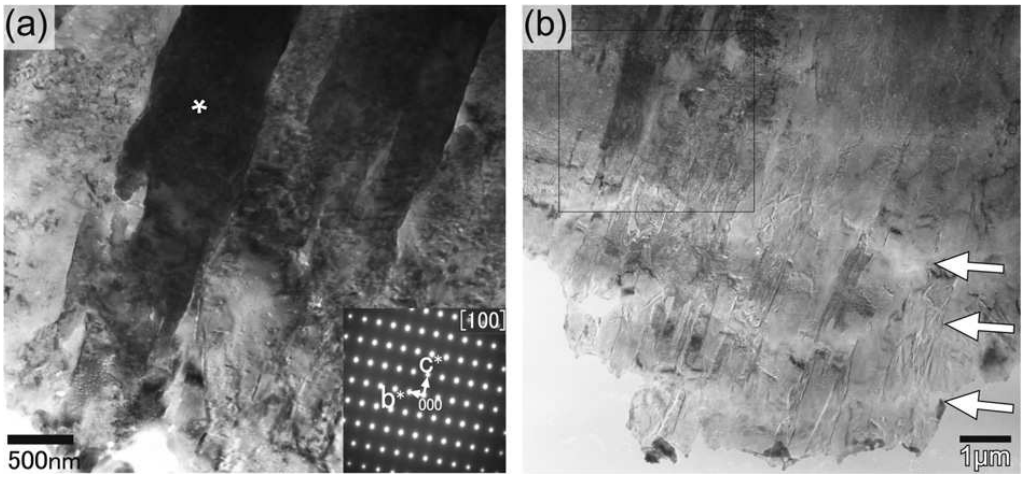

Figure 2-5. Skeletal microstructure of fiber area with several growth lines in *Porites lobata* by ATEM in the black frame of Fig.1a. (a) Crystals are in the fiber area. There is stripe contrast (white arrow), and the interval is approximately 1μm. (b) . Dark field transmission electron image of the crystals formed fibers. The lower image is the diffraction pattern of the [100] form of aragonite from as asterisk crystal.

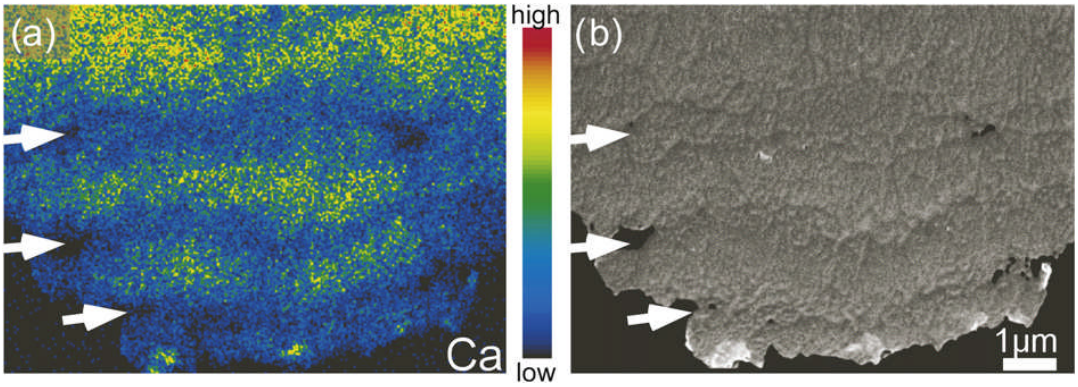

Figure 2-6. The element mapping image obtained by STEM on the same area shown in Fig. 2. (a) Ca, (b) SEM image. Redder hues indicate high concentrations, and bluer hues indicate low concentrations. The white arrows correspond to the stripe contrast.

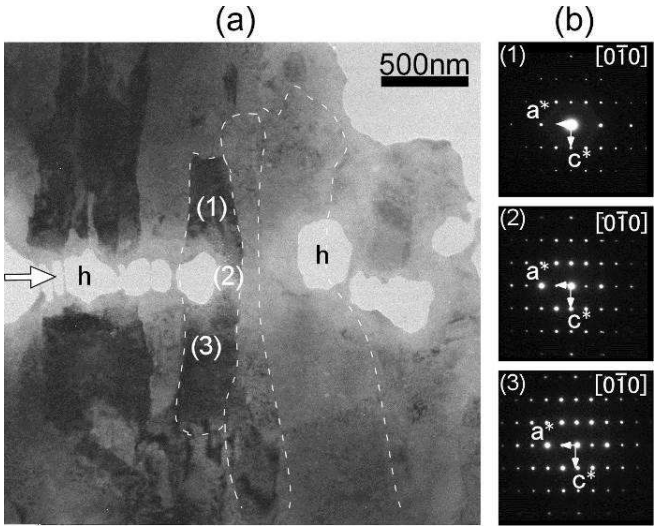

Figure 2-7. The detail view of growth lines. (a) Dark field transmission electron image of the fiber area. ‘h’ means hole of sample and the white arrows is corresponding to growth line. Dashed lines present outline form of a crystal. The dark contrast on crystals indicates that they have mostly the same crystallographic orientation. (b) Electron diffraction patterns. The number written in upper left means the diffraction pattern from the numbered area in (a). The all diffraction patterns show the [0-10] form of aragonite and same crystal orientation.

3. Precipitation of halite during calcification in massive reef-building coral, *Porites lobata*

3-1. Purpose

Mineral phases, their texture and crystallographic morphology of a material is important to understand how the material form, because these mineralogical and crystallographic feature indicate their growth environments. In the case of reef-building coral skeleton, Almost all of them is typically composed of only one mineral phase, pure aragonite ($CaCO_3$) from X-ray diffraction analysis for bulk coral skeleton. Although many kinds of minor element have been detected in coral skeleton from chemical analyses (e. g., Okai et al. 2002), the presence of such minor elements has been interpreted in several ways: as an impurity in aragonite, as a result of ion absorption into grain boundaries (e.g., Amiel et al. 1973; Mitsuguchi et al. 2010).

In chapter 2, the ATEM observation revealed that needle-like aragonite crystals with a few micron meters in length formed the fiber area of reef-building coral skeleton, *Porites lobata*. In a more detailed observation in the same fiber area of coral skeleton, I observe also nanosized granular crystals spread in the area. The nanosized crystals are different size and morphology from needle-like aragonite crystals which were reported past observation (Benzerara et al. 2011; Motai et al. 2012). In this chapter, I will identify mineral phases of these nanosized granular crystals and I will describe and discuss about nanosized crystals in fiber area of coral skeleton. That is because that crystalline inorganic materials other than aragonite are rarely observed, like following. The presence of calcite, which is another polymorph of $CaCO_3$ that is thermodynamically stable under conditions favorable for coral growth, had been reported previously (e. g., Vandermeulen and Watabe 1973). Greegor et al. (1997) identified strontianite

($SrCO_3$) as an independent phase of aragonite based on extended X-ray absorption fine structure (EXAFS) data, although later more precise EXAFS measurements concluded that small amounts of strontium ions could substitute for calcium ions in aragonite (Allison et al. 2001; Finch et al. 2003). It has also been suggested that brucite ($Mg(OH)_2$) is merely a secondary mineral encrusting the surface of coral skeleton based on scanning electron microscope with EDS and FT–IR observations (Nothdurft et al. 2005).

These reports about minerals other than aragonite seemed to be lack evidences for primary precipitation during coral calcification. However, when it could be noted the presence of other primary precipitated minerals except for aragonite during coral calcification, these minerals will yield important and new information about calcification medium.

3-2. Samples and methods

Sample Preparation

The skeletons of the massive coral *Porites lobata* used in this study were collected from Ishigaki Island, Okinawa Prefecture, Japan, in August 2007. This sample is same in chapter 1. *Porites lobata* was hemispherical, 6 cm in diameter, and cut perpendicularly to the growth axis. The following steps were performed following Shirai et al. (2008) to remove coral tissue, detrital materials, and salt from the surfaces of the skeletons. The samples were cleaned ultrasonically several times in distillated water and then in ethanol at room temperature, before being cleaned at 40°C for one hour using 30% H_2O_2 to remove organic materials from the surfaces of the coral skeleton. Finally, the samples were cleaned several times in distillated water at room temperature. After cleaning, the samples were dried at room temperature for a few days. The dried samples were imbedded in epoxy and polished into petrological thin sections. The sample was polished with water, until the thickness was less than 30 μm. This procedure was sufficient to remove contamination. An optical microscope was used to define observation areas following (Ogilvie 1986), which appeared as dark points under the microscope and are referred to as centers of calcifications (COC). Optical microscopy was also used to define fiber areas with growth lines: fiber areas were defined as fibrous bundles encompassing a COCs, with, growth lines typically arranged concentrically around COCs in fiber area.

ATEM observation

Nanoscale observations and elemental analysis were performed using an ATEM (JEOL JEM-2010) operated at 200 kV with NORAN system SIX (Thermo Electron) (Fig. 2-1). In preparation for the ATEM, the samples were cut out from where was located at around 2 mm and

15 mm from surface of coral skeleton. Then, samples were removed from the thin section glass, and mounted on molybdenum grids. The cut areas were approximately 1 mm^2, including fiber area with growth lines that were analyzed under an optical microscope. A Gatan Duomil 600 Ar ion milling apparatus (Fig. 2-3) was used to thin the central regions of these pieces to thicknesses of less than 100 nm. These samples were coated with carbon to eliminate charge-up on their surfaces.

FT–IR measurement

All IR transmission spectra were recorded at 4 cm^{-1} resolution by 32 scans with a Fourier transform infrared spectrometer (FT–IR 6100) and an IRT-5000 infrared microscope in the wave number range 4000–600 cm^{-1} under air conditions (Fig. 3-1). The size of the aperture was 50 × 50 μm, and the sample configuration was as for the ATEM sample. A background spectrum was measured for air.

3-3. Results and Discussions

Mineral Texture in Coral Skeleton

Fig. 3-2 presents an ATEM image of a fiber area, including some growth lines, in the coral skeleton. Aragonite grains are elongated along their crystallographic *c*-axis, exhibiting needle-like shape, and are typically a few hundred nanometers in width and several micrometers in length, as described by Motai et al. (2012). Growth lines are recognized as banding contrasts and are oriented almost perpendicular to the needle direction. More detailed ATEM observation of the same sample highlights the existence of dark granular contract in the aragonite crystals, at scales of 20 – 220 nm (Fig. 3-3). These dark contrasts are observed in both samples which were taken from surface and interior of coral skeleton. Furthermore, these contrasts exhibit diffraction patterns, indicating the presence of a crystalline material with cubic crystallographic symmetry (Fig. 3-2c). Elemental analysis results show that the material includes only sodium and chlorine. Thus, the dark contracts can be identified as crystalline NaCl, which forms the mineral halite. Each halite crystal exhibits the square cross-sectional form that is typical of euhedral halite. Halite grains are distributed inhomogeneously and seem to be independent of the arrangement of growth lines (Fig. 3-4).

Electron diffraction patterns from both aragonite and halite are shown in Fig. 3-5, which shows that halite has following two types of specific crystallographic orientation relationships with the aragonite host:

(a) $\{010\}_{aragonite}$ // $(220)_{halite}$, $\{203\}_{aragonite}$ // $\{\bar{2}20\}_{halite}$ and $<30\bar{2}>_{aragonite}$ // $<00\bar{1}>_{halite}$ (Fig. 3-4a),

(b) $\{111\}_{aragonite}$ // $(020)_{halite}$, $\{0\bar{2}1\}_{aragonite}$ // $\{\bar{2}00\}_{halite,}$ and $<\bar{3}12>_{aragonite}$ // $<00\bar{1}>_{halite}$ (Fig. 3-4b).

The crystallographic orientation relationship of type (a) suggests that halite (100), which is the external face of halite observed with ATEM (Fig. 3-5), contact with the aragonite $(\bar{3}\bar{1}3)$. As shown in Fig 3-5a, the arrangement of CO_3^{2-} ions on this aragonite surface is similar to that of Cl^- ions on halite (100). On this aragonite plane, the positions of triangular carbonate ion CO_3^{2-} could preset as corners of quadrangles which is similar to position of choline ion Cl^- of halite. Misfit between CO_3^{2-}-CO_3^{2-} distance in aragonite $(\bar{3}\bar{1}3)$ and Cl^--Cl^- distance in halite (100) is presented in table 3-1a. In the same way, the orientation relationship (b) shows that the aragonite $(\bar{2}21)$ connects on halite (100), and the misfit ratios of this case are presented in table 3-1b. These misfit ratios between the length of diagonal of cubic unit cell of halite and *b*-axis of aragonite are less than 10%, which shows that halite and aragonite could have hetero-epitaxial relationship.

It has been noted that salt evaporated from seawater could remain in coral skeleton during sample preparation (Swart 1981). However, the fact that halite has specific crystallographic orientation relationship with aragonite strongly suggests that halite grains observed in this study are not secondary formed during sample precipitation. The crystallographic relationship between aragonite and halite should be at random, if the halite precipitated as secondary mineral phases. Furthermore, the sample preparation process used in the present study was designed specifically to avoid such contamination. And, the halite grains here are found not only along grain boundaries, but also within the aragonite grains, and the halite appears to be in tight contact with the surrounding aragonite. In cases where secondary halite is precipitated during sample

preparation, the halite tends to assemble locally on the surfaces and/or along the boundaries of aragonite grains and in the spaces in which remaining water may have evaporated from fluid inclusion in coral skeleton. Thus, the evidence presented here strongly suggests that the halite grains observed are not secondary deposits; rather, they co-precipitated with aragonite in the calcification medium.

It is interesting the presence of mineral other than aragonite in coral skeletons. It has been believed that coral skeleton is typically composed of almost pure aragonite, such that other crystalline inorganic materials are rarely observed. The presence of calcite in skeletons of larval coral has been reported previously by using X-ray diffraction (e. g., Vandermeulen and Watabe 1973). Calcite is another polymorph of $CaCO_3$ that is thermodynamically stable under conditions favorable for coral growth. However, recent observations by Raman spectroscopy (Cuif and Dauphin 1998) and X-ray diffraction (Sowa et al. 2008) did not support the occurrence of the primary deposition of calcite in coral skeleton. Existence of strontianite and brucite have also been reported in coral skeleton (Greegor et al. 1997; Nothdurft et al. 2005), although both mineral phases have not been considered as primary precipitated mineral from calcification medium (Allison et al. 2001; Finch et al. 2003; Nothdurft et al. 2005). Therefore, the halite observed in this study represents the first evidence for primary deposition of a mineral phase other than aragonite in coral skeleton.

Sodium and chlorine contents of coral skeletons have been analyzed previously (Land and Hoops 1973; Oomori et al. 1982), and the presence of sodium has been interpreted in several ways: as an impurity in aragonite, as a result of ion absorption into grain boundaries, or as part of other organic compounds (e.g., Amiel et al. 1973; Mitsuguchi et al. 2010). Similarly, the presence of chlorine has been attributed to contamination from evaporated salt (Swart 1981).

However, the present study suggests that the presence of these elements in coral skeleton can be attributed, at least in partly, to halite grains that precipitated simultaneously with aragonite.

Implication of Halite for Calcification State

Although whether the calcification occur in extracellular or intracellular had been debated for long time, now extracellular calcification has been widely accepted rather than intracellular by more assured reasons; the observation of calcification space in coral by electron microscope (Johnston 1980). However, Tambutte et al. (2007) suggested that the calcification space may simply be an artifact of microscopic observation in a vacuum. The discovery of halite coprecipitated with aragonite is significant, because it indicates that the concentration of NaCl sometimes and/or locally reaches saturation in the calcification space. This supports the occurrence of calcification in the extracellular space, because the cells of marine invertebrates (and other organisms) usually kept lower level of sodium ions concentration intracellular than extracellular to avoid that high level of sodium ions concentrations inhibit cell activity (Kirschner 1991).

The ions utilized for calcification must be sourced from the external seawater into the calcification space. However, the seawater is typically undersaturated with respect to halite. Therefore, some additional mechanisms must be invoked to explain the over saturated for halite concentration during the transport of ions from seawater to the calcification space. Seawater includes abundant sodium, chlorine, and sulfate ions and sodium and sulfur are also abundant as trace elements in coral skeleton (Oomori et al. 1982; Mitsuguchi et al. 2010). It is possible that calcification medium keep the same chemical compositional ratio except $CaCO_3$ of seawater. The ratio of sodium and chlorine ions is almost same, so sodium/sulfate ion can act as a factor of

chemical proportion of seawater. Therefore, assuming that the calcification medium maintains the same sodium/sulfate ion ratio as seawater, gypsum ($CaSO_4 \cdot 2H_2O$) or anhydrite ($CaSO_4$) should be crystallized before sodium and chlorine can precipitate as halite (Scoffin 1987). In this ATEM observation and past XRD analysis, gypsum and anhydrite grains were not observed in coral skeleton, although that might be caused by characteristic of ATEM observation specialized for very narrow space in micron order and XRD analysis for detected main mineral phased in the sample. It is difficult to detect gypsum grains in the case that they are small amount in coral skeleton and locate in one spot.

Then, in the fiber area including both aragonite and halite was analyzed by the FT–IR measurements for confirming the existent of gypsum and anhydrite (Fig. 3-6). The FT-IR measurements can analyze selected area unlike bulk XRD and larger area than that of ATEM observation. FT–IR profiles indicated mineral aragonite by prominent absorption at 1490 cm^{-1} (ν3), 1080 cm^{-1} (ν1), 870–875 cm^{-1} (ν2), and 699 cm^{-1} and 712 cm^{-1} (ν4). The broad absorption peak at around 3400 cm^{-1} show organic band, the amide A and at 1550 cm^{-1} shows amide I and/or II. There are not major bands for gypsum 673 cm^{-1}, 1681 cm^{-1}, 1618 cm^{-1} and 3549 to 3242 cm^{-1} having doublet at least, or major bands for anhydrite doublet 676–616 cm^{-1}, then the FT-IR spectra suggest that no such sulfate mineral phases are present. These absorption profiles were confirmed by comparing the FT–IR measurements with those of Dauphin et al. (2006), who identified the mineral aragonite and indicated that the sulfur was included in organic composition of coral bulk skeleton. The absence of calcium sulfate suggests that the sodium/sulfate ion ratio in the calcification medium is much higher than that in seawater, indicating that a selective mechanism for the transport of ions from seawater to the calcification space may operate in coral skeleton.

Implication of mineral phases in coral skeleton for ion transport

It has recently been suggested that in the composition of the calcification medium is different to that of the surrounding external seawater, with higher calcium concentrations and pH values (Al-Horani et al. 2003). Tambutte et al. (1996) proposed a selective transport pathway for calcium ions in the production of aragonite and Al-Horani et al. (2003) proposed ATPase pump to elevates Ca^{2+} in the region of calcification. This study added the new chemical information that concentration of Na^{+} and Cl^{-} sometimes become over saturated and SO_4^{2-} ions are relatively small amount for these ions comparing with seawater. This implicates existence of selected paths for Na^{+}, Cl^{-} and SO_4^{2-}.

Annual fluctuations of the sodium/calcium ratio of coral skeleton were compared with environmental factors derived such as sea surface temperature, salinity, and so on (e.g., Land and Hoops 1973; Swart 1981). However, these fluctuations were found to exhibit little relationship with sea surface salinity, sea surface temperature, or seawater sodium/calcium (Mitsuguchi et al. 2010; Mitsuguchi and Kawakami 2012). This no relationship between sodium/calcium fluctuations and environment factors caused from coral selectivity for sodium ion concentration in the calcification medium. The selectivity would not allow sodium/calcium ratio in calcification medium affected directly or immediately by changes in the surrounding environment.

How do concentration of Na^{+}, Cl^{-} become higher and/or concentration of SO_4^{2-} become lower in calcification medium than that in seawater? Gagnon et al. (2012) and Tambutte et al. (2012) discussed about a pathway based on analysis of tracer elements, $^{43}Ca^{2+}$, $^{87}Sr^{2+}$, $^{136}Ba^{2+}$, Tb^{3+} and calcein neutral molecules, in coral skeleton. After the cultured period of coral in seawater with tracer is 20 minutes short in experiment with calcein and 155 hours long with other tracers, these

tracers could be detected in coral skeleton. These experiments suggest that coral allows the transport with diffusion of any component material of seawater into the calcification space regardless of their valency or size in cation and neutral.

In this case that any ions and molecules can transport regardless of their valency or size, Na^+, Cl^- and SO_4^{2-} could pass thought this diffusion pass, because size of these ions are smaller than above tracer molecules calcein. However, this pathway keep same Na^+/ SO_4^{2-} ratio between in calcification medium and seawater. For achieving the higher Na^+/ SO_4^{2-} ratio in calcification medium than that of seawater, there should be mechanism for accumulating Na^+ and Cl^-. It is also possible that coral have strict selectivity for anion ions, although there is no suggestion about pathway for anion ion.

3-4. Summary

ATEM observation revealed that there are halite grains in coral skeletal aragonite. The crystallographic relationship of halite and aragonite surrounded it seen similar to a kind of epitaxially-formed crystals. This indicates these halite grains precipitated during calcification. There is no gypsum and anhydrite in the area included both aragonite and halite grains suggest that Na^{+}/SO_4^{2-} ratio in calcification is higher than that of seawater.

Figure 3-1. Photo of Fourier transform infrared spectrometer (FT-IR) installed in our laboratory.

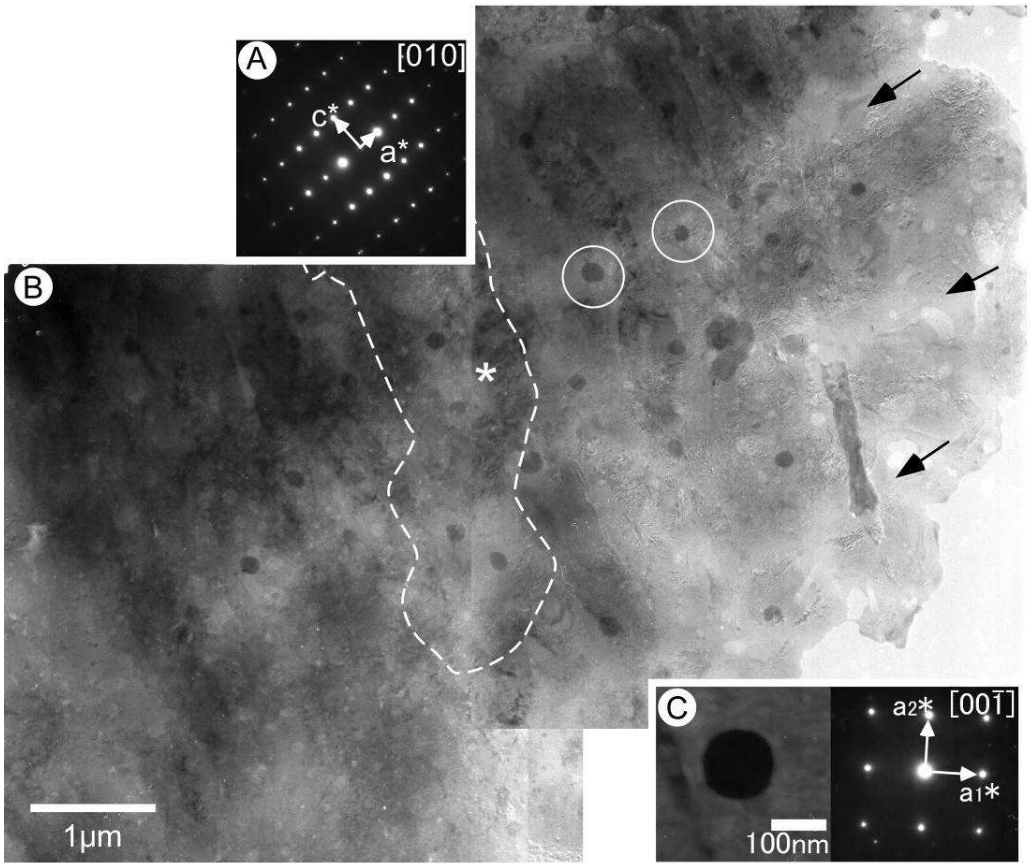

Figure 3-2. Skeletal microstructure of the fiber area with several growth lines in *Porites lobata*, obtained using an analytical transmission electron microscope (ATEM). (A) The diffraction pattern of the [010] zone axis of aragonite from around asterisk. (B) Overall view of a fiber area. Banding contrasts (black arrow) with an interval of approximately 1 μm correspond to growth lines. The dashed line represents the crystal outlines, determined from the dark field image. Some regions with dark contract are observed (enclosed by circles). (C) Magnified view of a regions with dark contract and its diffraction pattern. The pattern indicates the [00-1] zone axis of halite.

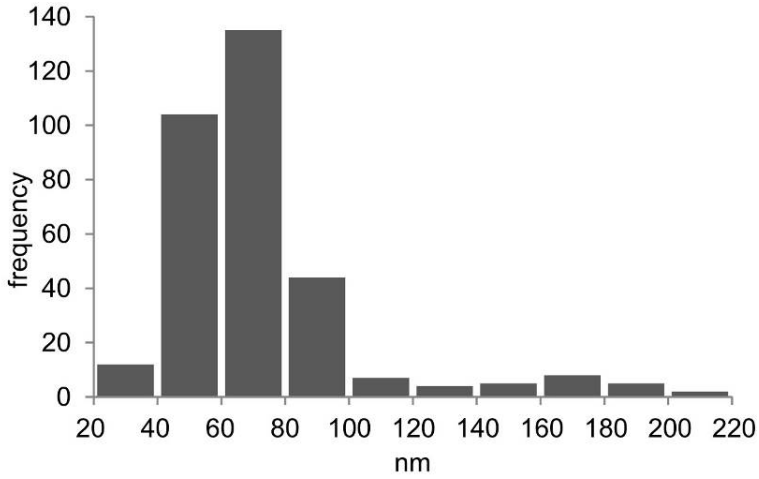

Figure 3-3. Size distribution of halite grains (n = 326) in fiber area of coral skeleton. Sizes represent length of sides of halite grains.

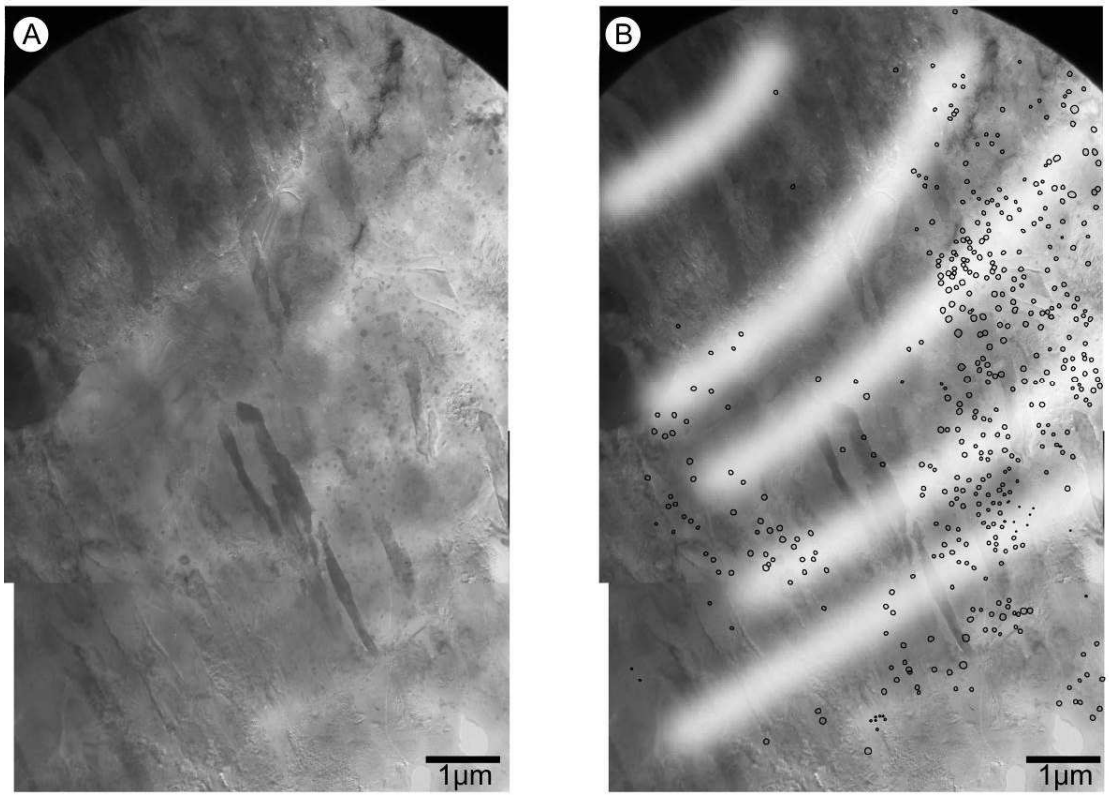

Figure 3-4. (A) Analytical transmission electron microscope (ATEM) image of the fiber area with several growth lines. (B) The view which is add position of the growth lines by white line and halite by enclosed the outline of these grains on (A) for clear the relationship between growth lines and distribution of halite. The fiber area is about 70 μm^2 and the halite occupies about 2.5% of this area.

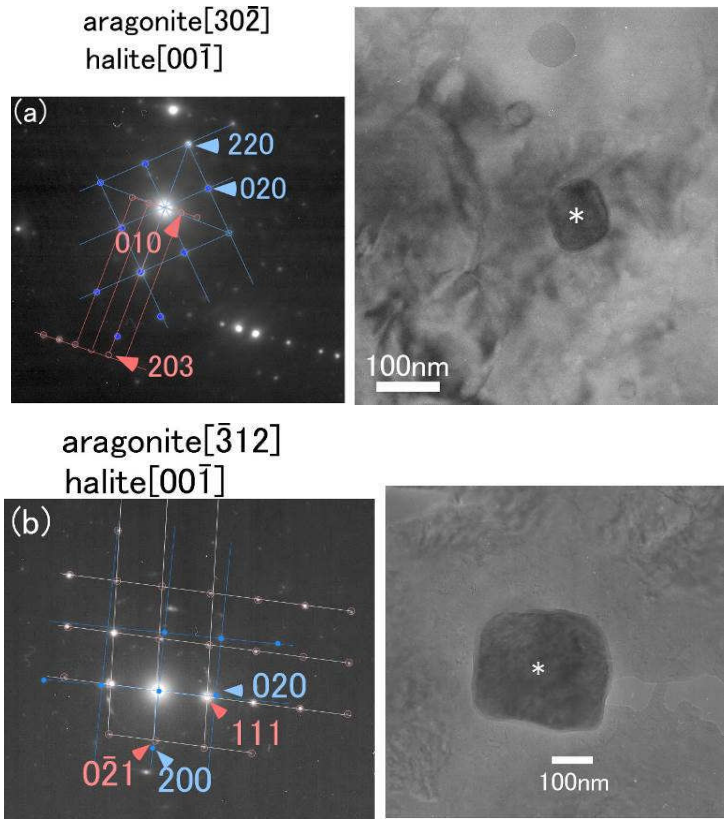

Figure 3-5. (a), (b) Diffraction patterns from the area including aragonite and halite grain. Pink-letters are reciprocal lattice from aragonite, and blue-letters are that form halite.

Table 3-1. The misfit ratio using by distance between sites of carbonates on aragonite and sites of choline on halite from Fig.4 (a) and (b) respectively.

(a)	aragonite	halite	Misfit ratio(%)
distance	7.59	7.98	**5.1**
distance	7.26	7.98	**9.9**
distance	7.96	7.98	**0.2**
angle	86	90	**5.3**

(b)	aragonite	halite	Misfit ratio(%)
distance	11.37	11.28	**-0.8**
distance	12.06	11.28	**-6.4**
angle	81.26	90	**10.8**

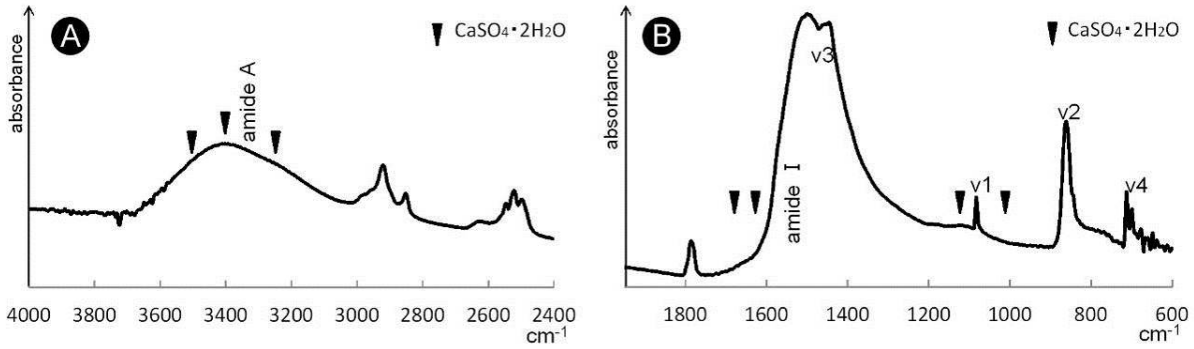

Figure 3-6. Fourier transform infrared spectrometer (FT–IR) profile of the coral fiber area illustrated in Fig. 1. The profiles cover (A) 4000–2400 cm^{-1} and (B) 2000–600 cm^{-1}. Mineral aragonite is indicated by prominent absorption at 1490 cm^{-1} (ν3), 1080 cm^{-1} (ν1), 870–875 cm^{-1} (ν2), and 699 cm^{-1} and 712 cm^{-1} (ν4). There are not major bands for gypsum 673 cm^{-1}, 1681 cm^{-1}, 1618 cm^{-1} and 3549 to 3242 cm^{-1} having doublet at least, or major bands for anhydrite doublet 676–616 cm^{-1}.

4. Mineral phase of skeleton in reef-building coral polyp cultured in artificial seawater with different Mg/Ca ratios

4-1. Purpose

Aragonite is a thermodynamically metastable phase in calcium carbonate in coral living environment. It was pointed out that the organic matrix in reef-building coral skeleton controls the coral calcification concerning about the aragonite formation and the skeletal texture (Cuif et al. 1999). On the other hand, extensive studies have been performed in order to find key factors controlling the abiotic precipitation of carbonate minerals (calcite or aragonite) in natural seawaters. They indicated that the determination of $CaCO_3$ polymorph precipitated are often influenced by temperature and chemical compositions of surrounding seawater, especially Mg/Ca molar ratio (e.g., Burton 1993; Morse and He 1993; Morse et al. 1997). In fact, it was reported that the polymorph of abiotic $CaCO_3$ (ooids, marine cements) changed corresponding with variations in the Mg/Ca ratio of seawater in Phanerozoic time (Sandberg 1983).

Morse et al. (1997) identified mineral phases of $CaCO_3$ precipitated inorganically from a series of artificial seawater with different Mg/Ca ratios from 0 °C to 35 °C. They showed that aragonite was likely to precipitate in the state of higher Mg/Ca molar ratio and temperature, and calcite was likely to precipitate in the state lower Mg/Ca ratio and temperature. From these results, aragonite would precipitated from seawaters with over 1.0 of Mg/Ca ratio and calcite would precipitated from seawater under 1.0 of Mg/Ca at 25 °C, which is temperature for coral living. As current Mg/Ca ratio in seawater is 5.2, aragonite phase can be precipitated from seawater inorganically. However, Ries et al. (2006) showed that reef-building coral produced both aragonite and calcite when the corals were cultured in seawater with Mg/Ca molar ratio at

lower than 3.5. This result was different from result of inorganically precipitation of Morse et al. (1997).

In Ries et al. (2006), the colony corals were cultured in artificial seawater. Colony corals already formed aragonite skeleton before the experiment. It is very difficult to be clear the boundary whether the coral skeleton formed before or after coral cultured in artificial seawater. Then, in this chapter, I observed and identified mineral phases of skeleton in cultured experiment under different Mg/Ca molar ratio from larvae of coral to polyp. Because corals do not form any skeleton during the larvae period of coral, it enables to estimate influence of Mg/Ca ratio in the case without substrate of skeleton. It is possible to put coral in minimum activity by using coral polyp, because the polyps have no symbiotic algae inside coral tissue. Coral have a symbiotic relationship with these algae and this relationship is considered to contribute to coral growth. It also could ignore the stress to coral activity by changing surrounding seawater because of tolerance of larvae to the changing.

4-2. Material and Methods

Collection of larvae

Corals in egg period have no algae. Then the polyps without symbiotic algae are used for experiment, before planulae take these algae from seawater into their tissue after extrication. Some branches of *Acropora digitefera* were collected at Sesoko, Okinawa, Japan, and were maintained in separate containers under laboratory conditions in Tropical Biosphere Research Center Sesoko Station, Institute for Marine Biological Laboratory, Ryukyu University. All branches spawned on 29 June 2013. After spawning, sperm and egg bundles from all colonies were mixed and gathered into a containers for fertilisation. The fertilized eggs were washed twice in filtered seawater (FSW) and kept in the containers. After 1.5 day, planulae were moved to plastic containers in preparation for settlement experiments.

Acclimating to artificial seawater

The Mg/Ca molar ratio (= 5.0, 2.5, 1.2) were modified by adding prepared seawater, which have the chemical composition in table 1, to FSW. Corals larvae were acclimated to these artificial seawater treatments over 1 week until the set chemical composition. After acclimating, the larvae were kept under 12 h light: 12 h dark conditions at room temperature (about 24 to 26°C). The seawater filled with containers was refilled with fresh FSW or artificial sweater daily.

Larval metamorphosis and settlement

For larval metamorphosis and settlement, Hym-248 was added to seawater following (Iwao et al. 2002). Hym-248, one of the Hydra-derived GLWamide-family neuropeptides, was

demonstrated to induce both settlement and metamorphosis in cultured planulae of nine Pacific Acropora species and mixed Acropora coral slicks with remarkable consistency (Iwao et al. 2002). Almost larval settled within 12 h.

ICP-MASS

Analysis of Mg, Ca, and Sr in seawater were performed with inductively coupled plasma-atomic emission spectrometry (iCAP 6300 ICP Spectrometer) (Fig. 4-1). The instruments are equipped with a single photomultiplier. Each element was determined in triplicate. Three and five standard solutions were prepared gravimetrically for the ICP-AES measurements. The instruments were calibrated with the standard solutions at intervals of five samples. Calibration curves were calculated by averaging the two calibrations bracketing the six samples, with a linear fit for ICP-AES.

Preparation for sample observation

The following steps were performed to remove coral tissue, detrital materials and salt from the surface of the skeletons. The samples were cleaned ultra- sonically in distillated water, then in ethanol at room temperature several times, followed by liquid sodium hypochlorite to remove organic materials from the surface of the coral skeleton at 40 °C for one hour, and lastly, in distilled water at room temperature several times. After cleaning, the samples were dried at room temperature for a few days.

Raman spectroscopy

The Raman spectra were analyzed at several points from each coral skeleton under different Mg/Ca ratio with a He-Ne laser. A background spectrum was measured for air.

Synchrotron X-ray diffraction measurements

X-ray diffraction (XRD) measurements were performed on bulk powder of samples. Synchrotron X-ray diffraction experiments were conducted at the Photon factory of high energy accelerator research organization (KEK, NE-1) in Tsukuba, Japan (Fig. 4-2). A monochromatic incident synchrotron X-ray beam collimated to a 40 μm diameter with a wavelength of 0.4178 Å was used. The two-dimensional diffraction intensity data recorded on an imaging plate (IP) were integrated along the Debye-Sherrer rings and converted to conventional one-dimensional intensity data using the software of IPAnalyzer and PDIndexer (Seto et al. 2010).

TEM observation

The dried samples were imbedded in epoxy resin. For ATEM sample preparation, an ultrathin section of 80 nm in thickness was prepared by an ultramicrotome EM-Supernova (Fig. 4-3) and mounted on cupper mesh grids.

Nanoscale observations were performed using an ATEM (JEOL JEM-2010) operated at 200 kV. These samples were coated with carbon to eliminate the buildup of charge on the sample (Fig. 2-1).

4-3. Results and discussion

Mineral phase of the cultured coral skeleton

Fig. 4-4 shows optical stereomicroscopic photographs of the skeletons produced in artificial seawaters. The skeletal features in natural seawater are characterized by corallites consisting of septum with the number of it increasing by a factor of 6 and endothecal dissepiments located under polyp. The cultured skeleton in artificial seawater also show basically same as these skeletal feature produced in natural seawater. This indicates that cultured corals could form their skeletons without stress from changing condition from natural seawater to artificial seawater.

XRD patterns of the skeletons produced in artificial seawaters are shown in Fig. 4-5. All diffraction peaks can be explained as aragonite. However, it is generally accepted that XRD may not detect small amount of calcite in the aragonite dominant sample, for example, less than a few volume %. Thus, Raman scattering measurements and ATEM observation were carried out complementarity. These techniques are considered to be favorable to find a small amount of calcite in aragonite dominant sample. Because the calcite precipitated from seawater tends to form solid solution with magnesium (Berner 1975), then sponge forming calcite with $Ca_{0.8}Mg_{0.2}CO_3$ composition was prepared as a standard (Ohmori et al. 2014). Figs. 4-6 and 4-7 show Raman scattering spectra. There are scattering peaks assigned as aragonite at 203, 708 and 712 cm^{-1}, but no calcite with chemical composition of $CaCO_3$ at 718 cm^{-1} nor $Ca_{0.8}Mg_{0.2}CO_3$ at around 270 cm^{-1} can be detected. ATEM photographs are shown in Fig. 4-8. SEAD patterns indicate that all grains observed are aragonite. Aragonite crystals show needle or granular shape with less than one micro meter in dimensions regardless of Mg/Ca molar ratio.

Mg/Ca ratio of calcification medium

X-ray diffraction analysis, Raman scattering analysis and ATEM observation show no calcite and only aragonite can be identified in all skeletons formed in artificial seawater with 5.0, 2.5 and 1.2 of Mg/Ca ratio in initial coral calcification without influence of already-made aragonite skeleton. This result is consistent with the results that only aragonite precipitates inorganically in model seawaters with over 1.0 of Mg/Ca ratio at 25 °C (Morse et al. 1997). It is suggested that the calcification medium has a near value to the current Mg/Ca ratio in the surrounding seawater.

On the other hand, when some colony coral specimens grown in artificial seawater with less than 3.5 of the Mg/Ca ratio, the calcite is found in the coral skeleton (Ries et al. 2006). It is possible that the Mg/Ca ratio in coral calcification medium become lower than that of external seawater in the case of Rise et al. (2006). The activity of coral polyp and colony coral seem to be same, because colonized coral is aggregate of coral polyp clone, which reproduced by asexually. The difference between present results and Rise et al. (2006) is absence or the presence of symbiotic algae inside coral. Coral polyps have no symbiotic algae and colonized corals got symbiotic algae during living in natural seawater before cultured experiment. Then, these results strongly suggest that symbiotic algae play an important role for decreasing of Mg/Ca ratio in calcification medium.

Ion transporting pathways from seawater to calcification medium

Generally, two pathways for ion transport are described: a paracellular and a transcellular pathway (Berridge and Loschman 1972). Paracellular pathway allows ions move through coral tissue by diffusion (Gagnon et al. 2012; Tambutte et al. 2012), and a transcellular pathway was selective a pathway for Ca transport (Tambutte et al. 1996; Al-Horani et al. 2003).

In the case of calcification by polyp coral without symbiotic algae, it is considered that ion transport by paracellular pathway is dominant. Thus, the calcification medium could have the chemical composition and state similar to external seawater. In the case of calcification in coral with symbiotic algae, transecellular pathway contributes a lot to form the high concentration of Ca^{2+} in calcification medium. In fact, Al-Horuni (2003) reported the concentration of Ca^{2+} in calcification medium of colonized coral would be higher than that in seawater. This activity of coral tissue results in lower Mg/Ca ratio in calcification medium than that of external seawater. Then, in the case of Ries et al. (2006), when the Mg/Ca ratio in external seawater is low, the Mg/Ca ratio in the calcification medium would become less than 1.0 which is unfavorable to crystallize aragonite (Morse et al. 1997). Coral is expected to collect the ions of Ca^{2+} into calcification medium for forming skeleton and making up for consumed amount. However, it is noted that incorporation of Ca into the calcification medium should enhance the aragonite formation. This precipitation of aragonite increases the Mg/Ca ratio in the calcification medium, because it is unlikely that Mg atoms are incorporated into aragonite.

4-4. Summary

The coral without symbiotic algae were cultured from polyp period in artificial seawater with Mg/Ca molar ratio = 5.0, 2.5, 1.2. The mineral phase of these skeletons is aragonite. These results are similar to the results of Morse et al. (1997) which calcium carbonate were precipitated inorganically from artificial seawater with different Mg/Ca ratios. This suggests that the Mg/Ca ratio in calcification medium of the cultured corals were similar to the ratio of the external seawater. On the other hand, mineral phases of coral skeleton in this chapter are different from experiment of Ries et al. (2006) which cultured colony coral with symbiotic algae inside of coral in artificial seawater with different Mg/Ca molar ratio. This suggests that symbiotic algae play a role in changing the Mg/Ca ratio in coral calcification medium.

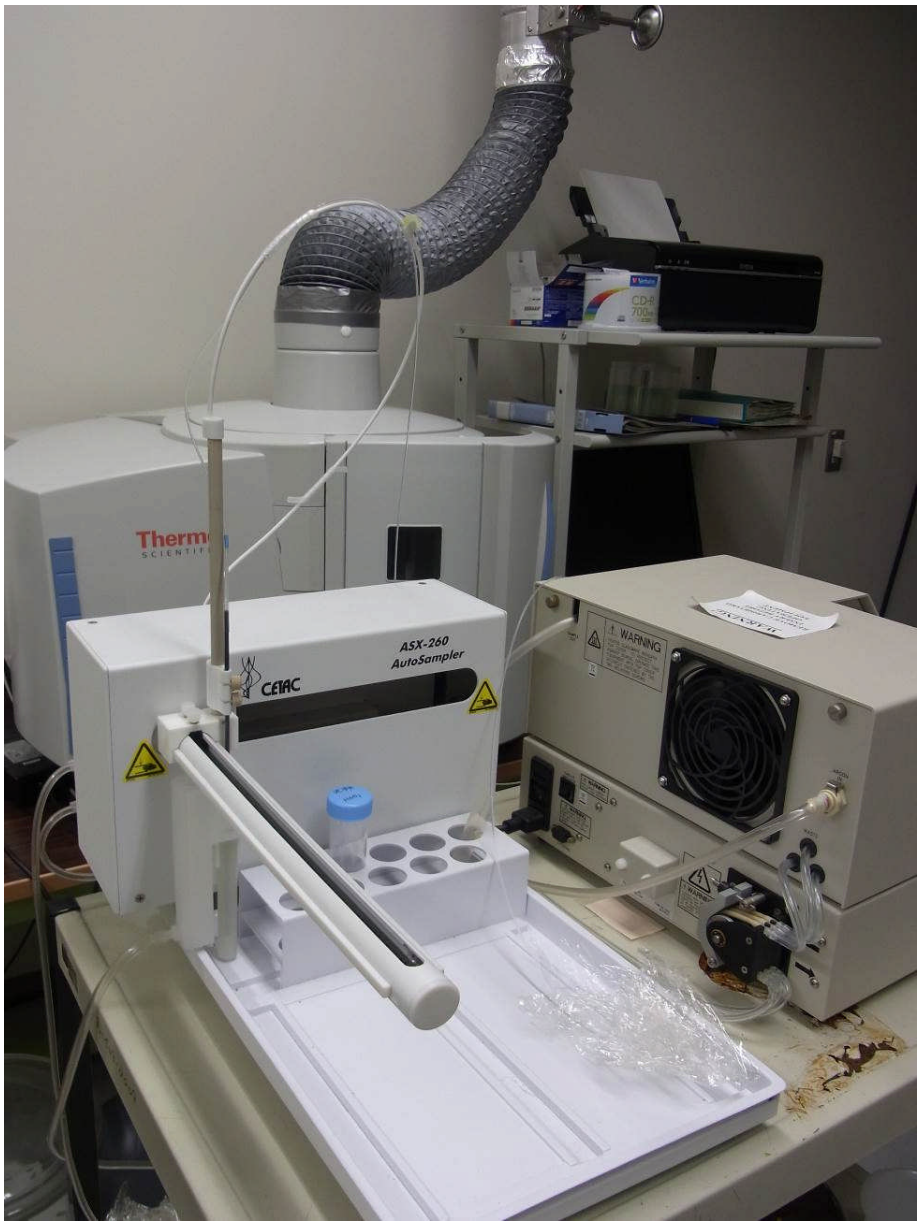

Figure 4-1. Photo of the inductively coupled plasma-atomic emission spectrometry (iCAP 6300 ICP Spectrometer) in Watanabe laboratory in faculty of science in Hokkaido University.

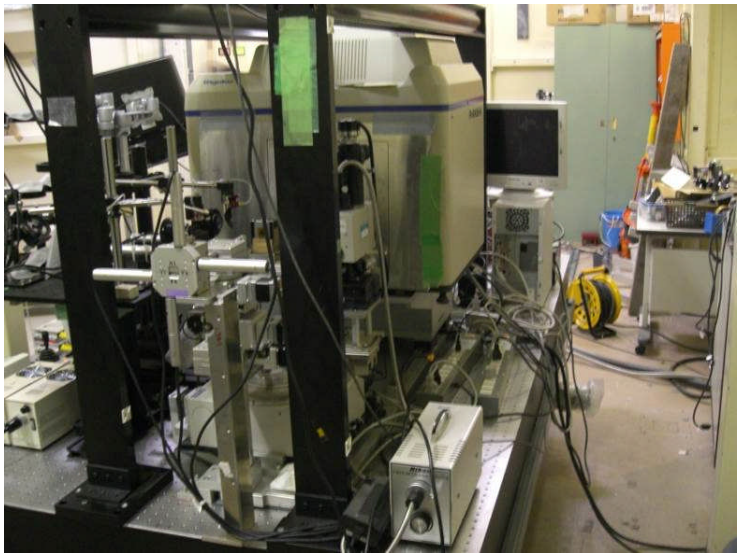

Figure 4-2. Photo of the beamline of NE1 at KEK

Figure 4-3. Photo of ultramicrotome EM-Supernova in division of biology, Department of Biological Science, in faculty of science in Hokkaido University.

Table 4-1. The amount of Mg, Ca, and Sr by ppm in 1L seawater, and artificial seawater.

	ppm		
		artifical seawater	
	seawater	Mg_bear	Mg_free
Ca	3.571	3.5	3.572
Mg	10.74	11.12	0.0006
Sr	0.0621	-0.0023	-0.0027

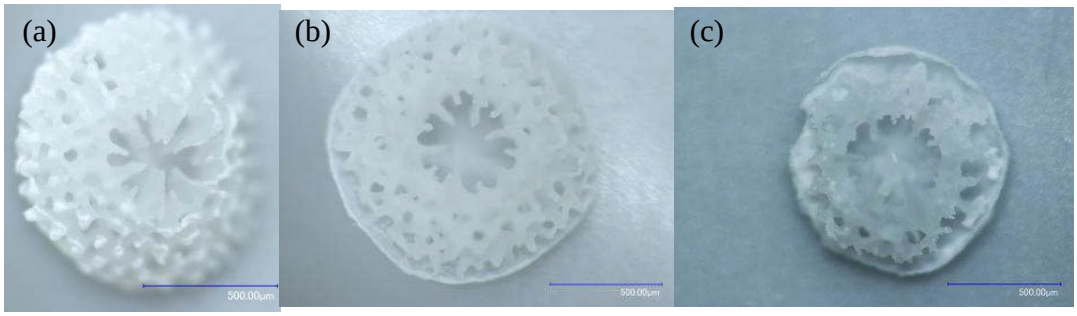

Figure 4-4. The observation of cultured coral skeleton in seawater (a), Mg/Ca molar =2.5 artificial seawater (b), and Mg/Ca molar =1.2 artificial seawater(c). The bottom plate and collalite are observed in all samples.

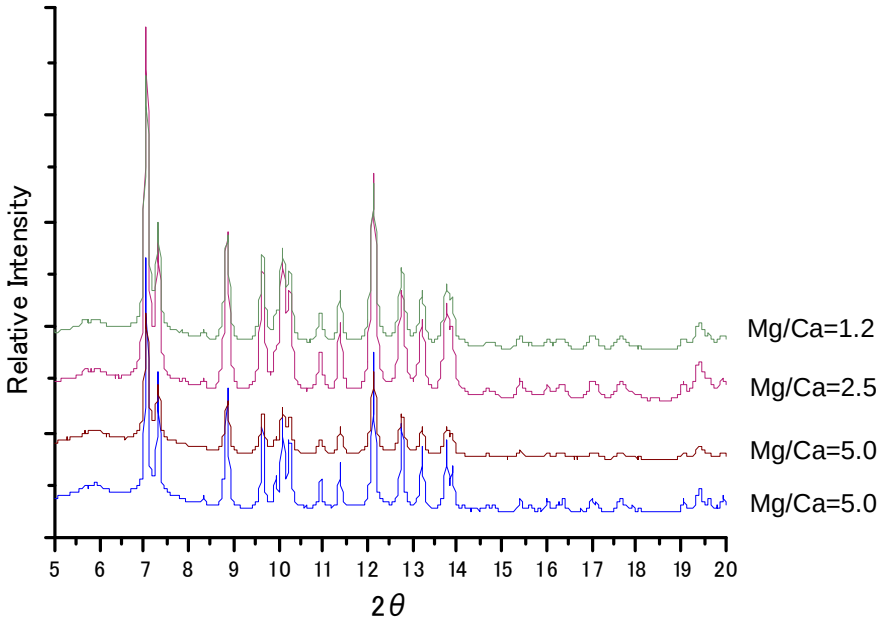

Figure 4-5. X-ray diffraction patterns of cultured coral skeletons. All peaks show aragonite phase.

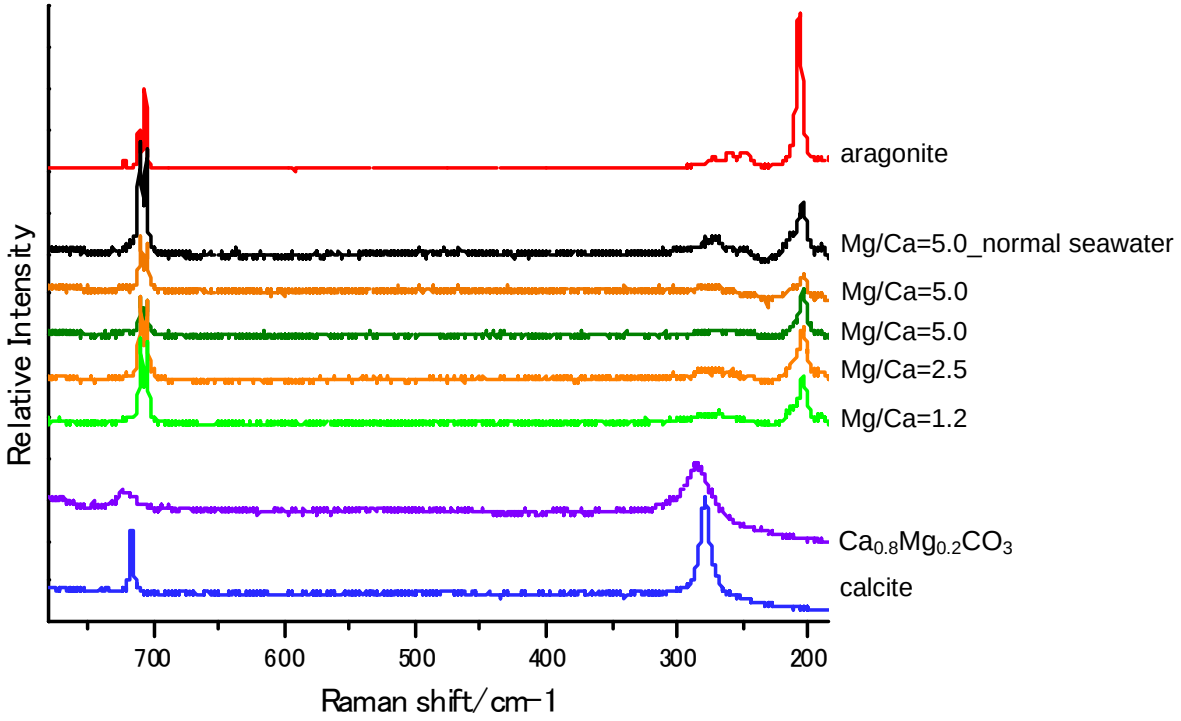

Figure 4-6. Raman patterns of cultured coral skeletons and standard sample, aragonite, calcite, $Ca_{0.8}Mg_{0.2}CO_3$ from sponge skeleton. All peaks from cultured coral skeletons seem similar to aragonite.

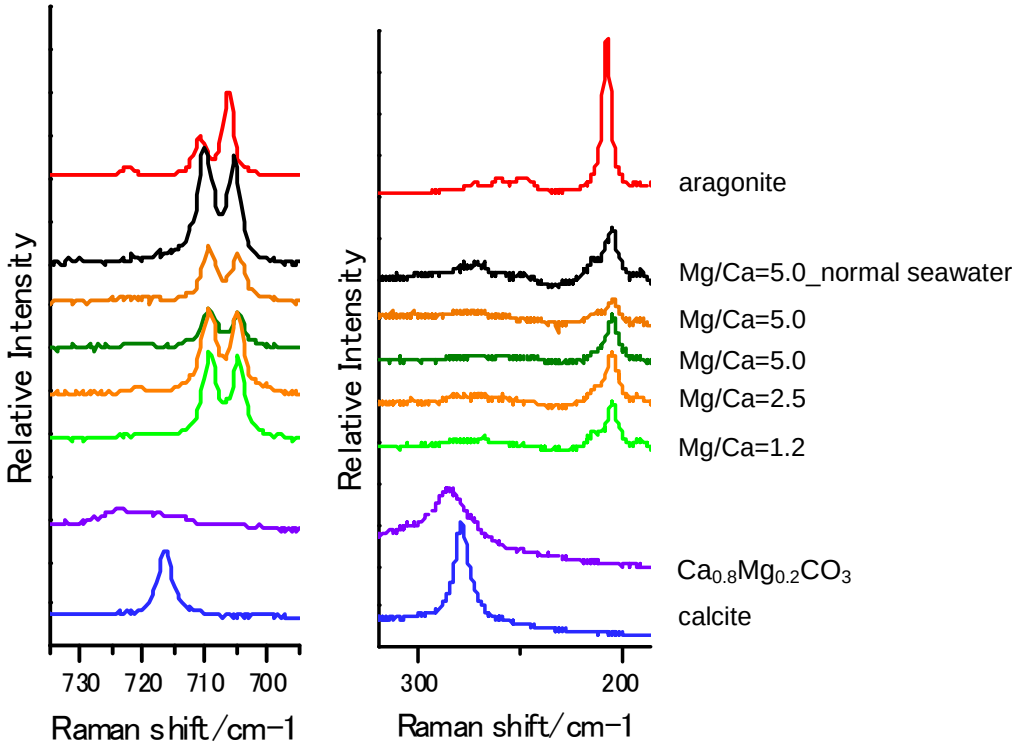

Figure 4-7. Extracts from Fig. 3 which is Raman patterns of cultured coral skeletons and standard sample, aragonite, calcite, $Ca_{0.8}Mg_{0.2}CO_3$ from sponge skeleton. All peaks from cultured coral skeletons seem similar to aragonite.

Mg/Ca molar ratio= 2.5 Mg/Ca molar ratio= 1.2

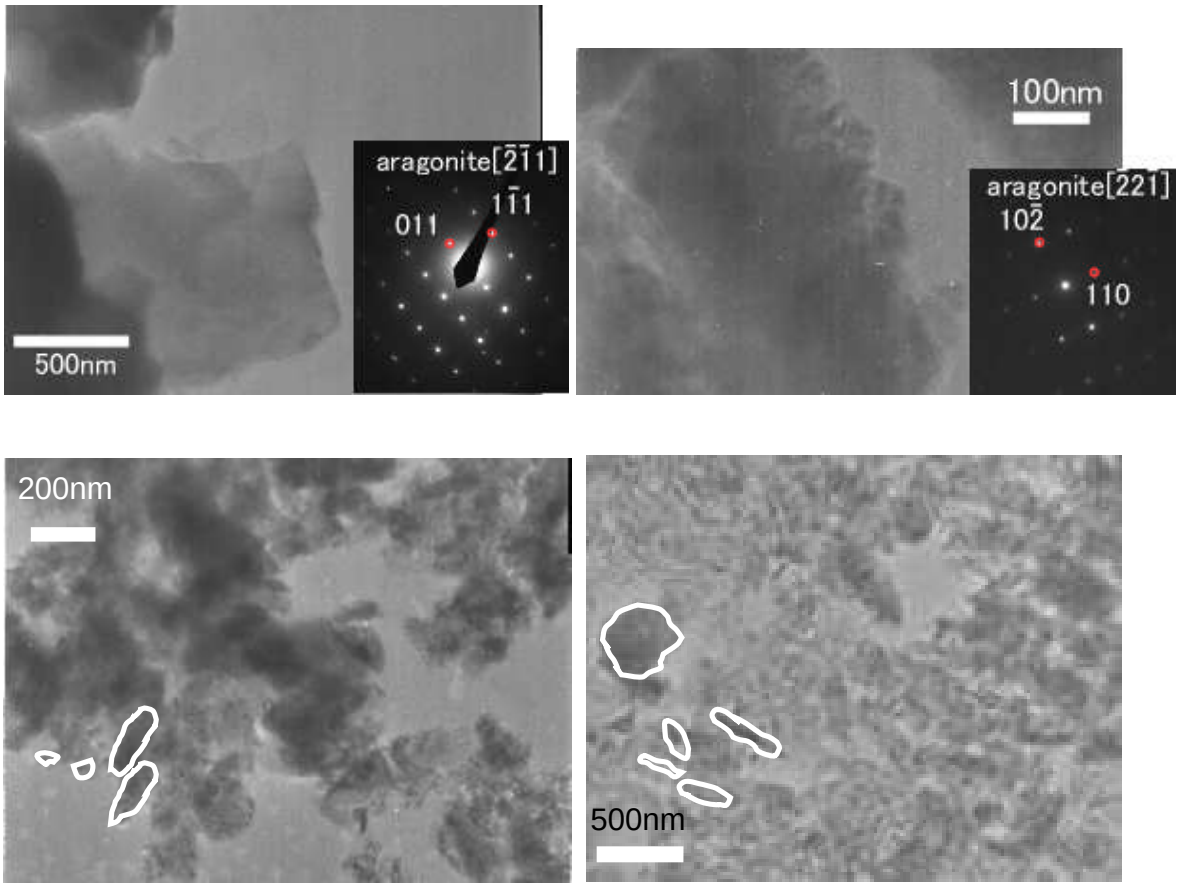

Figure 4-8. TEM photographs from cultured coral skeletons in Mg/Ca molar ratio is 2.5 and 1.2. SEAD patterns indicate the grains are aragonite. Aragonite crystals show needle or granular shape with several micrometer in dimensions.

5. Conclusions

In this thesis, reef-building coral skeletons were observed focused on the crystallographic texture and identification for mineral phases for understanding the compositional information of calcification medium. The fiber area including growth lines was mainly observed, because the area consisted of 97% in coral skeleton. The skeleton for observation is colonial massive coral with symbiotic algae in chapter 2 and 3, and branch coral in period a polyp without symbiotic algae in chapter 4.

Crystallographic observations revealed that the fibers consist of relatively equigranular needle-like aragonite crystal. Aragonite crystals across growth lines which are considered as place embedded organic matrix periodically in fiber area. In addition, when coral without symbiotic algae grow under Mg/Ca molar ratio = 5.0, 2.5, 1.2, this cultured experiment revealed that calcium carbonate in coral skeleton takes aragonite form and these results are corresponding with results of mineral phases inorganically precipitated from seawater (Morse 1997). These results support that crystals in coral skeleton could inorganically grow rather than strongly growth under organic strongly controlled condition.

As concerns about compositional information on calcification medium, this research can provide following new knowledge. There is no distribution of crystal size corresponding to growth lines characterized as the layered texture of fibers in coral skeletons and the growth lines are caused by different hardness in the same crystal. These suggest that coral holds $CaCO_3$ supersaturated fluid in the calcification space during fiber forming. The activity of coral tissue can change the elemental composition of calcification medium, that change the physical property of aragonite crystal. However, it does not affect strongly on changes of crystallographic morphology of crystals in fiber. ATEM observation for coral skeletal with symbiotic algae

revealed that there are halite grains in aragonite crystal, which would precipitate during calcification. On the other hand, there is no gypsum in the area included both aragonite. These evidences suggest that sometimes the concentration level of NaCl become supersaturated in the calcification medium in coral and Na^{+}/SO_4^{2-} ratio in the medium is higher than that of surrounding seawater. The Mg/Ca molar ratio in calcification medium would change by depending on symbiotic algae, because the mineral phase of $CaCO_3$ is different between cultured coral without and with symbiotic algae.

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