学位論文

Evaluation of paleotemperature proxy using coral genome biology

（サンゴゲノム生物学をつかった古水温プロキシの評価）

平成 29 年 12 月博士（理学）申請
東京大学大学院理学系研究科
地球惑星科学専攻

ベル　智子

Evaluation of paleotemperature proxy using coral genome biology

Tomoko Bell

Department of Earth and Planetary Science, The University of Tokyo

Submitted to the University of Tokyo
in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

December 2017

To my late grandfather

Ad as para per aspera

"To the stars through difficulty"

Kansas state motto coined by John James Ingalls in 1861 referring to the pioneering spirit of the early settlers and the difficult times Kansas went through before becoming a state.

Abstract

Corals have been used as temperature proxies since the 1970's, and especially coral skeletal Sr/Ca ratios are a robust tool to reconstruct past sea surface temperature (SST). However, it is reported that corals show strong individual variability such as growth rate difference. For example, Mg/Ca ratios have been also well studied as SST proxy, but most of the studies reported lower correlations between SST and Mg/Ca ratios compared to Sr/Ca ratios. Also, Mg/Ca ratios are correlated with growth rates. This is called vital effects, and it has been addressed as a downside for using corals as temperature proxies.

In this study, I set two specific objectives. First, I focus on the question why Sr/Ca ratios excel compared to other proxies. My second objective is elucidating the vital effects at molecular level. I address these two objectives by combining geochemistry and genome biology. To achieve these two objectives, I conducted four different interactive projects. In Chapter two, we conducted culture experiments using both adult and juvenile coral of *Acropora digitifera* to verify the robustness of skeletal Sr/Ca ratios as a temperature proxy. In Chapter three, I investigated the *Acropora digitifera* genome database (Zoophytebase), and I utilize the findings from the database to understand the result from Chapter two. In Chapter four, I reported *Acropora digitifera* genes that exhibited strong vital effects from two samples with different growth rates). In Chapter five, I summarized all the results from Chapter two through four and evaluate the criteria to be effective temperature proxies.

To conduct this study, I chose *Acropora digitifera* as the study species. This species has some advantages over *Porites* sp. which has been utilized most as a temperature proxy. First, the whole genome of *Acropora digitifera* has been sequenced and published. Secondly, it has been receiving attention as a new temperature proxy and four literatures have been published. Lastly, they live in both tropics and subtropics, thus they are able to provide sea temperature records from a wide range of the ocean. Therefore, I thought this species was most ideal for my study.

In chapter two, I confirmed that Sr/Ca ratios of *Acropora digitifera* are reliable temperature proxy by conducting two different culture experiments that have not been reported previously. As adult *in situ* samples are already influenced by various environmental factors, first we conducted temperature-controlled culture experiment (20, 22, 27, and 31 °C) using primary polyps of *Acropora digitifera* to accurately assess the impact of a wide range of temperatures on the growth rate and to methodically evaluate the skeletal trace elements (Sr/Ca and Mg/Ca). Water

temperatures positively affected the growth rate up to 31 °C, which exceeds the temperature threshold for this species. The growth rates also varied widely (>20%) during each of the four temperature treatments. The skeletal Sr/Ca ratios were strongly correlated with water temperature while Mg/Ca ratios showed moderate correlation (R^2 = 0.68, p < 0.001; R^2 = 0.53, p < 0.001, respectively). The variations in the skeletal Sr/Ca, and Mg/ Ca ratios at the four different temperatures were 0.36%–1.20% and 0.83%–3.76% respectively. Thus, the Sr/Ca ratios showed the least variation, despite the wide variations in the calcification rate. I suggest that the Sr/Ca ratios of *Acropora digitifera* juveniles are robust proxy of temperature, regardless of variations in the growth rate and the wide range of ambient temperatures.

Secondly, I conducted 12-month rearing experiments using 13 branches from five adult coral colonies of *Acropora digitifera* at outside aquaria (the common garden culture experiment) to compare the variations in skeletal elements observed among primary polyp samples. The sections of branches that grew during the experiment were analyzed for Sr/Ca and Mg/Ca ratios. These adult samples showed different growth rates (0.23–1.61 %/day) after the experiment. The growth rates were significantly different among colonies ($F(4, 8) = 23.898$; $p < 0.001$). I also confirmed a significant correlation between growth rates and Sr/Ca and Mg/Ca ratios. However, the correlations between skeletal elements and growth rates were - 0.69 ($p < 0.01$) and 0.24 ($p = 0.42$) for Mg and Sr, respectively. Thus Mg was largely influenced by the growth rate compared to Sr. Interestingly, the variation in the Sr/Ca ratios of *Acropora digitifera* was only 1.9%, which was one-sixth of the variation in the Mg/Ca ratios (11.9%). Thus, the influence of growth rate on Sr/Ca is much smaller than that on Mg/Ca in *Acropora digitifera* and makes Sr/Ca ratios more reliable SST proxy than Mg/Ca ratios.

In Chapter two, I could confirm that Sr/Ca ratios were correlated with water temperatures more than Mg/Ca ratios, but I was not able to explain why Mg/Ca ratios showed the lower correlation with temperature. In Chapter three, to approach this issue, I tried to establish the method to separate the vital effects from abiogenic one. In Chapter three, I utilized an open access and searchable gene database for coral *Acropora digitifera*, and examined the number of genes related to the elements in seawater to assess the origin of uncertainties in geochemical proxies. I found that *Acropora digitifera* has genes that might process at least 15 chemical elements as individual substances (Ca, Na, Zn, K, C, N, Cl, S, Fe, Mg, Mn, Cu, H, Mo, and Te) and transporters for 7 of these elements (Ca, Na, Zn, K, Cl, Cu, and H). The number of Ca-related genes was the highest (at least 428 genes,

including 53 transporters), whereas Sr, one of the most widely used geochemical proxies, was not found in the gene database. Therefore, the chemical elements that exist in seawater but were not found in the gene database (e.g., Sr, Li, and U) might be processed mainly abiogenically. I suggest that elements with no relevant coral genes could be good candidates for proxies with fewer vital effects (e.g., Sr/Ca, Li/Ca, and U/Ca). For example, Li/Ca and U/Ca were proposed as useful temperature proxies, moreover, Sr-U was recently introduced as a reliable temperature proxy by combining Sr/Ca and U/Ca. Thus, the number of genes in coral genomes related to specific elements may provide at least partial criteria for determining reliable proxies.

However, in Chapter three, it was still unclear why the high correlation between Mg/Ca ratios and growth rates was observed or why the variation in Mg/Ca was the highest. For example, in Chapter two, I analyzed skeletal samples of *Acropora digitifera* exhibiting different growth rates; their Sr/Ca ratios showed the lowest variation (1.9%), whereas other Mg/Ca showed much higher variation (11.9 %). Therefore, in Chapter four, I investigated genomes that are possibly related to metabolizing skeletal elements (Sr, Mg and Ca) using two different growth rate samples showing four fold differences. Intriguingly, I did not find Sr related genes, but I identified a Mg transporter (aug_v2a.04878) that showed higher gene expressions in the fast growth sample. I suggest this gene could possibly cause growth rate difference and might explain large individual variations reported in skeletal Mg/Ca ratios, which are problematic to be a reliable water temperature proxy. Therefore this gene could be related to the phenomena called vital effects in paleoclimatology. It has been long discussed that CaATPase, which is a transporter protein, played a significant roles in coral calcification by sending Ca ions to the center of calcification site. From my results, I identified six CaATPase. Interestingly I did not find the difference in CaATPase expressions between fast and slow growth samples, thus CaATPase might not be related to the growth rate differences. Moreover, I did not find significant difference in skeletal Ca values (mol) while there was one in skeletal Mg values (mmol) from different growth rate samples. That is to say, different expressions in Ca related genes might not be affecting the skeletal Ca values while aug_v2a.04878 expression could be related to the skeletal Mg values.

It is reported that global sea surface temperature is rising about 0.53℃ per 100 years. Therefore projecting future sea temperature change is one of the critical tasks to be resolved. In this study, I was able to confirm that Sr/Ca ratios of *Acropora digitifera* of both adult and juvenile samples were robust temperature proxy. My result will be useful to reconstruct sea temperature

from a wide range of the ocean. Moreover growth rate of *Acropora digitifera* is much faster than *Porites* sp. which has been widely used as temperature proxy since 1970's. Thus I can expect to collect temperature data with higher resolution.

One of the most important topics in biomineralization research is whether chemical elements in marine calcifiers are controlled biogenically or abiogenically when they are transported from seawater into the skeleton. To address this issue, I propose that a bioinformatics approach using genome information would be an effective method. I suggest that elements with no specific coral genes might make good candidates for reliable proxies. Genomic information can help us find new geochemical proxies with the fewest vital effects, and also explain the robustness of geochemical proxies that are already known to be effective in reconstructing past ocean environments.

Contents

Chapter 1

Introduction

1.1 Corals and related studies

Scleractininian corals appeared on the earth during the Triassic period, about 251 millions years ago, and they are thriving in tropic to subtropic shallow waters (Stanley, 2003). Coral reefs play a significant role in the ocean ecosystem and they cover an area of over 284,300 km^2 globally, which is approximately 0.08% of the entire ocean surface (Allemand, 2004). Coral reefs are considered as complex biological systems of high productivity (Stoddart, 2008), and Bellwood et al. (2015) stated corals increased fish diversity and reduced their extinction rate.

Not only are corals significant to local ecosystems, they play an essential role in the carbon cycle of the earth. As one of the main carbon reservoirs, corals absorb inorganic carbon from seawater in the form of HCO_3^- and CO_3^{2-} which they use to create immense calcium carbonate for their skeletons. It is estimated that their growth rate is 2 to 6 kg $CaCO_3\ m^{-2}\ yr^{-1}$ (Allemand, 2004). Corals, foraminifera and cocolithophores are responsible for 99.9% of calcareous deposits on the surface of the globe (Chalker and Barnes, 1990).

However, for the past decade, due to the rapid increase of sea temperature, the negative impacts on corals have been reported in a wide range of studies. According to the recent study (Descombes et al., 2015), it is predicted that coral reef habitat suitability will decrease up to 46 % by 2100 as a consequence of global warming. Along with these impact assessments for the near future, investigating past ocean environmental changes from a wide area of the ocean is becoming much more critical for climate change studies.

Because ocean environments influence the constitution and concentration of coral skeletons, they have been highly effective tools to investigate past sea temperature. For example, Sr/Ca ratios in coral skeletons have been widely used to reconstruct sea surface temperature (Gagan, 2000), and various chemical elements such as Li/Ca, and U/Ca ratios have been utilized as potential thermal proxies (Hathorne et al., 2013, Min et al., 1995). For these reasons, corals are commonly applied as robust tools in geochemistry. In the next section, we will review the coral geochemistry studies and address a common problem with them.

1.2 Coral geochemistry: vital effects and our approach

Corals have been used as geochemical proxies since the 1970's, and there are at least 61 studies that reported the correlation between skeletal Sr/Ca ratios and sea temperature. Correge (2006) and

Gagan et al. (2000) well summarized the previous studies using coral skeletal Sr/Ca ratios. According to Corrège (2006), there are 33 studies that investigated correlations between sea surface temperature (SST) and Sr/Ca ratios. Among them, 14 studies reported high correlations ($R^2 > 0.8$), 17 exhibited moderate correlations ($R^2 > 0.5$), and two reported low correlations ($R^2 < 0.5$) (Figure 1-1). The reason that coral Sr/Ca ratios are a reliable tool is explained by the distribution coefficients (K_d) from laboratory experiments using inorganic aragonite (Kinsman & Holland, 1969; Dietzel et al., 2004). Distribution coefficient formula is (Dietzel et al., 2004):

$K_d^{Sr,a} = 1.32 - 0.005091T\ (°C)$

When Temperature (T) = 25 °C, the distribution coefficients are $K_d^{Sr,a} = 1.19 \pm 0.03$

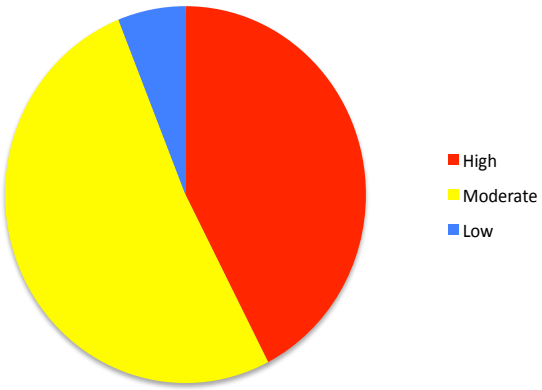

Figure 1-1. Correlations between sea surface temperature (SST) and coral (genus *Porites*) Sr/Ca ratios (Data from Corrège, 2006). High, moderate and low correlations represent $R^2 > 0.8$, $R^2 > 0.5$ and $R^2 < 0.5$ respectively.

However, it is reported that corals show strong individual variability such as growth rate difference. This is called "vital effects". For example, Hayashi et al. (2013) stated that the growth rate variations from five coral colonies in the uniform rearing environment were from 2.6 to 10.8 mm yr^{-1}. Also, Bell et al. (in press) reported the higher growth rate variations (seven fold difference) from two colonies in the same aquaria. This vital effect has been addressed as a downside for using corals as geochemical proxies. Nevertheless, there are many studies that report the robustness of skeletal Sr/Ca ratios. These studies focused on coral "growth rate" (Inoue et al., 2007; Hayashi et al., 2013), "growth pattern" (Gagan et al., 2012) and "crystal structures" (Cohen and McConnaughey, 2003).

Inoue et al. (2007) stated that Sr/Ca ratios were primarily controlled by temperature not by vital effects such as different growth rates. Gagan et al. (2012) suggested that there are two tissue

types to transport calcium ion, but this variation could be calibrated to reconstruct temperature history. Cohen and McConnaughey (2003) reported that the Sr/Ca ratios of crystals within the calcification center could indicate that the crystals were precipitated from a solution with a Sr/Ca ratio close to that of seawater. They stated that the Sr/Ca value in crystals within the center of calcification (K_d = 1.10) is close to that for Sr/Ca in aragonite precipitated experimentally at 25℃ (K_d = 1.13).

However, it should be noted Gagan et al. (2000) also reported that the calibration equations between coral skeletal Sr/Ca ratios and temperature exhibited the difference from the equation based on inorganic precipitation in terms of both slopes and intercepts. To consider the difference between organic and inorganic precipitation, we should be also aware that pH at the center of coral calcification fluctuates due to CaATPase activity which I will discuss in Chapter four. Therefore it seems to be difficult to explain coral calcification only with distribution coefficient. In addition, questions still remain such as why some proxies show more/less vital effects than others. For example, Mg/Ca ratios have been also well studied as SST proxy, but most of the studies reported lower correlations between SST and Mg/Ca ratios compared to Sr/Ca ratios (e.g. Inoue at al., 2007). To address this controversy issue, Wang and Xu (2001) made an energy partition model to explain the difference between Sr and Mg. They calculated the K_d of Sr, Mg and Ca in aragonite and the values of each are 1.26, 0.0087 and 1 respectively. This means that in inorganic aragonite crystals, both Sr and Mg substitute for Ca, but Sr should be more abundant than Mg.

To provide a different perspective, I thought gene compositions related to these elements (Sr, Mg and Ca) obtained by genome information would be able to help us understand the difference among these ions. Therefore, our study attempted to elucidate the system of vital effects using a fully innovative approach: coral genome biology combined with geochemical analysis. This approach was not feasible until the first coral genome was fully sequenced (Shinzato et al., 2011) and annotated for open access database (Shinzato et al., 2011, Dunlap et al., 2013). To my knowledge, this is the first study that combined coral genomic studies and geochemistry.

1.3 Coral genome biology

To conduct our strategy stated in section 1.2, it is essential to be familiar with published studies focusing on coral molecular biology. As previously mentioned, Shinzato et al. (2011) sequenced the very first coral genome of *Acropora digitifera,* and at this point, this is the only coral species whose

genome is entirely sequenced. As a result of this study, the open database called ZoophyteBase was published (Dunlup et al., 2013). In ZoophyteBase, proteins of *Acropora digitifera,* are annotated using hidden Markov models and the Kyoto Encyclopedia of Genes and Genomes (KEGG), which is widely used as a resource for investigating biological systems and linking genes to higher-level functions.

Since the whole *Acropora digitifera* genome has been sequenced, many coral biological studies using the novel RNA sequence technique have been conducted. Shinzato et al. (2014) investigated the relationship between corals and their symbiotic zooxanthella and reported that symbionts are able to provide most of their amino acids to the host corals. Meyer et al. (2009) analyzed heat shock proteins obtained from coral larvae to investigate the response to elevated temperatures. On the subject of the molecular basis of calcifications that produce hard tissues of corals, several molecules have been proposed (e.g., Ca^{2+} ATPase: Zoccola et al., 2004; Carbonic anhydrase: Moya et al., 2008; organic matrix protein: Watanabe et al., 2003).

However, there are no studies that explore relationships between coral soft and hard tissues. It is because molecular biologists are inclined to focus on soft tissue instead of hard tissue, such as skeletons, and vice versa for geochemists. To approach this problem and elucidate the system of vital effects, I researched both coral genome database and gene expressions that could possibly affect skeletal elements such as Sr/Ca and Mg/Ca ratios that are widely used in geochemistry (Figure 1-2). In the next section, I will explain an important part called, extracytoplasmic calcifying fluid (ECF), which significantly influences coral skeletons. I believe understanding the physiology of ECF will be imperative for conducting this study.

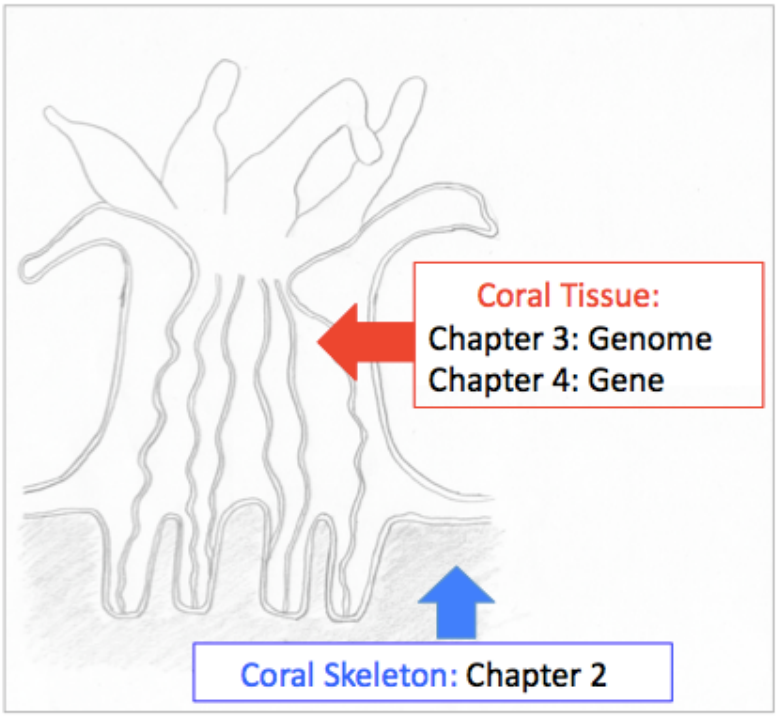

Figure 1-2. Anatomy of coral soft polyp and hard tissues (skeleton) and those relationship between Chapters in this study.

1.4 Coral calcification

To investigate chemical elements in coral skeletons as geochemical proxies, I believe understanding functions of ECF is critical. It is considered to have the most impact on skeletons and described as the center of coral calcification. It is located right next to calicoblastic cells and provides materials such as HCO^{3-} and Ca^{2+} to produce skeletons. Houlbre`que et al. (2009) stated that ECF is less than 10 μm (Figure 1-3). Also, Allemand (2004) stated that ECF is separated from the external seawater by four layers of cells, i.e. at least 40–50 μm thick. According to Gattuso et al. (1999), 1.7 mol/cm^2/h of calcium is transported to ECF, and considering the concentration of 10 mmol/cm^2 calcium exists in seawater, 170 times the concentration of calcium are transported in ECF per hour.

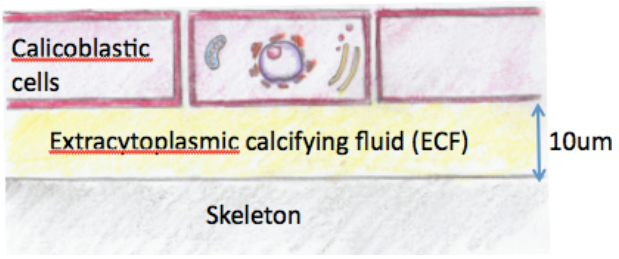

Figure 1- 3. Schematic diagram showing calicoblastic cells, ECF and skeleton.

Because ECF is very small (<10 μm), the direct chemical measurement of ECF is considered difficult. According to Dubinsky and Stambler (2010), there is only one paper that reports direct measurement of calcium concentration and pH of ECF (Al-Horani et al, 2003). In this situation, Alison et al. (2014) succeeded in estimating the pH level of ECF by analyzing B/Ca and B isotope ratios of coral skeletons. Most recently Kubota et al (2017) reported the pH declination in ECF since 1960 by measuring B isotopes in the skeleton. However, measuring all the chemical constitutions of ECF is considered unfeasible at this point.

Even if we fully understand all the chemical constitutions of ECF, we still need to understand what controls the ECF constitutions. Transport proteins are one of the factors that influence ECF constitutions. It is because they control the movement of ions across cell membranes by making channels or directly binding to ions. Therefore, transport proteins on calicoblastic cell membranes control ion inflows and outflows of ECF. In the next section, I will review the characteristics of transport proteins, which help us understand their significant role in coral skeletogenesis.

1.5 Transport proteins and CaATPase

According to Lodish et al. (2000), transport proteins are divided into three groups: (1) ATP powered pumps (2) ion channels and (3) transporters (Figure 1-4). The first class of transport proteins (ATP powered pumps) are able to carry 10^0 to 10^3 ions per second while the second class (ion channels) are able to carry 10^7 to 10^8 ions per second, and the third class (transporters) carry 10^2 to 10^4 ions per second. Alberts et al. (2002) defined ATP powered pumps and transporters as carrier proteins which are different from ion channels in which they interact with ions much more weakly. Ion channels form pores that extend across cell membranes and ions do not have to transform to pass through unlike carrier proteins.

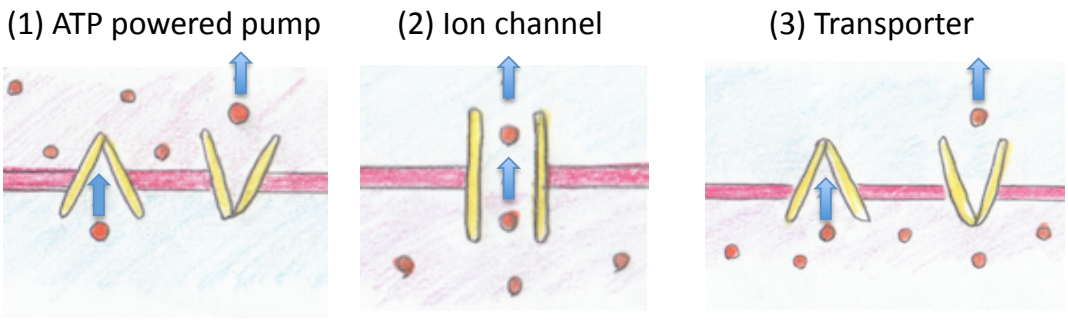

Figure 1-4. Schematic diagrams illustrating action of membrane transport proteins. Transport proteins are colored in

yellow and exist in cell membranes (red solid lines). Gradients are indicated by red color (higher concentration) and blue color (lower concentration). Circles represent specific molecules or ions and arrows indicate the direction of molecule or ion movements.

Transport proteins sense concentrations inside and outside of cells and try to keep the gradients consistent. This is called homeostasis and the distribution of each ion inside and outside of cells can be determined using the formula of membrane potential (Aliberts et al., 2002). For example, the magnesium equilibrium potential, E_{Mg} in volts can be written as the formula below:

$$E_{Mg} = \frac{R\,T}{Z\,F} \ln \frac{[Mg\,l\,]}{[Mg\,r\,]}$$

R (the gas constant) = 1.987 cal/(degree · mol), or 8.28 joules/(degree · mol)

T (the absolute temperature) = 293 K at 20 °C

Z (the valency) = +2

F (the Faraday constant) = 23,062 cal/(mol · V), or 96,000 coulombs/(mol · V)

[Mg l] and [Mg r] are the Mg^{2+} concentrations on two sides of a membrane respectively, at equilibrium.

As mentioned, transporters and ATP powered pumps carry fewer ions compared to ion channels because they physically bind ions and transform themselves to carry ions. Although the ATP powered pumps carry the least ions, this class of proteins plays a significant role in calcification. Among ATP powered pumps, CaATPase is the most discussed in geochemistry literatures (e.g. Cohen and McConnaughey, 2003; Allemand 2004). However, little is known how CaATPase influences the vital effects, thus we will discuss this issue in our study.

1.6 Study species and research objectives

As titled, my research is combining coral geochemistry and genome biology to evaluate coral as a thermal proxy. To conduct this study, I chose *Acropora digitifera* as the study species. This species has some advantages over *Porites* spp. which has been utilized most as a thermal proxy. Firstly, the whole genome of *Acropora digitifera* has been sequenced. Secondly, it has been receiving attention as a new thermal proxy and four literatures have been published (Gallup et al., 2006; Reynaud et al., 2007; Xiao et al., 2014; Sadler et al., 2015). Moreover, previous studies have been succeeding in culturing juvenile coral of *Acropora digitifera* (Inoue et al., 2011; Nishida et al.,

2014). Therefore, it is feasible to analyze primary polyps that have not been influenced by many environmental factors such as precipitation, extreme sea temperatures and anthropogenic effects. Lastly, they live in both tropics and subtropics, thus they are able to provide a sea temperature record from a wide range of the ocean. Therefore, I think this species is most ideal for our study.

In this study, I set two specific objectives. First, I focus on the question why Sr/Ca ratios excel compared to other proxies as mentioned in 1.2. My second objective is elucidating the vital effects, which were also discussed in 1.2. I address these two objectives using the information mentioned in 1.3, 1.4 and 1.5 and explore the possible relationships between specific genes and skeletal element ratios. To achieve these two objectives, I conducted four different interactive projects, and this dissertation consists of five Chapters. In Chapter two, we conducted culture experiments using both adult and juvenile *Acropora digitifera* to verify the robustness of skeletal Sr/Ca ratios as a thermal proxy. In Chapter three, I investigated the *Acropora digitifera* database (Zoophytebase) explained in 1.3, and I utilize the findings from the database to understand the result from Chapter two. In Chapter four, I reported *Acropora digitifera* genes from two types of samples that exhibited strong vital effects (i.e. different growth rates). In Chapter five, I summarize all the results from Chapter two through four and evaluate the criteria to be effective temperature proxies.

Chapter 2

Culture experiment and skeletal analysis

Chapter 2 Abstract

In this Chapter, we confirmed that Sr/Ca ratios of *Acropora digitifera* are reliable temperature proxy by conducting two different culture experiments that have not been reported previously. First we conducted temperature-controlled culture experiment (20, 22, 27, and 31 °C) using primary polyps of *Acropora digitifera* to accurately assess the impact of a wide range of temperatures on the growth rate and to methodically evaluate the skeletal trace elements (Sr/Ca and Mg/Ca). Water temperatures positively affected the growth rate up to 31 °C, which exceeds the temperature threshold for this species. The growth rates also varied widely (>20%) during each of the four temperature treatments. The skeletal Sr/Ca ratios were strongly correlated with water temperature while Mg/Ca ratios showed moderate correlation (R^2 = 0.68, $p < 0.001$; R^2 = 0.53, $p < 0.001$, respectively). The variations in the skeletal Sr/Ca, and Mg/ Ca ratios at the four different temperatures were 0.36%–1.20% and 0.83%–3.76% respectively. Thus, the Sr/Ca ratios showed the least variation, despite the wide variations in the growth rate. I suggest that the Sr/Ca ratios of *Acropora digitifera* juveniles are robust proxy of temperature, regardless of variations in the growth rate and the wide range of ambient temperatures.

Secondly, we conducted 12-month rearing experiments using 13 branches from five adult coral colonies of *Acropora digitifera* at outside aquaria (the common garden culture experiment). The sections of branches that grew during the experiment were analyzed for Sr/Ca and Mg/Ca ratios. *Acropora digitifera* showed different growth rates (0.23–1.61 %/day) after the experiment. The growth rates were significantly different among colonies ($F(4, 8) = 23.898$; $p < 0.001$). I also confirmed a significant correlation between growth rates and Sr/Ca and Mg/Ca ratios. However, the correlations between skeletal elements and growth rates were - 0.69 ($p < 0.01$) and 0.24 ($p = 0.42$) for Mg and Sr, respectively. Thus Mg was correlated with the growth rate compared to Sr. Interestingly, the variation in the Sr/Ca ratios of *Acropora digitifera* was only 1.9%, which was one-sixth of the variation in the Mg/Ca ratios (11.9%). Thus, the influence of growth rate on Sr/Ca is much smaller than that on Mg/Ca in *Acropora digitifera* and makes Sr/Ca ratios more reliable SST proxy than Mg/Ca ratios.

2.1. Introduction

As mentioned in Chapter one, what unique about this study is I investigated both hard and soft tissues that could be correlated each other to form coral skeletons. In this Chapter, I focus on analysis of hard tissues: coral skeletal analysis. As introduced in section 1.6, selecting *Acropora digitifera* as study species is very meaningful in terms of developing a new proxy. This species is relatively new as thermal proxies, and there are only four studies analyzing Sr/Ca ratios as temperature proxies (Table 2.1). As summarized in Table 2.1, these previous studies analyzed either *in situ* samples or conducted short-term (4 weeks to 89 days) culture experiments using adult samples. Therefore, there were no studies (1) analyzing skeletal ratios of juvenile samples (2) conducting long-term (more than 1 year) culture experiments using adult samples and analyzing their skeletal ratios. As mentioned in Chapter 1.6, it should be noted that any adult samples are already influenced by many environmental factors such as different salinity, nutrients and extreme temperatures. That is to say, it is difficult to see the correlation only with sea temperatures and skeletal elements. Therefore, in this study, I decided to conduct the culture experiment not only using adult but also juvenile samples to confirm the robustness of this species as temperature proxies.

Table 2-1. Literatures analyzing skeletal Sr/Ca and Mg/Ca ratios of *Acropora* sp.

	Reference	Temperature	Element/Calcium (mmol/mol)	Calbration equation*	Correlation
Sr/Ca	Bell et al, 2017	22-31	8.89-9.32	y = -0.0283x+9.763	r^2 = 0.68
	Sadler et al, 2016	21-28	9.54-9.90	y = - 20.989x (anomaly)	r^2 = 0.65
	Xiao et al, 2014	21-28	8.73-9.77	y = -0.04156x+10.59	r^2 = 0.62
	Reynaud et al, 2007	21-29	9.16-9.59	y = 0.007x^2-0.393x+14.807	r^2 = 0.97
	Gallup et al, 2006	24-31	9.12-9.51	y = -0.07072x+11.30	r^2 = 0.79
	Gallup et al, 2006	24-31	9.32-9.73	y = -0.06281x+11.32	r^2 = 0.62
Mg/Ca	Bell et al, 2017	22-31	4.29-4.90	y = 0.0342x+3.7741	r^2 = 0.53
	Reynaud et al, 2007	21-29	3.76-4.90	y = 0.138x + 0.898	r^2 = 0.98
	Xiao et al, 2014	21-28	3.39-3.95	y = 0.04974x + 2.339	r^2 = 0.21

* x = Temperature, y = Element/Calcium

Even though only adult samples are used in paleoclimate studies, I believe that the investigation of juvenile coral is important for accurate reconstructions of water temperatures. Clode et al. (2010) reported that the form of $CaCO_3$ in juvenile *Acropora milleopora* was aragonite, with no evidence of calcite, which was identical to the composition of adult corals. However, some studies have reported that the juvenile mineralogy of calcifying organisms differs from that of adults. For example, in some calcifying organisms, such as in gastropods, the Sr/Ca–temperature

calibration curves for juvenile samples differ from those of adults (Sosdian et al., 2006). Results of this study will therefore help us better understand coral calcification mechanisms during early life stages.

In our short-term culture experiment using juvenile samples described in 2-2, we were able to prepare clone samples by obtaining juvenile corals spawned and manipulated at our laboratory. We maintained our samples under controlled conditions, and we succeeded in eliminating other environmental impacts completely and strictly evaluating the correlation between sea temperatures and skeletal element ratios. Moreover it has been unknown at which stage of their lives, coral skeletal ratios resume recording surrounding temperature. By conducting culture experiment using juvenile samples, I was able to approach this question too.

For long-term experiment using adult samples described in 2-3, we were able to collect valuable samples that showed different growth rates cultured in the same aquaria. Reynaud et al. (2007) successfully proved that changing light level affect growth rates, but it did not influence skeletal Sr/Ca ratios. Inoue et al. (2007) also stated Sr/Ca ratios were not affected by growth rates while Mg/Ca ratios were significantly influenced. Therefore our samples showing a wide range of growth rates were ideal to discuss vital effects on skeletal proxies that were main topic in our study.

2.2 Temperature controlled experiment using juvenile coral

2.2.1 Method

Sample collection and experiment settings

Six *Acropora digitifera* colonies were collected from the fringing reef of Sesoko Island, Okinawa, Japan, on 4–5 June, 2014, and were maintained in an outside aquarium at Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. All the samples were collected in strict accordance with good animal practice, defined by the relevant national and/or local animal welfare bodies, and sampling was performed with the permission of the Japanese Municipality of Okinawa Prefecture (Permission Number: 25-69). Coral gametes were retrieved from these six colonies in the laboratory on 11 June, 2014, and the gametes were cultured in our laboratory with local reef water pumped directly from the fringing reef and filtered with 1 and 10 mm filters (PF Housing, Super Pure Filter, Organo Co., Ltd).

On 16 June, 2014, we observed planula larvae (Figure 2-1(a)), which were transferred to six-well culture plates. On 21 June, 2014, we added 5 ml of 1×10^{-3} M Hydra neuropeptide, Hym-248 (Iwao et al., 2002) to each well containing about 10 planula larvae in 16.4 ml of filtered seawater to induce metamorphosis. The settlement of the planula larvae on the bottom of each well was confirmed on 22 June, 2014 (Figure2-1 (b)), and 0.5 ml of solution containing zooxanthellae (*Symbiodinium*, clade A3), obtained from the giant clam *Tridacna crocea)* was added. We confirmed the infection of the larvae with the symbiotic zooxanthellae after 1 day under a microscope (Figure2-1(c)).

We prepared four temperature-controlled 4.6 liter aquaria (20, 22, 27, and 31°C) with metal-halide lamps that provided an average light intensity of 95.5 mmol m^{-2} s^{-1} for 12 h per day (08:00 h to 20:00 h). The maximum temperature treatment was at 31°C, which is almost 6°C higher than the average annual temperature (24.6°C) and the temperature (24.8°C) in the average spawning season (May to June; Hayashibara et al., 1993) in this area, based on data collected by the Japan Coast Guard from 1993 to 2014 (http://www1.kaiho. mlit.go.jp/KAN11/ suion/top_temp. htm). The seawater was constantly pumped directly from the fringing reef to each aquarium at an average flow rate of 41.2 ml/min. Salinity was measured with a TS Digital Salinometer (Model 3-G, The Tsurumi Seiki Co., Ltd.), and the average water temperature was controlled with thermostats, heaters, and chillers, and was measured every 15 min with loggers (Thermochron SL, KN Laboratories, Inc.).

The temperature, salinity, flow rate, and light (photon flux density) for each treatment are summarized in Table 2.2. The plates containing juvenile polyps, prepared with the method described above, were submerged in four different temperature-controlled aquaria for 21 days (25 June–16 July, 2014). After each plate was retrieved, the polyp tissues on the plates were removed with a water toothpick, thus yielding dry skeletal samples. The individual dry weights of the undamaged skeletal samples were measured with an ultramicrobalance (Cahn C-35, Thermo Electron). In this study, I considered this skeletal weight as the growth rate of 21 days.

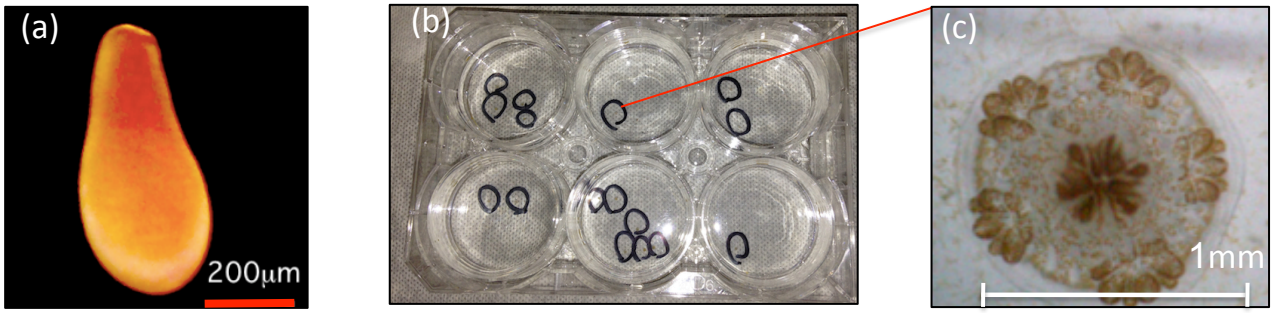

Figure 2-1. (a) Photo of planula larvae (5 days after the fertilization) (b) Six-well culture plate with settled planula larvaes (Black circles show the location of settled larvaes) (c) Photo of juvenile polyp infected with zooxanthellae.

Table 2-2. Temperature, salinity, flow rate, and light (photon flux density) for each treatment. ± Values for temperature indicate one standard deviation of the mean. The analytical error for salinity is less than 0.01.

Target Temperature (°C)	Actual Temperature (°C)	Salinity	Flow Rate (mL/min)	Light (μmol m^{-2} s^{-1})
20	19.5±1.0	34.43	40.5	96.5
22	21.9±0.8	34.52	41.0	95.5
27	26.9±1.1	34.46	39.0	92.0
31	31.0±0.1	34.45	45.0	98.0

Trace element and data analyses

After each plate was retrieved, the juvenile polyps were scraped from the sides of the plate and soaked in 30% H_2O_2 for 1 h to digest the organic materials. Seven to 10 skeletal samples from each aquarium were mixed and transferred to 1.5 ml tubes to create bulk samples. We set up four tubes for the 20°C and 31°C treatments and five tubes for the 22°C and 27°C treatments. The samples were washed with 18.2 Ω ultrapure water in an ultrasonic bath for 10 min, and this process was repeated five times. The samples were then dried overnight at 45°C in an oven, and an average of 500 μg of sample from each tube was dissolved in 5% HNO_3 for analysis.

I measured the Sr/Ca and Mg/Ca ratios in a total of 18 samples using inductively coupled plasma–atomic emission spectrometry (iCAP6300 Duo, Thermo Fisher Scientific, Inc.) and two types of standards: XSTC-13 (multi-element standard solution; SPEX) and JCp-1 (a coral standard material; Geological Survey of Japan, AIST). The data were analyzed with one-way ANOVA, the Tukey–Kramer HSD test, and Pearson's product-moment correlation test using the R software (R Foundation for Statistical Computing, 2013). The analytical uncertainties for Sr/Ca and Mg/Ca ratios based on a repetitive analysis of JCp-1 (N = 30) were 2.4% (± 0.22 mmol/mol) and 3.5% (±

0.79 mmol/mol) respectively.

2.2.2 Result

2.2.2.1 Growth rates at four different temperature treatments

The average skeletal dry weights of individual skeletal samples after 21 days were 154 ± 7 μg (N = 40, mean ± SE), 201 ± 7 μg (N = 41, mean ± SE), 217 ± 8 μg (N = 39, mean ± SE), and 256 ± 10 μg (N = 38, mean ± SE) for treatments at temperatures of 20, 22, 27, and 31°C, respectively; these values were significantly different from one another ($F(3, 154) = 26.564$; $p < 0.001$; Figure 2-2). The Tukey–Kramer test showed that the skeletal weight of polyps reared at 22 °C and 27 °C, which represented the boundaries of the normal seawater temperature range, did not differ significantly ($p = 0.49$), whereas all other possible pairs of treatments differed significantly ($p < 0.01$).

Therefore, growth rates of polyps reared at temperatures of 22 °C or 27 °C, the normal seawater temperature range in this area, did not differ significantly. The 31 °C treatment, which is almost 6 °C higher than the average local sea temperature, produced the highest growth rate, which was significantly different from that produced by the 27 °C treatment. The growth rates during all the treatments varied greatly, and the coefficients of variation were 26.9%, 23.4%, 24.5%, and 23.3% for the 20, 22, 27, and 31 °C treatments, respectively.

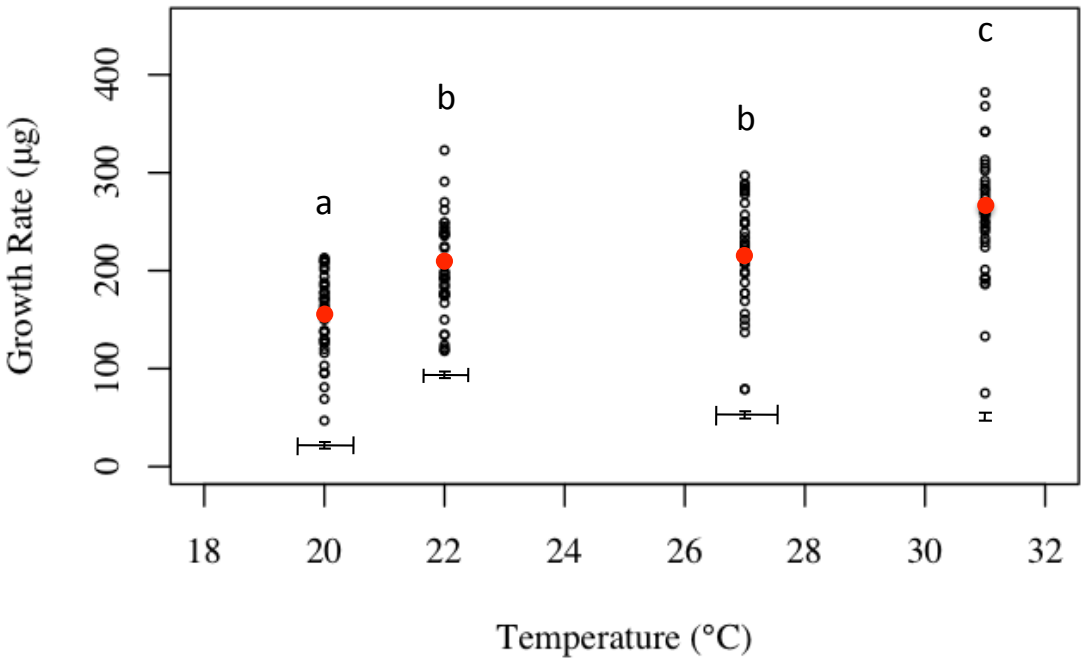

Figure 2-2. Juvenile polyp growth rate of each treatment.

Average juvenile polyp growth rates for four temperature treatments. Error bars for X axis represent the standard deviation (SD) of each mean temperature (±1 SD). Error bars for Y axis represent the standard error (SE) of each mean growth rate (±1 SE). The number of sample polyps are N = 40, 41, 39, and 38 at temperatures of 20, 22, 27, and 31 °C, respectively. Different letters on the bars indicate a statistically significant difference between treatments (one-way ANOVA test followed by the Tukey–Kramer HSD test, α = 0.05). The red solid circles represent the mean growth rates for each treatment.

2.2.2.2 Sr/Ca and Mg/Ca ratios and their relations to water temperature

The average Sr/Ca ratios were 9.26 ± 0.22 (N = 4, mean ± SD), 9.09 ± 0.22 (N = 5, mean ± SD), 8.97 ± 0.22 (N = 5, mean ± SD), and 8.92 ± 0.21 (N = 5, mean ± SD) mmol/mol; the average Mg/Ca ratios were 4.34 ± 0.13 (N = 4, mean ± SD), 4.61 ± 0.14 (N = 5, mean ± SD), 4.76 ± 0.14 (N = 5, mean ± SD), and 4.76 ± 0.14 (N = 4, mean ± SD) mmol/mol; in the 20, 22, 27, and 31 °C treatment groups, respectively. There were significant differences among the four treatment groups for these skeletal elements: Sr/Ca: $F(3, 14) = 17.319$, $p < 0.001$; and Mg/Ca: $F(3, 14) = 12.652$, $p < 0.001$. The calibration equations between temperature (x) and elemental ratios based on my results were, for Sr/Ca (y), y = − 0.0283x (± 0.0049) + 9.763 (± 0.123) ($R^2 = 0.68$; $p < 0.001$, $N = 18$), for Mg/Ca (y), y = 0.0342x (± 0.0081) + 3.7737 (± 0.204) ($R^2 = 0.53$; $p < 0.001$, $N = 18$) (Fig. 2-3).

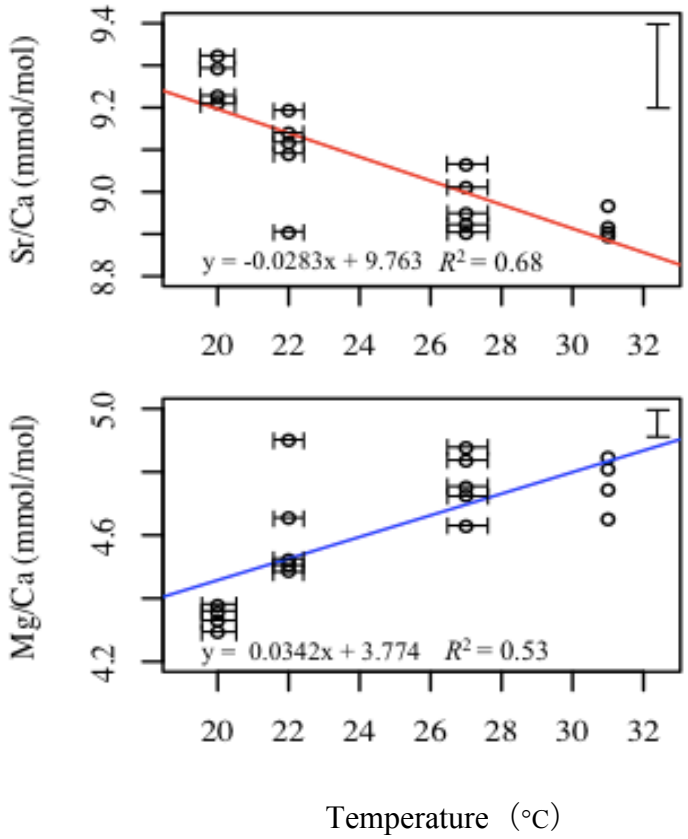

Figure 2-3. Linear regression between skeletal element ratios and water temperature (Bell et al., 2017).
Error bars for water temperatures represent one standard deviation of each mean. The temperature error bar for 31 °C was too small to show (±0.1°C). Error bars for elemental ratios represent the relative standard deviation based on repetitive analysis of JCp-1 (N = 30). Each circle indicates the bulk sample of 7 to 10 polyps.

2.2.2.3. Variations of Sr/Ca and Mg/Ca ratios

As mentioned before, the samples for the skeletal analysis were randomly chosen from each treatment, which showed high variations in growth rates. The coefficient of variations in Sr/Ca and Mg/Ca from the four treatments ranged from 0.36 to 1.2%, 0.80 to 3.7%, and 0.52 to 3.2%, respectively. That is to say, Sr/Ca ratios showed the lowest variation, and this is within the analytical uncertainties (cf. Sr/Ca = 2.4%). On the other hand, Mg/Ca ratios showed the highest variations, which are beyond the analytical uncertainties of Mg/Ca (2.5%).

2.2.2.4. Sr/Ca and Mg/Ca ratios and their relation to growth rates

I plotted Sr/Ca and Mg/Ca ratios and the average dry skeletal weights (growth rates) from each aquarium reported above (Figure 2-4). There were significant correlations between the growth rates and all the skeletal element ratios. Sr/Ca showed negative correlations and Mg/Ca ratios showed a positive correlation. Correlation coefficients of Sr/Ca and Mg/Ca ratios were $R^2 = 0.72$ ($p < 0.001$) and $R^2 = 0.61$ ($p < 0.001$) respectively.

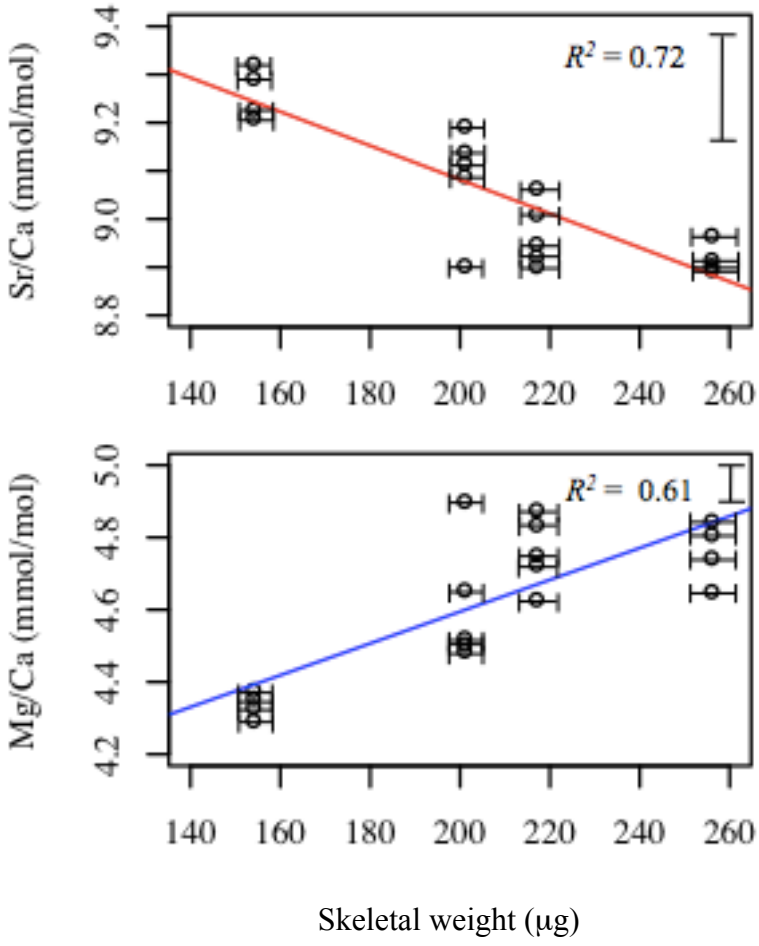

Figure 2-4. Skeletal element ratios and growth rate (Bell et al., 2017). Error bars represent one standard deviation based on repetitive analysis of JCp-1 ($N = 30$).

2.2.3. Discussion

2.2.3.1. Growth rates

The growth rates of juvenile *Acropora digitifera* observed in this study were consistent with the results of two previous studies conducted using culturing experiments with juvenile *Acropora digitifera.* (Inoue et al., 2012; Nishida et al., 2014). Inoue et al. (2012) suggested that water temperature had a positive effect on the growth rate, for temperatures up to 31 °C. My results and

those of Nishida et al. (2014) considered the maximum skeletal weight at a temperature of 31 °C, which was beyond the tolerance range of this species. Nishida et al. (2014) reported no significant difference in polyp growth rates between the 27 °C and 31 °C treatments, whereas our study showed a significant difference between these treatments. However, this may be explained by the difference in the experimental periods, which was 10 days in the study of Nishida et al. (2014) and 21 days in my study.

2.2.3.2. **Skeletal Sr/Ca ratios**

The skeletal Sr/Ca and Mg/Ca ratios and their calibration equations for *Acropora digitifera* are shown in Figure 2-5 and 2-6 respectively. My Sr/Ca ratios (8.89 ± 0.21–9.32 ± 0.22 mmol/mol) were very close to the values for adult *Acropora digitifera* (8.96 ± 0.17–9.49 ± 0.18 mmol/mol; Bell et al., in press) cultured in an outdoor aquarium at uncontrolled temperatures ranging from 19 to 31 °C in Okinawa, Japan (Hayashi et al., 2013), which will be discussed in section 2.3.2. My maximum Sr/Ca ratios observed in juveniles was slightly less than that observed in the adult sample (Bell et al., in press); however, this could be attributed to differences in the minimum temperatures examined, as the temperatures used in the adult and juvenile experiments were approximately 19 °C and 21 °C, respectively.

My measured Sr/Ca ratios were also similar to those obtained for adult *A. danai* and *A. formosa* specimens collected on the Great Barrier Reef, Australia, and Reunion Island, Africa (9.08–9.37 mmol/mol; Ribaud-Laurenti et al., 2001), for adult *A. robusta*, *A. humilis*, and *A. valida* from the South China Sea, cultured at temperatures of 21–28 °C (8.73–9.77 mmol/mol; Xiao et al., 2014), and for adult *Acropora sp*. from the Gulf of Aqaba, cultured at temperatures of 21–29 °C (9.592 ± 0.018– 9.157 ± 0.011 mmol/mol; Reynaud et al., 2007).

The slope and intercept values I derived for the relationship between Sr/Ca and temperature closely match those reported by Xiao et al. (2014). In contrast, interestingly, my calibration equation was dissimilar to that obtained in a previous study of coral samples collected from natural environments. For example, for *A. palmata* collected from the Dominican Republic and Puerto Rico, calibration equations were $y = -0.07072x + 11.30$ ($R^2 = 0.79$) and $y = -0.06281x + 11.32$ ($R^2 = 0.62$), respectively (Gallup et al., 2006). I attribute this inconsistency of intercepts to the differences between *in situ* and cultured samples. In Figure 2-5, calibration Eqs. (1) and (2) (Gallup et al., 2006) are from *in situ* samples and the others are from cultured samples. Although the slopes are

similar in all the studies, the large differences observed in the intercepts could be attributed to differences in light intensity, local seawater Sr/Ca ratios variations, sampling area on the coral skeleton, or the use of filters. In the culture-based experiments, the use of filters that eliminates particulates in pumped seawater is necessary to maintain a healthy environment for the corals.

According to Shen et al. (1996), the value for Sr/Ca in seawater changes depending on the filtration technique used, and filtration membranes reduce the Sr/Ca values because seawater with 0.45–5.0 μm particulates had the highest Sr/Ca ratios. Xiao et al. (2014) also reported seawater Sr/Ca ratios in aquaria were 0.16–0.47 mmol/mol lower than in local seawater. Gagan et al. (2012) reported that the Sr/Ca–SST sensitivity of adult *Porites* in reefs was suppressed by mass accumulations within the tissue layer, and that field samples were needed to calibrate the Sr/Ca paleothermometer for paleoclimate reconstruction. Gallup et al. (2006) used a sampling method for adult *Acropora* that spanned several years outside of the tissue layer, whereas our study used bulk samples of only 21-day-cultured juvenile corals with no bio-smoothing effect. However, the skeletal Sr/Ca ratios of Gallup et al. (2006) showed higher values than those obtained in this study. The bio-smoothing effect might be small in adult *Acropora* corals, meaning that differences between the results of the various studies may be controlled by factors other than temperature. Future work should examine the effect of the thickness of the tissue layer of polyps and analyzes calcium transport (e.g., using Alizarin Red) in longer duration culture experiments (e.g., 1 year) to test for Sr/Ca–SST sensitivity.

Another possible explanation of the varying intercepts is the different attributes of members of the genus *Acropora*. *Acropora* species are the most abundant and diversified group of corals, and inhabit shallow to deep photic zones (Wallace, 1999). Therefore, the different evolutionary and/or environmental histories of the *Acropora* species could affect the chemical compositions of their skeletons. The species used for the five calibration equations shown in Figure 2-5 were all different. Corrège (2006) reported a maximum difference of 1.9 mmol/mol in the intercepts based on 33 studies using different species of the genus *Porites* from multiple locations. Because the use of the genus *Acropora* as a paleothermal archive is relatively new, more studies are required to establish an average temperature calibration equation.

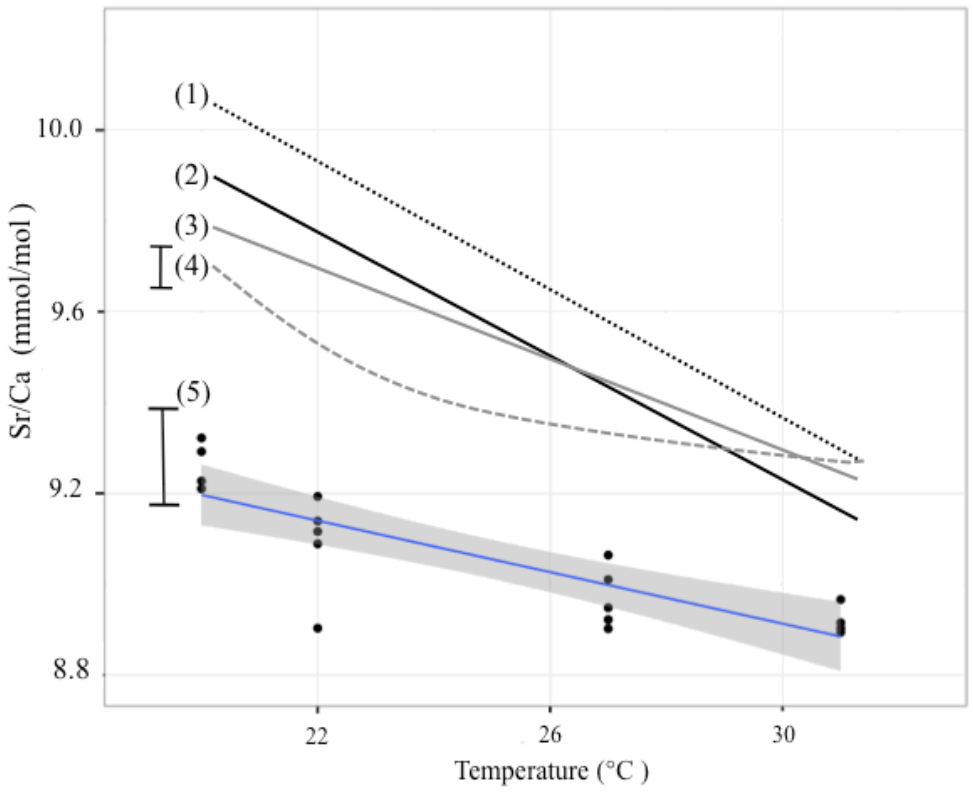

Figure 2-5. Comparison of Sr/Ca–temperature calibration equations. Equations in the Figure are: (1) Puerto Rico (*in situ*, adult, Gallup et al., 2006) (2) Dominican Republic (*in situ*, adult, Gallup et al., 2006) (3) South China Sea (Cultured, adult, Xiao et al., 2014) (4) The Gulf of Aqaba (Cultured, adult, Reynaud et al., 2007) (5) This study (Cultured, juvenile). Error bars for equation (1), (2) and (3) were too small to show in this Figure. Error bar next to equation (4) is analytical precision stated in Reynaud et al, 2007. Error bar next to equation (5) represent one standard deviation based on repetitive analysis of JCp-1 (N = 30). The grey zone represents the 95% confidence interval (the estimated coefficient ± two standard errors).

2.2.3.3 Skeletal Mg/Ca ratios

Few studies have investigated the Mg/Ca ratios in the genus *Acropora*, although the values obtained in our study (4.29–4.90 mmol/ mol) were within the range of those of adult *Acropora* sp. from the Gulf of Aqaba as obtained in culture experiments at temperatures of 21–29 °C (3.759 ± 0.029–4.901 ± 0.037 mmol/mol; Reynaud et al., 2007). The calibration equations derived in my study and by Reynaud et al. (2007) were y = 0.0342x+ 3.7737 (R^2 = 0.53) and y = 0.138x+ 0.898 (R^2 = 0.98), respectively. Interestingly, my results showed a lower slope of the calibration equation and higher Mg/Ca ratios than those obtained by Reynaud et al. (2007). It is noteworthy that the

most recent study of adult *A. robusta*, *A. humilis*, and *A. valida,* cultured at 21–28 °C (Xiao et al., 2014), reported lower Mg/Ca values of 3.20–3.95 mmol/mol, although the slope of the calibration equation was y = 0.04974x + 2.339 (R^2 = 0.21), which was similar to that derived in my study.

I can compare my results with the results of only two previous studies of the genus *Acropora*, using the regression lines displayed in Figure 2-6. Possible explanations of the large inconsistencies among the studies could include: (1) differences between the juvenile and adult samples; (2) skeletal Mg/Ca ratios are more heterogeneous in aragonite than Sr/Ca ratios; and/or (3) skeletal Mg/Ca ratios exist in multiple forms, generated by different pathways that depend on environmental factors other than temperature (e.g., light). Scenario (1) is possible because our study of adult *Acropora digitifera.* (Bell et al., in press) showed higher values for Mg/Ca (3.99–6.00 mmol/mol) than the study by Reynaud et al. (2007), even though the same 30% H_2O_2 pretreatment was used in both studies. In scenario (2), the amounts of samples could bias the Mg/Ca values. I dissolved an average of 500 μg of sample to make a 5 ml solution (100 ppm). Although the amount of sample used by Reynaud et al. (2007) was not stated, they reported that the coral powders were dissolved to create 20–30 ppm Ca. Xiao et al. (2014) dissolved 1 mg of sample to make a 10 ml solution (100 ppm). These differences in the samples analyzed could have contributed to the observed differences in Figure 2-6. In terms of scenario (3), Reynaud et al. (2007) reported that a light intensity of 400 μmol m^{-2} s^{-1} significantly increased the skeletal Mg/Ca ratios. The light intensities in our study and those of Reynaud et al. (2007) and Xiao et al. (2014) differed greatly (92–98 μmol m^{-2} s^{-1}, 400 μmol m^{-2} s^{-1}, and 190 W [about 870 μmol m^{-2} s^{-1}], respectively), which could also have contributed to the discrepancies in Figure 2-6.

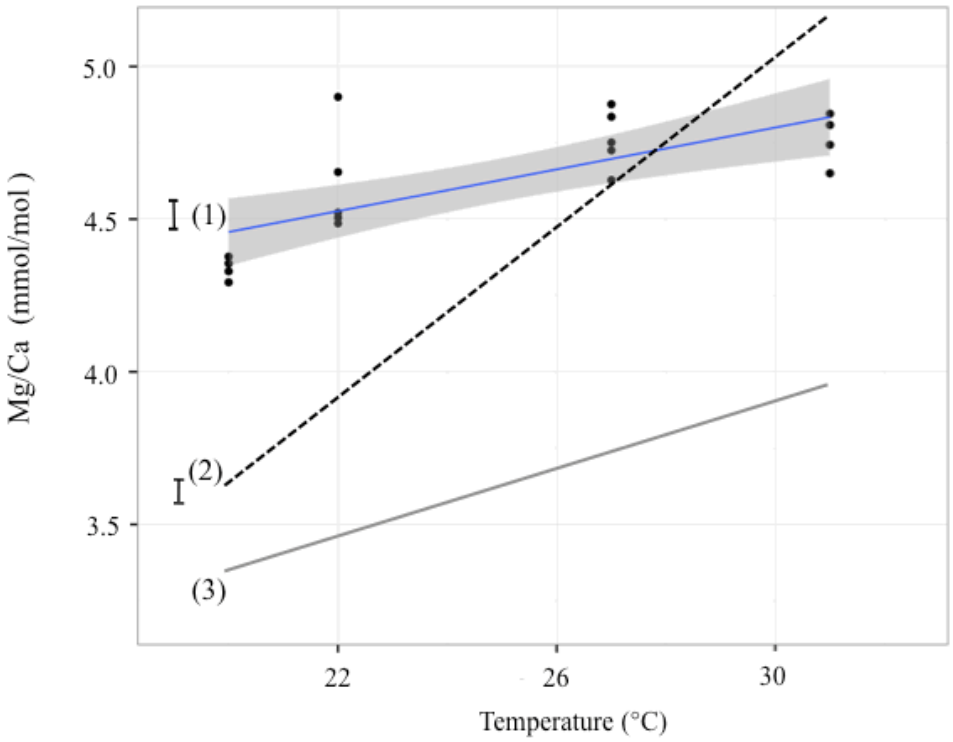

Figure 2-6. Comparison of Mg/Ca–temperature calibration equations. Equations in the Figure are: (1) This study (Cultured, juvenile) (2) The Gulf of Aqaba (Cultured, adult, Reynaud et al., 2007) (3) South China Sea (Cultured, adult, Xiao et al., 2014). Error bar next to equation (1) represent one standard deviation based on repetitive analysis of JCp-1 (N = 30). The grey zone represents the 95% confidence interval (the estimated coefficient ± two standard errors). Error bar next to equation (2) is analytical precision stated in Reynaud et al, 2007. Error bar for equation (3) is too small to show in this Figure.

2.2.3.4 Skeletal Sr/Ca and Mg/Ca ratios and growth rates

Few studies have reported on the effects of growth rates on coral Sr/Ca ratios (e.g., de Villiers et al., 1995; Reynaud et al., 2007). On the other hand, many studies have reported significant correlations between coral growth rates and skeletal Mg/Ca ratios (e.g., Allison and Finch, 2007; Inoue et al., 2007; Reynaud et al., 2007). There is only one study, which has reported on the relationship between growth rates and skeletal elements using the genus *Acropora* (Reynaud et al., 2007). In Reynaud et al.'s study, both Sr/Ca and Mg/Ca ratios showed very high correlations with growth rates (R^2 = 0.93 for Sr/Ca and R^2 = 0.98 for Mg/Ca). They cultured adult members of the genus *Acropora* and altered the light levels while maintaining a constant temperature. They found

that higher light levels significantly increased the growth rate but did not affect the Sr/Ca ratios, and that the small variations in the Sr/Ca ratios were within the range of the analytical error. Therefore, they successfully ruled out the possible impact of the growth rate on the Sr/Ca ratios, even though there was a moderate correlation between the growth rate and the skeletal Sr/Ca ratios (R^2 = 0.54) in their experiments. That is to say, they could rule out the influence of growth rates on Sr/ Ca, but they still observed the significant correlation between growth rates and Sr/Ca ratios statistically. The correlation between Sr/Ca ratios and growth rates could be an artifact in their study.

However, it should be noted that Reynaud et al. (2007) used only five averaged values to calculate this correlation coefficient. My result from the same genus also showed significant correlations between growth rates and Sr/Ca and Mg/Ca (R^2 = 0.72 and R^2 = 0.61 respectively), but our analysis also used average growth rates determined from each treatment and from bulk skeletal samples. Thus, a more detailed analysis, for example measurements of growth rates of individual polyps and skeletal elements, will be required in future studies. As described above, Sr/Ca and Mg/Ca showed significant correlations with growth rates, and some variations were found. To explain these variations, it would be useful to understand how ions are incorporated into calcium carbonate skeletons; i.e., how Sr, Mg, flow to extracytoplasmic calcifying fluid (ECF). I discuss this possibility in the next section.

2.2.3.5. **Variations of the growth rates and skeletal elements**

A proxy based on the elements in the coral skeleton should be consistent among individuals from the same environment, regardless of any variations in their growth rate. My results show that the variation was lowest in the Sr/Ca ratios, regardless of the variation in the growth rate, so the Sr/Ca ratios is an ideal proxy for temperature. Watanabe et al. (2001) reported that H_2O_2 treatment altered Mg/Ca ratios, while it did not affect Sr/Ca ratios; our results are consistent with these findings. The H_2O_2 treatment effects of both coral and synthetic aragonite Sr/Ca ratios tested by Holcomb et al. (2015) were also small (<0.1 mmol/ mol). Why the Sr/Ca ratios are superior to other proxies, such as the Mg/Ca ratios, can be explained in terms of biological, chemical, and mineralogical perspectives. First, Sr is unlikely to be controlled by biological processes, such as specific enzymes (Bell et al., in press), because Sr can be transported via paracellular pathways (pathways lacking active transport or ion specific channels), as suggested by Tambutté et al. (2012). Therefore, Sr transport is mainly abiogenic. In contrast, Mg is controlled more biogenically (e.g., Mg transporters) than abiogenically, and which will be discussed in Chapter three and four.

I must also consider the biomineralization process of coral $CaCO_3$ in terms of the organic skeletal matrix. Mitsuguchi et al. (2001) reported that the small ionic radius of Mg^{2+} and the ionic charge on Na^{+} might cause loose bonding to the aragonite lattice. Mg is also known to exist in the organic matrix (Meibom et al., 2004), acting as a glue to cement $CaCO_3$, and this Mg cannot be eliminated by pretreatment, so the amount of skeletal organic matter present can also cause variations in the growth rate and Mg/Ca ratios. In my results, fast growing polyps at high temperatures comprising more volume of centers of calcification indicated higher bulk skeletal Mg/Ca ratios. To further understand our skeletal Mg/Ca, observations on the distribution of possible ionic forms within coral skeletons and within organic matter are needed. For the reasons discussed above, Mg/Ca is not ideal parameters in coral skeletons for recording past temperatures, but it can provide information on biomineralization mechanisms, such as on ion transport systems, possible ionic forms, and their distributions.

2.2.4. Conclusion

In this study, I have shown that the Sr/Ca ratios in the primary polyps of *Acropora digitifera* are responsive to the ambient temperature, including at temperatures as high as 31 °C, which exceeds the temperature threshold for this species at our study site. Moreover, the relationship between the Sr/Ca ratios and temperature is reliable, regardless of variations in the growth rate. The Mg/Ca ratios showed a lower correlation with temperature than did the Sr/Ca ratios; Therefore, I have confirmed the robustness of the Sr/Ca ratios as a proxy for temperature, and have shown that this proxy is not affected by the stage of growth or variations in the growth rate, and is reliable over a wide range of temperatures.

2.3. Culture experiments using adult coral

2.3.1 Method

Sample collection and experiment settings

Five colonies of *Acropora digitifera* were selected from a fringing reef of Sesoko Island, Okinawa, Japan, in 2012. Branches of *Acropora digitifera* were collected from these colonies, which were at least 3 m apart and within 5m x 5m grid squares. All the samples were collected in strict accordance with good animal practice defined by the relevant national and/or local animal welfare bodies, and the sampling was performed with permission from the Japanese Municipality of Okinawa Prefecture (Permission Number: 23-25). Three branches from these colonies were randomly selected and attached to plastic bolts for the rearing experiment and cultured for ~12 months in an outside aquaria at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Japan.

The rearing experiment was performed under the same conditions as previous experiments using other coral species (Hayashi et al., 2013). From these experimental colonies, 13 branches survived from 5 colonies were used for the following experiments. The coral skeletal weight was measured using the buoyant weight technique (Davies, 1989). The increase rate was calculated as the percentage change in skeletal weight relative to the initial weight per day during the experiment. In this study, I defined this percentage as coral growth rate.

Trace element and data analysis

The sections of the branches that grew over the plastic bolts, growth area during 12 months rearing experiment, were clipped and soaked in 30% H_2O_2 for seven days to dissolve organic materials. Figure 2-7 showed the bottom of the plastic bolt and the growth area of the sample used for the trace element analysis. Then, the fragment samples were washed with 18.2 Ω ultrapure water in an ultrasonic bath for 10 minutes, and this washing process was repeated five times. Afterwards, the fragment samples were dried at 45°C in an oven overnight. Ten-milligram powdered samples were prepared and dissolved with 5% HNO_3 (Tamapure AA, Tama Chemicals) for the analysis. I measured Sr/Ca and Mg/Ca ratios for all 13 samples using inductively coupled plasma–atomic emission spectrometry (iCAP6300 Duo, Thermo Fisher Scientific) and employing two types of standards: XSTC-13 (multi-element standard solution, SPEX) and JCp-1 (a coral standard material, Geological Survey of Japan) (Okai et al., 2002).

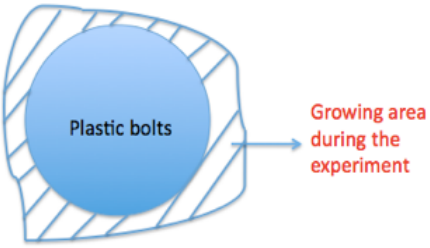

Figure 2-7. Schematic diagram showing the sample growth area during the culture experiment. The sample from lined area was collected and analyzed for trace element analysis.

2.3.2 Result

2.3.2.1 Growth rates

A total of 13 branches from 5 different colonies of *Acropora digitifera* showed different growth rates (0.23–1.61 %/day) after the common-garden culture experiment (Figure 2-8, 9 and 10). The average skeletal dry weight and the standard deviation of 13 samples were 0.83/%/day and 0.44/%/day respectively. The average growth rate of each colony was 0.68 ± 0.04%/day (N = 2, mean ± SD), 0.43 ± 0.19%/day (N = 3, mean ± SD), 1.04 ± 0.04 %/day (N = 3, mean ± SD), 1.43 ± 0.16 %/day (N = 3, mean ± SD), 0.38 ±0.21 %/day (N = 2, mean ± SD) for colony a, b, c, d, and e respectively; The growth rates were significantly different among 5 colonies ($F(4, 8) = 23.898$; $p < 0.001$).

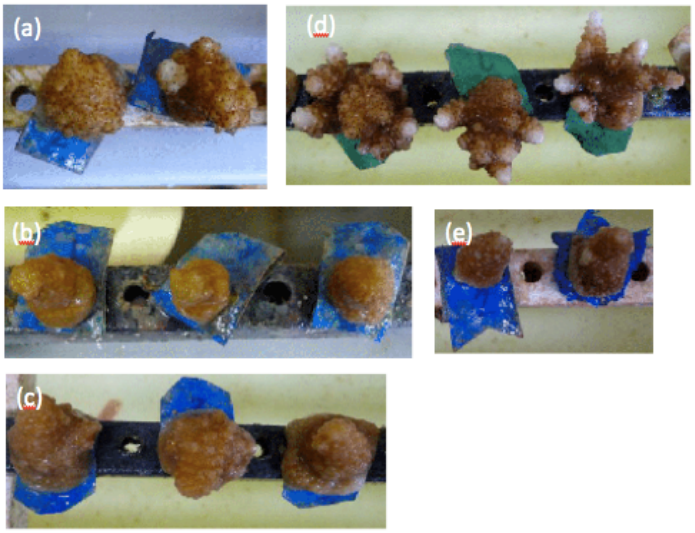

Figure 2-8. Top view photo of the 13 samples from the five colonies (a, b, c, d, and e) in the outside aquarium, 7 months after starting the experiment. Note that the blue and green plastic tapes attached to the samples are 2cm long.

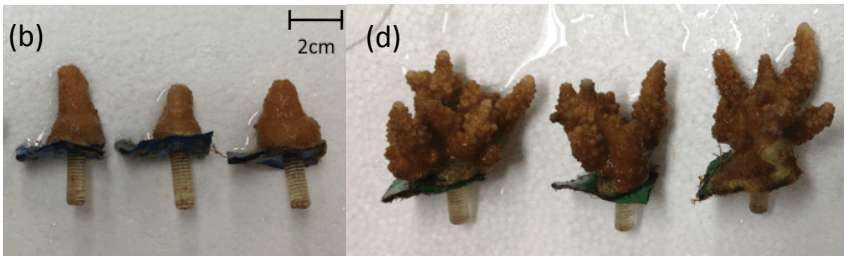

Figure 2-9. Side view photo of colony (b) and (d) after the completion of the one-year experiment.

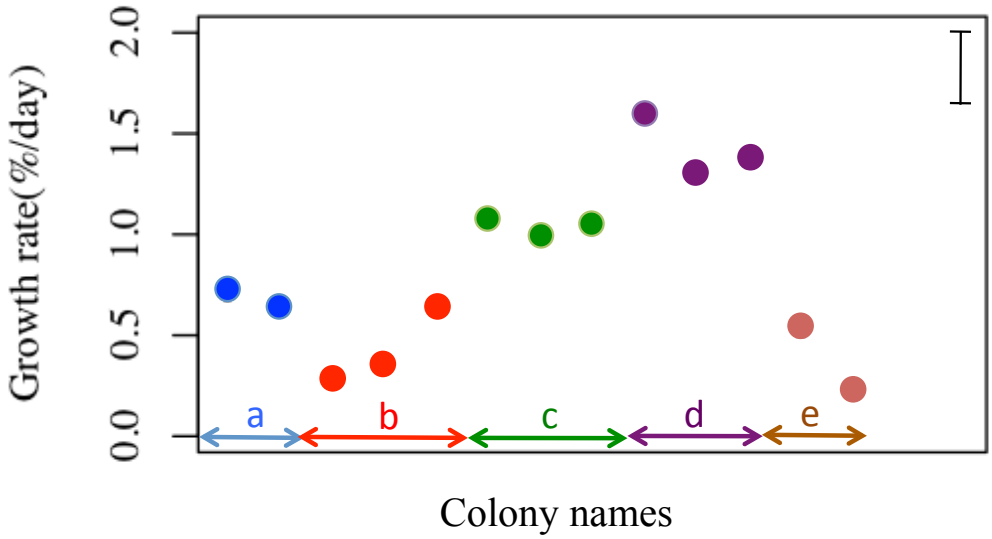

Figure 2-10. Growth rates from five colonies. Each color represents the different colony, and the number of samples are N = 2, 3, 3, 3 and 2 for colony a, b, c, d, and e respectively. Error bar represents the standard deviation of the mean of the 13 samples from five colonies.

2.3.2.2 Skeletal Sr/Ca and Mg/Ca ratios and their variation

The average Sr/Ca ratios were 9.19 ± 0.18 (N = 13, mean ± SD) and Mg/Ca ratios were 4.55 ± 0.54 (N = 13, mean ± SD). The values of these proxies were not significantly different among colonies ($F(4, 8) < 3.69$). The relative standard deviations of Sr/Ca and Mg/Ca were 1.9% and 11.9%, respectively. Thus, the variation in the Sr/Ca ratios was one-sixth of the variation in the Mg/Ca ratios (Figure 2-11).

2.3.2.3 Skeletal Sr/Ca and Mg/Ca ratios and growth rates

There were significant correlations between skeletal elements and growth rates, and they were

0.55 (p = 0.05) for Sr/Ca and -0.70 (p = 0.01) for Mg/Ca. There was no correlation between Sr/Ca and Mg/Ca (R = -0.53, p = 0.06).

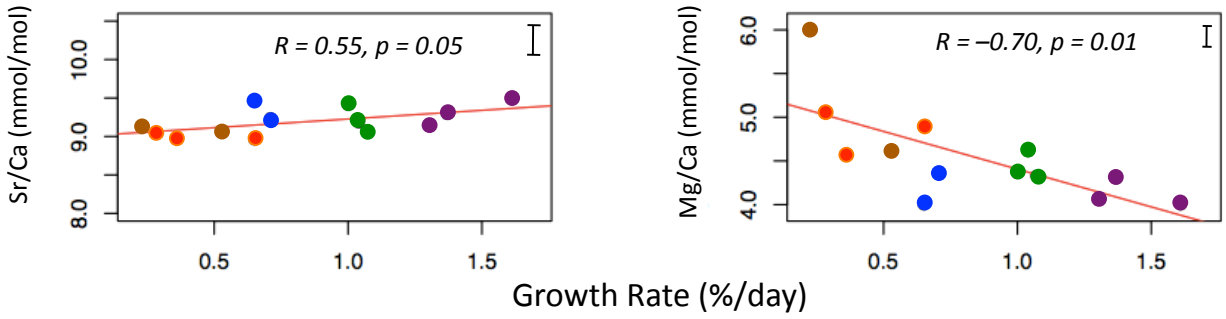

Figure 2-11. Skeletal element ratios of 13 *A. digitifera* samples from five colonies that showed varied growth rates. Each color represents the different colony, and samples from colony a, b, c, d and e are shown in blue, red, green, purple and brown respectively. The values of these proxies were not significantly different among colonies ($F(4, 8) < 3.69$). The error bar indicates the maximum difference observed between two kinds of standards: XSTC-13 (multi-element standard solution, SPEX) and JCp-1 (a coral standard material, Geological Survey of Japan).

2.3.3 Discussion

2.3.3.1 Growth rates

It is known that juvenile corals grow faster than adult (Buddemeier and Kinsie, 1976). The average growth rate from our experiment using juvenile coral (section 2.2.3.1) was 4.71%. Therefore our adult coral samples grew six times as slow as juvenile ones. However, weights of juvenile samples are lighter than adult ones, thus it is difficult to compare these two types of samples accurately. According to Moorsel (1988), juvenile corals form solid bases for the colonies that could arise from them eventually, and this force them to build massive skeleton to survive (i.e. faster growth). This could be one of the reasons why juvenile corals grow faster than adult ones.

According to Trapon et al. (2013), Juvenile coral is commonly defined as <50 mm maximum diameter, which is more than two years old for genus *Acropora* (Yucharoen et al., 2015), thus adult *Acropora digitifera* samples used for temperature reconstruction shall have at least two year worth

of skeletal samples crystallized during juvenile periods. Therefore it is of importance to compare juvenile and adult growth rates in the view of geochemistry too, and it can greatly improve our understanding of coral calcification system.

Compared to adult samples, juvenile samples are more ideal to observe calcification in real time, as it is feasible to observe calcium transportation over microscope by using staining method. Ohno et al (2017) observed active alkalization at calcification sites (subcalicoblastic medium) of aposymbiotic *Acropora digitifera* primary polyp, and they reported that pH regulate transporters are active in juvenile samples. Although we were not able to observe calcium transportation using adult corals in real time, I analyzed the gene expressions from two different growth rate samples reared in this experiment (Chapter four).

2.3.3.2 Skeletal Sr/Ca ratios

Sr/Ca ratios (8.96 ± 0.49 – 9.49 ± 0.52 mmol/mol) were very close to the values from juvenile coral of *Acropora digitifera*, (8.89 ± 0.21 – 9.32 ± 0.22 mmol/mol) in our study (section 2.2.2.2). As mentioned before, our maximum Sr/Ca ratios observed in the juvenile coral was slightly less than the values observed in the adult sample. However, this could be attributed to differences in the minimum temperatures examined, as the temperatures used in the adult and juvenile coral experiments were approximately 19 °C and 21 °C, respectively.

There are some concerns that juvenile mineralogy of calcifying organisms is possibly different from adult one. For example, other calcifying organisms such as shells have been reported to undergo significant morphological, microstructure and mineralogical changes from juvenile to adult stages (Auzoux-Bordenave et al., 2015). As mentioned above in 2.3.3.1, corals used for temperature reconstruction shall have at least two year worth of skeletal samples crystallized during juvenile periods. Therefore, it is of importance to compare juvenile and adult coral skeletal element ratios. This is the first study that confirmed that both skeletal Sr/Ca ratios exhibited the similar values, which made skeletal Sr/Ca of *Acropora digitifera* ideal as geochemical proxy.

The values of skeletal Sr/Ca ratios in my experiment were higher than those of *Porites* sp., and this trend was consistent with the previous reports (Sadler et al, 2015; Xiao et al, 2014; Reynaud et al, 2007; Gallup et al, 2006). It is known that *Acropora digitifera* grow much faster than *Porites* sp. and have different crystal structures. One of the possible scenarios is: crystal structure of *Porites* sp.

and *Acropora digitifera* could be different and cause the difference in their Sr/Ca ratios. Shirai et al. (2008) reported that microscale element analyses revealed that main part of the infilling skeleton of *Acropora nobilis* had higher Sr/Ca and U/Ca than the framework skeleton. From this reason, they suggested that chemical composition of each skeletal component of *Acropora* sp. needed to be investigated. Sadler et al. (2015) stated that near the base of *Acropora* sp. showed the clear annual oscillation, which meant this part of the skeleton had significant potential as paleoclimate proxy. My results from the base part of *Acropora digitifera* showed small variations among 13 samples, which can be supportive to Sadler's statement. However, Sadler et al. (2015) also stated 6 out of 12 samples did not have significant volume for solution based analysis. Thus this skeletal part needs further investigation to establish the relationship between Sr/Ca and water temperature, and the different analytical method such as laser ablation analysis might be necessary. It is noteworthy that Wall and Nehrke (2012) stated that *Porites lutea* are relatively porous and less differentiated into macro-morphological elements. Studies focusing on the relationship between these structural difference and elemental ratios would also provide more information about the taxon difference in skeletal Sr/Ca ratios too.

2.3.3.3. **Skeletal Mg/Ca ratios**

Mg/Ca ratios (3.99 ± 0.26 - 6.00 ± 0.39 mmol/mol) were higher than that of juvenile *Acropora digitifera* (4.34 ± 0.13 – 4.76 ± 0.14 mmol/mol) in this experiment. Reynaud et al (2007) also reported approximately 3.8 to 5.0 mmol/mol of Mg/Ca ratios from *Acropora sp*. cultured in thermostatic seawater at five temperature settings between 21°C and 29°C. On the other hand, Xiao et al (2014) reported 3.39 to 3.95 mmol/mol Mg/Ca ratios from *Acropora sp*. cultured in thermostatic seawater at six temperature settings between 21°C and 28°C.

There are no articles reporting Mg/Ca ratios of juvenile corals to compare with our results of adult corals. However, previous studies report wide range of Mg/Ca ratios in adult corals of *Porites* sp. Inoue et al (2007) reported approximately 3.5 to 6.9 mmol/mol of Mg/Ca ratios from *Porites* sp. cultured in thermostatic seawater at five temperature settings between 21°C and 29°C. They stated this variation was the result of vital effects, which might be quantified by what could not be explained from different distribution coefficients (K_d). They also stated that their growth rates are related to the Mg/Ca ratios in their skeletons. Therefore, I report the influence of growth rates on skeletal elements in the next section. It should be noted that we were able to compare growth rates and skeletal elemental ratios individually for each adult samples.

2.3.3.4 Growth rates and skeletal Sr/Ca and Mg/Ca ratios

Reynaud et al (2007) reported the significant correlation between Mg/Ca ratios and growth rates of adult *Acropora digitifera*. In this study, I also confirmed a significant correlation between growth rates and Sr/Ca and Mg/Ca ratios of adult coral of *Acropora digitifera*. They were R = 0.55 (p = 0.05) and R = - 0.70 (p = 0.01) respectively. Intriguingly, the correlations coefficients between skeletal elements and growth rates were 0.24 (p = 0.42), - 0.69 (p < 0.01) for Sr and Mg, respectively. Thus the correlation between Sr/Ca ratios and growth rates could be the result of Ca standardization. However, as I observed the weak correlation between Sr/Ca ratios and growth rates, we should look into the possibility that Sr/Ca ratios are also influenced by vital effects too. Orlowski and Champeil (1993) conducted the biochemical experiment and proved that CaATPase can be bind to Sr ions. That is to say, I can assume that Sr/Ca might be affected by vital effects to some extent. At this point, I do not know if there are Sr specific enzymes (e.g. strontium transporter), thus I will address this issue in the next chapter.

The correlation between growth rates and Mg/Ca ratios in this study was evident, as previously reported using *Porites* sp.. Also, Tanaka et al. (2015) conducted *Acropora digitifera* culture experiments under three different pH levels and reported that the variation of Mg/Ca among pH conditions was statistically insignificant, and the variation of Mg/Ca among three colonies was wider than other two colonies (4.16-5.21mmol/mol), This imply strong biological control on skeletal Mg/Ca under abiogenic fluctuations (pH). They suggested that incorporations of Mg/Ca is to some extent also affected by physiological, colony-specific factors. However, in the view of molecular physiology, it is unclear why the high correlation between Mg/Ca and growth rates was observed or why the variation in Mg/Ca was higher than Sr/Ca ratios. Therefore, in Chapter four, I discuss these uncertainties of skeletal Mg/Ca ratios by analyzing gene expressions from the samples used in this Chapter.

2.3.3.5 Variations of the growth rate and skeletal elements

Interestingly, the variation in the Sr/Ca ratios of our adult coral samples was only 1.9%, which was one-sixth of the variation in the Mg/Ca ratios (11.9%); thus, the influence of growth rates on Sr/Ca ratios is much smaller than that on Mg/Ca ratios. In geochemical studies, only one or two coral samples are typically collected for skeletal element analysis because geochemists are required to collect the most samples possible from one coral colony to obtain a few hundred years'

worth of data. Thus, it is not feasible or realistic to collect more than three coral samples. Therefore, to be a reliable geochemical tool, skeletal proxy should be consistent among individuals from the same environment, regardless of variations in growth rates. The Sr/Ca values had a variation of only 1.9%, regardless of the growth rate of the samples, and the Mg/Ca values showed the largest variation (11.9%). The stable Sr/Ca ratios among *Porites australiensis* samples with a four fold growth rate variation have been also reported (Hayashi et al, 2013), and this growth rate variation is a smaller range than found in this study (a sevenfold variation), which imply the robustness of Sr/Ca ratios as proxy.

2.4. Conclusion

I confirmed that adult coral of *Acropora digitifera* shows a wide range of growth rates in the same aquaria after one-year culture experiment. The range of growth rate was 0.23 - 1.65 % and this was much wider than three-week culture experiment using juvenile *Acropora digitifera.* Despite of a wide range of growth rates, Sr/Ca ratios of adult *Acropora digitifera* showed the least variation and Mg/Ca ratios showed the highest variation thus strong vital effects. Along with previous reports, I also found significant correlations between growth rates and Mg/Ca ratios. As abiogenic parameters are consistent in each culture experiment, Mg/Ca ratios are certainly affected biogenically, and I explore the possibility to identify genes that are related to these vital effects from next Chapter.

Chapter 3

Analysis of coral genome database

Chapter 3 Abstract

Coral skeletons are robust tools to examine past environments. However, their vital effects during skeletal formation cause uncertainties in paleoclimate reconstructions. Thus establishing the method to separate the vital effects from abiogenic one during skeletal formation is required. Here I utilized an open access and searchable gene database for coral *Acropora digitifera*, and examined the number of genes related to the elements in seawater to assess the origin of uncertainties in geochemical proxies. I found that in the predicted proteome database published as Zoophyte database, *Acropora digitifera* has genes that might process at least 15 chemical elements as individual substances (Ca, Na, Zn, K, C, N, Cl, S, Fe, Mg, Mn, Cu, H, Mo, and Te) and transporters for 7 of these elements (Ca, Na, Zn, K, Cl, Cu, and H). The number of Ca-related genes was the highest (at least 428 genes, including 53 transporters), whereas Sr, one of the most widely used geochemical proxies, was not found in the gene database. In Chapter two, I analyzed skeletal samples of *Acropora digitifera* exhibiting different growth rates; their Sr/Ca ratios showed the lower variation (1.9%), whereas Mg/Ca ratios showed higher variation (11.9%), which might be linked to the number of genes related to the proxies (namely, the magnitude of biogenic and/or abiogenic effects).

3.1 Introduction

Vital effects are defined as differences between the compositions of carbonates accreted by living organisms and predictions for carbonate minerals in equilibrium with seawater (Cohen and Gaetani, 2010), and quantification of vital effects is considered quite difficult. When geochemical proxy does not reflect environmental information, vital effects are listed as one of the reasons. However, as previously discussed in Chapter one and two, there were no methods to evaluate or quantify the impact of these effects at molecular level.

In this Chapter, I focused on coral genome biology (i.e., bioinformatics). Bioinformatics is defined as the application of computational techniques to understand and organize the information associated with biological macromolecules (Luscombe, 2001). As full genome of *Acropora digitifera* has been sequenced, I attempted to utilize this coral genome information into coral geochemistry. I believe that this information can provide new insights about vital effects.

Owing to the recent progress of coral genomic information through the increased throughput of next-generations sequencing technologies (Shinzato et al., 2011), molecular-based research on biological processes of corals has made rapid progress (e.g. Moya et al., 2012; Vidal-Dupio et al., 2013). Previously, several molecules have been proposed to form the molecular basis of coral calcification (e.g., Ca^{2+} ATPase (Zoccola et al., 2004), carbonic anhydrase (Moya et al., 2008), and organic matrix proteins (Watanabe et al., 2003; Drake et al., 2013; Takeuchi et al., 2016)), yet the molecules related to seawater elements except for Ca have not been fully explored.

Therefore in my study, I searched for genes possibly related to elemental metabolism using genomic information of *Acropora digitifera*. What is unique about our study is that I investigated each element in the periodic table and determined if it was processed more biogenically or abiogenically. I attempted to identify if there were any enzymes that were specialized in metabolizing each element.

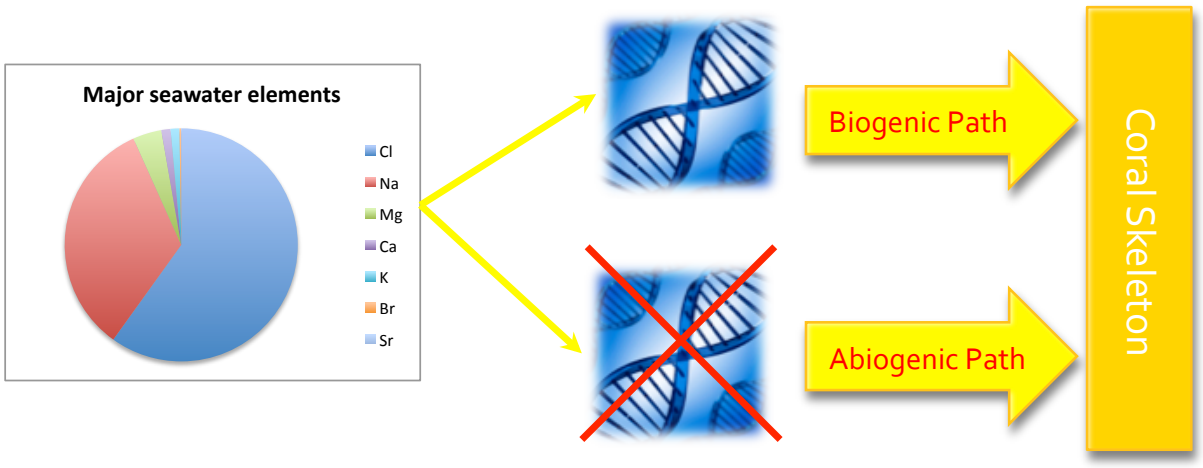

Figure 3-1. Concept of our study about biogenic and abiogenic paths for seawater elements to foam coral skeleton.

In general, increase in gene number is partially related to increase in biological complexity (Alberts et al., 2002). Also, Weng & Noel (2012) stated that metabolism is a complex network of chemical transformations mediated by multitude enzymes. Metabolic pathways generally contain a set of enzymes catalyzing sequential reactions. Therefore, I think the number of genes can be an index of complicity of metabolic process. Simply, I hypothesized that the number and type of genes specific to each element are correlated with the magnitude of the relevant biological functions, which may contribute to uncertainties in skeletal proxies affected by biological processes. To my knowledge, this is the first study to combine coral genomic studies and geochemistry, which could possibly elucidate the robustness of geochemical proxies.

3.2 Method

To identify genes that are specific to seawater elements from a coral genome, I used the published database ZoophyteBase (Dunlap et al., 2013), which allowed us to search for genes of *Acropora digitifera* that were predicted to be functional proteins performing metabolic processes involving seawater elements. In this database, proteins of *Acropora digitifera* are annotated using hidden Markov models and the Kyoto Encyclopedia of Genes and Genomes (KEGG), which is widely used as a resource for investigating biological systems and linking genes to higher-level functions. With this search engine, I used chemical elements found in seawater as keywords to search for functional proteins that are specific to each element. Although not all of the elements in the periodic table exist in seawater, I chose to investigate every element. It should be noted that chemical compounds, such as bicarbonate, were not investigated in our analysis although bicarbonate transporters have been studied using coral (*Acropora millepora*) (Moya et al., 2012).

Also, a threshold cutoff of $1e^{-5}$ was applied to detect genes specific to each element as this database shows all the genes including the ones with e-values more than $1e^{-5}$.

For example, there was one Mg transporter found in the Zoophyte database, but the e-value was 0.054, so it was not counted in this study. We should be also aware that the genes with e-value higher than $1e^{-5}$ in this database have possibilities to show different e-values depending on the selection of the databases. Each database has its advantage to calculate a sequence similarity (e-value), and it should be noted that Zoophytebase focuses on overall sequence similarities while most of other databases using the algorithm called BLAST (basic local alignment search tool), which evaluate local sequence similarities.

When one gene was found to have multiple local sequences expressing different functional proteins (transcript variants), that gene was counted based on the number of functional proteins. For example, the gene adi_v1.09241 appeared three times when the search for "calcium" was conducted because this gene consisted of three partial amino acid sequences that could express three different functional proteins. In this case, adi_v1.09241 was counted as three genes in this study.

In addition, the number of transporters was counted for each element from ZoophyteBase, as transporters are found to regulate ion flows in various types of eukaryotic cell membranes (Figure 1-4) and might have a functional role in calcification. This is because transporters control the movement of ions across cell membranes by binding to the ions, and if present in calicoblastic cell membranes, they control ion flows to extracytoplasmic calcifying fluid (ECF), which is the center of coral calcification. According to Lodish et al. (2000), transporters can carry ~10^4 ions per second; thus, the inventory of transporter genes is informative for estimating the possible ion transport from seawater to ECF too.

3.3. Results

3.3.1 Metabolic Genes and Seawater Chemistry

There are 90 elements occurring in nature, and 15 of these had hits in the ZoophyteBase search. Therefore, *Acropora digitifera* might have metabolic genes to process at least these 15 seawater elements as simple substances: Ca, Na, Zn, K, C, N, Cl, S, Fe, Mg, Mn, Cu, H, Mo, and Te. The numbers of metabolic genes for seawater elements were summarized using the periodic table (Table 3.1). The number of genes for each element was highly variable (from 0 to 428 hits), and the

chemical elements were aligned in order according to these numbers (Table 3.2). As we are interested in these elements as geochemical tools, the elements that have been previously used or studied as thermal proxies are marked with asterisks in Table 3.1.

Among the 15 seawater elements that were suggested to be processed by metabolic genes of *Acropora digitifera*, 5 elements are major seawater elements whose concentrations are >1 ppm in seawater (Cl, Na, Mg, Ca, and K), and seven elements (Zn, S, Fe, Mn, Cu, Mo, and Te) are trace elements that are defined as those with concentrations of <1 ppm. There are eight major elements in seawater that occur as simple substances: Cl (545.87 mmol/kg), Na (469.00 mmol/kg), Mg (52.82 mmol/kg), Ca (10.27 mmol/kg), K (10.21 mmol/kg), Br (0.842 mmol/kg), Sr (0.090 mmol/kg), and F (0.068 mmol/kg). Of these 8 major seawater elements, 4 were within the top 10 elements with the highest number of genes in Table 3-1: Ca (428 genes), Na (173), K (131), and Cl (44). Genes related to three other major elements in seawater (Sr, Br, and F) were not found in the ZoophyteBase search. Thus, at least using our method, we found no specific metabolic genes to process these elements in spite of their high concentrations in seawater.

It was noteworthy that Sr, which is one of the major seawater elements and the element most used as a geochemical proxy, was not found in the database by our survey. Although Ca had only the fourth highest concentrations among the five major seawater elements, the number of genes related to Ca was the highest (428 genes). Also, the number of Ca-related metabolic genes was more than two times greater than the number of genes for the second-ranking element, Na (173 genes).

Table 3-1. Number of metabolic genes for elements identified in Zoophyte database usuing our method.

H (6)																	He
*Li	Be											B	C (127)	N (54)	*O	F	Ne
*Na (173)	*Mg (1)											Al	Si	P	S (43)	Cl (44)	Ar
	*Ca (428)	Sc	Ti	V	Cr	Mn (1)	Fe (3)	Co	Ni	Cu (7)	Zn (149)	Ga	Ge	As	Se	Br	Kr
Rb	*Sr	Y	Zr	Nb	Mo (3)		Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te (1)	I	Xe
Cs	Ba		Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Ti	Pb	Bi	Po	At	Rn
Fr	Ra																
		La	Ce	Pr	Nd		Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu	
		Ac	Th	Pa	*U												

Using the Zoophyte search engine (http://bioserv7.bioinfo.pbf.hr/Zoophyte/browse/), we used each natural element in the periodic table as a key word to search for functional proteins. When a single gene had multiple local sequences to express different functional proteins, that gene was counted based on the number of functional proteins. Elements that have been previously used or studied as thermal proxies are marked with asterisks. A threshold cutoff of $1e^{-5}$ was applied to detect genes related to each element, and it should be noted that the genes with e-value higher than 1e-5 in this database have possibilities to have different e-values depending on the selection of the databases.

3.3.2. Transporter Genes and Seawater Chemistry

Of the 15 elements that are related to metabolic genes of *Acropora digitifera*, we found that 7 elements had related transporters: Na, K, Cl, Zn, Ca, Cu, and H (Table 3-2). This suggests that at least these seven elements obtained from seawater physically pass transporters of *Acropora digitifera* across membranes, possibly to reach ECF in *Acropora digitifera*. In the ZoophyteBase search, the most relevant transporters were found for Na (76 transporters), Ca (53), and K (39). These three elements are all major seawater elements, and the transporters related to these elements may import or export these elements between cells and seawater. Interestingly, the trace elements Zn and Cu, which are heavy metals known to have important biological roles (Morel and Price, 2003) such as working as cofactors of many enzyme (Biscéré et al., 2015), were among the seven elements for which relevant transporters were found.

Table 3-2. Numbers of metabolic and transporter genes identified in Zoophytebase using our method.

Key word in ZoophyteBase	Chemical element	Number of hits	Number of transporters
Calcium	Ca	428	53
Sodium	Na	173	76
Zinc	Zn	149	20
Potassium	K	131	39
Carbon	C	127	0
Nitrogen	N	54	0
Chloride	Cl	44	24
Sulfur	S	43	0
Copper	Cu	7	4
Hydrogen	H	6	6
Iron	Fe	3	0
Molybdenum	Mo	3	0
Magnesium	Mg	1	0
Manganese	Mn	1	0
Tellurium	Te	1	0

3.4. Discussion

3.4.1. Genes and seawater chemistry

As mentioned in introduction, in this study, I hypothesized that the number of genes can be possibly an index for biological complexity. Considering the larger number of Ca related genes in coral genome, this implies that a stable and well-developed Ca metabolic system appears to exist in coral *Acropora digitifera* and Ca are metabolized in a regular and steady manner biogenically although we need some cautions that these genes related to Ca are not always involved in biogenic processes of coral calcification. For example, calmodulin, which in one of the Ca^{2+}-sensing proteins and found in coral genome (Dunlap et al., 2013), has many roles in regulations for cytoskeleton organization, cellular metabolism, cell differentiation, etc (Benaim and Villalobo, 2002; Cyert, 2001; Vetter and Leclerc, 2003). Thus, calcium is involved in many metabolic processes in addition to skeletal formation in corals mentioned above.

I could assume Ca, Na, and K possess a large number of genes, and they are clearly controlled biogenically by *Acropora digitifera.*'s metabolic process. The chemical elements that exist in seawater but were not found in the gene database, such as Sr, B, and F, might be processed mainly abiogenically. However, in the next section (section 3.4.2.), I decided to conduct further investigation using different database regarding strontium to see if there were truly no genes that are specialized in metabolizing strontium. Strontium was analyzed in Chapter two from both juvenile and adult skeletal samples, and one of our main interests throughout this dissertation. Moreover, I expanded our research from dry work (database search) to wet work (molecular biological experiment), and I collected RNA from the *Acropora digitifera* samples used in Chapter two. In Chapter four, I investigated if there are any expressed genes specialized in metabolizing strontium (e.g. strontium transporter).

3.4.2. Validation regarding strontium specific genes

Although I could not find any strontium specific genes using our method in Zoophytebase, I thought I needed much more caution to interpret this result. Our result from Zoophytebase regarding strontium does not necessary mean that genes that are specialized in metabolizing strontium do not exist in *Acropora digitifera*. There are two scenarios that we should concern; (1) there are strontium related genes that we cannot find in Zoophytebase with our method, but they actually exist in other *Acropora digitifera* databases (2) there are actually proteins that strontium can specifically bind to (i.e. strontium transporter), but their gene annotations do not include the

word of strontium. To approach these two issues, I decided to utilize the following two databases from Europe and Japan (*Acropora digitifera* ver. oist_v1.1 and Protein data bank).

First, I conducted the survey using Protein data bank (PDB; https://www.ebi.ac.uk/pdbe/). The great advantage of using this database is that they have a feature named "interactive compounds". This feature is useful because it allows us to investigate proteins that interact with strontium ions but do not have the word of strontium in the annotation. In PDB database, there were 127 hits for the keyword of "strontium" under "proteins". As Zoophytebase is a proteome database, I searched the genes under "proteins". Intriguingly 87 out of the 127 genes clearly stated strontium as interactive compounds. Also 10 out of these 87 genes were specific only to strontium ions. The annotations of these 10 genes were not described as strontium specific genes, but the detailed investigation of these 10 genes were necessary as they could be possibly genes that were specialized in metabolizing strontium.

Next, I downloaded these ten protein sequences from PDB and conducted if similar sequences exist in *Acropora digitifera* ver. oist_v1.1 (http://marinegenomics.oist.jp/coral/). Four out of the 10 sequences show the similarity against *Acropora digitifera* sequences with the e-value less than $1e^{-5}$. They are; (1) Calmodulin in complex with strontium (2) Fiblin stablizing factor with strontium bond (3) Calcium binding protein in complex with Strontium (4) TPR domain of pex5p (peroxisomal cycling receptor) (Table 3-3). Therefore, I investigated each protein one by one as follows:

(1) Calmodulin and (3) Calcium binding proteins are not specifically synthesized to process strontium. Calmodulin is also part of calcium binding proteins, and they are known to have many roles including coral calcifications by binding to calcium, which I will discuss in 4.4.2 and Table 4-4. It should be noted that calcium specific proteins can also bind to strontium due to the similarity of the ionic radii (Orlowski and Champeil, 1993). According to Kushinir (1980), the ionic radius of Ca^{2+} and Sr^{2+} are 0.99 Å and 1.13 Å respectively.

Regarding (2) Fiblin stabilizing factor (PDB ID 1qrk, *Homo sapiens*), this protein is known to be activated by calcium (Fox et al, 1999). I can assume that this protein can also interact with strontium due to the radius similarity between Ca^{2+} and Sr^{2+}. Also it is reasonable to say that PDB showed interactive compounds only as strontium because it is reported as "Fiblin stablizing factor

with strontium bond".

For (4) peroxisomal cycling receptor (PDB ID 2j9q, *Homo sapiens*), Stanley et al. (2007) stated that this protein was confirmed to bind to Sr but also have the site resembles to Ca binding site. Therefore, it is reasonable to assume that this protein can interact with both Sr and Ca too. As further study, it will be of interest to extract these proteins listed in Table 3-3 from *Acropora digitifera* and conduct biochemical experiments to see if they can actually bind to strontium too.

Although I did not find strontium specific genes even in PDB database, I should look into other 77 proteins listed strontium as one of interactive compounds in PDB. I summarized the details of these proteins in Appendix. They are not specialized in metabolizing strontium, but these *Acropora digitifera* genes listed from OIST database may be capable of binding to strontium. In case, there are some proteins that can actually interact with strontium, it might explain the weak vital effects observed in Chapter two (i.e. weak correlation between adult coral Sr/Ca and growth rates).

Table 3-3. List of proteins from PDB that report strontium as only interactive compounds.

Interactive compunds : only Sr (Ttotal of 10)

PDB ID	*Annotation*	*Acropora digitifera (Gene with highest similaity in* OIST database)
4bw7	**Calmodulin in complex with strontium**	**30 genes (aug_v2a.01102.t1)**
1g1j	Oligomelization domain	No hits
1qrk	**Fiblin stablizing factor with strontium bond (XIII)**	**5 genes (aug_v2a.17869.t1)**
3ilg	Humnan insulin Sr^{2+} complex	No hits
1dd9	DNAG (DNA primase) catalytic core	No hits
2X53	Phage baseplate with strontium	No hits
3pX1	**Calcium binding protein in complex with Strontium**	**13 genes (aug_v2a.06504.t1)**
1oq7	Stearoyl Acyl Carrier Protein Desaturase	No hits
2j9q	**TPR domain of pex5p (peroxisomal cycling receptor)**	**2 genes (aug_v2a.00682.t1)**
4jkr	RNA Polymerase in complex with ppGpp (guanosine pentaphosphate)	No hits

The gene sequences from PDB were compared to *Acropora digitifera* ver. oist_v1.1. by blastp. The cutoff value was $1e^{-5}$, and the genes with e-value less than $1e^{-5}$ are written in bold letters. The gene with the highest similarity was shown in the parenthesis.

3.4.3. Relationships between the numbers of gene, growth rate and skeletal elements

I could discuss the relationships between the numbers of gene, growth rate and skeletal elements using the result from Chapter two and three. As previously stated, the correlation between growth rate and Mg/Ca ratios in this study was evident. In addition, the variation in the Sr/Ca ratios of *Acropora digitifera* was only 1.9%, which was one-sixth of the variation in the Mg/Ca ratios (11.9%). From these results regarding the numbers of gene, growth rate and skeletal elements, we

can suggest that considering elements with no specific coral genes could be criteria for reliable proxies (e.g. Sr/Ca, Li/Ca and U/Ca). For example, 31 out of 33 studies reported high correlations ($R^2 > 0.5$) between Sr/Ca ratios and SST (Correge, 2006). In addition, Li/Ca and U/Ca have been suggested to be useful thermal proxies (e.g. Hathorne et al, 2013 and Min et al, 1995), and moreover, DeCarlo et al. (2016) recently introduced Sr-U thermometer as a reliable temperature proxy by combining Sr/Ca and U/Ca.

On the other hand, it should be noted the results focusing on the numbers of genes need to be interpreted with some caution because some unknown genes found in coral genome (Shinzato et al., 2011) may contribute to coral skeletons' elemental compositions. For example, Takeuchi et al. (2016) recently reported the genes expressing organic matrix proteins that have unique sequences, which cannot be related to other cnidarians. Thus, there are possibilities that more new genes will be discovered, and we need to be keen about these new findings and the latest updates of the gene database. At this point, in the view of molecular physiology, it was unclear why the high correlation between Mg/Ca and growth rate was observed or why the variation in Mg/Ca was the higher than Sr/Ca ratios. These uncertainties of Mg/Ca can possibly be explained by Mg-related gene expression among samples with different growth rates. Thus I report comprehensive gene expression profiling using RNA-seq using coral branches with different growth rates in the next chapter.

3.5 Conclusion

One of the most important topics in biomineralization research is to clarify whether chemical elements in marine calcifiers are controlled biogenically or abiogenically when they are transported from seawater to skeleton. To explicate this issue, I propose a bioinformatics approach using genome information as an effective method. I suggest that elements with no specific coral genes could be selected as candidates for reliable proxy. I also propose that genomic information can help us find new geochemical proxies with the fewest vital effects and also explain the robustness of proxies that are already known to be effective in reconstructing past ocean environment.

Chapter 4

Analysis of genes related to different growth rates

Chapter 4 Abstract

Coral skeletal Sr/Ca ratios are widely used as a reliable water temperature proxy. On the other hand, Mg/Ca ratios show lower correlations with water temperatures, and they are considered to be impacted by growth rates. In this Chapter, I exploratory investigated genes that are possibly related to metabolizing skeletal elements (Sr, Mg and Ca) using two different growth rate samples (fast and slow) showing four fold differences. Intriguingly, I did not find Sr related genes, but I identified Mg transporter, aug_v2a.04878, that showed higher gene expressions in the fast growth sample. I suggest this gene could possibly be related to growth rate difference and large individual variations reported in skeletal Mg/Ca ratios, which are problematic to be a reliable water temperature proxy. Therefore, this gene may be related to the phenomena called vital effects in paleoclimatology. It has been long discussed that CaATPase played significant roles in coral calcification. Interestingly, I did not find the difference in CaATPase expressions between fast and slow growth samples, thus CaATPase might not be related to growth rate differences. I also report the candidate genes that could be related to differences in growth rates.

4.1. Introduction

Corals have been used as geochemical proxies since the 1970's and have had prominent roles in paleoclimatology (Correge, 2006). However, in geochemistry, individual variation among corals has not been well elucidated, thus hampering corals to be fully reliable proxies. Some research has been conducted to investigate the correlation between the individual variability and the robustness of geochemical proxies.

Most of these studies focus on different growth rates and elemental ratios in coral skeletons. For instance, Hirabayashi et al. (2013) and Inoue et al. (2007) reported growth rate variability does not impact coral Sr/Ca ratios, and Bell et al. (in press) also stated Sr/Ca ratios showed low standard deviations among coral samples that exhibited a wide range of growth rates. On contrast, coral Mg/Ca ratios show significant correlations with growth rates (Inoue et al., 2007) and they are controlled more biogenically (Tanaka et al., 2015; Reynaud et al., 2007). Thus, it is widely known that coral Sr/Ca ratios are more reliable than Mg/Ca ratios and Mg/Ca ratios show strong vital effects.

In Chapter two, I confirmed that the Sr/Ca ratios of juvenile coral of *Acropora digitifera* is a robust proxy of temperature, regardless of variations in the growth rate and the wide range of ambient temperatures. Also, I verified that Mg/Ca ratios of both juvenile and adult coral of *Acropora digitifera* showed strong individuality and high correlations with growth rates. However, I have not traced the cause of vital effects or quantified the strengths of the effects at this point.

To approach this issue, in Chapter three, I explored the possible relationship between the number of metabolic genes and skeletal elements by investigating the coral genome database. However, since the genes are not always expressed, the relationship between actual expressed genes (i.e., RNA) and skeletal proxies has not been clarified yet. I decided to use RNA-seq to approach this issue. RNA-seq is often used to identify specific genes that cause certain phenotypes and to investigate which genes are active and how much they are transcribed as RNA. It is a very powerful tool to identify individual variability within one species regarding a certain phenotype such as different growth rates.

Since the entire coral genome of *Acropora digitifera* has been sequenced (Shinzato et al., 2011), many coral genomic studies using RNA-seq have been conducted. As mentioned in Chapter one,

Shinzato et al. (2014) investigated the relationship between corals and their symbiotic zooxanthella and found out that symbionts are able to provide most of the amino acids to host corals. Meyer et al. (2011) analyzed heat shock proteins obtained from coral larvae to investigate the response to elevated temperatures. Zoccola et al. (2004) studied the coral CaATPase that plays a significant role to producing coral skeletons.

On the other hand, to our knowledge there are no studies that analyze expressed genes in the context of geochemistry and explore the relationship with skeletal growth rates and skeletal elements such as Sr/Ca and Mg/Ca ratios. As mentioned in Chapter one, *Acropora digitifera* has been recently receiving attention not only in molecular biology but as a reliable temperature proxy (Reynaud et al., 2007; Xiao et al., 2014; Bell et al., in press). Thus it is an excellent species to conduct this study, which combines molecular biology and geochemistry. I believe that this exploratory study will provide some indications about coral growth rate variability. These indication could trigger the research regarding possible impacts of specific gene expressions on skeletal proxies, making corals more robust tools for reconstructing past water temperature.

4.2. Materials and Method

Sample selection and preparation

The total of two samples, one each from the fast and slow growth colonies (Figure 2-9.) were selected for RNA-seq. The fast and slow growth samples showed 0.36 and 1.61 % growth rate per day respectively. Since I was interested in the relationship between gene expressions in the tissue and skeletal elements, we needed to extract RNA only from the soft tissue. Therefore, I was not able to freeze and crush whole coral samples with liquid nitrogen which has been reported as a successful method (Hinriches et al., 2013). In this study, the tissue of each sample was isolated by a utility knife and immediately treated by 1ml of Trizol reagent (Invitrogen). The total RNA was extracted following the manufacture's instruction of Trizol and the RNA samples were treated with DNAse to remove residual DNA (Invitrogen). Then RNA quantity was measured by NanoDrop (BioSpec-nano, Shimazu) and the quality was checked by gel electrophoresis. This RNA extraction process is summarized in Figure 4.1. It should be noted that the manufacturer's instruction stated to store RNA samples below -70°C after an extraction process. I found that the coral samples appeared to degrade during this storage process using the -80°C freezer as the stored samples could not be used for RNA-seq. Thus this storage process needed to be skipped to obtain a sufficient amount of RNA samples for RNA-seq analysis.

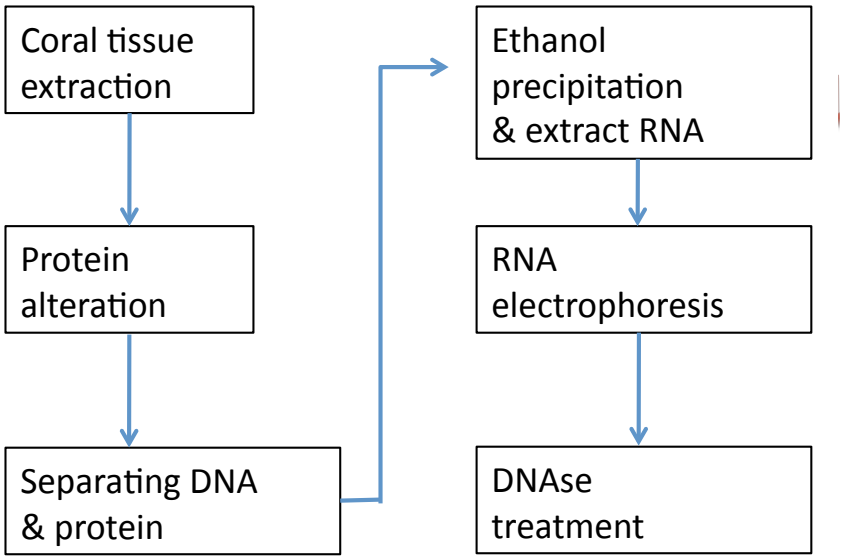

Figure 4-1. Workflow of RNA extraction process from coral tissue. This figure was made based on Invitorogen user guide.

RNA sequencing and quality filtering

RNA sequencing was conducted by illumina Hiseq2000 platform (100bp paired-end reads) at Hokkaido System Science Corporation, Japan. From the raw sequence, low -quality reads (Quality scores < 30) were excluded. The ratios of the low quality to total read for fast and slow growth samples were 4.5% and 4.3% percent respectively.

Mapping to reference transcriptome

85,598,060 and 91,099,740 high quality pair-end reads for the fast and slow growth samples were obtained respectively after the filtering process, and they were mapped against *Acropora digitifera* genome assembly (Shinzato et al., 2011) via TopHat 2.0.11 (Trapnell, 2009). Then, these reads were normalized per million mapped fragments and counted for each reference gene via Cufflinks 2.2.1 to calculate fragment per kilobase of exon per million reads (FPKM) (Trapnell, 2010).

Data organization

Using the organized data prepared from the method above, I simply use the name of the major skeletal elements (calcium, strontium, magnesium) as key words to identify genes that are annotated

with the name of each chemical element.

Validation using q-PCR

The leftover samples from RNA-seq were saved for q-PCR analysis. We designed specific primer for aug_v2a.04878 by the Software Primer3 (Rozen & Skaletsky, 2000) and it was constructed at GeneDesign, Inc. Japan (F：CGTTTTCCCACTTCCTTGAC, R：TGTCGCAGAAACACACATTG). These primers were diluted to 10 μM for q-PCR. cDNAs were made with PrimeScript® RT reagent Kit (Takara) following the manufacture's instruction. Three replicate samples for each individual (first and slow samples) were prepared, and q-PCR was conducted for each sample with 7500 Real Time PCR System (Applied Biosystems). The conditions of PCR were the following: 95℃ for 30 seconds 1 rep, 95℃ for 30 seconds 50 reps, 60℃ for 34 seconds 1rep, 95℃ for 15 seconds 1rep. GAPDH was used as a reference gene since this gene is known to have constant expression in RNA-seq (Barber et al., 2005), and it is reported to be the ideal reference gene for Scleractinian coral (Seneca et al., 2009).

4.3. Result

4.3.1. Annotation of genes related to Sr, Ca and Mg

The total of 14875 genes were identified as expressed genes from RNA-seq results. To evaluate the difference from two samples (fast and slow), I categorized genes that showed two fold difference as 1.5 to 2 are reported to be a good index to search for changes in gene expressions (Brazma and Vilo, 2000). Among 14874 identified genes, 3288 genes showed more than two fold difference in gene expressions. As I was interested in the relationship between gene expressions and skeletal elements, I investigated the number of genes that are related to metabolize the elements found in coral skeletons (Ca, Mg and Sr). I found out that the numbers of Ca and Mg related genes were 96 and 3 respectively. Out of 96 Ca related genes, 10 genes showed more than two fold difference in gene expressions and 86 genes did not. Out of three Mg related genes, only one gene showed more than two fold difference and the other two genes did not (Figure 4-2). I did not find any specific genes related to strontium, which was consistent with the finding from Chapter three, thus there were no differences between slow and fast growth samples. The ratio of genes that showed more than two fold differences was 10.4 % and 33.3% for Ca and Mg respectively.

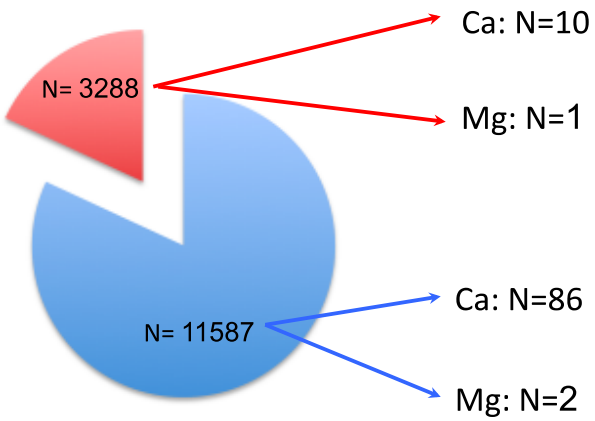

Figure 4-2. A circle chart showing the number of identified genes and Ca and Mg related genes among them. The red section indicates the genes that showed more than two fold difference and the blue section indicates genes that showed less than two fold difference between the fast and slow growth samples.

4.3.2. Ca and Mg related genes

I summarized the annotation of Mg, and Ca specific genes that showed more than two fold expression differences (Table 4-1). I also cross-plotted these gene expressions between fast and slow growth samples for Ca and Mg (Figure 4-3).

The highest expressions of genes are marked with green arrows in Figure 4-3. One of the marked genes, Mg related gene, aug_v2a.04878, showed almost four fold difference in gene expression between the fast and slow growth samples. This is a magnesium transporter protein, and it showed a higher gene expression in the fast growth sample. However, the other two Mg related genes, aug v2a.03007 and aug v2a.24016, are also transporter proteins, but their expression differences were less than two fold.

Ca related genes showed a more consistent trend: 7 out of 10 Ca related genes exhibited higher gene expressions in the fast growth sample. The Ca binding protein, aug_v2a.20373, marked with an arrow in Figure 4-3, showed the highest gene expression. For this gene, the gene expression from the fast growth sample was also four times as high as the slow growth sample. Out of 10 Ca related genes, five of them were calcium channels and exchangers that are responsible for Ca transportation. Three of them were calcium-sensing receptors and two of them were calcium-binding proteins that were related to homeostasis.

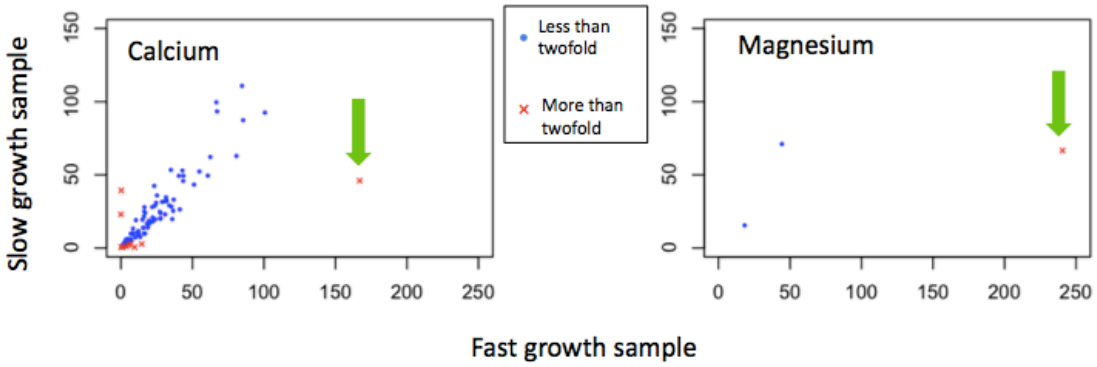

Figure 4-3. Cross plot of gene expressions (FPKM) from the fast and slow growth samples. Blue circles indicate genes showing less than two fold difference and the red × represent genes showing more than two fold difference between the two samples. The green arrow shows the highest expression gene in each figure.

Table 4-1. List of Mg specific genes, and Ca related genes that showed more than two fold difference between fast and slow growth samples. The sequences of the samples were annotated against the Swiss-Prot (http://www.uniprot.org) by blastx. Genes colored in yellow (blue) showed higher (lower) expression in fast growth sample.

Ca specific gene	Fast growth (FPKM)	Slow growth (FPKM)	Fast/Slow (fold change)	Annotation
aug_v2a.19644	9.6	0.6	16.1	Extracellular calcium-sensing receptor
aug_v2a.12207	14.6	2.8	5.2	Sodium/potassium/calcium exchanger
aug_v2a.20373	166.9	46.0	3.6	Probable calcium-binding protein
aug_v2a.01364	3.9	1.1	3.5	Two pore calcium channel protein
aug_v2a.24631	5.4	2.3	2.4	Two pore calcium channel protein
aug_v2a.19404	6.8	3.0	2.3	Calcium release-activated calcium channel protein
aug_v2a.22795	1.2	0.5	2.2	Extracellular calcium-sensing receptor
aug_v2a.18450	0.2	0.8	0.2	Extracellular calcium-sensing receptor
aug_v2a.23587	0.2	39.3	0.006	Calcium-binding mitochondrial carrier protein
aug_v2a.21348	0.0	23.0	N/A	Voltage-dependent L-type calcium channel subunit alpha-1C

Mg specific gene	Fast growth (FPKM)	Slow growth (FPKM)	Fast/Slow (fold change)	Annotation
aug_v2a.04878	240.5	66.6	3.6	Magnesium transporter protein
aug_v2a.24016	18.3	15.6	1.2	Magnesium transporter
aug_v2a.03307	44.4	71.0	0.6	Magnesium transporter protein

4.3.3. Real-time PCR validation for Mg transporter

In order to verify if the Mg transporter aug_v2a.04878 could be possibly related to faster growth as reported above, I conducted real-time PCR validation for this gene using the same RNA

sample analyzed by RNA-seq. The result indicated the fast growth sample showed relative quantity of 5 to 18 (fold difference) while the slow growth sample showed 0.6 to 1.6 (fold difference)(Figure 4-4). The difference between fast and slow growth samples from RNA-seq was approximately four fold. Although, I could not get the same exact result from RNA-seq and real-time PCR, I confirmed that the expression difference was in the same direction.

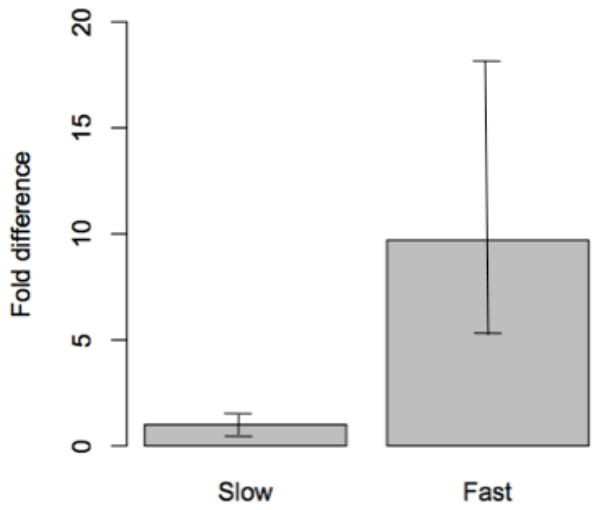

Figure 4-4. Quantitative comparison (Fold difference) of aug_v2a.04878 gene expression obtained from real time PCR. Slow and Fast represent the slow and fast growth samples (Growth rate of 0.36 and 1.61 %/day, respectively). The replicated samples (N=3 for each Slow and Fast sample) were prepared using leftover RNA-seq samples.

4.4. Discussion

4.4.1. Comparison between gene expression, skeletal Mg/Ca ratios and growth rates

To obtain the fast and slow growth samples used for RNA-seq, the culture experiment was conducted in Chapter two. In the Chapter, I analyzed skeletal Mg/Ca ratios from 13 samples (Figure 2-8) including the two samples used for RNA-seq in this chapter. Here, I calculated the correlation coefficients between the Mg/Ca ratios, Mg, Ca and growth rates from the 13 samples to investigate the possible relationship between Mg related gene expressions, skeletal elements and growth rates (Table 4-2). The correlation between Mg/Ca ratios and growth rates are apparent as many literatures previously reported (Reynaud et al., 2007; Tanaka et al., 2015; Inoue et al., 2007). There was no correlation between Ca and growth rates, but there was a significant correlation between Mg and growth rates. Additionally, Mg values (0.072-0.098 mmol) were significantly different among

colonies ($F(4,8) > 5.4329$; $p < 0.05$) while Ca ones (0.0156-0.0192 mol) were not ($F(4,8) < 0.2755$; $p > 0.05$). Therefore, I could infer that the observed correlation between Mg/Ca ratios and growth rates was caused by magnesium values not by calcium ones, which might be possibly related to the gene expression of Mg transport protein (Figure 4-5).

Even though our study is still exploratory and more data from RNA-seq will be necessary, one possible scenario is that aug_v2a.04878 could be related to faster growth rates and also lowers Mg/Ca ratios. In Chapter two, the fast growth sample of adult corals showed lower skeletal Mg and Mg/Ca ratios. One possible reason for this could be the amount of symbiotic algae resides within coral cells. Symbiotic algae are reported to promote coral growth rates (Inoue, 2012) and they need magnesium to synthesize chlorophyll (Adey and Loveland, 2004). Thus, the more symbiotic algae exist in the coral, the more the magnesium will be necessary inside coral host cells. This can result in less Mg for skeletal materials accumulated in ECF, which is outside of cells. Therefore, Mg transporter could enhance the flow to remove Mg ions from ECF, which resulted in lower skeletal Mg/Ca ratios and higher amount of Mg transporter expression. Zoccola et al. (2004) succeeded in visualizing the CaATPase expression on the calicoblastic membranes. If we could do such visualization using Mg transporter as further study, that would help us understand the Mg flux between ECF and other cells.

Interestingly, Hill and Hill (2012) suggested the magnesium inhibition hypothesis between host corals and symbiotic zooxanthellae. According to them, the increases in the Mg/Ca ratio in seawater that occurred over the last 100 million years created a situation where Mg^{2+} inhibited Ca^{2+} transport to zooxanthellae. This hypothesis proposes that there is an intracellular niche within host coral cells and zooxanthellae residing in them. Also, Reynaud (2007) reported that skeletal Mg/Ca decreased when light intensity changed from 100 to 200 μmol m^{-2} s^{-1}, but it increased significantly when the light intensity was 400 μmol m^{-2} s^{-1}. This suggests that the level of photosynthesis due to zooxanthellae might impact the fluctuations of skeletal Mg/Ca ratios. In Chapter two, I conducted a culture experiment using symbiotic algae. As further study, the analyzing skeletal Mg/Ca ratios and RNA-seq from the aposymbiotic samples will be necessary to discuss if symbiotic algae influence the variation of skeletal Mg/Ca ratios.

It should be noted that Mg/Ca ratios are known to be an effective paleoclimate proxy among foraminifera samples (Elderfield and Ganssen, 2000) unlike corals. On the other hand, some

research also reports high individual variability of foraminifera Mg/Ca ratios (Nooijer et al., 2014). It will be interesting to investigate Mg related gene expressions using different growth rate foraminifera, bivalves or other biological carbonates. To my knowledge, there are no previous studies about coral magnesium transporter, however many reports significant role of CaATPase transporting outside of calicoblastic cells (Allemand, 2004). Therefore in the next section, I discuss our result regarding CaATPase and other Ca related genes.

Table 4-2. Correlation coefficients between Mg/Ca, Mg, Ca and growth rates.
Bold letters show significant correlations.

	Mg/Ca	Mg	Ca
Growth Rates	**-0.70 (p < 0.01)**	**-0.70 (p < 0.01)**	0.16 (p = 0.60)

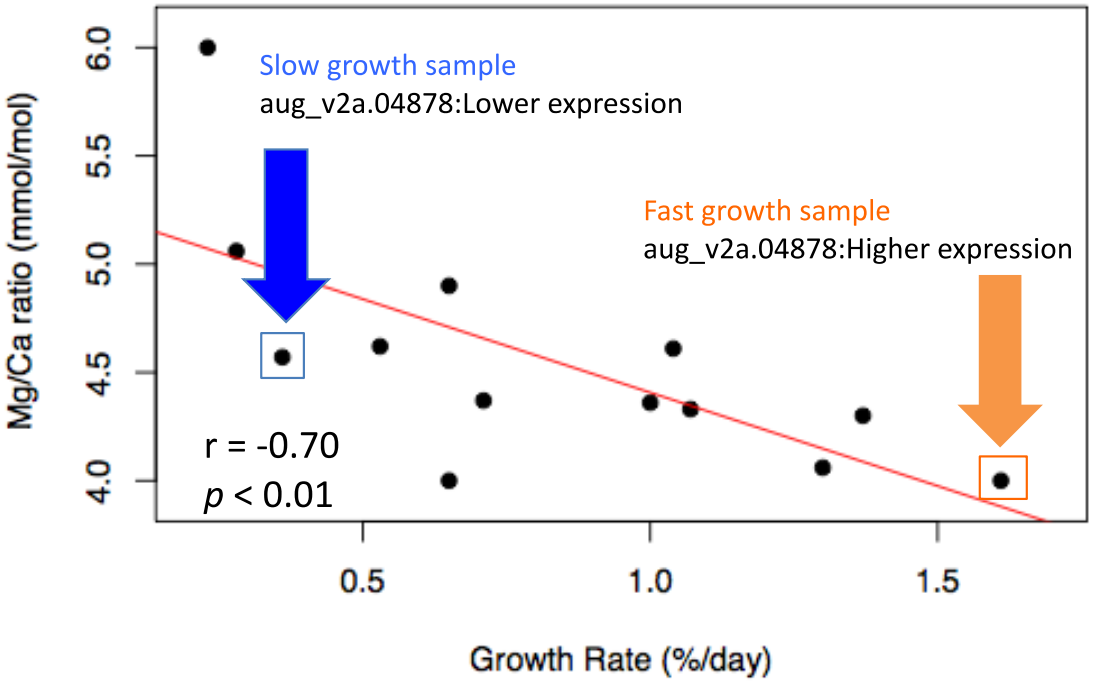

Figure 4-5. Plot of growth rates (%/day), skeletal Mg/Ca ratios (mmol/mol) of 13 samples including two samples (Fast and Slow growth samples) used for RNA-seq. These two samples are marked in the figure with arrows and squares (blue and orange arrows/squares for slow and fast growth sample respectively). Gene expression of aug_v2a. 04878 (Mg transporter) were described either as higher or as lower expression for these two samples. Note that all the samples in this figure were reared in the same exact conditions.

4.4.2. CaATPase and other Ca related genes

It has been long discussed that CaATPase is playing dominant roles in coral skeletons (Allemand, 2004). Zoccola et al. (2004) succeeded in visualizing and localizing CaATPase from coral *Stylophora pistillata*, and they confirmed that CaATPase were strongly expressed in calicoblastic cells. That is to say that CaATPase are transporting large amount of calcium from inside to outside of calicoblastic cells for efficient Ca transportation to form skeletons. They also stated that the coral Ca^{2+} pump is more closely related to vertebrate CaATPase than to *C. elegans* CaATPase. Bell et al. (2014) reported that there was 53% similarity between coral and human CaATPase sequences. In addition, I created the phylogenetic tree using coral CaATPase (v1.19074 and v1.22365), and it also showed that coral CaATPase could be related to vertebrate (Figure 4-6). This indicates that corals have well developed calcium transport systems which is consistent with our result from Chapter three: Considering the larger number of Ca-related genes in the coral genome, a stable and well-developed Ca metabolic system exists in corals, and that Ca is metabolized in a regular and steady manner biogenically.

Inoue et al. (2016) reported that CaATPase expressions appeared to have no influences on the growth rate of coral *Porites*. Intriguingly, I did not find any significant differences of CaATPase expressions either between the fast and slow growth samples, and the differences of all the CaATPase were less than two fold. Therefore, I can assume that CaATPase might not be the factor to determine the growth rates of coral *Acropora digitifera* too. I summarized the gene expression of CaATPase in Table 4-3.

I found out that out of ten Ca related genes; five of them were channel, transporters and exchanger that were responsible for Ca transportations. Three of them were calcium-sensing receptors that were in charge of calcium homeostasis. Two of them were calcium-binding proteins (CaBP) that could regulate the amount of calcium. What intriguing was aug_v2a.23587 that showed 170 fold difference, and slow grow samples showed higher gene expression. This is calcium-binding mitochondrial carrier protein, and it could be related to coral growth rates by regulating calcium concentrations in inside of calcobalstic cells. Isa and Okazaki (1987) reported that Ca^{2+} binding phospholipids of the skeletons might serve as seeding sites for $CaCO_3$ depositions. Extracting and quantifying these calcium-binding materials from fast and slow growth skeletal samples may provide more information to elucidate the gene expression of CaBP.

Even though I confirmed the expression difference of Ca related genes between fast and slow

growth samples, there were no correlations between skeletal Ca values and growth rates as mentioned above (Table 4-2). That is to say, skeletal Ca may not be impacted by gene expressions of Ca related genes unlike magnesium.

Table 4-3. List of CaATPase and their gene expressions from fast and slow growth samples

CaATPase	Fast growth (FPKM)	Slow growth (FPKM)	Fast/Slow (fold change)	Annotation
aug_v2a.22367	4.0	2.8	1.4	Plasma membrane calcium-transporting ATPase
aug_v2a.22365	100.7	92.4	1.1	Plasma membrane calcium-transporting ATPase
aug_v2a.01670	40.5	49.4	0.8	Calcium-transporting ATPase
aug_v2a.22366	21.8	28.0	0.8	Plasma membrane calcium-transporting ATPase
aug_v2a.01669	16.1	21.6	0.7	Calcium-transporting ATPase
aug_v2a.19074	67.2	93.2	0.7	Sarcoplasmic/endoplasmic reticulum calcium ATPase

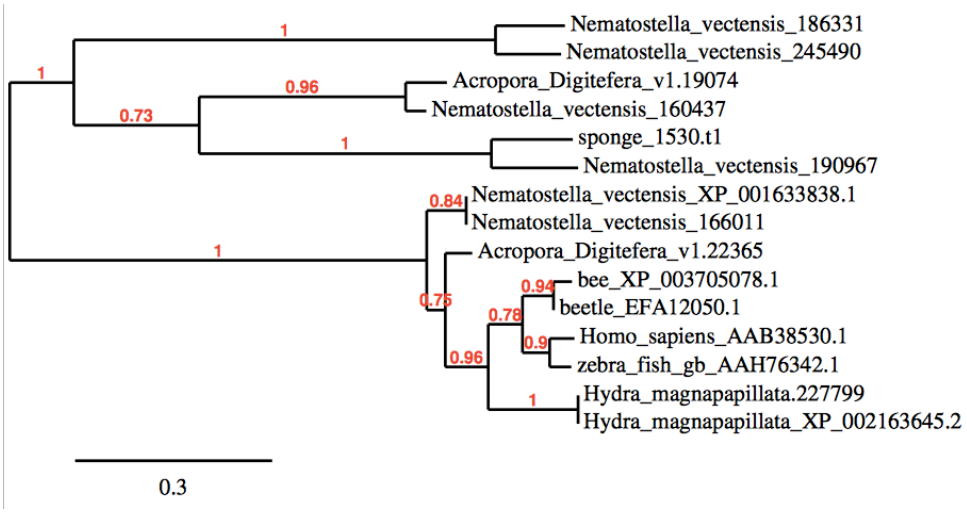

Figure 4-6. Phylogenetic tree showing sequence relationships between the *Acropora digitifera* CaATPase (v1.19074 and v1.22365). The tree was made using the Phylogeny.fr website (http://www.phylogeny.fr/index.cgi) using maximum-likelihood method. The numbers written in red represent bootstrapping values and the scale in the bottom left represents the number of substitutions per amino-acid site.

Although I did not find the expression difference in CaATPase between the fast and slow growth samples in the same aquaria (i.e. same temperature and pH), many studies reported that coral Ca related genes express differently due to temperature and pH changes (Desalvo et al., 2008; Meyer et al., 2011; Polato et al., 2013; Kaneiwska et al., 2012). The results from these studies are summarized in Table 4.4. As further study, it will be necessary to investigate the relationship between CaATPase gene expressions and skeletal Ca values using the samples from different level

of pH and temperatures.

Table 4-4. Ca related gene expressions from previous studies changing temperatures and pH.

Gene	Function	Coral	Experiment Period	pH 7.2	pH 7.4	pH 7.7	pH 7.8	29℃	31℃	32℃
Voltage gated Ca channel	Ca^{2+} transporter	*Pocillopora*	3weeks	↑	↑		↑			
Na/Ca exchanger	Ca^{2+} transporter	*Pocillopora*	3weeks	↓	↑		↓			
Plasma membrane Ca ATPase	Ca^{2+} transporter	*Pocillopora*	3weeks	↓	↑		↑			
Calmodulin	Ca^{2+} binding protein	*Acropora*	28 days			↓				
FKBP12	Ca^{2+} binding protein	*Acropora*	28 days			↓				
EGF- hand protein	Ca^{2+} binding protein	*Acropora*	28 days			↓				
Cacna1s	Ca channel regulation	*Acropora juvenile*	5days					↑	↑	
Calretinin	Ca^{2+} binding protein	*Acropora juvenile*	48h					↓		
Calretinin 2	Ca^{2+} binding protein	*Acropora juvenile*	48h					↓		
GI18443	Ca^{2+} binding protein	*Acropora juvenile*	48h					↓		
EF-hand domain protein	Ca^{2+} binding protein	*Montastraea*	10 days							↓
Calmodulin	Ca^{2+} binding protein	*Montastraea*	10 days							↓
FKBP12	Ca channel regulation	*Montastraea*	10 days							↓

Upward and downward arrows show upregulation and downregulation respectively.

4.5. Conclusions

In this study, we conducted RNA-seq using two skeletal samples that showed four fold difference of growth rates. As coral skeletons are used for elemental analysis in geochemistry, we explored genes that possibly affect the skeletal elements. A Mg transporters (aug_v2a.04878) showed four fold difference in gene expression between fast and slow growth samples. This implies that this gene could be possibly related to coral skeletal formation. As skeletal Mg/Ca ratios were lower in fast grow corals, this gene might be related to the lower value of skeletal Mg/Ca ratios too. One possible scenario is Mg transporter enhanced the flow to remove Mg ions from ECF, which resulted in lower skeletal Mg/Ca ratios and higher amount of Mg transporter expression. In any case, I can infer the expressions of this aug_v2a.04878 could possibly cause such an individual variability, so called vital effects, and leading skeletal Mg/Ca to be less reliable proxies than others. On contrast, there were no genes related to Sr metabolisms and that can be the robust reason why Sr/Ca is considered to be the most reliable proxy.

In biological and ecological studies, it will be beneficial to know which genes can lead fast or slow growth rates. In geochemistry, to understand the robustness of skeletal proxies in details, I need to further identify which gene could possibly control the value of skeletal proxies. Such an attempt to compare between gene expressions and geochemical skeletal proxies can provide valuable information about the robustness of geochemical proxies using bicarbonate materials. In addition, further study will be needed to investigate what triggered the different gene expressions such as genetic variations among colonies.

Chapter 5

General discussion and future application

5.1. Achievements of this study

I analyzed Sr/Ca and Mg/Ca ratios of *Acropora digitifera* from juvenile and adult coral samples. We fertilized the egg and sperm cells of *Acropora digitifera* and cultured juvenile samples for three weeks under controlled conditions. For adult samples, we cultured them about one year at outside aquaria, and to our knowledge, there are no other studies that conducted culture experiments using both juvenile and adult samples to validate corals as temperature proxy.

I was able to confirm that Sr/Ca ratios of juvenile samples strongly correlated with water temperature and showed less individual variability while Mg/Ca ratios showed weaker correlation with water temperature and high individual variability (i.e. vital effects). I also confirmed that both juvenile and adult *Acropora digitifera* growth rates significantly vary even in the same aquaria, and Mg/Ca ratios showed strong correlation with growth rates.

To explain these phenomena and approach the difference of ions in coral calcification process, I researched open gene database of *Acropora digitifera* and conducted RNA-seq using the two coral samples that showed the fast and slow growth. My results indicated that *Acropora digitifera* has genes that could process at least 15 chemical elements as individual substances (Ca, Na, Zn, K, C, N, Cl, S, Fe, Mg, Mn, Cu, H, Mo, and Te) and transporters for 7 of these elements (Ca, Na, Zn, K, Cl, Cu, and H). The number of Ca-related genes was the highest (at least 428 genes, including 53 transporters), whereas Sr, one of the most widely used geochemical proxies, was not found in the gene database.

Considering the larger number of Ca related genes in coral genome, this implies that a stable and well-developed Ca metabolic system exists in corals, and Ca are metabolized in a regular manner biogenically. On the other hand, chemical elements that exist in seawater but were not found in Zoophyte database, such as Sr, B, and F, might be processed mainly abiogenically. Notably, there was only one Mg related gene identified in Zoophyte database that implies Mg metabolic system was not well developed.

What unique about this study is that, in addition to this gene data base research, I was able to analyze both tissue and skeletal samples from the same samples to find relationships between gene expressions and skeletal elements. Our RNA-seq results suggested that the Mg transporter showed higher expression in the fast growth sample than slow growth one. As reported in this study and

many literatures, growth rate and Mg/Ca ratios are strongly correlated. Therefore the expression difference in this Mg related gene could be related to individual variations in skeletal Mg/Ca ratios. Even though our RNA-seq results are still exploratory, I could infer the expression difference in this gene could possibly explain why skeletal Mg/Ca ratios showed strong vital effects than other proxies such as Sr/Ca.

In addition, I confirmed that Sr/Ca ratios of *Acropora digitifera* are not strongly affected by growth rates. Although many studies have been conducted using adult coral of *Porites* sp., this was the first study analyzing both juvenile and adult coral of the same species (i.e. *Acropora digitifera*) as temperature proxy. It is also known that *Acropora* sp. grow much faster than *Porites* sp; *Porites* sp. grow 0.5-3.0cm/year (Lough and Cantin, 2014) where growth rates of *Acropora* sp. is 3.0-11.5cm/year (Boulon, 2005). Also, this species lives in both tropics and subtropics (Wallace, 1999; Yara et al., 2012). Thus Sr/Ca ratios of *Acropora digitifera* must be useful to reconstruct sea temperature with higher resolution from a wide range of the ocean. I can expect that this species will be able to provide the sea surface temperature data to global climate model.

It is reported that global sea surface temperature is rising about 0.53℃ per 100 years. Therefore projecting future sea temperature change is one of the most critical tasks that researchers in our generations need to resolve. In this study, I was able to confirm that Sr/Ca ratios of *Acropora digitifera,* relatively new temperature proxy*,* were robust temperature proxy. Moreover, I was able to suggest the possible reason at molecular level why Sr was processed mainly abiogenically thus reflecting ambient temperature.

5.2. Future application

I suggest my genetic approach can be applied to all the calcifying organisms that are used as geochemical proxy. For example, foraminifera and bivalves that are also utilized as temperature proxies can be investigated with the same method as this study. At this point, foraminifera do not have whole genome database, and the progress of genome study in calcifying organisms can be combined to the geochemistry using my approach. It is reported that Mg/Ca ratios of clone foraminifera samples also show more vital effects than Sr/Ca ratios as temperature proxy (Nooijer et al., 2014), which is consistent with my results. To elucidate this phenomenon, it would be interesting to investigate Mg related genes in foraminifera. The consolidate gene dataset for calcifying organisms would be very helpful to investigate how elements in seawater are possibly

processed in different organisms.

I could utilize our findings from this study to coral ecology studies to monitor coral reef growth rates because the genes discussed in Chapter four could be indexes to assess the coral growth rates. Currently, quadrat or line transect method is widely used (Rogers et al., 2001) and they are labor intensive to monitor coral reef growth rate. They require regular *in situ* monitoring, frequent visit of monitoring sites for long terms. With the knowledge of specific genes that cause faster growth, we would be able to collect tissue samples to project coral growth. For example, we can conduct the random sampling of coral tissue samples from study sites to analyze specific gene expressions and predict coral growth.

Another interesting utilization of this finding is screening corals before core extracting in paleoclimate studies. Currently, coral cores are extracted without any specific criteria, and usually only one coral colony that is relatively convenient for extraction process is randomly selected (e.g. accessible and/or flat surface). With the knowledge of genes that cause faster growth rate, we can collect tissue samples to identify which coral colony grow faster in the study area, and that will lead to the ideal coral skeleton that can provide higher resolution records.

Along with exploring all the possible applications, it is imperative to be aware of the limitation of this study and approach. It should be noted that ZoophyteBase is an open access and searchable database of complete annotation of the predicted proteome of the coral *Acropora digitifera,* and 81% of the predicted proteome was assigned (Dunlap et al., 2013). Thus, the coverage is not 100% as other gene databases, and we should be careful that some unknown/unidentified genes might be related to processing some elements. To overcome this problem, we need to be keen about that new coral genes discovered and its database update.

Also, it is reported that CaATPase is able to carry Sr instead of Ca. Orlowski and Champeil (1993) stated that this happens about one in 100 Ca ions. Therefore I cannot state that Sr are processed completely abiogenically. To my knowledge, there are no studies reporting that magnesium ions being able to bind calcium transporter, and this could be possibly explained by the ion diameter. The ionic radii of Sr^{2+} and Mg^{2+} are 1.13 and 0.65 Å, respectively while the ionic radius of Ca^{2+} is 0.99 Å. The radius of Mg^{2+} is about 35% smaller than the radius of Ca^{2+} (Kushnir, 1980).

Lastly, it should be noted that the following further studies are needed. In Chapter three, I suggested that considering elements with no specific coral genes could provide effective criteria for reliable proxies (e.g., Sr/Ca, Li/Ca and U/Ca). Therefore, skeletal samples should be analyzed for Li/Ca, U/Ca and U/Sr as they are expected to show less vital effects. As well as skeletal samples, RNA-seq data from more tissue samples should be analyzed as the data in Chapter four are still exploratory and much more data are needed to reach robust conclusion. In addition to analyzing different growth rate samples for RNA-seq, I also need to conduct biochemical experiments for the genes listed in Chapter three as they are the results of homology search using databases. León and Markel (2006) stated that sequence and structural similarity of proteins do not always mean functional similarity, and the computational results require experimental confirmation. In this study, I did not find strontium specific gene from both databases and RNA-seq. However, we should be aware that there might be some genes that are not specific to metabolize strontium but capable to bind to strontium. Ultimately, it will be ideal if I am able to construct figures about the relationship between specific genes and skeletal elements (Figure 5.1).

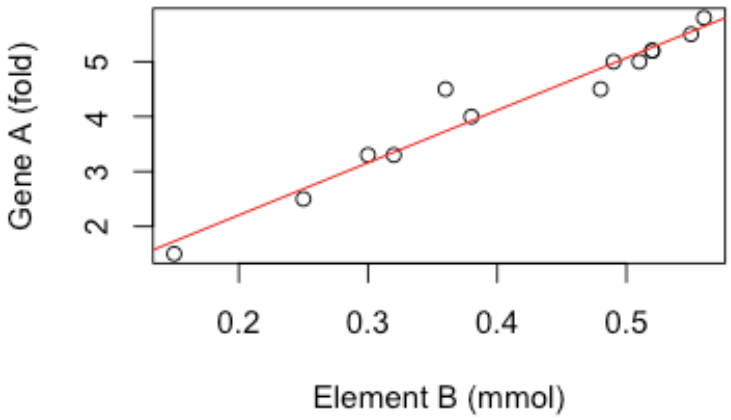

Figure 5-1. Depiction of the future study: finding the correlation between specific gene and skeletal element.

As conclusion, combining the genetic information from open databases and RNA-seq can certainly be utilized to increase the robustness of geochemical proxy. I propose a bioinformatics approach using genome information as an effective method. I suggest that genomic information can help us find new geochemical proxies with the fewest vital effects and also explain the robustness of proxies that are already known to be effective in reconstructing past ocean environments.

Acknowledgements

I would like to thank my most ambitious advisor Dr. Yusuke Yokoyama and big-hearted Dr. Akira Iguchi for their continuous support and patience for my research. I'm grateful for Dr. Atsushi Suzuki and Dr. Kozue Nishida for lots of constructive advice and great encouragement. Also, I would like to thank Dr. Kazuhiko Sakai for inspiring conversation and Dr. Takashi Nakamura for cooperation. In addition, I would like to thank Ms. Kei Ishikawa, Mr. Yoshikazu Oono, Mr. Yuki Yoshioka and Mr. Ituputa Tada for conducting this exciting research together. I truly appreciate all the amazing members at Dr. Yokoyama's lab for being supportive and helping me get through this Ph.D program. Also, I would like to thank my committee members, Dr. Toshihiro Kogure, Dr. Kazuyoshi Endo, Dr. Kayane Hajime, Dr. Chuya Shinzato for spending their valuable time to review this paper.

This research was supported by the CANON foundation (R12-Z-0013) and JSPS KAKENHI (26220102, 26247085, JP15KK0151). We thank the Japanese Municipality of Okinawa Prefecture for the permit to collect the coral samples, Hiromi Kinjyo for suggestions on culture methods, and Yumiko Yoshinaga and Mizuho Sato at AIST for the analysis of the water samples and the measurement of coral skeletal weights.

Words cannot express how thankful I am to my parents, Kazuo and Toshie Kasahara and my beautiful son Heagen Bell. I am so blessed that I have all the amazing and the kindest people in my life, especially; Jack, Ruth-Ann, Jim and Jesse Womble, Riza, Jonah, CJ and Kiara Kinney, Misaki, Victor and Monet Charney, Yuko Suzuki, Fereshteh Zadeh, Nahid Holmes, Bert Conyers, Susie Krehbiel, Ann and Rob Ramseyer, Jane Yourdon, Mary Stephens, Blaz Miklavick, Maria Kottermair, Shoko Hirabayashi, Maaike, Han and Tobias de Natris. Thank you very much for the continuous encouragement to help me get through the most challenging time in my life for the past year and half. Also, high paws to Charlie, Archie and Bowser.

References

Adey, W. H. and Loveland, K. (2007) *Dynamic aquaria: building living ecosystems*. Academic Press.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, H. and Walter, P. (2002) *Molecular Biology of the Cell*, *4th edition*. Garland Science, Chap. 7.

Al-Horani, F. A., Al-Moghrabi, S. M. and Beer, D. D. (2003) The mechanism of calcification and its relation to photosynthesis and respiration in the scleractinian coral Galaxea fascicularis. *Mar. Biol.* **142**(3), 419-426. doi:10.1007/s00227-002-0981-8

Allison, N. and Finch, A. A. (2007) High temporal resolution Mg/Ca and Ba/Ca records in modern *Porites lobata* corals. *Geochem. Geophys. Geosyst.*, **8**, Q05001.

Allison, N., Cohen, I., Finch, A. A., Erez, J. and Tudhope, A. W. (2014) Corals concentrate dissolved inorganic carbon to facilitate calcification. *Nat. Commun.* **5**, 5741. doi:10.1038/ncomms6741

Allemand, D., Ferrier-Pagès, C., Furla, P., Houlbrèque, F., Puverel, S., Reynaud, S., Tambutté, É., Tambutté, S. and Zoccola, D. (2004) Biomineralisation in reef-building corals: from molecular mechanisms to environmental control. *C. R. Palevol*, **3**(6-7), 453-467.

Auzoux-Bordenave, S., Brahmi, C., Badou, A., Rafélis, M. D. and Huchette, S. (2015). Shell growth, microstructure and composition over the development cycle of the European abalone Haliotis tuberculata. *Mar. Biol.* **162**, 687-697.

Barber, R. D., Harmer, D. W., Coleman, R. A. and Clark, B. J. (2005) GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiological Genomics,* **21**(3), 389-395. doi:10.1152/physiolgenomics.00025.2005

Bell, T., Iguchi, A., Suzuki, A., Seki, A. and Yokoyama, Y. (2014) Coral genes related to elements in seawater and their potential influence on variations in skeletal climate proxies. Presented at Japan Geoscience Union, Yokohama, Japan.

Bell, T., Iguchi, A., Suzuki, A., Seki, A. and Yokoyama, Y. (in press). Testing possible relationships between *Acropora digitifera* genes, seawater chemistry and skeletal elements. *Geochem. J.*

Bell T., Nishida, K., Ishikawa, K., Suzuki, A., Nakamura, T., Sakai, K., Iguchi, A. and Yokoyama, Y. (2017) Temperature controlled culture experiments using primary polyps of coral *Acropora digitiferaa*: their growth rate variations and skeletal Sr/Ca, Mg/Ca, and Na/Ca ratios. *Paleogeogr. Paleoclimatol. Paleoecol.* **484**(15), 129-135, https://doi.org/10.1016/j.palaeo.2017.03.016

Bellwood, D., Goatley, C., Cowman, P. and Bellwood, O. (2015). The evolution of fishes on coral reefs: Fossils, phylogenies, and functions. *Ecology of fishes on coral reefs*. Cambridge, CUP.

Benaim, G. and Villalobo, A. (2002) Phosphorylation of calmodulin. Functional implications. *Eur. J. Biochem.* **269**, 3619–3631.

Boulon, R. (2005) *Atlantic Acropora status review*. Silver Spring, MD: U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service.

Brazma, A. and Vilo, J. (2000) Gene expression data analysis. *FEBS Lett*. **480**, 17-24.

Buddemeier, R. W. and Kinsey, R. A. (1976) Coral growth. *Oceanogr. Mar. Biol. Ann. Rev.* **14**, 183-225.

Biscéré, T., Rodolfo-Metalpa, R., Lorrain, A., Chauvaud, L, Thébault, J., Clavier, J. and Houlbrèque, T. (2015) Responses of Two Scleractinian Corals to Cobalt Pollution and Ocean Acidification. *PLOS ONE* **10**, e0122898, doi: 10.1371/journal.pone.0122898.

Chalker, B. and Barnes, D. (1990) Gamma densitometry for the measurement of skeletal density.

Coral Reefs, **9**(1), 11-23.

Clode, P. L., Lema, K., Saunders, M. and Weiner, S. (2010) Skeletal mineralogy of newly settling *Acropora millepora* (Scleractinia) coral recruits. Coral Reefs , **30**(1), 1-8.

Cohen, A. and McConnaughey, T. A. (2003) Geochemical Perspectives on Coral Mineralization. *Rev. Mineral. Geochem.* **54**(1), 151-187.

Cohen, A. L. and Gaetani, G. A. (2010) Ion partitioning and the geochemistry of coral skeletons: solving the mystery of the vital effect. *Ion Partitioning in Ambient-Temperature Aqueous Systems*. European Mineral-ogical Union and the Mineralogical Society of Great Britain & Ireland, London.

Correge, T. (2006) Sea surface temperature and salinity reconstruction from coral geochemical tracers. *Paleogeogr. Paleoclimatol. Paleoecol*. **232**(2-4), 408-428.

Cyert, M. S. (2001) Genetic analysis of calmodulin and its targets in Saccharomyces cerevisiae. *Annu. Rev. Genet.* **35**, 647–672.

Davies, P. S. (1989) Short-term growth measurements of corals using an accurate buoyant weighing technique. *Mar. Biol.* **101**, 389–395.

Decarlo, T. M., Gaetani, G. A., Cohen, A. L., Foster, G. L., Alpert, A. E. and Stewart, J. A. (2016) Coral Sr-U thermometry. *Paleocean. Paleoclimatol.* **31**(6), 626-638.

Desalvo, M. K., Voolstra, C. R., Sunagawa, S., Schwarz, J. A., Stillman, J. H., Coffroth, M. A., Szmant, A. M., Medina, M. (2008). Differential gene expression during thermal stress and bleaching in the Caribbean coralMontastraea faveolata. *Mol. Ecol.* **17**(17), 3952-3971

Descombes, P., Wisz, M., Leprieur, F., Parravicini, V., Heine, C., Olsen, S., Swingedouw, D., Kulbicki, M., Mouillot, D. and Pellissier, L. (2015). Forecasted coral reef decline in marine biodiversity hotspots under climate change. *Global Change Biology*, 21(7) 2479-2487.

de Villiers, S., Nelson, B. K. and Chivas, A. R. (1995) Biological controls on coral Sr/Ca and δ ^{18}O reconstructions of sea surface temperatures. Science **269**(5228), 1247-1249.

Dietzel, M., Gussone, N. and Eisenhauer, A. (2004) Co-precipitation of Sr^{2+} and Ba^{2+} with aragonite by membrane diffusion of CO_2 between 10 and 50 ℃. *Chem. Geol.* **203**, 139–151.

Drake, J. L., Mass, T., Haramaty, L., Zelzion, E., Bhattacharya, D., and Falkowski, P. G. (2013) Proteomic analysis of skeletal organic matrix from the stony coral Stylophora pistillata. *PNAS* **110**(10), 3788-3793.

Dubinsky, Z., and Stambler, N. (2010) *Coral Reefs: an Ecosystem in Transition: An Ecosystem in Transition*. New York: Springer.

Dunlap, W. C., Starcevic, A., Baranasic, D., Diminic, J., Zucko, J., Gacesa, R., van Oppen, M. J. H., Hranueli, D., Cullum, J. and Long, P. F. (2013) KEGG orthology-based annotation of the predicted proteome of *Acropora digitifera*: ZoophyteBase - an open access and searchable database of a coral genome. *BMC Genomics* **14**, doi:10.1186/1471.

Elderfield, H. and Ganssen, G. (2000) Past temperature and ^{18}O of surface ocean waters inferred from foraminiferal Mg/Ca ratios. *Nature*, **405**, 442-445.

Fox, B. A., Yee, V. C., Pedersen, L. C., Trong, I. L., Bishop, P. D., Stenkamp, R. E. and Teller, D. C. (1999). Identification of the Calcium Binding Site and a Novel Ytterbium Site in Blood Coagulation Factor XIII by X-ray Crystallography. *J. Biol. Chem.,* **274**(8), 4917-4923.

Gagan, M. K., Dunbar, G. B. and Suzuki, A. (2012) The effect of skeletal mass accumulation in *Porites* on coral Sr/Ca and δ ^{18}O paleothermometry. Paleoceanography **27**(1), PA1203.

Gagan, M. K., Ayliffe, L. K., Beck, J. W., Cole, J. E., Druffel, E. R. M., Dunbar, R. B. and Schrag, D. P. (2000) New views of tropical paleoclimates from corals. *Quat. Sci. Rev.* **19**, 45–64.

Gallup, C. D., Olson, D. M., Edwards, R. L., Gruhn, L. M., Winter, A. and Taylor, F. W. (2006) Sr/Ca–Sea surface temperature calibration in the branching Caribbean coral *Acropora palmata*. *Geophys. Res. Lett*. **33**(3), L03606.

Gattuso, J., Allemand, D. and Frankignoulle, M. (1999). Photosynthesis and Calcification at Cellular, Organismal and Community Levels in Coral Reefs: A Review on Interactions and Control by Carbonate Chemistry. *American Zoologist,* **39**(1), 160-183. doi:10.1093/icb/39.1.160

Hathorne, E., Gagnon, A., Felis, T., Adkins, J., Asami, R., Boer, W., Caillon, N., Case, D., Cobb, K., Douville, E., deMenocal, P., Eisenhauer, A., Garbe-Schönberg, D., Geibert, W., Goldstein, S., Hughen, K., Inoue, M., Kawahata, H., Kölling, M., Cornec, F., Linsley, B., McGregor, H., Montagna, P., Nurhati, I., Quinn, T., Raddatz, J., Rebaubier, H., Robinson, L., Sadekov, A., Sherrell, R., Sinclair, D., Tudhope, A., Wei, G., Wong, H., Wu, H. and You, C. (2013). Interlaboratory study for coral Sr/Ca and other element/Ca ratio measurements. *Geochemistry, Geophysics, Geosystems*, **14**(9), 3730-3750.

Hayashi, E., Suzuki, A., Nakamura, T., Iwase, A., Ishimura, T., Iguchi, A., Sakai, K., Okai, T., Inoue, M., Araoka, D., Murayama, S. and Kawahata, H. (2013) Growth-rate influences on coral climate proxies tested by a multiple colony culture experiment. *Earth Planet. Sci. Lett.* **362,** 198-206.

Hayashibara, T., Shimoike, K., Kimura, T., Hosaka, S., Heyward, A., Harrison, P., Kudo, K. and Omori M. (1993) Patterns of coral spawning at Akajima Island, Okinawa, Japan. *Mar. Eco.-Prog. Ser*. **101**, 253-262.

Hills, M. and Hills, A (2012). The magnesium inhibition and arrested phagosome hypotheses: new perspectives on the evolution and ecology of Symbiodinium symbioses. *Biol. Rev.* **87**(4), 804-821.

Hirabayashi, S., Yokoyama, Y., Suzuki, A., Kawakubo, Y., Miyairi, Y., Okai, T. and Nojima, S. (2013) Coral growth-rate insensitive Sr/Ca as a robust temperature recorder at the extreme latitudinal limits of *Porites*. *Geochem. J*. **47**, e1–e5.

Holcomb, M., DeCarlo, T. M., Schoepf, V., Dissard, D., Tanaka, K. and McCulloch, M. (2015) Cleaning and pre-treatment procedures for biogenic and synthetic calcium carbonate powders for determination of elemental and boron isotopic compositions. *Chem. Geol.* **398**, 11-21.

Hinrichs, S., Patten, N. L. and Waite, A. M. (2013). Temporal Variations in Metabolic and Autotrophic Indices for *Acropora digitifera* and *Acropora spicifera* – Implications for Monitoring Projects. *PLOS ONE* **8**(5): e63693.

Houlbrèque, F., Meibom, A., Cuif, J., Stolarski, J., Marrocchi, Y., Ferrier-Pagès, C., Domart-Coulon, C. and Dunbar, R. B. (2009). Strontium-86 labeling experiments show spatially heterogeneous skeletal formation in the scleractinian coral *Porites porites*. *Geophys. Res. Lett.* **36**(4). doi:10.1029/2008gl036782

Inoue, M., Suzuki, A., Nohara, M., Hibino, K. and Kawahata H. (2007) Empirical assessment of coral Sr/Ca and Mg/Ca ratios as climate proxies using colonies grown at different temperatures. *Geophys. Res. Lett*. **34**, L12611

Inoue, M., Suwa, R., Suzuki, A., Sakai, K. and Kawahata, H. (2011) Effects of seawater pH on growth and skeletal U/Ca ratios of *Acropor digitifera* coral polyps. *Geophys. Res. Lett*. **38**, L12809.

Inoue, M., Shinmen, K., Kawahata, H., Nakamura, T., Tanaka, Y., Kato, A., Shinzato, C., Iguchi, A., Kan, H., Suzuki, A. and Sakai, K. (2012) Estimate of calcification responses to thermal and freshening stresses based on culture experiments with symbiotic and aposymbiotic primary polyps of a coral, *Acropor digitifera. Global Planet. Change* **92-93**, 1-7.

Inoue, M., Yokoyama, Y., Hikabe, M., Shinzato, C., Nakamura, T., Iguchi, A., Kan, H., Suzuki, A. (2016) The 4th Cannon Symposium
http://www.canon-foundation.jp/common/pdf/aid_awardees/4/37_inoue_cfk4.pdf.

Isa, Y., Okazaki, M. (1987) Some observations on the Ca-binding phospholipid from scleractinian coral skeletons, *Comp. Biochem. Physiol. B: Biochem. Mol. Biol*. **87**(3), 507-512.

Iwao, K., Fujisawa, T. and Hatta, M. (2002) A cnidarian neuropeptide of the GLWamide family induces metamorphosis of reef-building corals in the genus *Acropora. Coral Reefs* **21**(2), 127-129.

Kaniewska, P., Campbell, P. R., Kline, D. I., Rodriguez-Lanetty, M., Miller, D. J., Dove, S. and Hoegh-Guldberg, O. (2012) Major cellular and physiological impacts of ocean acidification on a reef building coral. *PLoS ONE* **7**, e34659.

Kinsman, D. J. J. and Holland, H. D. (1969) The co-precipitation of cations with $CaCO_3$-IV. The co-precipitation of Sr^{2+} with aragonite between 16° and 96°C. *Geochim. Cosmochim. Acta* **33,** 1–17.

Kubota, K., Yokoyama, Y., Ishikawa, T., Suzuki, A. and Ishii, M. (2017). Rapid decline in pH of coral calcification fluid due to incorporation of anthropogenic CO2. *Scientific Reports* **7**(1). doi:10.1038/s41598-017-07680-0

Kushnir, J. (1980) The coprecipitation of strontium, magnesium, sodium, potassium and chloride ions with gypsum. An experimental study. *Geochim. Cosmochim. Acta* **44,** 1471-1482.

Luscombe, N. M., Greenbaum, D. and Gerstein M. (2001) What is bioinformatics? A proposed definition and overview of the field. *Methods Inf. Med.* **40**(4), 346-58.

León, D. and Markel, S. (2006) *In silico technologies in drug target identification and validation*. CRC/Taylor & Francis, Chap.3.

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D. and Darnell, J. (2000) *Molecular Cell Biology*. W. H. Freeman, Chap. 15.

Lough, J. M., and Cantin, N. E. (2014). Perspectives on Massive Coral Growth Rates in a Changing Ocean. *The Biol. Bull.* **226**(3), 187-202.

Meyer, E., Aglyamova, G. V., Wang, S., Buchanan-Carter, J., Abrego, D., Colbourne, J. K., Willis, B. L. and Matz, M. V. (2009) Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. *BMC Genomics* **10**, doi:10.1186/1471-2164-10-219.

Meyer, E., Aglyamova, G. V. and Matz, M. V. (2011) Profiling gene expression responses of coral larvae (Acropora millepora) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. *Mol. Ecol*. **20**, 3599-3616.

Meibom, A., Cuif, J.-P., Hillion, F., Constantz, B. R., Juillet-Leclerc, A., Dauphin, Y., Watanabe, T. and Dunbar, R. B. (2004) Distribution of magnesium in coral skeleton. *Geophys. Res. Lett.* **31**, L23306, doi:10.1029/2004GL021313.

Min, G. R., Lawrence Edwards, R., Taylor, F., Recy, J., Gallup, C. and Warren Beck, J. (1995). Annual cycles of in coral skeletons and thermometry. *Geochim. et Cosmochim, Acta*, **59**(10), 2025-2042.

Mitsuguchi T., Uchida, T., Matsumoto, E., Isdale, P. J. and Kawana, T. (2001) Variations in Mg/Ca, Na/Ca, and Sr/Ca ratios of coral skeletons with chemical treatments: Implications for carbonate geochemistry. *Geochim. Cosmochim. Acta* **65**, 2865-2874.

Moorsel, G. V. (1988). Early maximum growth of stony corals (Scleractinia) after settlement on artificial substrata on a Caribbean reef. *Mar. Ecol. Prog. Ser.* **50**, 127-135.

Morel, F. M. M. and N. M. Price (2003) The biogeochemical cycles of trace metals in the Oceans. *Science* **300**, 944–947.

Moya, A., Tambutté, S., Bertucci, A., Tambutté, E., Lotto, S., Vullo, D., Supuran, C. T., Allemand, D. and Zoccola, D. (2008) Carbonic anhydrase in the scleractinian coral *Stylophora pistillata* characterization, localization, and role in biomineralization. *J. Biol. Chem.* **283**, 25475–25484.

Moya, A., Huisman, L., Ball, E. E., Hayward, D. C., Grasso, L. C., Chua, C., Woo, H. N., Gattuso, J-P., Foret, S. and Miller, D. J. (2012) Whole transcriptome analysis of the coral *Acropora millepora* reveals complex responses to CO_2-driven acidification during the initiation of calcification. *Mol. Ecol.* **21**, 2440–2454.

Nishida, K., Ishikawa, K., Iguchi, A., Tanaka, Y., Sato, M., Ishimura, T., Inoue, M., Nakamura, T., Sakai, K. and Suzuki, A. (2014) Skeletal oxygen and carbon isotope compositions of *Acropora* coral primary polyps experimentally cultured at different temperatures. *Geochem. Geophys. Geosyst*. **15**, 2840-2849.

Nooijer, L. D., Hathorne, E., Reichart, G., Langer, G. and Bijma, J. (2014) Variability in calcitic Mg/Ca and Sr/Ca ratios in clones of the benthic foraminifer Ammonia tepida. *Mar. Micropaleontol.* **107**, 32-43.

Ohno, Y., Iguchi, A., Shinzato, C., Inoue, M., Suzuki, A., Sakai, K. and Nakamura, T. (2017) An aposymbiotic primary coral polyp counteracts acidification by active pH regulation. *Sci. Rep.* **7**, 40324.

Okai, T., Suzuki, A., Kawahata, H., Terashima, S. and Imai, N. (2002) Preparation of a new Geological Survey of Japan geochemical reference material: Coral JCp-1, *Geostand. Newsl.* **26**, 95–99.

Orlowski, S., and Champeil, P. (1993) Strontium binding to sarcoplasmic reticulum Ca -ATPase. *FEBS Lett.* **328**(3), 296-300.

Polato, N. R., Altman, N. S. and Baums, I. B. (2013). Variation in the transcriptional response of threatened coral larvae to elevated temperatures. *Mol. Ecol.* **22**(5), 1366-1382.

R Development Core Team. (2013) R: A language and environment for statistical computing, edited, R Foundation for Statistical Computing, Vienna, Austria.

Reynaud, S., Ferrier-Pagès, C., Meibom, A., Mostefaoui, S., Mortlock, R., Fairbanks, R. and Allemand, D. (2007) Light and temperature effects on Sr/Ca and Mg/Ca ratios in the scleractinian coral *Acropora* sp. *Geochim. Cosmochim. Acta* **71**(2), 354-362.

Ribaud-Laurenti, A., Hamelin, B., Montaggioni, L. and Cardinal, D. (2001) Diagenesis and its impact on Sr/Ca ratios in Holocene *Acropora* corals. *Int. J. Earth Sci.* **90**(2), 438-451.
Rogers, C. S., Garrison, G., Grober-Dunsmore, R., Hillis, Z. and Franke, M. A. (2001) *Coral reef monitoring manual for the Caribbean and Western Atlantic*. St. John, USVI: National Park Service.

Rozen, S. and Skaletsky, H. (2000). Primer3 on the WWW for General Users and for Biologist Programmers. *Methods mol. biol.* **132**, 365-386.

Sadler, J., Webb, G. E. and Nothdurft, L. D. (2015) Structure and palaeoenvironmental implications of inter-branch coenosteum-rich skeleton in corymbose *Acropora* species. Coral Reefs **34**(1), 201-213, 10.1007/s00338-014-1228-0.

Seneca, F., Forêt, S., Ball, E., Smith-Keune, C., Miller, D. and van Oppen, M. (2009) Patterns of gene expression in a Scleractinian coral undergoing natural bleaching. *Mar. Biotechnol.* **12**, 594–604.

Shen, C. C., Lee, T., Chen, C. Y., Wang, C. H., Dai, C.F. and Li, L. A. (1996) The calibration of *D*[Sr/Ca] versus sea surface temperature relationship for *Porites* corals. *Geochim. Cosmochim. Acta* **60**(20), 3849-3858.

Shinzato, C., Eiichi, S., Kawashima, T., Hamada, M., Kanako, H., Makiko, T., Fujie, M., Fujiwara, M., Koyanagi, R., Ikuta, T., Fujiyama, A., Miller, D. J. and Satoh, N. (2011) Using the *Acropora digitifera* genome to understand coral responses to environmental change. *Nature* **476**, 320–323.

Shinzato, C., Mungpakdee, S., Satoh, N. and Shoguchi, E. (2014). A genomic approach to coral-dinoflagellate symbiosis: studies of *Acropora digitifera* and Symbiodinium minutum. *Front. Microbio.* **5** (336), doi: 10.3389/fmicb.2014.00336.

Shirai, K., Kawashima, T., Sowa, K., Watanabe, T., Nakamori, T., Takahata, N., Amekawa, Y., Sano, Y. (2008). Minor and trace element incorporation into branching coral Acropora nobilis skeleton. *Geochim.Cosmochim. Acta.* **72**(22), 5386-5400.

Sosdian, S., Gentry, D. K., Lear, C. H., Grossman, E. L., Hicks, D. and Rosenthal, Y. (2006) Strontium to calcium ratios in the marine gastropod *Conus ermineus*: Growth rate effects and temperature calibration. *Geochem. Geophys. Geosyst*. **7**, Q11023.

Stanley, G. (2003). The evolution of modern corals and their early history. *Earth Sci. Rev.* **60**(3-4) 195-225.

Stanley, W. A., Pursiainen, N. V., Garman, E. F., Juffer, A. H., Wilmanns, M. and Kursula, P. (2007). A previously unobserved conformation for the human Pex5p receptor suggests roles for intrinsic flexibility and rigid domain motions in ligand binding. *BMC Struct. Biol.* **7**(1), 24. doi:10.1186/1472-6807-7-24

Stoddart, R. D. (2008). Ecology and morphology of recent coral reefs. *Biol. Rev.* **44**, 433 - 498. 10.1111/j.1469-185X.1969.tb00609.x.

Takeuchi, T., Yamada, L., Shinzato, C., Sawada, H. and Satoh, N. (2016). Stepwise evolution of coral biomineralization revealed with genome-wide proteomics and transcriptomics. *Plos One*, **11**(6).

Tambutté, E., Tambutté, S., Segonds, N., Zoccola, D., Venn, A., Erez, J. and Allemand, D. (2012) Calcein labelling and electrophysiology: insights on coral tissue permeability and calcification. *Proc. Biol. Sci.* **279**(1726), 19-27.

Tanaka, K., Holcomb, M., Takahashi, A., Kurihara, H., Asami, R., Shinjo, R., Sowa, K., Rnkenburg, K., Watanabe, T. and McCulloch, M. (2015) Response o*r Acropora digitifera* to ocean acidification: constraints from $d^{11}B$, Sr, Mg, and Ba compositions of aragonitic skeletons cultured under variable seawater pH. *Coral Reefs* **34**, 1139-1149.

Trapnell, C., Pachter, L. and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics,* **25**(9), 1105-1111.

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., Baren, M. J., Salzberg, S. L., Wold, B. J., Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechno.* **28**(5), 511-515.

Trapon, M. L., Pratchett, M. S. and Hoey, A. S. (2013) Spatial Variation in Abundance, Size and Orientation of Juvenile Corals Related to the Biomass of Parrotfishes on the Great Barrier Reef, Australia. *PLoS ONE,* **8(**2). doi:10.1371/journal.pone.0057788.

Vetter, S. W. and Leclerc, E. (2003) Novel aspects of calmodulin target recognition and activation. *Eur. J. Biochem.* **270**, 404-414.

Vidal-Dupiol, J., Zoccola, D., Tambutté, E., Grunau, C., Cosseau, C., Smith, K. M., Freitag, M., Dheilly, N. M., Allemand, D. and Tambutté, S. (2013) Genes related to ion-transport and energy production are upregulated in response to CO_2-Driven pH decrease in corals: new insights from transcriptome analysis. *PLoS ONE* **8**, e58652.

Wall, M. and Nehrke, G. (2012). Reconstructing skeletal fiber arrangement and growth mode in the coral *Porites lutea* (Cnidaria, Scleractinia): a confocal Raman microscopy study. *Biogeosciences,* **9**(11), 4885-4895.

Watanabe, T., Fukuda, I., China, K. and Isa, Y. (2003) Molecular analyses of protein components of the organic matrix in the exoskeleton of two scleractinian coral species, *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.* **136**, 767–774.

Watanabe, T., Minagawa, M., Oba, T. and Winter, A. (2001) Pretreatment of coral aragonite for Mg and Sr analysis: Implications for coral thermometers. *Geochem. J.* **35**(4), 265-269.

Wang, Y. and Xu, H. (2001). Prediction of trace metal partitioning between minerals and aqueous solutions: a linear free energy correlation approach. *Geochimica et Cosmochim. Acta*, **65**(10), 1529-1543.

Weng, J. K. and Noel, J. P. (2012) The remarkable pliability and promiscuity of specialized metabolism. *Cold Spring Harbor Symp. Quant. Biol*. **77**, 309-20.

Wallace, C. C. (1999) *Staghorn corals of the world: a revision of the genus Acropora.* Collingwood, Australia, CSIRO Publishing.

Xiao, Y., WeiGuo, L., YunQi, M., YanLing, Z., MaoYong, H., ChongGuang, L. and QingQiang, L. (2014) Correlation between $\delta^{18}O$, Sr/Ca and Mg/Ca of coral *Acropora* and seawater temperature from coral culture experiments. *Sci. China Earth Sci*. **57**(5), 1048-1060.

Yara, Y., Vogt, M., Fujii, M., Yamano, H., Hauri, C., Steinacher, M., Gruber, N. and Yamanaka, Y. (2012). Ocean acidification limits temperature-induced poleward expansion of coral habitats around Japan. *Biogeosci. Discuss.* **9**(6), 7165-7196.

Yucharoen, M., Yeemin, T., Casareto, B. E., Suzuki, Y., Samsuvan, W., Sangmanee, K., Klinthong, W., Pengsakun, S. and Sutthacheep, M. (2015). Abundance, composition and growth rate of coral recruits on dead corals following the 2010 bleaching event at Mu Ko Surin, the Andaman Sea. *Ocean Sci. J.* **50**(2), 307-315.

Zoccola, D., Tambutté, E., Kulhanek, E., Puverel, S., Scimeca, J. C., Allemand, D. and Tambutté, S. (2004) Molecular cloning and localization of a PMCA P-type calcium ATPase from the coral *Stylophora pistilla*. *Biochim. Biophys. Acta*, *Biomembr.* **1663**, 117–126.

Appendix. List of genes from PDB that report strontium as one of interactive compounds

(strontium and other compounds besides calcium)

Interactive compunds: Sr and other compounds besides Ca (Total of 71)

PDB ID	*Annotation*	*Acropora digitifera (Gene with highest similaity in* OIST database)
1y1h	**Formylglycine generating enzyme**	**2 genes (aug_v2a.05480.t1)**
4rmx	Colanidase tailspike protein	No hits
1hyf	Ribonuclease in complex with strontium	No hits
2hmp	**Uncomplexed actin**	**20 genes (aug_v2a.05332.t1)**
5lt0	**Kinesin-1 motor domain**	**at least 30 genes (aug_v2a.21257.t1)**
5lt1	**Kinesin-1 motor domain**	**at least 30 genes (aug_v2a.21257.t1)**
2glq	**Alkaline phosphatase in complex with strontium**	**8 genes (aug_v2a.05574.t1)**
3ws4	**Mutant BETA-LACTAMASE**	**3 genes (aug_v2a.23038.t1)**
4g25	**Proteinaceous RNase P 1**	**2 genes (aug_v2a.02312.t1)**
3lj0	**Endoplasmic reticulum transmembrane sensor**	**at least 30 genes (aug_v2a.16167.t1)**
1s8f	**GTP(guanosine triphosphate)ases**	**1 gene (aug_v2a.24612.t1)**
4kbn	Dihydrofolate reductase	No hits
5jdq	Sodium-calcium exchanger soaked with 100 mM Na^{+} and 10mM Sr^{2+}	No hits
4iay	**Protein kinase A in complex with high Sr^{2+} concentration**	**at least 30 genes (aug_v2a.14117.t1)**
5m8h	ATP phosphoribosyltransferase	No hits
1tjm	**Synaptotagmin I C2B Domain**	**at least 30 gene (aug_v2a.02778.t1)**
3ccu	**Ribosomal Subunit: 23S**	**2 genes (aug_v2a.17041.t1)**
4iak	Protein kinase A in complex with high Sr^{2+} concentration	No hits
4ds7	**Calmodulin bound to the C-terminal fragment of spindle pole body protein**	**1gene (aug_v2a.01102.t1)**
3ccv	**Ribosomal Subunit: 23S**	**2 genes (aug_v2a.17041.t1)**
3ccl	**Ribosomal Subunit: 23S**	**2 genes (aug_v2a.17041.t1)**
5hxs	**Sodium-calcium exchanger soaked with 100 mM Na+ and 10mM Sr^{2+}**	**6 genes (aug_v2a.01943.t1)**
5kli	**Endoplasmic reticulum transmembrane sensor**	**1 gene (aug_v2a.02257.t1)**
3i56	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
5kkz	**bc1**	**1 gene (aug_v2a.02257.t)**
3g71	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1oo0	**Core components of the EJC(exon junction complex)**	**11 genes (aug_v2a.10243.t1)**
5jdn	**Sodium-calcium exchanger soaked with 10 mM Na+ and 10mM Sr^{2+}**	**6 genes (aug_v2a.01943.t1)**
3cma	**Large Ribosomal Subunit**	**1 gene (aug_v2a.10806.t1)**
3ccq	**Ribosomal Subunit: 23S**	**2 genes (aug_v2a.10806.t1)**
1wc3	**Adenylyl cyclase**	**5 genes (aug_v2a.03050.t1)**
1yhq	**Ribosomal Subunit: 50S**	**2 genes (aug_v2a.10806.t1)**
3cme	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
3cc4	**Ribosomal Subunit: 50S**	**2 genes (aug_v2a.10806.t1)**
3ccm	**Ribosomal Subunit: 50S**	**2 genes (aug_v2a.10806.t1)**
3g6e	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
3cd6	**Ribosomal Subunit mutant**	**2 genes (aug_v2a.10806.t1)**
3e4p	Malonate	No hits
3ccr	**50S Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1vqp	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1vq8	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1vqo	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
5jdm	**Sodium-calcium exchanger soaked with 2.5 mM Na+ and 0.1mM Sr^{2+}**	**6 genes (aug_v2a.01943.t1)**
2box	**Epidermal growth factor in complex with strontium**	**at least 30 genes (aug_v2a.11224.t1)**
1vqn	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1g1s	**Selectin/lectin/epidermal growth factor**	**at least 30 genes (aug_v2a.13721.t1)**
2rio	**Endoplasmic reticulum transmembrane sensor**	**at least 30 genes (aug_v2a.16167.t1)**
1vql	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1vqk	**Ribosomal subunit**	**2 genes (aug_v2a.10806.t1)**
1vqm	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
2qjy	**Inhibitor of cytochrome**	**1gene(aug_v2a.02257.t1)**
3cc7	**50S Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
1u3e	Endonuclease	No hits
1vq9	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
3cpw	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
3ccs	**50S Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
3i5a	Signal transduction domain	No hits
3g4s	**Large Ribosomal Subunit**	**3 genes (aug_v2a.17041.t1)**
4nmn	Helicase	No hits
3i55	**Large Ribosomal Subunit**	**2 genes (aug_v2a.10806.t1)**
3cce	**50S Ribosomal Subuni**	**2 genes (aug_v2a.10806.t1)**
2qjk	**Endoplasmic reticulum transmembrane sensor**	**1gene(aug_v2a.02257.t1)**
2qjp	**Endoplasmic reticulum transmembrane sensor**	**1gene(aug_v2a.02257.t1)**
2hdf	Colicin I receptor	No hits
1sfc	**Neuronal synapticfusion complex**	**2 genes (aug_v2a.16533.t1)**
3ow2	**50S Ribosomal Subuni**	**2 genes (aug_v2a.17041.t1)**
5jdl	**Sodium-calcium exchanger soaked with 2.5 mM Na^{+} and 1mM Sr^{2+}**	**6 genes (aug_v2a.01943.t1)**
3ccj	**50S Ribosomal Subuni**	**2 genes (aug_v2a.10806.t1)**
2btf	**Crystalline profilin-beta-actin**	**20 genes (aug_v2a.09988.t1)**
2spt	**Prothrombin fragmen**	**5 genes (aug_v2a.08937.t1)**
5b5m	Light harvesting antenna-reaction center	No hits

Appendix. List of genes from PDB that report strontium as one of interactive compounds

(strontium, calcium and other compounds)

Interactive compunds:	Sr, Ca and other compounds (Total of 6)	
PDB ID	*Annotation*	*Acropora digitifera (Gene with highest similaity in* OIST database)
4jij	**Catalytic domain**	**8 genes (aug_v2a.02174.t1)**
5c02	Transmembrane domain	No hits
4jqg	**Catalytic domain**	**8 genes (ug_v2a.02174.t1)**
3ws5	**Beta-Lactamase**	**3 genes (aug_v2a.23038.t1)**
2woh	**Strontium soaked E. coli copper amine oxidase**	**4 genes (aug_v2a.04934.t1)**
2xrm	**Intracellular subtilisin**	**5 genes (aug_v2a.19361.t1)**

The gene sequences from PDB were compared to *Acropora digitifera* ver. oist_v1.1. by blastp. The cutoff value was $1e^{-5}$, and the genes with e-value less than $1e^{-5}$ are written in bold letters. The gene with the highest similarity was shown in the parenthesis.