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造礁珊瑚黑病之菌相分佈研究

Bacterial consortium associated with the "black disease" of reef-building corals

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95
1

摘要

目前全世界的珊瑚礁都受到破壞，而珊瑚疾病為主要威脅因素之一。然而受限於傳統微生物學方法限制，過去對於珊瑚疾病爆發的原因和感染機制仍不清楚。2006 年，台灣綠島發生大量珊瑚死亡事件，原因為受到如黑色覆蓋物之海綿（*Terpios hoshinota*）入侵所引起，簡稱「黑病」。本研究將利用電子顯微鏡方法與非依賴培養式的微生物分子生物技術，細菌 16S rDNA 序列和變性梯度電泳法(denaturing gradient gel electrophoresis, DGGE)，分析 *T. hoshinota* 特有菌相，並比較受海綿覆蓋之珊瑚（*Porites lutea*）與未受海綿覆蓋之珊瑚（*P. lutea*）其菌相變化情形，期望發現與疾病有關微生物，以利往後病態監測所用。

結果發現，不論珊瑚或海綿，其菌相分布都與周遭海水菌相不同。*T. hoshinota* 細菌群相專一，主要為具光合作用的單細胞球形藍綠菌，經分子序列及外觀比對，可能是新種藍綠菌。此藍綠菌數量極大應該是和 *T. hoshinota* 存有某種程度的共生關係。另外，利用 DOTUR、LIBSHUFF 軟體來分析珊瑚 *P. lutea* 的細菌群相之間的多樣性和組成相似度。結果發現未罹病珊瑚 *P. lutea* 的菌相分佈，主要以 *Gammaproteobacteria*、*Cyanobacteria*、unclassified bacteria 為主；而罹病珊瑚 *P. lutea* 的菌相是以 *Gammaproteobacteria*、*Alphaproteobacteria* 為主，而 *Cyanobacteria* 以及 unclassified bacteria 則大量減少。過去研究發現，珊瑚上有許多共生細菌，當珊瑚受到環境壓力時，可能改變或抑制原本正常菌叢，導致菌相會以某一類群細菌為主，而非專一性的細菌也會伺機而生；本研究發現罹病珊瑚上 *Gammaproteobacteria* 大量增加，可能和環境壓力有關(受海綿覆蓋或海水污染)，而 *Alphaproteobacteria* 則可能為伺機生長的細菌，而這樣的改變，影響正常菌叢對珊瑚的功能，推測可能影響珊瑚健康。由於珊瑚上共生細菌易受環境壓力而改變，未來若能建立珊瑚共生菌相長期監測資料，應可作為珊瑚的健康指標。

關鍵字: 海綿、藍綠菌、珊瑚疾病、珊瑚微生物菌相、非培養式微生物分子技術

Summary

Coral diseases are one of the major natural disturbances that threat the survival of coral reefs worldwide. However, the characterization and mechanism of infection have been difficult in understanding the outbreak of coral diseases due to the limitation of applying classical microbiological assays. In this thesis, the bacteria consortium of the “black disease” caused by a black mat-like sponge, *Terpios hoshinota*, was characterized by electronic microscopy, denaturing gradient gel electrophoresis (DGGE), and 16S ribosomal DNA library construction. In order to understand the possible pathogenic bacteria in “black disease”, the specificity of *T. hoshinota* associated bacterial community and the variation between sponge-infected (SI) and non-sponge-infected (NSI) *Porites lutea* associated bacterial community were investigated in the fringing reef of the Green Island (Lutao), where outbreak of *Terpios* sponge was first reported in 2006.

Result shows that the bacterial communities from seawater, sponge, and coral were specific. *T. hoshinota* associated bacterial community was specific and the dominant bacteria group was autotrophic *Cyanobacteria*. The *Cyanobacteria* associated with *T. hoshinota* were supposedly a new *Cyanobacteria* species as demonstrated by 16S rDNA sequence with a unique morphology. Furthermore, the high abundance of *cyanobacteria* in *T. hoshinota* may contribute positively to *T.*

hoshinota outbreak. On the other hand, the diversity and composition similarity of bacterial community associated with SI and NSI *P. lutea* were analyzed using DOTUR and LIBSHUFF. In NSI *P. lutea*, the dominant bacterial groups were *Gammaproteobacteria, Cyanobacteria,* and unclassified bacteria; however, in SI *P. lutea*, *Gammaproteobacteria* and *Alphaproteobacteria* were major groups and the abundance of *Cyanobacteria* and unclassified bacteria were low. In previous studies, there were diverse coral holobiont on a coral. The natural coral holobiont changed to dominant bacteria group with abundance of opportunistic bacteria when corals experience environmental stress; similar situation was observed in SI *P. lutea*. The increase of the abundance of *Gammaproteobacteria* is probably related to environmental stress (sponge or seawater pollution). In addition, the unexpected emergence of *alphaproteobacteria* could be opportunistic bacteria because of disturbance of natural bacterial community. Disturbance of coral holobiont possible loss the normal symbiont function for coral and it directly or indirectly supposed to result in coral disease. Coral associated microbes were variable in different environment and may be a good bioindicator for environmental stress; therefore, building up a bioindicator, a long term investigation is apparently essential.

Key words: sponge, *Cyanobacteria*, coral disease, coral microbiology, uncultured molecular method

Content

口試委員審定書 i
致謝 ii
中文論文摘要 iii
英文論文摘要 iv
1 Introduction ... 1
1.1 Coral reef ecosystem degradation and mortality ... 1
1.2 Coral disease ... 2
1.2.1 Definition of coral disease ... 3
1.2.2 Microbial diseases of corals ... 3
1.2.3 Microbial-induced coral disease: two examples ... 5
1.2.4 The limitation of coral disease research ... 8
1.2.5 Coral disease studies in Taiwan ... 9
1.2.5.1 Black disease outbreak in Green Island ... 10
1.2.6 *Terpios hoshinota*, “the coral killer sponge” ... 11
1.2.6.1 Biology of Terpios hoshinota ... 13
1.2.7 Symbiotic relationships between sponge and endogenous microbe 14
1.2.7.1 Sponges as hosts of single-celled autotrophs ... 17
1.2.7.2 Sponge as hosts of heterotrophic single-celled microbes ... 19
1.3 Aim of this thesis ... 22
2 Materials and Methods ... 30
2.1 Sampling ... 30
2.1.1 Seawater sampling ... 31
2.1.2 Coral sampling ... 31
2.1.3 Sponge sampling ... 32
2.2 Electron microscopy ... 32
2.3 Molecular Method for bacterial library construction ... 33
2.3.1 DNA extraction ... 33
2.3.2 Amplification of bacterial 16S ribosomal rRNA by polymer chain reaction 35
2.3.3 Clone library construction ... 36
2.3.4 Denaturing gradient gel electrophoresis analysis ... 38
2.3.5 Recovery of DNA from DGGE gel ... 39
2.3.6 Sequencing and phylogenetic analysis ... 39
2.3.7 Bacterial group assignment ... 40
2.3.8 Diversity estimation in DOTUR ... 40
2.3.9 Sequence library comparison ... 41

2.3.10 Phylogenetic analyses 41
2.3.11 DGGE Cluster analysis 42
3 Results 44
3.1 16S rRNA-DGGE analysis 44
3.1.1 *P. lutea* associated bacteria communities changed when *T. hoshinota* covered 44
3.1.2 *Gammaproteobacteria* were the major group in bacteria communities of seawater and *P. lutea* 44
3.1.3 *T. hoshinota* associated bacterial community was specific 45
3.1.4 *Cyanobacteria* were the major group in *T. hoshinota* associated bacterial community 45
3.2 16S rRNA library construction 50
3.2.1 Composition analysis of bacterial community 50
3.2.1.1 *Gammaproteobacteria* was the major group in seawater samples 50
3.2.1.2 Bacterial communities associated with *P. lutea* changed when *T. hoshinota* covered 50
3.2.1.3 *Cyanobacteria* were the major group in the sponge 51
3.2.2 Diversity analysis of bacterial 16S rRNA clone libraries 58
3.2.2.1 Higher diversity of bacteria community associated with SI *Porites lutea* (close-to sponge part, P^dI) 58
3.2.2.2 Low diversity of bacterial community associated with *T. hoshinota* 59
3.2.3 Comparing different bacterial communities 63
3.2.3.1 Different composition between Chai-Kou and Kun-Guan seawater clone libraries 63
3.2.3.2 Different composition between SI and NSI *P. lutea* clone libraries 63
3.2.3.3 Similar bacterial communities associated with *T. hoshinota* covered on five different coral species 64
3.3 Phylogenetic analysis 68
3.3.1 Bacterial community associated with *P. lutea* 68
3.3.2 Bacterial community associated with *T. hoshinota* 70
3.4 Electronic microscopy of *T. hoshinota* 79

4 Discussion 84

4.1 The role of bacterial community associated with *T. hoshinota* 84

4.2 New species of *Cyanobacteria* associated with *T. hoshinota* 85

4.3 The composition of bacterial community associated *P. lutea* 87

4.3.1 The role of *Cyanobacteria* associated with coral 89

4.3.2 Variation of bacterial community associated with coral 90

4.4 Future research 94

Reference 97

Appendix 107

Table Content

Table 1 Standard Systematically Describing Coral Disease/Syndrome......................... 24
Table 2 Coral diseases observed in Taiwan... 25
Table 3 Standard systematically description of black disease 25
Table 4 The bacteria taxonomy in samples based on 16S rRNA bacteria clone libraries using RDP II analysis.. 52
Table 5 Diversity indices for the bacterial communities (*Porites lutea* and seawater)... 60
Table 6 Diversity indices for the bacterial communities (sponge) 61
Table 7 Difference in bacteria 16S rRNA clone libraries composition.......................... 65
Table 8 Difference in bacteria 16S rRNA clone libraries composition.......................... 66
Table 9 16S rRNA gene sequence analysis of bacterial clones from the non-sponge-infected *Porites lutea* 72
Table 10 16S rRNA gene sequence analysis of bacterial clones from the sponge-infected *Porites lutea* (far-away sponge, $P^{d}H$) 73
Table 11 16S rRNA gene sequence analysis of bacterial clones from the sponge-infected *Porites lutea* (close-to sponge, $P^{d}I$) 74
Table 12 16S rRNA gene sequence analysis of bacterial clones from *Terpios hoshinota* (infected Porites lutea) 76

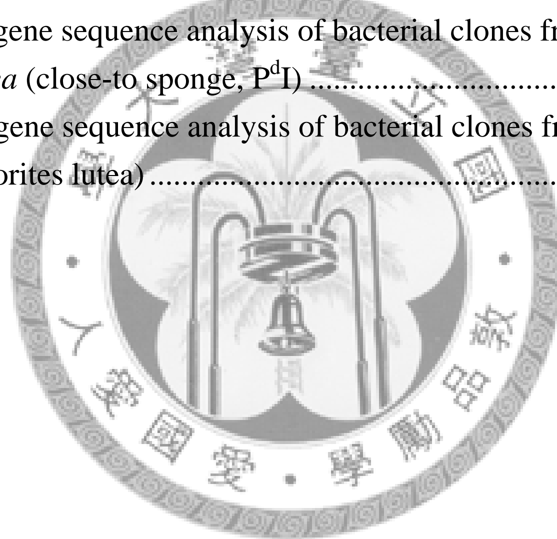


Figure Content

Fig. 1 Coral diseases observed in Taiwan 26
Fig. 2 A large area of coral reef was covered by the blackish mat........ 26
Fig. 3 Different morphologies of corals were covered by the blackish mat. 27
Fig. 4 Close-up of black disease infecting *Porites sp* 27
Fig. 5 Close-up of sponge crust. 28
Fig. 6 Portions of tylostyle head (spicule). 28
Fig. 7 Transmission electron microscopy of *T. hoshinota*. 28
Fig. 8 Progress rate of *T. hoshinota* in Green Island. 29
Fig. 9 Scars on *T. hoshinota* suspected by fish bite 29
Fig. 10 News of black disease outbreak in Taiwan 29
Fig. 11 *P. lutea* sampling procedure. 43
Fig. 12 *T. hoshinota* sampling procedure 43
Fig. 13 *T. hoshinota* covering on four kinds of coral species. 43
Fig. 14 Bacterial community composition (according to Fig. 15A 16S rRNA-DGGE gel and cluster diagram). 46
Fig. 15 Bacterial community composition on *Porites lutea*, *Terpios hoshinota* and seawater 47
Fig. 16 Bacterial community composition (according to Fig. 17 16S rRNA-DGGE gel and cluster diagram) 48
Fig. 17 Bacterial community composition in sponge-infected five different coral species 49
Fig. 18 Pie charts illustrating the diversity of bacterial groups on *Porites lutea* and seawater 55
Fig. 19 Pie charts illustrating the diversity of bacterial groups on *Terpios hoshinota* 56
Fig. 20 Comparison of predominant bacterial community among samples 57
Fig. 21 Rarefaction curves for the 16S rRNA bacteria clone libraries (*Porites lutea* and seawater). 62
Fig. 22 Rarefaction curves for the 16S rRNA bacteria clone libraries (*Terpios hoshinota* collected from five coral species) 62
Fig. 23 Non-metric multidimensional scaling plot (nMDS-2 dimensional) of *Porites lutea,* Seawater 67
Fig. 24 Non-metric multidimensional scaling plot (nMDS-2 dimensional) of *Terpios hoshinota* 67
Fig. 25 Maximum parsimony tree of sponge-associated and black band disease related cyanobacteria based on 16S rRNA. 77
Fig. 26 Neighbor-Joining tree of *Porites lutea* -associated and seawater related bacteria based on 16S rRNA 78
Fig. 27 *Terpios hoshinota* infected coral *Isopora palifera*. Black arrow: close-to coral,

white arrow: far-away coral. 80
Fig. 28 Transmission electron micrographs of *Terpios hoshinota* 81
Fig. 29 Transmission electron micrographs of *Cyanobacteria* 82
Fig. 30 Transmission electron micrographs of *Cyanobacteria* membrane 83
Fig. 31 Transmission electron micrographs of cyanobacterial symbionts in *Ircinia variabilis* from Marseille. 96

1 Introduction

1.1 Coral reef ecosystem degradation and mortality

Coral reefs, the rain forests of the sea, are the most diverse marine ecosystems; they not only provide the critically importance of the ecosystem goods but also service to maritime tropical and subtropical nations (Moberg and Folke, 1999). Under profound or uncertain threatening factors, however, world-wide coral reefs are in serious decline; an estimated 30% coral reefs are already severely damaged and close to 60% may be lost by 2030 as ever suggested by C. Wilkinson, 2002. As a result, what are these threatening factors? These factors include natural factors, such as hurricanes (alternatively typhoon or cyclones) attacking and global climate changing, and detrimental anthropogenic factors, such as local habitat degradation, over-fishing, pollutant rapidly accumulating, disease emerging, ocean eutrophication and increasing population. All of these factors are contributing to the recent declines of coral reef or to phase shifts in the coral reef's community structure (Jackson et al., 2001; Hughes et al., 2003; Bellwood et al., 2004).

1.2 Coral disease

Since the 1990s, an increase in coral disease has caused the changes in composition and structure of tropical reefs (Aronson et al., 1998; Harvell et al., 1999), most notably in the Caribbean (Green and Bruckner, 2000). In the Caribbean, the fast emergence, high prevalence and virulence of coral diseases and syndromes are detected. Moreover, the widespread geographic distribution and the frequent epizootic events have already resulted in significant coral mortalities. On most of the Caribbean reefs, loss of Acroporids is accompanied by an apparent ecological phase shift from coral-dominated substrata to algal-dominated substrata (Hughes, 1994).

Frequent outbreaks of coral disease seemed closely related to anthropogenic impacts including increased sedimentation and human sewage (Goreau et al., 1998). In the Caribbean Sea, the environment is a relatively small, partially enclosed and highly interconnected water body surrounded by dense human population. As increasing human pollution in combination with impacts of global warming and rapid currents in the basin all might facilitate the emergence and spread of pathogens and/or spread of other stress agents that could affect the natural susceptibility/resistance of coral reef organisms to their pathogens (Peter, 1997; Richardson, 1998; Harvell et al., 1999; Rosenberg and Ben-Haim, 2002). Currently, diseases of coral have become one of the most important factors in the decline of coral reefs throughout the entire region

(Porter et al., 2001; Weil, 2004). In addition, the number and distribution of coral disease across the Indo-Pacific is also increasingly reported recent years (Rosenberg and Loya, 2004).

1.2.1 Definition of coral disease

Coral disease is defined as "any impairment of a coral's vital organ, system and/or body functions". This definition includes both infectious diseases caused by biological parasites-pathogens and noninfectious diseases caused by genetic mutations, malnutrition and/or environmental factors (Peter, 1997).

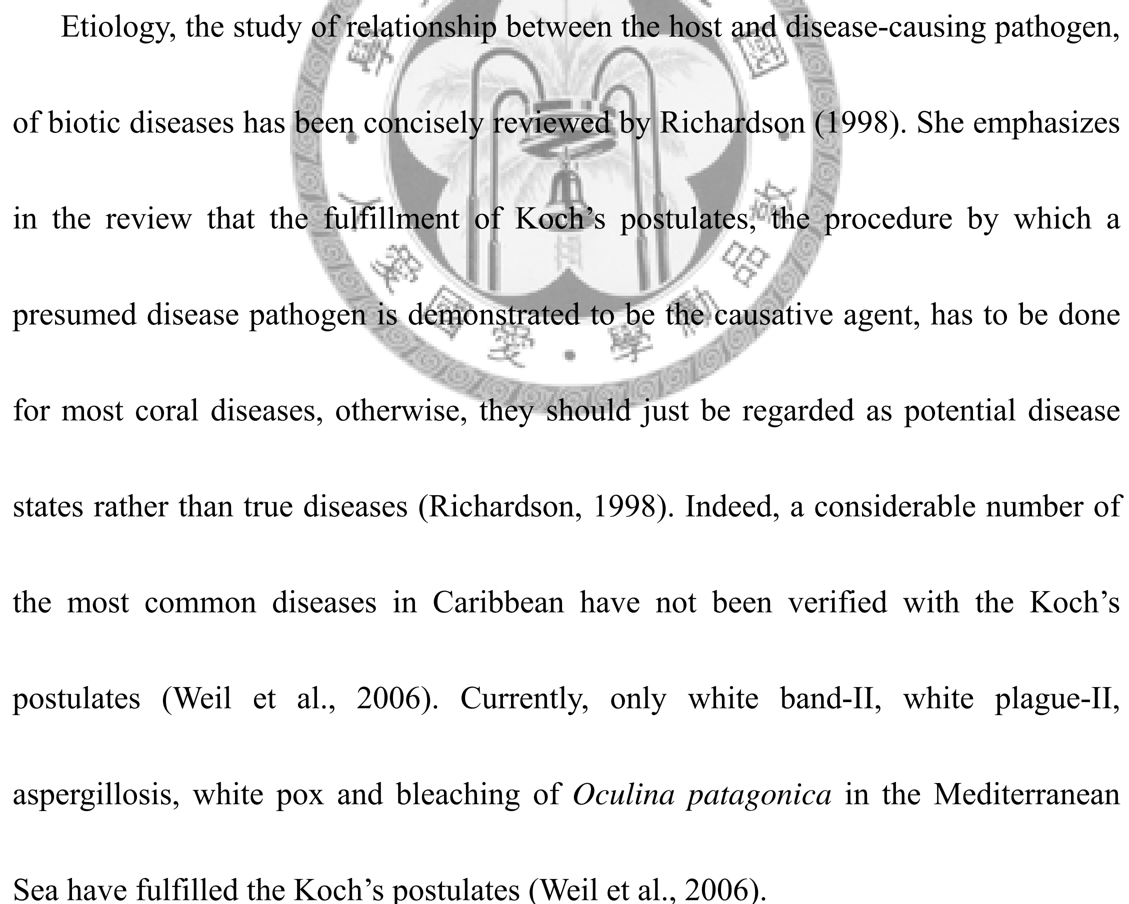
In general, the term "disease" is used for any affliction for which the causative agent has been identified and "syndrome" for those afflictions for which the causative agent is not known (Peter, 1997). Presently, however, the nomenclature to a coral disease is yet clearly defined according such definition.

1.2.2 Microbial diseases of corals

More and more microbe-induced coral diseases (MICD) are reported particularly in recent years and have become significant troubles, although non-microbial-induced coral diseases , such as bleaching, has long been paid more attention in the early years.

To date, a number of MICD-related studies have been excised. Unfortunately only few of coral specific pathogens are evidently and directly identified.

Part of the answer for why only few specific pathogens identified, some studies pointed out that microbe were opportunistic colonization on unhealthy corals rather than the specific, primary agent for the particular disease (Ainsworth et al., 2008). From this point of the view, a considerable number of reported coral diseases actually should be regarded as syndrome but not disease because their etiology remains uncertain.



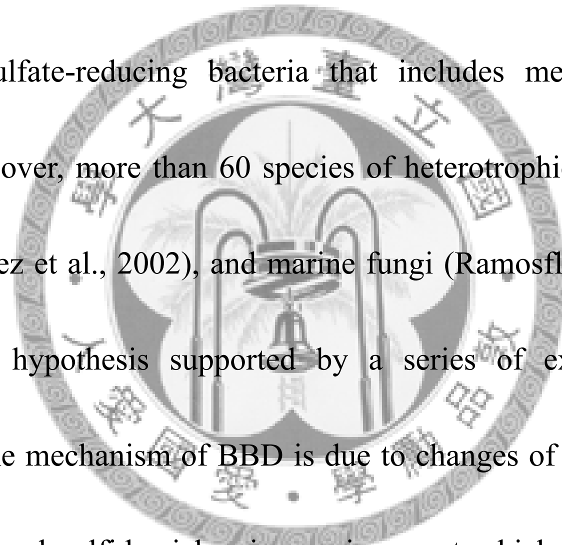
Etiology, the study of relationship between the host and disease-causing pathogen, of biotic diseases has been concisely reviewed by Richardson (1998). She emphasizes in the review that the fulfillment of Koch's postulates, the procedure by which a presumed disease pathogen is demonstrated to be the causative agent, has to be done for most coral diseases, otherwise, they should just be regarded as potential disease states rather than true diseases (Richardson, 1998). Indeed, a considerable number of the most common diseases in Caribbean have not been verified with the Koch's postulates (Weil et al., 2006). Currently, only white band-II, white plague-II, aspergillosis, white pox and bleaching of *Oculina patagonica* in the Mediterranean Sea have fulfilled the Koch's postulates (Weil et al., 2006).

1.2.3 Microbial-induced coral disease: two examples

The first coral disease was reported in 1965 and during the subsequent three decades, only 4 new diseases were ever reported (Sutherland et al., 2004). To date, the rate of discovery of new diseases has increased dramatically with more than 29 coral diseases now described (Green and Bruckner, 2000; Rosenberg and Loya, 2004; Weil et al., 2006) These diseases have been affecting at least 150 scleractinian, gorgonian, and hydrozoan zooxanthellate species, but only 6 of them have been identified etiologically (Rosenberg et al., 2007). Two characteristic examples of coral diseases, black band disease and coral bleaching, are selected to represent the study progress of microbes in coral diseases, in the coming paragraphs.

Black band disease (BBD), first reported by Antonius (Antouius, 1973), is now distributed globally, affecting at least 25 Caribbean and 45 Indo-Pacific coral species which mainly the massive-framework-building corals. (Frias-Lopez et al., 2004; Sutherland et al., 2004; Weil, 2004). Symptoms of BBD can be recognized as a dark band that moves across coral colonies destroying coral tissue. Rates of movement of band across coral colonies have been reported of up to 2 mm per day in average and can kill entire colonies in a matter of months (Rutzler and Santavy, 1983; Rutzler et al., 1983). Active disease progress during the warm summer months were observed in the field survey (Kuta and Richardson, 1996). Microscopic observation of BBD

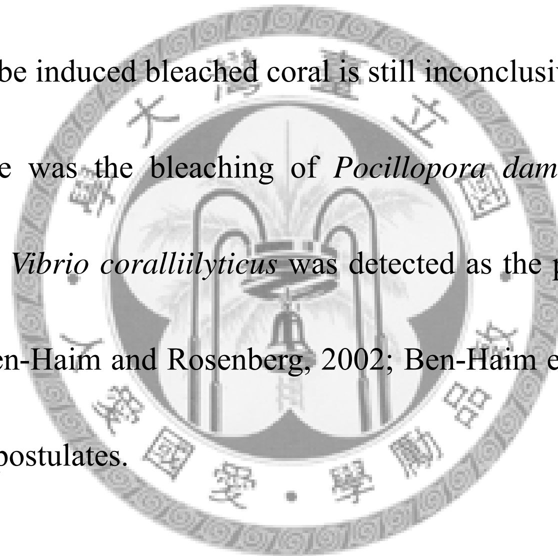
revealed a complex microbial consortium including heterotrophic and photosynthetic bacteria (Richardson, 1997, 1998). The recent progress in molecular techniques has enabled molecular characterization of the bacterial community associated with BBD. The BBD microbial community is dominated by filamentous *Cyanobacteria* whose identity has been the subject of recent controversy (Cooney et al., 2002; Frias-Lopez et al., 2002; Frias-Lopez et al., 2003; Sekar et al., 2006; Sussman et al., 2006; Myers et al., 2007), sulfide-oxidizing bacteria proposed to belong to the genus *Beggiatoa*, and a group of sulfate-reducing bacteria that includes members of the genus *Desulfovibrio*. Moreover, more than 60 species of heterotrophic bacteria (Cooney et al., 2002; Frias-Lopez et al., 2002), and marine fungi (Ramosflores, 1983) were also found in BBD. A hypothesis supported by a series of experiments has been demonstrated that the mechanism of BBD is due to changes of microbial community resulting in anoxic and sulfide rich microenvironment which in turn kill the coral tissue (Carlton and Richardson, 1995). Many of these microorganisms have been suggested as potential primary pathogens that cause BBD. However, Koch's postulates have not been fulfilled with any of the BBD consortium members. In the research, Voss JD *et al*. discovered that the BBD community composition was variable among different biogeographic areas (Voss et al., 2007). Therefore, although a large number of studies have been made on searching for the primary pathogen of



BBD, the answer still remains unclear. Perhaps, instead of a single primary pathogen, the BBD is actually due to polymicrobial infection (Richardson, 2004).

Coral bleaching, disruption of the symbiotic interaction between coral hosts and the endosymbiotic algae, has been considered a disease because of the physical stress, abnormal seawater temperature or salinity (Hoeghguldberg and Smith, 1989). However, there were two cases of coral bleaching likely caused by bacterial infection when the local seawater temperature turned warm (Kushmaro et al., 1998; Toren et al., 1998; Ben-Haim et al., 2003). The first case was observed from the bleaching of *Oculina patagonica* in the Mediterranean Sea. Koch's postulates were successfully applied to demonstrate that *Vibrio shiloi* was the causative agent of the bleaching disease (Kushmaro et al., 1996; Kushmaro et al., 1997). There were four steps employed as follows to complete the Koch's postulates of the bleaching of *O. patagonica.* 1) The bacterium was isolated from all 28 bleached coral samples but absent from all 24 unbleached corals; 2) the bacterium was purely cultivated and classified as a new species of *Vibrio*; 3) with controlled aquaria experiments, it demonstrated that *V. shiloi* could cause bleaching of healthy corals. At 29℃, 120 bacteria/ml induced 60% bleaching of *O. patagonica* in 10 days and 100% bleaching in 20 days. No bleaching occurred for several months under the same conditions if *V. shiloi* was not added or if antibiotics were administered at the same time as the

inoculum. 4) Finally, *V. shiloi* was reisolated form the tissues of the infected bleached corals (Kushmaro et al., 1997). Although the experimental procedures seemed reasonable, two uncertainties were still hidden. Firstly, this bacterium failed to be seen in the microbial diversity assays of the bleaching coral tissues (Ainsworth and Hoegh-Guldberg, 2008). Secondly, the other research team has lately also proved no *V. shiloi* involvement in the bleaching coral samples by fluorescence in situ hybridization and electronic microscopy (Ainsworth et al., 2008). Therefore, the mechanism of microbe induced bleached coral is still inconclusive.



Another example was the bleaching of *Pocillopora damicornis* in the Indian Ocean and Red Sea. *Vibrio coralliilyticus* was detected as the pathogen of bleaching of *P. damicornis* (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003), but it has not fulfilled Koch's postulates.

1.2.4 The limitation of coral disease research

Although more researches of coral disease are emphasized today, coral diseases remain unidentified. The epizootiology, etiology and pathology of coral pathogens are poorly understood. Epizootiology is the study focus on local and geographic distributions, environmental correlates, host ranges, prevalence, impact, vectors and natural reservoirs, and spatial and temporal variability. Pathology is the study of

isolation and identification of the pathogen.

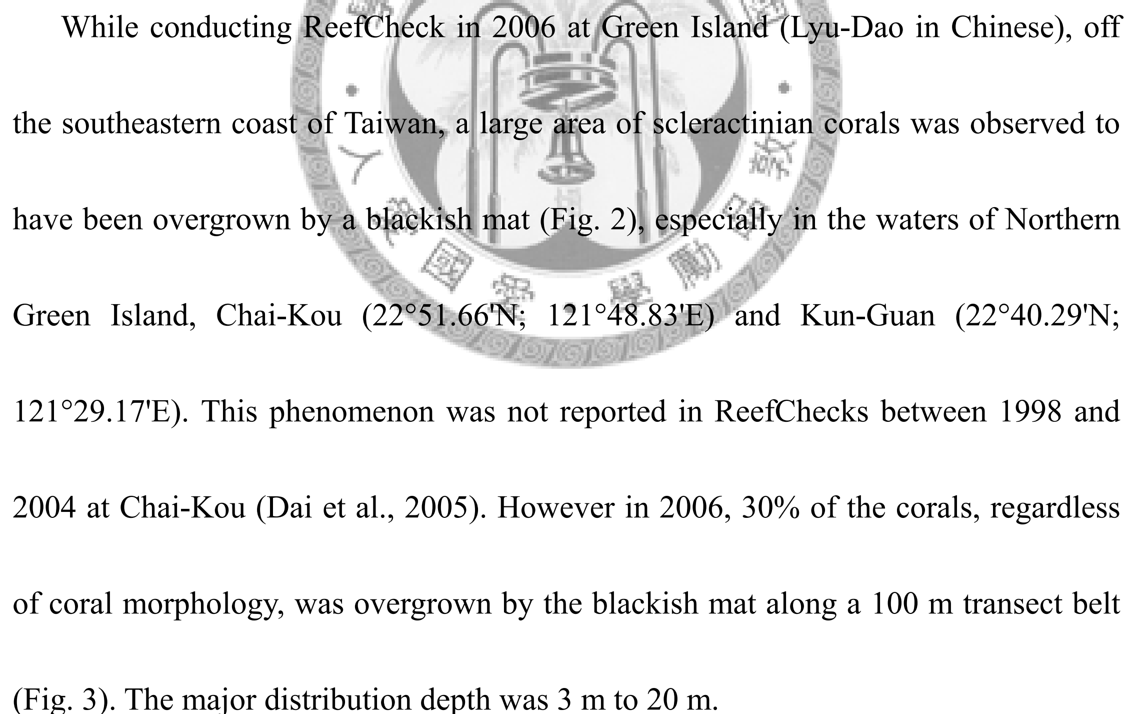
The reasons may be that the study of coral disease has suffered from a lack of standardization in nomenclature of gross lesions. Many existing descriptions of coral disease are ambiguous or open to subjective interpretation making geographic comparisons problematic, particularly between oceans. According to the paper by Work and Aeby, 2006, we designed the table (Table 1) to provide a structured approach to describing gross lesions in corals and attempt to bridge the gap between coral biology and veterinary pathology.

1.2.5 Coral disease studies in Taiwan

Coral communities around Taiwan are under severe stress from natural and anthropogenic impacts (Dai, 1997). However, there is a lack of understanding about the prevalence of coral diseases derived from the consequences of these impacts.

We have performed a qualitative survey of coral disease around Taiwan from July 2006 to September 2006 and July 2007. In this survey, systematic description (Table 1) of the symptoms and syndromes of coral diseases are conducted around the coral reefs and communities, including Dou-Fu-Chia, Ye-Liu in northeastern Taiwan, Yuan-An in southwestern Taiwan, Wang-Li-Tung, Tiao-Shi, outlet of the third nuclear power plant in south Taiwan and Green Island, an off-shore island. Eight previously described

diseases/syndromes, including black band disease, white plague disease, white band disease, pink line/spot syndrome, yellow blotch disease, Porites ulcerative white spot syndrome, skeleton anomalies and black disease were found in this preliminary survey (Table 2 and Fig. 1). No significant disease outbreak was observed except black disease in Green Island. Currently, quantitative surveys are been undertaking to confirm these observations (personal communication with Dr. A. Chen).



1.2.5.1 Black disease outbreak in Green Island

While conducting ReefCheck in 2006 at Green Island (Lyu-Dao in Chinese), off the southeastern coast of Taiwan, a large area of scleractinian corals was observed to have been overgrown by a blackish mat (Fig. 2), especially in the waters of Northern Green Island, Chai-Kou (22°51.66'N; 121°48.83'E) and Kun-Guan (22°40.29'N; 121°29.17'E). This phenomenon was not reported in ReefChecks between 1998 and 2004 at Chai-Kou (Dai et al., 2005). However in 2006, 30% of the corals, regardless of coral morphology, was overgrown by the blackish mat along a 100 m transect belt (Fig. 3). The major distribution depth was 3 m to 20 m.

The corals once covered by this blackish mat die rapidly. Such phenomenon therefore is suggested as a kind of coral disease, colloquially called “black disease” in Okinawa (Table 3 and Fig. 4) (Rutzler and Muzik, 1993)

This blackish mat was a kind of sponge, *Terpios hoshinota*, distinguished by canal system (astrorhizae, osculum) with more detailed researches (Fig. 5), lobed tyostyle spicules (Fig. 6), encrusting morphology, black or dark brown color and associated with the highly abundant *cyanobacteria* (Fig. 7) in a preliminary study. The progress rate of this sponge was measured about 1 mm per day (Fig. 8). Similar characteristics had been observed in the sponges in Okinawa (Ryukyus in Japan) and Guam (Bryan, 1973; Rutzler and Muzik, 1993).

1.2.6 ***Terpios hoshinota*, "the coral killer sponge"**

Sponges are important space competitors on coral reefs and are able to overgrow living corals. An outbreak of *T. hoshinota* was reported to be a "black disease" that killed and overgrowed live corals and is responsible for the death of large reef areas in the Ryukyus (Japan) and Guam in the last century (Bryan, 1973; Plucerrosario, 1987; Rutzler and Muzik, 1993). Furthermore, when the black disease has well colonized, it could last for over a decade occupying the substrates and preventing the recruitment of juvenile corals (Plucer-Rosario, 1987), and also weakened the carbonate skeleton resulting in coral reef destroyed (Bryan, 1973). There were few studies on interaction between *T. hoshinota* and corals. One study reported that sponge growing over live corals was significantly faster than growing over non-living substrate; corals retracted

the tentacles and closed the stomodaea as sponge covered it suggesting *T. hoshinota* may gain the nutrition from coral tissue (Bryan, 1973). However, there was a different hypothesis from G. Plucer-Rosario, 1987. He compared the growth rate of *T. hoshinota* between a living coral prong and an air-blasted prong. The sponge did not grow on the living coral prong in 11 out of 21 cases but grow faster on the air blasted prong. This result supported that *T. hoshinota* kills the corals in competition for space rather than for nutrients (Plucerrosario, 1987)..

Another interesting result was discovered by Teruya and his co-workers (2004) that *T. hoshinota* actually contains toxic chemicals for competition. Two novel cytotoxic alkaloid compounds, nakiterpiosin and nakiterpiosinone, were extracted from the Okinawa sponge *T. hoshinota* (Teruya et al., 2004). This observation also partially supported the competition hypothesis.

In addition, Bryan (1973) reported an intriguing observation that there were “scars” on the sponge. In our field survey, we also notice the similar scars appearing on the Green Island sponge (Fig. 9). Although there is yet no clue for this phenomenon, it is speculated that the scars were caused by browsing fish feeding. More research is required to verify the role of grazing reef fishes to the sponge, and to evaluate whether these fishes could serve as controlling agents for *T. hoshinota*.

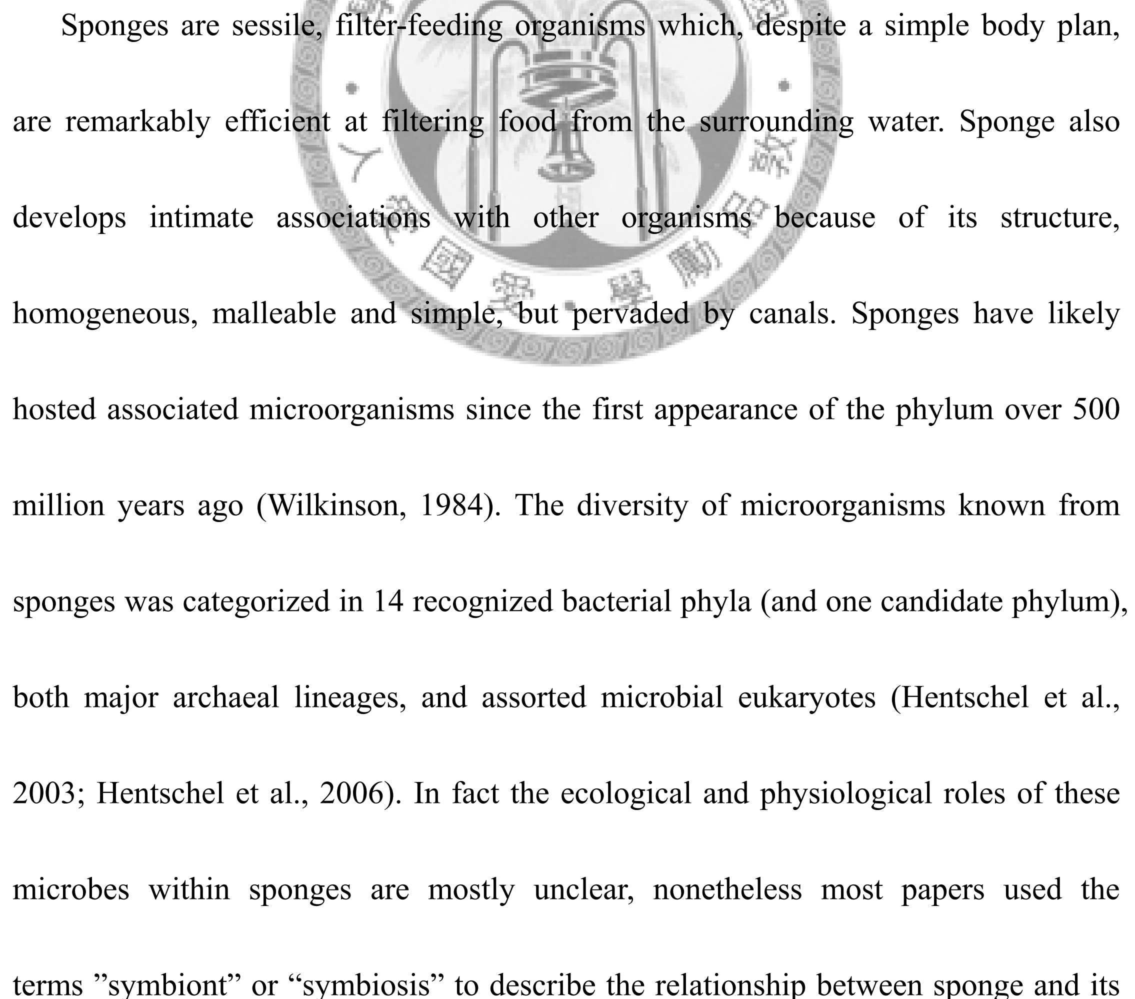
1.2.6.1 Biology of Terpios hoshinota

T. hoshinota, belongs to Phylum *Porifera*, Class *Demospongiae*, Order *Hadromerida*, Family *Suberitidae*, and Genus *Terpios*. The morphology of this sponge is extremely thin (typically less than 1 mm thick) encrustations. The sponge is gray or dark gray to brownish and black. Its canal system structure constitutes small oscula (3 mm) at the center of radiating, superficial exhalant networks (astrorhizae) and pores (50-300μm) situating in the meshes of those vein networks (Fig. 5A). All spicules of this species are tylostyles. The length and maximum shaft width of this tylostyle are 244.7μm and 3.0μm, respectively. All spicules are organized into radiating bundles near the ectosomal region. A typically developed head (tyle) consists of four knobs with axes perpendicular to each other and to the shaft. (Fig. 6A).

More interestingly, *T. hoshinota* harbors a massive amount of intercellular unicellular zoocyanellae, which fills almost all of intercellular space in sponge tissue. This zoocyanellae were identified as a kind of *Cyanobacterium*, *Aphanocapsa raspaigellae* based on morphology. However, the morphological similarity remains questionable because of no thin sheath observed in the *cyanobacteria* but in *Aphanocapsa raspaigellae* (Bryan, 1973; Plucerrosario, 1987; Rutzler and Muzik, 1993). Additionally the suggestion has no molecular phylogenetic evidence to support.

Despite the symbiotic relationship between *T. hoshinota* and the *cyanobacteria* remaining unclear, the fast-growing ability of *T. hoshinota* may be related to the *cyanobacteria*. Most of the successful space competitors often harbor large quantities of photosynthetic microorganisms. It is likely that the growth potential of these encrusting sponges is considerably enhanced by nutritional benefits derived from the association with photoautotrophs (Rutzler and Muzik, 1993).

1.2.7 Symbiotic relationships between sponge and endogenous microbe



Sponges are sessile, filter-feeding organisms which, despite a simple body plan, are remarkably efficient at filtering food from the surrounding water. Sponge also develops intimate associations with other organisms because of its structure, homogeneous, malleable and simple, but pervaded by canals. Sponges have likely hosted associated microorganisms since the first appearance of the phylum over 500 million years ago (Wilkinson, 1984). The diversity of microorganisms known from sponges was categorized in 14 recognized bacterial phyla (and one candidate phylum), both major archaeal lineages, and assorted microbial eukaryotes (Hentschel et al., 2003; Hentschel et al., 2006). In fact the ecological and physiological roles of these microbes within sponges are mostly unclear, nonetheless most papers used the terms ”symbiont” or “symbiosis” to describe the relationship between sponge and its

associated microbes.

Three decades ago, marine sponges might contain a specific microbe were noted (Vacelet, 1975; Vacelet and Donadey, 1977; Wilkinson, 1978a; Wilkinson, 1978c, 1978b; Wilkinson, 1984). Based on electron microscopy and bacterial cultivation studies, these pioneers of sponge symbiont research proposed the following three broad types of microbial associates in sponges: 1) abundant populations of sponge-specific microbes in the sponge mesohyl, 2) small populations of specific bacteria occurring intracellularly, and 3) populations of nonspecific bacteria resembling those in the surrounding seawater.

Although microbial communities can vary considerably among sponge species in both cultivation or uncultivation-based studies, many diverse sponge species, even from nonoverlapping geographic ranges, share a common set of unique, associated microbes (Hentschel et al., 2002; Hentschel et al., 2006). Indeed, subsequent studies have lent further weight to this notion (Thoms et al., 2003; Lafi et al., 2005; Hill et al., 2006; Thiel et al., 2007). Furthermore, distinct microbial communities between sponges and the surrounding seawater were demonstrated in some studies (Olson and McCarthy, 2005; Schleper et al., 2005; Hill et al., 2006). These results indicate that sponge-associated microbial communities are indeed unique and at least partially sponge specific. Currently, the existence of sponge-specific microorganisms has

consequently become something of a paradigm in this field (Taylor et al., 2007).

In some sponge species, microorganisms account for up to 60% of the sponge biomass (Wilkinson, 1978c, 1978b, 1978a;), exceeding seawater concentrations by two to three orders of magnitude (Friedrich et al., 2001). Reiswig coined the term “bacteriosponge” to describe sponge species with a significant biomass of microorganisms (Reiswig, 1973). Two types of associations between microbes and the host sponge were identified by Vacelet and Donadey according to 11 taxonomically diverse demosponge species using transmission electron microscopy (TEM) (Vacelet and Donadey, 1977). One is that sponges with dense tissues contain abundant, dense, and morphologically diverse microbial communities (i.e. bacteriosponges) and another is that sponge with well-irrigated tissues contain few bacteria and typically only a single morphotype; these two sponge types have recently been called High Microbial Abundance (HMA) sponges and Low Microbial Abundance (LMA) sponges (Hentschel et al., 2006).

Sponge and the microorganisms living within and around them display the different mechanism of interactions; for example microbes as parasitism which sometimes resulting in sponge death, or microbes as the major food source for heteroterophic sponges (Reiswig, 1975), or nutritional enhancements (Arillo et al., 1993), and mutualistic interaction that bacteria produce the secondary metabolite

production to help defense of sponge (Unson et al., 1994; Schmidt et al., 2000).

The sponge-microbe interaction is likely depended on what function the bacteria possess. In the coming sections, more details on the relationship between sponge and phototrophic or heterotrophic microbes are described.

1.2.7.1 Sponges as hosts of single-celled autotrophs

Photosynthetic single-celled organisms, from *Cyanobacteria* to dinoflagellates, are common symbionts of sponges and have been demonstrated to be beneficial in a wide variety of taxa. All of over 100 sponge species found to host *Cyanobacteria* are in only 26 of the 72 recognized demosponge families. Four genera (*Aplysina, Xestospongia de Laubenfels, Dysidea, Theonella Gray*) are particularly rich in these associations, with 5-10 species in each genus hosting *Cyanobacteria* (Diaz and Ward, 1999). Filamentous *Cyanobacteria* have been documented in the dictyoceratid *Oligoceras violacea* in the Caribbean (Rutzler, 1990) and *Dysidea herbacea* on Great Barrier Reef (Hinde et al., 1999). In both cases, the proportion of cyanobacterial biomass to overall biomass was particularly high, in *O. violacea* as much as half of the total cell volume of the association.

Many *Cyanobacterium*-containing species, flattened shapes, allowed optimal light reception for their photosynthetic symbionts (Wilkinson, 1983). Substantial

differences are in the proportion of photosymbiont-harboring sponge species, near shore (0%), middle distances from shore (20%-64%), far from shore (5%-90%) on the Great Barrier Reef. This observation suggested that these reflect differences in the nutrient content of the ambient water and therefore the relative ability of sponge to support themselves solely through filter feeding or photosymbiont (Wilkinson, 1987). As a result, the photosymbiont-harboring sponges more rely on the nutrient from photosymbiont. This idea was also emphasized with a study that shading of *Lamellodysidea chlorea* (sponge)-*Oscillatoria spongeliae* (filamentous *Cyanobacterium*) association for two weeks resulted in loss of 40% of sponge area covered by shaded individuals, indicating dependence of the host sponge on these symbionts (Thacker, 2005). Another study showed the sponge *Chondrilla nucula* underwent metabolic collapse and thiol depletion in dark conditions for 6 months. The reason was that symbiotic *Cyanobacteria* participated in controlling the redox potential of the sponge host cells by the transfer of reducing equivalent (Arillo et al., 1993). Therefore, metabolic integration is also important relationship between symbiotic *Cyanobacteria* and sponges. Moreover, there is evidence that secondary metabolic production is produced by symbiotic photosynthetic *Cyanobacteria* in *Dysidea herbacea* (Unson et al., 1994).

High degree of host specificity and potential coevolution between symbiotic

Cyanobacteria and their host sponges were demonstrated by phylogenic construction (Thacker and Starnes, 2003). Coevolution of sponges and *Cyanobacteria* is further indicated by vertical transmission of symbionts in *Chondrilla australiensis*, via incorporation into eggs (Usher et al., 2001) or via the unusual giant larvae in *Svenzea zeai* (Rutzler et al., 2003). Understanding of diverse relationship between sponge and symbiotic *Cyanobacteria* could offer more sponge ecology information.

The other symbiotic autotroph cell is dinoflagellate. Rutzler has pointed out one curious pattern that dinoflagellates (zooxanthellae) in sponges inhabit primarily excavating sponge species in the order *Hadromeria* (Rutzler, 1990). Rich brown colors, ranging from the golden medium brown of *Cliona varians* to an almost black brown in *Cliona caribbaea* indicate excavating species can be packed with zooxanthellae. Zooxanthellae had positive influence on growth rate of *Cliona viridis* (Rosell and Uriz, 1992) and may be coopted by sponge as sponge kills coral with producing alkaloids (Wulff, 2006).

1.2.7.2 Sponge as hosts of heterotrophic single-celled microbes

Heterotrophic prokaryotes appear to be ubiquitous symbionts of sponges, even though sponges are efficient consumers of bacteria (Reiswig, 1971, , 1973; Pile et al., 1997). An enormous variety of heterotrophic bacteria, including representatives of

seven divisions, has been identified by comprehensive surveys using 16S rRNA sequences. Three divisions were described as follows.

Archaea are the second well-understood sponge-associated microbial lineage next to the *Cyanobacteria*. Marine sponge-associated *Archaea, Cenarchaeum symbiosum*, was originally discovered by Preston et al., 1996. The worldwide distribution of the sponge-specific *C. symbiosum* clade (Webster et al., 2001; Margot et al., 2002; Lee et al., 2003) resembles the similarly cosmopolitan distribution of the free living marine *Crenarchaeote* group I. This phenomenon suggests that a free-living form of this archaeal sponge symbiont clade exists which is able to persist in sponges following uptake by filtration.

Proteobacteria, the complex picture of proteobacterial diversity within sponge was discovered using molecular phylogenetic method. More than two-thirds of the thousands of cultivated isolates from deep water and boreal sponges and other invertebrates belonged to the *Alphaproteobacteria* and *Gammaproteobacteria* (Graeber et al., 2004; Sandell et al., 2004) and *Gammaproteobacteria* dominated among the nearly 2000 isolates from *Halichondria panacea* (Imhoff and Stöhr, 2003). A sponge-specific gammaproteobacterial cluster showed closest homology to the ammonia-oxidizing *Nitrosococcus* clade, especially in mangrove sponges (Hentschel et al., 2006).

Actinobacteria, the phylum attracting scientist's eyes, are prolific secondary metabolite producers, which has obvious implications for natural products and drug discovery. Three sponge-specific actinobacterial clusters were described in *Theonella swinhoei* and *Aplusina aeropoba* and further extended to *Scleritoderma* spp. from deeper waters. Bioactive Salinospora previously only known from marine sediments (Kim et al., 2005) was isolated from the Australian sponge *Pseudoceratina clavata.*

One indication of the possibility of tight coupling between host sponges and their symbionts is the similarity of symbiotic bacteria over time, even after 11 days of starvation or antibiotic treatment of their sponge hosts (Friedrich et al., 2001). A hypothesis of co-speciation of sponges in the order *Halichondrida* and their bacterial symbionts has been supported by comparative phylogenetic analysis based on the gene coding for cytochrome oxidase subunit I (COI) (Erpenbeck et al., 2002).

The difficulty of culturing heterotrophic prokaryote sponge symbionts renders determination of their function in their host extremely challenging. Nitrifying symbionts add significant amounts of biologically meaningful nitrogen to tropical shallow benthic communities (Diaz and Ward, 1997). A variety of possible functions of prokaryote symbionts in sponges was listed by Hentschel et al., 2003. However, in most cases what the symbiosis provides for the sponges is largely mysterious, though these may be ancient associations.

1.3 Aim of this thesis

"Black disease" outbreak in the waters of Northern Green Island has been widely reported in several major Taiwan's newspapers (Fig. 10). This outbreak has also been highly paid attentions by the government, public and academic. Green Island not only is one of the hottest tourist point in Taiwan but also has recently been suggested to be upgraded onto a national reservation park because of its highly abundant in- or off-land's biodiversity and beautiful off-shore ecosystems. To combat this disease, an integrated research team has been formed by Construction and Planning Agent, Ministry of the Interior, in 2007, and is expected to obtain relevant information about this disease in a short time which will be helpful for disease control and prevention.

To be the member of this integrated team, we focus on understandings of the black disease associated microbes. As discussed above, the microbes play a crucial role on coral and sponge. The relationship of microorganism associated with sponge and coral is ranging from mutual benefit to parasitism and disease. Therefore, understanding the microbial community is important to explain the ecological interaction between *T. hoshinota* and corals. Hence, in this thesis, we would like to investigate the diversity and distribution of the microbial community of the "black disease" using culture-independent molecular techniques revealed a previously unseen microbial

diversity that encompasses an estimated >99% of the total microbial community of a given sample (Imhoff and Stöhr, 2003). Two goals are addressed as follows.

1) Identify the microbial community associated with *Terpios hoshinota*, and find out the major microorganisms in this sponge.

2) Realize variation of coral associated microbial community, and compare the difference of microbial community between healthy coral and unhealthy coral (sponge-infected). Furthermore search for any particular microbe(s) can be used as a bioindicator of unhealthy coral.

Table 1 Standard Systematically Describing Coral Disease/Syndrome, according to Work and Aeby, 2006

No:	Species name:	Colony/diameter:
Lesion/mucus:		
Structures affected:		Relief:
Distribution of lesion:		Size:
Location on colony:		Number:
Shapes:		Color:
Margins:		Extent:
Edges:		Rate:
Other creature:		

編號:	珊瑚名:	珊瑚大小/直徑:
病灶描述:		
影響結構:		病灶突起:
病灶分布:		病灶大小:
病灶位置:		病灶數量:
病灶形狀:		病灶顏色:
邊緣形狀:		嚴重程度:
界線:		惡化速度:
其他生物覆蓋:		

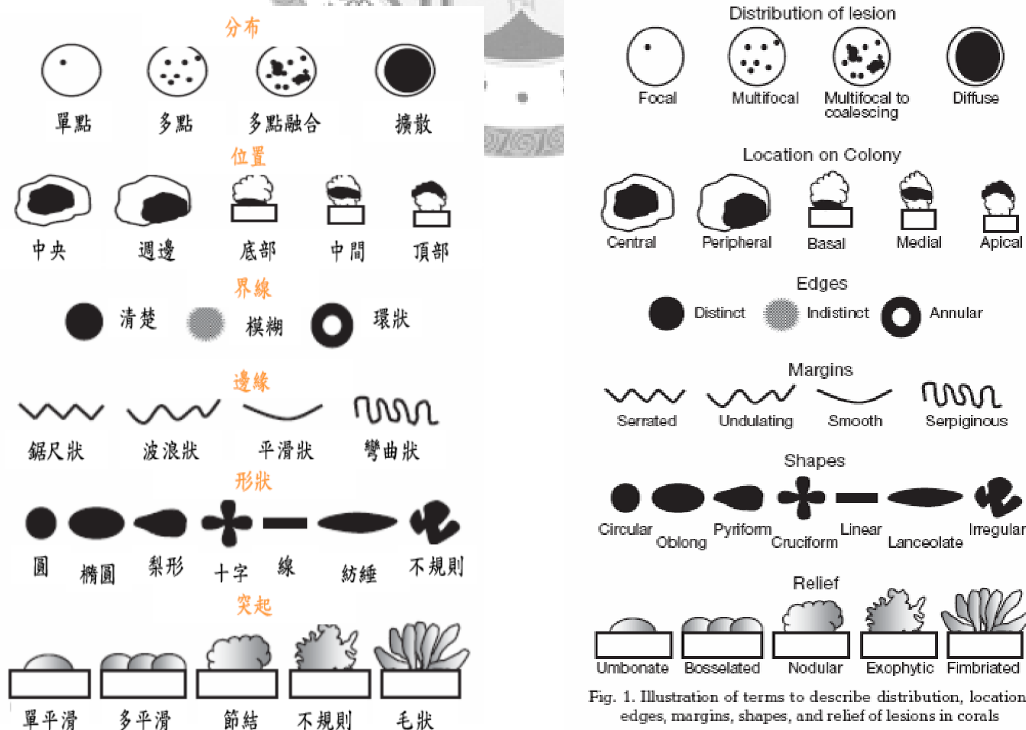


Fig. 1. Illustration of terms to describe distribution, location, edges, margins, shapes, and relief of lesions in corals

(Work and Aeby, 2006)

Table 2 Coral diseases observed in Taiwan

Disease \ Location	Ye-Liu	Dou-Fu-	Yuan-An	Wang-Li-Tung	Tiao-Shi	Outlet of the third nuclear power plant	Green Island
Black band						V	
Black disease				V			V
Skeletal anomalies				V	V		
White plague	V	V	V	V	V		
Whit band					V	V	
Skeleton eroding							
Pink line/spot		V	V		V		
Porites ulcerative white spot			V				
Dark spot							
Yellow blotch			V				
Unknown		V		V		V	

Table 3 Standard systematically description of black disease

<table>
<tr><td>編號:</td><td>珊瑚名:Porites</td><td colspan="2">珊瑚大小/直徑: 30 cm</td></tr>
<tr><td colspan="4">病灶描述: 黑色薄膜覆蓋珊瑚，覆蓋處珊瑚死亡，交界處產生白化</td></tr>
<tr><td colspan="2">影響結構: 侵蝕珊瑚蟲(無侵蝕骨骼，但骨骼易碎)</td><td colspan="2">病灶突起: 突起不規則(厚度約 1-3 公厘)</td></tr>
<tr><td colspan="2">病灶分布: 頂部、側邊</td><td colspan="2">病灶大小:最大直徑 15 公分</td></tr>
<tr><td colspan="2">病灶位置: 擴散</td><td colspan="2">病灶數量:1(一大片)</td></tr>
<tr><td colspan="2">病灶形狀: 不規則</td><td colspan="2">病灶顏色: 黑色、墨綠、灰色</td></tr>
<tr><td colspan="2">界線: 清楚</td><td colspan="2">嚴重程度: 50%</td></tr>
<tr><td colspan="2">邊緣形狀: 波浪狀</td><td colspan="2">惡化速度: 1 公厘/天</td></tr>
<tr><td colspan="4">其他生物覆蓋: 部分有藻類覆蓋，但大部分無</td></tr>
</table>

White plague, Tiao-shi

White plague, Wang-li-tung

Black band, Outlet of the third nuclear power plant

Black band, Outlet of the third nuclear power plant

White band, Tiao-shi

Skeletal anomalies, Wang-li-tung

Yellow blotch , Yuan-An

Yellow blotch , Yuan-An

Porites ulcerative white spot, Yuan-An

Pink spot, Dou-Fu-Chia

Unknown , Tiao-shi

Unknown , Yuan-An

Fig. 1 Coral diseases observed in Taiwan

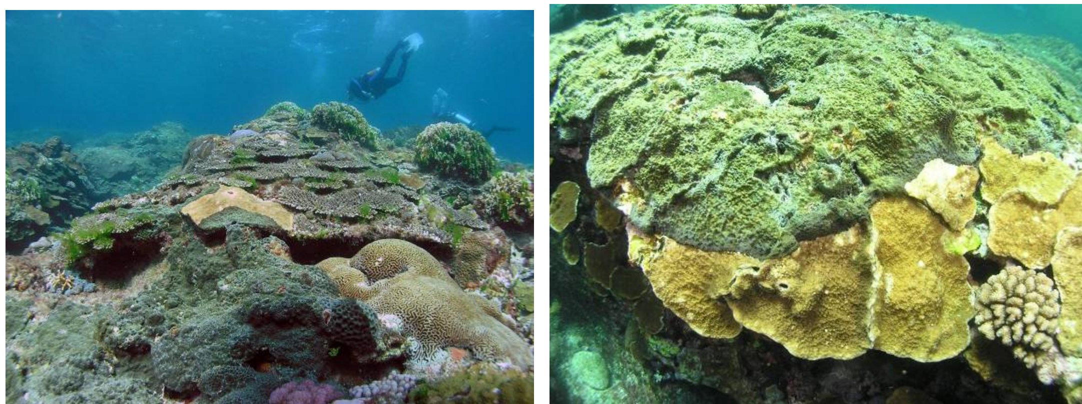


Fig. 2 A large area of coral reef was covered by the blackish mat.

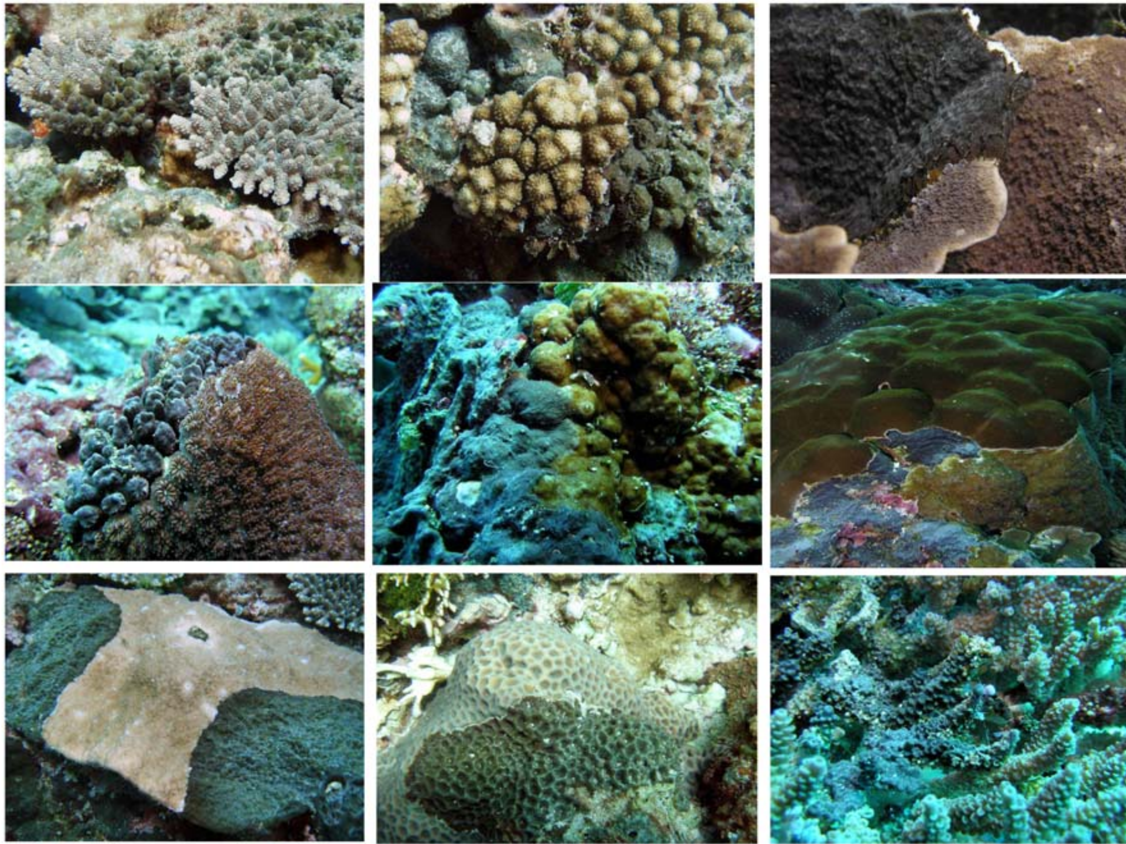


Fig. 3 Different morphologies of corals were covered by the blackish mat.

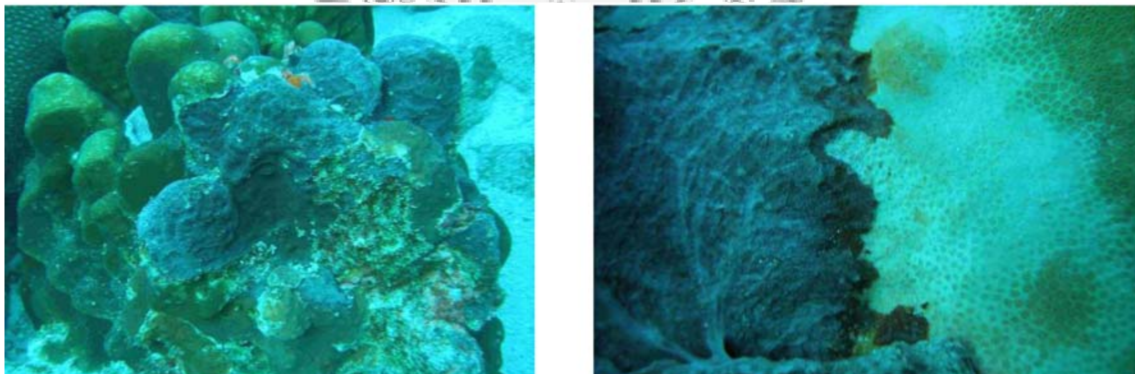


Fig. 4 Close-up of black disease infecting *Porites sp* .(Systematic descriptions in Table 3)

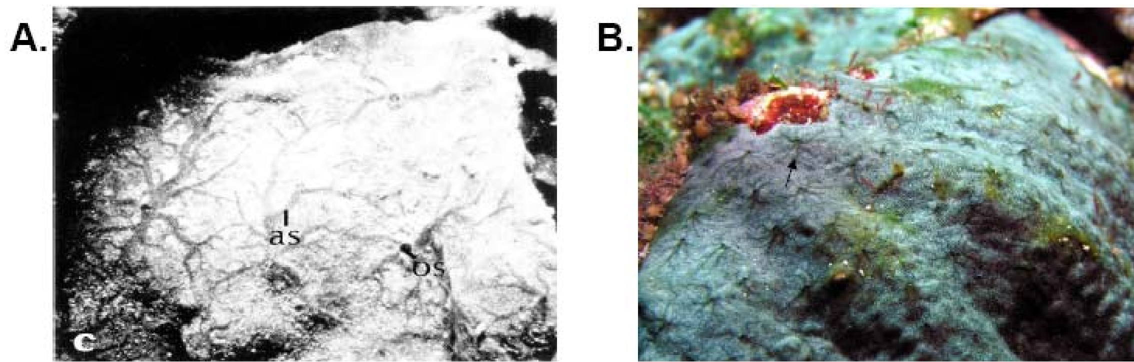


Fig. 5 Close-up of sponge crust.

A. as: astrorhizae, calcareous skeletons, superficial exhalant networks, os: oscula (3 mm, discernible in the field and center of radiating), *Terpios hoshinota* from Rutzler and Muzik, 1993. B. oscula like structure (black arrow), sponge specimen from Green Island.

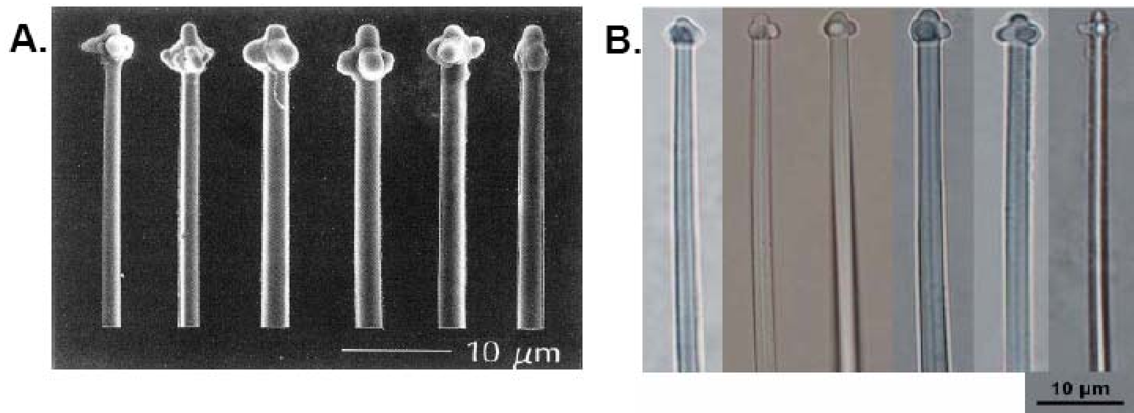


Fig. 6 Portions of tylostyle head (spicule).

A. Scanning electron micrograph, *Terpios hoshinota* from Rutzler and Muzik, 1993. B. electron micrograph, sponge specimen in this study.

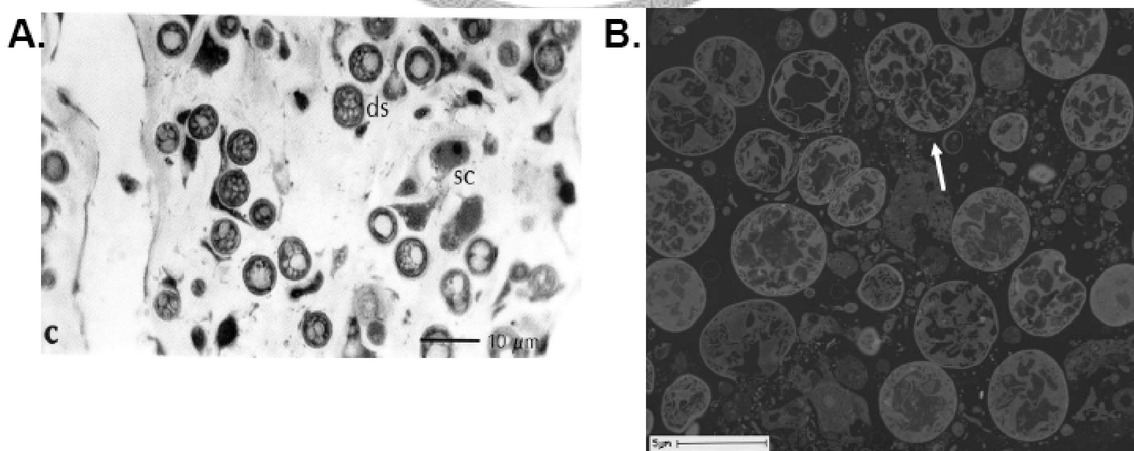


Fig. 7 Transmission electron microscopy of *T. hoshinota*.

A. zoocyanellae in choanosome (ds: dividing stage of zoocyanellae, sc: sponge cells), *Terpios hoshinota* from Rutzler and Muzik, 1993. B. zoocyanellae like cells in dividing stage (white arrow), sponge specimen from Green Island.

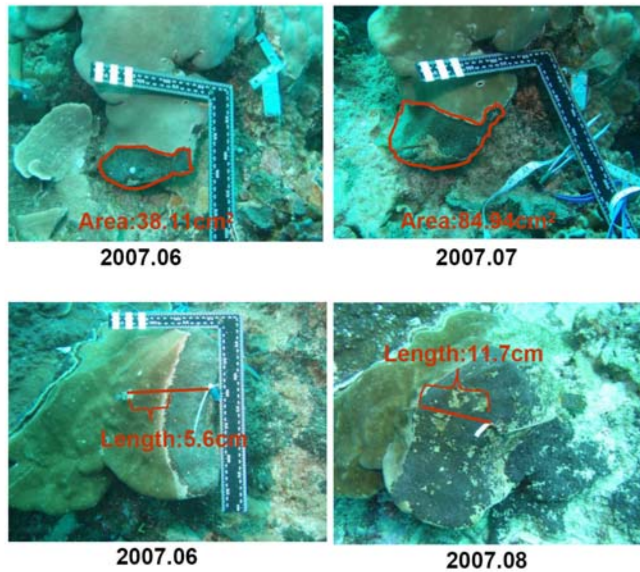


Fig. 8 Progress rate of *T. hoshinota* in Green Island.

Fig. 9 Scars on *T. hoshinota* suspected by fish bite

中華日報

生活萬象

疾病蔓延 綠島珊瑚變少了

中央社／台北十八日電

中國時報 A6/社會脈動 2006/11/19

外觀呈墨綠色壞死狀 國際首例 綠島珊瑚染怪病 海岸線全淪陷

【朱立群／台北報導】

綠島的珊瑚發生大規模病變！中華民國珊瑚礁學會昨天指出，綠島柴口海域的珊瑚自去年起陸續被發現染上「怪病」，呈現墨綠色的壞死狀，如今病變範圍蔓延整條綠島海岸線，並深達海底廿公尺。

學者指出，此一病變情形是國際上的首例，原因尚不明，若不搶救，綠島的珊瑚恐將全數壞死。

疑染真菌 蔓延海底廿公尺

「中華民國珊瑚礁學會」昨日在中研院生物多樣性中心發表《二〇〇六年台灣珊瑚體檢報告》，報告人之一、台大海洋研究所戴昌鳳教授指出，去年起陸續發現綠島柴口海域的珊瑚染上「真菌」或「黴菌」等菌類疾

自由電子報

www.libertytimes.com.tw

生活新聞

綠島石珊瑚染病 白化

不明病因籠罩 恐擴大

〔記者袁世忠／台北報導〕綠島生態拉警報！學者發現，綠島熱門浮潛區的石珊瑚，近年染上不知名疾病，厚厚一層真菌般的物質，讓石珊瑚紛紛死亡，數量已經比去年減少十二%，而且還在蔓延中，恐有擴大趨勢。

大紀元 > 新聞 > 台灣新聞

海域珊瑚染不明疾病 珊瑚覆蓋率降低

綠島珊瑚 黑死病蔓延

綠島珊瑚染「黑死病」，美麗外觀蒙上一層墨綠到黑色的薄膜。（圖：由中央研究院提供）

Fig. 10 News of black disease outbreak in Taiwan

[university seal watermark]

2 Materials and Methods

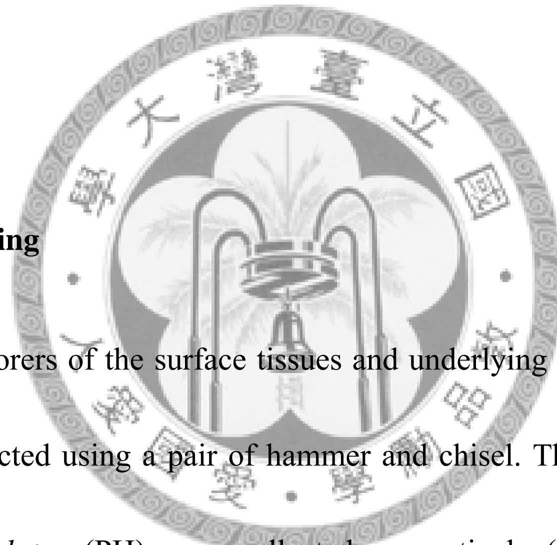
2.1 Sampling

In order to reveal the specificity of microbial community associated with *Terpios hoshinota*, Fourteen specimen of *T. hoshinota* covered on five different coral species 3 *Montipora efflorescens* (MM), 2 *Millepora sp.*(FM), 3 *Porites lutea* (PM), 3 *Favia complanata* (VM), 3 *Isopora palifera* (AM) colonies were collected from Chai-Kou (22°51.66'N; 121°48.83'E) and Kun-Guan (22°40.29'N; 121°29.17'E), Green Island in July 2007. The coral sampling strategy is to include the sponge-infected corals (SIC) and the non-sponge-infected corals (NSIC) for the comparison of the microbial community. Surrounding seawater was collected as a control for microbial diversity analyses.

While all sponge and coral samples were collected underwater, the samples were immediately placed in sterile 50 ml polypropylene centrifuge tubes. Back to the shore, each sample was washed twice with 0.2 μm filtered and autoclaved seawater to remove any loosely associated microbes. The detailed strategies for coral, sponge, and seawater sampling are described respectively in the following sections.

2.1.1 Seawater sampling

One liter seawater was collected from 10 cm above the coral colonies using the sterile Sanko high density polyethylene bottles in both sampling sites. The samples were kept on ice and stored at -20 ^{0}C as soon as returned to the field station. Seawater samples were filtered through sterile 0.22 μm pore diameter filters, Millex-GS (Millipore, USA). The microbes retained on the filters were collected for DNA extraction.



2.1.2 Coral sampling

2 cm diameter corers of the surface tissues and underlying skeleton to a depth of 0.5-1 cm were collected using a pair of hammer and chisel. Three SI and three NSI colonies of *Porites lutea* (PH) were collected, respectively (Fig. 11). These coral colonies lived at 6 m depths and were separated from each other by a distance of at least 10 m. In each of the SIC colonies, two separated parts were sampled. These two parts are the distal part (P^dH) (an area of the coral colony away from the sponge region) and proximal part (P^dI) (an area of the coral colony about 5 cm close to sponge). The samples were kept at 4 ^{0}C and transported back the field station less than 2hr, and stored in 100% ethanol at -20 ^{0}C until process.

2.1.3 Sponge sampling

T. hoshinota was collected using a pair of forceps or a 30ml sterile syringe (Fig. 12). Sponge specimens were taken from sponges covered on five kinds of coral species (Fig. 13). The samples were kept at 4 ^{0}C and transported back a field station less than 2h, and stored in 100% ethanol at -20 ^{0}C until use.

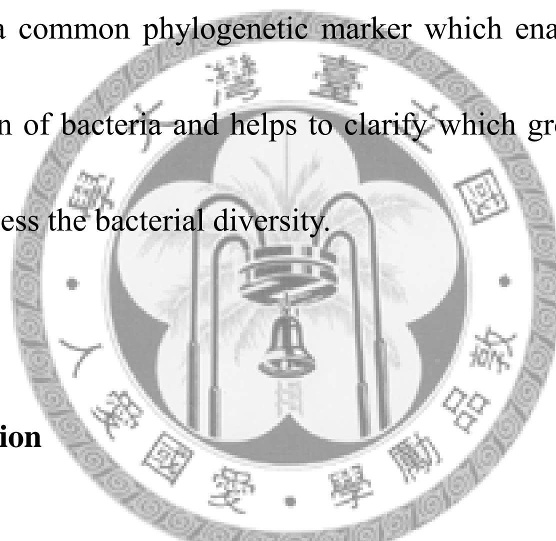
2.2 Electron microscopy

Small pieces of sponge tissue (c.f. 1 cm diameter) were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer and stored overnight at 4 ^{0}C for 24 hr. Fixed samples were rinsed with phosphate buffer 3 times, each time for 15 minute, placed in 1% (w/v) osmium tetroxide solution (prepared in 0.1M phosphate buffer) at room temperature for 4 h, and rinsed again with phosphate buffer 3 time for 15 min. Before embedded in Spurr’s resin, samples were subsequently dehydrated in a graded acetone series [30%, 50%, 70%, 85%, 95% and 100% (v/v) acetone]. For each concentration the dehydration was lasted for 20min. Sections were cut with a LEICA EM UC6 and stained with 5% (w/v) uranyl acetate in methanone followed by 0.5% (w/v) lead citrate. Sections were mounted on 50 mesh

copper grids coated with Formvar. Samples were visualized by transmission electron microscopy.

2.3 Molecular Method for bacterial library construction

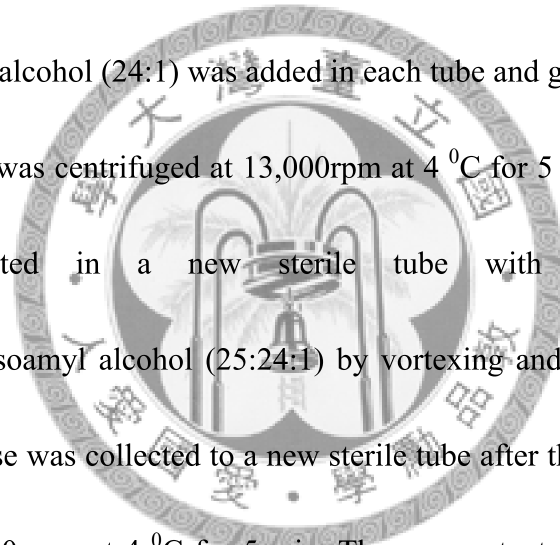
In this thesis, two molecular techniques, 16S ribosomal RNA (rRNA) library construction and denaturing gradient gel electrophoresis (DGGE), were applied to identify the bacterial community associated with sponges, corals and seawater. The 16S rRNA gene is a common phylogenetic marker which enables to determine the phylogenetic position of bacteria and helps to clarify which groups of bacteria in an ecosystem and to assess the bacterial diversity.



2.3.1 DNA extraction

Total DNA was extracted using modified standard phenol–chloroform procedure (Appendix 1) (Wilson, 1997) incorporating a grinding step in liquid nitrogen to lyse the cells mechanically. Frozen coral and sponge samples with liquid nitrogen were homogenized to a fine powder using sterile pestle and mortars. Ground sample in alcohol was transfer to a 1.5 ml sterile tube and centrifuged at 13,000 rpm for 5 min. The top alcohol layer was removed. The pellet was resuspended and votexed in 1 ml TE buffer and then centrifuged at 13,000 rpm for 5 min. The supernatant was

discarded and the pellet was washed in TE buffer again. This procedure was repeated three times and the pellet was ready for DNA extraction. The pellet was resuspended by 500 μl of Lysis buffer and added 30 μl of 10% SDS, 3 μl of 20 mg/ml proteinase K. The mixing sample was vortexed and kept at 37 ^{0}C for 1 hr. After that, the mixing sample was added 100 μl of 5M NaCl and 80 μl CTAB/ NaCl solution (Dissolve 4.1g NaCl and 10g of CTAB to 100ml). The mixing samples were vortexed and kept at 65 ^{0}C for 10 min. The supernatant was collected to two new Eppendorfs. 500 μl chloroform/isoamyl alcohol (24:1) was added in each tube and gently shaken for1 min. The mixing sample was centrifuged at 13,000rpm at 4 ^{0}C for 5 min. The top aqueous layer was collected in a new sterile tube with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) by vortexing and gently shaking for 1 min. The liquid phase was collected to a new sterile tube after the mixing sample was centrifuged at 13 000 rpm at 4 ^{0}C for 5 min. The supernatant was extracted with an equal volume of chloroform/iso-amyl alcohol (24:1) by vortexing and centrifuged at 13 000 rpm for 5 min at 4 ^{0}C. The supermentant was removed to new tube with adding 0.6 volume isopropanol and slowly mixed. The mixture was centrifuged at 13 000 rpm for 7 mins, the supernatant was then discarded and the pellet was washed with 70% pre-chilled ethanol. The pellet was air dried and suspended in 30 ml sterile Milli-Q water. Made 2 μl aliquot DNA samples in each PCR tube. The aliquot sample



was used for only once for avoiding DNA degradation result from repetitively taking a DNA sample in-and-out of freezer. Extraction of DNA from corals would also co-extract humic and phenolic materials that might inhibit PCR. To remove these interference factors, DNA was subsequently re-extracted by QIAamp® DNA Mini Kit (Qiagen) for further purification.

2.3.2 Amplification of bacterial 16S ribosomal rRNA by polymer chain reaction

Two molecular methods were used to study the bacterial community in the specimens. One is that using universal bacteria primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3') (Lane, 1991) for constructing clone library. Universal bacterial primers were used for amplification of the 16S rRNA genes from extracted genomic DNA of seawater, coral and sponge samples. A total of 1465 PCR product were obtained. The other method was denaturing gradient gel electrophoresis (DGGE). 16S rRNAs were amplified with DGGE-specific, bacterial primers 341F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3') (Sanchez et al., 2007). A 40-bp rich GC clamp was attached to the 5'end of the 341F primer to stabilize the melting behavior of the DNA fragments during running DGGE. These primers amplify a

606-bp section of the 16S rRNA genes of members of the domain Bacteria.

16S rRNA genes were amplified in a 50 μl reaction mixture consisting of 5 μl of 10× PCR buffer, 1 μl of a 2.5 mM total deoxynucleoside triphosphate mixture, 2.5 μl of 10 μM each primer, 1 μl of template DNA, and 1.2 U of DyNAzyme EXT DNA polymerase (FINNZYMES, Finland), according to the conditions recommended by the manufacturer. Amplification conditions for the bacterial library PCR consisted of an initial denaturation step of 94 ^{0}C for 5 min, followed by 35 cycles of 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, and 72 ^{0}C for 1.5 min. This was followed by a final step at 72 ^{0}C for 10 min and cooled at 4 ^{0}C. DGGE PCR amplification conditions consisted of an initial denaturation step of 94 ^{0}C for 5 min, followed by 30 cycles of 94 ^{0}C for 30 sec, 46 ^{0}C for 30 sec, and 72 ^{0}C for 1 min and a final extension step of 72 ^{0}C for 10 min, then cooled at 4 ^{0}C.

All PCR reactions were performed on a PxE Thermal Cycler (Thermo Electron Corporation, USA). The PCR products were checked using electrophoresis and visualised using a UV trans-illuminator, ImageQuant 300 (GE Healthcare, USA).

2.3.3 Clone library construction

The PCR products of bacterial 16S rRNA were purified using the QIAquick gel extraction kit (Qiagen, USA) for cloning. The purification procedure is followed

using the manufacture's instruction (http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000252). Finally the DNA was recovered in 20 μl sterile Milli-Q water (Q-Gard 1 Purification Pack, Millipore). The purified PCR products were ligated into TOPO-TA cloning vector (TOP10 genotypes of E.coli strain) by the invitrogen manufacturer's instructions (http://www.invitrogen.com/site/us/en/home/brands/Product-Brand/TOPO-PCR-Cloning/TOPO-TA-Kits-Selection-Guide.reg.tw.html). Positive colonies were selected by blue and white screening on Luria Bertani agar plates with ampicillin (50 μg/ml), IPTG (0.5 mM) and X-Gal (50 μg/ml). For finding the successful clones, each positive colony was examined by PCR with M13 forward (5'-GTA AAA CGA CGG CCA G-3') and reverse primers (5'-CAG GAA ACA GCT ATG AC-3'). The ampilification condition is the same as that with bacteria universal primer PCR condition (see section 2.3.2). Reaction products were checked using DNA agarose electrophoresis and visualized using a UV trans-illuminator.

To avoid the cloning bias, we therefore followed the bellow process to reduce the bias. In each transformation plate, the number of positive colonies (i.e. white colonies) must over 1000 (10-20 μl transformation solutions) which guarantees the transformation efficiency sufficient. According to a random number created by Microsoft Excel, we then randomly picked 30 white colonies from 100 white colonies

for sequencing (Singleton et al., 2001).

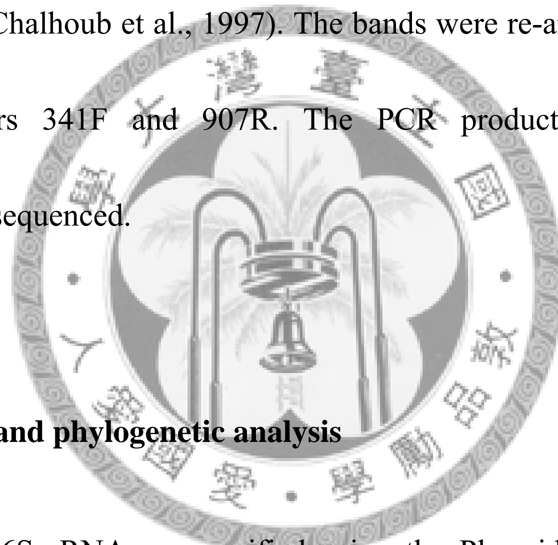
2.3.4 Denaturing gradient gel electrophoresis analysis

Denaturing gradient gel electrophoresis (DGGE) analysis was performed using the D-Code universal mutation detection system (Bio-Rad Laboratories, USA). The equal concentration (60 ng) of PCR products were loaded onto 7% (w/v) polyacrylamide gels run in 1× TAE buffer. A 22.5–35% linear gradient of urea and formamide was for 16S rRNAs from sponge samples and a 22.5–45% linear gradient was for 16S rRNAs from coral samples. The linear gradient range was decided by the result of perpendicular gel which contained 0 to 80% denaturant. The gel was prerun at 60 ^{0}C, 130V for 15 min. Samples were electrophoresed at 60 ^{0}C, 130V for 7 hr. After electrophoresis, the gels were washed three times by Milli-Q water for 5 min and visualized using silver staining. The silver staining composed of the following steps: 1) gel was soaked in 200ml solution of 0.1% $AgNO_3$ and slowly shaken for 20 min. Gel was washed once with Milli-Q water for 5 min. 2) gel was soaked and slowly shaken in 200 ml of the developing solution (3.0g of NaOH, 0.02g of $NaBH_4$, 0.8ml of formaldehyde) for 10 min. Gel was washed once with Milli-Q water for 5 min. 3) gel was soaked and slowly shaken in 200 ml stop solution (0.75% Na_2CO_3) for 5min. 4) Finally, the gels were washed with Milli-Q water and dried at room temperature. All

reactions were kept at 27 ^{0}C avoiding chemical sedimentation as low temperature (<25 ^{0}C)

2.3.5 Recovery of DNA from DGGE gel

From 16S rRNA-DGGE gels, distinct bands were excised from the dried gels using sterile blades and were rehydrated by placed in 10 μl of sterile Milli-Q water for 1 hr to elute DNA (Chalhoub et al., 1997). The bands were re-amplified by PCR with 16S rRNA- primers 341F and 907R. The PCR products were checked by electrophoresis and sequenced.



2.3.6 Sequencing and phylogenetic analysis

The amplified 16S rRNA was purified using the Plasmid DNA Extraction kit (Viogene, Taiwan) and its partial sequences (650–700 bp) were obtained using the 27F primer. The sequencing works were completed by Mission Biotech (http://16318279.boss.com.tw/show_page.asp?income=16318279) using ABI 3730 XL DNA Analyzer (Applied Biosystems, USA), and the sequencing reagents was the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, V3.1 (Applied Biosystems, USA).

2.3.7 Bacterial group assignment

Sequence cluster and approximate phylogenetic affiliations were characterized by software of the Ribosomal Database Project II (http://rdp.cme.msu.edu/) (Maidak et al. 1996).

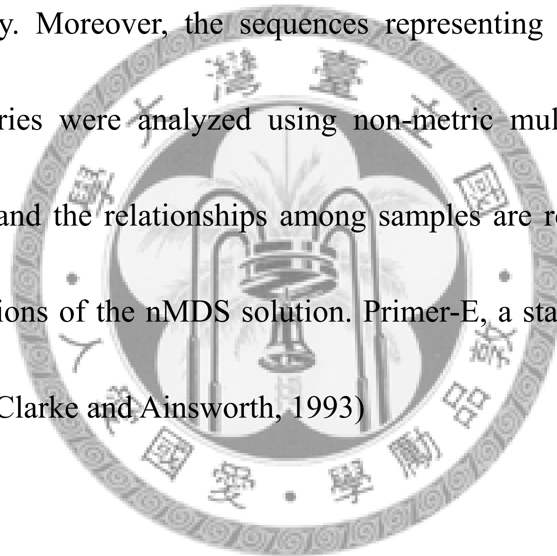
2.3.8 Diversity estimation in DOTUR

We calculate various diversity indices and richness of microbial diversity within each clone library of coral, sponge, and seawater samples using DOTUR software package (http://www.plantpath.wisc.edu/fac/joh/dotur.html) (Schloss and Handelsman, 2005). The results perform the number of sequences and their identity in each OTU as a function of distance. Basically we grouped those sequences that had >97% sequence identity together by DOTUR. However, 99% as threshold value for species identification of bacteria associated with sponge, because the variation of sponge bacterial sequences was small. DOTUR calculates richness indices including the Shannon-Weaver, the full bias corrected Chao1, abundance-based coverage estimator (ACE) and evenness indices including Simpson's eveness indices. The sampling completeness which how many sequences are required to obtain a desired level of

precision was estimated by the rarefaction curve.

2.3.9 Sequence library comparison

LIBSHUFF analyses (http://libshuff.mib.uga.edu) (Singleton et al., 2001) were employed to compare clone libraries created from the different parts of corals, sponge and ambient seawater samples to establish levels of similarity in the bacterial 16S rRNA gene diversity. Moreover, the sequences representing OTUs from different samples clone libraries were analyzed using non-metric multidimensional scaling (nMDS) ordination and the relationships among samples are represented in plots of the first two dimensions of the nMDS solution. Primer-E, a statistical tool, was used for nMDS analysis (Clarke and Ainsworth, 1993)

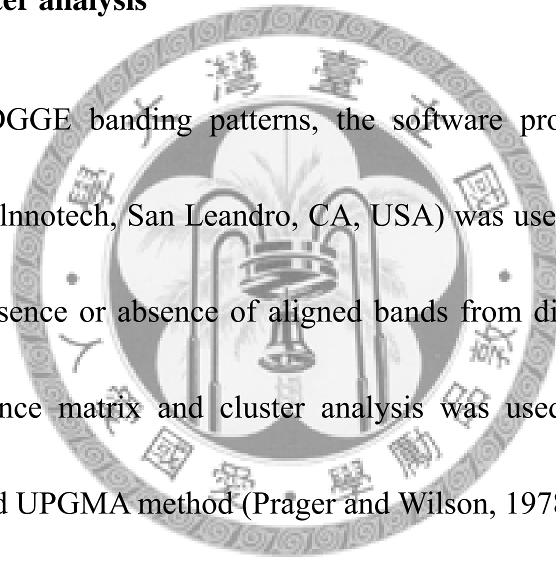


2.3.10 Phylogenetic analyses

Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007). Sequences were aligned with similar sequences of cultured organisms and environmental clones obtained from GenBank using a Clustal W algorithm in the MEGA version 4 (http://www.megasoftware.net/). Cloned *cyanobacteria* sequence from sponge were aligned with other sponge associated *cyanobacteria* (Steindler et al.,

2005) . Tree topologies were inferred from aligned sequences using neighbour-joining (Saitou and Nei, 1987) and maximum parsimony methods (Nei and Kumar, 2000). Evolutionary distances matrices were generated as described by Jukes and Cantor (Jukes, 1990). The robustness of inferred tree topologies was evaluated after 1000 bootstrap resamplings of the neighbour- joining and maximum parsimony data.

2.3.11 DGGE Cluster analysis



For 16S rRNA-DGGE banding patterns, the software program AlphaEase FC version 4.2.0 (Alphalnnotech, San Leandro, CA, USA) was used to make a similarity matrix based on presence or absence of aligned bands from different lanes in a gel. Moreover, the distance matrix and cluster analysis was used Pearson Coefficient (Symonds, 1926) and UPGMA method (Prager and Wilson, 1978).

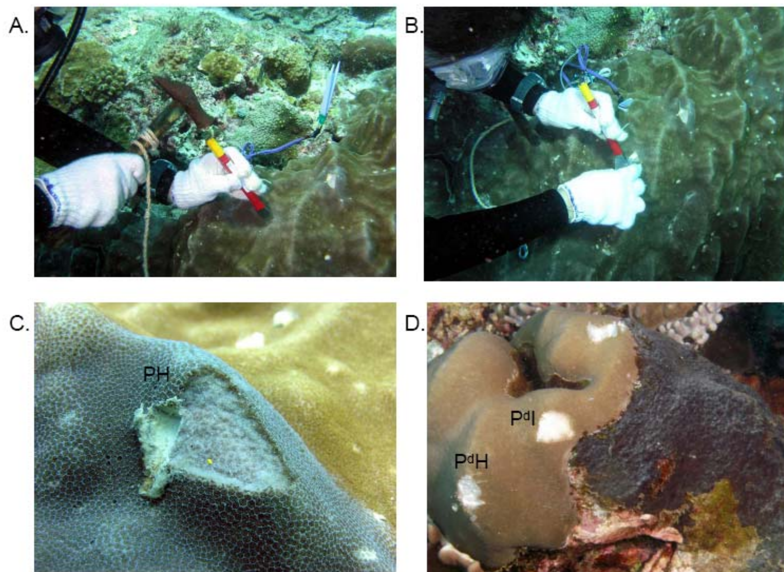


Fig. 11 *P. lutea* sampling procedure.

A.B. Using punch and hammer to collect coral samples. C. Non-sponge-infected *Porites lutea* (PH). D. Sponge-infected *Porites lutea*, two individual parts, far part (P^dH) (from an area of the coral colony away from the sponge region) and close part (P^dI) (from an area of the coral colony 5 cm near by sponge).

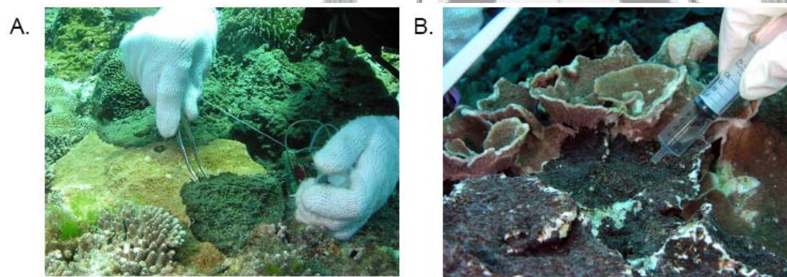


Fig. 12 *T. hoshinota* sampling procedure

Using a pair of forceps (A.) or a 30ml sterile syringe (B.)

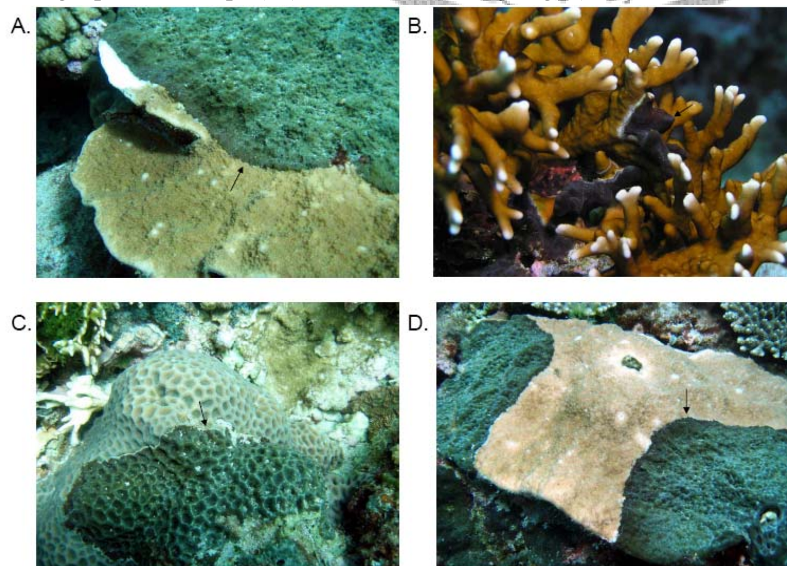


Fig. 13 *T. hoshinota* covering on four kinds of coral species.

A. *Montipora efflorescens* (MM), B. *Millepora sp.*(FM), C. *Favia complanata* (VM), D. *Isopora palifera* (AM).

3 Results

3.1 16S rRNA-DGGE analysis

DGGE is a convenient method to quickly demonstrate the distribution of bacterial community among samples. Therefore, using this method, we got the pictures of the variation of bacterial community associated with different parts of coral *Porites lutea* and the similarity of bacterial community associated with *Terpios hoshinota* covered on different coral species.

3.1.1 *P. lutea* associated bacteria communities changed when *T. hoshinota* covered

According to the DGGE banding distribution, the bacterial communities associated with non-sponge-infected (NSI) *P. lutea* (PH) were apparently different with sponge-infected (SI) *P. lutea* (P^dH, P^dI) and surrounding seawater (SW, KW) (Fig. 14). The bacterial communities associated with two parts of SI *P. lutea,* close-to sponge (P^dI) and far-away sponge (P^dH), clustered together and had very similar DGGE banding patterns. Moreover, the bacterial communities associated with SI *P. lutea* had similar DGGE banding patterns with surrounding seawater. The bacterial communities associated with *T. hoshinota* which infected *P. lutea* (PM) were unique with either coral or seawater samples.

3.1.2 *Gammaproteobacteria* were the major group in bacteria communities of seawater and *P. lutea*

Nineteen major DGGE bands were excised and sequenced from the gel of *P. lutea*

samples shown in Fig. 15. Most of sequences were belong to *Gammaproteobacteria*. *Vibrio sp*. was observed in Kun-Guan seawater and P^{d}H24 samples.

3.1.3 *T. hoshinota* associated bacterial community was specific

Bacterial communities associated with *T. hoshinota* infected different coral species clustered together and had similar DGGE patterns. Bacterial community was specific to sponge and no correlated with surrounding seawater (Fig. 16).

3.1.4 *Cyanobacteria* were the major group in *T. hoshinota* associated bacterial community

Thirty one DGGE bands were excised and sequenced from the gel of sponge samples shown in Fig. 17. There were two major bacteria groups (I, II) associated with *T. hoshinota*. In the first part, there were two major bands with similar location in both seawater and sponge but the sequences belonged to different bacteria groups. *Gammaproteobacteria* was observed in seawater samples and *Alphaproteobacteria* was observed in sponge samples. In the second part, two bands of seawater were observed as *Gammaproteobacteria* and three to five bands of sponge observed were *Cyanobacteria*. Large numbers of *Cyanobacteria* within *T. hoshinota* were observed. These DGGE result echoed the observation of the transmission electronic micrograph (See Section 3.4 Electron Microscopy of *T. hoshinota*).

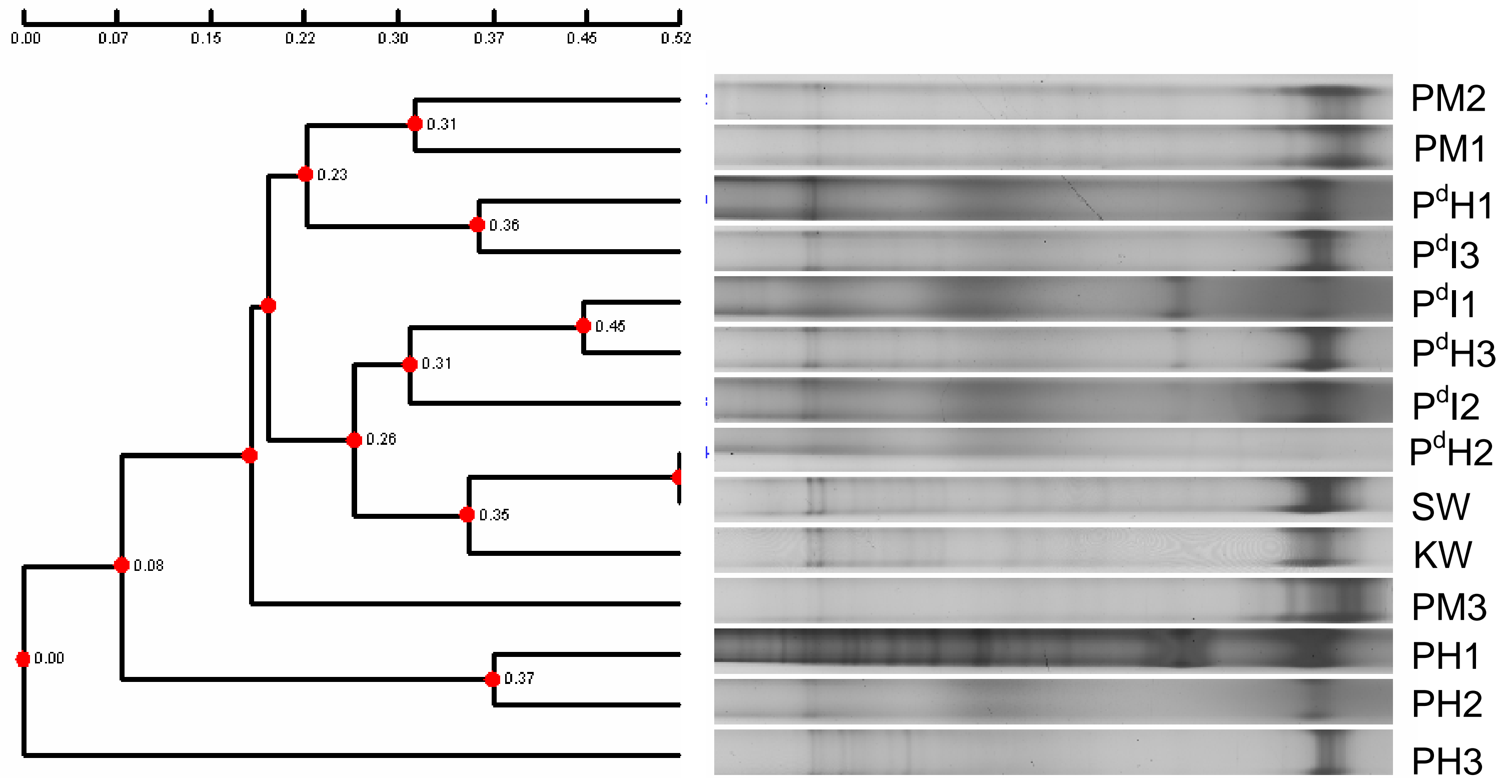


Fig. 14 Bacterial community composition (according to Fig. 15A 16S rRNA-DGGE gel and cluster diagram).
Sponge-infected *Porites lutea* colonies (P^dH: far-away sponge, P^dI: close-to sponge) and non-sponge-infected *Porites lutea* colonies (PH), sponges (PM) and seawater from Chai-Kou (SW) and Kun-Guan (KW).

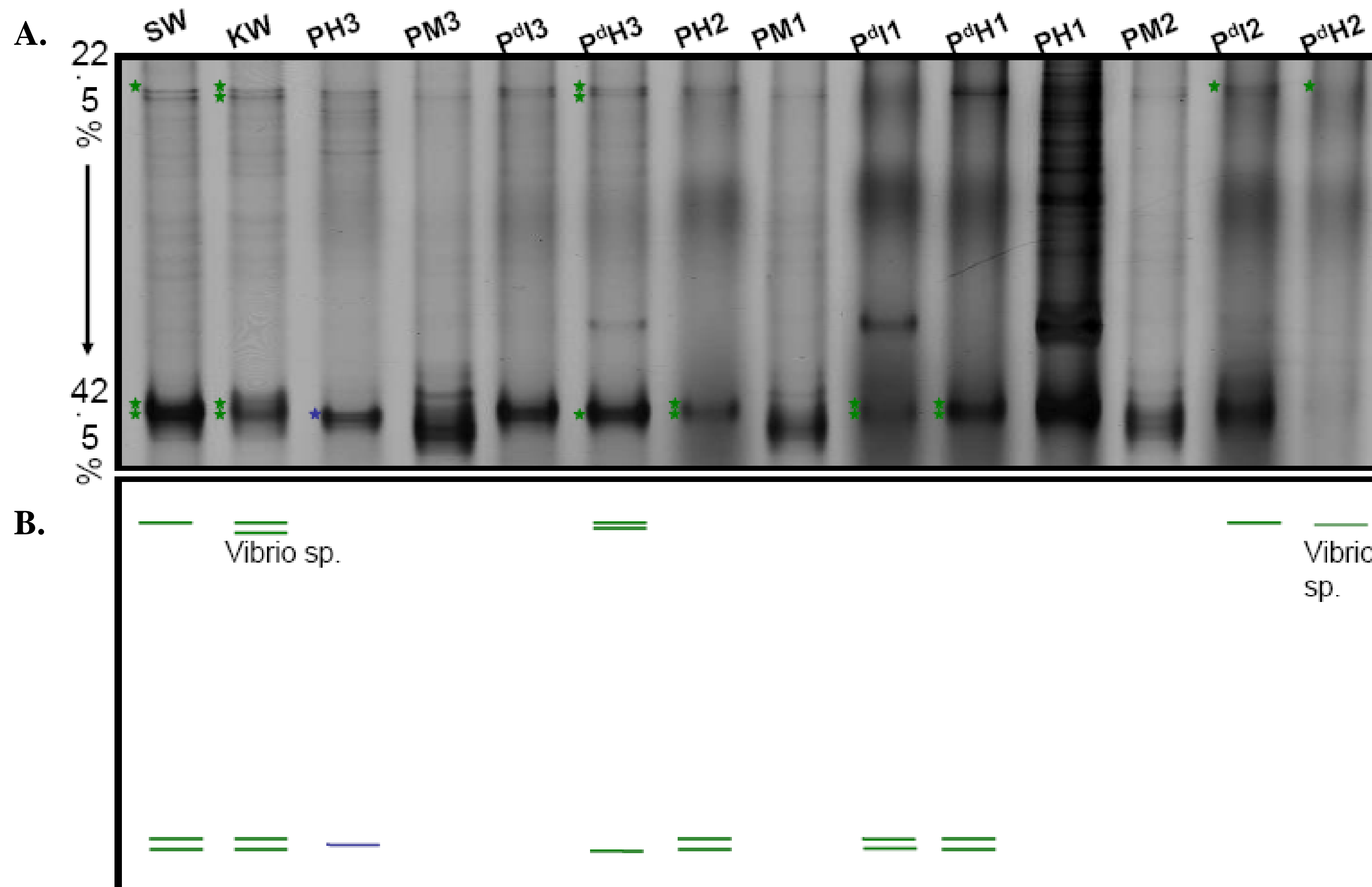


Fig. 15 Bacterial community composition on *Porites lutea*, *Terpios hoshinota* and seawater

SI (P^dH: far-away sponge, P^dI: close-to sponge) and NSI (PH) *Porites lutea* colonies, sponges (PM) and seawater from Chai-Kou (SW) and Kun-Guan (KW).

A. 16S rRNA DGGE gel. Asterisk indicate bands excised and the color indicate bacteria group. Green: *Gammaproteobacteria*, blue: *Alphaproteobacteria*.

B. The bacteria types and location of excised bands.

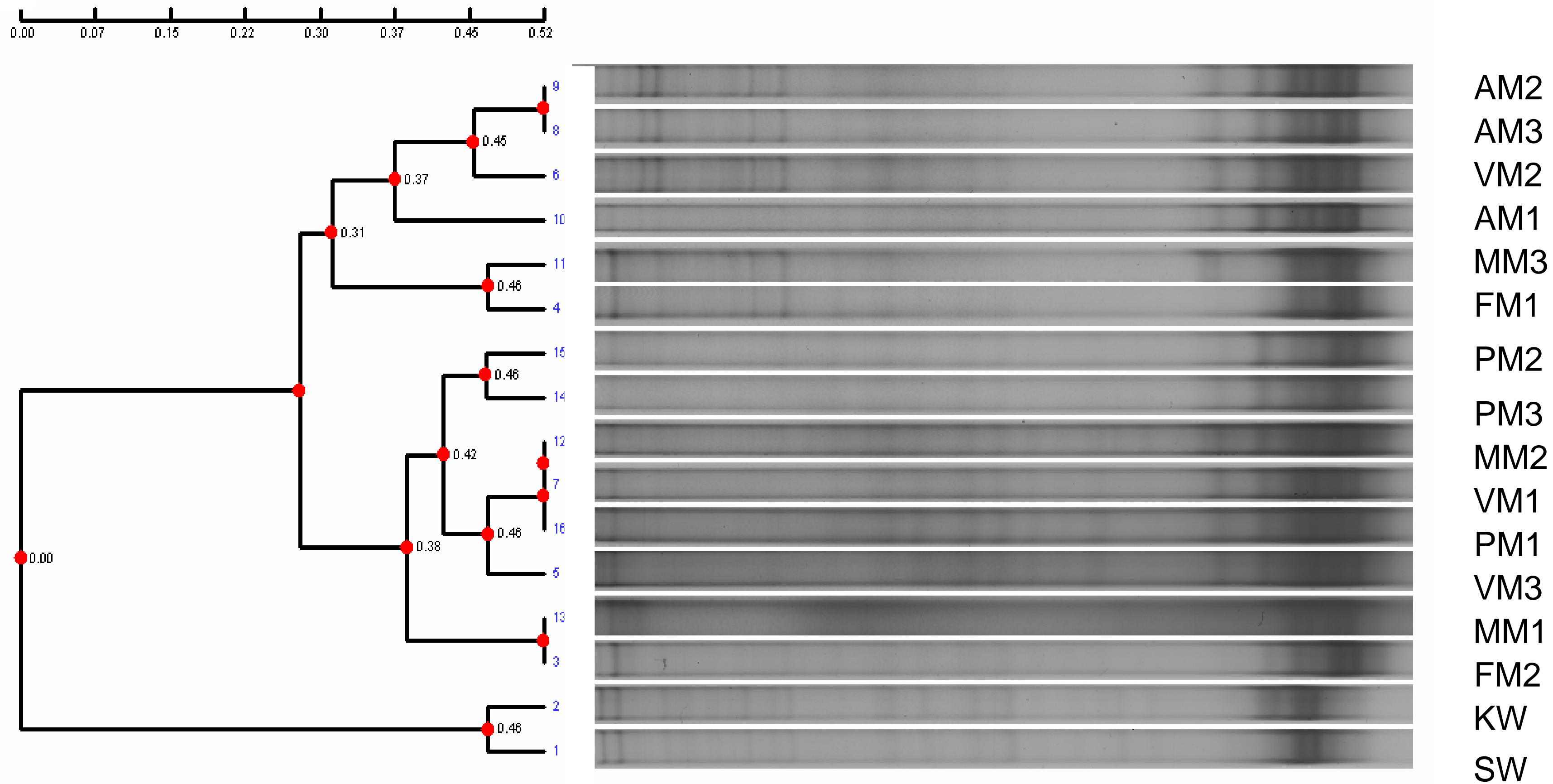


Fig. 16 Bacterial community composition (according to Fig. 17 16S rRNA-DGGE gel and cluster diagram)
Sponge-infected five different coral species (*Montipora efflorescens,* MM; *Millepora sp.*, FM; *Porites lutea*, PM; *Favia complanata,* VM; *Isopora palifra,* AM) and seawater from Chai-Kou (SW) and Kun-Guan (KW).

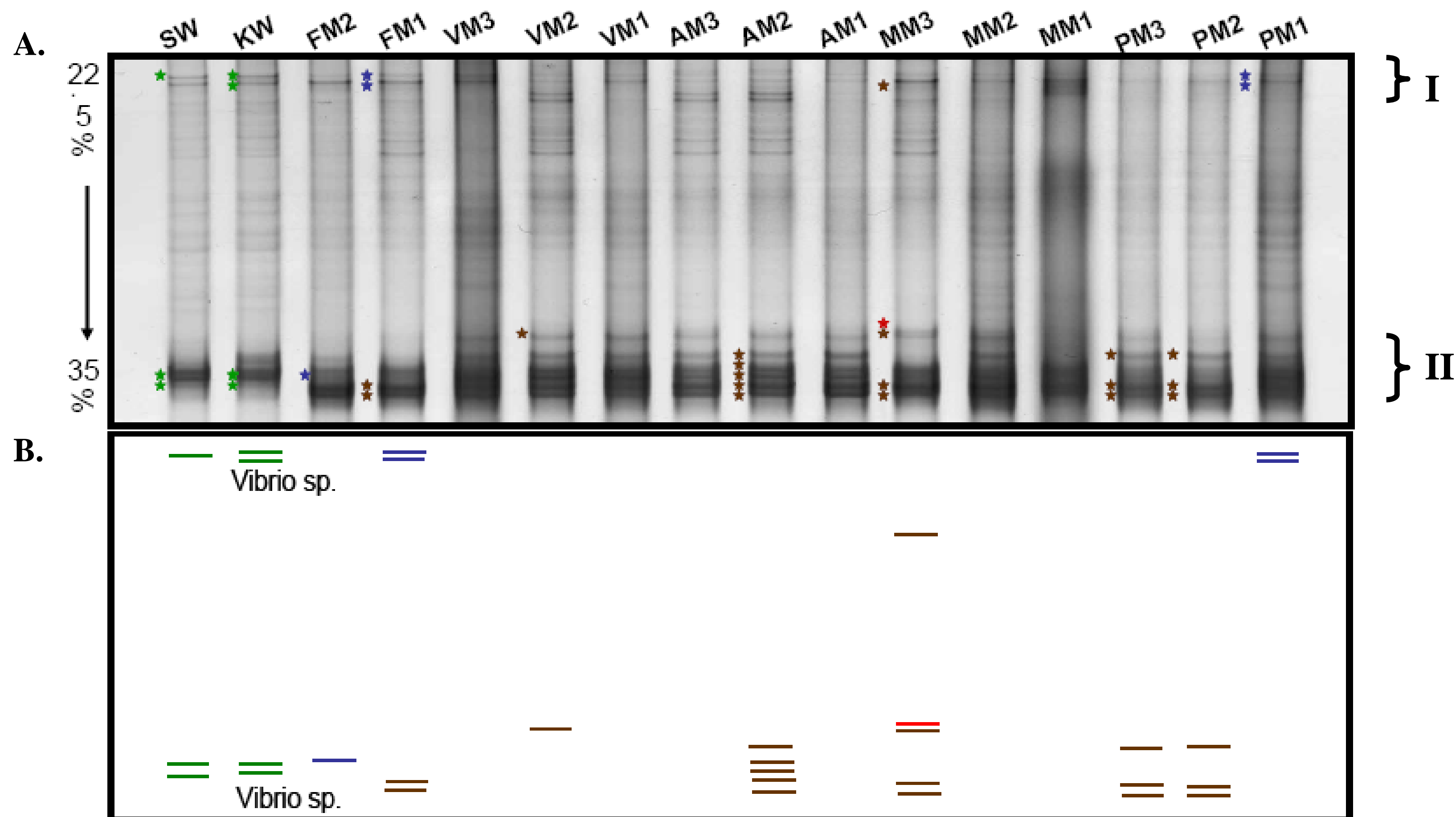


Fig. 17 Bacterial community composition in sponge-infected five different coral species (*Montipora efflorescens,* MM; *Millepora sp.*, FM; *Porites lutea*, PM; *Favia complanata,* VM; *Isopora palifra,* AM) and seawater from Chai-Kou (SW) and Kung-Guan (KW).

A. 16S rRNA DGGE gel. Asterisks indicate bands excised and the color indicate bacteria group. Green: *Gammaproteobacteria*, blue: *Alphaproteobacteria*, *brown: Cyanobacteria*, red: *Deltaproteobacteria*.

B. The bacteria types and location of excised bands.

3.2 16S rRNA library construction

3.2.1 Composition analysis of bacterial community

Molecular microbial composition analysis was performed on samples of seawater (SW, KW), NSI *P. lutea* colonies (PH), SI *P. lutea* colonies which divided two parts, close-to sponge (P^dI) and far-away sponge (P^dH) using Ribosomal Database Project II (RDP II). Moreover, to examine if there is any variation in the bacterial associates in *T. hoshinota,* we also compared the sponge samples collected from five different sponge-infected coral species (PM, MM, AM, VM, FM). The results were as below.

3.2.1.1 *Gammaproteobacteria* was the major group in seawater samples

Overall, *Gammaproteobacteria* (43%) were dominated among 46 clones library of Chai-Kou (SW) sample, followed by *Alphaproteobacteria* (17%) and *Cyanobacteria* (11%) (Fig. 18A). 47 clones library of Kun-Guan water (KW) sample was similar distribution as Chai-Kou water sample. The most common groups in KW were *Gammaproteobacteria* (57%), followed by *Alphaproteobacteria* (15%) and *Cyanobacteria* (13%) *Flavobacteria* (9%) (Fig. 18B).

3.2.1.2 Bacterial communities associated with *P. lutea* changed when *T. hoshinota* covered

The abundance of different bacterial groups varied substantially between SI and NSI *P. lutea* colonies. In total, 93 clones were obtained in NSI *P. lutea* (PH); 36% of the clones were clustered within the *Cyanobacteria*, 30% within the *Gammaproteobacteria*, 24% within the unclassified bacteria group (Fig. 18C).

However, among SI *P. lutea* colonies, close-to sponge ($P^{d}I$) and far-away sponge ($P^{d}H$), there were different compositions in clone libraries. For $P^{d}H$, 93 clones were sequenced and subjected to phylogenetic analysis. In general, 65% of the clones clustered within the *Gammaproteobacteria*, followed by unclassified bacteria (15%) and *Cyanobacteria* (10%) (Fig. 18D). For $P^{d}I$, 93 clones were obtained. The major groups were *Gammaproteobacteria* (67%), *Alphaproteobacteria* (18%) and *Cyanobacteria* (7%). Unclassified bacteria only were 1% in $P^{d}I$ (Fig. 18E).

DNA sequencing of microbial 16S rRNA of *P. lutea* indicated a shift in the bacterial population correlated with sponge infected. The abundance of *Cyanobacteria* and unclassified bacteria decreased and *Gammaproteobacteria* increased as *T. hoshinota* infected *P. lutea* (Fig. 20). Bacterial community of $P^{d}I$ was similar with seawater; however, *Gammaproteobacteria* of Chai-Kou seawater belonged to *Alteromonadales* but *Gammaproteobacteria* of $P^{d}I$ were unclassified *Oceanospirillales* (Table 4).

3.2.1.3 *Cyanobacteria* were the major group in the sponge

The bacterial community associated with sponge was consistence regardless of the sponge collected from different coral species (Fig. 19). Moreover, the bacterial community associated with sponge was unique to surrounding seawater (Fig. 20). Among two hundred eighteen sequences, the most abundant group was *Cyanobacteria* (78%), followed by *Alphaproteobacteria* (13%) and *Gammaproteobacteria* (8%). The *Alphaproteobacteria* and *Gammaproteobacteria* group associated with sponge found in surrounding seawater and coral that may be not symbiotic with sponge (Table 4).

Table 4 The bacteria taxonomy in samples based on 16S rRNA bacteria clone libraries using RDP II analysis.

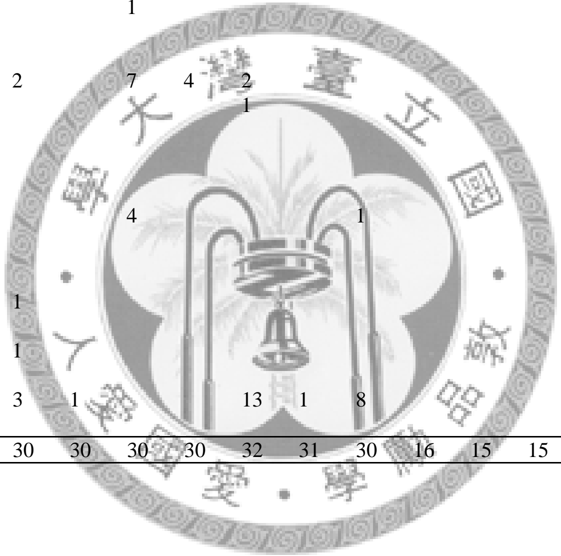
Phylum / Class / Order / Family	SW	KW	P^dH1	P^dH2	P^dH3	P^dI1	P^dI2	P^dI3	PH1	PH2	PH3	AM1	AM3	AM2	FM1	FM2	MM1	MM2	MM3	PM1	PM2	PM3	VM1	VM2	VM3
Actinobacteria																									
Actinobacteria																									
Acidimicrobiales																									
Acidimicrobiaceae											1														
Unclassified *Actinobacteria*								1																	
Firmicutes																									
Unclassified Firmicutes											2														
Cyanobacteria																									
Cyanobacteria																									
Prochlorococcus	5	2		2						1															
Unclassified *Cyanobacteria*		4		6	1		4	2	14	2	16	15	15	15	14	12	14	8	12	8	15	13	7	10	11
Acidobacteria																									
Acidobacteria																									
Acidobacteriales																									
Acidobacteriaceae											1														
Bacteroidetes																									
Sphingobacteria																									
Sphingobacteriales																									
Flexibacteraceae	1						1	1	2																
Flavobacteria																									
Flavobacteriales																									
Cryomorphaceae	2	1			2			2																	
Flavobacteriaceae	2	3																				1			1
Unclassified *Bacteroidetes*	3	1	3		1			1																1	

Table 4. (continued)

Phylum / Class / Order / Family	SW	KW	P^dH1	P^dH2	P^dH3	P^dI1	P^dI2	P^dI3	PH1	PH2	PH3	AM1	AM3	AM2	FM1	FM2	MM1	MM2	MM3	PM1	PM2	PM3	VM1	VM2	VM3
Lentisphaerae																									
Lentisphaerae																									
Lentisphaerales																									
Lentisphaeraceae							1																		
Proteobacteria																									
Gammaproteobacteria																									
Alteromonadales																									
Colwelliaceae		1																							
Pseudoalteromonadaceae		1																							
Incertae sedis		3																							
Alteromonadaceae	20	4																	1	1					
Chromatiales																									
unclassified *Chromatiales*							1																		
Thiotrichales																									
Thiotrichaceae																								1	
Piscirickettsiaceae							1																		
Vibrionales																									
Vibrionaceae		4																							
Oceanospirillales																									
Oceanospirillaceae		4																							
Hahellaceae			1				4	5									1								
Unclassified *Oceanospirillales*		1	23	12	12	29	6	11		25	1	1						1	1				1		1
Pseudomonadales																									
Pseudomonadaceae		2																							
Moraxellaceae				1																					
Unclassified *Gammaproteobacteria*		7		4	7			3		2								3				1	3	1	

Table 4. (continued)

Phylum / Class / Order / Family	SW	KW	P^{d}H1	P^{d}H2	P^{d}H3	P^{d}I1	P^{d}I2	P^{d}I3	PH1	PH2	PH3	AM1	AM3	AM2	FM1	FM2	MM1	MM2	MM3	PM1	PM2	PM3	VM1	VM2	VM3
Alphaproteobacteria																									
Parvularculales																									
Parvularculaceae																							1		
Rhizobiales																									
Hyphomicrobiaceae							1																		
unclassified *Rhizobiales*																				1					
Rhodobacterales																									
Rhodobacteraceae	5	6			2		7	4	2						1			4		5			3	3	2
Rhodospirillales									1																
Rhodospirillaceae																									
Sphingomonadales																									
Sphingomonadaceae	2																								
Unclassified *Alphaproteobacteria*	1	1					4				1				1	4		1	2						
Deltaproteobacteria																									
Myxococcales																									
Polyangiaceae					1																				
Bdellovibrionales																									
Bacteriovoracaceae					1																				
Uclassified *proteobacteria*	2	1																							
Unclassified bacteria	3	1	3	8	3	1			13	1	8									1					
total sequences	46	47	30	33	30	30	30	30	32	31	30	16	15	15	16	16	15	17	16	16	15	15	15	16	15



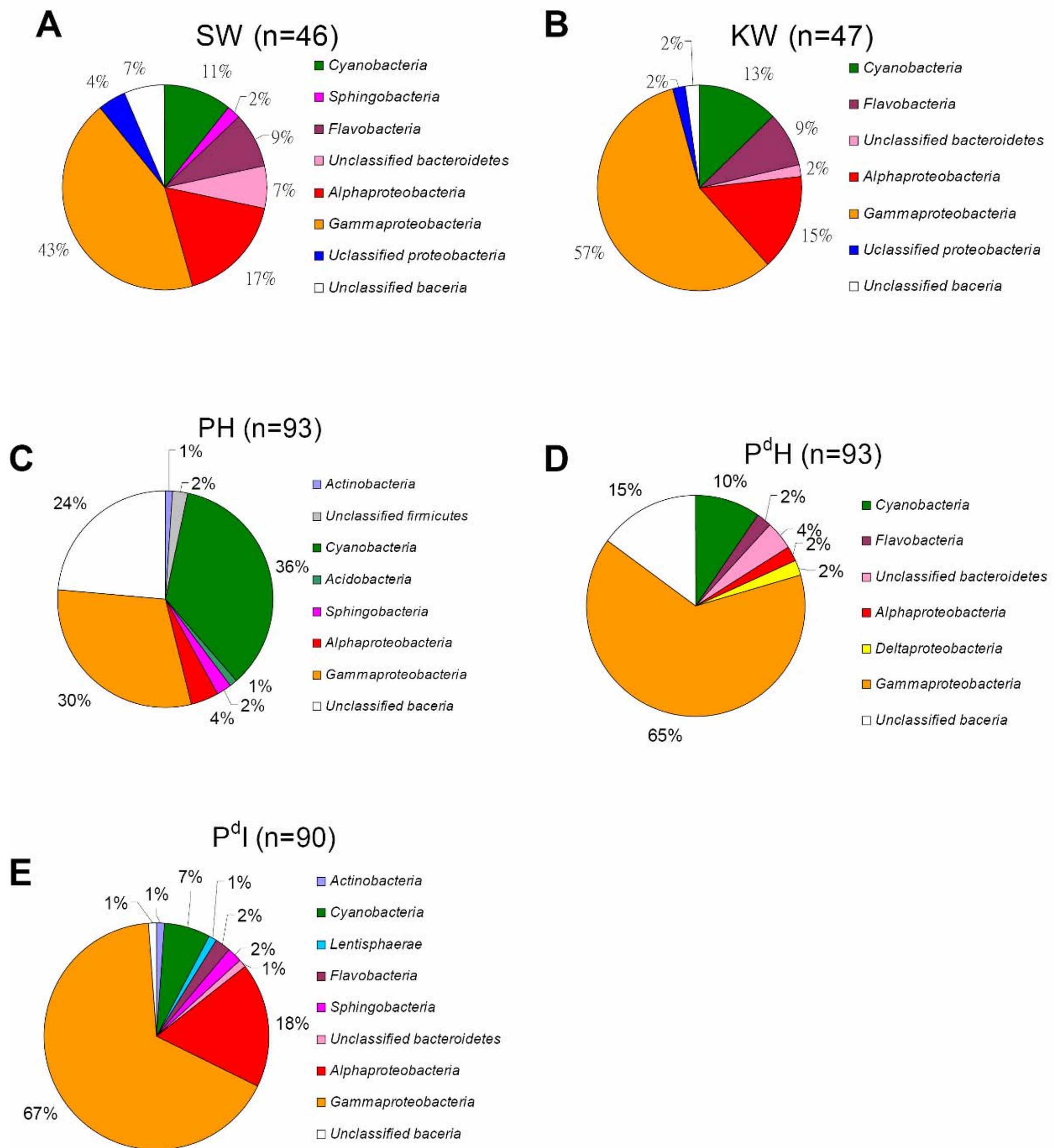


Fig. 18 Pie charts illustrating the diversity of bacterial groups on *Porites lutea* and seawater
Based on partial 16S rRNA gene sequences derived from clone libraries using RDP II analysis (n, clone numbers). A. SW, Chai-Kou sample. B. KW, Kun-Guan sample. C. PH, non-sponge-infected *Porites lutea* colonies. D. P^dH, sponge-infected *Porites lutea* colonies (far-away sponge). E. P^dI: sponge-infected *Porites lutea* colonies (close-to sponge).

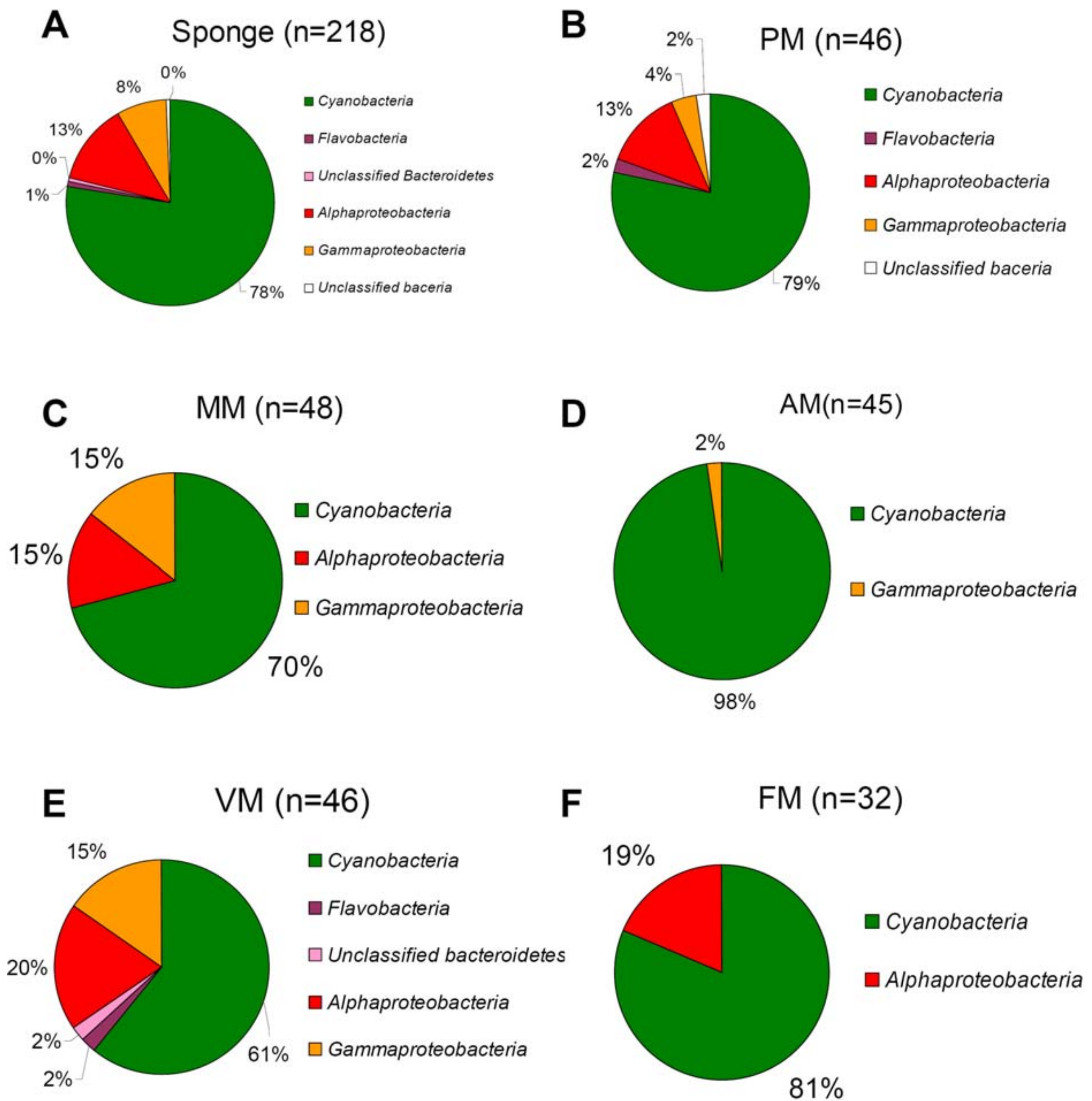
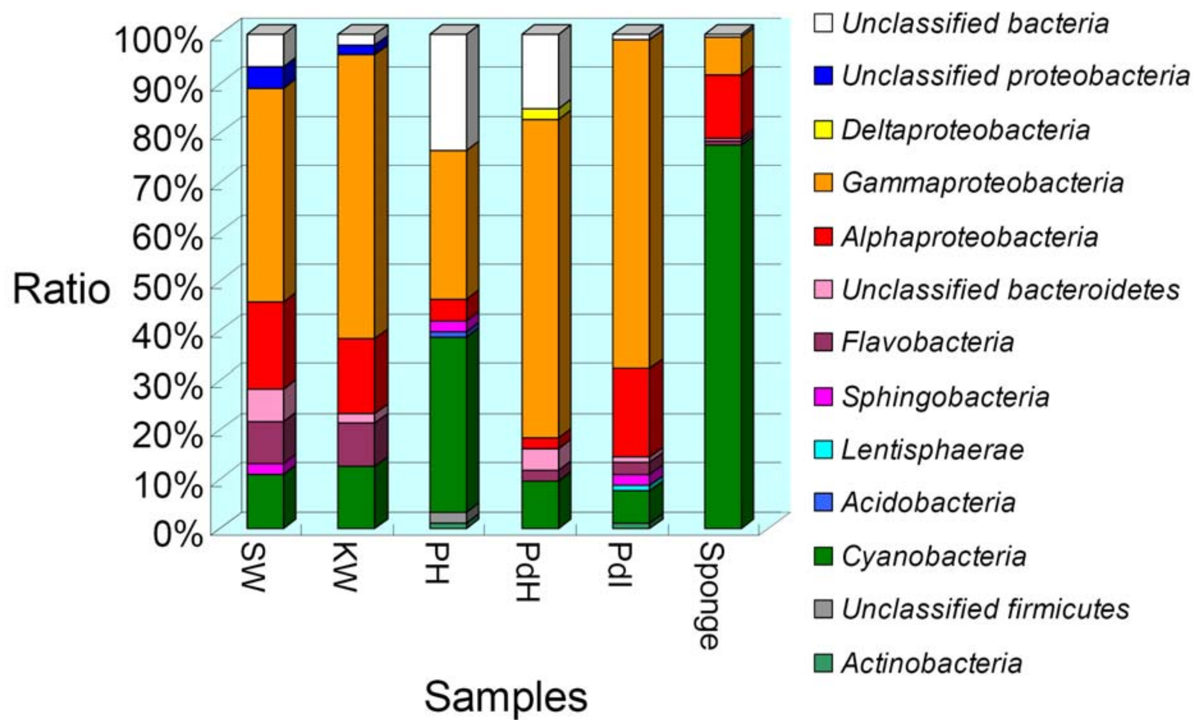


Fig. 19 Pie charts illustrating the diversity of bacterial groups on *Terpios hoshinota*
Based on partial 16S rRNA gene sequences derived from clone libraries using RDP II analysis (n, clone numbers). A. sponge, all sponge samples collected from five coral species. B. PM, *Porites lutea.* C. MM, *Montipora efflorescens.* D. AM, *Isopora palifera.* E. VM, *Favia complanata.* F. FM; *Millepora sp.*



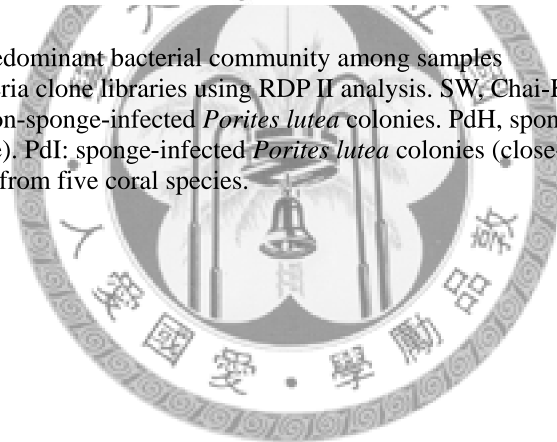


Fig. 20 Comparison of predominant bacterial community among samples
Based on 16S rRNA bacteria clone libraries using RDP II analysis. SW, Chai-Kou sample. KW, Kun-Guan sample. PH, non-sponge-infected *Porites lutea* colonies. PdH, sponge-infected *Porites lutea* colonies (far-away sponge). PdI: sponge-infected *Porites lutea* colonies (close-to sponge). Sponge, all sponge samples collected from five coral species.

3.2.2 Diversity analysis of bacterial 16S rRNA clone libraries

KW sample contained the highest number of operational taxonomic unit (OTU) groups. A total of 47 clones constituting 33 distinct OTU groupings were analyzed from KW samples, representing potential high bacteria diversity. SW library also had a high number of OTU groups, 17 distinct OTUs from 46 clones.

Both seawater samples did not reach to flatten towards an asymptote in rarefaction curve (Fig. 21). Rarefaction curve analysis statistically evaluated if screening of these clones provided a sufficient estimate of total diversity within each sample. The calculated rarefaction curves appeared to start to approach saturation for SW clone library but KW. Therefore, analysis of an increasing number of clones in both seawater samples would have revealed further diversity.

To realize the abundance and richness of the bacterial communities of each sample, we used the Shannon-Weaver index to quantify species diversity and we also calculated Chao1 index to estimate total species richness (Table 5). All indices revealed that both seawater samples clone libraries were diverse. However, the clone library of KW was more diverse than SW.

3.2.2.1 Higher diversity of bacteria community associated with SI *Porites lutea* (close-to sponge part, P^dI)

On NSI *P. lutea* colonies, the diversity of bacteria clone libraries PH1 and PH3 were higher than PH2, containing 3 OTUs from 31 clones. For PH1 sample, total 32 clones library contained 8 OTUs and 30 clones constituting 9 OTUs was analyzed in PH3 library (Table 5).

On SI *P. lutea* colonies, the diversity of bacteria clone libraries P^dI2 (close-to sponge)

was higher than P^dH2 (far-away sponge). However, the diversity of colonies P^dH1 and P^dI1, P^dH3 and P^dI3 had no statistically difference (Table 5).

Comparing diversity among the PH, P^dH and P^dI libraries, P^dI2, P^dI3 had higher diversity than PH1, PH2 and PH3. They also had higher diversity than P^dH1, P^dH2. The variation of diversity indices of bacteria communities among different parts of *P. lutea* may be linked to different environmental pressure (sponge infecting). Most of samples from *P. lutea* libraries approached saturation in calculated rarefaction curves except P^dI2, P^dI3 and P^dH3 (Fig. 21). It also demonstrated higher diversity of the P^dI samples though a larger number of clones require to be analyzed within the P^dI2, P^dI3 and P^dH3 libraries to present the actual diversity within these environments.

3.2.2.2 Low diversity of bacterial community associated with *T. hoshinota*

The diversity of 16S rRNA clone libraries of *T. hoshinota,* covering on different coral species, was simple (Table 6). All diversity indices calculated from most of samples were low. 15 clones constituting 1 OTU from AM3 was lowest diversity library. Most of libraries constructed by 15 to 17 sequences could represent the precision bacterial community associated with sponge confirmed by rarefaction curves which reach a clear saturation (Fig. 22). However, PM1, VM1 and VM2 had higher values in rarefaction curves, and PM1, VM1 samples also had higher distinct OTUs in clone libraries.

Table 5 Diversity indices for the bacterial communities (*Porites lutea* and seawater) as represented in the 16S rRNA gene libraries[a].

Index	Sponge-infected Porites						Non-sponge-infected Porites			Seawater	
	P^{d}H1	P^{d}I1	P^{d}H2	P^{d}I2	P^{d}H3	P^{d}I3	PH1	PH2	PH3	KW	SW
S[b]	4	2	5	16	13	13	8	3	9	33	17
N[c]	30	30	33	30	30	30	32	31	30	47	46
Evenness[d]	0.56	0.20	0.74	0.87	0.18	0.83	0.64	0.41	0.73	0.97	0.76
Richness[e]	2.03	0.68	2.63	10.15	8.12	8.12	4.65	1.34	5.42	19.14	9.62
Shannon	0.77	0.14	1.19	2.42	0.45	2.13	1.34	0.45	1.6	3.38	2.15
Chao 1[f]	4(4-4)	2(2-2)	6(5-18)	34(20-91)	31(16-94)	28(16-77)	23(11-75)	3(3-3)	24(12-77)	64(44-122)	26(19-55)
Simpson	0.5954	0.9333	0.3346	0.1011	0.1425	0.2804	0.3467	0.7612	0.2643	0.0175	0.2

[a]Calculations were based on OTUs formed at an evolutionary distance of <0.03 (or about 97% similarity).

[b]S defined as the number of OTUs.

[c]N defined as the number of sequences.

[d]Evenness defined as Shannon / Ln(the number of OTUs)

[e]Richness = (number of singleton OTUs-1)/logN. The maximum value is (N-1)/logN

[f]Confidence intervals for the Chao 1 estimator are shown in parenthesis.

Table 6 Diversity indices for the bacterial communities (sponge) as represented in the 16S rRNA gene libraries[a].

Index	Porites			Montipora			Isopora			Favia			Millepora	
	PM1	PM2	PM3	MM1	MM2	MM3	AM1	AM2	AM3	VM1	VM2	VM3	FM1	FM2
S[b]	8	2	3	2	5	7	5	3	1	7	5	4	3	4
N[c]	16	15	15	15	17	16	16	15	15	15	16	15	16	16
Evenness[d]	0.84	0.72	0.44	0.35	0.83	0.75	0.85	0.44	0.00	0.77	0.70	0.61	0.42	0.59
Richness[e]	5.81	0.85	1.70	0.85	3.25	4.98	3.32	1.70	0.00	5.10	3.32	2.55	1.66	2.49
Shannon	1.75	0.5	0.48	0.24	1.33	1.45	1.36	0.48	0	1.5	1.12	0.85	0.46	0.82
Chao 1	23(11-75)	2(2-2)	4(3-15)	2(2-2)	6(5-18)	12(7-39)	6(5-18)	4(3-15)	1(1-1)	12(7-39)	8(5-29)	4.5(4-12)	4(3-15)	4.5(4-12)
Simpson	0.175	0.6571	0.7428	0.8666	0.272	0.3083	0.25	0.7428	1	0.2761	0.3083	0.5333	0.7583	0.5583

[a]Calculations were based on OTUs formed at an evolutionary distance of <0.01 (or about 99% similarity).

[b]S defined as the number of OTUs.

[c]N defined as the number of sequences.

[d]Evenness defined as Shannon / Ln(the number of OTUs)

[e]Richness = (number of singleton OTUs-1)/logN. The maximum value is (N-1)/logN

[f]Confidence intervals for the Chao 1 estimator are shown in parenthesis.

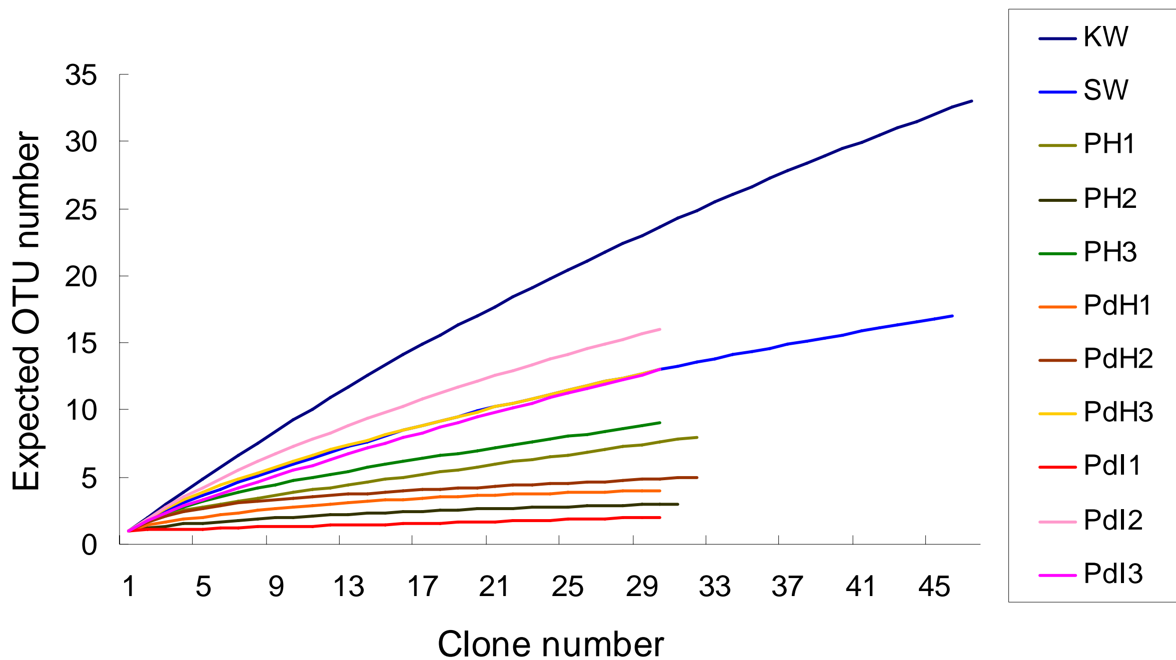


Fig. 21 Rarefaction curves for the 16S rRNA bacteria clone libraries (*Porites lutea* and seawater). SW, Chai-Kou sample. KW, Kun-Guan sample. PH1, 2, 3, non-sponge-infected *Porites lutea* colonies. P[d]H1, 2, 3, sponge-infected *Porites lutea* colonies (far-away sponge). P[d]I1, 2, 3, sponge-infected *Porites lutea* colonies (close-to sponge).

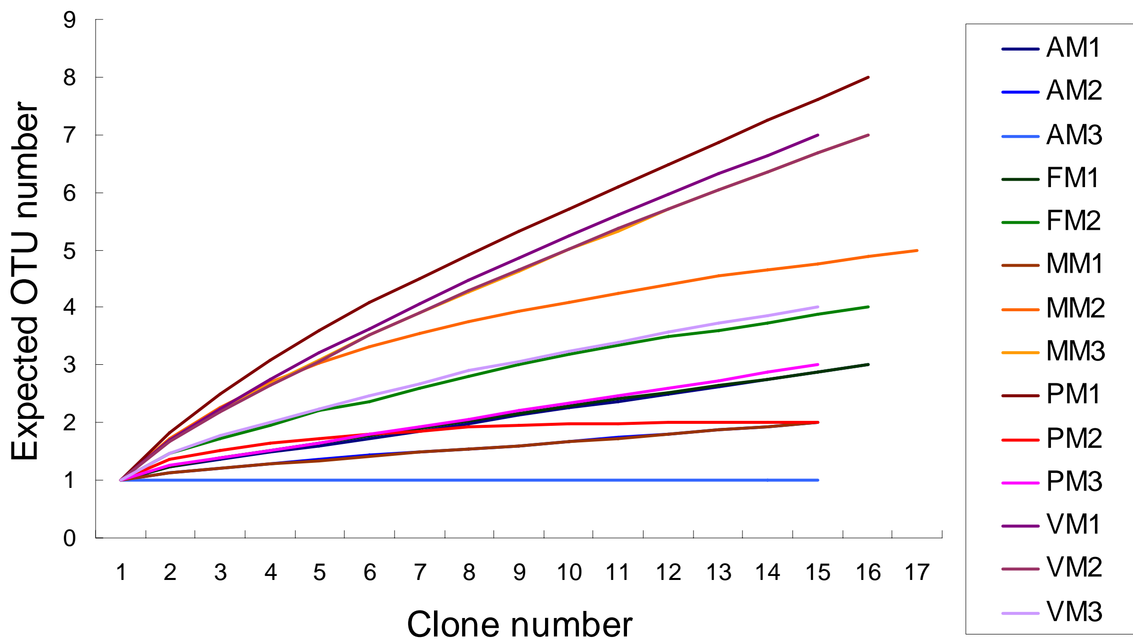
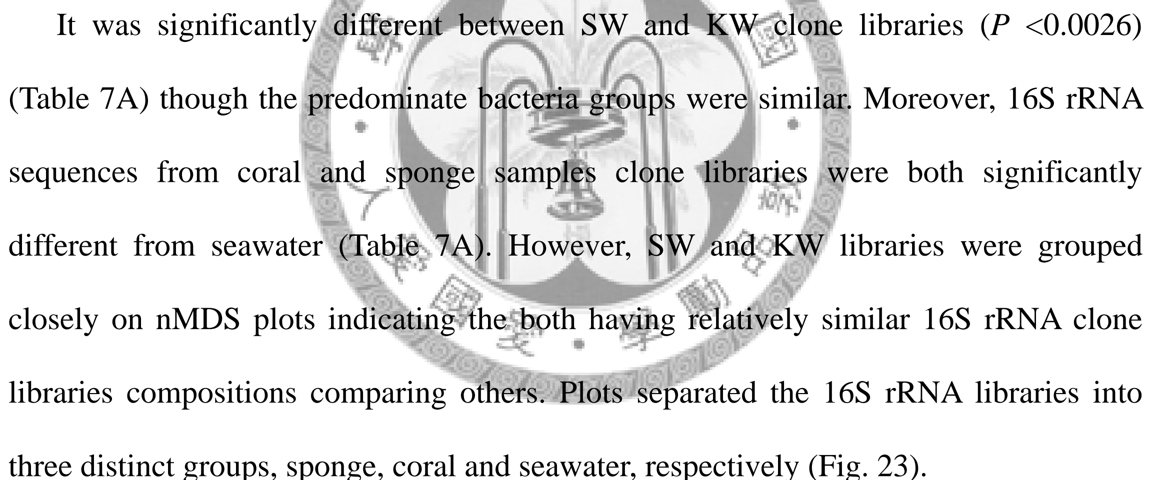


Fig. 22 Rarefaction curves for the 16S rRNA bacteria clone libraries (*Terpios hoshinota* collected from five coral species)
AM1, 2, 3, *Isopora palifera* colonies. FM1, 2, *Millepora sp.* colonies. MM1, 2, 3, *Montipora efflorescens* colonies. PM1, 2, 3, *Porites lutea* colonies. VM1, 2, 3, *Favia complanata* colonies.

3.2.3 Comparing different bacterial communities

We used LIBSHUFF to statistically analyze differences in two libraries of 16S rRNA gene sequences. Non-metric multidimensional scaling ordination was also employed to illustrate differences of bacterial communities on a 2-D scatter plot. On a nMDS plot, samples possessing the highest similarity in terms of community composition are grouped together.

3.2.3.1 Different composition between Chai-Kou and Kun-Guan seawater clone libraries



It was significantly different between SW and KW clone libraries ($P < 0.0026$) (Table 7A) though the predominate bacteria groups were similar. Moreover, 16S rRNA sequences from coral and sponge samples clone libraries were both significantly different from seawater (Table 7A). However, SW and KW libraries were grouped closely on nMDS plots indicating the both having relatively similar 16S rRNA clone libraries compositions comparing others. Plots separated the 16S rRNA libraries into three distinct groups, sponge, coral and seawater, respectively (Fig. 23).

3.2.3.2 Different composition between SI and NSI *P. lutea* clone libraries

Differences in 16S rRNA sequences between PH (93 sequences) and $P^{d}H$ (93 sequences) clone libraries and also between PH (93 sequences) and $P^{d}I$ (90 sequences) clone libraries are statisifically ($P < 0.0026$) significant as shown in Table 7A. However, it was not significantly different between $P^{d}H$ and $P^{d}I$ clone libraries ($P = 0.288$). Therefore, there was a distinct bacterial community existed in the PH samples. On the

nMDS plot, all the samples libraries from *P. lutea* colonies were grouped together, but PH libraries were far from P^dH and P^dI (Fig. 23), confirming the difference in 16S rRNA sequences libraries between PH and other *P. lutea* samples (P^dH and P^dI).

There was significant difference in bacteria 16S rRNA clone libraries among each NSI *P. lutea* colony (PH1, PH2, PH3) (Table 7B), indicating the bacterial community associated with the same coral species may be not consistence. We also observed that there was significant difference in each SI *P. lutea* colony (Table 7C, D).

Comparing 16S rRNA clone libraries compositions of close-to sponge part and far-away sponge part sampled from the same *P. lutea* colony, there were no significant difference in P^dH1 and P^dI1, $P^dH3$and P^dI3 but P^dH2 and P^dI2 (Table 7E).

3.2.3.3 Similar bacterial communities associated with *T. hoshinota* covered on five different coral species

There was almost no significant difference in all bacteria 16S rRNA sequence libraries among sponge samples collected from different coral species (Table 8A).

Among the sponge samples covered on the same coral species, there is no significant difference but *Protis lutea* (PM1 and PM2) and *Montipora efflorescens* colonies (MM1 and MM2) (Table 8B.).

The differences in bacterial community structure from sponge samples covered on different coral species inferred from nMDS analysis were showing identical bacteria groups associated with the sponge (Fig. 24).

Table 7 Difference in bacteria 16S rRNA clone libraries composition using LIBSHUFF analysis.
A. Similarity between *Porites lutea* (P^dH, P^dI, PH) and seawater (SW, KW).
B. Similarity among non-sponge-infected *Porites lutea* colonies (PH1, 2, 3).
C. Similarity among sponge-infected *Porites lutea* colonies (far-away sponge) (P^dH1, 2, 3).
D. Similarity among sponge-infected *Porites lutea* colonies (close-to sponge) (P^dI1, 2, 3).
E. Similarity between P^dH and P^dI from the same sponge-infected *Porites lutea* colony.

A.

	Y library				
X library	P^dH	P^dI	PH	KW	SW
P^dH	-	0.288	0.009	0.001*	0.001*
P^dI	0.05	-	0.012	0.001*	0.001*
PH	0.001*	0.001*	-	0.001*	0.001*
KW	0.575	1	0.001*	-	0.001*
SW	0.001*	0.001*	0.001*	0.098	-

P<0.0026

B.

	Y library		
X library	PH1	PH2	PH3
PH1	-	0.001*	0.001*
PH2	0.001*	-	0.162
PH3	0.001*	0.001*	-

p<0.0085

C.

	Y library		
X library	P^dH1	P^dH2	P^dH3
P^dH1	-	0.038	0.063
P^dH2	0.004*	-	0.012
P^dH3	0.007*	0.001*	-

p<0.0085

D.

	Y library		
X library	P^dI1	P^dI2	P^dI3
P^dI1	-	0.416	0.657
P^dI2	0.001*	-	0.03
P^dI3	0.001*	0.505	-

p<0.0085

E.

	Y library		
X library	P^dI1	P^dI2	P^dI3
P^dH1	0.094	-	-
P^dH2	-	0.001*	-
P^dH3	-	-	0.089

P<0.025

	Y library	
X library	P^dH1	P^dH2
P^dI1	0.37	-
P^dI2	-	0.002*
P^dI3	-	-

P<0.025

Table 8 Difference in bacteria 16S rRNA clone libraries composition using LIBSHUFF analysis.

A. Similarity among sponge samples infected different coral species (PM, MM, AM, VM, FM) and seawater (SW, KW).

	Y library						
X library	PM	MM	AM	VM	FM	KW	SW
PM	-	0.606	0.775	0.675	0.127	0.001*	0.001*
MM	0.042	-	0.732	0.014	0.692	0.001*	0.001*
AM	0.004	0.001*	-	0.707	0.382	0.001*	0.001*
VM	0.127	0.264	0.001*	-	0.117	0.001*	0.001*
FM	0.441	0.009	0.007	0.445	-	0.001*	0.001*
KW	0.001*	0.004	0.001*	0.001*	0.001*	-	0.001*
SW	0.001*	0.001*	0.001*	0.001*	0.001*	0.098	-

*P<0.0012

B. Similarity among sponge samples infected the same coral species but different colonies

	Y library		
X library	PM1	PM2	PM3
PM1	-	0.001*	0.008*
PM2	0.958	-	0.324
PM3	0.543	1	-

p<0.0085

	Y library	
X library	FM1	FM2
FM1	-	0.341
FM2	0.794	-

p<0.0085

	Y library		
X library	MM1	MM2	MM3
MM1	-	0.708	0.861
MM2	0.004*	-	0.031
MM3	0.508	0.794	-

p<0.0085

	Y library		
X library	AM1	AM2	AM3
AM1	-	0.028	0.902
AM2	0.337	-	1
AM3	0.365	1	-

p<0.0085

	Y library		
X library	VM1	VM2	VM3
VM1	-	0.094	0.796
VM2	0.864	-	0.473
VM3	0.457	0.591	-

p<0.0085

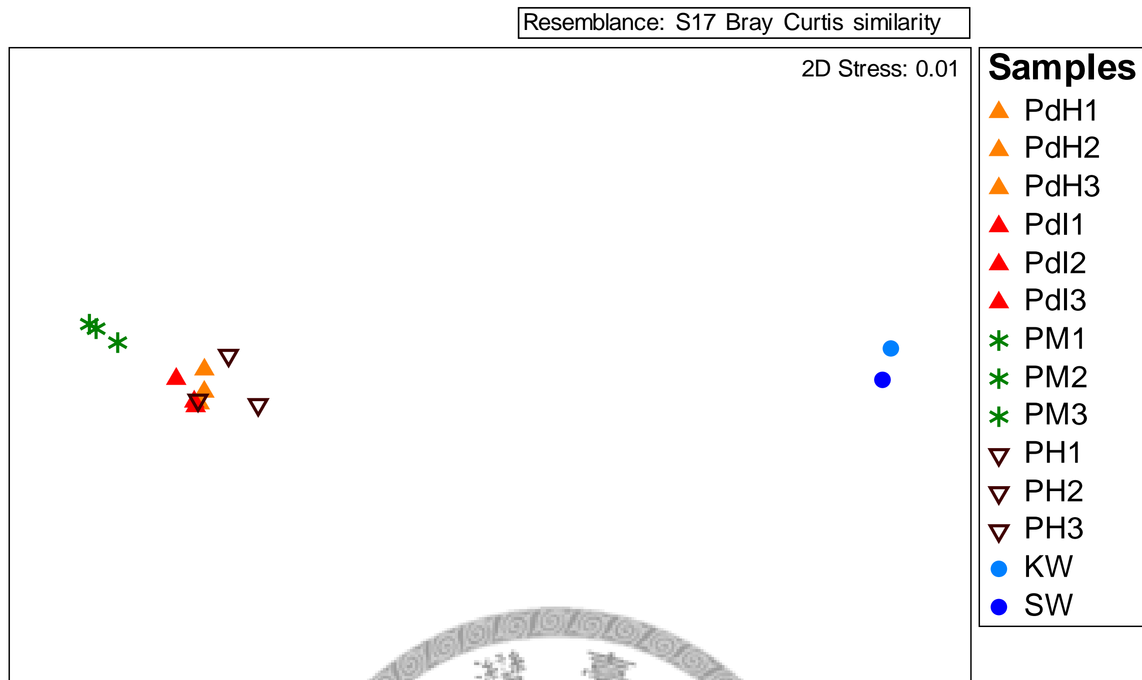


Fig. 23 Non-metric multidimensional scaling plot (nMDS-2 dimensional) of *Porites lutea,* Seawater Representation of the 16S rRNA partial sequences of the seawater (SW, Chai-Kou sample, KW, Kun-Guan), non sponge-infected *Porites lutea* colonies (PH1, 2, 3), sponge-infected *Porites lutea* colonies (far-away sponge) (PdH1, 2, 3), sponge-infected *Porites lutea* colonies (close-to sponge) (PdI1, 2, 3) and sponge collected from *Porites lutea* colonies (PM1, 2, 3)

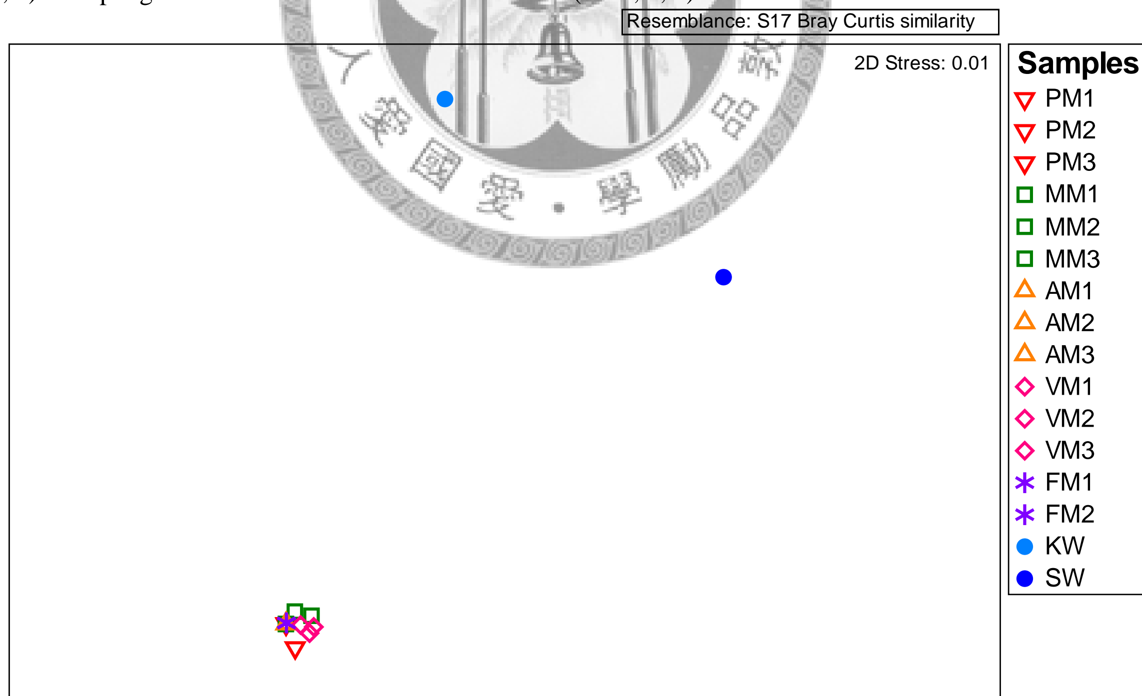


Fig. 24 Non-metric multidimensional scaling plot (nMDS-2 dimensional) of *Terpios hoshinota* Representation of the bacteria 16S rRNA partial sequences of *Terpios hoshinota* collected from five coral species
AM1, 2, 3, *Isopora palifera* colonies. FM1, 2; *Millepora sp.* colonies. MM1, 2, 3, *Montipora efflorescens* colonies. PM1, 2, 3, *Porites lutea* colonies. VM1, 2, 3, *Favia complanata* colonies.

3.3 Phylogenetic analysis

Phylogenetic analysis of representative clones from individual OTU groups in PH, P^dH, P^dI, and SW libraries is presented in Fig. 26. We summarized the nearest relative sequence within the public database (GenBank), the level of sequence similarity and the percentage occurrence of that OTU in each clone library.

3.3.1 Bacterial community associated with *P. lutea*

In NSI *P. lutea* clone library (Table 9), 71% clones were related to previously described bacteria associated with marine invertebrate. A number of clones were closely affiliated to coral associated bacteria. PH_OTU8 (30% of representative clones) was closely (97% 16S rRNA gene sequence similarity) related to an uncultured *Gammaproteobacterium* (GenBank accession number CD207G01) which was found from the healthy tissue of Caribbean coral *Montastraea annularis* and distantly (92% similarity) related to uncultured *Spongiobacter sp.* clone ME82 (GenBank accession number DQ917877) isolated from *Muricea elongate* (Order Gorgonacea). PH_OTU11 (19% of representative clones) was found to be closely related to an uncultured *Candidate* division CAB-I bacterium (GenBank accession number CD205F11) which also isolated from Caribbean coral *M. annularis*. Clone PH_OTU7 was similar to an uncultured *Alphaproteobacterium* clone DC-3 (GenBank accession number AY529881) which was related to corals disease outbreak in Magnetic Island. Moreover, two OTU groups (PH_OTU12 and PH_OTU13, total 16% of representative clones) were similar (92-93% similarity) to uncultured *Cyanobacterium* clone DPC025 and DPC164. Three ribotypes retrieved from PH were related to sponge symbiont bacteria. PH_OTU1, PH_OTU2 were closely affiliated to uncultured *Actinobacteria* (GenBank accession

number AF186414 and AM259924) and PH_OTU9 was related to uncultured *Bacteroidetes* bacterium clone MPCa6_D05, isolated from sponge *Microciona prolifera* from the Chesapake Bay. However, still 25% of 16S rRNA clones were low sequence similarity (<90%) within GenBank database.

In SI *P. lutea* libraries (Table 10,11), the major OTU group (P^{d}H_OTU4 and P^{d}I_OTU1) (53%-60% of 16S rRNA clones) in P^{d}H and P^{d}I libraries still was closely related (97-98% similarity) to uncultured *Gammaproteobacteria* isolated from the healthy tissue of Caribbean coral *M. annularis*. High percentage of OTU groups (P^{d}H_OTU3, 5, 10-15 and P^{d}I_OTU1-2, 4-6, 8-9,13, 15-17, 22-23 and 26-27) in P^{d}H and P^{d}I libraries were related to previously described bacteria associated with marine environmental samples (seawater or sediment).

There was a shift in the retrieved 16S rRNA sequences group from PH, P^{d}H to P^{d}I. The first, the clones similar to uncultured *Gammaproteobacteria* was increased in percentage in P^{d}H and P^{d}I libraries, especially the clones similar to uncultured *Gammaproteobacteria* isolated from the healthy tissue of Caribbean coral *M. annularis*. Only one out of 15 OTU groups constituting 30% of sequenced clones within the PH library affiliated with sequences of the *Gammaproteobacteria* division. Conversely, a total of 7 out of 18 OTU groups in P^{d}H library and 64% of representative clones sequenced within the P^{d}H library affiliated with sequence of the *Gammaproteobacteria*. Furthermore, in P^{d}I library, 7 out of 27 OTU groups constituting 66% of sequenced clones were closely related to *Gammaproteobacteria*. Secondly, the clones similar to *Candidate* division CAB-I and plastid uncultured phototrophic eukaryote both were decreased in P^{d}H and P^{d}I libraries. 19% and 14% of sequenced clones were within the PH library. However, 19% and 4% of sequenced clones were within the P^{d}H library and 1% and 6% within P^{d}I library. Thirdly, the OTU groups associated with *P. lutea* were

more close to bacteria from marine environment as sponge infected *P. lutea*. A total of 5 out of 15 OTU groups within the PH library were related to bacteria isolated form marine environment. Otherwise, 8 out of 18 OTU groups in P^dH library and 15 out of 27 OTU groups in P^dI library were similar to bacteria isolated form marine environment. Finally, there were no OTU groups similar to cyanobacteria detected in P^dH to P^dI libraries, except P^dI_OTU25.

3.3.2 Bacterial community associated with *T. hoshinota*

According to previous result that there were *cyanobacteria* groups specific to *T. hoshinota* and *P. lutea* samples, we constructed the phylogenetic tree of comparing representative cyanobacteria clones from individual OTU groups in this study with sponge-associated, black band disease related *Cyanobacteria* obtained from other studies (Steindler, 2005 and Frias-Lopez, 2004) (Fig. 25). The result showed that *Cyanobacteria* from *T. hoshinota* was different with other sponges-associated *Cyanobacteria* and had individual clade. This clade was more close to group V (*Chroococcales, Oscillatoriales, Pleurocapsales, and Prochlorales*) than group II consisting of four sequences from *Dysidea herbacea, Dysidea granulose, Lendenfeldia dendyi,* and *Aplysina gerardogreeni*, originating from Guam, the Mexican Pacific, and Zanzibar. *Cyanobacteria* related black band disease was close to group II because both of them belonged to filamentous *Cyanobacteria*. Furthermore, NSI *P. lutea* associated *Cyanobacteria* (PH_OTU12, 13) appeared being unrelated to any major group of *Cyanobacteria*. These sequences might not belong to *Cyanobacteria* or a new cyanobacterial group.

In PM clone library (Table 12), 76% clones were related (96% similarity) to uncultured *Prochloron sp*. clone NA-108 which was obligate symbiont in *Didemnid*

ascidians associated with marine invertebrate. A few other clones were related uncultured bacterium from marine environment.

Table 9 16S rRNA gene sequence analysis of bacterial clones from the non-sponge-infected *Porites lutea*, based on BLAST analysis

Bacterial clone	Representative % of 16S rRNA clones	No. of base pairs sequenced	Nearest relative	Accession number	Reference	Sequence similarity (%)	Bacterial group
PH_OTU1	1	872	Uncultured sponge symbiont PAUC38fp	AF186414	Sponge Theonella	99%	*Actinobacteria*
PH_OTU2	1	810	Uncultured *Actinobacterium* clone CN81	AM259924	Mediterranean Sponge Chondrilla nucula	98%	*Actinobacteria*
PH_OTU3	1	789	Uncultured *Alphaproteobacterium* clone CD204C11	DQ200432	Caribbean coral Montastraea annularis	97%	*Alphaproteobacteria*
PH_OTU4	1	820	Roseobacter sp. SPO804	DQ993342	Caribbean coral Montastraea annularis	99%	*Alphaproteobacteria*
PH_OTU5	2	878	Uncultured bacterium clone B8S-114	EU652589	Marine environment	95%	*Alphaproteobacteria*
PH_OTU6	8	848	Uncultured bacterium clone w3uc5	DQ416510	Marine environment	81%	*Alphaproteobacteria*
PH_OTU7	1	817	Uncultured bacterium clone DC-3	AY529881	Disease outbreak on corals from Magnetic Island	92%	*Alphaproteobacteria*
PH_OTU8	30	833	Uncultured *Gammaproteobacterium* clone CD207G01	DQ200629	Caribbean coral Montastraea annularis	97%	*Gammaproteobacteria*
PH_OTU9	1	760	Uncultured *Bacteroidetes* bacterium clone MPCa6_D05	EF414089	Sponge Microciona prolifera,Chesapeake Bay	90%	*Bacteroidetes*
PH_OTU10	1	790	Microscilla marina strain:IFO 16560	AB078080	Marine environment	95%	*Bacteroidetes*
PH_OTU11	19	796	Uncultured *Candidate division CAB-I* clone CD205F11	DQ200556	Caribbean coral Montastraea annularis	98%	*Candidate division CAB-I*
PH_OTU12	1	813	Uncultured *Cyanobacterium* clone DPC025	DQ269091	Marine eukaryotes	93%	*Cyanobacteria*
PH_OTU13	15	859	Uncultured *cyanobacterium* clone DPC164	DQ269106	Marine eukaryotes	92%	*Cyanobacteria*
PH_OTU14	3	826	Mycoplasma cottewii strain VIS	U67945	Goat	81%	*Firmicutes*
PH_OTU15	14	830	Uncultured phototrophic eukaryote clone CD205F08	DQ200553	Marine environment	88%	Plastid uncultured phototrophic eukaryote

Table 10 16S rRNA gene sequence analysis of bacterial clones from the sponge-infected *Porites lutea* (far-away sponge, P^{d}H), based on BLAST analysis

Bacterial clone	Representative % of 16S rRNA clones	No. of base pairs sequenced	Nearest relative	Accession number	Reference	Sequence similarity (%)	Bacterial group
P^{d}H_OTU1	1	739	Uncultured *Alphaproteobacterium* clone BBD216b_13f	EF123418	Black band disease microbial mats on Caribbean Siderastrea siderea	94%	*Alphaproteobacteria*
P^{d}H_OTU2	1	821	Uncultured bacterium clone DC-3	AY529881	Disease outbreak on corals from Magnetic Island	91%	*Alphaproteobacteria*
P^{d}H_OTU3	1	829	*Rhodobacteraceae* bacterium MOLA 442	AM990709	Marine environment	99%	*Alphaproteobacteria*
P^{d}H_OTU4	53	839	Uncultured *Gammaproteobacterium* clone CD207G01	DQ200629	Caribbean coral Montastraea annularis	97%	*Gammaproteobacteria*
P^{d}H_OTU5	1	720	Uncultured *Gammaproteobacterium* clone CD204G09	DQ200474	Caribbean coral Montastraea annularis	98%	*Gammaproteobacteria*
P^{d}H_OTU6	1	729	*Acinetobacter sp.* MUB1	AY273199	Marine environment	93%	*Gammaproteobacteria*
P^{d}H_OTU7	1	829	Uncultured bacterium clone A8S-109	EU652550	Marine environment	93%	*Gammaproteobacteria*
P^{d}H_OTU8	5	820	Uncultured *Gammaproteobacterium* clone Ax29_A1	EF092194	Marine environment	94%	*Gammaproteobacteria*
P^{d}H_OTU9	2	839	Uncultured *Gammaproteobacterium* clone CD207F03	DQ200619	Caribbean coral Montastraea annularis	95%	*Gammaproteobacteria*
P^{d}H_OTU10	1	839	Uncultured bacterium clone P7X3b1C05	EU491022	Marine environment	93%	*Gammaproteobacteria*
P^{d}H_OTU11	1	779	*Bdellovibrio sp.* JS5	AF084859	Coastal Marsh	91%	*Deltaproteobacteria*
P^{d}H_OTU12	1	794	Uncultured *Deltaproteobacterium* clone GASP-WC2W3_E07	EF075373	Marine environment	92%	*Deltaproteobacteria*
P^{d}H_OTU13	3	856	Uncultured *Bacteroidetes* bacterium clone: pKB3B-27	AB247902	Hydrothermal vents in Kermadec Volcanic Arc	84%	*Bacteroidetes*
P^{d}H_OTU14	1	849	Uncultured *Bacteroidetes* bacterium clone MPWIC_G09	EF414162	Sponge Microciona prolifera from the Chesapeake Bay	95%	*Bacteroidetes*
P^{d}H_OTU15	1	821	Uncultured *Cytophagaceae* bacterium clone 1-13	AY094494	Marine environment	90%	*Sphingobacteria*
P^{d}H_OTU16	1	822	Uncultured *Flexibacteraceae* bacterium clone EC206	DQ889876	Octocoral Erythropodium caribaeorum	90%	*Sphingobacteria*
P^{d}H_OTU17	19	788	Uncultured *candidate division CAB-I* clone CD205F11	DQ200556	Caribbean coral Montastraea annularis	98%	*Candidate division CAB-I*
P^{d}H_OTU18	4	749	Uncultured phototrophic eukaryote clone CD205F08	DQ200553	Marine environment	88%-91%	Plastid uncultured phototrophic eukaryote

Table 11 16S rRNA gene sequence analysis of bacterial clones from the sponge-infected *Porites lutea* (close-to sponge, P^dI), based on BLAST analysis

Bacterial clone	Representative % of 16S rRNA clones	No. of base pairs sequenced	Nearest relative	Accession number	Reference	Sequence similarity (%)	Bacterial group
P^dI_OTU1	1	850	Uncultured *Actinobacterium* pItb-RF-5	AB294984	Marine environment	96%	*Actinobacteria*
P^dI _OTU2	2	878	Uncultured *Roseobacter* AC2_A2	EF092158	Marine environment	99%	*Alphaproteobacteria*
P^dI _OTU3	1	828	Uncultured *Ruegeria* clone BME18	DQ917819	Bleached and wild-type Muricea elongata	99%	*Alphaproteobacteria*
P^dI _OTU4	3	799	Uncultured *Alphaproteobacterium* BBD_HS216b_07	DQ644016	Marine environment	99%	*Alphaproteobacteria*
P^dI _OTU5	1	840	*Alphaproteobacterium* MOLA 143	AM990917	Coastal NW Mediterranean ecosystem	93%	*Alphaproteobacteria*
P^dI _OTU6	1	782	Uncultured *Alphaproteobacterium* LC1-35	DQ289905	Permeable shelf sediments	98%	*Alphaproteobacteria*
P^dI _OTU7	3	828	Uncultured *Alphaproteobacterium* CD204H01	DQ200478	Caribbean coral Montastraea annularis	97%	*Alphaproteobacteria*
P^dI _OTU8	2	806	*Alphaproteobacterium* Y3F	AF253467	Marine Roseobacter	96%	*Alphaproteobacteria*
P^dI _OTU9	1	825	*Rhodobacteraceae* bacterium DG1297	DQ486508	Marine environment	95%	*Alphaproteobacteria*
P^dI _OTU10	1	858	*Pseudoruegeria aquimaris* SW-255	DQ675021	Isolated from seawater of the East Sea in Korea	95%	*Alphaproteobacteria*
P^dI _OTU11	1	879	Uncultured *Alphaproteobacterium* BBD_STXLK_42b	DQ644018	Production of cyanobacterial toxin Microcystin in black band disease of corals	96%	*Alphaproteobacteria*
P^dI _OTU12	60	837	Uncultured *Gammaproteobacterium* CD207G01	DQ200629	Caribbean coral Montastraea annularis	98%	*Gammaproteobacteria*
P^dI _OTU13	1	865	Uncultured *Gammaproteobacterium* Ax29_A1	EF092194	Marine environment	94%	*Gammaproteobacteria*
P^dI _OTU14	1	835	Uncultured *Gammaproteobacterium* CD207F03	DQ200619	Caribbean coral Montastraea annularis	94%	*Gammaproteobacteria*
P^dI _OTU15	1	825	*Cycloclasticus sp.* E	AF093003	Marine environment	86%	*Gammaproteobacteria*

P^{d}I _OTU16	1	844	*Gammaproteobacterium* L193	AY371439	Cultured microbial associates of deep-water marine invertebrates	98%	*Gammaproteobacteria*
P^{d}I _OTU17	1	811	Uncultured *Gammaproteobacterium* SARG_3	AM238545	Marine dimethylsulfide-consuming bacteria	97%	*Gammaproteobacteria*
P^{d}I _OTU18	1	860	Uncultured bacterium PDA-OTU12	AY700599	Coral Pocillopora damicornis from the Great Barrier Reef	97%	*Gammaproteobacteria*
P^{d}I _OTU19	1	855	Uncultured *Bacteroidetes* MPWIC_G09	EF414162	Sponge Microciona prolifera from the Chesapeake Bay	96%	*Bacteroidetes*
P^{d}I _OTU20	1	833	Uncultured *Flexibacteraceae* EC206	DQ889876	Octocoral Erythropodium caribaeorum	90%	*Bacteroidetes*
P^{d}I _OTU21	1	778	Mucus bacterium 86	AY654823	Culturable bacteria from the mucus of Oculina patagonica	94%	*Bacteroidetes*
P^{d}I _OTU22	1	876	Uncultured *Cytophaga* JTB248	AB015263	Sediments of Deepest Cold-Seep Area, the Japan	86%	*Bacteroidetes*
P^{d}I _OTU23	1	853	Uncultured *Bacteroidetes* IRD18C04	AY947919	Marine environment	90%	*Bacteroidetes*
P^{d}I _OTU24	1	795	Uncultured *candidate division CAB-I* CD205F11	DQ200556	Caribbean coral Montastraea annularis	98%	*Candidate division CAB-I*
P^{d}I _OTU25	1	820	*Leptolyngbya sp.* HBC3	EU249121	Cyanobacterial Diversity of a Marine Stromatolite	93%	*Cyanobacteria*
P^{d}I _OTU26	1	838	Uncultured bacterium PoritesC32clC05	EU636623	Marine environment	99%	*Lentisphaerales*
P^{d}I _OTU27	6	847	Uncultured phototrophic eukaryote clone CD205F08	DQ200553	Marine environment	88%	Plastid uncultured phototrophic eukaryote

Table 12 16S rRNA gene sequence analysis of bacterial clones from *Terpios hoshinota* (infected Porites lutea), based on BLAST analysis

Bacterial clone	Representative % of 16S rRNA clones	No. of base pairs sequenced	Nearest relative	Accession number	Reference	Sequence similarity (%)	Bacterial group
PM_OTU1	2	779	Bacterium s1cb31	DQ416551	Mucus and Tissues of the Coral *Oculina patagonica*	99%	*Alphaproteobacterium*
PM_OTU2	9	795	*Roseobacter sp.* Y2	DQ120728	Marine environment	94%	*Alphaproteobacterium*
PM_OTU3	2	749	Uncultured bacterium clone D8S-106	EU652584	Yellow Sea sediment	96%	*Alphaproteobacterium*
PM_OTU4	4	739	Uncultured bacterium clone 1-52	EF040508	Associated with Halichondria sp.	88%	*Gammaproteobacterium*
PM_OTU5	2	802	Uncultured *Proteobacterium* clone JL-ESNP-H53	AY664193	Eastern subtropical North Pacific	93%	*Gammaproteobacterium*
PM_OTU6	2	799	Uncultured *Bacteroidetes* bacterium clone: pItb-RF-14	AB294988	Shallow Submarine Hydrothermal System	99%	*Bacteroidetes*
PM_OTU7	76	779	Uncultured *Prochloron sp.* clone NA-108	DQ357945	Obligate symbiont in didemnid ascidians	96%	*Cyanobacteria*
PM_OTU8	2	780	Uncultured *Cyanobacterium* clone BB31NT16S-12	EF089520	Black band disease in Red Sea stony corals	90%	*Cyanobacteria*

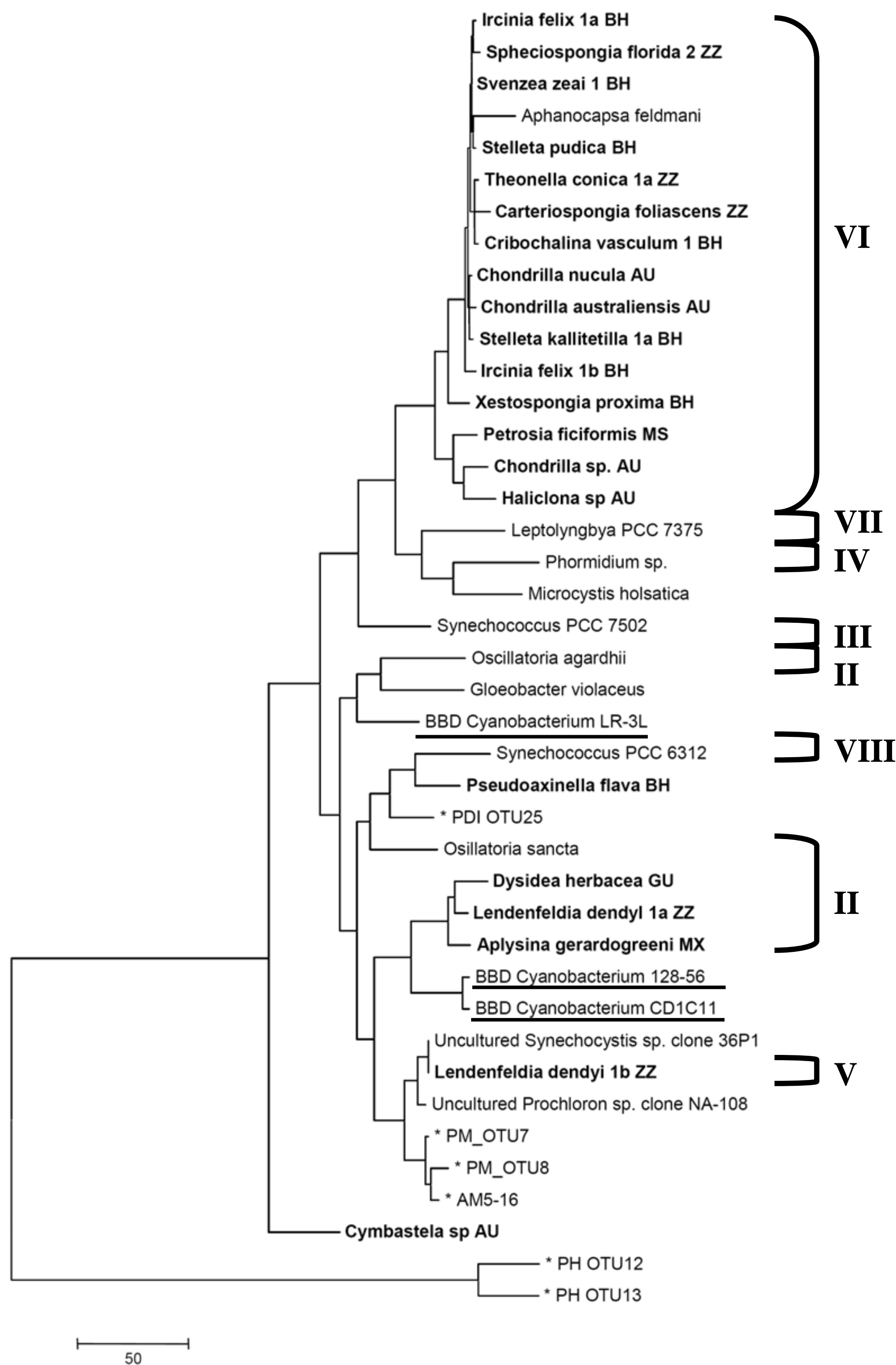


Fig. 25 Maximum parsimony tree of sponge-associated and black band disease related cyanobacteria based on 16S rRNA.
Bootstrap values above 50% are indicated. Sponge-associated cyanobacteria (which were named following their host species and sampling localities: AU, Australia; BH, Bahamas; GU, Guam; MS, Mediterranean Sea; MX, Mexican Pacific; ZZ, Zanzibar) are indicated in bold. Black band disease related cyanobacteria are indicated using underline. Sequences form this work are indicated by asterisk. The group numbers follow Steindler et al..

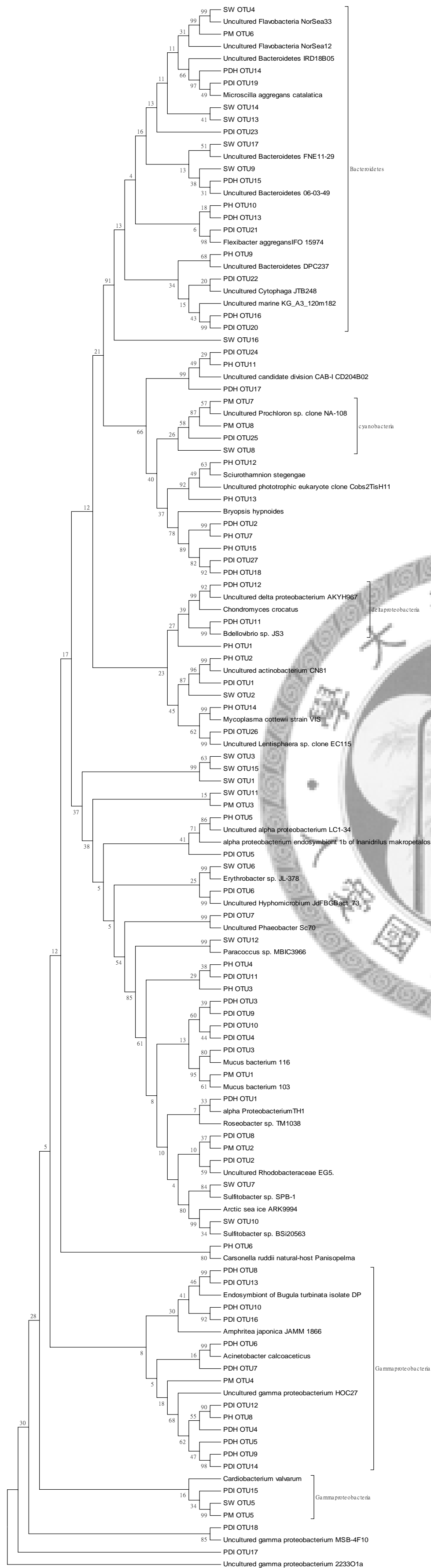


Fig. 26 Neighbor-Joining tree of *Porites lutea* -associated and seawater related bacteria based on 16S rRNA Bootstrap values above 50% are indicated. The labels SW, Chai-Kou sample. KW, Kun-Guan sample. PH, non sponge-infected *Porites lutea* colonies. P^{d}H, sponge-infected *Porites lutea* colonies (far-away sponge). P^{d}I, sponge-infected *Porites lutea* colonies (close-to sponge).

3.4 Electronic microscopy of *T. hoshinota*

T. hoshinota is a cyanobacteriosponge, contains a significant number of large unicellular *Cyanobacteria* throughout the sponge body. In the field survey, we observed that the sponge's edge was thicker and dark color in the boundary with coral compared to the rest part (Fig. 27). In order to clarify if there is any difference in the cellular morphology (including *Cyanobacteria* and sponge) between the sponge close-to and far-away parts to coral, transmission electron microscopy was used for examination

On micrographs, *Cyanobacteria* were easily observed throughout the entire sponge tissue (Fig. 28A). In histological sections, 5%-10% of the cyanobacteria were observed in different stages of division by binary fission (Fig. 28B, D). The relatively thick layer (up to 100 nm in width) of the septum was formed in end of dividing stage (Fig. 28E). The thylakoids were arranged around the periphery of the cells. The number of thylakoid layers sometimes varied and the width of the thylakoid lumen could also vary markedly. The cell centre was finely granulated and often had spherical, 300 nm, one or more electron-dense granules (resembling polyphosphate granules) and electron-transparent areas. Electron-dense granules resembling cyanophysin granules occurred between the thylakoids (Fig. 29A). All *Cyanobacteria* found in these sections were intercellular position although the sponge tissue was rather difficult to be distinguished.

Furthermore, comparing the cyanobacterial cellular morphology between two difference specimens, the section from "far-away coral" specimen exhibited that the thylakoid membrane became relatively indistinct and the out membrane was deckle edge (Fig. 29B, 30B), even in diving stage (Fig. 28C).

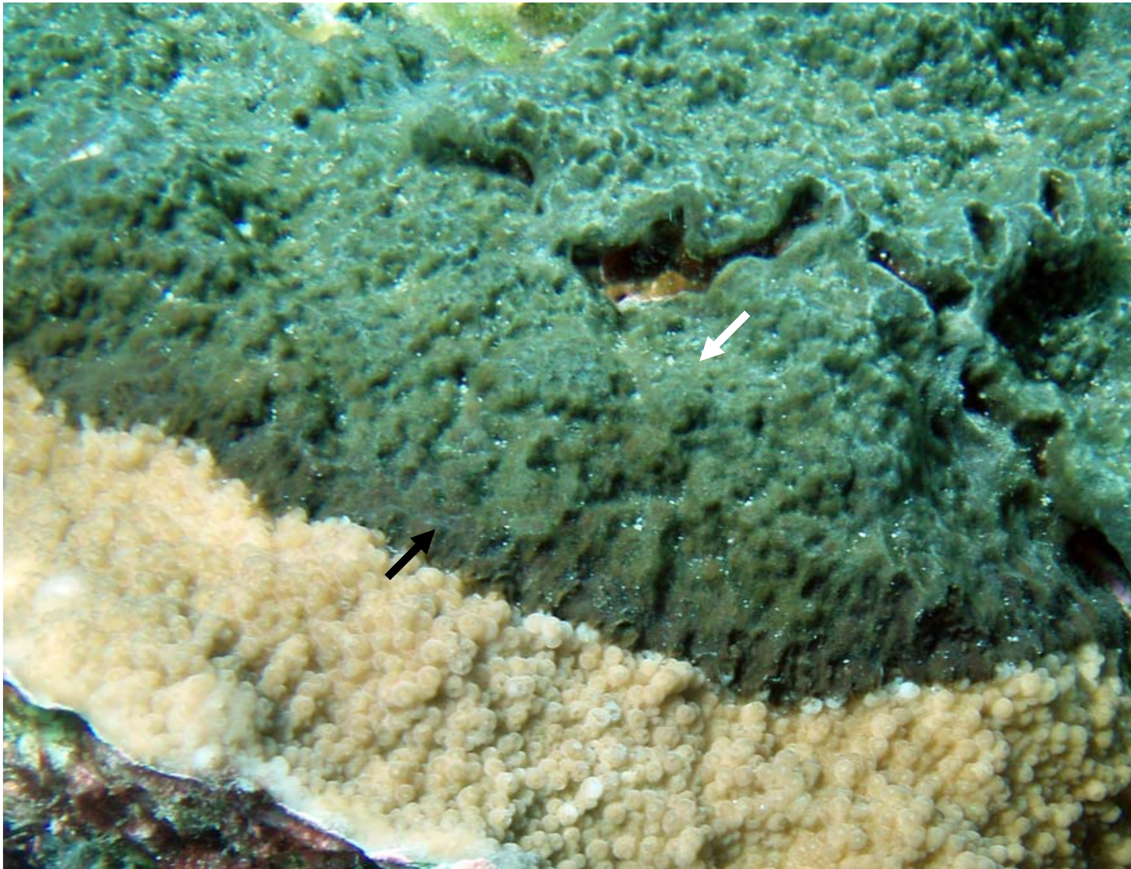


Fig. 27 *Terpios hoshinota* infected coral *Isopora palifera*. Black arrow: close-to coral, white arrow: far-away coral.

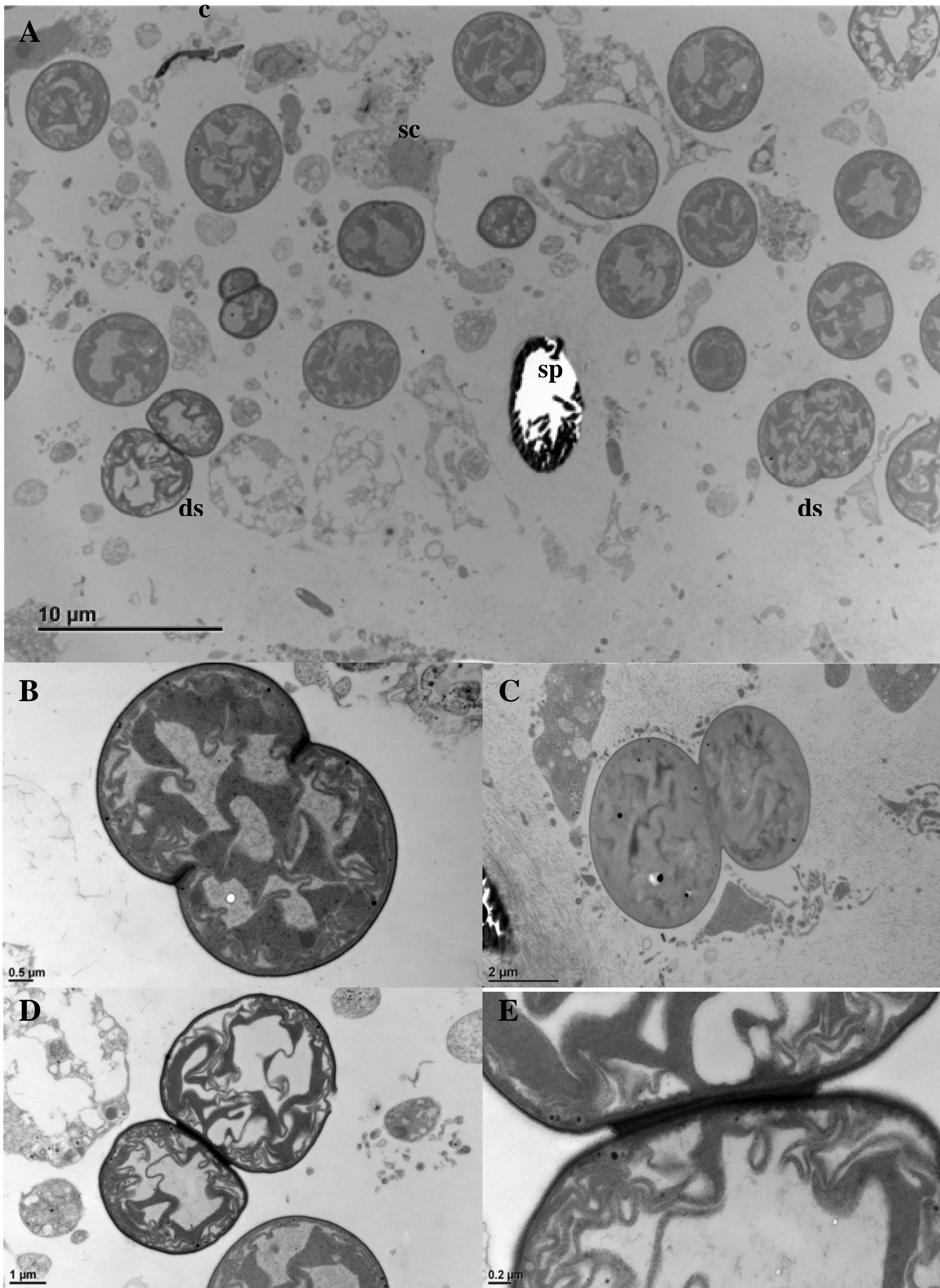
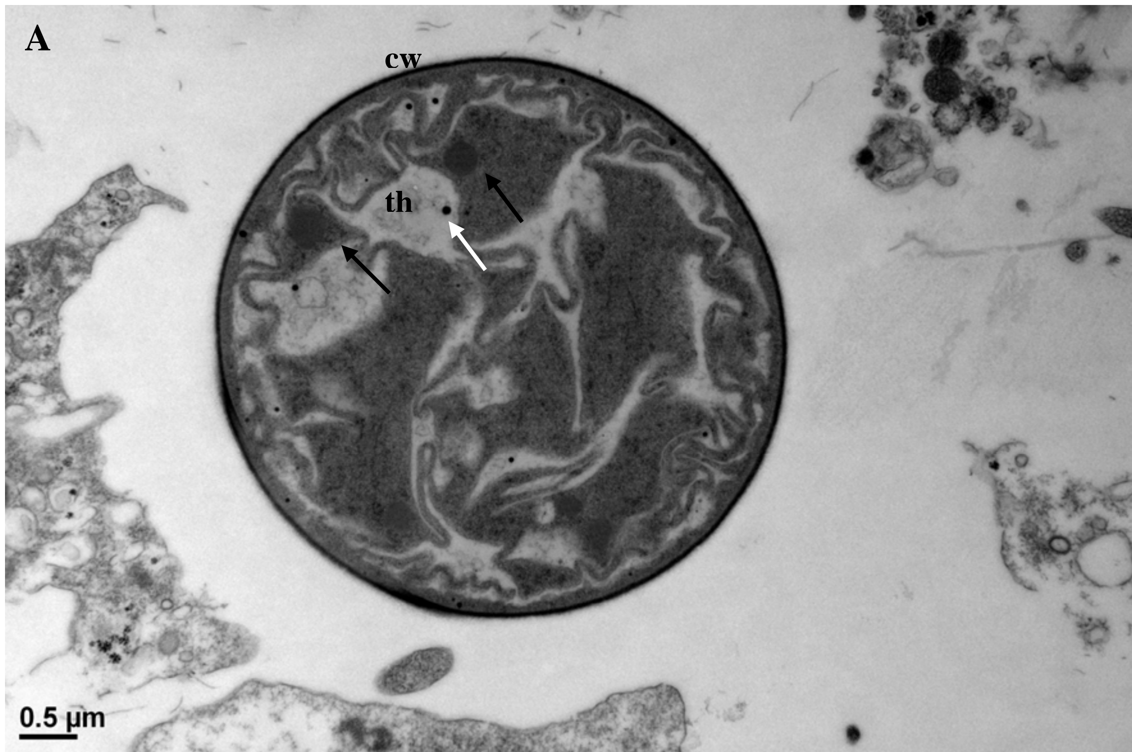


Fig. 28 Transmission electron micrographs of *Terpios hoshinota*
A. *Cyanbacteria* in choanosome (c: *Cyanobacteria*, dc: dividing *Cyanobacteria*, sp: spicules position, sc: sponge cell). B. dividing stage of *Cyanobacteria* (close-to-coral specimen). C. dividing stage of *Cyanobacteria* (far-away-coral specimen). D. end of dividing stage. E. close up the septum in end of dividing stage.



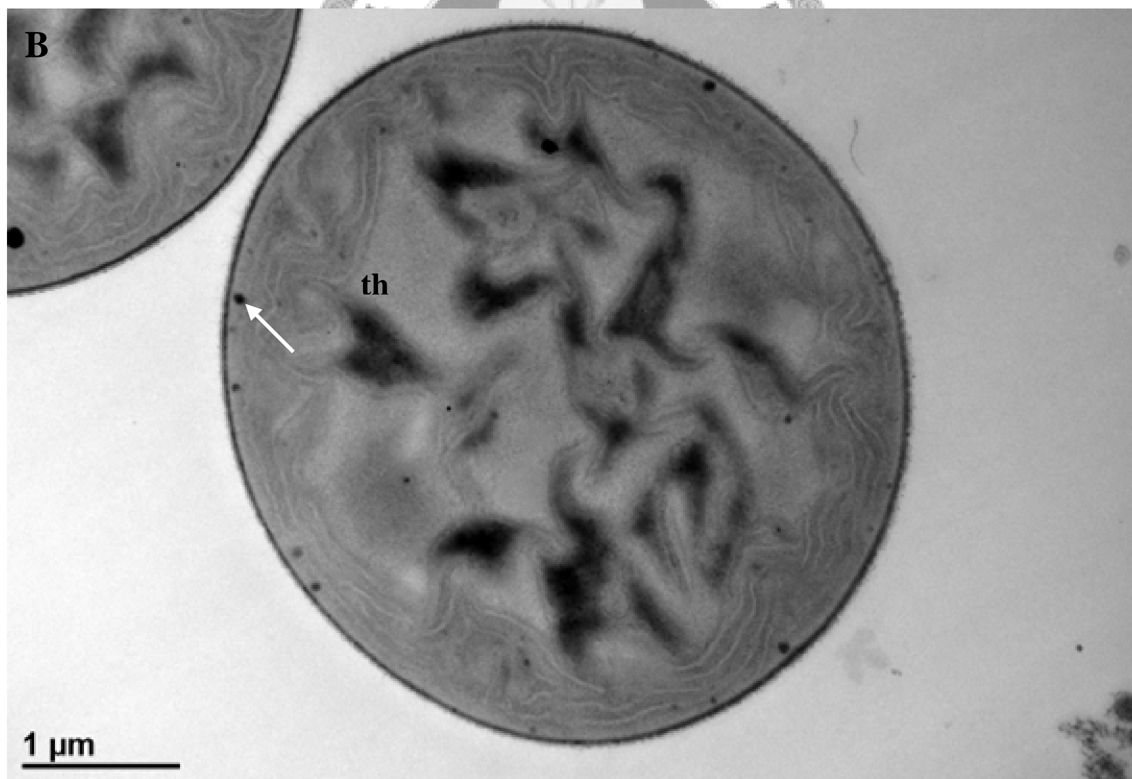


Fig. 29 Transmission electron micrographs of *Cyanobacteria*
A. *Cyanobacteria* in “close-to-coral” specimen. B. cyanobacteria in “far-away-coral” specimen. (cw: cell wall, th: thylakoid, black arrow: polyphosphate like granules, white arrow: resembling cyanophycin granules)

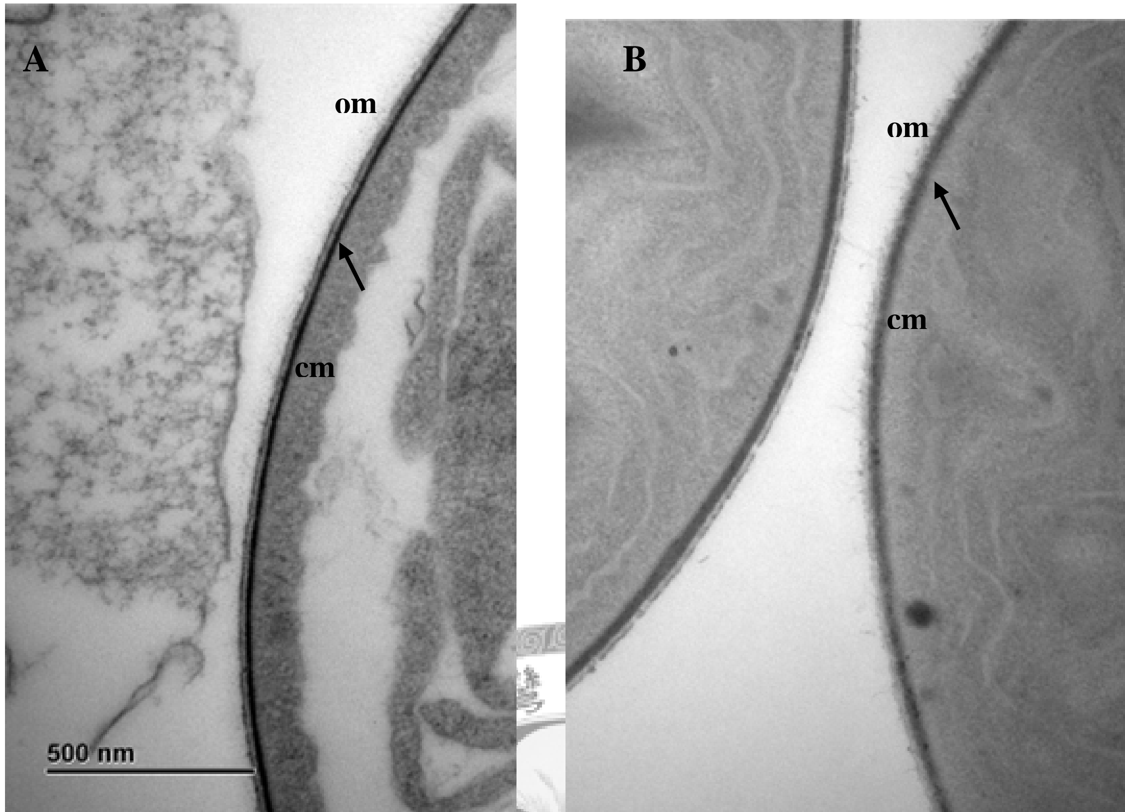


Fig. 30 Transmission electron micrographs of *Cyanobacteria* membrane
A. *Cyanobacteria* in "close-to-coral" specimen. B. *Cyanobacteria* in "far-away-coral" specimen. (om: out membrane, black arrow: cell wall, cm: cell membrane)

4 Discussion

4.1 The role of bacterial community associated with *T. hoshinota*

The bacterial community of *T. hoshinota* was unique, specific and low diversity. Undoubtedly, the major group was *Cyanobacteria* demonstrated by the results of DGGE and 16S rDNA library. Heterotrophic bacteria were also discovered within *T. hoshinota* and surrounding seawater. Therefore, those heterotrophic bacteria might be as food for *T. hoshinota* or existed in canal system of *T. hoshinota* (Pile et al., 1997).

T. hoshinota, harboring a large number of unicellular *Cyanobacteria*, has a flattened shape similar to the phototrophic sponges allowed maximum exposure to sunlight. Currently, there are four genera of *Cyanobacteria* observed within 38 genera of sponges. These *Cyanobacteria* may benefit sponges through fixation of atmospheric nitrogen, fixation of carbon as glycerol, producing two types of sunscreen compounds for UV protection and synthesis of the secondary metabolites for defensive toxins. Although real function of the *Cyanobacteria* in *T. hoshinota* remains unknown, such a considerable number of bacteria dwelling in the sponge may be functionally associated with why the sponge grow so rapidly. Interestingly, the sponge would not grow if sunlight was blocked in a field experiment as suggested by a preliminary test (in a personal communication with Dr. K.-Y. Soong). This result

apparently emphasizes a very close, symbiotic relationship likely existing between the sponge and *Cyanobacteria*.

Another indirect evidence for functional interactions among C*yanobacteria*, sponge and coral is that the morphology of *Cyanobacteria* is different comparing the sponge samples between close-to coral part and far-away coral part. Thylakoid structure of the *Cyanobacteria* in "close-to-coral" specimen has own clearer shape and smooth margin of out membrane whereas that of the *Cyanobacteria* in the "far-away coral" sponge part have blur membrane organization and rough margin of out membrane, suggesting the *Cyanobacteria* in the "close-to-coral" sponge may be more healthy in appearance because of more nutrition supplied from coral. However, this observation was limited in qualification evaluation of cyanobacterial morphology. The ecological relationship among coral, sponge and *Cyanobacteria* has to be demonstrated by more experimental evidences.

4.2 New species of *Cyanobacteria* associated with *T. hoshinota*

T. hoshinota that harbor large quantities of *Cyanobacteria* was considered a "cyanobacteriosponge" and these symbiont was suspected of being a kind of *Aphanocapsa raspaigellae* because of its morphology (Rutzler and Muzik, 1993); However, according to TEM image of *T. hoshinota* isolated from Green Island, these

endogenous *Cyanobacteria* were morphologically different from *A. raspaigellae* although both of them were spherical and had similar cell size (5.7-6.3 μm) (Fig. 31). Firstly, the thylakoids membrane was less coiled in *T hoshinota* associated *Cyanobacteria* than that in *A. raspaigellae*. Secondly, there were no large electron-dense, angular shapes resembling carboxysomes in the centre of *T. hoshinota* associated *Cyanobacteria*. Moreover, through comparative morphology of other coccoid *Cyanobacteria*, *T. hoshinota* associated *Cyanobacteria* is suggested to be new species.

Furthermore, we have compared the 16S rDNA sequence of the *Cyanobacteria* with other *Cyanobacteria* and found that the most similar sequence of 16S rDNA was uncultured *Prochloron sp.* clone NA-108. However, the both sequences share only 96% similarity. Additionally, according to phylogenetic tree of *Cyanobacteria* associated with sponge, the *T. hoshinota* associated *Cyanobacteria* was a unique clade and differ from other sponge cyanobacterial symbiont. These results strongly support that the *T. hoshinota* associated *Cyanobacteria* is a new species, nonetheless more evidences are still required.

4.3 The composition of bacterial community associated *P. lutea*

Before discussing the composition of bacterial community associated *P. lutea*, it is

necessary for knowing that there are multiple relations between microbes and coral according to present knowledge. Coral are composed of two layers of cells, the epidermis and gastrodermis, covered by a surface mucus layer and connected to a large, porous calcium carbonate skeleton (Rosenberg et al., 2007). These structures also interact with diverse coral holobiont, the host organism and all of its associated symbiotic microorganisms. Three habitats for microbes in corals are briefly divided: surface mucus layer, coral tissue (including the gastrodermal cavity) and calcium carbonate skeleton, each of which harbors a distinct bacteria population (Ritchie and Smith, 2004; Bourne and Munn, 2005; Koren and Rosenberg, 2006). Microbes on the mucus layer of coral structure were diverse and abundant beneficial bacterial community, including antibacterial activity (Ritchie, 2006). Bacteria also colonize coral tissue. However, those bacterial species were different from the species colonizing the surface mucus layer (Koren and Rosenberg, 2006).

Furthermore, the diversity of bacteria species that are associated with a particular coral species is high including many novel species (Rohwer et al., 2002). The identity of the members of the coral bacterial community were distinctly different with surrounding seawater (Frias-Lopez et al., 2002), but there were similar bacterial populations associated with the same coral species, even if geographically separated (Rohwer et al., 2002). All information suggested here that the association between the

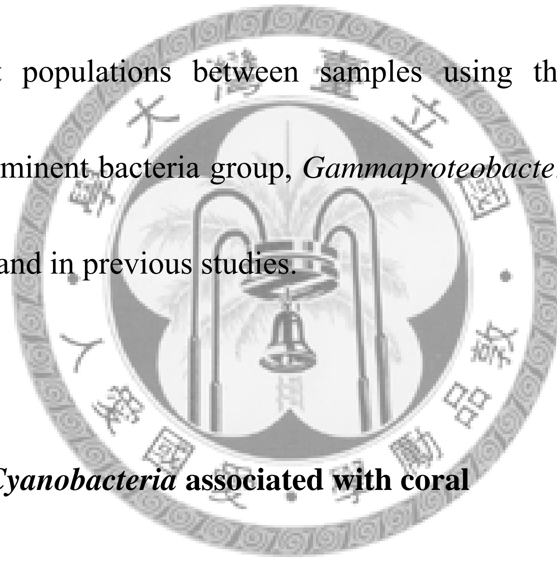
coral and its microbiota is specific.

Return to our results. According to analysis of bacterial clone libraries of NSI *P. lutea* (PH), the diversity of clone libraries was high that 3 coral samples yielded 15 distinct bacterial ribotypes, in spite of the degree of diversity was lower than that observed in other coral samples (Rohwer et al., 2002). However, the bacterial diversity in PH did not approach saturation in the calculated rarefaction curve; hence, higher diversity in PH is expected. Moreover, 24% unclassified bacteria were also observed in our data which support that the most of the coral associated bacteria are novel at the genus and species level (Rohwer et al., 2002).

Most prominent bacteria phyla associated with NSI *P. lutea* were *Cyanobacteria* (36%), *Gammaproteobacteria* (30%), and unclassified bacteria (24%). These results are similar to previous 16S rDNA analysis of *Porites* associated bacteria (Rohwer et al., 2002; Wegley et al., 2007). *Gammaproteobacteria* were the most abundant of all the bacterial sequences (31%, 33%) in both studies. However, the most prominent family was *Oceanospirillales sp.* in this thesis but *Acinetobacter sp.* in previous studies (Wegley et al., 2007). This difference may be explained by different coral species investigated between studies although the both corals belong to same genus.

Comparing the composition of bacterial clone libraries of each PH colony from the same sampling location, there was significant different among those libraries; this

result was converse to the idea of the consistence of bacterial communities from the same coral species in spit of separating by time or space (Rohwer et al., 2002). The possible reason might be different methods used to analyze the bacterial clone libraries. In our study, we used the method LIBSHUF with statistic basis which is specific software to compare the difference among bacterial clone libraries; however the method used by Rohwer's team was to simply group bacteria into rough taxonomic groups, calculated each taxonomic group with percentage and briefly compared dominant populations between samples using the percentage values. Nonetheless, the prominent bacteria group, *Gammaproteobacteria*, was still the same in each PH libraries and in previous studies.



4.3.1 The role of *Cyanobacteria* associated with coral

Another major bacteria group associated with *P. lutea* was *Cyanobacteria*. What the role of *Cyanobacteria* in coral holobiont? To review previous research, there is an interesting case where symbiotic *Cyanobacteria* were found within the Caribbean coral *Montastraea cavernosa* tissue. The expression of the nitrogen- fixing enzyme nitrogenase in *Cyanobacteria* suggests that nitrogen fixation may be an important source of this limiting element for the zooxanthellate-coral symbiotic association (Lesser et al., 2004). Furthermore, coral skeletons are porous structures also inhabited

by a variety of bacteria. *Cyanobacteria* or endolithic algae which have photosynthesis and nitrogen fixation ability were recognized the important nutrient regeneration source or alternative source of energy during coral bleaching (Fine et al., 2005; Magnusson et al., 2007). Therefore, *Cyanobacteria* have offered a great function to coral's life; however, we also found that the abundance of *Cyanobacteria* associated with *P. lutea* changed as sponge covered. Further discussion will be presented in the next paragraph.

4.3.2 Variation of bacterial community associated with coral

Comparing the bacterial clone libraries from different parts of *P. lutea*, PH, P^dI, P^dH, we found that the composition and diversity of bacterial communities associated with *P. lutea* changed as *T. hoshinota* covered on coral. P^dI showed greater ribotype diversity than the P^dH and PH. The P^dH clone library has lowest diversity. Distinct differences were also discovered between the bacterial composition of NSI and SI coral. This phenomenon was also observed in previous studies (Pantos et al., 2003).

With the investigation of microorganism-induced coral disease, more research found that there was also an important role of microorganism to health coral and suggested that the microbial community variation may be a bioindicator of environmental stress and disease. An important implication of the model was that

disrupting any one of these components may cause physiological changes and would result in coral disease or death (Rohwer et al., 2002). Therefore, when discussing about microbe as primary or secondary pathogens of coral disease, we need to realize nature dynamic relationship between coral and their associated microorganism in changeable environment.

In the past, yet only few attempts have so far been made at the research of dynamic relationship between microbes and corals in different environments. Currently, molecular methodology was applied on coral microbiology, more and more research focus on the variation of coral associated microbes with temporal and spatial dynamics. For example, there were shifts in the structure of bacterial communities inhabiting coral tissue with different environment factor or stress, including pollution (Klaus et al., 2005), seawater temperature (Koren and Rosenberg, 2006), seawater depth (Klaus et al., 2007), disease (Pantos et al., 2003). Increased dominance of specific bacterial strains (Klaus et al., 2005)or lower diversity of bacterial community associated with coral tissue (Pantos et al., 2003) were common responses observed in disturbed environments. This change may be due to either environmental factors acting directly on the bacterial communities, or indirectly via a physiological response of the coral, e.g. changes in the rate of photosynthesis or respiration, induced by these same environmental factors (Pantos et al., 2003). Rosenberg et al. proposed “the coral

probiotic hypothesis” that as corals were at different environmental conditions, symbiotic microorganisms were dynamic selects for the most advantageous coral holobiot in the prevailing conditions (Reshef et al., 2006). By altering the structure of its resident microbial community, the holobiont can adapt to changing environmental conditions more rapidly and flexibility than a process that is dependent on genetic mutation and selection of the coral host (Rosenberg et al., 2007). Furthermore, the perturbation in a normal microbial flora was ”whole coral response”, even if a relatively small part of infected coral and it occurred prior to the onset of visible signs of disease (Pantos et al., 2003). This evidence supported that changes in the microbial flora may serve as a bioindicator of environmental stress and disease.

Those researches, moreover, lead us further into a consideration of the role of the bacterial community associated with *P. lutea* in this study. The percentage of *Gammaproteobacteria* increased two times but unclassified bacteria and *Cyanobacteria* were decreased extremely when sponge covered on *P. lutea*. Interestingly, the percentage of *Alphaproteobacteria* increased especially in P^dI samples. Several explanations were proposed for such observation: 1) the natural bacterial community may be altered, either directly by an environmental stress (pollution) or by competition from *T. hoshinota*. 2) The physiology of the coral colony may change due to an environmental stress, resulting in further affecting the

coral-associated bacterial community. 3) Antibiotic properties possessed by corals (Ritchie, 2006) may be compromised under stress, allowing the colonization of different communities of bacteria. In our results, the change of the original population may result in the loss of the antibiotic protection and allow the colonization of the tissue by other bacterial species (i.e. *Alphaproteobacteria*). 4) The coral may be compromised by the loss of a symbiont to adapt new environmental condition. We also observed the similar phenomenon in our study where the *Cyanobacteria* associated with coral has been largely lost (Fig. 20.).

In addition, the most similar sequence of *Gammaproteobacteria* associated with *P. lutea* was uncultured *Gammaproteobacterium* isolated from the healthy tissue of Caribbean coral *M. annularis* (Klaus et al., 2007). When sponge covered on *P. lutea*, the bacterial group became dominantly by this *Gammaproteobacterium*. This phenomenon maybe related to disturbed environments (Pantos et al., 2003; Klaus et al., 2005). Another similar bacterium was also found in both PH and the Caribbean coral *M. annularis*. PH_OTU11 have 98% sequence similarity with the candidate division CAB-I from coral. CAB-I was high abundance in coral *M. annularis*, especially at shallow water depth and suspected to responsive to light and potentially phototrophic (Klaus et al., 2007). From this point of the view, PH_OTU11 may be have similar function for *P. lutea*; when sponge covered on coral, this bacteria group

decreased from 19% to 1%, suggesting sponge covering may severely influenced the bacteria function for healthy coral.

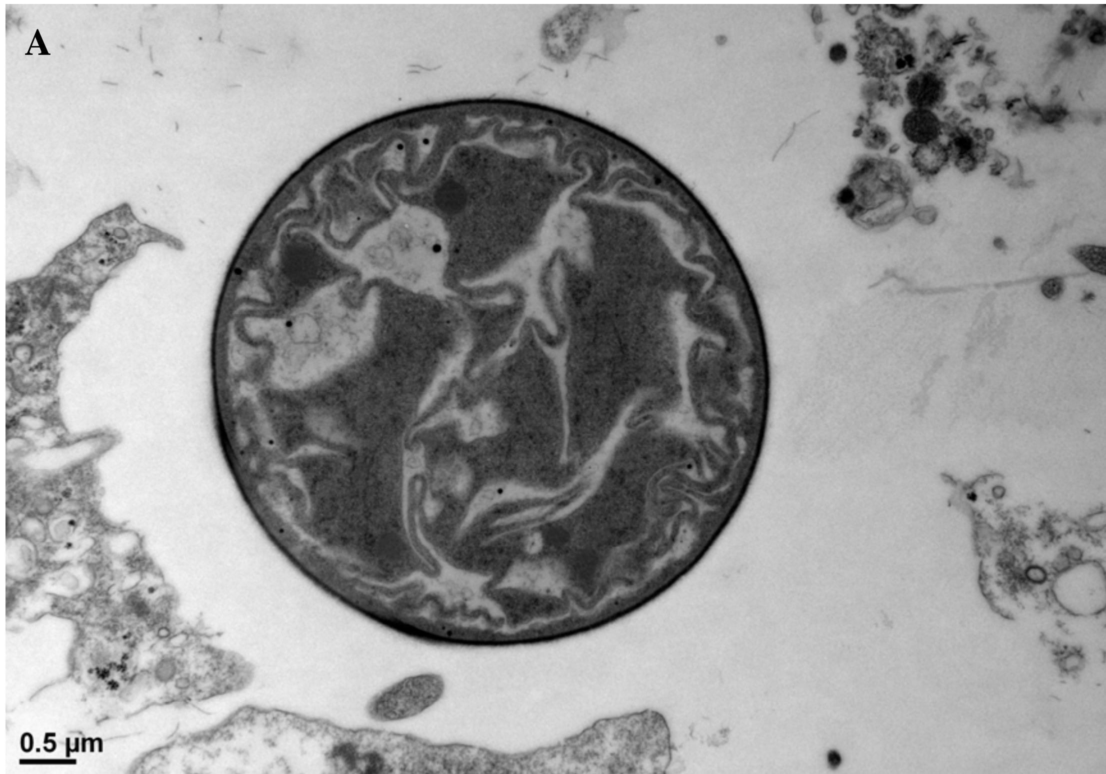
4.4 Future research

Coral microbiology is important for the understandings to the coral heath. In this thesis, we only identified the bacteria communities of *T. hoshinota* and *P. lutea*. The function of the bacterial community is still unknown; especially the role of the *Cyanobacteria* associated with *T. hoshinota*. The further study should pay more efforts on identification of the interactive function between the microbe and corals or sponge which will be helpful to clarify the microbial role and to provide better suggestions to coral maintenance and disease control.

On the other hand, the variation of bacterial communities associated with the NSI and SI *P. lutea* also offered a positive glimpse of the important information for being a possible bioindicator of the particular environmental stress. However, these snap-shot study data are in a limit for further understandings which bacterial population appearing and disappearing associates with what coral physiology and what environmental stresses. To build up a bioindicator, a long term investigation is apparently essential.

Finally, besides the function of bacteria community associated *T. hoshinota*, the

knowledge of the biology of *T. hoshinota* was also less known in the past. Reproduction and development of *T. hoshinota* is important to realize the possible reason of outbreak of *T. hoshinota.* At the same, fishing bite character on *T. hoshinota* was discovered in the field. Decease of sponge-eating fish resulted in food chain function unbalance are looking further research.



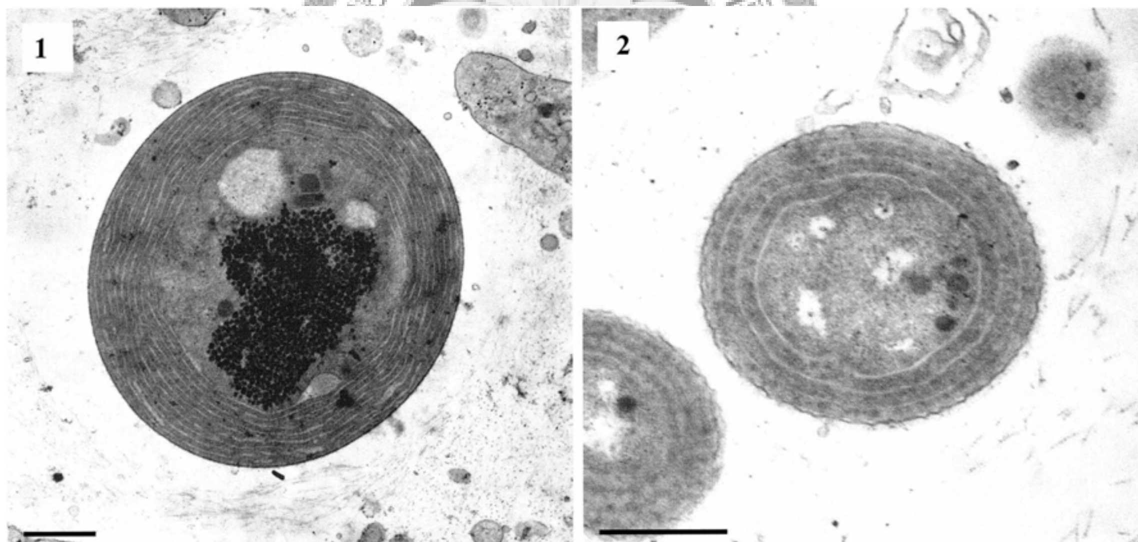


Fig. 31 Transmission electron micrographs of cyanobacterial symbionts in *Ircinia variabilis* from Marseille.

A. Cyanobacteria in Terpios hoshinota. 1.Aphanocapsa raspaigellae. 2. A. feldmannii. Scale bars: 1 mm (1) and 500nm (2).

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Materials

TE buffer

Add 1ml of 1M Tris-HCl (pH 7.5) in 98.8ml MILIQ-water and then add 0.2ml of 0.5M EDTA.

20mg/ml proteinase K

Add 0.2g of proteinase K in 10ml MILIQ-water.

Lysis buffer

10% SDS

Dissolve 20 g sodium dodecyl sulfate in MILIQ-water in a total volume of 100 ml with stirring. Filter sterilizes using a 0.45-µm filter.

5M NaCl

Dissolve 29.22g NaCl in 100ml MILIQ-water. Autoclave for 15-20mins at 121℃.

CTAB/NaCl solution

Dissolve 4.1g NaCl in 80ml MILIQ-water and then add 10g of CTAB with heating and stirring. Then adjust volume to 100ml.

Chloroform/isoamyl alcohol(24:1)

Phenol/chloroform/isoamyl alcohol(25:24:1)

Isopropanol

70% Ethanol

To 60 ml MILLIQ-water add 140 ml of 100% ethanol.

MILIQ Water (sterile)

Water bath (for incubations at 37℃, 65℃)

Pipettes, Tips

Microcentrifuge, Microcentrifuge tubes

Vortex

Method

1. 在實驗前先將 proteinase K 於冰上退冰。
2. 將樣品(約 2×2cm)加入液態氮磨碎。
3. 將樣品 1mL 離心 12000rpm 5 分鐘後倒掉上清液，加入 TE buffer 1mL，Vortex 離心 12000rpm 5 分鐘，重複三次。
4. pellet 用 500μL Lysis buffer 重新懸浮，加入 30μL of 10% SDS, and 3μL of 20mg/mL proteinase K（加入時 tip 要深入液面），Vortex，水浴 1 小時 at 37℃。
5. 加入 100μL of 5M NaCl，快速混合。
6. （使用寬口 tip）加入 80μL CTAB/NaCl solution（加入時 tip 要深入液面），Vortex，水浴 10 分鐘 at 65℃。
7. 加入 Chloroform/isoamyl alcohol 500μL，Vortex，離心 12000rpm 5 分鐘。
8. 取上清液至新的 tube，加入等比例(約 500μL)的 phenol/chloroform/isoamyl alcohol(下層液)，Vortex，離心 5 分鐘.
9. 取上清液至新的 tube，加入 0.6 倍的 2-propanol(約 300μL)，(不可 Vortex，只可輕輕混勻)，離心 7 分鐘。
10. 先倒掉上清液，加入 70%的酒精 300μL，離心 5 分鐘.
11. 倒掉上清液，加入滅菌過的超純水保存在 4℃冰箱裡。