

MICROBIAL COMMUNITY OF BLACK BAND DISEASE ON INFECTION, HEALTHY, AND DEAD PART OF SCLERACTINIAN *Montipora* sp. COLONY AT SERIBU ISLANDS, INDONESIA

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ABSTRACT

It is crucial to understand the microbial community associated with the host when attempting to discern the pathogen responsible for disease outbreaks in scleractinian corals. This study determines changes in the bacterial community associated with *Montipora* sp. in response to black band disease in Indonesian waters. Healthy, diseased, and dead *Montipora* sp. (n = 3 for each sample type per location) were collected from three different locations (Pari Island, Pramuka Island, and Peteloran Island). DGGE (Denaturing Gradient Gel Electrophoresis) was carried out to identify the bacterial community associated with each sample type and histological analysis was conducted to identify pathogens associated with specific tissues. Various *Desulfovibrio* species were found as novelty to be associated with infection samples, including *Desulfovibrio desulfuricans*, *Desulfovibrio magneticus*, and *Desulfovibrio gigas*, *Bacillus benzoovorans*, *Bacillus farraginis* in genus which previously associated with pathogenicity in corals. Various bacterial species associated with uninfected corals were lost in diseased and dead samples. Unlike healthy samples, coral tissues such as the epidermis, endodermis, zooxanthellae were not present on dead samples under histological observation. Liberated zooxanthellae and cyanobacteria were found in black band diseased *Montipora* sp. samples.

KEYWORDS: bacteria, black band disease, *Montipora* sp., Seribu Islands

INTRODUCTION

Vast research throughout the Caribbean over the past few decades has provided great insight into the epidemiology of coral disease,

providing a platform that can be utilised in other regions of the world. In the mid 1990s, 18 types of coral disease were recorded, infecting at least 150 species of scleractinian, gorgonian,

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and hydrozoans corals throughout the Caribbean and Indo-Pacific. Despite this, Koch's postulates (to determine the causal agent of a disease) have only been fulfilled for 5 of these 18 diseases (Sutherland *et al.*, 2004).

Bacterial infections of corals can lead to devastating large-scale losses on coral reefs, resulting in reef deterioration and a disturbance for the ecosystem as a whole (Harvell *et al.*, 2007).

Black Band Disease (BBD) is a classic example of such an infection, where by the disease can reduce and alter the structure of coral reef communities (Rosenberg & Loya, 2004). The extent of damage from BBD in Indonesian reefs has yet to be reported.

Changes in the bacterial communities associated with corals have been identified in various infectious diseases of corals and can be used to determine the causal agent of a given disease, including that of Black Band Disease. BBD can be found in various coral species (Voss, 2006), including, the massive corals (Frias-Lopez *et al.*, 2004), Faviidae (Barneah *et al.*, 2007) and coral foliose (Sato *et al.*, 2010). The disease has also been found to have a wide geographic distribution, having been identified in the Caribbean, Indo-Pacific waters, the Red Sea, the Great Barrier Reef (Willis *et al.*, 2004), as well as Indonesian waters (Haapkylä *et al.*, 2007).

Previous research has proposed that the primary cause of Black Band Disease is the bacteria *Phormidium corallyticum*, but further clarification is necessary to support this claim (Cooney *et al.*, 2002). It has also been suggested that BBD is caused by a consortium of microorganisms which are being dominated by cyanobacteria. Cyanobacteria are made up of an array of species, including filamental *Phormidium corallyticum*, heterotrophic bacteria (Garrett & Ducklow, 1975), marine fungi (Ramos-Flores, 1983) and bacteria that can oxidizes sulfite (Beggiatoa) and sulphate reducing (*Desulfovibrio*) (Ducklow & Mitchell, 1979; Richardson, 1996).

Black Band Disease is characterized by the presence of a progressive black band (width 5-30 mm) on the surface of a coral, leaving a bare skeleton with a lack of soft tissue behind. The rate at which these bands progress can reach up to 2 cm per day (Rutzler *et al.*, 1983), with exposed skeleton being quickly colonised

by algae. As BBD appears to be activated at temperatures exceeding 28°C, infections are found to increase during summer months and dramatically decrease in winter (Cooney *et al.*, 2002), suggesting the causal agent may be a thermophilic organism.

The use of molecular techniques to determine bacterial causal agents of coral diseases, (including BBD) has yet to be conducted for disease cases from Indonesia. Several studies have reported the different in bacterial communities found on corals with different surrounding environment such as aquaria and nature (Sweet *et al.*, 2011; Sweet *et al.*, 2012). This valuable approach can be used to analyse microbial community structure and species composition associated with diseased tissues, through the amplification of ribosomal DNA (rDNA) using specific 15S rRNA primers, via polymerase chain reaction (PCR), followed by sequence analysis of cloned rDNA (Muyzer *et al.*, 1993).

This study aims to identify the prevalence of BBD across three different Indonesian reefs, all at different distances from the mainland of Java Island. Additionally, the prokaryotic community associated with healthy, BBD diseased and dead tissue is also determined, helping to elucidate the causal agent of this disease at these locations.

MATERIALS AND METHODS

Sample Collection

Coral samples were taken from zone 1 (Pari Island), zone 2 (Pramuka Island) and zone 3 (Penjaliran Island) using hammer and punch. Three different types of sample were collected, based on disease state, including healthy tissue, BBD-affected tissue and dead tissue from the same coral colony. Samples were taken and measured 5 cm² in 4 replicates of each sample. Samples were collected for the purposes of identifying associated bacteria and histology (Figure 1).

Upon collection, samples for prokaryotic analysis were placed into separate sterile containers containing 70% ethanol, allowing samples to be preserved until DNA extraction could take place at the Coral Health and Disease laboratory at the Newcastle University School of Biology, United Kingdom.

Samples for histological analysis were suspended in seawater taken from the reef upon

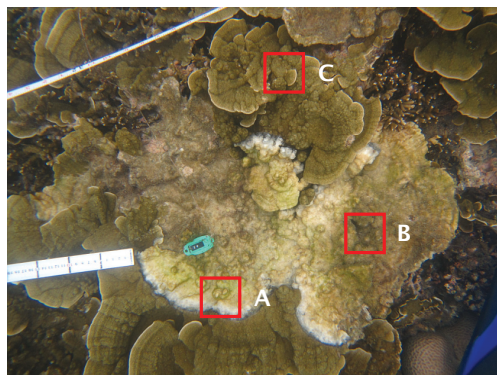


Figure 1. Photograph representing the manner in which samples were collected for the determination of bacterial communities associated with tissue type and histological analysis. Black band diseased sample (A), dead sample (B), and healthy sample (C)

collection before further sample preparation was conducted at Newcastle University.

Bacteria Identification

Coral samples were crushed with a sterile pestle and mortar and DNA was subsequently extracted using a Blood and Tissue DNA Extraction Kit (Qiagen). PCR was carried out, following the same protocol as Smith *et al.* (2014) using a Hyaid thermal cycler to amplify target 16S rRNA using the primers 357f-GC-518r (Muyzer *et al.*, 1993). Denaturing Gradient Gel Electrophoresis (DGGE) was utilised to profile the bacterial community based on the separation of 16S rRNA bands, which were subsequently purified using a Qiagen Gel Extraction Kit. DGGE was conducted according to Smith *et al.* (2014). Purified samples were sent for sequencing at Genevision (Newcastle University). Sequence data was analysed by comparing sequences with those in the BLAST database (<http://blast.ncbi.nlm.nih.gov/>) to identify the species of bacteria (Altschul *et al.*, 1997).

Histology

Healthy, BBD-affected, and dead coral samples were preserved in 5% paraformaldehyde (diluted with seawater) for a minimum period of one week, then decalcified using 10% EDTA (dissolved in distilled water) over a pe-

riod of weeks, with the 10% EDTA being replaced for a fresh 10% EDTA in every few days. The healthy, BBD-affected and dead *Montipora* sp. samples for histological analysis were prepared and sectioned following the same protocol used by Smith *et al.* (2014).

Data Analysis

Similarities in the microbial community associated with the different sample types (healthy, BBD-affected, and dead) were analyzed using BioNumeric software. This analysis identified 18 bands across all sample types, each band representative of a dominant prokaryotic member of the microbial community.

RESULTS

Bacteria Associated with Coral Samples

Based on DGGE analysis of the dominant prokaryotic community, 18 different bacterial species were identified across all three sample types (Figure 2). The diversity of bacteria in BBD-affected corals was higher than in healthy and dead corals samples.

Healthy Coral Samples

Species identified across all healthy samples include those represented by the following bands; 1 (*Halomonas anticariensis*), 8 (*Pseudoalteromonas denitrificans*), 13 (*Psychromonas ingrahamii*), 16 (*Shewanella benthica*), 17 (*Shewanella loihica*), and band 18 (*Vibrio fischeri*). Band 13 (*Psychromonas ingrahamii*) was only found in two healthy samples (H1 and H2) (Figure 2).

Bacteria of the *Halomonas* genus are halophilic bacteria, usually isolated from highly saline environments. They are gram-negative bacteria that live at temperatures range of 20°C-45°C and at pH 6-9 (Martinez-Canovas *et al.*, 2004). *Halomonas anticariensis* (Gammaproteobacteria) was first isolated from highly saline found in peatland environments. Bacterium belonging to the genus *Halomonas* have been found on the coral species *Acropora* sp. tissue. *Pseudoalteromonas denitrificans*, belongs to genus of bacteria often associated with a host organism from the marine environment, such as fish, mollusks, tunicates, sponges, and micro and macro algae. Some members of the genus *Pseudoalteromonas* (including *P. denitrificans*) have been found pro-

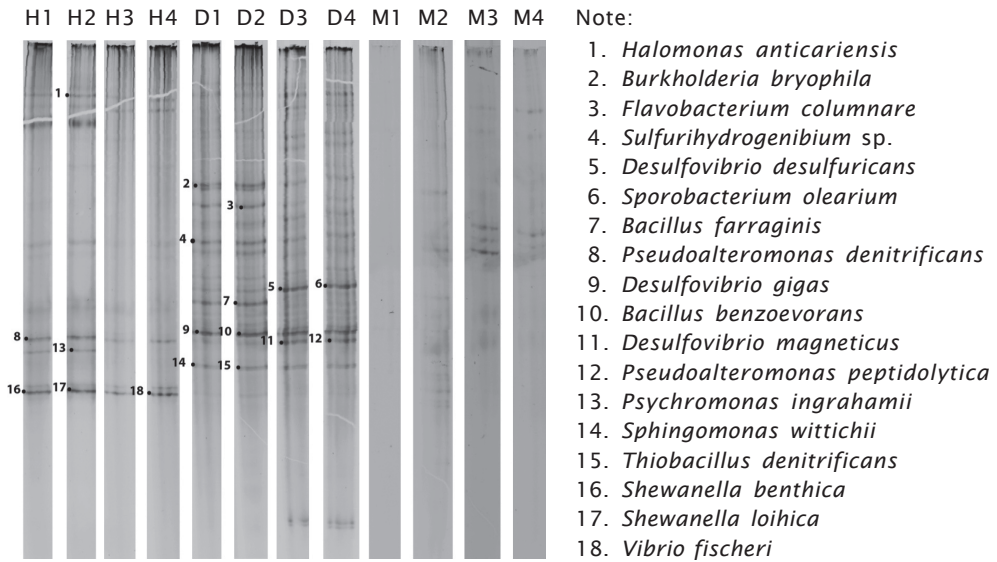


Figure 2. Band profile of DGGE result on DNA extraction of health (H1-H4), disease (D1-D4) and dead sample (M1-M4) by gen test using primer 16S rRNA and PCR application

ducing active compounds which also being isolated from seawater samples (Skovhus *et al.*, 2004). *Pseudoalteromonas ruthenica* has previously been isolated from the coral *Acropora* sp. (Raina *et al.*, 2009).

Vibrio fischeri (of the family Vibrionaceae) is a motile, gram-negative, rod-shaped bacteria found in both seawater and freshwater. *Vibrio fischeri* are also bioluminescent and are often found in symbiosis with marine animals such as squid (Small & McFall-Ngai, 1999). *Vibrio fischeri* has previously been identified in the coral *Acropora* sp. (Raina *et al.*, 2009). *Vibrio* spp. are often associated with coral pathogenicity, thus it is interesting to see how they are associated with healthy coral samples in this instance.

Shewanella spp. are gram-negative bacteria and have previously been identified across a range of environments, including marine environments, freshwater rivers and lakes, and soil. This genus has previously been discovered by other researchers on healthy corals such as *Shewanella* sp. (Sekar *et al.*, 2006), *Bacillus algicola* (Sabdono & Radjasa, 2006), *Halomonas* sp. (Ojima *et al.*, 2012), and *Sphingomonas wittichii* (Rosenber & Ben-Haim, 2002).

There are 9 genera bacteria associating with the coral i.e. *Vibrio* sp., *Alteromonas* sp.,

Psychrobacter sp., *Ralstonia* sp., *Silicibacter* sp., *Bacillus* sp., and *Cytophaga* sp. (Abdelnasser & Ibrahim, 2008).

Black Band Disease Samples

Bacterial bands associated with BBD-affected coral samples (Figure 2) i.e., *Burkholderia bryophila* (2), *Flavobacterium columnare*, (3) *Sulfurihydrogenibium* sp. (4), *Desulfovibrio desulfuricans* (5), *Sporobacterium olearium* (6), *Bacillus farraginis* (7), *Desulfovibrio gigas* (9), *Bacillus benzoovorans* (10), *Desulfovibrio magneticus* (11), *Pseudoalteromonas peptidolytica* (12), *Sphingomonas wittichii* (14), and *Thiobacillus denitrificans* (15).

Almost all of the bands associated with BBD samples were found across all BBD samples, including bands 2-6, and 9-15. Band 7 was only found in two BBD-affected samples (D1 and D2) (Figure 2).

Desulfovibrio spp. are gram-negative bacteria, generally associated with marine, brackish and freshwater environments. Their presence is also found to flourish as the environment becomes more anoxic. *Desulfovibrio* spp. belong to a class of bacteria that reduce sulfate and thus they have a role in the cycling of sulfur. In corals, this would cause localised anoxia and increased sulphide levels in host tissues, which would be harmful to the coral

and could ultimately result in colony death (Viehman *et al.*, 2006). Further, Viehman *et al.* (2006) state that many previous molecular-based studies have found several *Desulfovibrio* spp. to be associated with black band disease.

Bacillus spp. are ubiquitous in nature and this genus is found to include free-living and pathogenic species. *Bacillus* spp. are gram-positive, rod-shaped bacteria that can be grown in aerobic and anaerobic conditions. Spores from *Bacillus* spp. are resistant to heat (high temperature). These bacteria are known to degrade xylan and carbohydrates, as well as catalyze enzymes. *Bacillus* spp. have previously been isolated from the sponge species *Petromica citrina* and *Chelonaplysilla erecta* (Bastos *et al.*, 2013).

The genus *Burkholderia* belongs to the betaproteobacteria group, comprising more than 60 species which can be found in several habitats such as, water, plant roots, or it can be a causal disease in plants or humans (Santos *et al.*, 2013). Despite this, this is the first report of bacteria belonging to this genus in coral reefs and diseased corals.

Flavobacterium columnare is a gram-negative, rod-shaped bacteria which has previously been found to cause disease in freshwater fish. This species can produce an enzyme that degrades chondroitin sulfate (Figueiredo *et al.*, 2005). This is the first report of this species in a diseased coral.

Sulfurihydrogenibium spp. are motile, gram-negative, rod-shaped bacteria capable of oxidizing sulphur, such that they are believed to play an important role in the sulfur cycle (Nakagawa *et al.*, 2005). These bacteria have previously been associated with pathogenicity in corals.

Sporobacterium olearium bacteria can damage methoxylated aromatic compounds and syringate in significant amounts in the presence of sulfide. These bacteria can be found in both freshwater sediments and the marine environment (Lomans *et al.*, 2001), although there are no previous reports associating it with corals and pathogenicity in corals.

Pseudoalteromonas peptidolytica are motile, gram-negative bacteria belonging to the class Gammaproteobacteria. This bacteria is associated with aerobic environments and has previously been isolated from seawater in the Sea of Japan. Interestingly, bacteria of this ge-

nus have previously been reported as pathogens in fish (Vynne, 2011) and mussels (Venkateswaran & Dohmoto, 2000).

Pseudoalteromonas ruthenica has previously been isolated from the coral *Acropora* sp (Raina *et al.*, 2009).

Sphingomonas wittichii is an alphaproteo bacteria and was first isolated from river water. It is known to be a good metabolizer of dibenzop-dioxin (Yabuuchi *et al.*, 2001). *Sphingomonas* spp. are gram-negative, rod-shaped, chemoheterotrophic bacteria usually associated with an oxygenated environment. These bacteria can cause disease in humans (especially *Sphingomonas paucimobilis*). *Sphingomonas* bacteria are ubiquitous in nature, have been isolated from soil, water and plant roots, largely these bacteria are able to survive in low nutrient conditions. To date, these bacteria have not been associated with pathogenicity of corals.

Thiobacillus denitrificans are gram-negative, rod-shaped bacteria, belonging to the class betaproteobacteria. These bacteria have been found in seawater sediments and are able to oxidize sulfur to sulfate, a process in line with the absorption of oxygen and nitrate reduction (Aminuddin, 1979). *Thiobacillus denitrificans* is also able to reduce nitrate and nitrite (Haaijer *et al.*, 2006). This bacterium has not been reported to infect corals.

Out of the bacteria found in BBD-affected samples, many of them belong to the group Cyanobacteria, such as *Desulfovibrio desulfuricans*, *Desulfovibrio gigas*, *Desulfovibrio magneticus*, *Bacillus farraginis*, *Bacillus benzoovorans*. Cyanobacteria are a major cause of black band disease in corals (Richardson, 1998).

Dead Coral Samples

Few bands were found on dead coral samples and of those bands identified, none were found consistently across all dead samples (M1-M4).

It is evident that Black Band disease begins with high abundance of cyanobacteria, known as cyanobacterial patches (CP). CPs were found on several colonies of *Montipora* spp. in shallow waters at Pramuka Island which were overgrown with algae a few days later, indicating those coral colonies had died. Tissue damage between CP and BBD can be eas-

ily distinguished in the field, as CPs are not associated with a black belt. Colonies found to have a CP did not always develop BBD (Figure 3). These observations are similar to that reported by Sato *et al.* (2010) in the Great Barrier Reef.

CPs identified in *Montipora* sp. colonies (Figure 3A) were found to grow in size after 2 weeks (Figure 3B). Exposed skeleton was then subsequently colonised by algae (Figure 3C). Microscopic observations of CPs revealed a large numbers of cyanobacteria filaments.

Histology Observation

It was easy to indicate epidermis layer, gastrodermis, as well as zooxanthellae in healthy *Montipora* sp. samples, whereas these tissue structures were found to be broken down in BBD samples. Long, rod-shaped structures resembling filamental cyanobacteria were identified in abundance in BBD samples (Figure 4B). These cyanobacteria were not found in dead coral samples and zooxanthellae were also can not be found along with any healthy tissue structure.

Bacterial communities were grouped and undergo a treatment in accordance with the samples between healthy (H) and diseased (D) coral. Excluding the samples of dead coral that 2M2 is not clustered either to 3M1 and 2M2 samples. The test results explained that the bacteria live on the healthy coral is different with diseased and dead sample (Figure 5).

DISCUSSION

There are 3 species of *Desulfovibrio* spp. found on infected coral samples in the Seribu Islands i.e. *Desulfovibrio magneticus*, *Desulfovibrio gigas*, and *Desulfovibrio desulfuricans*. *Desulfovibrio* spp. are pathogenic because they produce and accumulate sulfide in high concentrations in black band disease, giving rise to anoxic conditions, which ultimately leads to coral mortality (Richardson *et al.*, 1997).

Another type of bacteria found in BBD infected samples at Seribu Islands were *Bacillus* spp. (*Bacillus benzoovorans* and *Bacillus farraginis*). Although these species have not been reported to be pathogenic to corals in the past, *Bacillus* spp. have been found to have

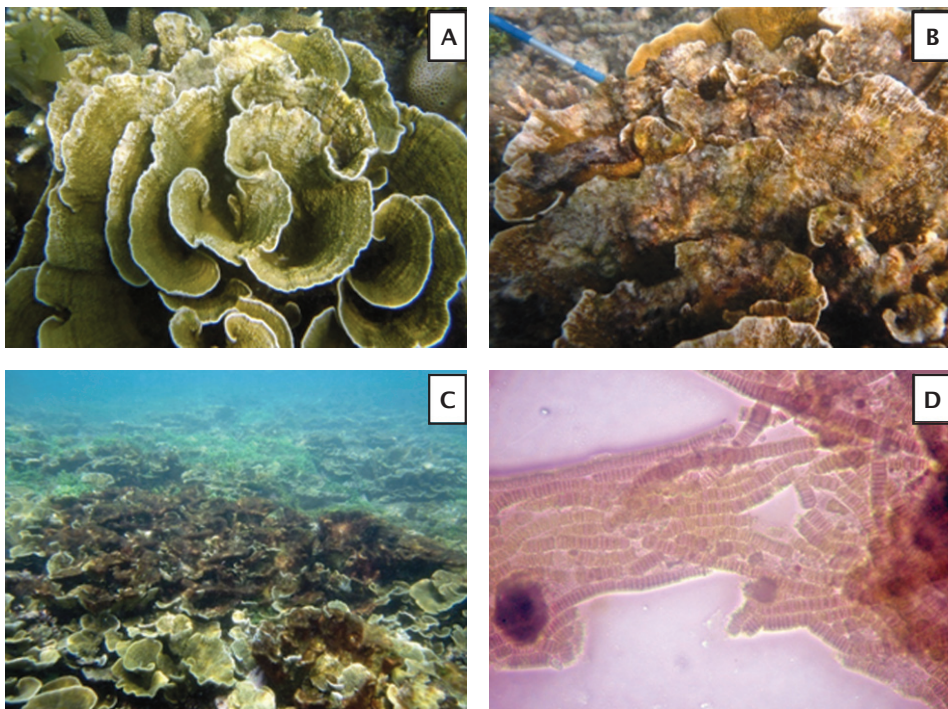


Figure 3. *Montipora* sp. with CP (A, B) after overgrown with algae (C) and cyanobacteria patches (CP) under microscope observation 40x cyanobacterial filaments (D)

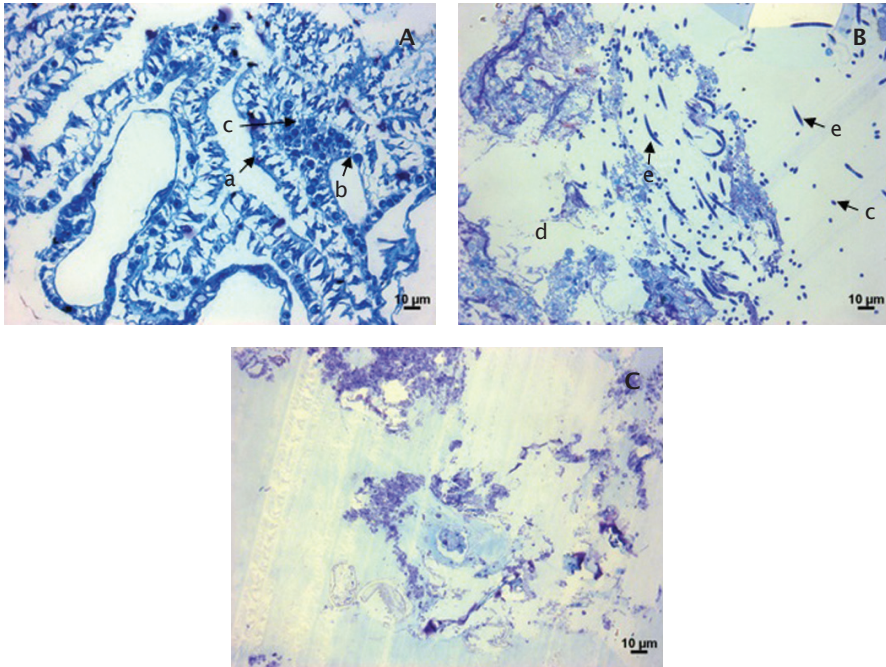


Figure 4. Histological sections of healthy (A), Black Band Disease (B), and dead (C) *Montipora* sp. tissue under light microscopy (magnification x 40), stained with toluidine blue. Specific coral tissues are labelled, including epidermis (a), endodermis (b), and zooxanthellae (c). Infected coral samples (B); damaged coral tissue (d) and cyanobacteria (e)

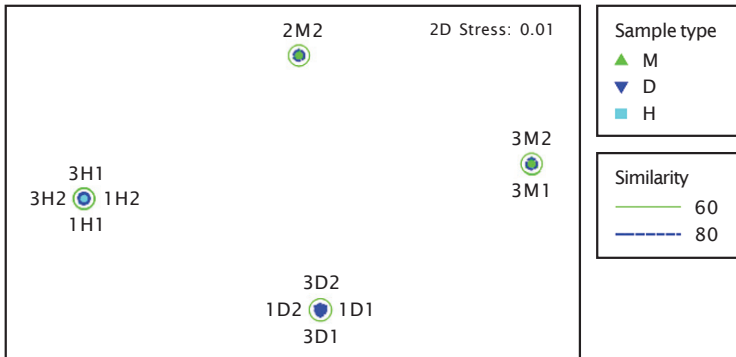


Figure 5. The similarity rate of bacteria on healthy, infected, and dead sample according to different sites by using Primer 6 programme

antibacterial properties against other bacteria and their influx could disrupt the native coral microbiota (Sabdono & Radjasa, 2006). This could potentially having a negative impact upon symbioses between the host coral and microbial members of the holobiont if key species are reduced/lost.

Sulfurihydrogenibium sp. is a bacteria that can oxidize hydrogen and sulfur (Hirayama *et al.*, 2005), but there is no report that describes it as a pathogenic bacteria in corals.

Shewanella sp. was found to be associated with healthy *Montipora* sp. samples in the

present study, coinciding with previous reports that associate this bacteria with healthy corals (Frias-Lopez *et al.*, 2002). Despite this, other studies have also found these bacteria to be associated with infected corals (Sekar *et al.*, 2006), thus there is the potential that healthy samples harbouring this bacterial species may become diseased following an influx associated with favourable environmental conditions (Frias-Lopez *et al.*, 2002; and Rohwer *et al.*, 2002).

Sphingomonas sp., found in BBD samples in the present study, have previously been associated with healthy coral (Rosenberg & Ben-Haim, 2002). That said, members of this genus have also been associated with corals suffering from White Plague in Florida, demonstrating their pathogenic potential (Ravindran *et al.*, 1998).

Although Black Band Disease (BBD) has been known for decades (since 1970), the main pathogenic bacteria of the disease has yet to be identified. Previously, it was considered that BBD was caused by the bacteria *Phormidium corallyticum*. But, the use of molecular techniques to identify the microbial community associated with this disease has led to the discovery of a consortium of bacteria associated with BBD (Frias-Lopez *et al.*, 2004). This consortium is comprised of various cyanobacteria, sulfate-reducing bacteria such as *Desulfovibrio* spp., sulfide-oxidizing *Beggiatoa* spp. and marine fungi. This makes it difficult to fulfil Koch's postulates for BBD, as this approach is more suited to diseases with single or minimal causal agents (Richardson, 1998; Sutherland *et al.*, 2004).

Sato *et al.* (2010) has identified 262 cases of CP in *Montipora* sp. in the Great Barrier Reef and 18.7% of these cases developing into BBD. In contrast to this, CP cases in *Montipora* spp. in the Thousand Islands (or Seribu Island) were not found to develop into BBD, with a recent outbreak of BBD reaching a recent peak (Johan, 2013, pers obs) appearing to exist as a separate disease.

CONCLUSION

Bacteria belonging to the genus *Bacillus* sp., *Sulfurihydrogenibium* sp. and *Desulfovibrio* spp. are associated with BBD in *Montipora* sp. in Indonesian reefs, with *Desulfovibrio* spp. appearing to be a strong causal agent for this disease. *Sphingomonas wittichii*, *Halomonas*

sp., *Bacillus algicola*, and *Shewanella* sp. are all associated with the healthy *Montipora* sp. holobiont in this region. Following histological analysis, there are marked differences between healthy, BBD-affected and dead *Montipora* sp. tissues. In BBD-affected samples, there is mass breakdown in host tissue, zooxanthellae are lost and filament-shaped bacteria are present. These filament-shaped bacteria are lost in dead samples, thus clearly demonstrating their presence is strongly associated with BBD tissues.

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