Phylogeny of the coral pathogen *Vibrio coralliilyticus* and the development of a qPCR-based diagnostic assay for its detection

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ABSTRACT

Coral disease has emerged over recent decades as a significant threat to coral reef ecosystems, with declines in coral cover and diversity of Caribbean reefs providing an example of the impacts of disease at regional scales. If similar trends are to be mitigated or avoided on reefs worldwide, a deeper understanding of the factors underlying the origin and spread of coral diseases, as well as the steps that can be taken to prevent, control, or reduce their impacts is required. In recent years, an increased focus on coral microbiology has revealed several coral pathogens that could serve as targets for a new generation of coral disease diagnostic tools. The development and implementation of novel diagnostic techniques, which specifically target these pathogens, will allow accurate coral disease detection and diagnosis, pathogen load monitoring, and identification of pathogen sources, vectors, and reservoirs. These capabilities will allow reef managers to discern the threats that may affect the occurrence, prevalence, or severity of diseases so their sources can be identified, and possibly reduced through better management practices. In the first chapter, I establish the need for sensitive and specific molecular-based coral pathogen detection, outline the emerging technologies that could serve as the basis of a new generation of coral disease diagnostic assays, and address the unique challenges inherent to the application of these techniques to environmentally-derived coral samples.

The coral pathogen *Vibrio coralliilyticus* represents a good model system to study coral disease. It has been implicated as the etiological agent responsible for bleaching and tissue lysis in a number of scleractinian coral species throughout the Indo-Pacific and has been the focus of research efforts characterizing the organism's genome, proteome, and metabolome. Little is known, however, about the population

genetics of *V. coralliilyticus*, its evolutionary history, or the population dynamics of this widely distributed species. In order to determine whether this bacterium exists as a single cosmopolitan clonal population, which might indicate rapid spread of a pandemic strain, or is grouped into endemic and genotypically distinct strains, a phenotypic and phylogenetic comparison of geographically disparate isolates was conducted. Five phylogenetic marker genes (*16S*, *rpoA*, *recA*, *pyrH*, and *dnaJ*) frequently used for discriminating closely related *Vibrio* species and a zinc-metalloprotease gene (*vcpA*) linked to pathogenicity were sequenced in thirteen isolates collected from the Red and Caribbean Seas, and Indian, Pacific, and Atlantic Oceans. No evidence of clonality or consistent lineage structure was observed, suggesting that *V. coralliilyticus* represents an endemic components of coral reef ecosystems that varies genetically among the globally distributed geographic locations sampled.

To gain a more complete understanding of the epidemiology of *V. coralliilyticus*, including information on its distribution, incidence of infection, and rates of transmission through populations, a real-time quantitative PCR (qPCR)-based detection assay for *V. coralliilyticus* was developed. The assay, which targets the *dnaJ* gene, a housekeeping gene encoding for heat shock protein 40, was highly sensitive, detecting as little as 0.1 pg of purified *V. coralliilyticus* DNA and 10^4 colony forming units (CFU) per reaction (20 μL) for pure bacterial cultures. Inhibition of the assay by DNA and cells derived from bacteria other than *V. coralliilyticus* was minimal. These findings support the utility of this assay to target the pathogen within the complex coral holobiont. This assay represents a novel approach to coral disease diagnosis and provides a useful tool for coral pathogen detection and accurate diagnosis, which will play a vital role in advancing the field of coral disease research.

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ABSTRACT

Coral disease has emerged over recent decades as a significant threat to coral reef ecosystems. Declines in coral cover and diversity of Caribbean reefs provide an example of the potential impacts of disease at regional scales and if similar trends are to be mitigated or avoided on reefs worldwide, a deeper understanding of the factors underlying the origin and spread of coral diseases, as well as the steps that can be taken to prevent, control, or reduce their impacts is required. Highly skilled individuals base current coral disease diagnosis almost exclusively upon observations of macroscopic disease signs, although often *in situ* diagnosis is difficult because different diseases present similar signs. In recent years, an increased focus on coral microbiology and the application of classic culture techniques and emerging molecular technologies has revealed several coral pathogens that could serve as targets for a new generation of coral disease diagnostic tools. The development and implementation of novel diagnostic techniques, which specifically target the microbial agents that have been identified or implicated in coral disease, will aid in elucidation of disease causation and the implementation of possible management strategies. These molecular assays will allow accurate coral disease detection and diagnosis, pathogen load monitoring in individuals and ecosystems, and identification of pathogen sources, vectors, and reservoirs. These capabilities will advance the field of coral disease research and contribute the knowledge necessary to effectively

manage the world's coral reefs. This paper establishes the need for sensitive and specific molecular-based coral pathogen detection, outlines the emerging technologies that could serve as the basis of a new generation of coral disease diagnostic assays, and addresses the unique challenges inherent to the application of these techniques to environmentally-derived coral samples.

1.1. **THE NEED FOR IMPROVED CORAL DISEASE DIAGNOSTIC TOOLS**

The world's coral reefs are in decline, with hard coral cover on Caribbean reefs decreasing by an average of 80% in the last 30 years (Gardener et al. 2003) and Indo-Pacific reefs suffering an average loss of 1% per year since the early 1980s (Bruno and Selig 2007). The causes of these declines are diverse and complex, including water pollution, habitat destruction, overfishing, invasive species, and global climate change (Walker and Ormond 1982; Bryant et al. 1998; Bellwood et al. 2004). In recent years, coral diseases have also emerged as a significant threat to the world's coral reef ecosystems (Rosenberg et al. 2007; Bourne et al. 2009). Since the first coral disease was described in 1973, evidence from field studies documenting the population- and community-level impacts of disease on coral reef ecosystems worldwide has been accumulating (reviewed in Weil 2004; Aronson and Precht 2001; Patterson et al. 2002; Willis et al. 2004; Weil et al. 2006; Harvell et al. 2007) and it is now clear that coral diseases have the potential to cause widespread mortality and significantly alter reef community structure (e.g. Aronson and Precht 2001; Porter et al. 2001; Sutherland et al. 2004; Miller et al. 2009).

Despite the serious threat that coral diseases pose to the health of reef ecosystems globally, little is known about many basic aspects of these diseases, including their etiologies, how they are spread, and the steps that can be taken to prevent, control, or reduce their impacts. This work has been greatly hindered by a lack of identified causative agents for many diseases, inadequate diagnostic tools, and insufficient application of diagnostic procedures following established biomedical principles (Work et al. 2008). Current diagnostics focus on documenting disease signs *in situ*, describing macroscopic characteristics such as species infected, extent and pattern of tissue loss (Antonius 1977), presence and appearance of microbial mats (Porter et al. 2001), abnormal coloration (Antonius 1981), or skeletal anomalies (Loya

et al. 1984). Corals display few macroscopic signs indicative of stress and consequently an array of maladies, including environmental stress, predation, and infectious disease, are often manifested as a paling or sloughing of the coral tissue. For example, more than six "white" diseases, which are characterized by a spreading zone of tissue loss, exposing white coral skeleton directly adjacent to healthy coral tissue, have been described in the Caribbean Sea alone (Sutherland et al. 2004; Fig. 1). Due to their nearly identical appearance, several of these diseases (e.g. white plague I and white plague II) are differentiated almost exclusively by the rate of lesion progression through the infected colony (Sutherland et al. 2004). Such difficulties have resulted in cases of misidentified diseases, repeated name changes for the same disease (Richardson et al. 1998), and even classification of predation scars as disease (Goreau et al. 1998; Bruckner and Bruckner 2002; Patterson et al. 2002). Currently, it is uncertain how many distinct coral diseases exist worldwide; in two articles published in the same year, one report identified 18 diseases while another put the number at 29 (Sutherland et al. 2004, Weil 2004). This confusion underlines the need for more robust coral disease diagnostic methods.
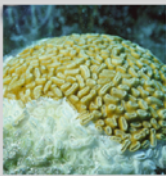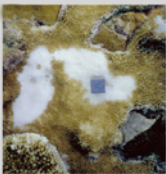
Disease	Appearance	Published Description	Reference
White Plague II		"A sharp line of tissue loss where healthy tissue is immediately adjacent to recently denuded skeleton"	Sutherland et al. 2004
White Band I		"A white band of recently denuded skeleton adjacent to a necrotic front of normally pigmented living tissue."	Sutherland et al. 2004
White Pox		"Irregularly shaped distinct white patches of recently exposed skeleton surrounded by a necrotic front of normally pigmented living tissue."	Sutherland et al. 2004

Figure 1. Examples of "white" diseases affecting Caribbean corals.

In recent years, an increased interest in coral microbiology, in combination with the application of histology and biomedical approaches to diagnosis and the development of new molecular approaches, has revealed several bacterial species linked to coral disease lesions (Rosenberg et al. 2007; Sussman et al. 2008). Debate exists as to the primacy of a compromised coral host versus opportunistically proliferating bacteria in causing coral diseases (Lesser et al. 2007, Ainsworth et al. 2008). However, since disease is classically considered to be the outcome of interactions among a causative agent, susceptible host and the environment (e.g. Wobeser 2006), debating the status of an etiological agent as either a primary or secondary pathogen is diversionary and does not negate the need to understand its role in pathogenesis (Jubb et al. 1993; Work et al. 2008). Using these potential pathogens as targets for a new generation of sensitive and highly specific, molecular-based diagnostic assays, we can begin to answer many of the basic questions that plague the field of coral disease research. The ability to detect specific coral pathogens in both the coral host and its environment will allow researchers and reef mangers to: 1) better understand disease etiologies, 2) make more accurate diagnoses, 3) monitor pathogen loads in coral hosts and their environment, 4) identify pathogen sources, vectors, and reservoirs, and 5) make better informed management decisions. This review outlines the need for novel, pathogen-specific coral disease diagnostics, the technologies that could serve as the basis for such tools, and the challenges inherent to the adoption of these technologies for pathogen detection within the complex coral holobiont.

1.2. **BENEFITS OF PATHOGEN-SPECIFIC DETECTION TOOLS**

In this section, I highlight the role that specific and sensitive pathogen detection will play in advancing our understanding of the etiology, spread, and ultimately management of coral diseases.

1.2.1. **EARLIER AND MORE ACCURATE DISEASE DIAGNOSIS**

The coral holobiont comprises a complex association among the coral animal and its microbial partners, including symbiotic dinoflagellates (zooxanthellae) (Muscatine et al. 1981), bacteria (Rohwer et al. 2002), Archaea (Wegley et al. 2004), viruses (Wilson et al. 2005), endolithic algae (Shasher et al. 1997), and fungi (Bentis et al. 2000). Numerous studies have examined these associations in both healthy and stressed corals and it has been suggested that shifts in these microbial communities can act as indicators of coral stress (Pantos et al. 2003; Bourne et al. 2008; Vega-Thurber et al. 2009). For example, Pantos et al. (2003) demonstrated bacterial community shifts throughout the entire coral colony, even when just a small part of the colony showed signs of disease, and Bourne et al. (2008) reported dramatic shifts in coral-associated microbial communities well before the appearance of visual signs of thermal bleaching. Using a metagenomics approach, Vega-Thurber et al. (2009) demonstrated functional gene shifts, including an increased abundance of virulence genes, in coral microbial partners during temperature, nutrient, and pH stress. Kimes et al. (2010) observed significant differences in biogeochemical cycling-related genes between healthy and yellow-band infected *Montastraea faveolata* colonies. Sagawa et al. (2009) also developed a 16S rRNA gene-based microarray to assess coral microbial associations and demonstrated shifts in bacterial communities in diseased corals. These community-level bacterial profiling approaches are capable of detecting microbial community shifts prior to visual disease signs, which would facilitate diagnosis at the earliest stages of infection when mitigation measures would be most

effective (Teplitski and Ritchie 2009). Efforts to develop rapid and sensitive assays to monitor coral-associated microbial communities as proxies for coral health are important and should be a research focus. The application of microarrays may facilitate the development of such tools (see section 3.5). More targeted, single pathogen assays can also build upon these community-level approaches and help to establish the link between disease causation and the presence of a specific microbial pathogen.

1.2.2. Better understanding of disease etiology

While some coral diseases are tightly linked with the presence of a specific pathogen, the causes of many other diseases and disease-like syndromes remain elusive (Teplitski and Ritchie 2009). Better tools with high specificity for target pathogens would enable investigations of the circumstances under which microbes that are normally found on coral surfaces become pathogenic and the conditions and mechanisms that trigger a switch from commensal or neutral to pathogenic. Moreover, there are cases where bacterial species, which were linked to specific diseases in early studies, no longer elicit the same response or are not associated with disease signs, potentially indicating development of disease resistance (Reshef et al. 2006; Rosenberg et al. 2007). For example, *Vibrio shiloi*, which was initially identified as the agent responsible for annual bleaching of the Mediterranean coral *Oculina patagonica*, no longer appears to cause bleaching in this coral species (Ainsworth et al. 2008). Additionally, *Aspergillus sydowii*, which was shown to cause disease in gorgonians, has also been found on healthy coral colonies, raising questions about its role in disease onset (Toledo-Hernandez et al. 2008). The development of tools to detect and quantify putative pathogens in both controlled laboratory experiments and environmentally-derived samples will help to establish the etiology of specific coral diseases and clarify the role of individual microbes in the onset of

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disease lesions. Once the link between a specific microbial entity and lesion onset is established, pathogen-specific assays can provide information on all aspects of the disease onset process.

1.2.3. MONITOR PATHOGEN LOAD

Emerging evidence suggests that the abundance of coral pathogens varies on reefs throughout the year and within coral hosts during the course of infection (Banin et al. 2000, Sussman et al. 2003; Vezzulli et al. 2010). The ability to quantify pathogen load in coral and environmental samples will allow researchers and reef managers to gauge the health status of individual corals, assess the impact of environmental parameters (e.g. temperature, nutrient load, sedimentation rate) on pathogen load, and better predict large-scale disease outbreaks. Some efforts have been made to establish links between environmental parameters and coral disease prevalence. Using high-resolution satellite datasets and long-term coral disease surveys, Bruno et al. (2007) established a link between coral disease outbreaks and warm temperature anomalies at sites with high coral cover. By monitoring bacterial communities *in situ*, Vezzulli *et al*. (2010) also discovered a link between mass mortality events of the coral *Paramuricea clavata*, seawater temperatures, chlorophyll concentrations, and the presence of culturable *Vibrio* spp. in the surrounding seawater. Better tools for monitoring pathogen density would provide a deeper understanding of how pathogen load and virulence respond to natural (e.g. seasonal, El Niño/La Niña) and anthropogenic (e.g. pesticide and nutrient influx, sedimentation) fluctuations, allowing researchers and managers to closely follow these dynamics and model pathogen response to environmental change.

1.2.4. IDENTIFY PATHOGEN SOURCES, VECTORS, AND RESERVOIRS

It is currently unclear if the recent emergence of diseases is associated with the introduction of pathogenic organisms from terrestrial environments, or whether

disease-causing microbes have always been present but have only recently become pathogenic due to deteriorating environmental conditions and/or reduced host resistance. To better understand the dynamics of coral disease outbreaks and ensure that they are effectively managed, information regarding pathogen sources, vectors, and reservoirs is needed. Pathogen sources are the avenues through which a pathogen enters the environment, reservoirs are the biotic or abiotic origins of a pathogen, and vectors are living entities that do not cause or suffer from a disease, but transmit a pathogen from one host to another (Wobeser 2006). The identification of the marine fireworm as the winter reservoir and spring/summer vector of the coral pathogen *Vibrio shiloi* nicely demonstrates the utility and importance of molecular-based pathogen detection techniques in the study of coral epidemiology (Sussman et al. 2003).

1.2.5. BETTER INFORMED MANAGEMENT DECISIONS

As coral diseases continue to increase in frequency, distribution, and intensity, reef managers will face increasing challenges if priority is not assigned to developing tools to manage, prevent or mitigate their impacts. In order to effectively manage coral disease outbreaks, we must first understand what causes the observed diseases, how these diseases are spread between colonies and populations, and how environmental parameters influence pathogen virulence and host susceptibility to infection (Bruckner 2002). Tools that increase our capacity to establish links between disease signs and the presence of a specific microbial pathogen would significantly advance current understanding of the classification of coral diseases and their underlying causes. Accurate diagnosis will direct research and management strategies to address the true cause of disease on reefs (Ainsworth et al. 2007) and facilitate the development of assays to detect and quantify the pathogens that underlie them. Such assays will help reef managers to discern the threats that may affect the occurrence,

prevalence, or severity of diseases so their sources can be identified, and possibly reduced through better management practices (Bruckner 2002). For example, habitat degradation, increased human impacts, and poor water quality are often speculated as causes of the recent rise in coral diseases (Bellwood et al. 2004), but linkages with specific factors have not been conclusively determined. By understanding the relationship between various stressors and the occurrence of coral diseases, managers may be able to identify potential threats in a timely manner and develop strategies to lessen their impacts (Bruckner 2002). Several biological controls for coral diseases, including bacteriophage therapy and probiotic addition, have recently been proposed (Effrony et al. 2009; Teplitski and Ritchie 2009) and pathogen-specific diagnostics could be used to identify where and when these controls should be implemented and assess their efficacy. In order to assist resource managers to combat disease epizootics, prevent future outbreaks, and reduce the time needed for recovery, the development of sensitive, specific, and robust coral disease diagnostics should be an essential research priority (Bourne et al. 2009).

1.3. PATHOGEN DETECTION METHODS

Effective diagnostic tools must be sensitive, reproducible and specific in their detection of targeted microbial organisms. In the field of human pathogen detection, culture and colony counting-, immunology-, and nucleic acid-based methods are the most commonly used (Lazcka et al. 2007). Here I provide a brief overview of these techniques and evaluate their potential for coral pathogen detection (summarized in Table 1).

Table 1: Summary of pathogen detection techniques and molecular diagnostics.

Technique	Principle	Advantages	Disadvantages	Used for coral pathogen detection?
Culture and colony counting	Samples are plated onto selective growth media, incubated, and resulting colonies counted	• Well established in human disease diagnosis • Low cost	• Extensive development and testing of selective media • Long wait time for test result • Low sensitivity	Sutherland et al. 2010 Vezzulli et al. 2010
Antibodies	Samples are hybridized with pathogen-specific antibodies and antibody/antigens complexes are detected	• Well established in human disease diagnosis • High specificity of monoclonal antibodies	• Monoclonal antibodies are slow to develop • Low specificity of polyclonal antibodies • Antibody-producing cell lines difficult to maintain	Israely et al. 2001
Fluorescent *in situ* hybridization (FISH)	Samples are hybridized with custom-synthesized nucleic acid probes attached to fluorescent reporter molecules and then visualized on imaging microscope	• Allows localization and visualization of microbes within host tissue • Use of different fluorescent reporters allows for simultaneous detection of multiple microbes	• Low specificity of FISH probes • Time consuming and labor intensive processing • Innate fluorescence of zooxanthellae necessitates specialized imaging microscopy equipment	Ainsworth et al. 2006 Ainsworth et al. 2007 Ainsworth et al. 2008
Polymerase chain reaction (PCR)	Samples are subjected to PCR amplification with specific primer sets and PCR products are visualized on agarose gel	• High sensitivity • High specificity	• Not quantitative • High contamination risk • Potential for non-specific primer binding and amplification	Richardson et al. 2001 Ritchie et al. 2001 Bourne et al. 2007 Polson et al. 2008
Real time quantitative polymerase chain reaction (qPCR)	Samples are subjected to PCR amplification incorporating a fluorescent reporter that emits a signal proportional to the quantity of PCR product synthesized	• High sensitivity • High specificity • Low contamination risk • Quantitative results	• High cost • Requires specialized thermocycler	Pollock et al. 2010

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1.3.1. CULTURE-BASED DETECTION

The culture and plating method is the oldest bacterial detection technique and remains a cornerstone of human pathogen detection. This method involves plating of samples onto selective growth media followed by an incubation period and then colony counting. Specialized growth media can contain inhibitors of non-target species/strains, substrates that only the targeted microbe can degrade, and/or substances that confer a particular color to the growing colonies (Lazcka et al. 2007). However, selective media take time to develop and test, and even when selective media are available for a pathogen of interest, culture and plating techniques are excessively time consuming, in some cases requiring up to 16 days for confirmation of a positive result (Brooks et al. 2004). In addition, these methods are less sensitive than immunologic or genetic-based techniques (Feng 2001). For corals, standardized diagnostics based on culture-dependent methods are currently unavailable, largely because of a lack of selective media capable of promoting growth of specific pathogens amongst the highly complex, diverse, and abundant microbial populations associated with compromised coral tissues. For example, selective media, such as thiosulfate-citrate-bile salts-sucrose (TCBS), have been developed to discriminate *Vibrio* bacteria from other bacterial species. While known coral pathogens, including *Vibrio coralliilyticus* and *Vibrio shiloi*, can be grown on TCBS agar, they cannot be effectively discriminated from other *Vibrio* species, which are known to co-exist within the coral holobiont.

In some cases, culture protocols have been developed to selectively grow microbial species of interest. For example, Sutherland et al. (2010) developed a technique to isolate *Serratia marcescens*, the presumed etiological agent of white pox in the Caribbean, involving two subsequent colorimetric culture steps followed by inoculation onto non-selective media. Interestingly, this method revealed human

sewage to be a likely source of the pathogen on reefs in the Florida Keys. Where appropriate selective media exist, most probable number (MPN) methods can be used to estimate the concentration of bacteria (Vezzulli et al. 2010). MPN involves serially-diluting samples into appropriate media, further dividing these dilutions into replicate aliquots, culturing, and assigning a binomial (growth vs. no growth) score to the resulting cultures. This method can be used to estimate the concentration of certain bacterial groups in a given sample, however the dilution, aliquoting, and culturing steps can be time consuming and reproducibility is often an issue. Due to the high diversity of microbes present in coral samples, lack of appropriate media for many coral pathogens, and the low sensitivity and long processing time required, culture-based diagnostic methods are not the ideal platform for coral pathogen detection.

1.3.2. IMMUNOLOGY-BASED DETECTION

The use of antibody technology is well established in human medical diagnostics and has been applied with some success to the detection of coral pathogens. Immunology-based pathogen monitoring involves the production of either polyclonal or monoclonal antibodies and the detection of antibody/antigen complexes, which indicate the presence of the targeted pathogen within a sample. The use of *Vibrio shiloi*-specific antibodies has revealed the reservoir and transmission vector of this coral bleaching pathogen and provided insight into the dynamics of pathogen invasion and spread within the *Occulina patogonica* coral host (Israely et al. 2001; Sussman et al. 2003). However, polyclonal antibodies often have low specificity, and highly specific monoclonal antibodies are generally slow to develop and expensive to produce and maintain. While immunology-based coral pathogen detection is certainly feasible, the cost and effort required to develop and maintain antibody-producing cell lines may limit its utility in routine monitoring.

1.3.3. NUCLEIC ACID-BASED DETECTION

Nucleic acid-based techniques using molecular probes and/or polymerase chain reaction (PCR) are rapidly replacing culture- and immunology-based methods because of their potential for high specificity and sensitivity. Therefore, fluorescent *in situ* hydridization (FISH) and PCR-based techniques, both of which have been most widely utilized in coral pathogen detection to date, are discussed in more detail next.

1.3.3.1. FLUORESCENT *IN SITU* HYBRIDIZATION

Fluorescent *in situ* hybridization (FISH) allows identification, localization, and visualization of individual microbial cells within healthy and diseased tissue (Moter and Gobel 2000), through targeting of these microbes with custom-synthesized nucleic acid probes attached to fluorescent reporter molecules. Ainsworth et *al.* (2006, 2007, and 2008) utilized FISH to assess the microbial composition of diseased corals in the Mediterranean (2008), Red Sea (2007), and on the Great Barrier Reef (2006). While these studies provide useful information on the spatial arrangement of microbes in healthy and diseased coral tissue, the low specificity of FISH probes limits their utility in detecting pathogenic microbes beyond the genus level. In addition, the method is time-consuming and labor intensive, and also requires specialized imaging microscopy equipment. Extensive processing of samples may also result in the loss of loosely attached microbes including the pathogen cells themselves. Therefore, although applicable for helping to elucidate disease etiology, the utility of FISH as a routine coral disease diagnostic is limited.

1.3.3.2. POLYMERASE CHAIN REACTION (PCR)-BASED METHODS

PCR-based methods allow high sensitivity and specificity by targeting and amplifying short nucleic acid (DNA or RNA) sequences within the genomes of coral-associated microbes. These methods are far less time consuming than culture- or immunology-based approaches, yielding result in hours rather than days or even

weeks with some culture-based techniques (Goarant and Merien 2006). Several community-level PCR techniques, including denaturing gradient gel electrophoresis (DGGE) (Bourne et al. 2008), 16S rRNA clone libraries (Bourne et al. 2008), and microarrays (Sunagawa et al. 2009; Kimes et al. 2010), have provided insights into the microbial communities associated with healthy and stressed corals. While this information can be used to detect shifts in community structure, these changes cannot be linked to specific pathogens. Even when specific pathogens have been identified, standard PCR-based methods do not provide accurate quantification of individual microbial species/strains.

1.3.3.3. REAL-TIME QUANTITATIVE PCR (QPCR)

The combination of high sensitivity and specificity, low contamination risk, ease of performance, and speed make real-time, quantitative PCR (qPCR) technology an appealing option for specific coral pathogen detection (Espy et al. 2006). qPCR allows for accurate quantification of microbe densities in a given sample by incorporating a fluorescent reporter in the PCR reaction that emits a signal proportional to the quantity of PCR product. This information can then be used to infer the amount of target gene and relative number of pathogen cells in a given sample (Hough et al. 2002; Ruzsovics et al. 2002; Tondella et al. 2002). qPCR assays have been designed for a number of bacterial (Panicker and Bej 2005; Gubala 2006), fungal (Haugland et al. 2004), and viral (Butler et al. 2001) pathogens. For example, a real-time PCR assay targeting *Vibrio vulnificus* was developed to confirm the pathogen-free status of raw oysters and was used to investigate possible treatment methods to reduce *V. vulnificus* loads in seafood (Panicker et al. 2004; Panicker and Bej 2005; Vickery et al. 2007). Similarly, a real-time PCR assay was developed to detect *Vibrio penaeicida* in the prawn *Litopenaeus stylirostris* and aquaculture facilities in New Caledonia (Goarant and Merien 2006). This single-day technique

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provided information on infection level, served as a decision-making tool for prawn farmers, and provided a research tool for understanding the dynamics of this pathogen within aquaculture facilities. Analogous assays targeting known coral pathogens could easily be developed (Pollock et al. in 2010b).

Pathogen detection in complex, coral-derived samples provides a unique set of challenges, which are markedly different from those facing human, veterinary, and even aquaculture based researchers. Due to the complex nature of the coral holobiont, care must be taken when choosing a qPCR platform. Fluorescent chemistries used for qPCR fall into two broad categories: 1) intercalating dyes and 2) oligonucleotide-specific probes (Table 2). In the following sections, I describe the qPCR platforms available to researchers and evaluate the feasibility of adopting these assays to detect coral pathogens and/or microbes closely linked with disease lesions (for specific details of qPCR chemistries available, see reviews by Espy et al. 2006; Zhang and Fang 2006).

1.3.3.3.1. INTERCALATING DYES

The cheapest and most commonly utilized qPCR chemistries are intercalating dyes, such as SYBR Green, which detect double-stranded DNA (dsDNA) that is synthesized during PCR amplification (Hernandez et al. 2004). As the quantity of dsDNA increases during subsequent PCR cycles, the fluorescence signal increases proportionally (illustrated in Table 2) (Morrison et al. 1998). The use of a dsDNA binding dye in qPCR detection is advantageous because: i) it can be used with normal PCR primer sets, ii) it does not require the time and labor-intensive design of specific probes, and iii) it is cheaper than probe-based chemistries. However, since dsDNA-binding dye fluoresces in the presence of any dsDNA, it is not specific and must therefore be accompanied by melting curve analysis to differentiate PCR products based upon the length and G-C content of the amplified product (Espy et al. 2006).

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This process, which is similar, but not equivalent to gel electrophoresis, provides some insight into the identity of the PCR-amplified product, but without further analysis (e.g. sequencing or restriction fragment length polymorphism), it is difficult to validate the identity of the amplified nucleotide. Due to the high microbial diversity of both coral-derived samples and the overlaying seawater, the potential for non-target DNA amplification is high. To ensure specific pathogen detection in environmentally-derived coral samples, it may be necessary to use a more selective qPCR chemistry.

1.3.3.3.2. OLIGONUCLEOTIDE-SPECIFIC PROBES
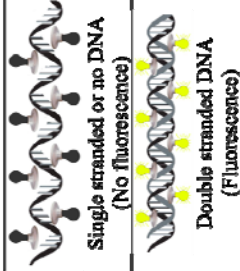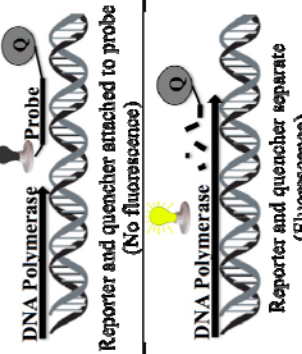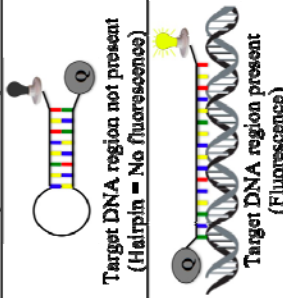
Oligonucleotide probe technologies, including TaqMan and Molecular Beacon, add an additional layer of specificity to the qPCR assay by incorporating a sequence-specific probe, which must anneal to a particular region within the PCR amplicon for fluorescence.

1.3.3.3.2.1. TAQMAN PROBES

The TaqMan probe is a short, linear oligonucleotide that contains a 5'-fluorescent reporter molecule and 3'-quencher. When the two are kept in close proximity, the fluorescence emitted by the reporter is greatly reduced through a fluorescence resonance energy transfer (FRET) mechanism (Uhl and Cockerill 2004). Following the DNA denaturation stage of PCR, the TaqMan probe anneals to the target DNA sequences and, as *Taq* DNA-polymerase extends through this target region during the extension phase, the TaqMan probe is hydrolyzed, releasing the reporter molecule into solution. Now separated from the quencher, the reporter's fluorescence is dramatically increased. As more reporter molecules are liberated during successive PCR cycles, the fluorescence intensity increases proportionally to the amount of PCR amplicon produced (Holland et al. 1991; Heid et al. 1996).

Incorporation of a sequence-specific probe makes TaqMan qPCR more specific than intercalating dye chemistries. Also, the ability to multiplex TaqMan reactions by including several distinct primer/probe sets labeled with different colored fluorescent reporters in a single reaction make TaqMan chemistry ideal for detecting several pathogens simultaneously. Unfortunately, TaqMan probes are more expensive than intercalating dye chemistries and the need for custom probes makes their design slightly more involved.

Table 2. Summary of qPCR chemistries.

Chemistry	Principle	Illustration	Specificity	Ease of design	Cost	Requires melt curve analysis?
Intercalating dye	When the dye binds to double-stranded DNA (dsDNA) molecules produced during PCR amplification its fluorescence increases dramatically		Very low	Easy	Very low	Yes
TaqMan probe	When the probe anneals to a specific DNA region during the extension phase of PCR, DNA-polymerase removes the fluorescent reporter and quencher molecules from the probe causing them to separate and greatly increasing fluorescence		High	Difficult	High	No
Molecular beacon	When the probe anneals to a specific DNA region it loses its hairpin structure, separating the fluorescent reporter and quencher molecules, greatly increasing fluorescence		Very high	Very difficult	High	No

1.3.3.3.2.2. **MOLECULAR BEACONS**

Like TaqMan probes, molecular beacons contain a 5'-fluorescent reporter and a 3'-quencher. However, molecular beacons also contain regions at either end of the probe that are complementary to one other. At low temperatures, these regions anneal, forcing the probe into a "hairpin" structure, which maintains the reporter and quencher in close proximity and minimizes fluorescence. Between these self-complementary regions, molecular beacons also contain an oligonucleotide sequence that is custom-designed to be complementary to a region within the PCR product. During the annealing step of PCR, this region binds to its complement within a denatured, single stranded PCR amplicon. This forces the molecular beacon into a linear configuration, which separates the reporter and quencher and dramatically increases fluorescence. In the absence of PCR product, the molecular beacon regains its "hairpin" structure, preventing fluorescence. Thus, the fluorescent intensity is directly proportional to the concentration of PCR product.

Molecular beacons provide an even greater degree of specificity than TaqMan chemistry. Due to the high stability of its hairpin structure, nearly perfect hybridization between the molecular beacon and its PCR amplified complement is required for fluorescence. This specificity makes molecular beacon chemistry ideal for pathogen detection in complex, coral-derived samples. However, since even a single nucleotide polymorphism (SNP) can greatly reduce the probe's binding efficiency (Mhlanga and Malmberg 2001), the design of molecular beacons is extremely important and can be quite demanding.

1.3.4. **DNA TARGET SELECTION**

For the development of molecular diagnostic assays, the choice of a nucleic acid target is just as important as the molecular assay platform used to detect it. To allow for high specificity, nucleic acid targets must be both well-conserved within the

genome of the target species/strain and unique from non-target sequences. Therefore, a great deal of care must be taken in genetic target selection, primer/probe design, and assay optimization. Ribosomal and mitochondrial DNA are the most common targets for nucleic acid-based microbe detection because of their genetic stability and high copy number within cells (Woese 1987). However, other genes, including housekeeping genes and virulence factors that are present as only a single copy in the genome, may also serve as useful targets.

1.3.4.1. RIBOSOMAL GENES

Several ribosomal RNA genes, including 16S, 18S, 23S, and internal transcribed spacer region (ITS) genes, have served as targets for nucleic acid-based detection. With the public availability of over one million 16S rRNA sequences, spanning both the variable and more highly conserved regions of this ubiquitous bacterial gene, the 16S rRNA gene provides an obvious nucleic acid target. Since its introduction in the late 1980's, most FISH applications have targeted rRNA genes because of their high copy number in bacterial cells. However, low genetic divergence in closely related species/strains often hinders the utility of the 16S rRNA gene in differentiating beyond the genus level (Thompson et al. 2005). For example, the known coral pathogen *Vibrio coralliilyticus* shares greater than 98% 16S sequence similarity with its closest phylogenetic neighbor, *V. neptunius* (Thompson et al. 2005). Although some variation (2%) exists between these closely related species, it is likely inadequate to design sufficiently specific primers and/or probes.

1.3.4.2. GENOMIC PHYLOGENETIC MARKER GENES

The accelerated use of genetic sequencing as a means of differentiating closely related bacterial species and strains has led to the proliferation of sequence information from a large number of non-ribosomal phylogenetic marker genes in a diverse sampling of microbial species. In some of the more well-studied groups, such

as the vibrios, which contain 4 of the 7 known coral pathogens, sequence data from several phylogenetic marker genes are available for all described species (Thompson et al. 2005) and even multiple strains of the identified coral pathogen *Vibrio coralliilyticus* (Pollock et al. 2010a). This information is useful for selecting genes with the greatest discriminatory power based on phylogenetic reconstructions and also provides the raw sequence data to identify specific oligonucleotide sequences within these genes, which can be targeted by custom-designed molecular primers and probes.

1.3.4.3. VIRULENCE FACTORS

An increasing number of molecular-based pathogen detection assays have targeted genes directly involved in virulence. For example, the thermostable direct hemolysin gene (*tdh*), has been used as a target for detection of *Vibrio parahaemolyticus* and the gene is also inferred as a direct marker of pathogenicity (Blackstone et al. 2003). Similarly, the hemolysin gene (*vvh*) has been targeted for the specific detection of *V. vulnificus* (Panicker and Bej 2005). Directly targeting strain-specific virulence factors provides a means of differentiating pathogenic and benign strains. Gubala (2006), for example, designed a qPCR-based assay capable of exclusively detecting potentially toxigenic strains of *V. cholerae* by targeting a 2kb fragment of the *rtxA* gene, a cytotoxin coding gene that is unique to virulent strains.

Specific virulence factors have been described in two coral pathogens, the Zn-metalloprotease gene (*vcpA*) in *Vibrio coralliilyticus* and Toxin P gene in *Vibrio shiloi*, both of which could serve as molecular targets (Banin et al. 2001; Sussman et al. 2009; Pollock et al. 2010a). As researchers develop a deeper understanding of the genetic basis of coral pathogen virulence, it is likely that more virulence targets will become available.

1.3.5. EMERGING DIAGNOSTIC TECHNIQUES

Application of the technological advances outlined above will undoubtedly enhance our ability to study coral diseases, however, a variety of new and emerging technologies will further revolutionize the field in decades to come. High resolution microarrays offer one method for rapid assessment of shifts in coral-associated bacterial community structure. As previously highlighted, Sunagawa et al. (2009) utilized a 16S rRNA gene microarray (PhyloChip G2) to characterize the bacterial community structures of healthy and diseased corals and investigate the etiology of the observed disease. If known bacterial groups or indicator organisms are indentified that are important to coral health, these shifts can be used to infer potential changes in coral health or, additionally, detect identified pathogens associated with disease lesions. Vega Thurber et al. (2009) assessed changes in overall bacterial community structure and abundance of functional genes in response to environmental stressors using a 454 pyrosequencing platform. Comparative genomic approaches such as these will continue to provide insights into the bacterial community-level changes that accompany coral stress and potentially facilitate coral disease outbreaks. Transcriptomic approaches offer further advantages for targeting expressed virulence genes, provided this methodology can be adapted to coral samples. Additionally, metabolomic techniques, which use NMR and mass spectroscopy to detect chemical fingerprints left behind by specific chemical processes, show great promise for improving disease diagnosis and pathogen detection (Gowda et al. 2008; Boroujerdi et al. 2009). While genomic, metagenomic, transcriptomic, and metabolomic approaches have the potential to generate extensive, detailed data, these techniques require expensive, specialized equipment and often the desired information is hidden within immense datasets that require specialized software to decipher. However, just as quantitative PCR, which was only available to a handful of well-funded

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laboratories just a decade ago, is becoming increasingly more affordable and accessible, the prohibitive cost of emerging technologies will certainly fall, increasing their availability to coral researchers.

1.3.6. VALIDATION OF DIAGNOSTICS FOR CORAL PATHOGEN DETECTION

The validity of any diagnostic test is determined by its ability to distinguish host organisms that have the disease from those that do not. Validity is comprised of two key components: sensitivity and specificity. Sensitivity describes the test's ability to correctly identify those with the disease and is expressed as the proportion of affected animals that are correctly identified as disease positive by the test compared to the total number tested. Specificity is the ability of the test to correctly identify those that do not have the disease and is expressed as the proportion of animals that do not have the disease that are correctly identified as disease negative (Wobeser 2006). In order to calculate the specificity and sensitivity of a test, we must first know which animals are actually infected with the disease. Such knowledge is usually gained by comparing the test's results with the results of a so-called "gold standard", which theoretically has both a sensitivity and specificity of 100% (Wobeser 2006). For example, the gold standard for *Chlamydia* diagnosis in humans is isolation of the causative agent, the bacteria *Chlamydia trachomatis*. It is important to realize that while gold standards are the best evidence available, they are not infallible and that gold standards providing full certainty are rare, particularly in a young field like coral disease research. Generally, the challenge is to find a standard that is as close as possible to the theoretical gold standard, but until effective gold standards are established for coral pathogen detection, it may be useful to use several of the diagnostic techniques described previously to cross-validate test results.

Coral researchers are faced with a unique set of challenges when developing disease diagnostics for the detection of specific pathogenic microbes among the

diverse and complex coral holobiont. One major challenge is reproducibly obtaining

high purity microbial DNA (or RNA) from coral-derived samples. The complex

nature of the coral holobiont, which contains genetic material from the coral host as

well as its associated algae, bacteria, and viruses, in combination with the presence of

high concentrations of PCR inhibitors (e.g. salts and DNAses) make successful DNA

extraction and pathogen detection from coral tissue extremely difficult. Several

extraction methods have been developed to overcome these limitations, but

consistently obtaining high quality DNA from coral samples remains a persistent

challenge to coral researchers. Early pathogen detection assays will therefore require

extensive testing to confirm their specificity and sensitivity. It may also be advisable

to use commercially available DNA extraction kits to standardize extraction

efficiencies between samples and among labs. For example, early trials have shown

the MO Bio Plant DNA Extraction Kit to be a robust and reproducible method for

extracting coral-associated microbial DNA (Pollock et al. 2010b). Current application

of RNA-based expression studies directly on diseased coral samples is limited, except

for certain band diseases such as Black Band Disease (Frias-Lopez et al. 2004), which

can first be separated from the coral. mRNA is inherently unstable and is rapidly

degraded, particularly in the presence of the extensive exogenous enzymes present

within coral-derived samples. This is a real challenge for samples collected in the

field, often in remote locations far from adequate specialized laboratory facilities.

Such transcriptomic approaches, however, offer enormous potential for selective

identification both of active organisms involved in the infection process and virulence

genes directly involved in disease progression, and should be a current and future

research focus.

1.4. CONCLUSIONS

Further development and application of molecular-based diagnostic tools for coral pathogen detection is limited by lack of knowledge of the organisms and genes involved in the onset and progression of most coral diseases. In particular, current knowledge of the causes of a large number of coral diseases is rudimentary, with only a few actual pathogens identified (reviewed in Harvell et al. 2007; Bourne et al. 2009). Therefore, further research into coral disease ecology, in combination with robust biomedical approaches to describe diseases at gross and cellular levels is needed to develop an understanding of the pathogenesis of coral diseases and the interactions between agent, host, and the environment (Work et al. 2008). Only after pathogens are identified and their mechanisms of virulence determined can the development of diagnostics that target certain microbial groups or important genes proceed. Coral disease investigations, like other human, veterinary, or wildlife disease investigations, require an interdisciplinary approach, including the use of both traditional and developing technologies.

As coral diseases continue to threaten reefs worldwide, there is increasing urgency for tools to understand and control their spread. Several approaches, including phage therapy and probiotic addition, have been proposed to control coral disease outbreaks (Teplitski and Ritchie 2009); however the success of any of these strategies will depend upon rapid and reliable disease detection and diagnosis. With the extensive cost and potential environmental risk of certain control measures (e.g., phage therapy), it will be critical that diagnoses are made with very high degree of certainty. In this review, I have evaluated several approaches for the detection of specific coral pathogens and I have found real time quantitative PCR to be most promising due to its high sensitivity, high specificity, and low contamination risk. However, the high monetary and environmental cost of false results underlines the

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need for a careful evaluation of the available diagnostics options to best address the question of interest and the extensive validation of any coral disease diagnostic tool. Accurate coral disease diagnosis will help to direct research and management strategies to address the true cause of disease on reefs and aid reef managers in their efforts to control the occurrence, prevalence, and severity of coral disease on reefs worldwide. Therefore, the development and testing of highly sensitive and specific pathogen-specific coral disease diagnostics should be a major research priority.

1.5. SPECIFIC RESEARCH OBJECTIVES

This study will provide a phylogenetic classification and comparison of geographically diverse strains of the coral pathogen, *Vibrio coralliilyticus*, implicated in bleaching and disease of corals globally. The study will also aim to use this phylogenetic information to develop molecular diagnostic tools to detect and quantify this pathogen in pure cultures, controlled infection trials and environmental samples. Specifically, my aims are:

1. **To investigate the phylogeography of coral-associated *V. coralliilyticus* strains from the Red Sea, Caribbean Sea, and Indian, Pacific, and Atlantic Oceans using multi-locus sequence analysis (Chapter 2).** Understanding phylogenetic relationships among different strains of *V. coralliilyticus* will provide insights into the origin and spread of this pathogen. Comparative sequence analysis will also help to identify potential targets for molecular-based detection assays.

2. **To develop a qPCR-based assay to detect and quantify potentially pathogenic *V. coralliilyticus* strains (Chapter 3).** A *V. coralliilyticus*-specific assay will allow for the early detection of disease outbreaks and aid in

the identification of pathogen reservoirs and transmission vectors. Also, the quantitative aspect will allow for detailed study of the dynamics of white syndrome infection and spread both in the laboratory and the field.

3.   **To determine the specificity and sensitivity of this qPCR-based diagnostic tool *in vitro* (Chapter 3).** Prior to implementation *in vivo,* it will be important to assess the tool's ability to discriminate *V. coralliilyticus* strains from other closely related bacteria and determine its detection limits in pure and mixed bacterial cultures.

CHAPTER 2. PHYLOGENY OF THE CORAL PATHOGEN *VIBRIO CORALLIILYTICUS*

The work described in this chapter has been published as:

ABSTRACT

A phenotypic and phylogenetic comparison of geographically disparate isolates of the coral pathogen *Vibrio coralliilyticus* was conducted to determine whether the bacterium exists as a single cosmopolitan clonal population, which might indicate rapid spread of a pandemic strain, or is grouped into endemic and genotypically distinct strains. All strains included in this study displayed similar phenotypic characteristics to those of the typed *V. coralliilyticus* strain LMG 20984^{T}. Five phylogenetic marker genes (*16S*, *rpoA*, *recA*, *pyrH*, and *dnaJ*) frequently used for discriminating closely related *Vibrio* species and a zinc-metalloprotease gene (*vcpA*) linked to pathogenicity were sequenced in thirteen *V. coralliilyticus* isolates collected from corals, bivalves, and their surrounding seawater in the Red and Caribbean Seas, and Indian, Pacific, and Atlantic Oceans. A high level of genetic polymorphism was observed with all isolates possessing unique genotypes at all six genetic loci examined. No consistent lineage structure was observed within the marker genes and homologous recombination was detected in the *16S* and *vcpA* genes, suggesting that *V. coralliilyticus* does not possess a highly clonal population structure. Interestingly, two geographically distinct (Caribbean/south-Atlantic and

Indo-Pacific/north-Atlantic) and highly divergent clades were detected within the zinc-metalloprotease gene, but it is not known if these clades correspond to phenotypic differences in virulence. These findings stress the need for a multi-locus approach for inferring *V. coralliilyticus* phylogeny and indicate that populations of this bacterium are likely an endemic component of coral reef ecosystems globally.

2.1. INTRODUCTION

Bacteria from the family Vibrionaceae play an important though poorly elucidated role in both the maintenance and disruption of coral health. Some coral-associated vibrios have a commensal or even mutualistic relationship with their coral host, contributing to nutrient cycling within coral mucus (Bourne and Munn 2005; Olson et al. 2009) and providing defense against invading microbes (Ritchie 2006; Chimetto et al. 2008; Shnit-Orland and Kushamaro 2009). However, several *Vibrio* species have also been shown to disrupt the normal function of coral hosts and their algal symbionts, leading to a decline into a diseased state (Kushmaro et al. 2001; Ben-Haim et al. 2003; Rosenberg et al. 2007; Sussman et al. 2009). Of the eight coral pathogens implicated in the onset of disease lesions, half belong to the Vibrionaceae family (Rosenberg et al. 2007; Bourne et al. 2009), though little is known about their geographic origins or distributions, phylogenetic relationships between virulent and avirulent strains, or the genetic basis of their virulence.

Vibrio coralliilyticus has recently emerged as a coral pathogen of concern on reefs throughout the Indo-Pacific. First implicated as the aetiological agent responsible for bleaching and tissue lysis of *Pocillopora damicornis* corals off the coast of Zanzibar in the Indian Ocean (Ben-Haim et al. 2003), it was more recently identified as a possible causative agent of outbreaks of the coral disease white syndrome (WS) at several locations throughout the Indo-Pacific (Sussman et al. 2008). WS is a collective term to describe tissue loss resulting in a spreading white band of exposed skeleton occurring on scleractinian corals in the Indian and Pacific Oceans (Willis et al. 2004). In recent years, WS epizootics have been reported on reefs throughout the Indo-Pacific, including the Great Barrier Reef (Willis et al. 2004), Christmas Island (J.P. Hobbs, pers. comm.), Rowley Shoals (Long et al. 2009), Marshall Islands (Sussman et al. 2008), Palau (Sussman et al. 2008), NW Hawaiian

Islands (Aeby 2005) and American Samoa (G. Aeby in press). Although a direct link between *V. coralliilyticus* and widespread WS outbreaks has not been established, aquarium-based infection experiments have demonstrated that this bacterium does have the potential to cause WS in several Pacific coral species (Sussman et al. 2008) and isolates of this potentially pathogenic bacterium have recently been collected from diseased corals in the Indo-Pacific and beyond (Table 1) including the Red Sea (Ben-Haim et al. 2003), Caribbean Sea (Vizcaino et al. in review), Indian (Ben-Haim and Rosenberg 2002), Pacific (Thompson et al. 2005; Sussman et al. 2008), and Atlantic Oceans (Ben-Haim et al. 2003; Thompson et al. 2005). Despite the global distribution of this potentially pathogenic bacterium, little is known about the population genetics of *V. coralliilyticus*, which represents a critical first step towards understanding the evolutionary history and population dynamics of this species.

Multilocus sequence typing (MLST), a method for characterizing microbial isolates by means of sequencing internal fragments of phylogenetic marker genes (Maiden et al. 1998), provides an effective means for inferring evolutionary relationships of pathogenic bacteria (Jolley et al. 2000; Feil et al. 2001; Godoy et al. 2003; Jolley et al. 2004). MLST has been used to identify populations and sub-populations of pathogens with distinct geographic distributions (Falush et al. 2003) and has also been used to correlate this genetic variability with important phenotypic differences, most notably virulence (Kreiswirth et al. 1993; Maiden et al. 1998; Claus et al. 2002; Shnit-Orland 2009). Analyses of meningococcal populations, for example, directly linked the virulence of certain pathogenic lineages to specific polysaccharide capsule morphologies (Claus et al. 2002; Dolan-Livengood et al. 2003). This information has proven invaluable in identifying and tracking invasive genotypes and has been used to design highly specific protein-based vaccines (Pollard and Maiden

2003). At present, MLST is an important method for characterizing hyper-virulent and antibiotic-resistant clones of several pathogenic bacteria, including *Neisseria meningitides* (Maiden et al. 1998), *Streptococcus pneumoniae* (McGee et al. 2001), and *Staphylococcus aureus* (Enright et al. 2002) and MLST schemes exist for 22 other bacterial species (www.mlst.net).

In this study, we provide a phenotypic and phylogenetic comparison of geographically disparate isolates of the putative coral pathogen, *V. coralliilyticus*. A total of thirteen *V. coralliilyticus* isolates were collected from healthy, bleached, and WS-infected corals as well as healthy and diseased bivalve larvae from diverse geographic locations including the Pacific Ocean (Great Barrier Reef, Australia; the Republic of the Marshall Islands; Palau), Atlantic Ocean (United Kingdom; Brazil), Indian Ocean (Zanzibar), Caribbean Sea (Puerto Rico), and Red Sea (Israel) (Fig. 1, Table 1). Phenotypic comparisons of isolates was performed using API 20E and API 20NE kits (bioMérieux). Evolutionary relationships between isolates were inferred from the nucleotide sequences of a zinc-metalloprotease gene (*vcpA*) (GeneBank accession number GQ452012), which has been linked to *V. coralliilyticus* pathogenicity through photoinactivation of *Symbiodinium* endosymbionts and direct lysis of coral tissue (Sussman et al. 2008), and five phylogenetic marker genes with demonstrated potential for discriminating closely related *Vibrio* strains: 16S ribosomal RNA gene (*16S*), RNA polymerase alpha subunit gene (*rpoA*), uridylate kinase gene (*pyrH*), recA protein gene (*recA*), and heat shock protein 40 encoding gene (*dnaJ*) (Thompson et al. 2005; Nungh et al. 2007). Genetic sequences were obtained from either the National Center for Biotechnology Information's (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/) or via PCR amplification and sequencing using specific PCR primers (Table 2).

Table 1. Geographic and host information of *Vibrio coralliilyticus* isolates analysed in this study. All strains (with the exception of the Caribbean strains) are available from the BCCM/LMG Bacteria Collection at Ghent University (Ghent, Belgium).

| Isolate	BCCM/LMG Accession No.	Collection Location	Host species	Host condition	Reference	Date of Isolation
P1	LMG 23696	Nelly Bay, Magnetic Island, Australia	*Montipora aequituerbculata*	WS Infected	Sussman et al. 2008	2003
P2	LMG 23691	Majuro Atoll, Republic of the Marshall Islands	*Acropora cytherea*	WS Infected	Sussman et al. 2008	2004
P4	LMG 23693	Nikko Bay, Palau	*Pachyseris speciosa*	WS Infected	Sussman et al. 2008	2005
P5	LMG 23692	Nikko Bay, Palau	*Pachyseris speciosa*	WS Infected	Sussman et al. 2008	2005
P6	LMG 23694	Nikko Bay, Palau	*Pachyseris speciosa* (seawater above)	WS Infected	Sussman et al. 2008	2005
BH1	LMG 20984T[a]	Indian Ocean, Zanzibar, Tanzania	*Pocillopora damicornis*	Bleached	Ben-Haim and Rosenberg 2002	1999
BH2	LMG 21348	Red Sea, Eilat, Israel	*Pocillopora damicornis*	Bleached	Ben-Haim et al. 2003	2001
BH3	LMG 21349	Red Sea, Eilat, Israel	*Pocillopora damicornis*	Bleached	Ben-Haim et al. 2003	2001
BH4	LMG 21350	Red Sea, Eilat, Israel	*Pocillopora damicornis*	Bleached	Ben-Haim et al. 2003	2001
BH5	LMG 10953	Kent, United Kingdom	*Crassostrea gigas* (oyster) larvae	Diseased	Ben-Haim et al. 2003	1980s
BH6	LMG 20538	Atlantic Ocean, Florianópolis, Brazil	*Nodipecten nodosus* (bivalve) larvae	Apparently healthy	Ben-Haim et al. 2003	1998
C1	n/a	Caribbean Sea, La Parguera, Puerto Rico	*Pseudopterogorgia americana*	Diseased	Vizcaino et al. in review	2006
C2	n/a	Caribbean Sea, La Parguera, Puerto Rico	*Pseudopterogorgia americana*	Diseased	Vizcaino et al. in review	2006

[a] Typed strain
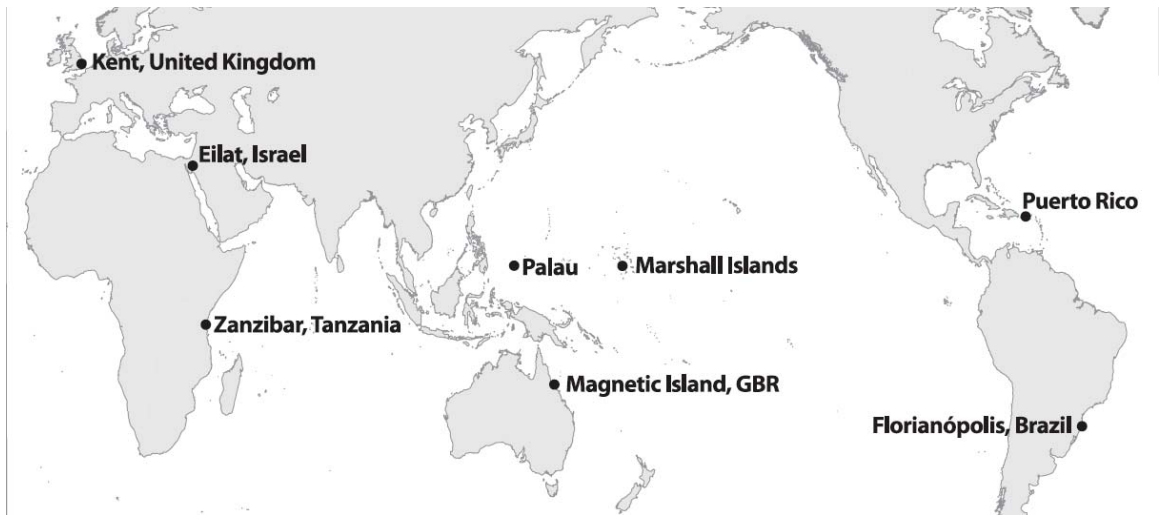
Figure 1. Collection locations of *Vibrio coralliilyticus* isolates used in this study. Strains were isolated from healthy, bleached, and WS-infected corals as well as healthy and diseased bivalve larvae using methods previously described (Thompson et al. 2001; Ben-Haim and Rosenberg 2002; Sussman et al. 2008; Vizcaino et al. in review).

Table 2. PCR amplification and sequencing primers for *16S* (1,300 nt), *rpoA* (928 nt), *pyrH* (443 nt), *recA* (613 nt), *dnaJ* (558 nt), and *vtpA* (1840 nt) genes, which were used to infer evolutionary relationships between *Vibrio corallilyticus* strains.

Gene product	Primer	Sequence [5' – 3']	Reference
Zinc-metalloprotease (*vcpA*)	vcpAF	ATGAAACAACGTCAAATGCTTTG	Wilson et al. in review
	vcpAR	CCCTTTCACTTACGATGTTGTG	
Uridylate kinase (*pyrH*)	pyrH-04-F	ATGASNACBAAYCCWAAACC	Thompson et al. 2005
	pyrH-02-R	GTRAABGCNGMYARRTCCA	
RNA polymerase alpha subunit (*rpoA*)	rpoA-01-F	ATGCAGGGTTCTGTDACAG	Thompson et al. 2005
	rpoA-03-R	GHGGCCARTTTCHARRCGC	
recA protein (*recA*)	recA-01-F	TGARAARCARTTYGGTAAAGG	Thompson et al. 2005
	recA-02-R	TCRCCNTTRTAGCTRTACC	
Heat shock protein 40 (*dnaJ*)	805-VibrioMF2	TTTTAYGAAGTDYTDGGYGT	Nungh et al. 2007
	806-VibrioMR	GACAVGTWGGACAGGYYTGYTG	
16S ribosomal RNA (*16S*)	63f	CAGGCCTAACACATGCAAGTC	Marchesi et al. 1998
	1387R	GGGCGGWGTGTACAAGGC	

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2.2. RESULTS AND DISCUSSION

All *V. coralliilyticus* isolates displayed similar phenotypic characteristics to those of the typed LMG 20984[T] strain. Cells were Gram-negative, motile, and rod shaped similar to those reported by Ben-Haim et al. (2003). Biochemical characteristics as assessed by the API 20E and API 20NE kits (bioMérieux), were similar for all strains with a list of these patterns presented in Table 3.

Genotypic characterization revealed *V. coralliilyticus* to be highly polymorphic, with all thirteen isolates possessing unique genotypes at all six genetic loci examined. Mean nucleotide divergence (p-distance; Nei and Kumar 2000) ranged from 0.2% in the 16S rRNA gene to 3.7% in the zinc-metalloprotease gene (*vcpA*) with an average divergence of 1.3% over the five phylogenetic marker genes (*16S*, *rpoA*, *recA*, *pyrH*, and *dnaJ*). Phylogenetic reconstructions based on these gene sequences revealed no consistent lineage structure (Fig. 2A and Fig. 3) and exact tests of population differentiation (Raymond and Rousset 1995; Goudet et al. 1996) revealed that neither host species nor geographic origin explained the phylogenetic relationships between isolates ($p > 0.05$). Additionally, phylogenetic trees deduced from individual genes indicated many discrepancies between topologies (Fig. 3), which may result from variable selective pressures acting on the individual genes examined and/or homologous recombination between strains (Denamur et al. 1993; Salaun et al. 1998). Pairwise homoplasy index (PHI) tests (Bruen et al. 2006) implemented in SplitsTree4 (Huson and Bryant 2006; http://www.splitstree.org/) did suggest some level of recombination within the 16S rRNA gene ($p < 10^{-6}$) and zinc-metalloprotease gene ($p < 10^{-14}$), which is consistent with the conflicting phylogenetic splits (parallelograms) observed in the split tree decomposition networks, another indicator of recombination (Fig. 4).

Table 2: Consensus results for phenotypic/biochemical characterisation of *Vibrio coralliilyticus* isolates using API® 20E and 20NE test strips.

API® 20E [a]	Result:	API® 20 NE [a]	Result:
ß-galactosidase	+[b]	Nitrate reduction to nitrite	+
Arginine-dihydrolase	±	Indole production	+
Lysine decarboxylase	-	Glucose fermentation	+
Ornithine decarboxylase	-	Arginine-dihydrolase	±
Citrate utilisation	-	Urease	-
H$_2$S production	-	Esculin hydrolysis	-
Urease	-	Gelatin hydrolysis	+
Tryptophane deaminase	+	ß-galactosidase	+[b]
Indole production	+	Glucose assimilation	+
Voges-Proskauer	+	Arabinose assimilation	-
Gelatinase activity	+	Mannose assimilation	+
Glucose fermentation	+	Mannitol assimilation	+[c]
Mannitol fermentation	±	N-acetyl-glucosamine assimilation	±
Inositol fermentation	-	Maltose assimilation	+
Sorbitol fermentation	-	Potassium gluconate assimilation	+
Rhamnose fermentation	-	Capric acid assimilation	-
Sucrose fermentation	+	Adipic acid assimilation	-
Melobiose fermentation	-	Malic acid assimilation	+[c]
Amygdalin fermentation	-	Citrate assimilation	-
Arabinose fermentation	-	Phenylacetic acid	-
Cytochrome oxidase	+	Cytochrome oxidase	+

[a] Tests performed in duplicate for each strain in this study. Tests performed according to the manufacturer's instruction (bioMérieux) with the exception that strains were suspended from Marine Agar plates into 2% NaCl solution and auxiliary media was supplemented to a final concentration of 2% NaCl.
[b] Isolate BH5 negative
[c] Isolate P1 negative
± Weak positive result

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A.

B.
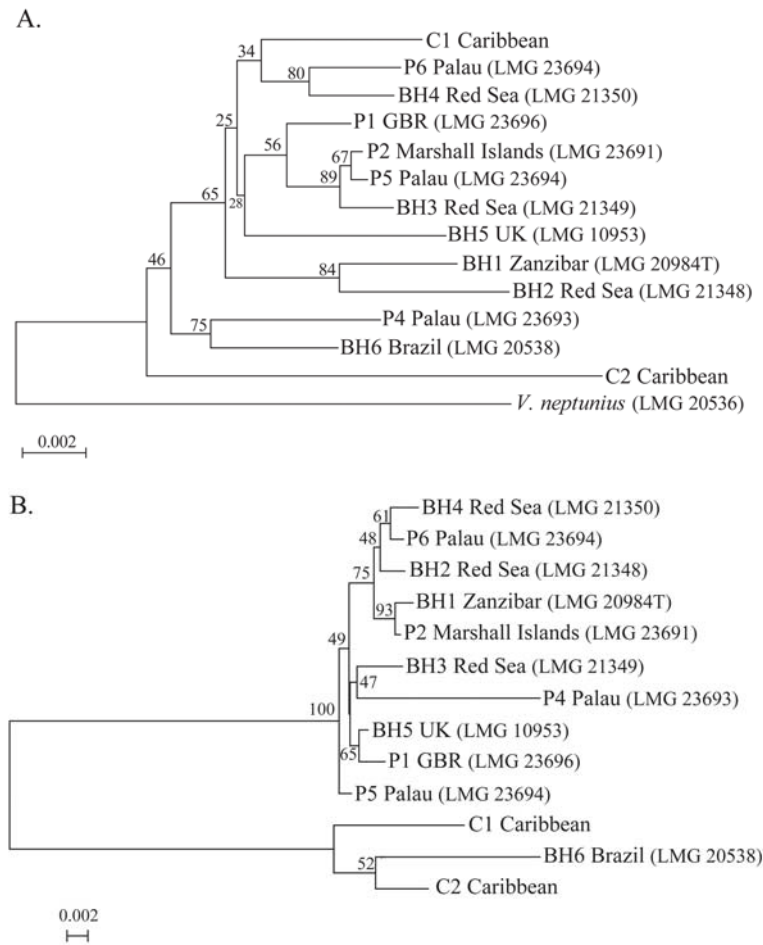
**Figure 2.** Consensus phylogenetic trees based on neighbor-joining method showing the relationships between *Vibrio coralliilyticus* isolates. Evolutionary trees were inferred by aligning concatenated gene sequences using ClustalW (Higgins et al. 1996) and calculating similarity matrices and phylogenetic trees using MEGA4 (Tamura et al. 2007). Evolutionary distance estimations were obtained using the Jukes-Cantor model (Jukes and Cantor 1969). Bootstrap percentages (≥ 25) after 500 simulations are depicted above their respective branches. Scale bars represent the average number of base substitutions per site. **A)** Concatenated phylogeny using *16S* (1,300 nt), *rpoA (*928 nt)*, pyrH* (443 nt)*, recA* (613 nt) and *dnaJ* (558 nt) gene sequences. *Vibrio neptunius* (LMG 20536) served as the outgroup. **B)** Phylogeny based on metalloprotease encoding (*vtpA*) (1840 nt) gene sequences. This tree is unrooted.

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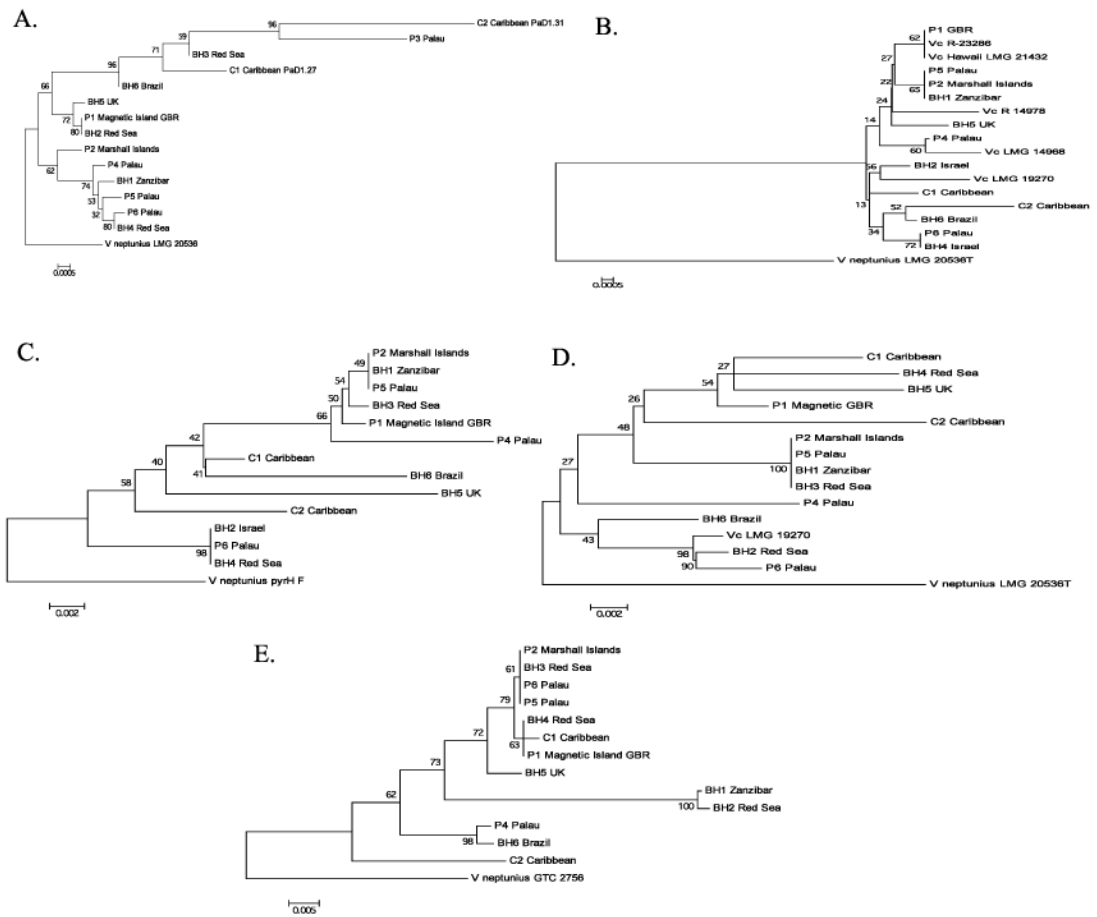
Figure 3. Phylogenetic trees of individual genes based on neighbor-joining method showing the relationships between *Vibrio coralliilyticus* isolates. Evolutionary trees were inferred by aligning concatenated gene sequences using ClustalW (Higgins et al. 1996) and calculating similarity matrices and phylogenetic trees using MEGA4 (Tamura et al. 2007). Evolutionary distance estimations were obtained using the Jukes-Cantor model (Jukes and Cantor 1969). Bootstrap percentages (≥ 25) after 500 simulations are depicted above their respective branches. Scale bars represent the average number of base substitutions per site. Phylogenies using **A)** *16S* (1,300 nt), **B)** *rpoA (*928 nt), **C)** *pyrH* (443 nt), **D)** *recA* (613 nt) and **E)** *dnaJ* (558 nt) gene sequences. *Vibrio neptunius* (LMG 20536) served as the outgroup.
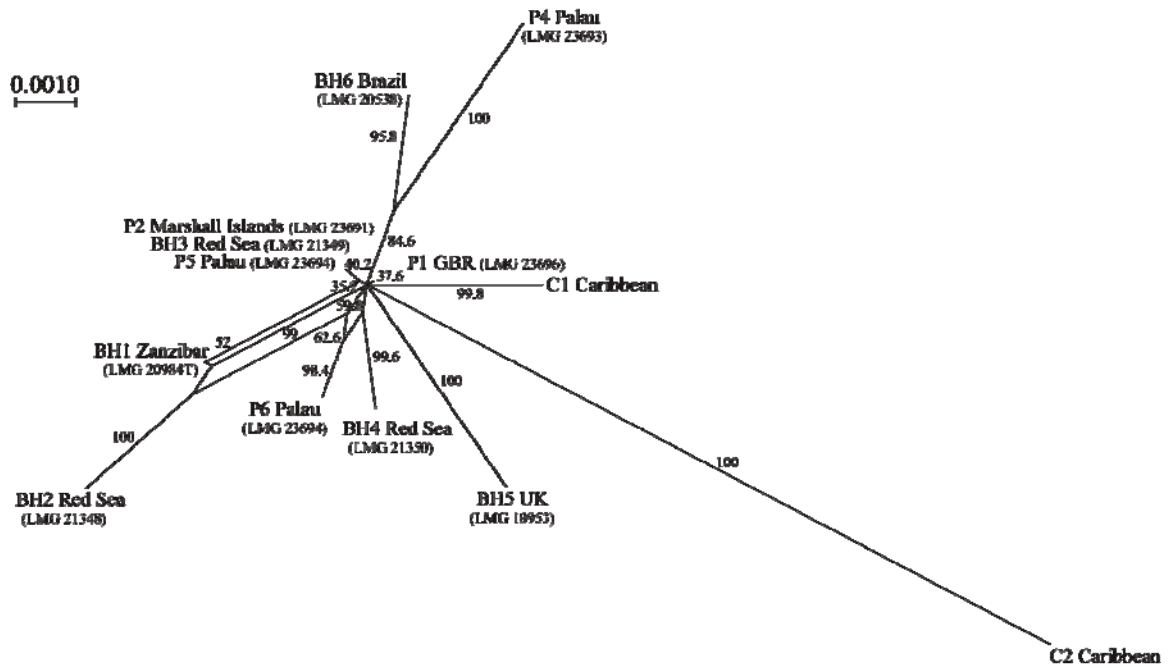
Figure 4. Splits tree decomposition networks in *Vibrio coralliilyticus* on the basis of *16S* (1,300 nt), *rpoA* (928 nt), *pyrH* (443 nt), *recA* (613 nt) and *dnaJ* (558 nt) gene sequences using SplitsTree4 (http://www.splitstree.org/). Bootstrap percentages (≥ 50) after 500 simulations are shown. The scale bar represents 10% estimated sequence divergence.

The high genetic diversity and lack of consistent lineage signal suggest that *V. coralliilyticus* does not possess a strong clonal structure. It is possible that high recombination rates or the limited number of isolates that were analyzed from each site might obscure the presence of clonal complexes within *V. coralliilyticus*. Although much larger sample sizes from each region would be needed to detect clonal population structures, it is unlikely that recombination would be sufficiently frequent to erase clonal signals over years or decades, or even during a rapid geographic spread (Maiden 2006). Therefore, it is doubtful that the geographically disparate *V. coralliilyticus* isolates examined in this study represent a rapidly spreading pandemic strain. Instead, results suggest that *V. coralliilyticus* populations represent endemic components of coral reef ecosystems that vary genetically among the globally distributed geographic locations sampled.

Interestingly, a geographic trend was observed in the clustering of the zinc-metalloprotease gene (*vcpA*) sequences, with the Indo-Pacific and northern-Atlantic isolates forming one closely related clade and the Caribbean and southern-Atlantic isolates forming another (Fig. 2B). Analysis of molecular variance (AMOVA; Excoffier and Smouse 1994) revealed that this split explains nearly 85% of the genetic variation within this gene and that the nucleotide sequence is more highly conserved in the Indo-Pacific/northern-Atlantic isolates, with a mean nucleotide divergence of only 1% compared to 2.4% in the Caribbean/southern-Atlantic isolates. The sequence divergence between clades, however, was much greater (7.8%), which suggests that these clades may code for two distinct forms of the metalloprotease. The *V. vulnificus* metalloprotease encoding gene, *vvp*, exists in two variants, types A and B, which are typically isolated from avirulent and virulent strains, respectively (Wang et al. 2008). These two gene types differ by 4.8% (Wang et al. 2008) yet the resulting

enzymes have indistinguishable biological activities (Watanabe et al. 2004; Miyoshi et al. 2006). While the level of sequence divergence between the *V. coralliilyticus* metalloprotease clades in this study is greater still, it is currently unknown if this genetic variability correlates with virulence. This is the subject of ongoing investigations.

The results presented here provide a framework for inferring the evolutionary history of the putative coral pathogen *V. coralliilyticus* via MLST. This species was found to be genetically diverse, with little evidence of clonality among the isolates analyzed. While only a limited number of isolates were evaluated, they do suggest that the recent emergence of white syndromes on corals from widely separated reef regions is unlikely to have been caused by a rapidly spreading pandemic strain. Given the potential of *V. coralliilyticus* to cause significant coral mortality, this study highlights the need for further phylogenetic studies that incorporate sampling from a greater diversity of geographic locations and host species to better understand the origin and spread of this putative coral pathogen. It will also be important to include information on pathogenicity in order to understand how genetic diversity, particularly of known virulence factors such as the metalloprotease, relates to coral mortality in different reef regions.

CHAPTER 3. DETECTION AND QUANTIFICATION OF THE CORAL PATHOGEN *VIBRIO CORALLIILYTICUS* BY USE OF REAL-TIME PCR WITH TAQMAN FLUORESCENT PROBES

ABSTRACT

Coral diseases represent an emerging threat to coral reefs worldwide. To enhance understanding of the impact, spread, and underlying causes of coral disease, rapid and highly sensitive diagnostic tools are required to specifically detect and quantify coral pathogens. *Vibrio coralliilyticus* represents a good model system for the development of novel coral pathogen diagnostic tools since it has been implicated as the etiological agent responsible for bleaching and tissue lysis in a number of scleractinian coral species throughout the Indo-Pacific. A real-time quantitative PCR (qPCR)-based detection assay for *V. coralliilyticus* has been successfully developed, which targets the *dnaJ* gene, a housekeeping gene encoding for heat shock protein 40. The assay was highly sensitive, detecting as little as 0.1 pg of purified *V. coralliilyticus* DNA and 10^4 colony forming units (CFU) per reaction (20 μL) for pure bacterial cultures. Inhibition of the assay by DNA and cells derived from bacteria other than *V. coralliilyticus* was minimal, validating the applicability of this assay when targeting the pathogen within the complex coral holobiont. This assay represents a novel approach to coral disease diagnosis and provides a useful tool for coral pathogen detection and accurate diagnosis that will play a vital role in advancing the field of coral disease research.

3.1. INTRODUCTION

      Coral reefs represent one of the most vulnerable ecosystems on the planet and are increasingly threatened by anthropogenic impacts, including climate change, overfishing, and pollution (Bryant et al. 1998; Bellwood et al. 2004; Hoegh-Guldberg et al. 2007). Disease epizootics on coral reefs have also been implicated in significant declines in coral cover and shifts in community structure, particularly in the Western Atlantic (Aronson and Precht 2001; Patterson et al. 2002; Weil et al. 2006). Such observations have led to concern about the impact of coral disease outbreaks on coral reef ecosystems worldwide. While the current focus on disease ecology and declines in reef ecosystems has been instrumental in identifying coral disease as an emerging threat, further understanding of biological and environmental drivers of disease would be significantly advanced by the development and implementation of microbial diagnostic techniques to detect and diagnose coral disease. Rapid and reliable diagnostics are virtually non-existent, due to a combination of factors, including a lack of systematic case definitions describing disease lesions, poor understanding of the causative agents of disease, and limited knowledge of the complex physiology of the coral holobiont (Work et al. 2008; Bourne et al. 2009). Effective disease management will always be difficult in an open system such as the coral reef environment, but development of advanced diagnostic tools for sensitive, reproducible, and specific detection of coral disease pathogens can allow for rapid assessment of disease outbreaks and may aid in the monitoring, control, and prevention of wide-spread coral epizootics.

      Vibrio coralliilyticus has recently emerged as a coral pathogen of particular concern on reefs throughout the Indo-Pacific. First implicated as the etiological agent responsible for bleaching and tissue lysis of the coral *Pocillopora damicornis* off the coast of Zanzibar in the Indian Ocean (Ben-Haim and Rosenberg 2002), *V.*

coralliilyticus has more recently been identified as the causative agent of outbreaks of white syndromes (WS's), a collective term describing coral diseases characterized by tissue loss resulting in a spreading band of exposed white skeleton on Indo-Pacific scleractinian corals (Work et al. 2008). Isolates of this potentially pathogenic bacterium have been collected from diseased corals in the Red Sea (Ben-Haim et al. 2003), Caribbean Sea (Vizcaino et al. in review), Indian (Ben-Haim and Rosenberg 2002), Pacific (Thompson et al. 2005; Sussman et al. 2008), and Atlantic Oceans (Ben-Haim et al. 2003; Thompson et al. 2005). *V. coralliilyticus* represents an emerging model pathogen for understanding the mechanisms linking bacterial infection and coral disease (Sussman et al. 2009) and therefore provides an ideal model for diagnostic assay development, which in future studies will allow accurate pathogen detection, coral disease diagnosis, and pathogen load monitoring in individuals and ecosystems, as well as the identification of pathogen sources, vectors, and reservoirs.

Traditionally, pathogen detection has depended upon culture-based and biochemical assays, which are time-consuming and labor-intensive. More recently, PCR-based methods, which amplify short nucleic acid sequences specific to the pathogen of interest, have begun to replace traditional techniques, providing increased sensitivity and specificity while greatly reducing sample processing time (Espy et al. 2006). Real-time quantitative PCR (qPCR) builds upon the PCR platform by incorporating into the reaction a fluorescent reporter, which emits a signal proportional to the quantity of PCR product produced during consecutive thermal cycles. This information is then used to infer the amount of target gene and relative number of pathogen cells in a given sample (Hough et al. 2002; Tondella et al. 2002). Due to its specificity, sensitivity, and speed, qPCR has become the detection method

of choice for a range of pathogenic bacteria, including numerous *Vibrio* species (Espy et al. 2006; Gubala 2006; Goarant and Merien 2006). Many qPCR chemistries are available, but the high specificity of TaqMan® probes (Applied Biosystems) along with the ability to detect several pathogen strains or species in a single multiplex reaction, make these probes an appealing choice for specific pathogen detection (Espy et al. 2006), particularly given the complex and diverse microbial assemblage associated with the coral holobiont and overlaying seawater.

In this paper, we describe a TaqMan®-based real-time quantitative PCR assay targeting a segment of the heat shock protein 40 encoding gene (*dnaJ*) of *V. coralliilyticus*. This assay provides the ability to detect and quantify this pathogen in coral and water samples with high specificity and sensitivity.

3.2. MATERIALS AND METHODS

Bacterial strains and growth medium

All bacterial strains used in this study are listed in Table 1. Strains were cultured on marine agar plates (MA, Difco, 55.1 g L^{-1}) at 28°C or in marine broth (MB, Difco, 47.4 g L^{-1}) at 28°C with shaking at 160 rpm.

DNA extraction and purification

Genomic DNA was extracted from liquid cultures of single bacterial strains using the Promega Wizard® Prep DNA Purification Kit (Promega, Syndey, Australia) following the manufacturer's directions for Gram-negative bacteria.

Selection of oligonucleotide primers and probes

Nucleotide sequences of the heat shock protein 40 encoding gene (*dnaJ*) were retrieved from a diverse selection of *Vibrio* species, including *V. coralliilyticus* (LMG 20984), using the National Center for Biotechnology Information's (NCBI) Entrez Nucleotide Database search tool (http://www.ncbi.nlm.nih.gov/). In addition, the *dnaJ*

gene was PCR amplified in several *V. coralliilyticus* strains (LMG 21348, LMG 21349, LMG 21350, LMG 10953, LMG 20538, LMG 23696, LMG 23691, LMG 23693, LMG 23692, LMG 23694) using primers and thermal cycling parameters described by Nhung et al. (2007). The PCR products were sequenced (Macrogen, Seoul, Korea) and these sequences compiled with those downloaded from the NCBI database using ClustalW (Higgins et al. 1996). Compiled sequences were imported into BioEdit (Hall 1999) and manually screened for single nucleotide polymorphisms (SNPs) that were conserved within *V. coralliilyticus* strains, but differed from non-*V. coralliilyticus* strains. Oligonucleotide primers and TaqMan® probes were designed to target regions with high densities of these nucleotide mismatches following the guidelines for custom TaqMan® assays outlined by Applied Biosystems. A 128-bp region of the *dnaJ* gene located between nucleotides 363 and 490 was targeted using primers Vc_dnaJ_F1 and Vc_dnaJ_R1 (Table 2). These primer sequences were analyzed using the IDT OligoAnalyzer software (Integrated DNA Technology, Inc., Coralville, IA) to determine their G+C content and test for the presence of primer dimers and secondary structures that might adversely affect PCR yield. A TaqMan® Minor Groove Binder (MGB) probe was designed to target a 13-bp sequence between nucleotides 427 and 439 using the Primer Express® Software (Version 2.0, Applied Biosystems). The specificity of the primer and probe sequences was evaluated via BLAST analysis using NCBI's GenBank database (http://www.ncbi.nlm.nih.gov/). Oligonucleotide primers were custom synthesized by Invitrogen (Victoria, Australia) and the 6-carboxyfluoresceine (6-FAM)-labeled oligonucleotide TaqMan® probe was custom synthesized by Applied Biosystems (Victoria, Australia).

Table 1: Species, strain, and threshold cycle (Ct) for all bacterial strains tested. Origin, host organism, and *dnaJ* gene sequence accession numbers are shown for *V. coralliilyticus* strains.

Species	Strain	Origin/reference	Host organism	$C_t \pm$ SEM	*dnaJ* gene sequence accession number	Reference
Vibrio coralliilyticus	LMG 23696	Nelly Bay, Magnetic Island, Australia	*Montipora aequituberculata*	12.43 ± 0.20	HM215570	Sussman et al. 2008
Vibrio coralliilyticus	LMG 23691	Majuro Atoll, Republic of Marshall Islands	*Acropora cytherea*	14.07 ± 1.33	HM215571	Sussman et al. 2008
Vibrio coralliilyticus	LMG 23693	Nikko Bay, Palau	*Pachyseris speciosa*	10.83 ± 2.76	HM215572	Sussman et al. 2008
Vibrio coralliilyticus	LMG 23692	Nikko Bay, Palau	*Pachyseris speciosa*	9.40 ± 0.36	HM215573	Sussman et al. 2008
Vibrio coralliilyticus	LMG 23694	Nikko Bay, Palau	*Pachyseris speciosa* (seawater above)	12.54 ± 0.24	HM215574	Sussman et al. 2008
Vibrio coralliilyticus	LMG 20984^T	Indian Ocean, Zanzibar, Tanzania	*Pocillopora damicornis*	12.80 ± 0.71	HM215575	Ben-Haim and Rosenberg 2002
Vibrio coralliilyticus	LMG 21348	Red Sea, Eilat, Israel	*Pocillopora damicornis*	13.81 ± 0.49	HM215576	Rosenberg et al. 2003
Vibrio coralliilyticus	LMG 21349	Red Sea, Eilat,	*Pocillopora damicornis*	12.98 ± 0.94	HM215577	Rosenberg et al. 2003
Vibrio coralliilyticus	LMG 21350	Red Sea, Eilat,	*Pocillopora damicornis*	11.49 ± 0.19	HM215578	Rosenberg et al. 2003
Vibrio coralliilyticus	LMG 10953	Kent, United Kingdom	*Crassostrea gigas* (oyster) larvae	10.53 ± 0.40	HM215579	Rosenberg et al. 2003
Vibrio coralliilyticus	LMG 20538	Atlantic Ocean, Florianópolis, Brazil	*Nodipecten nodosus* (bivalve) larvae	12.13 ± 0.50	HM215580	Rosenberg et al. 2003
Vibrio coralliilyticus	C1	Caribbean Sea, La Parguera, Puerto Rico	*Pseudopterogorgia americana*	14.53 ± 0.28	HM215568	Vizcaino et al. 2010
Vibrio coralliilyticus	C2	Caribbean Sea, La Parguera, Puerto Rico	*Pseudopterogorgia americana*	NA	HM215569	Vizcaino et al. 2010
Vibrio alginolyticus	ATCC 17749			33.74 ± 0.33		
Vibrio brasiliensis	DSM 17184			37.84†		
Vibrio calviensis	DSM 14347			27.06 ± 0.52		
Vibrio campbellii	ATCC 25920^T			39.10†		
Enterovibrio campbellii	LMG 21363			37.33 ± 2.41		
Allivibrio fischeri	DSM 507			31.36 ± 1.42		

Vibrio fortis	DSM 19133	NA
Vibrio furnissii	DSM 19622	NA
Vibrio harveyi	DSM 19623	NA
Vibrio natriegens	ATCC 14048	28.56 ± 0.60
Vibrio neptunius	LMG 20536	NA
Vibrio ordalii	ATCC 33509	25.56 ± 0.41
Vibrio parahaemolyticus	ATCC 17802	NA
Vibrio proteolyticus	ATCC 15338	30.00 ± 0.89††
Vibrio rotiferianus	LMG 21460	NA
Vibrio splendidus	ATCC 33125	32.31 ± 0.82
Vibrio tubiashii	ATCC 19109	NA
Vibrio xuii	LMG 21346	NA
Escherichia coli	ATCC 25922	NA
Psychrobacter sp.	AIMS (1618)	NA
Shewanella sp.	AIMS (C041)	25.34 ± 0.45

†† = Amplification in 2 of 3 reactions
† = Amplification in 1 of 3 reactions
NA = No amplification

* Strain designations beginning with LMG were derived from the Belgian Coordinated Collections of Micro-organisms, ATCC from the American Type Culture Collection, DSM from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH culture collection, AIMS from the Australian Institute of Marine Science culture collection, and C1 and C2 were provided by Dr. Pamela Morris.

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Table 2. Oligonucleotide primers and probes within the *dnaJ* gene used for the qPCR detection of the coral pathogen, *Vibrio coralliilyticus*

Primer or probe	Sequence [5' – 3']	Amplicon size (bp)	Location	Length (bp)	G+C content (%)
Vc_dnaJ_F1	5'-CGG TTC GYG GTG TTT CAA AA-3'	128	363-382	20	47%
Vc_dnaJ_R1	5'-AAC CTG ACC ATG ACC GTG ACA-3'		470-490	21	52%
Vc_dnaJ_TMP	5'-6-FAM-CAG TGG CGC GAA G-MGBNFQ-3'		427-439	13	69%

Assay optimization

A RotoGene 3000 real time analyzer (Corbett Research Pty. Ltd., Sydney, Australia) was used in tandem with a CAS-1200 robotic workstation (Corbett Robotics Pty. Ltd., Brisbane, Australia) for all qPCR experiments. For optimization of the real-time reaction, purified DNA from *V. coralliilyticus* strain LMG 23696 was used. The optimal concentrations of reagents were determined using the following qPCR reaction conditions: 1 X TaqMan Buffer A, 0.5 U of AmpliTaq Gold® DNA polymerase, 200 μM of deoxynucleotide triphosphates (with 400 μM of deoxyuridine triphosphate replacing deoxythymidine triphosphate), 0.2 U of AmpErase Uracil N-glycosylase (UNG), 2 to 5 mM $MgCl_2$, each primer at concentrations of 0.2 to 0.8 μM, fluorophore-labeled TaqMan® probe at concentrations of 0.1 to 0.4 μM, 1 μL of template, and an appropriate amount of sterile, deionized MilliQ (Millipore Australia, North Ryde, NSW) water to bring the total reaction volume to 20 μL. The PCR cycling parameters were as follows: 50°C for 120 s (UNG activation), 95°C for 10 min (AmpliTaq Gold® DNA polymerase activation), and then 40 cycles of 95°C for 15 s (denaturation) and 60°C for 60 s (annealing/extension). During the annealing/extension phase of each thermal cycle, fluorescence was measured in the FAM channel (470 nm excitation/510 nm detection).

Testing assay sensitivity and specificity

The qPCR assay was validated using *V. coralliilyticus* isolates, non-target *Vibrio* species, and other bacterial species (Table 1). The assay's DNA detection limit was determined by subjecting 10-fold serial dilutions (diluted in sterile, deionized MilliQ water) of purified genomic *V. coralliilyticus* DNA (LMG 23696), ranging from 100 ng to 0.01 pg per reaction, to qPCR amplification. A qPCR mixture containing no DNA was used as a negative control. In addition, the assay's single strain detection limit was assessed using overnight liquid cultures of *V. coralliilyticus*

strain LMG 23696, 10-fold serially diluted to concentrations ranging from 10^8 CFU mL^{-1} (optical density at 600 nm = 0.78) to extinction in marine broth. Total bacterial cell numbers were determined by triplicate colony forming unit (CFU) counts on marine agar plates. Purified genomic DNA was extracted from these dilutions, 10-fold diluted with sterile, deionized MilliQ water (to increase qPCR efficiency), and 1 µL was used as template for qPCR amplification. A qPCR mixture containing no bacterial cells or DNA was used as a negative control.

To test the ability of the qPCR assay to accurately quantify *V. coralliilyticus* DNA in the presence of competing, non-*V. coralliilyticus* DNA, 10-fold serial dilutions of *V. campbellii* (ATCC 25920^T) DNA, ranging from 0.01 to 100 ng per reaction, were spiked into a qPCR reaction containing 10 ng of *V. coralliilyticus* (LMG 23696) DNA. A qPCR mixture containing 10 ng *V. coralliilyticus* DNA and no *V. campbellii* DNA was used as a positive control and a qPCR mixture containing no bacterial DNA served as a negative control. Additionally, pure cultures of *V. coralliilyticus* (LMG 23696) and *V. campbellii* (ATCC 25920^T) were grown to optical densities at 600 nm (OD$_{600}$) of 0.80 and 0.40, respectively. *V. coralliilyticus* was diluted to approximately 10^7 CFU per mL and 0.5 mL aliquots were combined with an equal volume of *V. campbellii* culture corresponding to 0, 10, 10^4, or 10^7 CFU mL^{-1}. DNA was extracted as detailed above with the PowerPlant® DNA Isolation Kit and 1 µL of this purified DNA served as the template for qPCR amplification. A qPCR mixture free of *V. coralliilyticus* and *V. campbellii* DNA and cells served as a negative control.

3.3. RESULTS

Assay optimizations and specificity

A qPCR assay was developed to specifically target the housekeeping gene

dnaJ of the coral pathogen *Vibrio coralliilyticus* and optimized with regards to

$MgCl_2$, oligonucleotide primer, and TaqMan probe concentrations (Fig. 1) with the

ideal reagent concentrations summarized in Table 3. The qPCR assay specifically

detected 12 out of 13 isolated *V. coralliilyticus* strains tested in this study (Table 1).

The exception was one Caribbean strain (C2), which failed to give specific

amplification despite repeated attempts. Positive detection of the target gene segment

was determined by the increase in fluorescent signal beyond the fluorescence

threshold value (normalized fluorescence = 0.010) at a specific cycle, referred to as

the threshold cycle (C_t). Specific detection was further confirmed by gel

electrophoresis, which revealed a PCR product of the correct theoretical size (128 bp,

data not shown), and DNA sequencing, which confirmed the target amplified product

to be a segment of the *dnaJ* gene. No amplification with the assay was detected for 13

other closely related *Vibrio* strains including the closely related *Vibrio neptunis* and 2

non-*Vibrio* species (see Table 1). A total of 5 other *Vibrio* strains and one non-*Vibrio*

strain (*Shewanella sp.*) exhibited C_t values less than the cutoff of 32 cycles. However,

the C_t values for these strains (average $C_t \pm$ standard error of the mean [SEM]) (27.96

$\pm$ 2.40) were much higher than for the *V. coralliilyticus* strains (12.30 $\pm$ 1.52) and no

amplicons were evident in post-qPCR gel electrophoresis (data not shown).
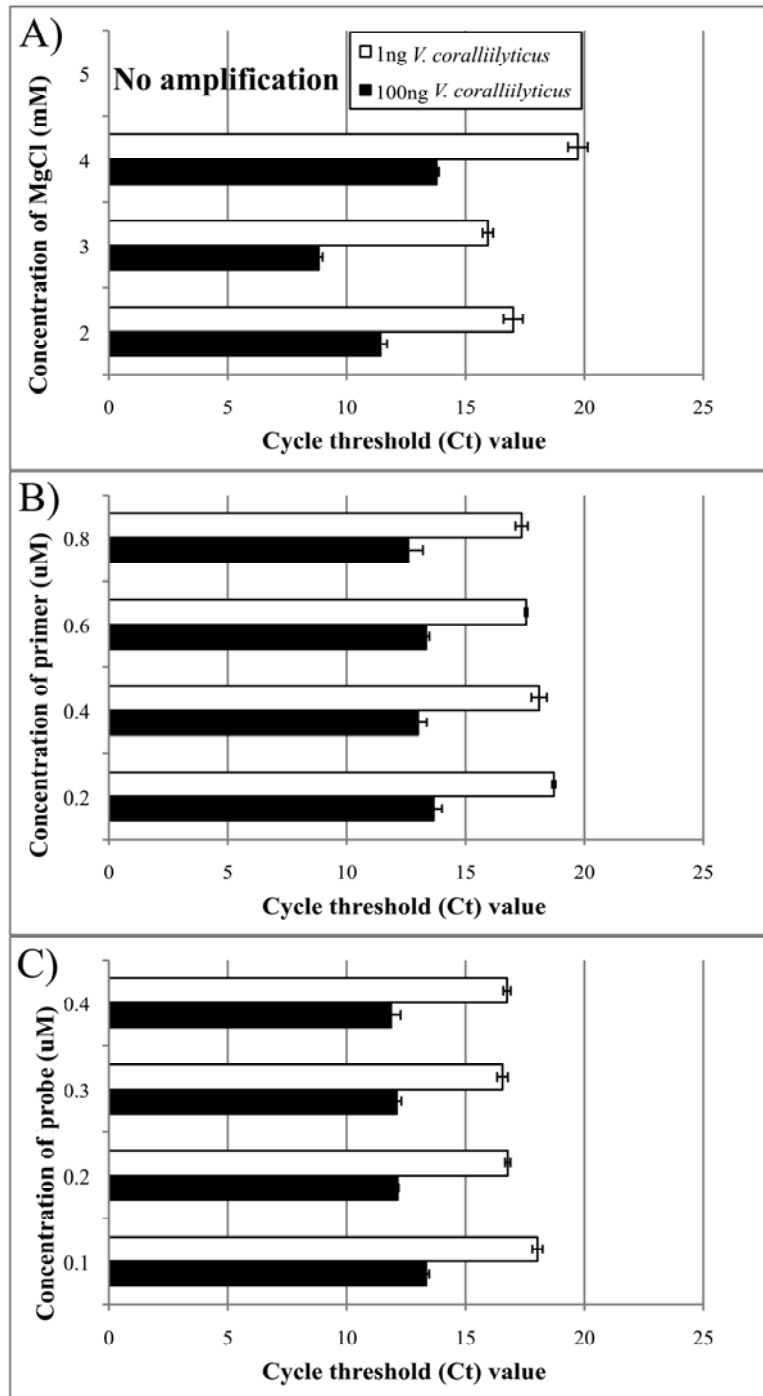
Figure 1. qPCR reaction optimization. Concentrations of: A) $MgCl_2$, B) oligonucleotide primer, and C) TaqMan probe versus C_t values of the fluorescent signal are shown. White and black columns represent qPCR reactions spiked with 1 ng and 100 ng of purified *V. coralliilyticus* DNA, respectively. Error bars indicate standard error of the mean for two replicate qPCR reactions.

Table 3. Optimized reagent concentrations for the qPCR *Vibrio coralliilyticus* assay.

Reagent	Single rxn volume (μL)	Final concentration
Deionized water	9.9	—
10X TaqMan Buffer A	2	1 X
25 mM MgCl2 Solution	2.4	3 mM
2.5 mM dNTPs (5mM dUTP)	1.6	200 μM (400 μM dUTP)
Vc_dnaJ_F1 Primer (10μM)	1.2	0.6 μM
Vc_dnaJ_R1 Primer (10μM)	1.2	0.6 μM
Vc_dnaJ_TMP1 Probe (10 U/μL)	0.4	0.2 μM
AmpliTaq Gold (5 U/μL)	0.1	0.025 U μL^{-1}
AmpErase UNG (1U/μL)	0.2	0.01 U μL^{-1}
DNA Template	1	—
Total Mix Volume	20	—

Assay sensitivity

The detection limit for the qPCR assay, determined by spiking the reaction with purified *V. coralliilyticus* genomic DNA, was 0.1 picogram (Fig. 2A). The lowest detection limit for single cultures of *V. coralliilyticus* grown in nutrient-rich media was 10^4 CFU mL^{-1} (Fig. 2B). Standard curves revealed a strong linear negative correlation between C_t values and *V. coralliilyticus* DNA and cell concentrations over several orders of magnitude, with r^2 values of 0.998 and 0.953 for DNA and cells, respectively (Fig. 2). Thus, as concentrations of *V. coralliilyticus* DNA or cells in the reaction mixture increased, the C_t value decreased (Fig. 2).
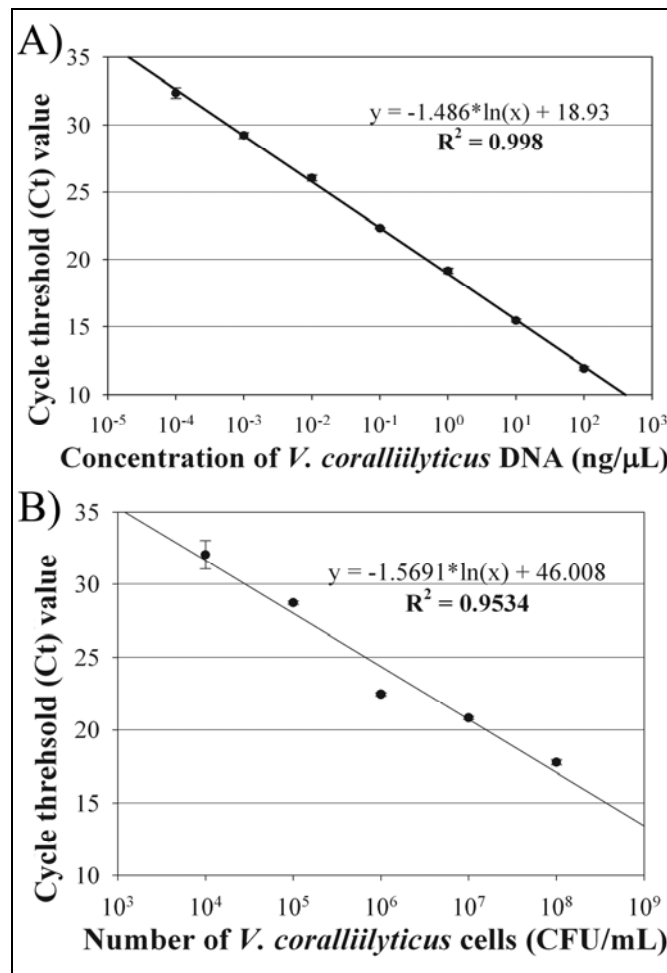
Figure 2. Standard curves delineating threshold (C_t) values of fluorescence for indicators of pathogen presence: A) concentration of *V. coralliilyticus* DNA and B) number of *V. coralliilyticus* cells in pure culture. Error bars indicate standard error of the mean for three replicate qPCR reactions.

Little interference of the qPCR assay was observed when purified *V. coralliilyticus* DNA (10 ng) was combined with varying concentrations of non-*V. coralliilyticus* DNA (0.01 to 100 ng). Over the entire range of non-target DNA concentrations tested, the resulting C_t values (17.76 ± 0.53) were not significantly different from that of a control treatment containing 10 ng *V. coralliilyticus* DNA and no non-specific DNA (16.75 ± 0.18; ANOVA; $p = 0.51$, Table 4). Similarly, detection of *V. coralliilyticus* cells (10^4, 10^5, 10^6, 10^7, or 10^8 CFU per mL) in a background of non-*V. coralliilyticus* cells (i.e. *V. campbellii* [ATCC 25920^T] at 0, 10, 10^4, 10^7 CFU per mL) showed little reduction in assay sensitivity (Fig. 3). For example, when V. coralliilyticus was seeded at 107 cells with similarly high concentrations of non-target cells, little inhibition of the assay was observed (Table 4).
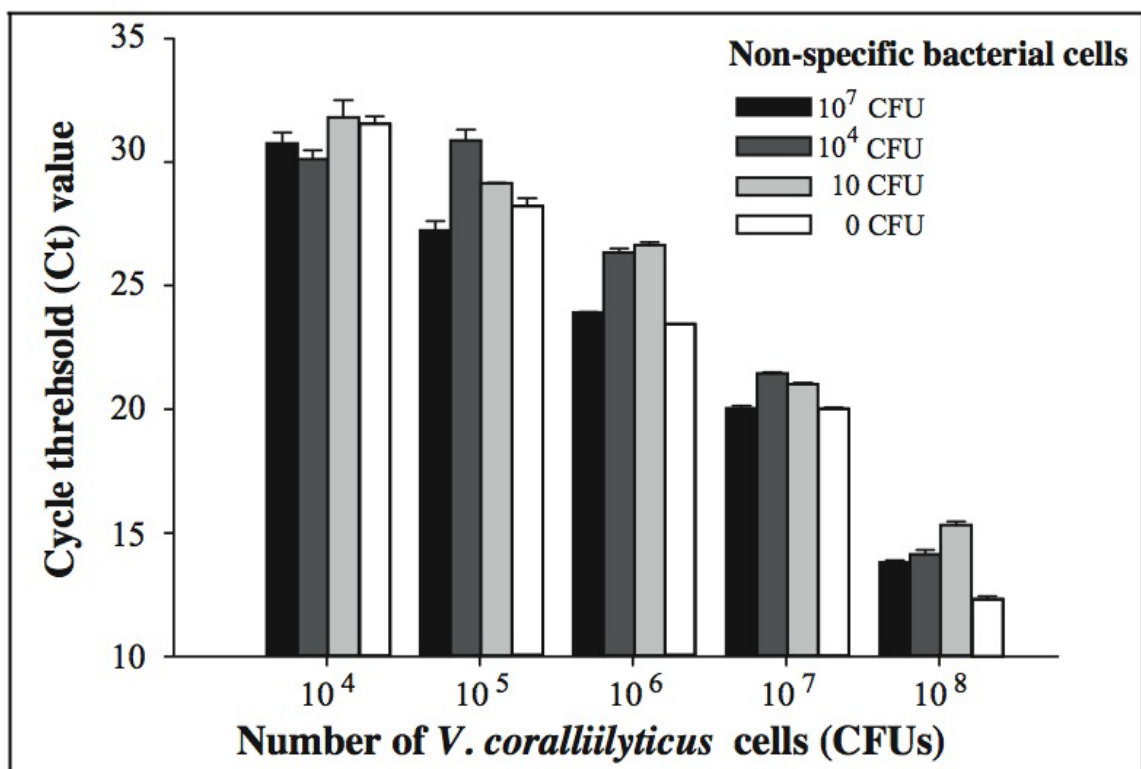
Figure 3. Detection of *V. coralliilyticus* in the presence of competing, non-*V. coralliilyticus* cells. Columns illustrate the fluorescence signal (C_t) resulting from the qPCR amplification of pure cultures of *V. coralliilyticus* (10^4-10^8 CFU) combined with non-specific bacterial cells (0-10^7 CFU). Error bars indicate standard error of the mean for three replicate qPCR reactions.

Table 4. Effect of non-target bacterial (i.e. *V. campbellii* (ATCC 25920^T)) DNA and cells on the detection of *V. coralliilyticus* (LMG 23696) purified DNA (10 ng) or bacteria cells (10^7 CFU). *V. coralliilyticus* DNA (10 ng) and bacteria cells (10^7 CFU) free of non-target DNA and cells served as positive controls and a qPCR mixture containing no bacterial DNA or cells served as a negative control.

Non-target DNA (ng)	C_t (mean ± sem)	Non-target cells (CFU)	C_t (mean ± SEM)
100	16.97 ± 0.33	10^7	21.02 ± 0.06
10	16.9 ± 0.08	10^4	21.44 ± 0.04
1	16.74 ± 0.10	10^1	20.04 ± 0.10
0.1	17 ± 0.09	0 [a]	20.03 ± 0.06
0.01	16.37 ± 0.43	NTC[b]	No Amplification
0 [a]	16.75 ± 0.18		
NTC[b]	35.04 ± 0.02		

[a] Positive control
[b] NTC = No template control = Negative control

3.3. Discussion

The current study describes the first assay developed to detect and quantify a coral pathogen using a real-time quantitative PCR (qPCR) approach. While previous studies have utilized antibodies or fluorescent *in situ* hybridization (FISH) to detect coral pathogens (Israely et al. 2001; Ainsworth et al. 2006), the combination of high sensitivity and specificity, low contamination risk, and ease and speed of performance (Espy et al. 2006) make qPCR technology an ideal choice for rapid pathogen detection in complex hosts, such as corals. Diseases have contributed significantly to recent declines in coral reef ecosystems worldwide (Green and Bruckner 2000; Sutherland et al. 2004; Harvell et al. 2007), although compared to investigations of other wildlife, the study of coral disease is in its infancy (Work et al. 2008). Traditional coral diagnosis has been based almost exclusively upon observations of macroscopic disease signs (Ainsworth et al. 2007), often resulting in cases of misidentified diseases, repeated name changes for the same disease, and even misclassification of predation scars as disease (Bruckner and Bruckner 1997; Bruckner and Bruckner 2002; Patterson et al. 2002). This confusion highlights the need for more systematic morphological disease descriptions (Work et al. 2008) and

the development of robust techniques to diagnose the underlying pathologies of diseases. Detection of microbial agents that cause coral diseases using specific probes will provide important insights into the dynamics of pathogen invasion and spread within populations (e.g. Israely et al. 2001), while also aiding in the identification of disease vectors and reservoirs (e.g. Sussman et al. 2003).

The TaqMan®-based qPCR assay, optimized to target the coral pathogen *Vibrio coralliilyticus*, detected 12 out of 13 tested isolates. The assay was fairly specific with no signal for 15 out of 21 phylogenetically closely-related, non-*V. coralliilyticus* strains. A total of 6 other non-*V. coralliilyticus* strains exhibited some positive signal, although importantly, the fluorescence appeared late in the thermocycle protocol (mean ± SEM: 27.96 ± 2.40), well beyond the threshold cycles (C_t) of the *V. coralliilyticus* isolates (mean ± SEM: 12.30 ± 1.52). The assay was also highly sensitive; capable of detecting as little as 0.1 picogram of purified *V. coralliilyticus* DNA or 10^4 CFU mL^{-1} for pure cultures. These detection limits are likely to be within biologically relevant pathogen concentrations. For example, antibodies for specific detection of the coral bleaching pathogen *Vibrio shiloi*, showed that bacterial densities reached 8.4 x 10^8 cells cm^{-3} one month prior to maximum visual bleaching signs on the coral species *Oculina patagonica* (Israely et al. 2001). During disease onset, pathogens commonly replicate at high numbers, especially at the lesion interface, and therefore the sensitivity of this assay will be valuable for specific detection and quantification of *V. coralliilyticus* cell numbers, potentially even before disease signs can be visually observed. This is important since coral-associated microbial populations, which are typically stable in healthy corals, have been documented to undergo dramatic shifts well before disease signs are visible macroscopically (Israely et al. 2001; Pantos et al. 2003; Bourne et al. 2008). For

example, colony-wide bacterial shifts were detected on *Montastrea annularis* even though only a small part of the colony exhibited disease signs (Pantos et al. 2003). Similarly, shifts in the microbial community structure of corals have been detected well before the appearance of visual signs of bleaching (Israely et al. 2001; Bourne et al. 2008). The assay developed in this study provides a means of detecting and quantifying cell numbers of an identified coral pathogen, potentially facilitating coral disease diagnosis at the earliest stages of infection.

V. coralliilyticus, like *Vibrio shiloi,* is becoming a model pathogen for the study of coral disease. Recent research efforts have characterized the organism's genome (PJ Morris pers comm; FL Thompson pers comm), proteome (Kimes et al. 2009), and metabolome (Boroujerdi et al. 2009) and enhanced our understanding of the genetic (Meron et al. 2009; Pollock et al. 2010a) and physiological (Meron et al. 2009; Sussman et al. 2009) basis of its virulence. Before effective management response plans can be formulated, however, continuing research on the genetic and cellular aspects of *V. coralliilyticus* must be complemented with knowledge of the epidemiology of this pathogen, including information on its distribution, incidence of infection, and rates of transmission throughout populations. The *V. coralliilyticus*-specific qPCR assay developed in this study will contribute significantly to the generation of this knowledge, by facilitating the identification of pathogen reservoirs, as well as mechanisms and vectors involved in disease transmission between individuals and populations (Sussman et al. 2003). Development of this assay represents a critical step towards a more complete understanding of the epidemiology of this pathogen and will overcome key stumbling blocks in the capacity of reef managers to control the spread of coral disease. In particular, this assay can be used as an early warning sign of a potential disease outbreak, providing managers with

critical information of when and where control measures should be used. Although phage-mediated control remains a controversial solution to disease outbreaks (Teplitski and Ritchie 2009), this tool could be utilized to assess the success of such measures, illustrating another important role of pathogen-specific detection in the management decision-making process.

3.4. CONCLUSIONS

This study details the development of a qPCR-based coral pathogen detection and quantification tool that is sensitive, specific, and robust. The assay was capable of detection and quantification of *V. coralliilyticus* in the presence of competing, non-*V. coralliilyticus* bacterial cells and DNA, an important consideration for accurate detection within complex coral holobiont samples, particularly where the target organism is present within a matrix of other microbial and host cells. This assay provides a novel tool for the study of coral diseases, and applied in the field, will provide insights into the epidemiology of the emerging model pathogen, *V. coralliilyticus*. The qPCR platform allows rapid sample analysis, with the entire process from DNA extraction to probe detection taking less than a day. In addition, TaqMan® chemistry provides the ability to multiplex the reaction, facilitating the simultaneous detection of multiple pathogenic species or target genes in a single reaction. The assay presented here represents a novel tool for coral pathogen detection and quantification, which will play an important role in advancing the field of coral disease research and the effective management of coral reefs worldwide.

GENERAL DISCUSSION

Vibrio coralliilyticus has emerged as a coral pathogen of great concern, to both coral reef researchers and managers alike, in light of its identification as the causative agent of bleaching and tissue lysis of corals in the Indian Ocean (Ben-Haim et al. 2003) and outbreaks of white syndromes (WS) in coral populations at several locations throughout the Indo-Pacific (Sussman et al. 2008). Despite the global distribution of *V. coralliilyticus* and the toll that WS has already taken on coral reefs throughout the Indo-Pacific, little is known about the population genetics of this pathogen or its role in large-scale WS outbreaks. Currently, it is unclear if the recent emergence of WS on reefs throughout the Indo-Pacific is the result of a rapidly spreading, pandemic strain of *V. coralliilyticus* or if this bacterium is even associated with WS in all cases. Scientists lack the epidemiological information required to control or contain WS outbreaks and our current diagnostic methods are only capable of detecting WS at the latest stages of infection, when control measures are least effective. This knowledge gap highlights the need for a deeper understanding of *V. coralliilyticus*, the development of sensitive diagnostic tools for its detection, and in-depth field-based studies utilizing these tools to gain insight into the dynamics of WS outbreaks.

V. coralliilyticus is an excellent model pathogen for the study of coral disease, given recent research efforts that have characterized the organism's genome (Johnson et al. in review), proteome (Kimes et al. in review), resistome (Vizcaino et al. 2010),

and metabolome (Ben-Haim et al. 2003). Moreover, the basis for its virulence has been explored in both genetic (Meron et al. 2009; Pollock et al. 2010a) and physiological (Meron et al. 2009; Sussman et al. 2009) studies. Although it has been established that *V. corallliilyticus* is globally distributed (Chapter 2), otherwise, little is known about the population dynamics, population genetics, or evolutionary history of this bacterium. Such information is invaluable for identifying and tracking invasive genotypes and can also be used to correlate genetic variability with important phenotypic differences, most notably virulence (Kreiswirth et al. 1993; Maiden et al. 1998; Claus et al. 2002; Shnit-Orland 2009).

In order to bridge this knowledge gap, biochemical characterization assays and multilocus sequence typing (MLST) were used to infer phenotypic and phylogenetic relationships between geographically diverse isolates of *V. coralliilyticus*. This study revealed *V. coralliilyticus* to be highly genetically diverse, with little evidence of clonality among the isolates analyzed (Chapter 2). Furthermore, phylogenetic reconstructions suggest that the recent emergence of white syndromes on corals from widely separated reef regions is unlikely to have been caused by a rapidly spreading pandemic strain. Instead, the results suggest that *V. coralliilyticus* populations are an endemic component of coral reef ecosystems that vary genetically among the globally-distributed locations sampled.

Interestingly, a geographic trend was observed in the clustering of the zinc-metalloprotease (a known *V. coralliilyticus* virulence factor) gene sequences, with the Indo-Pacific and northern-Atlantic isolates forming one closely related clade and the Caribbean and southern-Atlantic isolates forming another. While the high level of sequence divergence between these clades suggests that they may code for two distinct forms of the metalloprotease, this assertion cannot be made without further

study. Future investigations should examine if and how this genetic variability correlates with virulence by measuring the degree of *Symbiodinium* photosystem II inactivation (the presumed target of the zinc-metalloprotease) resulting from inoculation with genetically distinct *V. coralliilyticus* isolates. Controlled, aquarium-based coral infection experiments would also help to illuminate how different strains affect coral health at the holobiont level.

The current study was limited by the small sample size available for phylogeographic studies. Although all 13 *V. coralliilyticus* isolates publically available at the time of this study were used, inclusion of multiple isolates from each of the geographic regions examined, as well as the inclusion of more locations, would increase the resolution of this analysis and further test the robustness of the observed distinction between the Indo-Pacific/North Atlantic and the Caribbean/South Atlantic clades. As more *V. coralliilyticus* strains are isolated and their genes sequenced, we will gain a deeper understanding of the phylogeny of this pathogen at both the local and global scales.

Traditionally, coral disease diagnosis has been based almost exclusively upon visual observation of macroscopic disease signs (Work and Aeby 2006; Ainsworth et al. 2007). While visual disease identification is rapid and convenient, this method is often inadequate for definitive diagnosis and reveals little about the pathologies underlying the observed disease signs. Development and implementation of a new generation of coral disease diagnostic assays that specifically target identified coral pathogens (Chapter 3) will allow researchers to better discern the factors that affect the occurrence and prevalence of diseases and, potentially, control them. A review of the technologies that could be used for such an assay suggest that real time

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quantitative polymerase chain reaction (qPCR) is an ideal platform, due to its low

contamination risk, high specificity, and high sensitivity (Chapter 1).

In order to provide an early indicator of infection, aid in the identification of

disease vectors and reservoirs, and assist managers in developing strategies to prevent

the spread of coral disease outbreaks, a novel assay was developed to detect and

quantify *V. coralliilyticus* (Chapter 2). This qPCR assay was found to be specific,

detecting 12 out of 13 tested *V. coralliilyticus* isolates while producing no signal for

15 out of 21 phylogenetically closely-related, non-*V. coralliilyticus* strains. The 6

remaining non-*V. coralliilyticus* strains exhibited some positive signal, although

importantly, the fluorescence appeared late in the thermocycle protocol, well beyond

the threshold cycles (C_t) of the *V. coralliilyticus* isolates.

The assay was also highly sensitive, capable of detecting as little as 0.1

picogram of purified *V. coralliilyticus* DNA or 10^4 CFU mL^{-1} for pure cultures. These

detection limits are on par with other assays for marine pathogen detection, including

the fish pathogen, *Aeromonas salmonicida*, which can be detected at concentrations

as low as 0.5 picogram of purified DNA (Balcázar et al. 2007) and *Vibrio*

parahaemolyticus, which can be detected at 10^4 CFU mL^{-1} for pure cultures (Ward

and Bej 2005). These detection limits are also likely to be within biologically relevant

pathogen concentrations. For example, antibodies for specific detection of the coral

bleaching pathogen, *Vibrio shiloi*, showed that bacterial densities reached 8.4 x 10^8

cells cm^{-3} one month prior to maximum visual bleaching signs on the coral species

Oculina patagonica (Israely et al. 2001). Since coral-associated microbial

populations, which are typically stable in healthy corals, have been documented to

undergo dramatic shifts well before disease signs are visible macroscopically (Israely

et al. 2001; Pantos et al. 2003; Bourne et al. 2008), this assay has the potential to detect *V. coralliilyticus* even before disease signs can be visually observed.

While the assay developed in this study provides a useful tool to investigate the epidemiology of *V. coralliilyticus*, extensive validation of the assay is required. qPCR has been used widely for pathogen detection in a range of organisms, but the coral host provides a unique set of challenges, which are markedly different from those facing researchers in the fields of human, veterinary, and even aquaculture-related diseases. This assay aims to detect *V. coralliilyticus* within environmental samples derived from the coral holobiont, which contains genetic material from the coral animal, as well as its microbial partners, including symbiotic dinoflagellates (zooxanthellae) (Muscatine et al. 1981), bacteria (Rohwer et al. 2002), Archaea (Wegley et al. 2004), viruses (Wilson et al. 2005), endolithic algae (Shasher et al. 1997), and fungi (Bentis et al. 2000). In addition, the presence of high concentrations of PCR inhibitors, including salts and DNAses, make successful DNA extraction and qPCR amplification from coral tissue extremely difficult. While the use of specialized DNA extraction protocols and extensive assay optimization increase the resolution of qPCR-based detection (Chapter 3), the potential for PCR inhibition and sample-to-sample variability highlight the need for cross-validation of this assay with a second technique, such as antibodies or fluorescent *in situ* hydridization (FISH) (details of assay validation are highlighted in Chapter 1). Currently, efforts are underway to develop antibodies specific to *V. coralliilyticus* that could serve as a secondary technique for cross-validation of the qPCR assay (D Bourne pers. comm.).

Effective management of coral disease outbreaks will require a better understanding of the underlying causes of disease outbreaks, how diseases are spread between colonies and populations, and the relationship between environmental

parameters, pathogen virulence, and susceptibility of the coral host to infection (Bruckner 2002). Once appropriate steps have been taken to ensure that the tool is sensitive, specific, and robust, it can be used in the field to answer many of these important questions. The ability to detect and quantify *V. coralliilyticus* will provide information on the concentration of this pathogen on healthy and diseased corals, giving researchers and managers a novel proxy for coral health. In addition, this tool could provide data on the relationship between environmental parameters (e.g. temperature, salinity, nutrient load) and pathogen load. These data could then be incorporated into detailed mathematical models to better predict large-scale disease outbreaks. Finally, this *V. coralliilyticus*-specific assay could be used to identify when and where biological controls for coral diseases (e.g. bacteriophage therapy and probiotic addition) (Effrony et al. 2009; Teplitski and Ritchie 2009) should be implemented and to assess their efficacy. Taken together, these capabilities will assist resource managers to combat disease epizootics, prevent future outbreaks, and reduce the time needed for reef recovery (Bourne et al. 2009).

The research presented in this thesis builds upon recent investigations into the genetic and physiological basis of *V. coralliilyticus* virulence by providing a study of the phylogeography of this globally distributed pathogen (Chapter 2) and developing a novel assay to detect and quantify this bacterium in seawater and coral samples (Chapter 3). The assay described in this thesis has the potential to provide accurate coral disease diagnosis and pathogen load monitoring, which will help to direct research and management strategies to address the causes of coral diseases on reefs and aid reef managers in their efforts to control the occurrence, prevalence, and severity of coral disease outbreaks.

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