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Genetic Profiling of the White Pox Disease Coral Pathogen Serratia marcescens from the Florida Keys

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Genetic profiling of the white pox disease coral pathogen *Serratia marcescens* from the Florida Keys

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Abstract

Since its first known outbreak in 1996, white pox disease has decimated the reef building coral species *Acropora palmata* throughout the Florida Keys and the Caribbean. The only known cause of white pox disease is the fecal enterobacterium *Serratia marcescens*. Two *S. marcescens* strains have been previously extracted from infected *A. palmata*: PDL100 and PDR60. One strain of this bacterium (PDR60) found in infected *A. palmata* has been traced back to human sewage, and is the only known marine reverse zoonotic disease. In this study, bacterial isolates from canals, wastewater, infected *A. palmata*, and non-host corals from Florida Key reefs were analyzed to determine which strains, if any, of *S. marcescens* were present in this area during July 2013. Potential *S. marcescens* isolates identified using MacConkey Sorbitol Agar amended with the antibiotic colistin (MCSA) and DNase with Toludine Blue agar amended with the antibiotic cephalothin (DTC), were confirmed as *S. marcescens* using *Serratia*-specific polymerase chain reaction. These *S. marcescens* positive isolates were fingerprinted using pulsed-field gel electrophoresis (PFGE). Unlike study results from the past decade, neither the PDL100 nor the PDR60 strain was isolated during this study. Four different *S. marcescens* strains were found from a combination of canal and wastewater. A fifth strain was found from the non-host coral species *Porites porites*, but this strain is an unknown species of bacteria, not *S. marcescens*, because it tested negative on DTC. Although bacterial isolates were taken directly from *A. palmata* lesions, none of these samples were positive for *S. marcescens*, leading to the conclusion that there may be other unknown causes of white pox disease that have yet to be identified.

Introduction

Current state and importance of coral reefs

The amount of coral cover loss that has occurred throughout the world over the last 35 years is historically unprecedented (Greenstein et al. 1998, Aronson et al. 2002, Gardner et al. 2006). As of 2000, 11% of the world's historic coral cover had receded (Wilkinson 2000), and as of 2006, hard coral cover had diminished by 80% from its 1970 amount (Gardner et al. 2006). The occasional report of site-specific increases in coral cover may be misleading. There has been a visible shift from framework building hard corals, such as the *Acropora* and *Montastrea* that encourage reef complexity and diversity, toward non-framework building soft corals and sponges, such as *Agaricia* and *Porites*, that do little to advance overall reef health (Edmunds et al. 2001, Cho et al. 2002, Gardner et al. 2006). This geologically sudden decrease in the amount of hard corals has caused a decrease in reef structural complexity, lowering diversity, and severely flattening coral reefs. Within the Caribbean, reef flattening increased by over 55% from the early 1970s to 2009 (Alvarez-Filip et al. 2009).

The disappearance rate of hard corals is alarming since coral reefs are one of the most important ecosystems in the world. Reefs are home to at least 60,000 flora and fauna which is a larger range of organisms than any other habitat (Reaka-Kudla 1994, Moberg & Folke 1999). Many species use coral reefs as a sheltered nursery for their young before they are large enough to enter the open ocean. Humans depend on coral reefs as a breeding ground for a large number of commercial fish species and as the source of most beach resort sand. Coral reefs are important storm barriers as well, diffusing wave energy away from coastal communities. If coral reefs continue to degrade, beach cities will experience a greater erosion problem on shore than they are already having. Furthermore, destruction of coral reefs would mean the likely extinction

of thousands of the organisms that live there which could have undiscovered consequences for the rest of the world's ecosystems (McClanahan 2002, Levinton 2009, Trujillo & Thurman 2011).

Threats to Coral Reefs

Coral reefs are at risk due to a multitude of reasons, the greatest of which are overfishing, global climate change, natural disasters, and disease. Overfishing is a threat to coral populations because many commercial fish are dominant grazers of algae that compete with corals for space. A decrease in the number of algae-eating fish causes a subsequent increase in microalgae proliferation, which in turn prevents coral growth and recruitment. Over time, extensive algal coverage can cause coral colony mortality (Knowlton 1992, Connell et al. 1997, McCook et al. 2001, Grober-Dunsmore et al. 2006). This "top-down" effect occurs when any significant species of coral reef algal feeder is suddenly removed from its habitat (Hughes et al. 1999, Aronson & Precht 2000, Porter et al. 2001). A mass die-off of the Caribbean sea urchin *Diadema antillarum* in 1983, most likely due to a species-specific disease, created algal overgrowth throughout affected areas that quickly outcompeted corals for space and is partially responsible for the sudden rise in coral reef death in the mid-1980s (Lessios 1988, Hughes 1994, Grober-Dunsmore et al. 2006). Increased nutrient levels from coastal runoff also cause a proliferation of algae, which outcompete corals for space. This is called the "bottom-up" effect (Muller et al. 2008).

Natural disasters such as hurricanes are also responsible for coral reef destruction. It is argued that corals that can asexually reproduce through fragmentation welcome this type of disturbance because storms break off their branches and help spread their populations to new

areas (Richmond 1993). However, many current populations of these types of hard corals throughout the Caribbean are already so small that destructive storms are more of a hindrance than a help. Hurricanes not only break up coral colonies, but also stir up a lot of sediment particulates. When these particulates settle after the storm, they often bury fragmented coral colonies. Burial blocks coral endosymbiotic microalgae called zooxanthellae from the sun and causes the entire coral to eventually die (Wolanksi et al. 2003, Grober-Dunsmore et al. 2006). The Caribbean Hurricane Allen is responsible for destroying a large portion of the coral reef, specifically branching *Acroporids*, in that area in 1980 (Gardner et al. 2006).

Global climate change, the incremental increase of the world's atmospheric temperature due to increased greenhouse gas emissions, also gradually increases ocean temperatures. Heat trapped in the atmosphere melts the polar ice caps, reducing the amount of energy reflected back into the atmosphere and increasing the heat absorbed by the oceans (Walsh 1991, Grotefendt et al. 1998). Corals are very temperature sensitive, so even slightly elevated ocean temperatures stress corals, causing them to expel their endosymbiotic zooxanthellae in a process called bleaching. Bleaching can make corals more susceptible to infection from environmental pathogens (Brown 1997, Hoegh-Guldberg 1999, Porter et al. 2001, Grober-Dunsmore et al. 2006). Abiotic disease caused by environmental stressors such as ocean warming can destroy an entire reef (Muller et al. 2008). Furthermore, it is very likely that climate change is prolonging the disease season for species-specific biotic pathogens (Patterson et al. 2002).

Environmental stressors can support biotic diseases by 1) encouraging the pathogen's growth and reproduction, 2) hindering the host species' defenses against the pathogen, and 3) increasing the rate of infection (Peters 1997, Toren et al. 1998, Kushmaro et al. 1998, Israely et al. 2001, Banin et al. 2001, Alker et al. 2001, Kuta & Richardson 2002, Ben-Haim & Rosenberg

2002, Richardson & Kuta 2003, Ben-Haim et al. 2003, Sutherland et al. 2004). Rising ocean temperature increased the hard branching elkhorn coral *Acropora palmata*'s susceptibility to disease by stressing it to the point of expelling its zooxanthellae and decreasing its protective mucus antibiotic activity (Ritchie 2006, Lesser et al. 2007, Muller et al. 2008). As of 2004, there were 22 coral species worldwide infected with at least four different types of coral disease, including 82% of all Caribbean coral species (Sutherland et al. 2004).

Acropora palmata

Acropora palmata is a stony, branching coral. Due to its quick growth rate and calcium carbonate skeletal deposition, it is considered a primary reef foundation species that must grow before many other reef species can inhabit the area (Gladfelter et al. 1997, Mayor et al. 2006). Requiring a hard bottom habitat less than 10 meters deep, *A. palmata* is the dominant coral in high reef zones, consistently exposed to intense waves and surge (Mayor et al. 2006). Since branches are often broken off by wave action, one of *A. palmata*'s primary distribution techniques is asexual regeneration from fragmentation. Colonies also reproduce sexually via broadcast spawning. Spawning requires the successful fertilization of an egg and the settlement of the resulting polyp on an uninhabited and sunlit hard surface (Heyward et al. 1987, Quinn & Kojis 2005).

In the 1970s, white band disease killed over 90% of *Acropora palmata* throughout the Caribbean (Burke et al. 1976, Aronson & Precht 2001, Mayor et al. 2006, Muller et al. 2013). Broadcast spawning, which *A. palmata* depends upon to increase its genetic diversity, became increasingly difficult. Diseased corals stopped producing gametes and the few healthy adult coral colonies grew too far apart from one another to effectively fertilize each other. This situation has expounded over time, creating a negative Allee Effect in which poor individual

fitness is correlated with a decrease in population density (Allee 1931, Stephens et al. 1999, Quinn & Kojis 2005). In addition, the few fertilized eggs that do develop often die before finding a suitable settlement site. Failure to settle can be caused by an overabundance of macro-algae growing on most substrate habitats due to the historically recent absence of herbivorous predators (Quinn & Kojis 2005). Stressors such as increased water temperatures, salinity, and/or pollutants also negatively impact *A. palmata* sexual reproduction by 1) decreasing fertilization rates, 2) preventing larval development or settlement, even if proper substrate is available, and 3) killing newly settled coral polyps (Richmond 1997, Lirman 2000, Grober-Dunsmore et al. 2006). Lack of fertilization and larval settlement reduces fecundity, leading to smaller population sizes and densities of one of the most important reef-building corals in the Caribbean (Knowlton 2001).

Infrequent sexual reproduction puts this species in danger by limiting its gene flow. Throughout the 1970s and 80s *Acropora palmata* populations declined by over 96% in the Caribbean. (Rogers et al. 1991, Aronson & Precht 2002, Grober-Dunsmore et al. 2006). As of 2013, there has been no documented significant increase in this species' overall population density in this area (Muller et al. 2013). Live coral cover of *A. palmata* at Discovery Bay, Jamaica and the Florida Keys Marine National Sanctuary (FKMNS) is now less than 1% of the cover that existed for this species in 1994 (Sutherland & Ritchie 2004, Quinn & Kojis 2005, Zubillaga et al. 2007). Due to this alarming population decimation, this species was officially listed as "threatened" in 2006 under the United States Endangered Species Act of 1973 (Hogarth 2006, Muller et al. 2008) and it is currently being reviewed for endangered species status (Muller et al. 2013, NOAA Federal Register 77 FR 73220).

Fortunately, a few reefs in the Caribbean are still disease-free and it is possible for reefs to recover over time. Seventy five percent of the *Acropora palmata* located at Los Roques in the Southern Caribbean was undamaged by disease or predators as of 2007, with a large amount of small colonies (Zubillaga et al. 2007). However, it is unclear whether this abundance of small colonies indicates increased sexual production and a growing population of newly formed colonies or a declining population with a high mortality rate of large existing colonies (Zubillaga et al. 2007, Roth et al. 2013).

Off St. John, US Virgin Islands, *Acropora palmata* populations and colony sizes rose from 2001 to 2003. This may have been caused by an increase in larval settlement thanks to better survivorship due to the availability of hard substrate in high current locations. Increasing larva settlement meant that sexual reproduction of *A. palmata* was still successful at this location at this time. By 2010, St. John, US Virgin Islands reported larger *A. palmata* colonies than were present in 2004 despite the presence of white pox disease, a severe thermal-stressing occurrence in 2005, and multiple large storms. Increases in colony size indicates that growth of these colonies is faster than their combined stressors are at counteracting growth, which is surprising since it can take a reef up to 11 years to fully recover from any stressor (Grober-Dunsmore et al. 2006, Muller et al. 2013). Large colonies (greater than 120 cm in size) can take even longer to recover because they have a greater surface area subjected to stressors. Although *A. palmata* colony size has increased since 2004 at St. John, there has been no increase in population density, suggesting low polyp recruitment (Muller et al. 2013). *A. palmata* recovery rate is being delayed as the population of sexually mature corals, as well as their genetic diversity, are continuing to decrease (Grober-Dunsmore et al. 2006, Rogers et al. 2008).

Coral diseases: causation and transmission

Although other stressors make corals susceptible to infection and death, disease is the number one stressor of corals in the Caribbean, causing more coral mortality than any other stressor (Rogers et al. 2008). Many of the existing coral disease outbreaks have become epizootics: animalian epidemics that can potentially cause extinction of the species infected (Stedman 2000, Sutherland et al. 2004). It is critical that coral diseases, specifically those that infect reef-building foundation species such as *Acropora palmata*, be studied in order to learn how to stop the formation of such diseases.

In order to prove causation of a coral disease, Koch's Postulates must be followed. These postulates, published in 1890 by the bacteriologist Robert Koch, have four criteria in which the suspected pathogen must: 1) be found in all cases of the disease, 2) be isolated and successfully grown in a pure culture, 3) cause the disease in a healthy organism infected with the pure culture, and 4) be re-isolated from this infected organism as the same pure isolate obtained previously (Rivers 1937). However, Koch's postulates are not always feasible with endangered species because the morality of purposefully infecting an already threatened species is questionable at best. Furthermore, it is possible for a disease-causing pathogen to be unable to grow in pure culture (Fredericks & Relman 1996, Sutherland et al. 2004, Wiley et al. 2008). Since many ecological diseases are caused by a consortium of pathogens, a modern approach to Koch's postulates requires that the pathogen in question must 1) be found in environments with the disease, 2) be isolated from an environment with this disease and grown in pure culture, 3) cause a process associated with the disease in the laboratory, and 4) cause this same process in the environment that disease naturally occurs (Staley et al. 2007).

There are currently approximately 18 known coral diseases, half of which exist exclusively in the Caribbean (Rodriguez-Martinez et al. 2001 Sutherland et al. 2004). Environmental factors such as elevated water temperatures, pollution, and reduced water turbulence can increase coral susceptibility to disease and the virulence of the pathogen (Rodriguez-Martinez et al. 2001, Kuta & Richardson 2002, Ben-Haim & Rosenberg 2002, Richardson & Kuta 2003, Ben-Haim et al. 2003, Sutherland et al. 2004). Skeletal anomalies of normal polyp structure such as a loss of mucus secretory cells, zooxanthellae, or nematocysts can also increase a coral's susceptibility to a disease (Porter et al. 2001). Disease signs often include exposed coral skeleton, increased mucus production, and polyp disintegration, all of which are caused by active tissue necrosis (Porter et al. 2001).

There are two types of disease transmission: horizontal and vertical. Diseases spread horizontally from one adult colony to another through an organism known as a vector or through other carriers such as organic matter or inorganic material. Diseases spread vertically when an infected individual passes its disease down to its offspring (Lipsitch et al. 1996, Sutherland et al. 2004). Both of these transmission processes are part of pathogenesis, which is "the process by which infection leads to disease" (Stedman 2000). A pathogen's success in causing a disease is dependent on its successful transmission to new hosts (Lipsitch et al. 1996, Stedman 2000, Sutherland et al. 2004).

Coral diseases: white plague, white band, and white pox

The white diseases (white plague, white band, and white pox) in the Caribbean cause progressive tissue loss in their hosts and are all infectious (Porter et al. 2001, Sutherland et al. 2004). The percentage of all coral species infected with any of these three diseases increased

drastically from 1996 to 1998 (Porter et al. 2001). White diseases get their name from the exposed white coral skeleton that is visible where infection exists. White plague disease (WPL) infects a variety of corals and occurs throughout the Caribbean and the Indo-Pacific. WPL shows signs of tissue necrosis at the perimeters of a white lesion usually found at the base of the coral that can expand to consume the entire coral over time (Peters 1984, Bythell et al. 2002, Sutherland et al. 2004). Corals affected by this disease are usually round boulder types. White band disease (WBD) exclusively infects Caribbean *Acropora palmata* and *Acropora cervicornis* corals (Sutherland et al. 2004). WBD shows signs of tissue necrosis in a white concentric ring of exposed skeleton that can extend from the coral's base to its branching tips. White pox disease (WPD) is the most specific, showing signs of infection only in the species *Acropora palmata*. It is possible that WPD affects other coral species as well, but manifests itself in an as-yet unrecognizable way on these corals (Patterson et al 2002). WPD's visible effects, or gross signs, on *A. palmata* are multiple, unevenly shaped and sized white blotches of tissue necrosis all over the coral that can expand over time to consume and kill the entire coral colony (Patterson et al. 2002, Sutherland et al. 2004). In all white diseases, the infected areas are directly adjacent to living tissue (Mayor et al. 2006, Patterson et al. 2002).

White pox disease was first discovered in 1996 in Key West Florida on Eastern Dry Rocks Reef (Holden 1996). It spreads rapidly, with a maximum lesion growth rate of 10.5 cm^2 per day. Its transmission method is unknown (Porter et al. 2001, Patterson et al. 2002). Faster growing lesions are more harmful to a coral's survival than are the more abundant, slower growing lesions (Patterson et al. 2002). Within just a 3-year period between its discovery in 1996 and 1999, WPD contributed to the 85% decline in *Acropora palmata* found in seven reefs throughout the Florida Keys (Patterson et al. 2002). By 2002, WPD had contributed to the

disappearance of 88% of *A. palmata* coverage in the Florida Keys National Marine Sanctuary (Sutherland & Ritchie 2004). Currently, the effects of WPD are found throughout the Caribbean, including Puerto Rico, the Florida Keys, the Bahamas, the Virgin Islands, and Mexico (Goreau et al. 1998, Rodriguez-Martinez et al. 2001, Muller et al. 2013)

Transmission of white pox disease

Invertebrates such as corals only have an innate immune system including physio-chemical barriers and cellular defenses against pathogens (Roch 1999, Sutherland et al. 2004). These cellular defenses include self-non-self recognition that allows amoebocytes to migrate from uninjured tissue to the healing front where they engulf and destroy microbial invaders, phagocytizing bacteria and other pathogens (Meszaros & Bigger 1999, Sutherland et al. 2004). This process makes healing wounds possible, but a corals' immunity is temporary, only lasting between 2-4 weeks on average (Raison et al. 1976).

Mucus sloughing is a strategy used by many coral species to remove unwanted sediment and particulates that land on them and also to protect the coral's surface tissue from pathogenic bacteria (Rublee et al. 1980, Sutherland et al. 2004). This sticky mucus layer can sometimes aid in bacterial infections as it helps the bacterium attach to the coral's surface (Toren et al. 1998). A bacterium can reach a coral in the first place passively through the water column or actively through a biological carrier within proximity to both healthy and diseased corals (Grober-Dunsmore et al. 2006). Coral diseases may be transmitted by direct contact between a diseased and a healthy coral or through indirect contact between a diseased coral, a carrier such as a vector or a reservoir, and a healthy coral. Vectors are living organisms that transmit a disease and vector borne transmission has already been documented for the coral-eating fireworm *Hermodice carunculata* (Bruckner et al. 1997, Sussman et al. 2003). Possible modes of

transmission of elkhorn coral disease that have been studied so far are: 1) contaminated water, 2) free-floating mucus sloughed from contaminated coral reservoirs, and 3) living, marine, corallivorous vectors such as damselfish, the fireworm *Hermodice carunculata*, or snails (Bruckner et al. 1997, Sussman et al. 2003, Grober-Dunsmore et al. 2006, Sutherland et al. 2010). Predatory vectors may carry the pathogens themselves, obtained from a previously eaten infected coral. Another hypothesis is that the skeletal lesions created from corallivorous feeding expose the coral to pathogens already in the water column (Kaufman 1977, Grober-Dunsmore et al. 2006).

The corallivorous snail *Coralliophila abbreviata* preferentially eats *Acropora palmata* and is hypothesized to make this coral more susceptible to disease by directly transmitting a disease to the coral or by creating substantial tissue loss in the coral (Grober-Dunsmore et al. 2006). This gastropod is a threat to *A. palmata* because one snail can eat 6.5cm^2 per day, completely destroying the coral tissue in this area and leaving it at risk of infection (Miller et al. 2002, Zubillaga et al. 2007). *C. abbreviata* feed by everting their guts, consuming both coral and pathogen. When a snail secondarily everts its guts onto another colony, or even a different area on the same colony, it may infect that area with the pathogen obtained from the previous feeding. Studies from both 2003 and 2006 show that the mantle fluid of many of these snails tested positive for *Serratia marcescens* strain PDR60, the same strain of the bacterium found in *A. palmata* infected with WPD (Sutherland et al. 2010). PDR60 has also been found in other coral species not within the *Acropora* genus. These non-host coral species may be capable of harboring WPD in their outer mucus layer and transmitting it to any *A. palmata* within range when this mucus is sloughed and gets carried off by currents. Reservoir corals do not appear to

be negatively affected by the pathogen themselves and show no signs of disease, even when their mucus surface layers test positive for the WPD bacterium (Sutherland et al. 2010).

A cause of white pox disease: *Serratia marcescens*

The fact that all of the white diseases occur more frequently on reefs closer to shore than on reefs further out to sea support the hypothesis that human-associated factors may be the cause of these diseases. It has been confirmed that the enterobacterium *Serratia marcescens* found in the human gut is a direct cause of WPD in *Acropora palmata* (Patterson et al. 2002, Sutherland et al. 2011). *Serratia marcescens* is a common human pathogen causing mostly hospital-acquired infections of the eyes, respiratory tract, urinary tract, and other diseases in immune deficient patients (Hejazi et al. 1997). This gram-negative member of the Enterobacteriacea family is opportunistic and shown to have pathogenic effects on a variety of terrestrial species in both the plant and animal kingdoms. It is also prevalent in soil and freshwater (Hejazi and Falkiner 1997, Grimont and Grimont 2006).

It is theorized that the *Serratia marcescens* bacterium leaks into the limestone bedrock of the Florida Keys from septic tanks and trickles out to sea with other runoff. Of the 54 *Serratia marcescens* strains discovered in human sewage, only one (PDR60) has been isolated from WPD infected *Acropora palmata* samples thus far (Sutherland et al. 2010). Via adherence to Koch's postulates, it has been shown that the *S. marcescens* strain PDR60 isolated from human wastewater and WPD lesions can cause WPD in *A. palmata* in as little as four days (Sutherland et al. 2011). *S. marcescens* strain PDL100, isolated form WPD lesions in 1999 and confirmed as a WPD pathogen, has only been found in infected *A. palmata* (Patterson et al. 2002). Strain PDR60 seems to currently be the dominant strain because it is the only strain of *S. marcescens*

isolated from both reef and sewage samples from 2002 to 2012. PDR60 has also been isolated from the protective mucus layer of four non-host coral species (*Siderastrea siderea, Solenastrea bournoni, Porites porites,* and *Porites atreoides)* as well as the predatory snail *Coralliophila abbreviata* (Sutherland et al. 2010, Sutherland et al. 2011, Broom 2013).

It is likely that *Serratia marcescens* strain PDR60 is using the nutrients associated with the mucus of non-host corals as a growing medium. Coral mucus is a holobiont polymer made by the symbiotic relationship between polyp mucocytes and photosynthetic dinoflagellates (Brown & Bythell 2005, Bythell & Wild 2011). Each coral has a community of symbiotic microbes within its mucus layer that determines the overall health of the coral and its ability to resist pathogenic infection (Reshef et al. 2006, Rosenberg et al. 2007, Teplitski & Ritchie 2009, Krediet et al. 2012). Coral pathogens and commensals both utilize glycosidases and N-acetyl-glucosaminidase to access carbon and nitrogen sources in coral mucus (Krediet et al. 2012). When a pathogen lands on a coral's mucus layer it must compete for resources with the coral's native mucus inhabitants. A bacterium's ability to outcompete these microbial symbionts and increase their own population in a host depends on quorum sensing (QS) cell-to-cell signaling (Fuqua et al. 2001). QS usually takes the form of swarming or biofilm formation and is responsible for the timing of host attachment, cultural spreading, and production of antibiotics and exoenzymes (Van Houdt et al. 2007, Alagely et al. 2011). Sometimes QS activities are dependent on environmental conditions.

Serratia marcescens strain PDL100 is more effective at utilizing the glycoprotein components of *Acropora palmata* mucus than other isolates of *S. marcescens* or even the coral's own microbial symbionts, allowing PDL100 to outcompete and outgrow the coral's own microbiota (Sharon & Rosenberg 2008, Fabich et al. 2008, Krediet et al. 2009, Krediet et al.

2009). This may stem from *S. marcescens*' symbiotic relationship with many other invertebrates such as the sugar beet maggot (Iverson et al. 1984, Krediet et al. 2009,). The enzymatic activity of strain PDL100 is most similar to coral commensals within its early stages of mucus colonization and becomes more similar to pathogenic human mucosal strains later in its colonization (Fabich et al. 2008, Krediet et al. 2009). It is hypothesized that PDL100 does not infect coral species other than *A. palmata* because it is unable to build a high enough population within the mucus surface layer (Krediet et al. 2009, Krediet et al. 2012). When a non-host coral sloughs mucus and associated *S. marcescens*, the *S. marcescens* may be capable of surviving by consuming non-host coral mucus until it happens to land on an *A. palmata* coral that it can infect (Sutherland et al. 2010, Krediet et al. 2012).

In addition to *Serratia marcescens* strains PDL100 and PDR60, Sutherland et al. (2010) also found various other strains of *S. marcescens* in canal water, beach water, and sea bird guano. Strain type was verified by similarities in PFGE banding patterns and showed that strains PDL100 and PDR60, are only about 45.5% similar to one another (Sutherland et al. 2010). Though some very similar *S. marcescens* strains may have evolved from one another in response to the evolution of coral mucus microbiota to resist *S. marcescens* pathogens (Krediet et al. 2011), PDL100 and PDR60 are likely too genetically different to have evolved from one another in less than one decade. PDL100 and PDR60 are likely unrelated strains (Sutherland et al. 2010).

Regardless of strain specificity, sewage contamination of near-shore waters in the Florida Keys is a problem in Florida where untreated wastewater disposed through septic tanks and other in-ground receptacles seeps through the porous limestone substrate and escapes into nearby shores (Lapointe et al. 1990, Paul et al. 1997, Sutherland et al. 2011). Once waterborne, fecal

contaminants can move between 0.5 to 41 miles an hour in strong currents (Paul et al. 2000). Oceanic sewage leaks are also a concern in less developed Caribbean countries that cannot afford to fund wastewater treatment facilities (Sleasman 2009). The spread of *Serratia marcescens* strain PDR60 from human fecal waste to corals has made WPD the first known case of a marine reverse zoonosis, a disease introduced into the marine wildlife by humans (Woolhouse & Gaunt 2007, Sutherland et al. 2010).

Research Objective

This study is part of a long-term investigation of the ecology of WPD in the Florida Keys, taking into account its potential sources, reservoir non-host corals, and vectors, such as *Coralliophila abbreviata*. The objective of this research is to continue identifying, through genetic fingerprinting analysis, the *Serratia marcescens* strains present in WPD-affected *Acropora palmata* as well as potential sources and non-host coral reservoirs. Due to the evidence that strain PDR60 has been present in the reef environment and in human wastewater for a decade (2002-2012), it is hypothesized that PDR60 will continue to be identified from reef and wastewater sources in the Florida Keys. It is also possible that a new strain of *S. marcescens* associated with the reef environment may be identified. Although *S. marcescens* is currently the only bacterium known to cause WPD, there may be other causes of the disease.

Methods

In July 2013, Dr. Sutherland collected samples of coral mucus from WPD-affected *Acropora palmata*, healthy *A. palmata*, and non-host corals *Acropora cervicornis*, *Siderastrea siderea, Porites porites, Porites astreoides, Orbicella annularis*, *Orbicella faveolata*, and *Orbicella franksi* [*Orbicella* previously known as *Montastrea* (Budd et al. 2012)]. Raw sewage

and treated wastewater samples were collected from wastewater treatment plants in Key Largo, Key West, and Marathon Key. Water samples were collected from Looe Key reef, canals on Big Pine Key (Tropical Lane, Sexton Cove, Doctor's Arm and Eden Pines), and a Big Pine Key freshwater sink hole (Blue Hole) (Figure 1). Coral mucus, canal water, sink hole water and treated wastewater samples were filtered (10 ml in duplicate) onto nitrocellulose membrane filters and the filters were transferred to MacConkey Sorbitol Agar (MCSA, Becton Dickinson Co., Sparks MD) amended with the antibiotic colistin (Research International Product Corp., Mt. Prospect, IL). Raw sewage was directly spread plated (10 µl and 100 µl) onto MCSA. All MCSA plates were incubated overnight at 37ºC. Bacteria characteristic of *Serratia marcescens* resisted colistin and fermented sorbitol, turning the potential *S. marcescens* bacteria on the MCSA plate pinkish/red. These MCSA positive *S. marcescens* bacteria were then plated onto DNase with Toludine Blue agar amended with the antibiotic cephalothin (DTC) (Becton Dickinson, Sparks MD) and incubated overnight at 41ºC. Probable *S. marcescens* had a DNase-positive reaction on DTC, indicated by a red halo on the DTC plate (Farmer et al. 1973, Sutherland et al. 2010).

Probable *Serratia marcescens* (positive reaction on both MCSA and DTC) (Sutherland et al. 2010) were stored in Tryptic Soy agar stabs. In addition, bacteria collected from the reef environment (WPD lesions, non-host corals, and reef water) that demonstrated a positive reaction on MCSA, but a negative reaction on DTC were also stored in Tryptic Soy agar stabs. These MCSA positive, DTC negative bacteria were known not to be *S. marcescens* because they failed to test positive for both MCSA and DTC. These unknown bacteria with a positive reaction on MCSA may be novel pathogens associated with WPD and warrant investigation.

A total of 49 probable *Serratia marcescens* and 21 unknown bacteria were plated for pure culture using Trypic Soy agar (Becton Dickinson Co., Sparks MD) at room temperature. One bacterial colony from each plate was frozen in glycerol for long-term storage. DNA was extracted from a second bacterial colony from each plate using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) (Sutherland et al. 2010). Extracted DNA was diluted to 20% of its original concentration using PCR grade water for polymerase chain reaction (PCR).

PCR isolated and amplified DNA fragments unique for *Serratia* species (Sutherland et al. 2010). Primers *S. marcescens* 16SV (GGGAGCTTGCTCACTGGGTG) and *S. marcescens* 16SWR (GCGAGTAACGTCAGTTGATGAGCGTATTA) annealed to the 410 base pair region on the 16S rRNA gene specific for *Serratia* species, allowing for amplification of this fragment (Wilson et al. 1999). Although the primers used were specific to the *Serratia* genus and not the *S. marcescens* species, the combination of culture on MCSA followed by DTC followed by this *Serratia* specific PCR method confirmed positive bacterial isolates as *S. marcescens* (Sutherland et al. 2010). A master mix of the *S.mar* 16SV and *S.mar* 16SWR primers, dNTPs (180µL of PCR water, 20µL of 25mM dNTP stock), *Taq* DNA polymerase (5 Prime Inc., Gaithersburg), and PCR buffer with magnesium (5 Prime Inc., Gaithersburg) was prepared and added to 1µl of each diluted DNA sample. A positive control with 1µl of known *S. marcescens* DNA (*S. marcescens* PDR60) (diluted 20%) and a negative control with 1µl of PCR grade water were also prepared. The *S. marcescens* DNA in these PCR mixes was then copied multiple times in a Thermal Cycler (Bio Rad MJ Mini) by denaturing the existing DNA at 94°C for 32 seconds, annealing primers to their 3' ends at 60°C for 32 seconds, and commencing nucleotide extension with the binding of Taq polymerase for 32 seconds at 72°C. A final extension of the DNA was

done at 72°C (Sutherland et al. 2010). This cycle was repeated 40 times. PCR products were refrigerated at 4°C until gel electrophoresis.

Gel electrophoresis separated PCR products into DNA fragments based on size. The filtering gel medium was a 1.5% agarose gel (Acros Organics, NJ). Using an electric field of 80 volts for 1 hour, smaller fragments were pushed faster through agarose gel towards the positive electrode while larger fragments moved more slowly. A band (410 bp fragment) resulted for each sample containing *Serratia marcescens* DNA. Staining the gel with ethidium bromide made these bands visible by ultra-violet transillumination.

Pulsed-field gel electrophoresis (PFGE) was carried out for bacteria isolates confirmed as *Serratia marcescens* as well as for the unknown bacteria isolates from non-host corals and wastewater that tested negative on DTC, but positive with *Serratia*-specific PCR (Table 1). Fingerprints for each isolate were compared to confirmed *S. marcescens* fingerprints isolated from diseased elkhorn coral (strains PDL100 and PDR60). The PFGE fingerprinting method is the most reliable for strain typing *S. marcescens* and has been used to diagnose medical *Serratia* strain outbreaks (Shi et al. 1997, Maragakis et al. 2008, Sutherland et al. 2010). Plug preparation and plug processing for PFGE was performed on only 25 PCR positive isolates due to time restraints. These 25 isolates included all of the PCR positive coral (n=3) and wastewater samples (n=14) with the remaining positive samples that were tested coming from canal water (n=7) and the freshwater sinkhole (n=1) (Table 1).

Bacteria isolates were incubated with shaking overnight in Tryptic Soy broth (Becton Dickinson Co., Sparks MD) at 37°C. The resulting bacterial cultures were washed twice with 5µl of PFI buffer [10mM Tris-HCl (pH 7.6), 1M NaCl] stained with crystal violet and centrifuged for 10 minutes at 2400 rpm to separate the bacteria from the broth. Bacterial cells

were resuspended using 1µl of PFI buffer and counted using a hemacytometer. Cell concentrations must be equal in all samples in order to compare PFGE fingerprint patterns (Sutherland et al. 2010). All bacterial samples were diluted to a 10^6 cell concentration in PFI buffer and embedded in agarose gel plugs (Cambrex Bio Science, Rockland, ME). PFGE plugs were stored in $T_{10}E_1$ buffer (10mM Tris-HCl, 1mM EDTA) at 4°C until processing (Marty et al. 2002, Sutherland et al. 2010).

Lysis buffer [1M Tris-HCl (pH 7.6), 5M NaCl, 0.5M EDTA (pH 8.0), 10% Brij-58 solution, and 4% deoxycholate] lysed bacterial cells, separating DNA from cell material during shaker incubation at 37°C for 16 hours. DNA plugs were then washed to remove cell material: two washes via incubation in ESP buffer [0.5M EDTA (pH8.0), 1% Sarkosyl, and 1mg/mL Proteinase K] at 50°C for 24 hours followed by three washes with incubation in $T_{10}E_1$ buffer at 25°C for 30 minutes. Further shaking incubation in 1.5mM Pefablock proteinase inhibitor (Acros Organics, NJ) at 37°C for 2.5 hours prevented DNA degradation from cell lysis. Plugs were washed three times in $T_{10}E_{50}$ buffer (10mM Tris-HCl, 50mM EDTA) through shaking incubation at 50°C for 1 hour to remove all traces of lysis buffer and proteinase inhibitor. Processed plugs were stored in $T_{10}E_{50}$ buffer at 4°C until restriction digestion.

Restriction enzyme digestion and pulsed-field gel electrophoresis (PFGE) separated DNA fragments larger than 30-50 base pairs. Cells were washed three times in $T_{10}E_1$ buffer incubated for one hour with shaking at 25°C, and then washed once in 1X Mulit-Core buffer (Promega) for two hours with shaking at 25°C. Of the 25 isolates for which plugs were prepared and processed, plugs for only 15 isolates remained intact after processing and were subjected to restriction enzyme digestion with *SpeI* (Promega) at 37°C for 20 hours to cut DNA into fragments. *SpeI* has been shown to be the best choice for *Serratia marcescens*, but it is also capable of

fragmenting DNA from other bacteria (Marty et al. 2002, Sutherland et al. 2010). Digested plugs were washed with $T_{10}E_1$ buffer for two hours with shaking at 50°C. Enzyme digested plugs were stored in $T_{10}E_{50}$ buffer at 4°C until PFGE.

The 15 fragmented DNA isolates in plugs were run through PFGE on a 1.0% SeaChem GTG agarose gel (Acros Organics, NJ) in 0.5 x TBE (Tris Borate EDTA) buffer with Thiourea (Acros Organics, NJ) to reduce smiling curves of the resulting fingerprints. Using an electric field of 6 volts, alternating directions every 5-60 seconds for 16 hours at 14°C, the pulsed-field forced these limited mobility fragments to change conformation with the directional change of the electric charge. The larger a fragment, the longer it took to re-conform (Electrophoresis 2000, Alaidan et al. 2009). The gel was stained with ethidium bromide and viewed under UV transillumination. Of the 15 isolates run through PFGE, only eight produced band patterns that were bright enough under UV illumination for analysis and comparison to PFGE band patterns produced in other studies (Sutherland et al. 2010, Sutherland et al. 2011, Broome 2013). .

Results and Discussion

In order to conclusively identify *Serratia marcescens,* bacterial isolates must test positive with MCSA, DTC, and *Serratia*-specific PCR (Sutherland et al. 2010). Of the 70 plated bacterial isolates, 25 tested positive for *Serratia marcescens* with MCSA, DTC, and PCR. None of these 25 *S. marcescens* were from corals. Twenty two of these *S. marcescens* were chosen for PFGE fingerprinting. The unknown bacteria (MCSA positive, DTC negative) from WPD lesions, non-host corals, and reef water totaled 21. Of these 21 unknown bacteria, four were PCR positive and included three non-host coral isolates and one wastewater isolate. PFGE fingerprinting was completed for one of the three unknown bacteria from a non-host coral (Table

2). In all, PFGE fingerprinting was attempted for 25 bacteria isolates, but due to dissolution of PFGE plugs during processing and restriction enzyme digestion, PFGE fingerprints were successfully produced for only eight of these 25 isolates.

All collection sites had PCR positive isolates, but 85.2% of the PCR positives came from wastewater and canals. This abundance of *Serratia marcescens* in wastewater and canals, but the lack of *S. marcescens* on coral reefs may be due to several factors. Sewage contamination is common in residential canals and in wastewater (Porter et al. 2001). In 2000 it was discovered that reefs nearest Key Largo (upper Keys) and Key West (lower Keys) city centers were more affected by white diseases than reefs in the middle Keys (Porter et al. 2001). The reef samples collected in this study came from the middle Keys (Looe Key reef), and none of the bacteria collected from the reef were confirmed as *S. marcescens*. The bacterium may be present in the open ocean, but not at high enough concentrations to be routinely detected.

Serratia marcescens may have been undetectable at Looe Key reef in July 2103 due to low concentrations in seawater or the limited survival of this bacterium in seawater. The two strains of *S. marcescens* linked to WPD, PDL100 and PDR60, are capable of surviving for a limited time in seawater (Looney et al. 2010, Nys 2013). While *S. marcescens* PDL100 apparently has not evolved to survive independently in seawater for extended periods of time, *S. marcescens* PDR60 is longer lived. The presence of vectors and coral mucus have been hypothesized to increase the survival time of these strains (Looney et al. 2010, Sutherland et al. 2010, Nys 2013). Looney et al. (2010) showed that the life span of the *S. marcescens* strain PDL100 in seawater was dependent on ocean temperature, nutrient levels, and the type of species-specific coral mucus it resides in. Seawater alone allowed the PDL100 to survive for an average of 15 hours, but seawater plus *Siderastrea siderea* mucus provided a much longer

survival time of about 88 hours. Additional nutrients including dissolved phosphates and organic carbon, as well as an increase in water temperature from 30ºC to 35ºC increased *S. marcescens* PDL100 life span further to an average of 120 hours. Glucose amended seawater allowed PDL100 to survive for up to 43 days (Nys 2013). This is much longer than the bacterium can survive in seawater plus either *Orbicella faveolata* or *Acropora palmata* mucus, both of which only allowed the pathogen to live an average of 17 hours (Looney et al. 2010). Therefore, there may be other, as of yet undiscovered, strains present in nearby environments that cannot survive to infect reefs offshore.

Serratia marcescens may have found a livable niche within non-host coral surface mucus layers that allow it to live on in the oceanic environment without reintroduction when it would have otherwise died exposed in the water column. PDL100 specifically has been shown to survive longer periods of time in non-host coral mucus than in *A. palmata* mucus (Looney et al. 2010) and PDR60 has been shown to survive more than 24 days in seawater alone (Nys 2013). It is possible that PDR60, like PDL100, is adapting to survive within non-host coral reservoirs.

Another possible explanation for the low *Serratia marcescens* levels on the reef is that sampling for this study was done at random and on threatened corals. This limits the number of coral mucus samples that may be collected. The relatively small sample size in this study may not be indicative of the amount of corals and ocean water truly infected with *S. marcescens*. Very few samples of *Acropora palmata* mucus were taken to begin with due to *A. palmata's* threatened species status. It is also possible that WPD signs may be caused by more than one pathogen (Sutherland et al. 2010). Although the *S. marcescens* strains PDL100 and PDR60 are the only known causes of WPD, this does not rule out the possible effects of any other strain of *S. marcescens* or any other pathogen. While details are not yet known about these unknown

potential pathogens, the unknown bacteria (MCSA positive, DTC negative) collected in this study (Table 2) are an important first step in identifying a novel pathogen associated with WPD. The unknown bacteria (MCSA positive, DTC negative) collected from non-host corals (*O. faveolata* and *P. porites*) tested positive with *Serratia*-specific PCR (Table 2). The unknown bacteria collected from the host coral *Acropora palmata* tested negative with PCR. According to Sutherland et al. (2010), MCSA positive, DTC negative, and PCR positive tests can be used to identify three different *Serratia* species (*S. liquefaciens, S. odorifera*, and *S. plymuthica*) while MCSA positive, DTC negative, and PCR negative tests identifies unrelated pathogens (e.g., *Escherichia coli*).

The eight PFGE fingerprints generated in this study were from bacteria collected from wastewater, canals, and a non-host coral (Figure 2). None of these fingerprints matched the PDR60 or PDL100 *Serratia marcescens* strains. PDR60 has been identified in wastewater and has been the dominant strain found on reefs since 2002. This strain was identified in 2002, 2003, August 2011, and August 2012 (Sutherland et al. 2010, Broome 2013). This study did not identify *S. marcescens* strain PDR60 in wastewater or reef samples collected in July 2013, and these results are consistent with those for wastewater samples collected in May 2011 for which no PDR60 was identified (Sutherland personal communication). Five different PFGE fingerprint patterns (strains A – E) were generated in this study. Strain B was found in two different wastewater treatment plants, Key Largo and Marathon. Strains A and E were each represented by two *S. marcescens* isolates from single locations, Eden Pines and Tropical Lane canals, respectively. Strains C and D were each produced for one *S. marcescens* isolate. Strain C came from Key West wastewater and Strain D was the one non-host coral isolate that came from Looe Key Reef (Figure 2, Table 1).

Recent research shows that *Acropora palmata* may be developing resistance to *Serratia marcescens.* Krediet et al. (2012) discovered that 8% of tested *A. palmata* microbial symbionts can actually inhibit glycosidase use of the pathogen *Serratia marcescens*, strain PDL100. A coral commensal Exiguobacterium species was shown to reduce the pathogen's ability to grow on coral mucus by inhibiting the pathogen's ability to catabolize enzymes. It is hypothesized that many commensal compounds are capable of producing antibiotics and inhibiting QS swarming and biofilm capacities (Ritchie 2006, Tait et al. 2010, Alagely et al. 2011). When tested on *A. palmata* mucus, several proteobacteria prevented biofilm formation of the *S. marcescens* strain PDL100, but encouraged swarming, while several native commensal bacteria and Marinobacter species successfully prevented both swarming and biofilm formation (Alagely et al. 2011). It is possible that *A. palmata* is developing a resistance to the PDR60 *S. marcescens* strain over time, as it has been doing with the PDL100 strain (Krediet et al. 2012). Sutherland et al. (2011) suggested that some *Acropora palmata* are apparently resistant to the PDR60 strain. It is encouraging that *A. palmata* colonies and their symbionts may be evolving to better resist *S. marcescens* infection and WPD.

Selective pressures by *Acropora palmata* against *Serratia marcescens* could be creating a resistance to this pathogen. The Coral Probiotic Hypothesis was proposed by Reshef et al. (2006) to explain the development of apparent host resistance to a confirmed coral pathogen and has been applied to white plague disease. This hypothesis states that symbiotic microorganisms in coral mucus have a dynamic relationship with their environment, which allows the corals they live in to adapt to environmental changes more quickly than they would be able to using mutation or natural selection alone. These symbionts can potentially become resistant to diseases, despite the coral itself not possessing an immune system (Reshef et al. 2006).

In this study, PFGE fingerprinting was successfully completed for only eight of the 70 isolates plated for pure culture. While *S. marcescens* was not identified from the reef environment, PFGE analyses must be completed with all 70 of the isolates in order to make a conclusion regarding the presence or absence of *S. marcescens* on Looe Key reef in July 2013. This study represents only one month in one year of this five-year research project (2010 to 2015). The results of this research do not support the hypothesis that *S. marcescens* PDR60 is still present in both human wastewater and coral reefs of the Florida Keys. Strain PDR60 was not among the four *S. marcescens* strains identified in this study, however, it cannot be concluded from this limited data that *S. marcescens* strain PDR60 is no longer present on the reef or in wastewater. The identification of unknown bacteria from white pox affected *Acropora palmata* supports the hypothesis that there may be other unknown pathogens that cause WPD.

It should be noted that the apparent absence of *Serratia marcescens* in reef samples in this study may be attributed to wastewater treatment improvement in the Florida Keys. *S. marcescens* is mostly introduced into the marine environment via poor waste disposal systems. The state of Florida recently passed new laws under its Sanitary Wastewater Master Plan requiring more advanced wastewater treatment at an estimated cost of $939 million (Sleasman 2009, CH64E-6 Florida Administrative Code 2009, Sutherland et al. 2011). The majority of the Florida Keys has upgraded from septic tanks, cesspits and injection wells to treatment of wastewater at facilities including those in Key Largo, Marathon, and Key West. However, as of 2013, 20% of the inhabited Florida Keys still used Site Disposal Systems (SDS) that may allow sewage to seep into the water column (Broome, 2013). The fact that strain PDR60 was found in Florida wastewater as well as the surface mucus layer of non-host corals as recently as August 2012 (Broome 2013) may indicate that there is still sewage runoff into the ocean from poorly

treated sewage sites. This sewage may be supplying the *S. marcescens* pathogen to nearby coral reefs where it aggregates on coral mucus surface layers (Lipp et al. 2002).

Future research should include the testing of more potential non-host coral reservoirs and vectors as well as the wastewater treatment plants and canals within the Florida Keys. Specific attention should be given to the investigation of the bacteria unknowns identified in this study to explore the possibility of novel WPD pathogens. This information will allow researchers to better understand *Serratia marcescens* and other potential pathogens in the hopes of managing WPD infected *Acropora palmata* populations.

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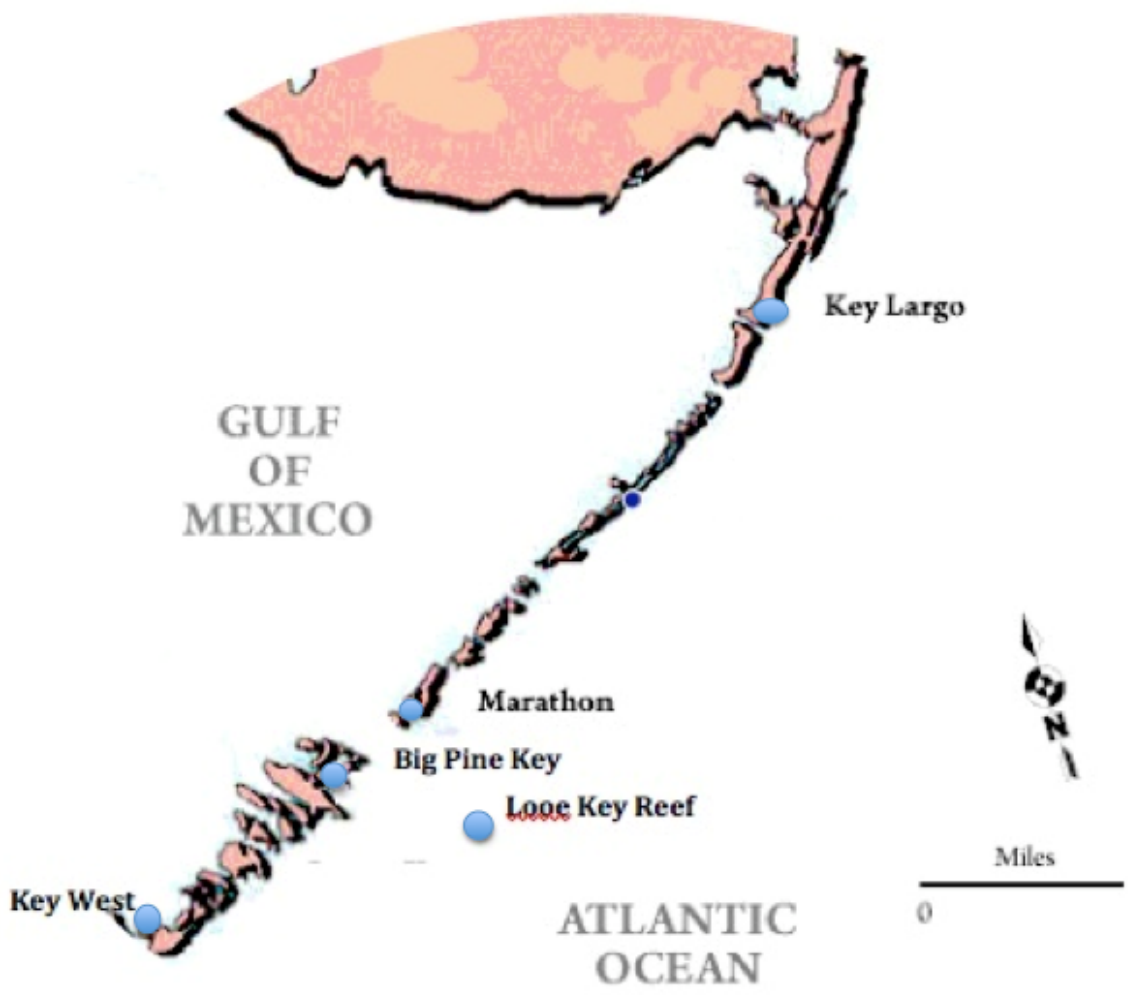

Figure 1: Sites of Sample Collection in the Florida Keys. Wastewater was collected from wastewater treatment plants located on Key Largo, Marathon Key, and Key West. Host and non-host coral mucus samples were collected from Looe Key Reef. Canal water was collected from Key Largo and Big Pine Key. One sample from Blue Hole's freshwater sinkhole was also collected from Big Pine Key.

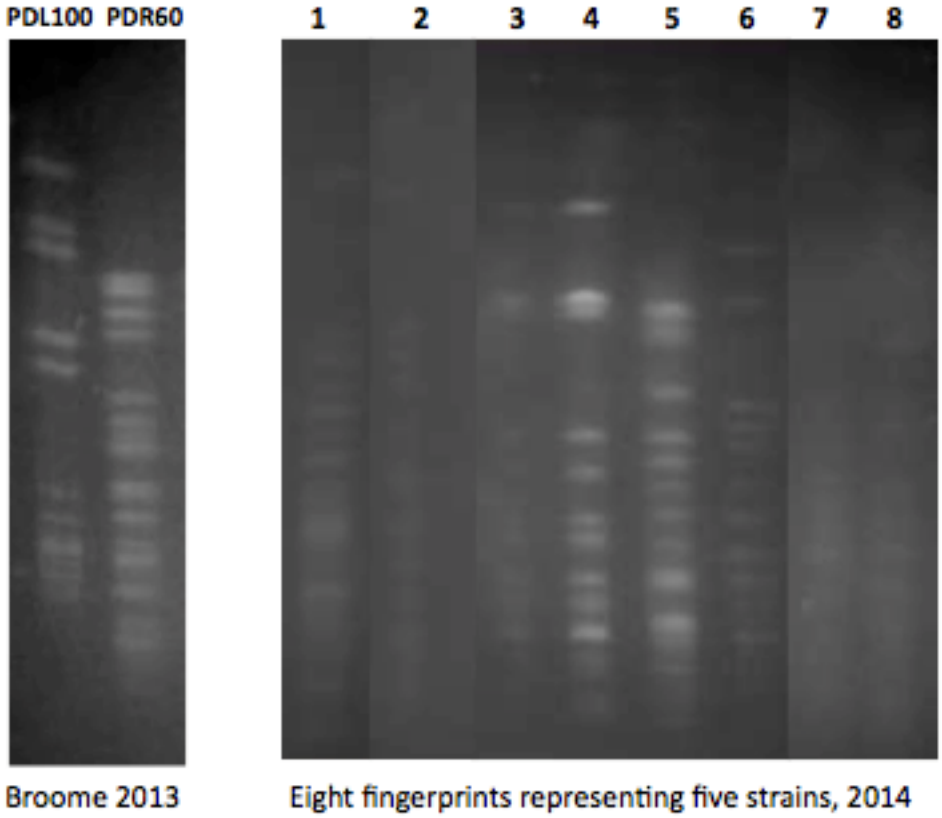

Figure 2: PFGE gels showing fingerprint band patterns for July 2013 isolates and for strains PDL100 and PDR60. The left image shows PFGE fingerprints of strains PDL100 and PDR60 (Broome 2013). The right image shows the eight PFGE fingerprints representing the five different unknown strains found in this study. Lane 1 is Strain D: non-host coral from Looe Key testing negative for DTC, and therefore not *Serratia marcescens*. Lanes 2 through 6 were positive on MCSA, DTC, and PCR, and therefore are all strains of *S. marcescens*. Lanes 2 and 6 are Strain B: wastewater isolates from Marathon (lane 2) and Key Largo (lane 6). Lanes 3 and 4 are Strain E canal water isolates from Tropical Lane Canal. Lane 5 is Strain C wastewater isolate from Key West. Lanes 7 and 8 are Strain A canal water isolates from Eden Pines Canal.

Table 1: Results for the 25 bacterial isolates for which PFGE was attempted, including their MCSA, DTC, and PCR results as well as their collection site information. 70 isolates were obtained from all major Keys and 31 of these isolates tested positive for PCR. PFGE fingerprinting was attempted for 25 *Serratia marcescens* PCR positive isolates; priority was given to coral and wastewater (WWI) samples over canal samples. "n/a" PFGE samples did not remain intact through the fingerprinting process. "Not Visible" PFGE samples were fingerprinted but no band pattern was visible. Five different strains (A through E) were fingerprinted on PFGE.

Collection Site	Bacterial Isolate	MCSA	DTC	PCR	PFGE	Total
Looe Key Reef	*O. faveolata* (1-1)	+	-	+	Not Visible	3 Coral
	P. porites (8-1)	+	-	+	Strain D	
	P. porites (6-2)	+	-	+	n/a	
Key West Wastewater Treatment Plant	WWI 1	+	-	+	Not Visible	14 Wastewater
	WWI 8	+	+	+	n/a	
	WWI 10	+	+	+	n/a	
	WWI 2A	+	+	+	Strain C	
Marathon Key Wastewater Treatment Plant	WWI 10	+	+	+	Strain B	
	WWI 9	+	+	+	Not Visible	
	WWI 4	+	+	+	Not Visible	
	WWI 1	+	+	+	Not Visible	
	WWI 2	+	+	+	n/a	
	WWI 5	+	+	+	n/a	
Key Largo Wastewater Treatment Plant	WWI 4	+	+	+	n/a	
	WWI 8	+	+	+	n/a	
	WWI 10	+	+	+	Strain B	
	WWI 11	+	+	+	n/a	
Tropical Lane Canal	5	+	+	+	n/a	7 Canals
	6	+	+	+	Strain E	
	7	+	+	+	Strain E	
Eden Pines Canal	11 2A	+	+	+	Not Visible	
	14 A	+	+	+	Strain A	
	15 A	+	+	+	Strain A	
	16 A	+	+	+	Not Visible	
Blue Hole Freshwater	4	+	+	+	n/a	1 Freshwater Sinkhole

Table 2: *Serratia marcescens* test results for the 21 DTC negative unknown bacterial isolates. None of these isolates are *S. marcescens*, which requires a positive MCSA, DTC, and PCR test result. (Sutherland et al. 2010).

Collection Site	Bacterial Isolate	MCSA	DTC	PCR
Key West Wastewater Treatment Plant	WWI 1	+	-	+
Looe Key Reef	*P. porites* (6-2)	+	-	+
	P. porites (8-1)	+	-	+
	O. faveolata (1-1)	+	-	+
	O. faveolata (3-1)	+	-	-
	A. palmata 30D	+	-	-
	A. palmata 64D	+	-	-
	A. palmata 64 D 1	+	-	-
	A. palmata 64 D 2	+	-	-
	A. palmata 64 D 3	+	-	-
	S. siderea (6-1)	+	-	-
	S. siderea (8-1)	+	-	-
	S. siderea (9-1)	+	-	-
	Water 1	+	-	-
	Water (1-1)	+	-	-
	Water (1-2)	+	-	-
	Water 2	+	-	-
	Water (2-3)	+	-	-
	Water (2-6)	+	-	-
	Water 3	+	-	-
	Water 4	+	-	-

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