CHARACTERIZATION OF *MYCOBACTERIUM CHELONAE* COMPLEX ISOLATES IN VERTEBRATES WITH SPECIAL FOCUS ON PATHOLOGY AND TRANSCRIPTOMICS IN SYNGNATHID FISH

by

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ABSTRACT

Mycobacterium chelonae is an important, rapidly growing, acid-fast bacterium that causes opportunistic disease in humans and fatal disease in aquatic animals. Organisms in the *M. chelonae-abscessus* complex (MCAC), including *M. chelonae* and *M. abscessus* subsp. *abscessus*, are difficult to differentiate. Many proposed methods to identify isolates in the MCAC exist but have been proven inadequate in diagnostic settings. Furthermore, standard break-points for species identification have yet to be established. As a result, these bacteria are often misidentified.

Of the numerous aquatic species held in captivity, fish in the Family Syngnathidae, the seadragons, seahorses, and pipefish, are highly susceptible to disease caused by *M. chelonae.* Syngnathids infected with *M. chelonae* present with disseminated disease that appears more acute, typically without granuloma formation, when compared to other vertebrates. The objectives of this study were to: (1) evaluate clinical isolates previously identified as *M. chelonae* cultured from biofilms, syngnathids, teleosts, reptiles, and mammals, including humans by comparing 11 targeted genetic

loci, *hsp65* PRA, and minimum inhibitory concentrations to antimicrobial drugs, (2) consider whole genome sequencing with core extraction to evaluate true phylogenetic placement of *M. chelonae* isolates and their relationship to each other, as well as type strains of nontuberculous mycobacteria, (3) characterize the histologic lesions of mycobacteriosis in syngnathids, and (4) describe the transcriptome of the lined seahorse, *Hippocampus erectus*, naturally infected with *M. chelonae*. Results identified whole genome sequencing as an ideal method for phylogenomic placement of closely related *M. chelonae* isolates, several of which had been previously misidentified. Findings indicate the current *M. chelonae* reference strain, ATCC 35752, is not representative of current clinical isolates, while *M. chelonae* ATCC 19237 is representative and represents a subspecies of *M. chelonae*. Breakpoints for species designation using targeted gene sequencing were proposed. A non-tuberculous species was identified in two syngnathids. There was no host specificity with relation to strain, but host lesions did vary by species affected. Syngnathids have a different presentation of mycobacteriosis than other teleosts. While components of the innate immune system were identified, components in the adaptive system important to controlling mycobacteriosis were not and may underlie their unique pathological presentation.

INDEX WORDS: Syngnathids, mycobacteriosis, *Mycobacterium chelonae*, whole genome sequencing

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DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2016

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December 2016

DEDICATION

I dedicate this dissertation to my loving and supportive family, Stephen, Elayne, and David Fogelson. Their perpetual support has been a catalyst for my achievements.

ACKNOWLEDGEMENTS

I would like to acknowledge the many people who enabled this research to move forward in a timely manner and assisted in the production of meaningful results. Many thanks to my PhD committee members for their guidance and encouragement, John Leary for his assistance with qPCR assay development and interpretation, the Mycobacteria/Nocardia Laboratory at Tyler,TX for their expertise in mycobacterial phylogeny and phylogenetic interpretation, Dr. Walt Lorenz for his knowledge and expertise in linux code and bioinformatics program usage, Shan-Ho Tsai and Yecheng Huang for their support with linux command line trouble shooting, and the staff at the Georgia Aquarium, Dr. Shane Boylan, and Dr. Emily Christiansen for assistance with sample collection.

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Chapter 1

INTRODUCTION

Nontuberculous mycobacteria (NTM) have the ability to cause disease in a diverse host range and represent a substantial health issue for fish, reptiles, birds, and mammals, including humans. Over the past 15 years, reports of mycobacteriosis have increased across species (1-3). Although normally considered a minor secondary pathogen in immune compromised humans (4), recent reports of immune competent individuals contracting mycobacteriosis have been identified (5, 6). In contrast to human patients, NTM are major pathogens in fish species. Hundreds of fish species are affected in fresh, salt, and brackish water habitats. Furthermore, mycobacteriosis has not only contributed to the decline of natural fish populations, such as wild striped bass *Moroxone saxatalis* in Chesapeake Bay (7), but has also been documented as an important disease of cultured species like seabass and seabream (8). All fish species are considered susceptible to mycobacteriosis, but some appear more susceptible than others. One family with an increased susceptibility to mycobacteriosis is the Syngnathidae (9).

The syngnathids are a group of fish composed of the seahorses, pipefish, and weedy and leafy seadragons. Although the husbandry of these animals has been well described (9), little information exists on the immune system of these fish. Regarding syngnathid health, there are several reports of diseases affecting this family, including

acanthacephala, digeneans, myxozoans, scuticocilliates, and bacteria (10-15). From the few large scale studies of disease prevalence in syngnathids, one of the most important diseases is mycobacteriosis and the most common species cultured from affected fish is *Mycobacterium chelonae* (9, 16).

M. chelonae is a NTM and is part of the *M. chelonae-abscessus* complex (MCAC). Within the complex are several closely related species that cannot be readily differentiated by routine methods and more species are currently being discovered. *M. chelonae*, *Mycobacterium abscessus* subsp. *abscessus*, *Mycobacterium immunogenum*, *Mycobacterium salmoniphilum*, *Mycobacterium franklinii*, and the non-validated species, *Mycobacterium saopaulense,* compose this group of closely related organisms (17-20). In addition to being difficult to differentiate, these NTM cause similar pathology in most vertebrates, usually consisting of a localized soft tissue infection characterized by granuloma formation in the affected tissues (21-23). Bacterial numbers can vary within lesions from few to numerous and dissemination rarely occurs in immunocompromised patients (24). Pathologic changes associated with *M. chelonae* infection across the syngnathid family have yet to be thoroughly described, but they have been characterized in a few members of the group, including weedy (*Phyllopteryx taeniolatus*) and leafy (*Phycodurus eques*) seadragons (11). Lesions were characterized by focal or multifocal histiocytic inflammation with rare granuloma formation. NTM numbers were high within lesions and bacterial embolism was noted in the gills. The presence of high numbers of bacteria, as well as dissemination, is an unusual presentation for NTM and calls into question whether syngnathids are being

infected with a unique strain of *M. chelonae* or if they have an altered cell-mediated immune response incapable of containing the infection.

In this research, isolates of *M. chelonae* cultured from biofilms, fish, reptiles, and mammals, including humans, were investigated for strain variation, accurate phylogenomic identification, antimicrobial susceptibility, and host specificity under the hypothesis that strains of *M. chelonae* infecting syngnathids are genetically similar to those infecting other vertebrates. In addition, a second hypothesis, that syngnathids have a different pathologic presentation of mycobacteriosis due to a limited cell-mediated immune response, was also investigated.

This work is important as *M. chelonae* is ubiquitous in the environment and can cause disease in a number of species. The data generated from this research reveals unique distinctions between bacterial strains applicable to the development of rapid and reliable diagnostic methods of NTM species delineation. The pathologic presentation of disease in syngnathids is described in conjunction with the evaluation of NTM isolates. A detailed evaluation of the lined seahorse *Hippocampus erectus* transcriptome and descriptions of components of the immune response to mycobacteriosis are included. A better delineation of the NTM strains that affect syngnathids, as well as a novel evaluation of the immune response of one syngnathid, the lined seahorse, to pathogenic intra-cellular organism is presented.

Chapter 2

LITERATURE REVIEW

Mycobacteria taxonomy

Mycobacteria are aerobic, intracellular, gram-positive, acid-fast, 0.2-0.6 µm by 1-10 µm, bacilli, with a thick mycolate-rich cell wall (25), which accounts for many of the unique properties of this genus, including resistance to dehydration, fluctuations in pH, antibiotics, and disinfectants, as well as the ability to stimulate immune responses (26, 27). According to the 2014 version of Bergey's Manual of Systematic Bacteriology, 169 species of mycobacteria have been identified (28). Members of the order Actinomycetales, the mycobacteria group together with other genera containing high G+C content, such as *Nocardia*, *Rhodococcus,* and *Corynebacterium*.

The family Mycobacteriaceae is divisible into two main groupings that include the tuberculous and nontuberculous (NTM) species. When compared to *Escherichia coli* growth rates on agar plates, as defined by Runyon (29), members of the genus *Mycobacterium* are considered "slow growing". However, within the genus, an additional division between growth rates can also be made. Traditionally, tuberculosis causing species, such as *M. tuberculosis, M. bovis, M. intracellulare* and *M. microti,* are characterized as slow growing, with growth appearing after 7 days of incubation. In contrast, the NTM mycobacteria, such as *M. chelonae, M. abscessus* subsp. *abscessus*, and *M. abscessus* subsp. *massiliense*, are characterized as rapidly growing (RGB), with growth appearing before 7-days of incubation (30).

Nontuberculous mycobacteria epidemiology

In general, NTM are ubiquitous in the environment and inhabit a wide variety of ecosystems (31). Many species have been readily cultured from soil, water, aerosol, and biofilms (32, 33). Isolation of NTM from aquatic environments, such as municipal water supplies, household plumbing, and biofilms within drinking water distribution systems highlights the pervasive nature of these organisms (34-36). Their ability to inhabit a variety of habitats likely facilitates interactions with a diverse range of hosts, contributing to the epidemiology of disease and also makes NTM relevant for study under the One Health initiative (37).

Many species of NTM produce extracellular polysaccharide matrix proteins that enable them to form biofilms and thus can persist under adverse environmental conditions (35, 38-40). The benefits of biofilm formation are numerous, one being a physical barrier against disinfectants. Studies with the NTM, *Mycobacterium marinum*, show high levels of resistance to commonly employed disinfectants, such as sodium hypochlorite (41). Another important benefit of biofilm formation is the close interactions of bacteria within the biofilm, which can facilitate horizontal exchange of genetic material (42-44) and the propagation of mutations that can confer antimicrobial resistance (45). Biofilms play an important role in the pathogenesis of mycobacteriosis in animals where a direct link between biofilm strains of NTM and disease have been established (46, 47).

NTM rarely cause overt disease in humans but can cause fatal disease in birds, reptiles, amphibians, and aquatic animals (48, 49). However, reports of human NTM, along with identification of new mycobacterial species, have been increasing in

frequency over the last 15 years (50, 51). In humans, significant morbidity from opportunistic *Mycobacterium* spp. infection is most often attributed to immunosuppression by concurrent disease processes like, HIV, diabetes, and leukemia or congenital impaired interferon gamma (IFN-γ) mediated immunity and cystic fibrosis (4, 52, 53). Although immunosuppression underlies many clinical cases of NTM infections, reports of immune competent individuals with NTM infections do exist, many of which involve medical implants or inoculation by contaminated fomites (40, 54).

In addition to immunosuppressive conditions, patients with specific genetic abnormalities appear to have increased susceptibility to infection (55, 56). Kartalija et al. (57) observed that pulmonary NTM was more prevalent in patients with taller stature, thin body frame, scoliosis, and pectus excavatum, all of which are features of the congenital disease, Marfan syndrome. In addition to the morphologic abnormalities, the immune modulatory molecules adiponectin and leptin were abnormally regulated in these patients, potentially causing a downstream suppression of the cytokines IFN-γ and IL-10, which are important molecular defenses against mycobacteriosis.

In contrast to human infection, mycobacteriosis caused by NTM in fish is a commonly reported fatal disease linked to environmental stress, trauma, high environmental bacterial presence, and contaminated food (3, 58). Hundreds of fish species are affected by this disease (59). Due the ubiquitous nature of these bacteria within diverse aquatic environments and their resistance to common disinfectants, these organisms frequently challenge fish. Consequently, mycobacteriosis has not only contributed to declines in wild fish populations, such as striped bass *Moroxone saxatilis* in Chesapeake Bay (7), it is also an important disease of cultured species, including sea

bass *Dicentrarchus labrax* and Atlantic salmon *Salmo salar* (8, 60). Infections of fish by NTM are well recognized and a correlation between environmental presence and infection exists. Using repetitive-sequence-based polymerase chain reaction fingerprinting (rep-PCR), Yanong et al (61) demonstrated that the same strain of *M. marinum* was isolated from tank biofilms and clinically ill pompano. Also considered zoonotic pathogens, NTM from aquatic settings, including aquaculture facilities and aquariums, put humans at risk for disease. (62-64).

Similar to humans, a link between genetic susceptibility and mycobacteriosis exists in fish. Although all fish are considered susceptible, members of the family Anabantidae (bettas and gouramis), Characidae (tetras and piranhas), Cyprinidae (danios, goldfish, and barbs), Cichlidae (cichlids), and Syngnathidae (seahorses, seadragons, and pipefish) appear to have increased susceptibility (14, 59, 65, 66). Differences in host susceptibility were demonstrated by Broussard and Ennis (67), who found zebrafish *Danio rerio* to be 10 times more susceptible than medaka *Oryzias latipes* inoculated with *M. marinum.*

Although some species of fish appear to have a genetic predisposition to mycobacterial disease, NTM mycobacteriosis does not necessarily have host specificity. Several studies have identified a lack of host specificity in wild fish, including one by Stine et al. (68), where 29 different mycobacterial isolates, not all of which were associated with disease, were recovered from 12 species of fish. The majority of isolates were present in more than one fish species and identified from more than one location in the Chesapeake Bay or its watershed. Another study by Sanchez et al. (unpublished data), evaluating *M. chelonae* isolates from fish, reptiles, terrestrial

mammals, and humans, also provided evidence that NTM species show little host preference.

***Mycobacterium chelonae-abscessus* complex**

Classified in the *M. chelonae-abscessus* complex (MCAC) is a specific group of NTM of concern to both humans and aquatic animals. *M. chelonae*, *Mycobacterium abscessus* subsp. *abscessus*, *Mycobacterium immunogenum*, *Mycobacterium salmoniphilum*, *Mycobacterium franklinii,* and the non-validated species, *Mycobacterium saopaulense,* compose this group of closely related organisms (17-20). This group, like other NTM, is ubiquitous and has been cultured from a wide variety of environments, as well as from clinically ill patients worldwide (69, 70). Like many other bacterial species, MCAC can form biofilms that aid in their persistence within the environment or a host (39, 71). In addition, MCAC have high resistance to common disinfectants, such as chlorine, and tolerate a wide range of pHs and temperatures (72).

Organisms in the MCAC are closely related phenotypically and difficult to differentiate (73). In 1992, based on DNA-DNA hybridization methods (74), two of the most important members of the MCAC, *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* were both elevated to species status as *M. chelonae* and *M. abscessus* subsp. *abscessus*, respectively. Only two biochemical tests are reported to dependably separate species within the complex, sodium chloride tolerance and citrate utilization (75, 76), but these are time consuming, results can be vague, and biochemical testing has become antiquated with the advent of molecular diagnostics.

Isolates of the MCAC are typed using a combination of methods, including phenotypic characteristics, targeted gene sequencing, PCR restriction fragment length

polymorphism analysis of the heat shock protein-65 gene (*hsp65)*, and high-performance liquid chromatography (HPLC) of mycolic acids (77). Many of these methods are also time consuming, labor intensive, may be difficult to assess or reproduce and require large databases with in-house validation due to the lack of commercial systems. In addition, there is a lack of consensus among laboratories regarding percent identity break points used to differentiate closely related species. One method currently used in diagnostic laboratories is targeted gene sequencing. Targeted gene sequencing has advantages over the previously stated modalities in several ways. Most notably, it produces unambiguous, easily interpreted data.

New technologies, including matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) has been a huge leap forward for rapid reliable species identification. However, issues remain in the database for members of the MCAC and identifications can be equivocal (78). For example, *M. abscessus* subsp. *abscessus* has identical spectra to *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (79).

Due to the intricacies of identification, many clinical laboratories only identify isolates to the *M. chelonae-abscessus* complex level (80). This generic classification can pose a serious concern to both human and veterinary patients, as treatment protocols may vary due to differing antibiotic susceptibilities among members of the complex (81-83). For example, it is now known that approximately 80% of *M. abscessus* subsp. *abscessus* isolates carry an erythromycin resistance methylase gene [*erm*(41)] that enables them to resist treatment with macrolide antibiotics such as clarithromycin and azithromycin (84, 85). In contrast, *M. chelonae* lacks an *erm*(41) and all wild type

(untreated) isolates are macrolide susceptible. In addition, *M. chelonae* isolates have MICs >128 µg/mL for cefoxitin and have lower MICs to tobramycin than most isolates of *M. abscessus* subsp. *abscessus.*

By applying recent advancements in automated gene sequencing, identification of closely related species has become significantly less challenging and many mycobacterial isolates are identifiable by 16S rRNA gene sequencing. This is untrue for MCAC isolates, as they show little variability in this region, making the 16S rRNA locus unsuitable for discrimination between these species (86). For instance, use of the partial 16S rRNA gene sequence fails to separate the most important species in the MCAC, *M. chelonae* and *M. abscessus*, as there is 100% identity between the two at this locus (86-88). Other genes purported to differentiate between closely related species include regions 3 and 5 in the β-subunit of the RNA polymerase gene (*rpoB*), the Telenti et al. (89) sequence of the 65 kD heat shock protein gene (*hsp65*), DNA gyrase subunits A (*gyr A*) and B (*gyr B*), translation elongation factor Tu (*EF-Tu*), manganese dependent superoxide dismutase (*SodA*), *Escherichia coli* secretion gene (*SecA*), and the 16S-23S internal transcribed spacer region (ITS) (40-43). At present, diagnostic laboratories employ a combination of techniques, most notably the Nocardia/Mycobacteria Research Laboratory (Tyler, TX) combines targeted sequencing of *erm*(41) and *rpoB*. However, uncertainty remains in the definitive classification of MCAC species (personal communication, Dr. Richard Wallace and Barbara Brown-Elliot).

A pilot study by Arnold et al. (90) exemplifies the uncertainty surrounding classification of MCAC strains. Using the housekeeping gene, *rpoB*, to evaluate 94

isolates previously identified *M. chelonae,* 41% were more closely related to *M. abscessus* subsp. *abscessus* at this locus. Although these results suggest that *rpoB* may be helpful for species identification, a study by Sanchez et al. (UGA unpublished data) revealed that *rpoB*, *hsp65*, and ITS analysis of *M. chelonae* complex isolates did not uniformly separate MCAC isolates into the same groupings. Of the three methods used in that study, *hsp65* most consistently identified isolates correctly using *M. chelonae* ATCC19237 and *M. abscessus* subsp. *abscessus* ATCC 19977^T type strain as references.

Whole genome sequencing (WGS) represents an all-encompassing method for identification of closely related bacterial species. As costs decrease and availability increases, WGS is increasing in popularity and use. As of August 2016, Genbank included 29 *M. abscessus* and three *M. chelonae* complete genomes. The genetic markers *rpoB*, *hsp65*, and a variable number of tandem repeat analyses were used for genotyping of these isolates before WGS was performed (91, 92). Two of the three whole genome sequences for *M. chelonae* are of a type strain isolated from a clinically ill turtle, *M. chelonae* ATCC 35752^T. The third whole genome (strain 1518) was constructed from 14 antibiotic resistant human isolates. Although some certainty exists regarding the correct identification of the type strains in GenBank, clinical isolate *M. chelonae* strain 1518, identified possibly at only one genetic locus before whole genome sequencing was performed, may be misidentified in the public database. As molecular identification techniques evolve, accurate species identification of MCAC isolates will be illuminated. While this clarity will allow for proper species identification, it will be

increasingly more apparent that information in public databases, like GenBank, are inaccurate and should be used with caution for diagnostic purposes.

Clinical signs and pathology in fish

Piscine mycobacteriosis is a significant cause of mortality in both wild and captive fish. The first case of disease caused by a NTM was reported over a hundred years ago in a carp *Cyprinus carpio* (93). Since then, the disease has been extensively reviewed and hundreds of fish species have been identified as being affected by mycobacteriosis (3, 59). Generally described as a chronic progressive disease, clinical signs can include weight loss, anorexia, lethargy, epidermal discoloration, and skin ulceration (59). Although chronic mortality is often reported in relation to mycobacteriosis, disease can also be acute or even clinically silent (94).

Apparently healthy aquarium species have been identified with clinically silent mycobacterial infection, including striped panchax *Aplocheilus lineatus,* freshwater angelfish *Pterophyllum scalare,* pearl gourami *Trichogaster leeri,* and Siamese fighting fish *Betta splenden* (95). In addition, laboratory species, such as zebrafish, have been extensively studied and asymptomatic *M. chelonae* infections found in up to 34% of the colonies evaluated (96). Inapparent infections are also present in wild fish populations, as demonstrated by Densmore et al. (97), who identified an uncharacterized species of mycobacteria in snakehead collected during routine electrofishing for species health assessments.

Primarily seen in the chronic form, piscine mycobacteriosis has traditionally been attributed to three main species of NTM: *M. marinum*, *M. fortuitum* and *M. chelonae*. In recent years, additional species of NTM mycobacteria have also been identified as

significant contributors to disease in fish, including *Mycobacterium shottsii* (98) and *Mycobacterium pseudoshottsii (99)* in striped bass *Morone saxatilis* from Cheseapeake Bay, and *Mycobacterium gordonae* from captive goldfish (100), as well as *Mycobacterium peregrinum* (101) and *Mycobacterium haemophilum* (102) in zebrafish.

Disease caused by NTM has been documented in hundreds of fresh, salt, and brackish water fish species (61, 66, 103-105). The pathogenesis of NTM in fish is poorly understood, but transmission is thought to occur primarily by ingestion of contaminated feed and water or by bacterial invasion of damaged epidermis. Once the bacteria gains entry into the body, it can manifest disease in any organ system, but in the majority of fish cases, the spleen, kidney, and liver are the most severely affected. On gross examination of infected animals, mycobacteriosis is often described as producing discrete white nodules, histologically granulomas, scattered throughout affected organ tissues.

Microscopic evaluation reveals a progression in the morphology of the lesions caused by NTM over time, starting from an inflammatory focus to a discrete granuloma. Gautier et al. (106) described the chronic progression of disease in striped bass *Morone saxatilis* infected intraperitoneally with 10^5 CFU of *M. marinum.* These stages were described as: (1) a pre-granulomatous inflammatory focus, (2) an epithelioid granuloma, (3) a spindle cell granuloma, (4) bacillary granuloma, and (5) a resolving lesion. However, it is uncertain how many fish actually recover from mycobacteriosis, as it has not been well studied. In addition, a dose dependent response is also associated with the onset and clinical signs of disease (i.e. acute or chronic). In a study by Talaat et al. (94), goldfish inoculated with high doses of *M. marinum* (10^8 to 10^9 CFU) presented with

acute disease characterized by necrosis, inflammation, and systemic dissemination of the bacteria, with mortality in less than 17 days post inoculation. Whereas lower doses produced systemic granuloma formation with little to no necrosis and survival of the fish to the end of the experiment (56 days).

A large number of studies focus on the pathogenesis of *M. marinum* in fish, due to its genetic similarities to human tuberculosis, caused by *Mycobacterium tuberculosis*, as well as disease presentation and progression. However, some reports do investigate the pathogenesis of other NTM infections. One study evaluated *M. chelonae* in Atlantic salmon *Salmo salar* and identified a pathologic presentation similar to striped bass with *M. marinum*. Infected farmed fish, Atlantic salmon, had miliary nodules in the liver, spleen, and kidney characterized by densely packed epithelioid macrophages with intracellular acid-fast bacteria surrounded by a thin fibrous capsule.

In general, chronic mycobacteriosis in fish usually presents with mature discrete granulomas characterized by central areas of caseous necrosis and cellular debris containing variable numbers of acid-fast bacteria. These areas are surrounded by epithelioid macrophages rimmed by flattened macrophages or concentric layers of fibrous connective tissue sometimes with scattered peripheral lymphocytes (66). This presentation of mycobacteriosis is different from that seen in members of the Family Syngnathidae. In syngnathids, discrete granulomas are a rare finding and microscopically, lesions often appear more acute and necrotizing (11, 15).

Syngnathids

Syngnathids are in the class Actinopterygii (ray finned fish) under the order Syngnathiformes and family Syngnathidae. Most recently, Betancur-R et al. (107)

reclassified syngnathids from the suborder Syngnathoidei into a new order within the Scombrimorphia. Examining 21 molecular markers in 1184 bony fish taxa elucidated this new classification. Scombrimorphia groups several morphologically disparate fishes into one clade containing a host of marine reef-dwellers, including syngnathids, mullids, cllionymids, and most surprisingly the scombrids (mackerels, bonitos, and tunas). Reports of mycobacteriosis are sparse in these wild species. However, one report of acid-fast bacterial granulomatous peritonitis in a tuna fish *Thunnus* spp. has been documented (108). Of the numerous species held in captivity, fish of the family Syngnathidae, which includes the seadragons, seahorses, and pipefish, hold high educational and economic value (109).

Nontuberculous Mycobacteria in Syngnathids

Syngnathids are susceptible to a variety of disease etiologies including acanthacephala, digeneans, myxozoans, scuticocilliates, and multiple bacteria (10-15). From the few large scale studies of disease in syngnathids, one of the more prevalent diseases is mycobacteriosis and one of the most common species of mycobacteria isolated is *M. chelonae* (14, 110). In addition to their increased susceptibility, syngnathids present with histologic lesions significantly different from those of mycobacteriosis in other teleost species. Lesions are characterized by extensive coagulative necrosis of the affected organ, with large nodular aggregates or sheets of macrophages that have cytoplasm laden with numerous acid-fast bacterial rods (11, 13). This pathologic presentation of necrosis is more akin to previously described acute mycobacterial infections in fish than the more typical chronic form of disease. Furthermore, large numbers of free bacteria may be present within lesions and in

circulation (11). Bacterial embolism is uncommonly reported in other teleosts with mycobacteriosis who tend to form discrete granulomas that wall off comparatively low numbers of bacteria (111). At present, it is unclear if these interesting lesions in syngnathids are a product of an ineffective cell mediated immune response, pathogen virulence, or potentially a combination of the two.

Pathogenesis of *Mycobacterium* spp.

The pathogenesis of obligate slow growing mycobacteria, such as *M. tuberculosis*, has been studied extensively. However, pathogenic mechanisms of NTM causing mycobacteriosis in fish are poorly described. In reviews by Koul et al. (112) and Sakomoto (113), the basic mechanisms by which the obligate pathogen *M. tuberculosis* infects and persists in a mammalian host are discussed. Mechanisms include the targeting of macrophages through cell surface receptors, such as Fc receptors, toll-like receptor 2 (TLR2), mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), and compliment receptor 3 (CR3), the inhibition of host cell processes such as phagosomal-lysosomal fusion, alteration of antigen presentation, regulation of apoptosis, alterations in intracellular signaling by Ca^{2+} and MAPK, and the inhibition of the anti-microbial cytokine interferon-gamma (IFN-γ).

As stated earlier, the primary modes of infection are through defects in the epithelium or ingestion. To enter a fish host and establish an infection, the bacteria must evade the host innate and adaptive immune responses. Many innate and adaptive immune system components have been identified in fish and are described in more detail in the following sections.

Depending on the route of entry, mycobacteria encounter many innate immune barriers. If the mycobacteria invade through the skin, they first come into contact with the fish's innate epidermal barrier which provides physical protection, as well as production of mucus rich in anti-microbial peptides, like beta-defensin, among others (114). With compromise of the epidermal barrier, mycobacteria can enter into the skin. Fish are more commonly infected through ingestion of bacteria from the water column or via feed contamination. Harriff et al. (115) clearly demonstrated the oral route of inoculation in zebrafish *Danio rerio* intubated with *M. marinum* was more effective in producing disease than immersion challenge. Similarly, Sodchit et al. (116) found that ingestion of contaminated *Artemia* and water fleas *Cyclops* lead to mycobacteriosis caused by *Mycobacterium spp., M. fortuitum, M. marinum and M. chelonae* in experimentally challenged Siamese fighting fish *Betta splendens*. A mucus coat instilled with antimicrobial peptides that protects against invasion also covers the gastrointestinal mucosa (117). Another innate protective mechanism helpful in moving pathogens out of the system is the regular sloughing and regeneration of mucosal epithelial cells.

Once mycobacteria penetrate the mucosal barriers and enter the host, macrophages performing immune surveillance identify potential pathogens through surface pattern recognition receptors, such as toll-like receptors (TLRs). In humans, *Mycobacterium tuberculosis* is recognized by the cell membrane receptors TLR2 and TLR4 along with numerous other membrane bound receptors such as mincle, dectin-1, compliment receptor 3, and mannose receptor (118-120). *M. tuberculosis* can also bind to TLR9 within the endosome and cytosolic NOD-like receptors (NLRs) (121). Species

of NTM infecting fish can also bind to a TLR2 homologue, which elicits a signaling cascade that results in transcription of nuclear factor-kappa beta (NF-κB) and cytokine production. NF-κβ has been identified in zebrafish infected with *M. marinum,* as well as the upregulation of TLR-21 in response to infection (122).

After antigenic components of the mycobacteria are recognized by receptors, macrophages start producing inflammatory cytokines through NF-κβ transcription and the inflammasome pathway (123). Both of these pathways have been identified in fish, as well as the products made by them, which include the innate cytokines tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and interleukin 12 (IL-12) (subunits IL12-p40 and IL12-p35). TNF is extremely important in the pathogenesis of mycobacteriosis as it can attract inflammatory cells to the affected area and bind to TNF receptors (124). In turn, TNF receptors can produce many different outcomes, including transcription of NF-κβ, induction of apoptosis via caspase mediated cell death, and induction of the caspase independent cell death pathway, necroptosis (125). Additionally, TNF can also elicit the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), which are instrumental to the intracellular killing of microbes. Downstream activation of ROIs and inducible nitric oxide synthetase (iNOS) to produce NO is all upregulated by TNF (126). ROI and NO induction as a result of cytokine signaling has been identified in several teleost species including goldfish. More specifically, goldfish macrophages stimulated with recombinant IFN-γ proteins show ROI and NO upregulation (127, 128).

Ultimately, macrophages will phagocytize the bacteria, process its antigens and present them on the surface in a major histocompatibility complex receptor (MHC),

more specifically MHC II. Mycobacteria can escape the phagosome and enter the cytosol, at which time the antigens may be presented in MHC I receptors (129). Infected macrophages migrate to T-cell rich tissues, such as the kidney and spleen, to present mycobacterial antigen to naïve T-cells. Production of the cytokine IL-12 from infected macrophages promotes transformation of naïve T-cells into effector T-cells skewed toward a T helper 1 response (130), with subsequent IFN-γ production. IFN-γ production promotes a delayed type hypersensitivity where granulomas form to contain the infection. Alternatively, infected cells can also be targeted for death by CD8+ cytotoxic T lymphocytes or can be damaged by ROIs or NO at which time bacteria can be released into the extracellular space and infect additional cells.

T cells are essential for the mitigation of mycobacteriosis in the host. Two signals, CD28/B7 and CTLA-4, are necessary for naïve T cells to begin cytokine production (131), which includes the upregulation of interleukin-2 (IL-2) and others needed for the proliferation of differentiated CD4+ Th1 cells (132). Increased surface expression of IL-2 receptors on activated T-cells can then auto-trigger for further proliferation by stimulating production of additional IL-2. Another important cytokine produced by Th1 cells is IFN-γ, which also triggers the naïve CD4+ T cells to differentiate into a Th1 lineage, as well as the activate macrophages.

Often granulomas are associated with T-cells at their periphery. With *M. marinum* infection, the numbers of bacteria within these granulomas are often low. Granulomas are maintained by many factors including TNF-α, TGF-β, and IFN-γ. There is a delicate balance between control and disseminated disease. Anti-inflammatory cytokines, such as IL-10, suppressors of cytokine signaling (SOCS3), and TGF-β,

produced by macrophages, limit host cell damage by the immune response, as well as counteract pro-inflammatory cytokines. In humans, if anti-inflammatory cytokines are overexpressed or if pro-inflammatory cytokines are under expressed, un-controlled disseminated disease can occur, as many pathways in the Th1 response are altered (126, 133, 134). Aberrant expression of cytokines may be responsible for the acute disseminated disease observed in syngnathids, but reports of cytokine response to mycobacteriosis do not exist at this time. However, there is one report of “pregnancy” in male broad-nosed pipefish *Syngnathus typhle* and experimental exposure to *Vibrio* spp. where inflammatory cytokines, such as Interleukin-10 and tumor necrosis factor (TNF), were observed, demonstrating that these fish possess cytokines orthologous to humans involved in the inflammatory pathway during mycobacterial infection (135).

Although the adaptive immune response to mycobacterial infection is primarily cell-mediated, in humans there can also be a humoral component to the adaptive response. When IFN-γ activates macrophages, it also triggers B-cells to produce IgG2a, which aids in bacterial opsonization and their recognition by Fc receptors on the macrophage (136). IgG2a binding of Fc and C3 receptors targets the bacteria for macrophage killing, but has been shown to be an inefficient process in a murine model of tuberculosis infection (137). Regardless, fish have a limited repertoire of immunoglobulins (IgM, IgD, Ig T/Z, IgNAR) and it is unlikely that IgG plays a role in the control of piscine mycobacteriosis, although other less characterized antibodies could potentially participate in the response to infection.

Like many other pathogens, mycobacterial species possess a variety of virulence determinants that enable them to cause disease in a susceptible host. Numerous

virulence determinants associated with invasion and intracellular persistence have been described in both slow and rapidly growing mycobacterial species. These determinants are often characterized based on their function, molecular features or cellular localization

Approximately 5% of the *M. tuberculosis* genome, which corresponds to hundreds of genes, encodes proteins associated with intracellular growth and survival of the mycobacteria (138). Although much is unknown regarding the pathogenesis of mycobacteria infection, many known virulence factors of *M. tuberculosis* have been reviewed, including mycolic acid biosynthesis, genes involved in fatty acid and lipid metabolism, metal transporter proteins, cell envelope proteins, and protein kinases were reviewed by Forrellad et al. (139). An important factor involved in intracellular persistence is the cell wall lipoarabinomannan (LAM). LAM acts upon several pathways that involve scavenging of cytotoxic oxygen radicals, inhibition of protein kinase C activity, and blocking of the transcriptional activation of IFN-γ (140).

Virulence genes also play a major role in the pathogenicity of mycobacteria. *M. tuberculosis* and *M. marinum* contain the genes Rv1477/Rv/1478 and iipA/iipB, respectively, linked to the separation of dividing bacterial cells through the production of peptidoglycan hydrolases (141, 142). Additionally, the virulence genes, Rv3874/Rv3875, within the regions of difference locus (ΔRD1), have been characterized. The ΔRD1 locus encodes for the virulence effector proteins early secreted antigen 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). Part of the mycobacterial secretion system, these proteins are associated with the promotion of macrophage aggregation and intracellular bacterial spreading (73). Ripoll et al (43)

identified 30 homologs of *M. tuberculosis* virulence factors, as well as additional "mycobacterial" and "non-mycobacterial" factors in *M. smegmatis* and *M. abscessus* subsp. *abscessus*. "Myocbacterial" factors highlighted included the ESX gene clusters, which are responsible for the ATP-dependent secretion systems containing characteristic proline-glutamate (PE) or proline-proline-glutamate (PPE) N-terminal motifs, mammalian cell entry (MCE) proteins, LpqH-like proteins, a 19 kD protein recognized by T-cells during mycobacterial infection, and regulators of the mycobacterial virulence factors including SigA, SigC, SigD, SigE and SigH. *M. abscessus* also contains "non-mycobacterial" factors of virulence, such as phospholipase C, an enzyme used by intracellular pathogens to escape phagosomal vacuoles. As *M. chelonae* and *M. abscessus* subsp. *abscessus* are very closely related, it stands to reason that many virulence determinants described in *M. abscessus* subsp. *abscessus* may be present in *M. chelonae* and contribute to the pathogenesis of piscine mycobacteriosis.

Fish innate immunity with respect to mycobacteriosis

Well conserved amongst vertebrate species, including fish, the innate immune system confers non-specific resistance against disease agents and is divided into cellular and humoral components. The cellular component represents physical barriers such as skin, mucus, acidic or alkaline microenvironments, and phagocytic cells. In contrast, the humoral component is made up of soluble proteins/glycoproteins, antimicrobial peptides, proteases, complement proteins, and transferrins (143). Some specific cellular and humoral components include phagocytic cells including macrophages, the mucous layer protecting the skin, antimicrobial peptides such as

lectins, and neutrophils, and compliment components including C3, and the MAC attack complex, C9. In teleosts, the recognition of and responses to pathogenic microbes is thought to be well conserved. The presence of macrophages and cytotoxic, pattern recognition receptor (PRR) orthologues, pro-inflammatory cytokine orthologues, antimicrobial peptides, and complement has been identified in a variety of species (144).

Although much is unknown regarding the immune system of fish, the presence of orthologous molecules suggests conserved functions when compared to higher vertebrates. One important microbicidal molecule is NADPH oxidase. Teleost phagocytes possess NADPH oxidase and the ability to produce microbicidal reactive oxygen intermediates (ROIs) by activation of the NADPH oxidase complex, as demonstrated by Mayumi et al. (128), using molecular cloning and expression analysis of the NADPH oxidase genes gp91^{phox}, p22^{phox}, p46^{phox}, p67^{phox}, and p40^{phox} in carp *Cyprinis carpio*. Boltana et al. (127) identified the same NADPH oxidase genes in trout *Oncorhynchus mykiss* by stimulating macrophages to produce ROIs through LPS, poly (I:C) and zymosan injection, demonstrating similar pathways for microbial killing in fish and higher vertebrates.

A total of 17 types of TLRs have been identified in fish species and the mechanisms used for intracellular signaling have also been established as similar to mammalian systems, including the presence of myeloid differentiation factor 88 (MYD88), TIR domain –containing adaptor protein, and interferon regulatory factor 3 and 7 (IRF3 and IRF7) (145, 146).

The numerous conserved cytokines in that participate in the innate pathway in fish were extensively reviewed by Aoki et al. (144). These include members of the Class I cytokines, Class II cytokines, TNF superfamily cytokines and interleukin family cytokines. Pro-inflammatory cytokines in the Class I cytokine designation, such as IL-1, have been identified in a variety of species including carp, rainbow trout, goldfish, flounder *Paralichtyes olivaceous*, European sea bass *Dicentrarchus labrax*, gilthead seabream *Sparus aurata*, turbot *Scophthalmus maximus*, common dentex *Dentex dentex*, sharpsnout seabream *Diplodus puntazzo*, zebrafish, Japanese medaka, pufferfish *Fugu rubripes*, and small spotted catshark *Scyliorhinus canicula* (147). Anti-inflammatory cytokines have also been described in *C. auratus,* including transforming grown factor beta (TGF-β), suppressor of cytokine signaling-3 (SOCS-3) and Interleukin-10 (IL-10) (148).

A delicate balance between pro-inflammatory and anti-inflammatory mechanisms exists during infection with mycobacteria. Hodgkinson et al. (148) described the expression of acute phase pro-inflammatory cytokines IL-1β, IL12p35, IL12p40, and IFN-γ in *C. auratus* experimentally infected with *M. marinum*. This study revealed pro-inflammatory cytokines are elevated early in infection, but within 7 days decrease, except for IL-1β, which remains elevated at 56 dpi. A spike of the anti-inflammatory cytokines TGF-β, IL-10, and SOCS-3 is observed at the time of pro-inflammatory decrease, but these cytokines also drop below control levels at 56 dpi. The anti-inflammatory cytokine TGF-β has both pro-inflammatory and anti-inflammatory properties and has been suggested as having a significant role in the immunopathogenesis of piscine mycobacteriosis (149).

Components of the innate immune system often overlap with the adaptive immune system. One such group that bridges the innate and adaptive system is composed of the pro-inflammatory cytokines known as interferons (IFNs). Interferons play an integral role in mycobacterial host resistance. Primary sources for IFN include CD4+ and CD8+ T-cells, however innate lymphocyte populations including γδT cells, natural killer T-cells, and natural killer cells can produce interferons (150).

Fish adaptive immunity

The adaptive immune system requires cellular memory and centers on T-cell expression of genes, proteins, and biochemical messages in response to antigens, antibodies, and effector cells. Uribe et al. (151) reviewed specific immunity in teleosts and, similar to the innate immune system, found many components are conserved from higher vertebrates. As part of the adaptive immune system, teleosts can produce antibodies, primarily IgM, immunologic memory, cellular cytotoxicity, and adaptive cytokines (152, 153).

One group of well-known cytokines, in the adaptive pathway, comprise the Interferon family, secreted pro-inflammatory cellular proteins that play a crucial role in both the innate and adaptive immune system. Primarily expressed by T-lymphocytes, IFNs are intricately involved in the adaptive response to intracellular viral, protozoal and bacterial pathogens (154, 155). Furthermore, IFNs have a complex interaction with the host immune system by which they can induce cellular changes. These include the stimulation of phagocytic cell bactericidal activity, stimulation of antigen presentation through Class I and Class II major histocompatibility complex (MHC) molecules, attraction of leukocytes to an affected area, alteration of cell proliferation, and apoptosis,

as well as activation of macrophages, natural killer cells, and cytotoxic T cells (156, 157). Activation of the aforementioned immune cell population leads to up-regulated expression of various transcription factors and cytokines such as TNF-α, IL1-β, IL-6 and IL-12 (155, 158).

Mammals and fish produce Type I, Type II, and Type III classes of IFNs. Type I IFNs are divided into multiple subtypes (IFN-α, IFN-β, IFN-δ, IFN-3, IFN-k, IFN-t and IFN-u and limitin) and act with type III IFNs to trigger the innate immune system to defend cells against viral replication (159). Whereas, type II IFNs contain one subtype, IFN-γ, which promotes a cell-mediated immune response against viral, protozoal, and bacterial pathogens (160). In fish, a putative IFN-γ gene has been identified in several species including Atlantic cod *Gadus morhua*, channel catfish *Ictalurus punctatus*, fugu, goldfish, Atlantic salmon, Rainbow trout, and zebrafish (158, 161-166).

Although IFN-γ in teleosts shows low sequence homology to mammalian IFN-γ, the bioactivity of teleost IFN-γ has similar functions to its mammalian counterpart. Previous studies in trout, goldfish, and Atlantic salmon provide evidence that teleost macrophages respond to IFN-γ by up-regulation of MHC associated antigen presentation, transcription stimulation, increased phagocytic ability of macrophages, and elevated production of inducible nitric oxide synthase (iNOS) within macrophages (167).

IFN-γ plays a crucial role in the resistance and control of a host to infection by intracellular bacterial pathogens, like mycobacteria (154). As a critical mediator in the TH1 mediated macrophage activation and killing of mycobacteria, IFN-γ also plays an essential role in granuloma formation. Macrophages activated by IFN-γ differentiate

into epithelioid macrophages, which can aggregate to form granulomas and/or fuse to produce multinucleated giant cells(168). An increased susceptibility to mycobacterial infection has been linked to decreased expression of IFN-γ as a result of either immunosuppression, concurrent disease, or impaired functional activity of IFN-γ resulting from receptor gene mutations (169). Casanova et al. (170) provided a concise review of the known congenital mutations associated with mycobacterial susceptibility in humans that included: STAT1, IFNGR1, IFNGR2, IRF8, IL12B, IL12B1 IKBKG, CYBB, and TYK2 genes STAT1, IFNGR1, and IFNGR2. All are part of the signaling pathway for IFN-γ production. Although a number of teleosts possess some of the essential components involved in defense against mycobacteriosis, the role of cytokines in the syngnathid adaptive immune response has yet to be elucidated.

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Chapter 3

Evaluation of Human, Veterinary, and Environmental *Mycobacterium chelonae* Isolates by Core Genome Phylogenomic Analysis, Targeted Gene Comparison, and Anti-microbial Susceptibility Patterns: A Tale of Mistaken Identities[1]

[1] Fogelson, S.B., Camus, A.C., Lorenz, W.W., Vasireddy, R., Vasireddy, S., Smith, T., Brown-Elliott, B.A., Wallace Jr., R.J., Hasan, N.A., Reischl, U. and Sanchez, S. To be submitted to *Journal of Clinical Microbiology*

Abstract

Mycobacterium chelonae is a member of the *Mycobacterium chelonae-abscessus* complex (MCAC), which causes opportunistic disease in fish, reptiles, birds and mammals. MCAC isolates are difficult to identify, have differing antimicrobial susceptibilities, and successful treatment protocols are limited to non-existent in many species. Thirty previously identified *M. chelonae* clinical isolates cultured from biofilms, fish, reptiles, mammals, including humans, and three ATCC reference strains were evaluated. Core phylogenomic analysis, targeted gene comparisons, and *in-vitro* antimicrobial susceptibility patterns were assessed to develop a rapid and reliable method for species identification and understand the epidemiologic significance of the isolates. Results revealed previous identifications of clinical isolates to be inaccurate and reference sequences were mislabeled in public databases. Core genomic alignment and SNPs pattern of the complete 16S rRNA sequence of *M. chelonae* isolates clearly separated the turtle type strain ATCC 35752^T from the clinical isolates and human reference strain ATCC 19237, providing evidence of two separate subspecies. Concatenation of the partial *rpoB* (752 bp) and complete *hsp65* (1,626 bp) sequence produced the same sub-clusters as the core phylogeny. The partial *rpoB* and partial *hsp65* sequences reliably identified isolates to species level when respective cut-offs of 98% and 98.4% identity to the *M. chelonae* type strain ATCC 35752^T were employed, but the human reference strain ATCC19237 was clearly the type representative for current clinical strains. Additionally, two isolates were identified as *Mycobacterium saopaulense,* representing the first report of this species in the United States. A new *Mycobacterium* sp. was isolated from two different fish species.

Introduction

The *Mycobacterium chelonae-abscessus* complex (MCAC) is comprised of a group of Gram-positive, acid-fast, nontuberculous bacteria classified within the Family Mycobacteriaceae. *Mycobacterium chelonae*, *Mycobacterium abscessus*, *Mycobacterium immunogenum*, *Mycobacterium salmoniphilum*, *Mycobacterium franklinii*, and the non-validated species, *Mycobacterium saopaulense,* compose this group of closely related organisms (1-4). Members of the MCAC cause disease in a variety of species including fish, reptiles, birds and mammals, including humans (5-7). MCAC are ubiquitous in the environment and have been identified in municipal water sources, soil, and biofilms (8-10). Municipal water supplies are often subject to disinfection, however many isolates form biofilms that protect against commonly used disinfectants (11-13). Many cases of mycobacteriosis have been directly linked to environmental sources (14, 15) and the potential for zoonotic disease by MCAC organisms is a significant concern. The ability of these bacteria to populate a variety of habitats likely facilitates interactions with a diverse host range and contributes to the epidemiology of disease, making these MCAC organisms relevant for study under the One Health perspective (studying diseases at the nexus of human, animal, and the environment) (16).

Animals, including humans, infected by MCAC species present with an array of acute to chronic disease signs and lesions, ranging from localized soft tissue involvement to fulminant systemic infection (7, 17-19). Human susceptibility to non-tuberculous mycobacterial (NTM) infection and the severity of disease is often correlated to and observed in immune compromised individuals (20-22). In particular,

M. chelonae, is a known opportunistic pathogen that is being increasingly reported in humans (23, 24). Susceptibility to mycobacteriosis varies among families of fish, but a similar link has also been made between immune compromise and susceptibility (17, 25, 26). One highly susceptible group is the Family Syngnathidae, which includes seahorses, seadragons, pipehorses, and pipefish (27, 28). Mycobacteriosis in fish is uniquely challenging as infections are typically fatal and pose a risk of zoonotic infection to immune compromised humans contacting diseased fish, contaminated water, and biofilms (29). At present, treatment options are limited in humans and almost non-existent in fish (30, 31).

Members of the MCAC complex are closely related and difficult to differentiate. The two most commonly reported species, *M. chelonae* and *M. abscessus* subsp. *abscessus*, were considered subspecies of the same species prior to 1992. However, new identification strategies have provided compelling evidence that these two mycobacteria are different species that share common phenotypic characteristics, but divergent antimicrobial susceptibilities (32, 33). At this time, biochemical identification methods are antiquated, expensive, time consuming and results can be equivocal. (34, 35). Furthermore, biochemical testing has largely been replaced by molecular techniques. Due to the intricacies of identification, many clinical laboratories only identify isolates to the *M. chelonae-abscessus* complex level (36). This generic classification can pose a serious concern to both human and veterinary patients, as treatment protocols may vary due to differing antibiotic susceptibilities among members of the complex (32, 37, 38). For example, approximately 80% of *M. abscessus* subsp. *abscessus* carry an erythromycin resistance methylase gene [*erm* (41)] that confers

resistance to macrolide antibiotics such as clarithromycin and azithromycin (39, 40). In contrast, *M. chelonae* lacks *erm* (41) and wild type (untreated) isolates are macrolide susceptible.

Other methods used to differentiate species within the MCAC, include phenotypic characteristics and restriction fragment length polymorphism analysis of *hsp65* (*hsp65* PRA) (41). Similar to biochemical identification methods, these approaches are time consuming, labor intensive, often difficult to assess or reproduce, and require large databases with in-house validation due to the lack of commercial systems. In addition, there is a lack of consensus among laboratories regarding percent identity break points used to differentiate closely related species. New technologies, including matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF), have facilitated a huge leap forward for rapid and reliable species identification. However, issues of ambiguity with respect to identification of MCAC members remain in the public databases (42). For example, *M. abscessus* subsp. *abscessus* has the identical spectra to *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (43).

Newer strategies, such as targeted gene sequencing, have several advantages over traditional identification modalities. Most notably, targeted gene sequencing produces unambiguous, easily interpreted data. Advancements in automated sequencing have made this approach more accessible and timely for the identification of closely related species. In general, 16S rRNA gene sequencing has been useful for identifying NTM mycobacterial isolates (44). However, partial 16S rRNA gene sequencing fails to separate *M. chelonae* and *M. abscessus* subsp. *abscessus,* making

it unsuitable for discriminating between MCAC isolates (30, 45, 46). Other genes purported to differentiate closely related species include regions 3 and 5 of the β-subunit of the RNA polymerase gene (*rpoB)*, the Telenti sequence of the 65 kDa heat shock protein gene (*hsp65*), DNA gyrase subunits A (*gyr A)* and B (*gyr B*), translation elongation factor *Tu* (EF-*Tu*), manganese dependent superoxide dismutase (*Mn-SodA*), *Escherichia coli* secretion gene (*SecA*), and the 16S-23S internal transcribed spacer region (ITS) (46-49). At present, diagnostic laboratories employ a combination of techniques. Most notably, the Nocardia/Mycobacteria Research Laboratory (Tyler, TX) combines targeted sequencing of *erm(41)* and *rpoB*, but uncertainty remains in the definitive classification of MCAC species (personal communication, Barbara Brown-Elliott).

Numerous reports describe *M. chelonae* infections in individual hosts, as well as epizootics within the same species (50-53). However, there is little published information regarding the epidemiology of infection among different animal species. The goal of this study was to compare *M. chelonae* isolates from humans, veterinary species, and the environment in an effort to understand strain variability across isolates from different sources as well as investigate the true phylogenetic placement of *M. chelonae* strains by using whole genome sequence analysis. In addition, we aimed to describe an optimal gene target to accurately identify *M. chelonae* in a diagnostic setting when a rapidly growing nontuberculous mycobacteria within the *Mycobacterium chelonae-abscessus* group is suspected. Antimicrobial susceptibility testing was used to investigate the epidemiologic significance of isolates in relation to their phylogenomic position and source. To accomplish this, *M. chelonae* and *M. abscessus* subsp.

abscessus reference strains, three biofilms, and 27 clinical *M. chelonae* isolates from diseased fish, reptiles and mammals, including humans, were subjected to whole genome sequencing (WGS) and annotation with core genome extraction. Isolates were evaluated by core phylogenomic analysis, targeted gene sequence phylogenetic analysis, *hsp65* PRA, and antimicrobial broth microdilution for determination of minimum inhibitory concentrations (MICs) (54). The information gathered provides a standard method for *M. chelonae* identification and proposed break-points for species delineation within the MCAC. The antimicrobial patterns observed give insight into potential treatment options for isolates of importance.

Materials and methods

Sample preparation. Twenty-seven clinical subcultured isolates, previously identified as *M. chelonae*, from fish, reptiles and mammals, including humans, along with three biofilm and three reference strains (*Mycobacterium chelonae* ATCC 35752^T from a turtle, *M. chelonae* ATCC 19237 from a human, and *M. abscessus* subspecies *abscessus* ATCC 19977^T, were harvested from Middlebrook 7H11 agar plates (Table 3.1). Genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA) following the manufacturer's protocol. Approximately 15-28 ng/µL of DNA was submitted from each sample to the Georgia Genomics Facility (The University of Georgia, Athens, GA) for DNA library preparation using Illumina TruSeq adaptors. Paired end (PE) 300-base reads were generated on an Illumina MiSeq PE300 sequencer (Illumina Inc., San Diego, CA).

Sequence preparation and assembly. Sequence read quality for each sample was assessed using FastQC (55). Raw reads were trimmed using Trimmomatic

software (56) run with the following settings: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:20 TRAILING:10 SLIDINGWINDOW:4:20 MINLEN:50. Draft level genomes were assembled from trimmed reads using SPAdes software (version 3.6.2) (57). Assembly metrics were evaluated using the Quality Assessment Tool for Genome Assemblies (QUAST) (58). Automated genome annotation was performed using the RAST (Rapid Annotations using Subsystems Technology) server (59).

Core genome alignment and phylogenomic analysis. A pair-wise genome content distance matrix was produced for the genome assemblies of 27 clinical samples, three biofilm samples, three ATCC reference strains and six sequences obtained from GenBank (*M. chelonae* ATCC 35752^T from a turtle, *M. chelonae* ATCC 19237 from a human, *M. chelonae* CCUG 47445^T from a turtle, *M. abscessus* subspecies *abscessus* ATCC 19977^T from a human, *M. abscessus* subsp. *massiliense* G0 06 from a human, *M. abscessus* subsp. *bolletii* MC1518 from a human, and *M. chelonae 1518* from a human) using Progressive Mauve aligner (60). Extraction of a core genomic region from the 39 whole genomes was performed and the extracted genes concatenated using a custom perl script. Phylogenomic analysis of a 1,183,170 bp core sequence, composed of 1,041 annotated regions, was performed to assess phylogenomic position using RAxML, employing a GTR Gamma rapid bootstrapping and search for best scoring Maximum Likelihood model with 1000 bootstrap replications (61).

Sequence analyses and phylogenetic comparisons. All assembled and annotated genomes were imported into Geneious for in-silico targeted gene evaluation (62). Keyword searches were employed to identify genes of interest whose DNA

sequences were then extracted from the annotated draft and reference genomes. Once found, the targeted sequence was extracted. For gene extraction of partial *rpoB* (752 bp), partial *hsp65* (441 bp), and partial ITS (245-257 bp), published primers were utilized in-silico (9, 46, 63, 64). A multisequence nucleotide alignment at 8 gene targets: *gyrA* (2,118 bp), *gyrB* (1,935-2,013 bp), EF-*Tu* (1,259 bp), Mn *sodA* (624 bp), *rec*A (1,041 bp), ITS (838 bp-854 bp), *hsp65* (1,626 bp), *rpoB* (752 bp), and *erm* (41) (673 bp) was performed and percent identity between sequences achieved using the default settings in MUSCLE program with a maximum of 10 iterations (65). GenBank sequences for *M. abscessus* subsp. *abscessus* ATCC 19977^{T}*, M.chelonae* ATCC 35752^{T}*, M. abscessus* subsp *massiliense* GO 06*, M. franklinii,* and *M. saopaulense* were included for partial *rpoB*, partial *hsp65*, and ITS when available.

The *rpoB* locus was further evaluated using an additional 170 sequences contributed by the Mycobacteria/Nocardia Reference Laboratory. The additional sequences were extracted from human isolates and separated into potential sequevars by evaluation of single nucleotide polymorphisms (SNPs) in the 752 bp sequence. The *M. chelonae* ATCC 35752^{T} reference strain was designated as sequevar 1 and subsequent sequevars were identified by comparing single nucleotide polymorphisms in relation to the reference sequence. These sequences were then translated for evaluation of amino acid discrepancies at loci of nucleotide difference.

RAxML (version 7.2.8) was used to estimate phylogenies and produce phylogenetic comparison matrices (61). Phylogenetic trees were obtained from the DNA sequences by employing a GTR Gamma rapid bootstrapping and search for best scoring Maximum Likelihood model with 1000 bootstrap replications. In addition,

concatenated sequences, partial *hsp65* (441 bp) and *rpoB,* as well as the complete *hsp65* (1,626 bp) and *rpoB* (752 bp) were evaluated as described above and compared to the core genomic phylogeny for evaluation of potential methods of delineation for diagnostic use.

***Erm*(41).** All isolates were evaluated for presence of the *erm*(41) by generating a custom BLAST database for each individual assembly followed by Blastn using the 673 bp *erm*(41) GenBank *M. abscessus* subsp. *abscessus* ATCC 19977^{T} NC 010397 query sequence (39).

***hsp65* and PCR-restriction fragment length polymorphism analysis of *hsp65* (*hsp65* PRA).** Extraction of the partial *hsp65* (441 bp) and the complete *hsp65* (1,626 bp) from the annotated genome assemblies was performed in-silico. Primers Tb11 and Tb12 (63) were used to identify and extract a 441 bp region of interest including flanking sequence. Primer sequences were included in the analysis as minor variation in primer binding areas of sequences did occur. The complete *hsp65* sequence was identified in Geneious by generating a custom BLAST database of each annotated genome assembly and performing a keyword search for the heat shock protein 60 gene family chaperone GroEL with subsequent extraction of the complete 1,626 bp sequence for further evaluation.

In-silico restriction length polymorphism analysis of the partial and complete *hsp65* locus was performed targeting restriction sites for enzymes *Bst*EII and *Hae*III. A virtual gel was used to evaluate all fragments larger than 35 bp and a second to evaluate fragments larger than 60 bp. Using the algorithm of Taylor et al. (66), additional reference *Mycobacterium* spp. (*M. franklinii*, *M. fortuitum*, *M. massiliense, M.*

septicum, M. farcinogenes, and *M. saopaulense)* were selected to more thoroughly evaluate isolates at the partial *hsp65*.

Minimum inhibitory concentrations (MIC) and colony morphology. Antimicrobial susceptibility testing was performed using a Sensititre RAPMYCO panel (Thermofisher Thermo Scientific, Oakwood Village, OH) on isolates harvested from Middlebrook 7H11 plates, following Clinical and Laboratory Standards Institute (CLSI) recommendations(54). Clarithromycin was evaluated at day 3 and day 14 of incubation. Sensititre RAPMYCO uses a standard-ordered broth microdilution panel for susceptibility testing and previously established breakpoints (RGM) (67, 68). In addition to MIC data, colony morphologies were observed and recorded.

GenBank accessions. Accessions used for analysis: NC_010397 *M. abscessus* subsp. *abscesssus* ATCC 19977^{T}, CP010946 *M. chelonae* ATCC 35752^{T}, CP007220 *M. chelonae* CCUG 47445^{T}, NZ_JAOI00000000.1 *M. chelonae* 1518, NZ_HG964481 *M. farcinogenes*, NZ CP011269 *Mycobacterium fortuitum,* KM392060 *M. franklinii*, NC_018150 *M. abscessus* subsp. *massiliense* GO 06, CP009613.1 *M. abscessus* subsp. *bolletii,* NZ_HG322951 *Mycobacterium septicum*, and KM973026 *M. saopaulense*. A total of 33 whole genome sequences produced from this study have been deposited in GenBank under Bioproject: PRJNA347845, Biosamples: SAMN05897971- SAMN05898003**.**

Results

Core genomic analysis. The core genomic analysis (Figure 3.1 A) clearly discriminated between species and provided the accurate identification of species previously misidentified as *M. chelonae.* The core genomes of the 27 clinical isolates,

three biofilms, three ATCC reference strains, and six genome sequences from GenBank were 1,183,170 bp in length and had 1,041 coding sequences (CDS) separated by intergenic sequence. Of the CDS, 865 were confirmed by RAST as a gene, 147 were hypothetical, and the remaining 28 were probable CDS. Within the 865 CDS, 16S rRNA, *rpoB, hsp65* partial (441 bp), *hsp65* whole (1,626), *gyrA*, EF-*Tu*, Mn-*sodA*, and *rec*A were present. The sequenced reference strains, *M. chelonae* ATCC 35752^T, and *M. abscessus* ATCC 19977^T, were 100% and 99.99% identical to the uploaded GenBank strains CP010946 *M. chelonae* ATCC 35752, *M. chelonae* CCUG 47445^T and NC_010397 *M. abscessus* subsp. *abscessus* ATCC 19977^T, respectively. As a result, from this point forward, *M. chelonae* ATCC 35752^T and *M. abscessus* ATCC 19977^T will refer to both the GenBank sequence and sequenced isolates. The previously misidentified, *M. chelonae* 1518, had 100% identity to *M. abscessus* subsp. *abscessus* ATCC 19977^T and *M. abscessus* subsp. *bolletii* MC 1518. *M. abscessus* subsp. *massiliense* had 97.9% identity to *M. abscessus* ATCC 19977^T, but only 86.7% identity to CP010946 *M. chelonae* ATCC 35752^T.

Six clinical isolates did not group with *M. chelonae* ATCC 35752^T using the core genomic analysis and were determined as other species. These isolates included: seakrait, cow, turtle, H9, seahorse1, and pipefish (Figure 3.1 A). The seakrait isolate and five reference strains (sequenced ATCC 19977^T, GenBank NC_010397 *M. abscessus* subsp. *abscessus* ATCC 19977^T, GenBank CP009613.1 *M. abscessus* subsp. *bolletii* MC 1518, GenBank NC_018150 *M. abscessus* subsp. *massiliense* GO 06, and GenBank NZ_JAOI00000000.1 *M. chelonae* 1518) were 99.5-99.9% identical. The cow and turtle isolates clustered together with 100% identity to each other and 87.8

% identity to *M. abscessus* subsp. *abscessus* ATCC 19977^T, but with only 88.1% identity to *M. chelonae* ATCC 35752^T. Human isolate H9 (*M. franklinii*) was 88.5% identical to *M. abscessus* subsp. *abscessus* ATCC 19977^T and 87.8% identical to *M. chelonae* ATCC 35752^T. Two clinical isolates, seahorse1 and pipefish, were 99.4% identical to each other. The closest reference strain, *M. chelonae* ATCC 35752^T, shared only 74.6% identity to the seahorse1 and pipefish isolate. Blastn searches of the NCBI databse placed these two isolates closest to the whole genomes of *Mycobacterium fortuitum* strain CT6 and *Mycobacterium* sp. VKM AC-181 Ac-1817D, with only 88% identity, indicating the seahorse1 and pipefish isolates are a previously undescribed species.

Twenty-four of 32 isolates clustered with the sequenced *M. chelonae* ATCC 35752^T with 96.5-96.6% identity (Figure 3.1 B). A mixture of human, fish, reptile and biofilm isolates clustered in this large group, all with greater than 98.0% identity to each other but not *M. chelonae* ATCC 35752^T. The *M. chelonae* cluster formed five sub-branches, designated Groups A-E. The turtle *M. chelonae* ATCC 35752^T reference strain comprised Group A. Group B included a reptile (python) and two human isolates (H8, H17) that were 98.8% identical. These three isolates were epidemiologically divergent as they were identified in Ohio, California, and North Carolina. (Table 3.1). Group C was comprised entirely of human isolates (H7, H10, H11, H15, H18, H19, H20) all with greater than 98.3% identity to each other. Similar to Group B none of the isolates were identified in the same geographic location. Group D contained a biofilm (biofilm1) and two fish isolates (seadragon1, seadragon2) that were 99.99%-100% identical. Group E included the human *M. chelonae* ATCC 19237 sequenced reference

strain, two biofilms (biofilm2, biofilm3), three human (H12, H13, H14), and six fish isolates (cichlid, trumpetfish, seahorse2, seahorse3, seahorse4, seahrose5) that clustered with greater than 99.2% identity. Several members from Group E were identified within the same geographic location but not the same environment.

Targeted gene analysis. Several gene targets were evaluated by multisequence alignment with the production of an identity matrix. Alignments of 16S rRNA, *gyrA*, *gyrB*, EF-*Tu, recA*, and Mn-*sodA* produced erroneous clustering or separation of the isolates and/or reference strains evidenced by inaccurate phylogenetic placement of the human isolates (EF-*Tu*, Mn-*sodA*, *gyrA*, *gyrB*) or lack of species separation (16S rRNA, *recA*) when compared to the core genomic results. Therefore, evaluation based on these alignments were not pursued further. However, the sequences for the clinical isolates and ATCC 19237 had at least three single nucleotide polymorphisms in the complete 16S rRNA sequence that distinctly separated them from the type strain ATCC 35752^T (Supplementary Figure 3.1 and Supplementary Table 3.1)

ITS. The ITS locus was extracted as a 245-257 bp sequence (Figure 3.2). Multi-sequence alignment of the clinical isolates and reference strains revealed adequate grouping into species-specific branches. Isolates with greater than 97.7% (833/854 bp) identity to *M. chelonae* ATCC 35752^T were considered *M. chelonae*. All isolates in this grouping had greater than 99.5% (846/850 bp) identity to each other. The seakrait was 100% (853/853 bp) identical to *M. abscessus* subsp. *abscessus* ATCC 19977^T. The cow and turtle isolates were 100% identical to each other and 99.1 % identical to human isolate H9, although they were placed on separate branches. The pipefish and seahorse1 isolates were highly divergent from the *M. abscessus* subsp. *abscessus* and

M. chelonae groups, with only 46.1-49.0% identity among them. Their extraction product was only 245 bp in comparison to all other isolates that had products of 256-257 bp. The two outliers branched closely together and were 100% identical at the ITS locus.

hsp65. Targeted extraction of the 441bp partial *hsp65* gene sequence successfully reproduced the *M. chelonae* ATCC 35752^T cluster generated by core genome analysis. Isolates with greater than 98.4% identity (434-441/441 bp) to the turtle reference strain, *M. chelonae* ATCC 35752^T, were considered *M. chelonae* (Figure 3.3). However, there was little discrimination within two large sub-clusters, each containing strains 99.8-100% identical to each other. One sub-group contained exclusively human isolates (H7, H10, H11, H15, H18, H19, H20) and the other a mixture of reference strain *M. chelonae* ATCC 19237, human (H8, H12, H13, H14), fish (cichlid, trumpetfish, seadragon1, seadragon2, seahorse2, seahorse3, seahorse4, seahorse5) and biofilm (biofilm1, bioflm2, biofilm3) isolates. The partial *hsp65* sequence of human isolate H9 was 98.4% identical (434/441 bp) to KM392060 *M. franklinii*. The turtle and cow isolates also branched separately from the *M. chelonae* cluster and were 99.5% identical (439/441bp) to KM973026 *M. saopaulense*. The seakrait isolate, originally identified as *M. chelonae*, had 100% identity to *M. abscessus* subsp. *abscessus* ATCC 19977^T. The pipefish and seahorse1 isolates had only 90.2% identity (398/441 bp) to *M. chelonae* ATCC 35752^T.

The complete, 1,626 bp, *hsp65* multisequence alignment was more discriminating than the partial sequence and produced some clusters mirroring the core genome phylogeny (Figure 3.4). All isolates with greater than 95.3% identity (1,549-1,626 bp) to *M. chelonae* ATCC 35752^T were considered *M. chelonae*. As with the core

genome and partial *hsp*65 phylogenies, the same group of human isolates branched together (H7, H10, H11, H15, H18, H19, H20) and shared 99.9-100% (1,625-1,626/1,626 bp) identity. The second cluster from the partial *hsp*65 gene analysis was split into two groups when the complete *hsp65* sequence was evaluated. One biofilm and two seadragon isolates (biofilm1, seadragon1, and seadragon2) shared 100% identity (1,626/1,626 bp), while another mixed group of fish isolates (cichlid, trumpetfish, seahorse2, seahorse3, seahorse4, and seahorse5), biofilm isolates (biofilm 2 and biofilm3), and the sequenced human ATCC 19237 reference strain were 99.9-100% identical. Human isolate, H9, diverged from the *M. chelonae* grouping, with only 95.3% identity (1,549/1,626 bp). The turtle and cow isolates branched together and were only 93.3% identical (1,517/1,626 bp) to *M. chelonae* ATCC 35752^T. The pipefish and seahorse1 isolates had the lowest identity to the reference strain, with only 90.9% (1,478/1,626 bp) shared identity.

rpoB. Phylogenetic analysis of *rpoB* (752 bp) produced similar phylogenetic positioning as the core genome (Figure 3.5). Isolates with identities greater than 97.9% (736/752 bp) to *M. chelonae* ATCC 35752^T were considered as *M. chelonae*. The main grouping consisted of multiple fish, biofilm and human isolates, all of which had 100% identity to each other. One biofilm and two seadragon (biofilm1, seadragon1, and seadragon2) isolates branched separately and were 100% identical, but were closely related to the first group. The remaining human isolates split into three sub-clusters, each with greater than 99.0% (745/752 bp) identity to each other. Similar to *hsp*65*,* human isolate H9 branched separately, as did the turtle and cow isolates and the pipefish and seahorse1 isolates.

Among 202 *rpoB* sequences evaluated, single nucleotide polymorphisms (SNPs) ranged from zero in *M. chelonae* ATCC 35752^T, up to 5 in some clinical isolates. Seventeen unique sequevars were recognized based on SNPs consistently identified at positions 24 (A-to-G), 36 (C-to-G), 90 (C-to-T), 99 (C-to-T), 100 (C-to-T), 102 (C-to-G), 123 (C-to-T), 126 (C-to-A), 204 (G-to-A), 237 (T-to-C), 363 (T-to-C), 384 (C-to-T), 385 (C-to-T), 430 (G-to-A), 444 (G-to-A), 480 (C-to-G), 559 (C-to-T), 654 (C-to-A), and 723 (G-to-T). However, sequence translations revealed only one amino acid change in a single human isolate from the sequence database, where a G-to-A substitution at codon 430 resulted in a glutamic acid substitution for lysine. Multisequence alignment of the additional *rpoB* sequences showed greater than 99.2% identity to *M. chelonae* ATCC 35752^T.

hsp65* whole sequence and *rpoB. Concatenation of partial *hsp65* (441 bp) and *rpoB* (752 bp) produced a 1,193 bp sequence (Figure 3.6). The phylogenetic positioning of several isolates was not consistent with that of the core genome and no further analysis was performed. A concatenation of the complete *hsp65* (1,626 bp) and partial *rpoB* (752 bp) created a 2,378 bp sequence. Clustering of clinical isolates was almost identical to the core genome phylogeny. However, unlike the core genome phylogeny, the *M. chelonae* ATCC 35752^T reference strain branched at a different location. All isolates with greater than 96.1% (2,285/2,387 bp) identity to *M. chelonae* ATCC 35752^T reference strains were considered *M. chelonae.*

***Erm* (41).** The erm (41) gene was only found in GenBank reference strains *M. abscessus* subsp. *abscessus* ATCC 19977^T, NZ_JAOI00000000.1 *M. chelonae* 1518,

and the seakrait isolate. All other clinical isolates and reference strains lacked this gene.

Restriction fragment length polymorphism analysis (*hsp65* PRA). The partial and complete *hsp65* sequences were also evaluated for diagnostic relevance in comparison to the core genome phylogeny (Figure 3.7 and 3.8). Results from both are representative of the clusters observed in the phylogenetic analysis for each sequence. A two-step restriction analysis was performed using *Bst*EII and *Hae*III on the partial *hsp65* sequence using fragments over 60 bp. Restriction with *Bst*EII produced three groups of clinical isolates, each with 2-4 fragments: 310/131 bp, 231/210 bp, and 231/116/84 bp. If these groupings are followed, isolate H9 would be considered *M. chelonae*. Restriction using HaeIII provided a more accurate representation of isolate phylogenetic positions. The 310/131 digestion is further broken down into Groups 1 (H9 and Genbank KM392060 *M. franklinii*), Group 2 (H8, H12, H13, H14, H17, python1, cichlid, seahorse2, seahorse3, seahorse4, seahorse5, seadragon1, seadragon2, biofilm1, biofilm2, biofilm3, trumpetfish, sequenced ATCC 35752^T, Genbank CP010946 ATCC 35752^T, ATCC 19237) and Group 3 (H7, H10, H11, H15, H18, H19, H20). In this analysis, the Group 3 isolates no longer clustered with *M. chelonae* ATCC 35752^T (Figure 3.7).

PRA of complete (1,626 bp) *hsp65* divided the clinical isolates into 4 groups using the *BstE*II restriction site (Figure 3.8). This restriction identifies the cow and turtle isolates as *M. abscessus* subsp. *abscessus.* However, further restriction with *Hae*III, places the cow and turtle isolates into a group separate from the *M. abscessus* subsp. *abscessus* reference strains. Restriction with *Hae*III separates the *M. chelonae* isolates

into 4 groups, where Groups 1, 3 and 4 are separated from the reference strains in Group 2. The unidentified pipefish and seahorse1 isolates are also separated into different groups.

MIC susceptibility and colony morphology. Twenty-six *M. chelonae* clinical isolates and three reference strains were evaluated using the Sensititre RAPMYCO panel (Table 3.2). Observations of colony morphologies revealed subtle phenotypic differences between isolates. The majority (22/31), had nonpigmented, smooth, glossy, raised colonies. The cow and turtle isolates also produced nonpigmented, smooth, raised colonies, but turned the 7H11 media brown after 7 days of incubation. The pipefish and seahorse1 outliers grew as nonpigmented, granular, glossy, raised, colonies, different from all others. Isolates H12, H13, H17, seahorse5 and python1 produced nonpigmented, rough, crusting, raised colonies.

MICs of the NTM isolates were classified using CLSI recommended categories: susceptible, intermediate, or resistant. While a high degree of antimicrobial resistance was generally observed among the isolates, the broadest resistance was found in the aquatic biofilm and fish isolates.

Of the 29 isolates evaluated, 97% (28/29) were susceptible to the macrolide, clarithromycin (Table 3.2). *M. abscessus* subsp. *abscessus* ATCC 19977^T was the only isolate with resistance to clarithromycin at the 14 day inducible reading point. For the *M. chelonae* isolates, 69% (20/29 isolates) and 59% (17/29 isolates) were susceptible to the aminoglycosides tobramycin and amikacin, respectively. Only 50% of the isolates were susceptible to linezolid, the majority of which were of human origin (n=9). Susceptibilities of *M. chelonae* isolates were low for cefoxitin,

trimethoprim/sulfamethoxide, imipenem, moxifloxicin, and ciprofloxacin at 3%, 10%, 3%, 14% and 21% (1/29, 3/29, 1/29, 4/29, 6/29), respectively. The turtle ATCC 35752^T and human ATCC 19237 had a slightly different antimicrobial susceptibility profile from each other. The human strain of *M. chelonae* ATCC 19237 had a pattern more similar to the fish (cichlid, seahorse2, seahorse3, seahorse4, seahorse5, seadragon1), human (H10, H11, H12, H14, H17, H19, H20), and biofilm (biofilm1, biofilm2, biofilm3) clinical isolates than the turtle reference strain ATCC 35752^T.

Discussion

Mycobacterium chelonae is a ubiquitous environmental bacterium capable of causing disease in both human and veterinary species. An uncommon pathogen in humans, disease is usually associated with immunocompromised patients. However, the incidence of infection, including reports in healthy adult humans, is increasing (24, 69-71). In the aquatic realm, *M. chelonae* is commonly diagnosed and causes significant losses in certain groups of fish, such as the syngnathids (25, 27). The increasing number of reports and the zoonotic potential of NTM, such as *M. chelonae*, warrants further interdisciplinary research into the dynamics of infection to optimize methods of prevention and treatment.

Isolates of *M. chelonae* are commonly misidentified or are identified only to genus level. Complicating identification, novel species and sub-species within the MCAC continue to be described (1, 2, 10, 72). Whole genome sequencing and core genome evaluation of mycobacterial species from animal and environmental sources were employed to investigate whether *M. chelonae* isolates from fish, reptiles, and aquatic biofilms are distinguishable from human clinical isolates and to determine if

different epidemiologically related subtypes exhibit host preference. Since current identification methods are focused on gene targeted approaches and break points for species delineation have not been identified, a second objective of this research was to develop an inexpensive and reliable method for identification of *M. chelonae*. The methods proposed are applicable to human and veterinary diagnostic laboratories and could potentially assist in the formulation of more effective treatment options.

Core genome evaluation of six GenBank whole genome sequences, three ATCC reference strains, and 30 clinical isolates from an array of species and the aquatic environment provided a clear phylogenomic discrimination of relatedness. In addition to accurate species identification, it was readily apparent that several isolates, including GenBank sequences were previously misidentified as *M. chelonae*. The power of this core genomic analysis was high, due to the use of approximately one quarter of the whole genome for analysis instead of individual genes. The *M. chelonae* ATCC 35752^{T} genome is 4,898,027 bp and is comprised of 4,489 protein-coding genes (73). In the core alignment, 24.1% of the genome and 23.1% of the conserved coding regions of *M. chelonae* were used for analysis. Furthermore, many of the commonly used housekeeping genes that are individually employed for species identification were also included, such as EF-*Tu*, *SecA*, *gyrA,* Mn-*SodA*, 16S rRNA, *rpoB,* and *hsp65* (partial and complete).

Isolates that were accurately identified in the core genome analysis, but not in GenBank included a human antibiotic resistant strain originally identified as *M. chelonae* 1518. Core genome alignment and the presence of *erm* (41) clearly delineate this isolate as *M. abscessus* subsp. *bolletii* and not *M. chelonae* as listed in GenBank. Two

syngnathid fish isolates, pipefish and seahorse1, were highly divergent from the *M. chelonae* and *M. abscessus* subsp. *abscessus* groupings using core genome alignment, targeted gene sequencing and PRA analysis (Figures 3.1 A and Figure 3.7). Blastn searches of the NCBI database did not match these two isolates to a documented species. The two isolates were most closely related to *Mycobacterium fortuitum* strain CT6, 94.8% identity at *hsp*65, and *Mycobacterium farcinogenes* NZ_HG964481, 96.9% identity at *rpoB*, indicating a previously undescribed *Mycobacterium* sp. outside the MCAC.

Interestingly, in the core genome alignment, the turtle type strain *M. chelonae* ATCC 35752^T branched separately from the clinical isolates with which they shared only 96.5-96.6% identity. Whereas the human reference strain *M. chelonae* ATCC 19237, was 98.4-99.6% identical to environmental, animal, and human isolates. In addition, *M. chelonae* ATCC 35752^T had a slightly different antimicrobial sensitivity profile, with susceptibility to ciprofloxacin, moxifloxicin, linezolid, and amikacin (Table 3.2). Phylogenetic and antimicrobial differences between the turtle reference strain, *M. chelonae* ATCC 35752^T, clinical isolates, and *M. chelonae* ATCC 19237 suggest the presence of two subspecies within the genus *M. chelonae*. Furthermore, the antimicrobial susceptibility profile as well as the genetic sequence of *M. chelonae* ATCC 19237 are representative of the current clinical strains observed in patients with disease, making this isolate more relevant as a type strain when trying to differentiate clinical species in a diagnostic setting.

Multisequence analysis has been proven as a useful and effective diagnostic tool for bacterial identification (74). Although whole genome sequencing provided the

greatest discrimination among isolates, it is not yet practical in diagnostic laboratory settings. Comparison of several commonly targeted genes did provide insight as to the most useful gene for diagnostic purposes. Most notably, concatenation of complete *hsp65* and partial *rpoB* sequences produced results with phylogenetic positioning and discrimination between isolates that was highly similar to the core genome. When compared to the turtle reference strain, *M. chelonae* ATCC 35752^T, isolates with identities greater than 98.4% were considered *M. chelonae*. While this technique has significant promise for use in species identification and epidemiologic investigation, there is no published data regarding the proposed breakpoint and a larger sample pool is needed to substantiate the method and this finding. Moreover, using the concatenated complete *hsp65* and partial *rpoB* sequences both the turtle type strain *M. chelonae* ATCC 35752^T, and the human reference strain *M. chelonae* ATCC 19237 had greater than 99.1% identity to the main grouping of *M. chelonae* isolates that included the biofilms, reptile, fish, and human isolates making differentiation between the reference strains difficult.

As previously reported, 16S rRNA analysis did not completely distinguish between species of the MCAC (30) (Supplementary Figure 3.1 and Supplementary Table 3.1). However, the SNP pattern of clinical and environmental isolates designated as *M. chelonae* was the same as ATCC 19237 and not the currently used turtle type strain ATCC 35752^T, which further supports the theory of two subspecies within the *M. chelonae* genus. Other commonly used genes for bacterial identification (*gyrA*, *gyrB*, EF-*Tu*, *RecA*, and Mn-*Sod*) either did not reliable identify species or produced inaccurate phylogenetic positioning. However, some individual genes did prove to be

diagnostically useful. Specifically, the ITS, partial and complete *hsp65,* and *rpoB* loci were the most discriminating of the individual housekeeping genes tested and grouped isolates similarly to the core genomic phylogeny (Figures 2-5). When employing the partial *hsp65*, complete *hsp*65, and *rpoB* sequences the cow and turtle isolates were clearly identified as *M. saopaulense*. *rpoB* and partial *hsp65* analysis also unmistakably delineated H9 as *M. franklinii*. However, the ITS sequence did not have the same discriminatory power as the complete *hsp65* or partial *rpoB* alignments. Contradictory to the core genome analysis, partial and complete *hsp65*, and the *rpoB* phylogenies, the ITS analysis resulted in high sequence homology (99.1% identity) of the cow and turtle isolates to H9 (*M. franklinii*), which in a diagnostic setting would not appropriately split these species apart.

The complete *hsp65* sequence placed H12, H13, H14, and *M. chelonae* ATCC 35752^{T} in different positions within the phylogenetic tree, when compared to the core genome phylogeny. Nevertheless, they were still over 99.8% identical (1,623/1,626 bp) to the main group of fish and biofilm isolates, placing them within the *M. chelonae* grouping. These positional discrepancies are likely a factor of the small sequence length evaluated when using the complete *hsp65* gene as compared to 1,183,170 bp considered in the core alignment.

Regardless of the positional differences of isolates across the phylogenetic comparisons of *hsp65* (complete and partial)*, rpoB* and the core genome, these three methods can be used for identification of *M. chelonae* and its close relatives by employment of specified breakpoints. These breakpoints would be akin to taxonomic thresholds applied to other bacterial genera that use the16S rRNA locus for

identification and a 98.7% identity as a basic cut-off level (75). For MCAC isolates, *hsp65* or *rpoB* could be used as the genetic sequence for comparison due to the lack of diversity in 16S rRNA. Breakpoints for the proposed genetic loci would include identities greater than 98.4% for partial (441 bp), 95.4% for complete *hsp65* and 97.9% for *rpoB* sequences when compared to the turtle reference strain *M. chelonae* ATCC 35752.

Examination of a larger 202 sequence dataset provided by the Mycobacteria/Nocardia Laboratory confirmed the *rpoB* breakpoint of 97.9% results in the separation of *M. chelonae* from other closely related species. These results are in agreement with two previous reports by Adekambi et al. (76, 77). Although a proposed breakpoint was found for *hsp65*, ideally additional partial and complete *hsp65* sequences are needed to confirm the validity of these breakpoints. Further evaluation of SNPs from the 202 *rpoB* sequences separated isolates into sequevars. Translation of the sequences confirmed that gene function was likely not affected by these point mutations, as amino acid sequences were unchanged in all but one sequence. The utility of identifying specific *rpoB* sequevars among isolates may be limited to small scale epidemiologic tracking of outbreaks, but no such connection could be made in the present data set.

Using the partial *hsp65* PRA algorithm proposed by Telenti et al. (63) and revised by Taylor et al. (66), restriction analysis of partial and complete *hsp65* sequences did not accurately classify all species in the data set. This could be a function of “in-silico” analysis, which can identify single base pair differences in fragment sizes, versus up to 10 bp associated with human interpretation of gel fragments. Fragments produced from

the sequences evaluated were 9-15 bp different than previously reported algorithms for identifying *Mycobacterium* spp. For example, the PRA pattern for *M. chelonae* is 325/140 bp for *Bst*EII and 210 bp for *Hae*III, compared to the "in-silico" restriction pattern of 310/131 bp for *Bst*EII and 197/60 bp for *Hae*III (66). Fragments under 60 bp were not assessed using traditional methods, but if differences between agarose gel methods and "in-silico" restriction analysis of up to 15 bp are present, there may have been inadvertent exclusion or inclusion of fragments in previous analyses.

PRA analysis of the partial *hsp65* gene classified multiple human isolates as species different from the *M. chelonae* ATCC 35752^T and *M. chelonae* ATCC 19237 reference strains (Figure 3.7). The same protocol used with the complete *hsp65* sequence produced four distinct subgroups, all classified as *M. chelonae* based on core genome phylogeny (Figure 3.8). PRA analysis was inaccurate in species identification and should no longer be used for identification of bacteria in the MCAC.

The clustering of clinical isolates using the core genome phylogeny, partial and complete *hsp65* and partial *rpoB* sequences provided some insight into their epidemiologic relatedness (Figures 3.1,3.3, 3.4, 3.5). The most interesting epidemiologic finding observed was the unexpected clustering of the type strain ATCC 35752^T away from the majority of clinical and environmental isolates. The type strain ATCC 35752^T was separated out as its own strain in the core genome, but was also significantly separated from the majority of clinical isolates when using partial and complete *hsp65* and partial *rpoB* sequences for comparison. Whereas, the human reference strain ATCC 19237 was reliably clustered with the bulk of the clinical and environmental isolates. These results provide further evidence that the current type

strain is not truly representative of the current clinical isolates regardless of isolate origin. Furthermore, these findings support the existence of two subspecies within the genus *M. chelonae*.

Isolates from diverse aquatic and terrestrial hosts, as well as the environment, all group as *M. chelonae*, suggesting little or no host preference. Nevertheless, certain human isolates tended to group together, while others repeatedly grouped with the aquatic animal or biofilm isolates. Although no human isolates had less than 98.1 % identity to other members of the *M. chelonae* group, the consistent clustering of isolates H7, H10, H11, H15, H18-H20, suggests an epidemiologic link. No known geographic or environmental associations existed among the isolates, but all were derived from soft tissue sources (Table 3.1). Additional patient epidemiologic data and investigation of expressed virulence factors would be necessary to identify a connection between the cases. In contrast, when evaluating the core genome, human isolates H12-H14 grouped with the aquatic animal, biofilm, and human *M. chelonae* ATCC 19237 isolates. While a direct relationship could not be determined, MCAC infections in humans have been linked to environmental biofilms (78-80) and it is reasonable to speculate that these three human isolates originated from an aquatic source.

Numerous reports describe MCAC infections in fish, which is not unexpected, considering the ubiquitous presence of NTM in aquatic systems. Seven of the 9 syngnathid fish isolates evaluated were *M. chelonae*, the most common mycobacterial species reported to affect syngnathid fish (81). In addition to *M. chelonae*, syngnathids can also be infected by other non-tuberculous mycobacteria, such as the recently described *Mycobacterium hippocampi* (72) and the newly identified pipefish and

seahorse1 isolates in this data set. Although a correlation between biofilm associated *M. chelonae* organisms and fish disease have yet to be demonstrated, other NTM have been linked with disease in fish and provide evidence that environmental contamination is a significant source of infection. One such study used rep-PCR banding patterns to identify identical *M. marinum* strains in diseased pompano *Trachinotus carolinus* and their associated tank biofilms (15).

Significant antimicrobial resistance has been documented in MCAC isolates (82). The *erm* (41) locus present in strains of *M. abscessus* subsp. *abscessus*, including the *M. abscessus* subsp. *abscessus* ATCC 19977^T reference strain and *M. abscessus* subsp. *bolleti* MC 1518^T, but not *M. chelonae*, is known to confer inducible macrolide resistance (39, 83). The presence of *erm* (41) in two isolates originally identified as *M. chelonae* (NZ_JAOI00000000.1 *M. chelonae* 1518 and the seakrait isolate), support their identification as *M. abscessus* subsp. *abscessus* by complete genome sequencing. In a clinical setting, sensitivity to macrolides such as clarithromycin could be used as an ancillary diagnostic assay to help identify closely related species within the MCAC.

Characterization of colony morphology and other phenotypic traits can be an adjunct to conventional and molecular based diagnostic methods (84, 85), but as evidenced here, rarely provides sufficient evidence for definitive identification. The majority of clinical strains evaluated in this study produced similar raised, nonpigmented colonies that varied from smooth to dry and flaky, which are virtually impossible to distinguish from each other without side by side culture. Exceptions were the unidentified pipefish and seahorse1 isolates, which produced granular rough colonies, and *M. saopaulense*, which turned the agar media brown after several days of

incubation (2). This morphologic variance substantiated the identification of the turtle and cow isolates as *M. saopaulense* and not *M. chelonae*, as they had been originally been reported. Both isolates turned media brown after 7 days, but in a diagnostic setting rapidly growing mycobacteria may not be allowed to incubate this long before identifications are attempted and this unique feature may be missed.

Similar to previous reports, the evaluated clinical isolates had a relatively resistant antimicrobial pattern (37, 67, 86). Isolates cultivated from biofilms had the broadest antimicrobial resistance, which could be due to their exposure to other environmental bacteria, and the potent antimicrobials produced by other members of the biofilm community causing specific microbial resistance genes to be expressed. In addition, strains of *M. chelonae* can produce extracellular polysaccharide matrix proteins to form biofilms, which can physically protect a bacterial colony from antimicrobial penetration (87). In the present study, biofilm strains were not evaluated for production of extracellular matrix proteins but this could account for their more resistant pattern. Regardless of isolate origin, 100% of *M. chelonae* strains were susceptible to clarithromycin. Although MICs for all antimicrobials were produced *in-vitro, in-vivo* testing is warranted as many of the evaluated drugs in this study may have differential efficacy *in-vivo*. The information provided here has provided a starting point to investigate effective treatments for *M. chelonae* infections in a diverse array of species.

M. chelonae is an opportunistic environmental pathogen with zoonotic potential. Mycobacteriosis is of concern in a diverse number of species, including fish, reptiles, and humans. Accurate species identification is necessary to understand their

epidemiological significance and to formulate appropriate treatment regimens. The current study used whole genome sequencing to assess the phylogenomic position of 27 MCAC isolates cultured from fish, reptiles, mammals, including human, three biofilms and three ATCC reference strains in relation to six GenBank reference sequences. Core genome and multilocus sequence analysis was used to identify the most reliable method to identify closely related isolates of *M. chelonae.* The core genome was the most discriminatory and ideal method to identify species, but concatenation of the complete *hsp65* and *rpoB* genes reliably split the isolates into subclusters similar to the core and thus could be used for isolate identification purposes. Single locus analysis revealed *rpoB,* partial *hsp65* and complete *hsp65* most reliably identified isolates to species level using one gene. In addition, breakpoints for partial *rpoB,* partial *hsp65* and complete *hsp65* of 98%, 98.4% and 95.4%, respectively, are proposed.

This study has demonstrated by core and 16S rRNA sequences that the turtle strain ATCC 35752 is a different taxonomic group than the human strain ATCC 19237 and that *M. chelonae* is not a homogeneous species. Furthermore, the current type strain ATCC 35752 ^{T}, should be used in conjunction with ATCC 19237 for all studies as they appear to be representatives of two *M. chelonae* subspecies. Within this study we also presented the first report of *M. saopaulense* in the United States. *M. chelonae* isolates from different geographic locations and host origin had high sequence homology, which suggests a common epidemiologic link, but none were identified. Presence of the *erm* (41) locus in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* MC 1518 proved helpful in differentiating isolates from *M. chelonae. Hsp65* PRA did not reliably identify species and this method is not recommended for

diagnostic purposes. Colony morphology and phenotypic characteristics were of limited use, but were helpful adjuncts to the identification of *M. saopaulense*. *In-vitro* MIC values were determined to aid in the selection of treatment regimens for MCAC infections. In particular, findings indicate clarithromycin could be an option for further study as an effective treatment against *M. chelonae* infections.

In summary, this study represents the largest whole genome evaluation of *M. chelonae* isolates to date, with consideration of environmental, non-mammalian, and mammalian isolates. The comparison of core genomes demonstrated enough variability within the species to warrant a division into subspecies. The data presented here will enable both veterinary and human diagnostic laboratories to accurately identify species within the MCAC, so that medical professionals can ultimately formulate appropriate treatment regimens and provide the best care for their patients.

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Table 3.1 *Mycobacterium chelonae* isolates sequenced in this study with original identification and host information.

Isolate	Host species	Tissue origin [a]	Geographic location [b]	Original ID Method [c]	Original ID [c]	WGS ID [c,d]
ATCC 19977^T	*Homo sapiens*	soft tissue (knee)	ATCC	Phenotyping/Hybridization	*M. abscessus*	*M. abscessus*
ATCC 35752^T	*Chelona corticata*	lung	ATCC	Phenotyping	*M. chelonae*	*M. chelonae*
ATCC 19237	*Homo sapiens*	gastric lavage	ATCC	Phenotyping/Hybridization	*M. chelonae*	*M. chelonae*
Seakrait	*Laticauda colubrina*	NA	Texas	Phenotyping/*hsp65*	*M. chelonae*	*M. abscessus*
Cichlid	*Cichlidae*	Spleen	Georgia	16S rRNA	*M. chelonae*	*M. chelonae*
Pipefish	*Syngnathoides biaculeatus*	ovary	South Carolina	*hsp65*	*Mycobacterium spp.*	New species
Seahorse1	*Hippocampus erectus*	tail	Georgia	*hsp65*	*Mycobacterium spp.*	New species
Seahorse2	*Hippocampus erectus*	skeletal muscle	Georgia	*hsp65*	*M. chelonae*	*M. chelonae*
Seahorse3	*Hippocampus whitei*	tail	Georgia	*hsp65*	*M. chelonae*	*M. chelonae*
Seahorse4	*Hippocampus erectus*	ovary	Georgia	*hsp65*	*M. chelonae*	*M. chelonae*
Seahorse5	*Hippocampus reidi*	ovary	Georgia	*hsp65*	*Mycobacterium spp.*	*M. chelonae*
Seadragon1	*Phyllopteryx taeniolatus*	soft tissue	Georgia	16S rRNA	*M. chelonae*	*M. chelonae*
Seadragon2	*Phycodurus eques*	liver/mesentery	Georgia	16S rRNA	*M. chelonae*	*M. chelonae*
Trumpetfish	*Aulostomus maculatus*	soft tissue	South Carolina	Phenotyping/*hsp65*	*M. chelonae*	*M. chelonae*
Turtle	*Platysternon megacephalum*	NA	Maryland	Phenotyping/*hsp65*	*M. chelonae*	*M. saopaulense*
Python1	*Morelia boeleni*	NA	Ohio	Phenotyping/*hsp65*	*M. chelonae*	*M. chelonae*
Biofilm1	Biofilm	aquarium system	Georgia	*hsp65*	*M. chelonae*	*M. chelonae*
Biofilm2	Biofilm	aquarium system	Georgia	*hsp65*	*M. chelonae*	*M. chelonae*
Biofilm3	Biofilm	aquarium system	Georgia	*hsp65*	*M. chelonae*	*M. chelonae*
Cow	*Bos taurus*	NA	Puerto Rico	Phenotyping/*hsp65*	*M. chelonae*	*M. saopaulense*
H7	*Homo sapiens*	sputum	Texas	*hsp65* PRA	*M.chelonae*	*M.chelonae*
H8	*Homo sapiens*	soft tissue (nasal)	North Carolina	*hsp65* PRA	*M. chelonae*	*M. chelonae*
H9	*Homo sapiens*	soft tissue (calf)	Massachussetts	*hsp65* PRA	*M. franklinii*	*M. franklinii*
H10	*Homo sapiens*	soft tissue (foot)	Minnesota	*hsp65* PRA	*M.chelonae*	*M.chelonae*
H11	*Homo sapiens*	sputum	Texas	*rpoB*	*M.chelonae*	*M.chelonae*
H12	*Homo sapiens*	soft tissue (axilla)	Kansas	*rpoB*	*M.chelonae*	*M.chelonae*
H13	*Homo sapiens*	eye	Massachussetts	*rpoB*	*M.chelonae*	*M.chelonae*
H14	*Homo sapiens*	knee (synovial fluid)	North Carolina	*rpoB*	*M.chelonae*	*M.chelonae*
H15	*Homo sapiens*	soft tissue (finger)	North Carolina	*rpoB*	*M.chelonae*	*M.chelonae*
H17	*Homo sapiens*	soft tissue (leg)	California	*hsp65* PRA	*M.chelonae*	*M.chelonae*
H18	*Homo sapiens*	soft tissue (skin)	Massachussetts	*hsp65* PRA	*M.chelonae*	*M.chelonae*
H19	*Homo sapiens*	soft tissue (leg)	Ohio	*hsp65* PRA	*M.chelonae*	*M.chelonae*
H20	*Homo sapiens*	NA	Maryland	*hsp65* PRA	*M.chelonae*	*M.chelonae*

[a] NA, information not available

[b] ATCC, American Type Culture Collection

[c] ID, identification

[d] WGS, Whole genome sequencing

^{T} Denotes type strain

Table 3.2. Drug susceptibility data of *Mycobacterium chelonae* clinical isolates reported as MICs.[a]

Isolate	Host species	Bacterial Identification	Antibiotic, mg/µL (interpretation)									
			Clarithromycin[b]	Tobramycin[b]	Amikacin	Linezolid[b]	Doxycylcline	Ciprofloxacin	Moxifloxicin[b]	Trimethoprim/ Sulfamethoxide[b]	Imipenem	Cefoxitin
ATCC 35752[T]	*M. chelonae* type strain	*M. chelonae*	1 (S)	2 (S)	16 (S)	<1 (S)	8 (R)	0.25 (S)	0.25 (S)	>8/152 (R)	32 (R)	>128 (R)
ATCC 19237	M. chelonae reference strain	*M.chelonae*	2 (S)	2 (S)	16 (S)	16 (I)	>16 (R)	4 (R)	4 (R)	>8/152 (R)	32 (R)	>128 (R)
Cichlid	*Cichlidae*	*M. chelonae*	2 (S)	4 (I)	32 (I)	16 (I)	>16 (R)	>4 (R)	8 (R)	>8/152 (R)	64 (R)	>128 (R)
Seahorse2	*Hippocampus erectus*	*M. chelonae*	0.5 (S)	1 (S)	8 (S)	8 (S)	16 (R)	> 4 (R)	8 (R)	>8/152 (R)	32 (R)	> 128 (R)
Seahorse3	*Hippocampus whitei*	*M. chelonae*	1 (S)	2 (S)	16 (S)	16 (I)	>16 (R)	>4 (R)	>8 (R)	>8/152 (R)	16 (I)	> 128 (R)
Seahorse4	*Hippocampus erectus*	*M. chelonae*	1 (S)	2 (S)	32 (I)	8 (S)	>16 (R)	>4 (R)	> 8 (R)	>8/152 (R)	64 (R)	> 128 (R)
Seahorse5	*Hippocampus reidi*	*M. chelonae*	0.12 (S)	2 (S)	16 (S)	4 (S)	>16 (R)	4 (R)	8 (R)	>8/152 (R)	32 (R)	>128 (R)
Seadragon1	*Phyllopteryx taeniolatus*	*M. chelonae*	0.5 (S)	2 (S)	16 (S)	32 (R)	>16 (R)	2 (I)	4 (R)	>8/152 (R)	> 64 (R)	>128 (R)
Python1	*Morelia boeleni*	*M. chelonae*	1 (S)	4 (I)	32 (I)	16 (I)	2 (I)	> 4 (R)	8 (R)	>8/152 (R)	>64 (R)	>128 (R)
Biofilm1	Biofilm	*M. chelonae*	1 (S)	2 (S)	32 (I)	32 (R)	>16 (R)	4 (R)	8 (R)	>8/152 (R)	64 (R)	>128 (R)
Biofilm2	Biofilm	*M. chelonae*	1 (S)	8 (R)	32 (I)	32 (R)	16 (R)	>4 (R)	8 (R)	>8/152 (R)	64 (R)	128 (R)
Biofilm3	Biofilm	*M. chelonae*	0.5 (S)	2 (S)	32 (I)	32 (R)	>16 (R)	4 (R)	>8 (R)	>8/152 (R)	32 (R)	>128 (R)
H7	*Homo sapiens*	*M. chelonae*	2 (S)	4 (I)	16(S)	8 (S)	0.5 (S)	2 (I)	2 (I)	4/76 (R)	16 (I)	>128 (R)
H8	*Homo sapiens*	*M. chelonae*	1 (S)	2 (S)	16(S)	8 (S)	≤0.12 (S)	1 (S)	2 (I)	8/152 (R)	32 (R)	>128 (R)
H10	*Homo sapiens*	*M. chelonae*	NA	2 (S)	64 (R)	NA	>16 (R)	> 8 (R)	NA	NA	8 (I)	>256 (R)
H11	*Homo sapiens*	*M. chelonae*	1(S)	<1 (S)	16 (S)	8 (S)	>16 (R)	>4 (R)	8 (R)	1/19 (S)	8 (I)	>128 (R)
H12	*Homo sapiens*	*M. chelonae*	0.12 (S)	2 (S)	16(S)	4 (S)	>16 (R)	4 (R)	8 (R)	4/76 (R)	16 (I)	>128 (R)
H13	*Homo sapiens*	*M. chelonae*	0.12 (S)	2 (S)	16(S)	8 (S)	>16 (R)	2 (I)	2 (I)	4/76 (R)	16 (I)	>128 (R)
H14	*Homo sapiens*	*M. chelonae*	1(S)	2 (S)	16(S)	16 (I)	>16 (R)	4 (R)	4 (R)	4/76 (R)	32 (R)	>128 (R)
H15	*Homo sapiens*	*M. chelonae*	1(S)	2 (S)	16(S)	4 (S)	0.5 (S)	0.5 (S)	1 (S)	4/76 (R)	16 (I)	>128 (R)
H17	*Homo sapiens*	*M. chelonae*	≤0.12 (S)	<1 (S)	32 (I)	≤ 2 (S)	32 (R)	4 (R)	2 (I)	NA	8 (I)	>256 (R)
H18	*Homo sapiens*	*M. chelonae*	2(S)	2 (S)	32 (I)	8 (S)	>16 (R)	4 (R)	4 (R)	8/152 (R)	16 (I)	128 (R)
H19	*Homo sapiens*	*M. chelonae*	2(S)	2 (S)	32 (I)	16 (I)	>16 (R)	1 (S)	2 (I)	4/76 (R)	16 (I)	>128 (R)
H20	*Homo sapiens*	*M. chelonae*	< 0.12 (S)	2 (S)	16(S)	8 (S)	0.5 (S)	8 (R)	NA	NA	16 (I)	>256 (R)
H9	*Homo sapiens*	*M. franklinii*	2 (S)	NA	4 (S)	16 (I)	>16 (R)	2 (I)	4 (R)	8/152 (R)	16 (I)	32 (I)
Turtle1	*Platysternon megacephalum*	*M. saopaulense*	2 (S)	16 (R)	64 (R)	16 (I)	>16 (R)	>4 (R)	8 (R)	>8/152 (R)	> 64 (R)	>128 (R)
Cow	*Bos taurus*	*M. saopaulense*	0.5 (S)	8 (R)	32 (I)	32 (R)	>16 (R)	2 (I)	4 (R)	>8/152 (R)	64 (R)	> 128 (R)
ATCC 19977[T]	*M. abscessus* subsp. *abscessus type strain*	*M. abscessus*	16 (R)	16 (R)	16 (S)	> 32 (R)	>16 (R)	>4 (R)	>8 (R)	>8/152 (R)	64 (R)	64 (I)
Seakrait	*Laticauda colubrina*	*M.abscessus*	1 (S)	8 (R)	16 (S)	32 (R)	>16 (R)	>4 (R)	>8 (R)	>8/152 (R)	32 (R)	32 I

[a] Green shading represents S, susceptible; Green to yellow shading represents I, intermediate susceptible; Red to yellow shading represents I, intermediate resistant Red shading represents R, resistant. Susceptibility patterns interpreted using CLSI recommendation.

[b] NA, information not available

[T] Denotes type strain

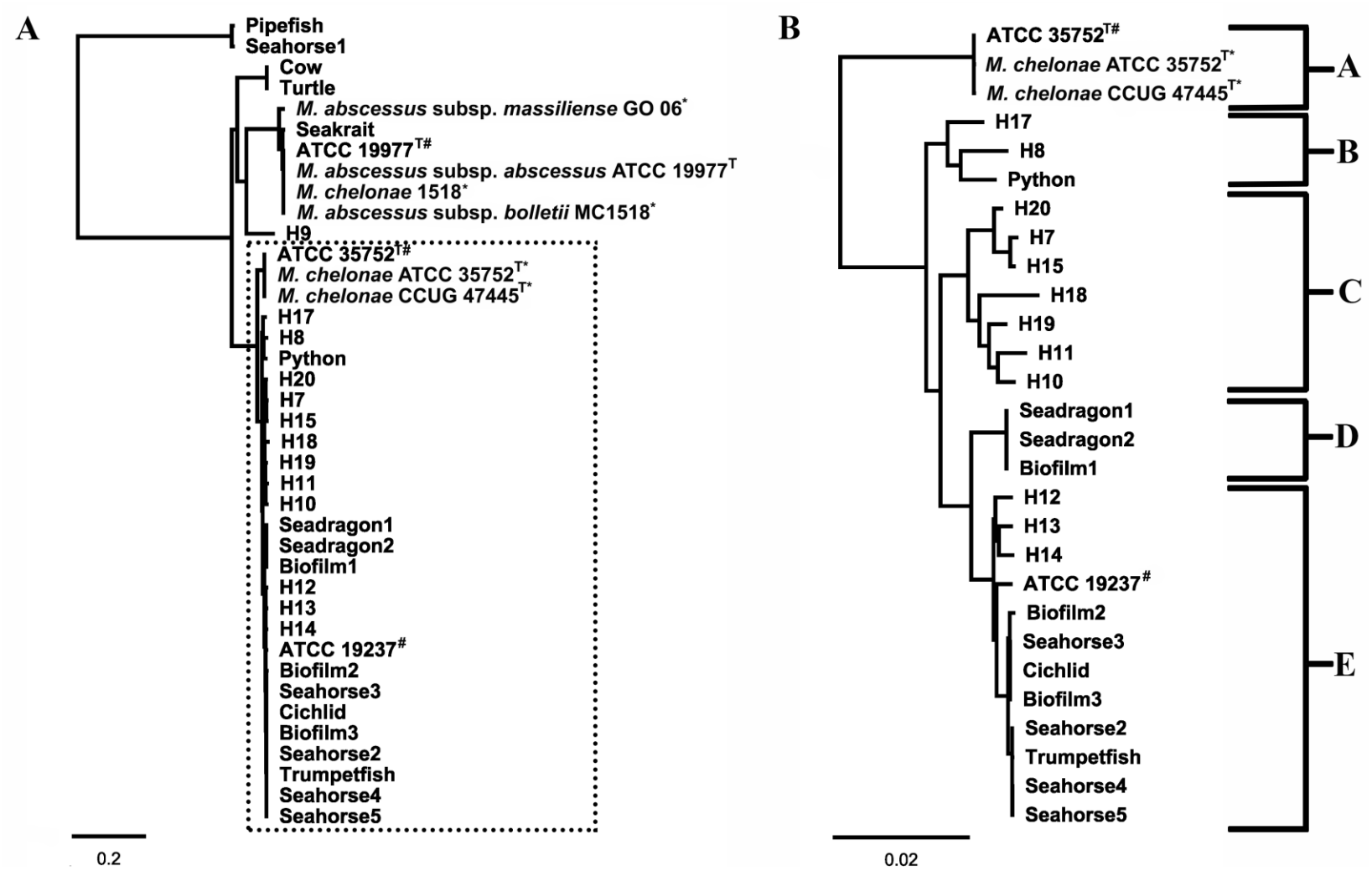


Figure 3.1. A. Phylogenetic comparison of *Mycobacterium chelonae* clinical isolates relative to six Genbank genome sequences using the **core genome** represented by 1,183,170 bp sequence and 1,040 coding regions. Phylogeny was

produced using the best scoring Maximum Likelihood model with 1000 bootstrap replications. Dotted box delineates *M. chelonae* clade. Scale bar represents average number of nucleotide substitutions per site. 0.02 represents 17,675 nucleotides which are not identical. **B.** Higher resolution view of *M. chelonae* isolates with delineation of sub-groupings within the *M. chelonae* clade.

[T] Denotes Type strain

[*] Denotes sequence used from GenBank.

[#] Denotes isolate sequenced in study

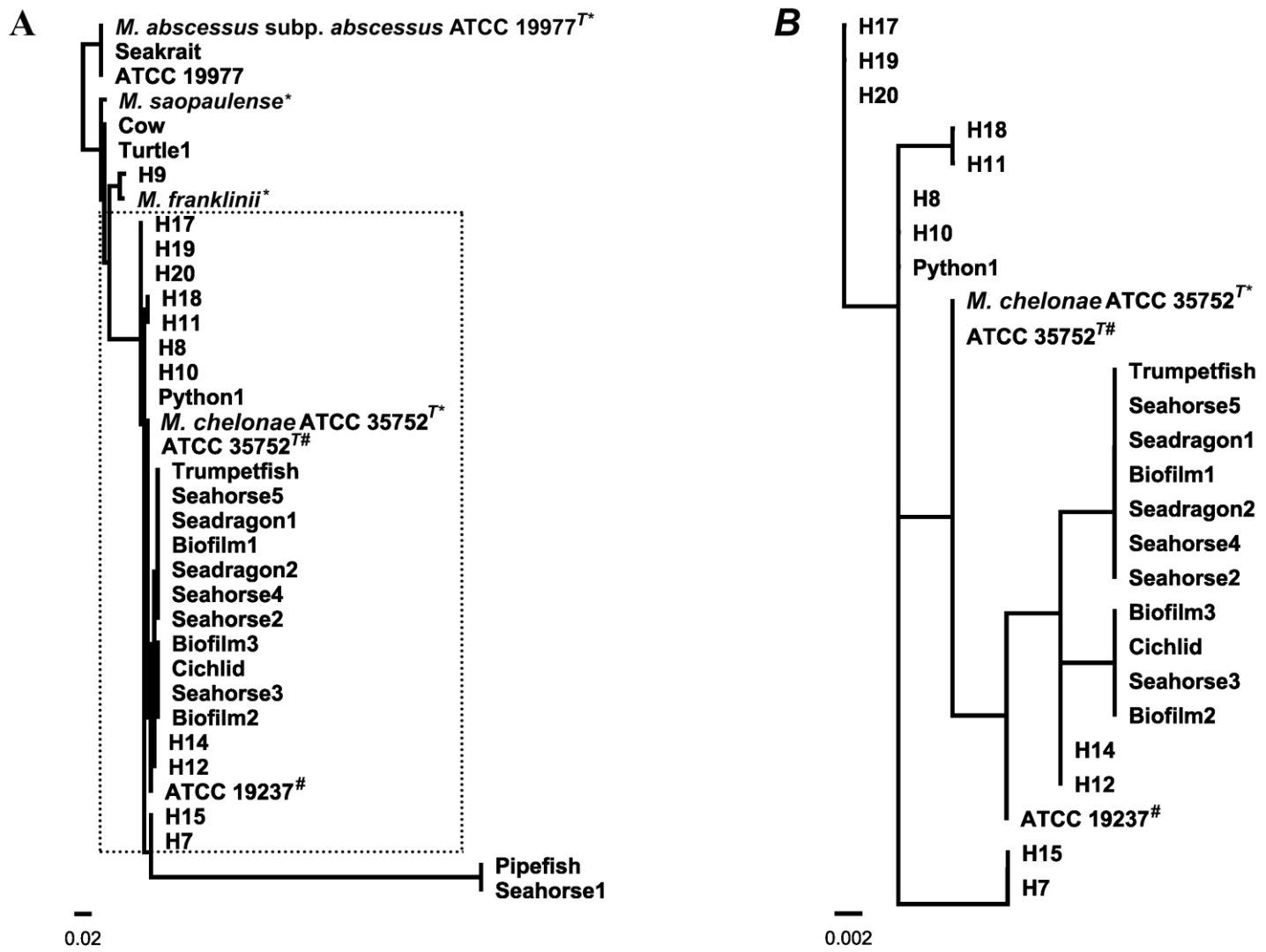


Figure 3.2. A. Phylogenetic comparison of *Mycobacterium chelonae* clinical isolates relative to four Genbank reference sequences at the **ITS** locus. Phylogeny was produced using the best scoring Maximum Likelihood model with 1000

bootstrap replications. Dotted box delineates branch with *M. chelonae* isolates. **B.** Higher resolution view of *M. chelonae* isolates with delineation of sub-groupings within the *M. chelonae* clade. Scale bar represents average number of nucleotide substitutions per site. 0.02 represents 3 nucleotides which are not identical.

[T] Denotes Type strain

[*] Denotes sequence used from GenBank.

[#] Denotes isolate sequenced in study

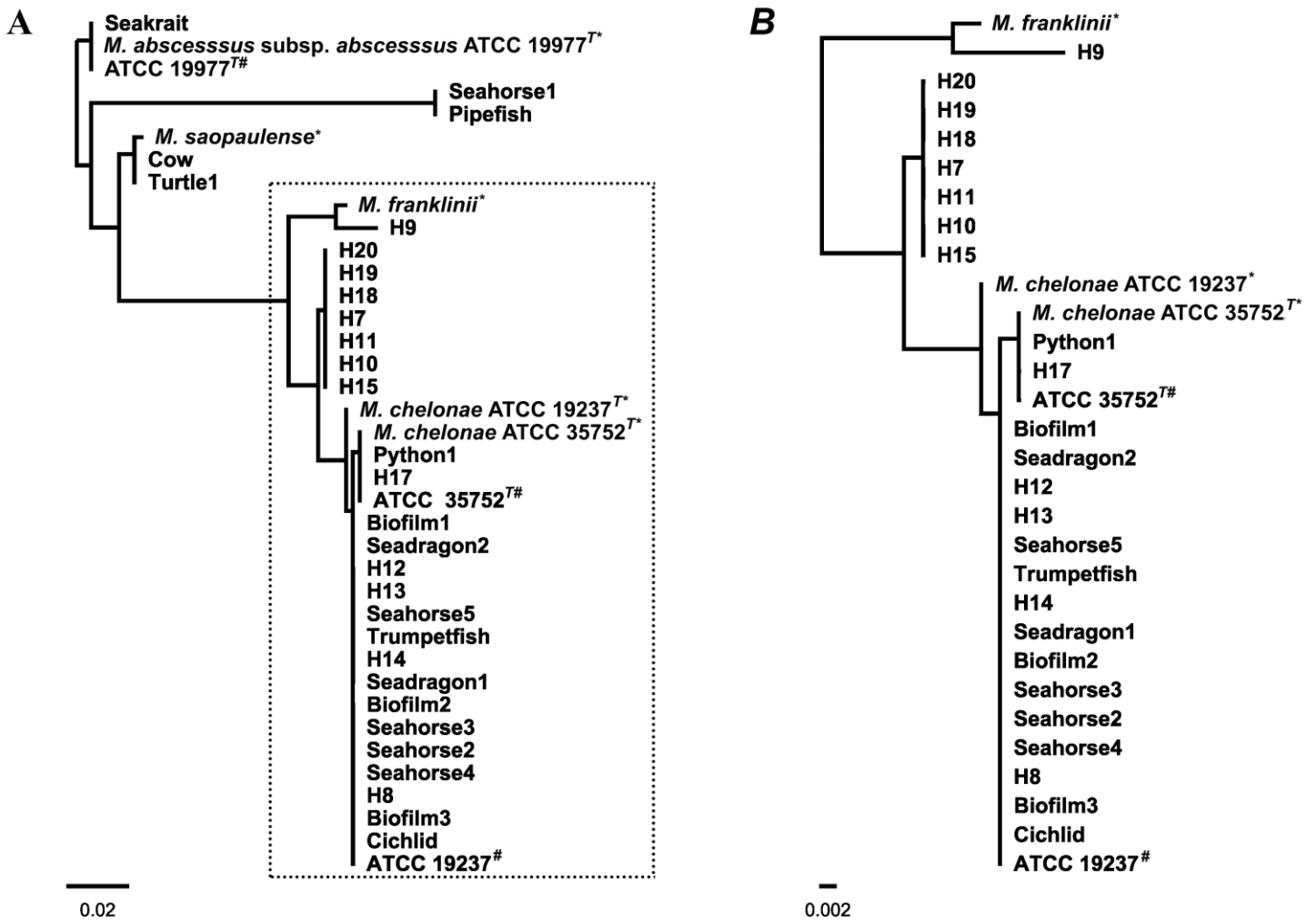


Figure 3.3 A. Phylogenetic comparison of *Mycobacterium chelonae* clinical isolates relative to five Genbank reference sequences at the **partial *hsp65* 441 bp** locus. Phylogeny was produced using the best scoring Maximum Likelihood model with 1000 bootstrap replications. Dotted box delineates branch with *M. chelonae* and *M. franklinii*. **B.** Higher

resolution view of *M. chelonae* isolates with delineation of sub-groupings within the *M. chelonae* clade. Scale bar represents average number of nucleotide substitutions per site. 0.002 represents 1 nucleotide which is not identical.

[T] Denotes Type strain

[*] Denotes sequence used from Genbank

[#] Denotes isolate sequenced in study

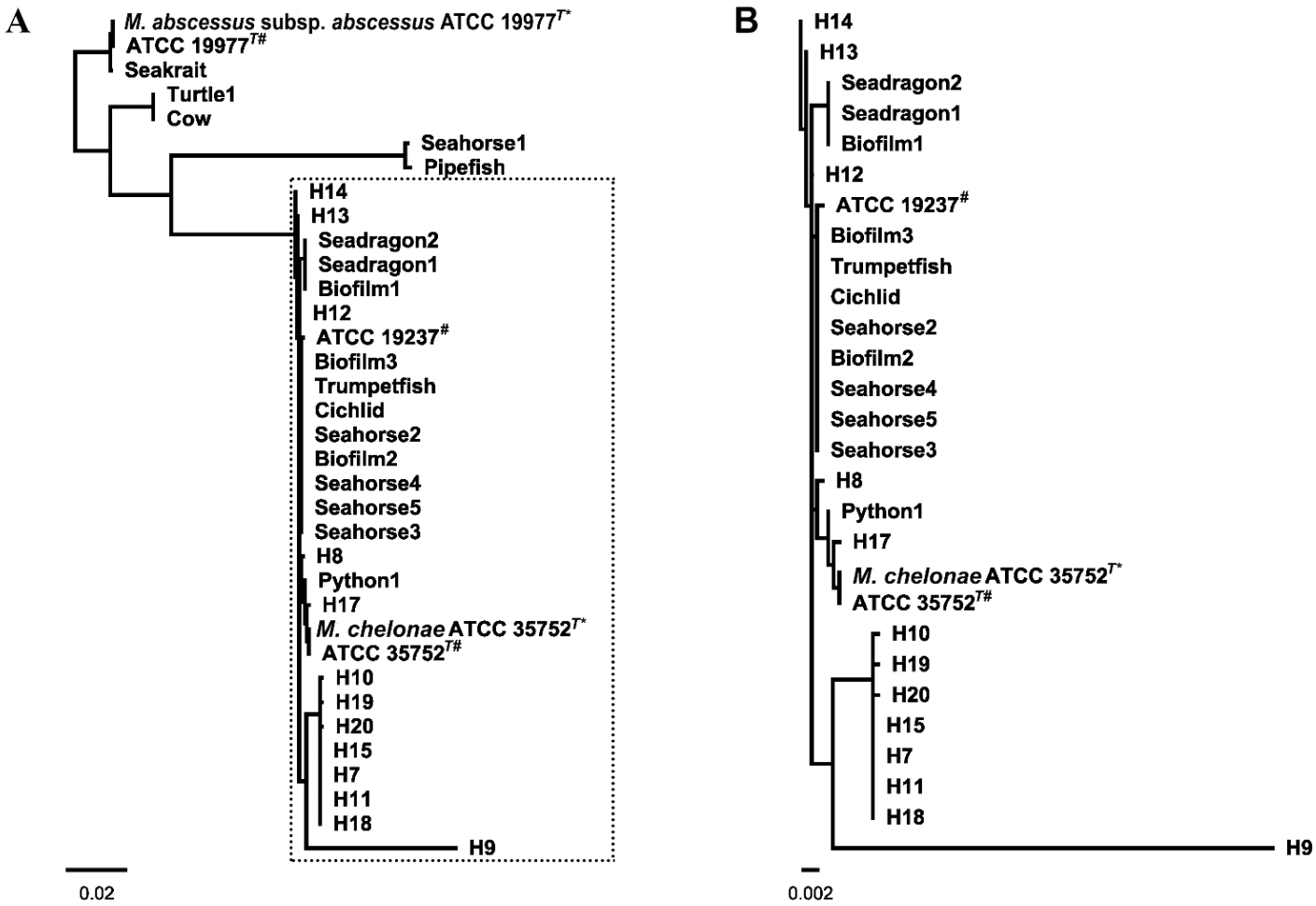


Figure 3.4. A. Phylogenetic comparison of *Mycobacterium chelonae* clinical isolates relative to two Genbank reference strains at the **complete *hsp65* 1,626 bp** locus. Phylogeny was produced using the best scoring Maximum Likelihood model with 1000 bootstrap replications. Dotted box delineates branch with *M. chelonae* and *M. franklinii*. **B.** Higher

resolution view of *M. chelonae* isolates with delineation of sub-groupings within the *M. chelonae* clade. Scale bar represents average number of nucleotide substitutions per site. 0.002 represents 3-4 nucleotides which are not identical.

[T] Denotes Type strain

[*] Denotes sequence used from Genbank.

[#] Denotes isolate sequenced in study

Figure 3.5. A. Phylogenetic comparison of *Mycobacterium chelonae* clinical isolates relative to five Genbank reference sequences at the **partial rpoB 752 bp** locus. Phylogeny was produced using the best scoring Maximum Likelihood model

with 1000 bootstrap replications. Dotted box delineates branch with *M. chelonae* and *M. franklinii*. **B.** Higher resolution view of *M. chelonae* isolates with delineation of sub-groupings within the *M. chelonae* clade. Scale bar represents average number of nucleotide substitutions per site. 0.002 represents 3-4 nucleotides which are not identical.

[T] Denotes Type strain

* Denotes sequence used from Genbank

[#] Denotes isolate sequenced in study

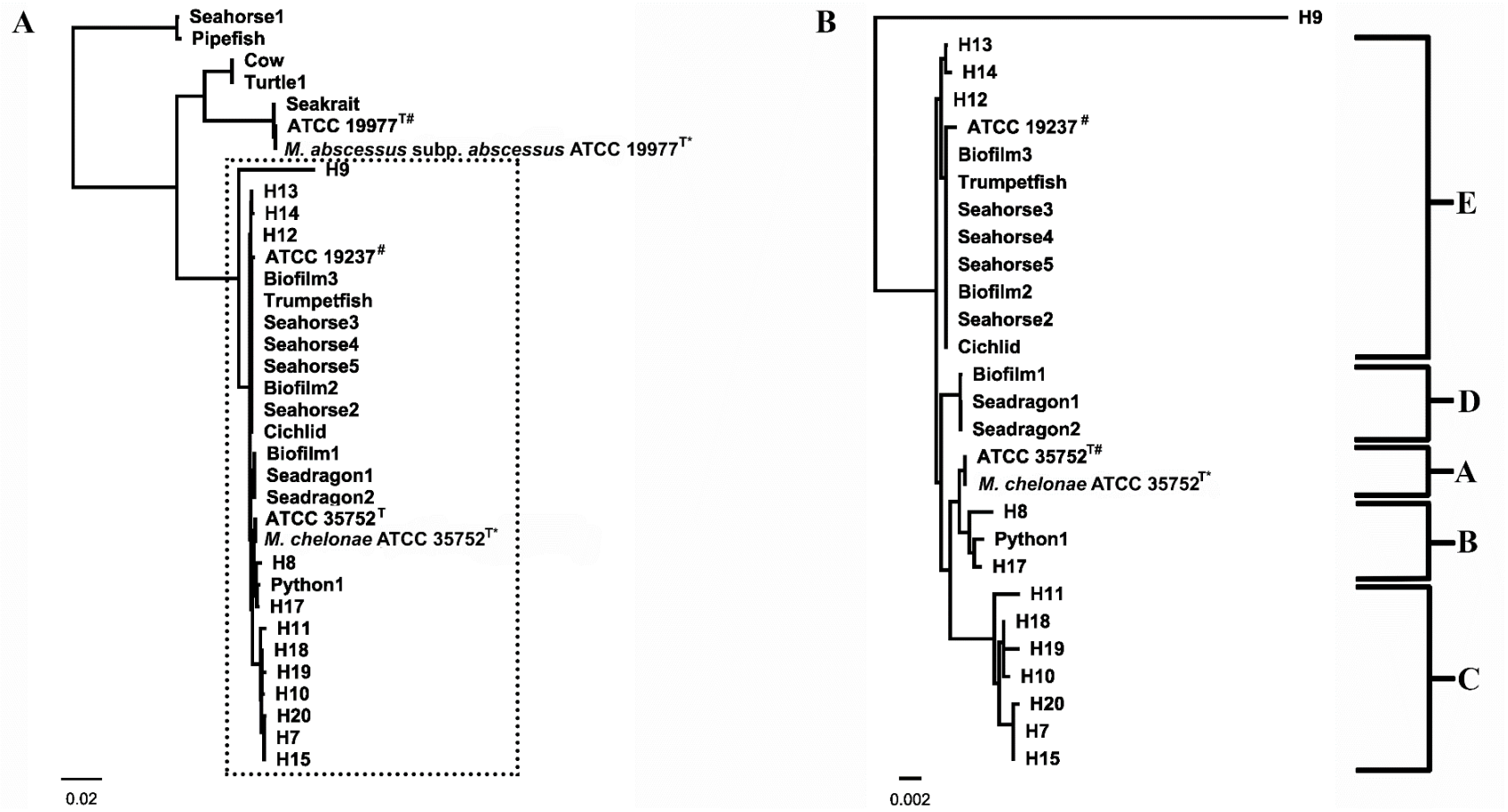


Figure 3.6. A. Phylogenetic comparison of *Mycobacterium chelonae* clinical isolates relative to two Genbank reference strains using the **concatenated whole *hsp65* 1,626 bp and partial *rpoB* 752** bp locus. Phylogeny was produced using the best scoring Maximum Likelihood model with 1000 bootstrap replications. Dotted box delineates branch with *M. chelonae* and *M. franklinii*. **B.** Higher resolution view of *M. chelonae* isolates with delineation of sub-groupings within the

M. chelonae clade. Scale bar represents average number of nucleotide substitutions per site. 0.002 represents 5 nucleotides which are not identical.

[T] Denotes Type strain

* Denotes sequence used from Genbank.

[#] Denotes isolate sequenced in study

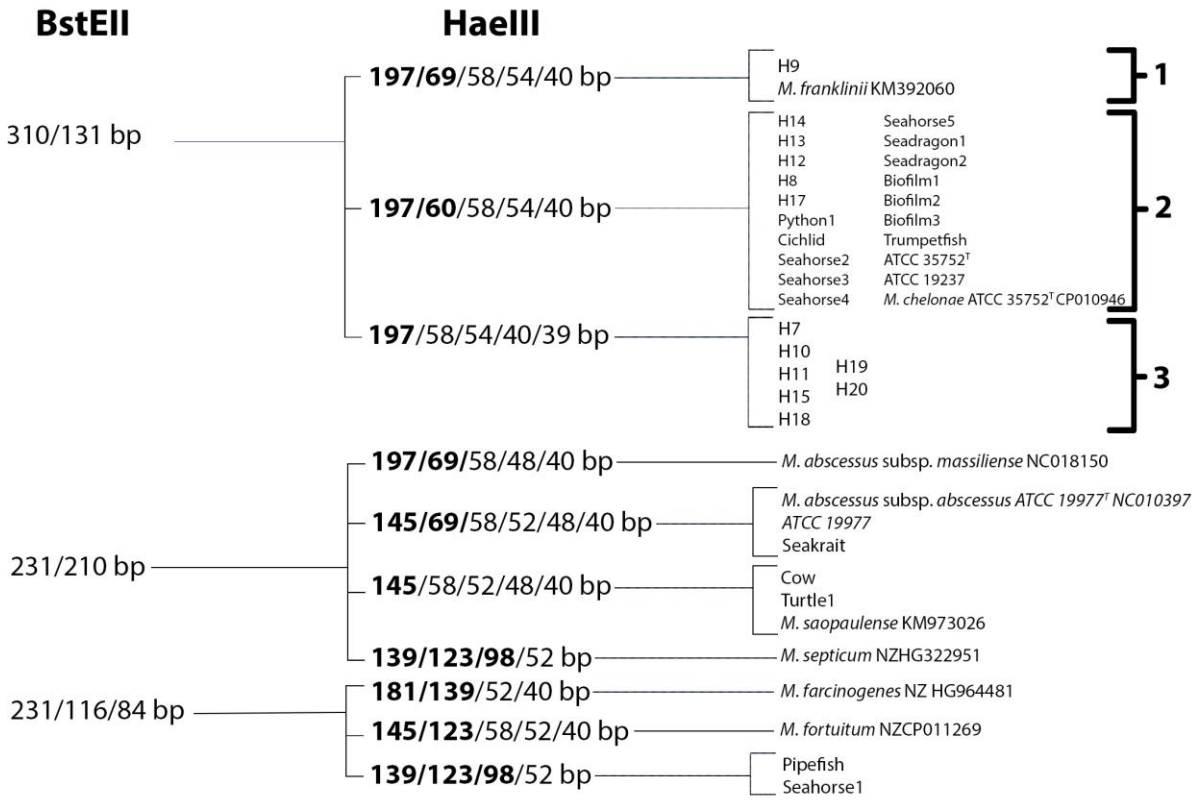


Figure 3.7. Summary of PCR-restriction length polymorphism analysis results performed on the partial *hsp65* (441 bp) fragment **(*hsp65* PRA)**. Results are arranged according to the Taylor et al. (63) algorithm with slight modification to account for fragment length created in-vitro and inclusion of fragments 35 bp or greater.

[T] Denotes type strain

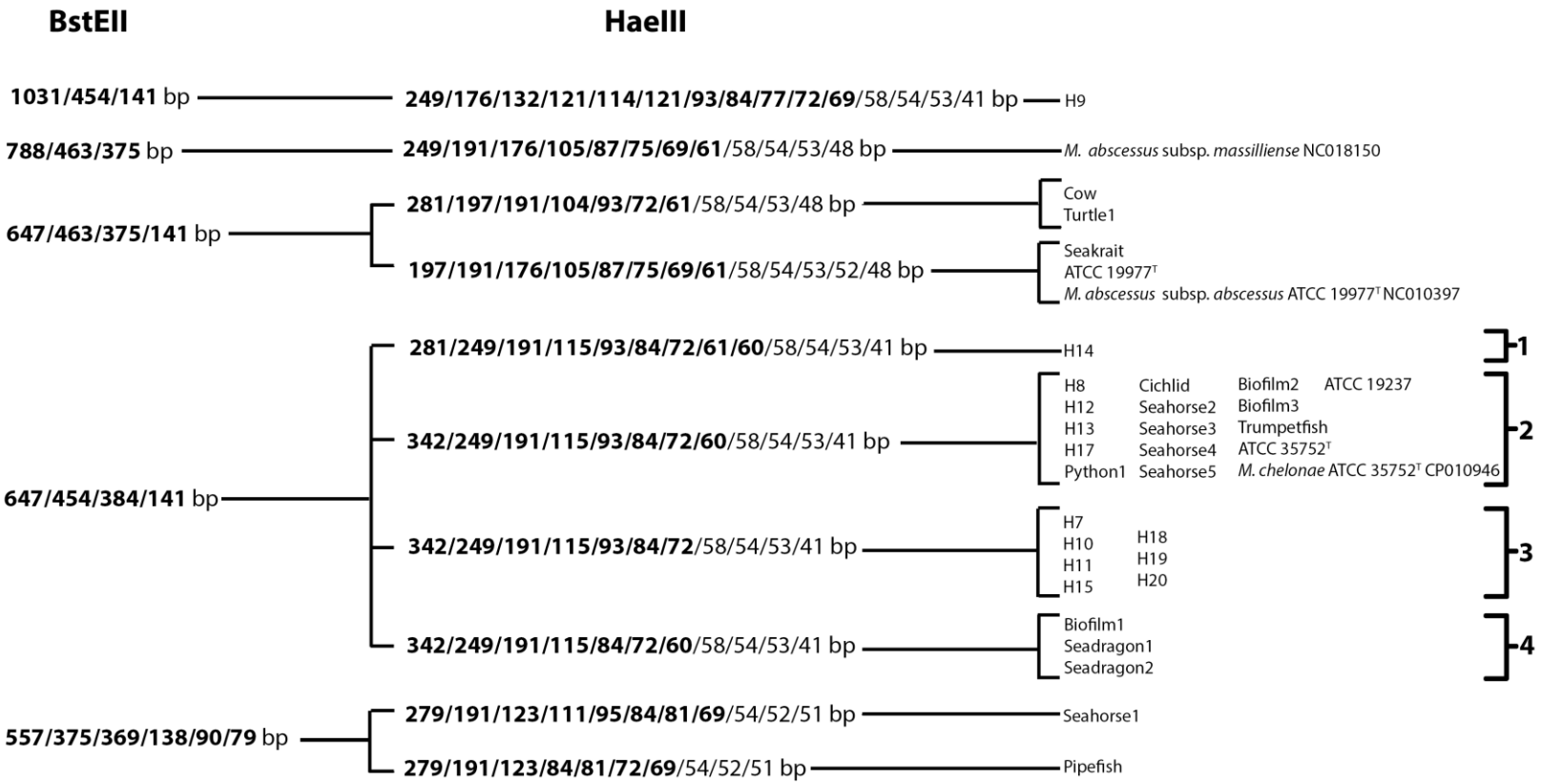
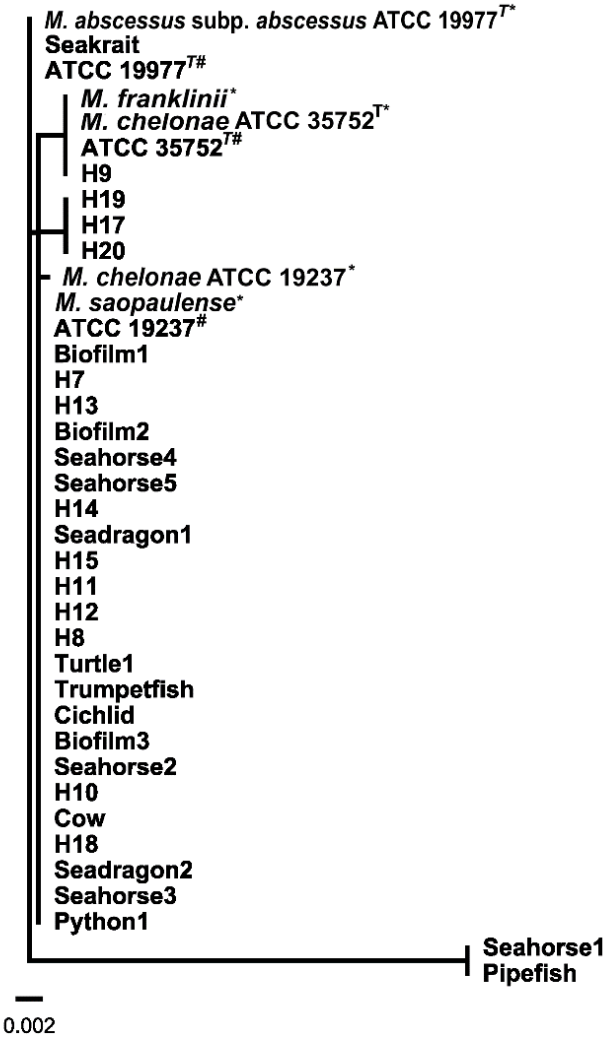


Figure 3.8. Summary of PCR-restriction length polymorphism analysis results performed on the whole *hsp65* (1,626 bp) sequence **(hsp65 PRA complete).**

T Denotes type strain



Supplementary Figure 3.1. Phylogenetic comparison of *Mycobacterium chelonae-abscessus* complex clinical isolates relative to six Genbank reference strains at the **16S rRNA** locus 1,522 bp. Phylogeny was produced using the best scoring Maximum Likelihood model with 1000 bootstrap replications. Scale bar represents average number of nucleotide substitutions per site. 0.002 represents 3 nucleotides which are not identical.

T Denotes Type strain

* Denotes sequence used from Genbank

# Denotes isolate sequenced in study

		Nucleotide location substitution						
Isolate	**Sequevar**	**1**	**11**	**571**	**588**	**978**	**988**	**1,017**
ATCC 35752[T]	1	A	G	G	T	G	T	C
ATCC 19237	2	*	*	*	*	A	C	T
Cichlid	2	*	*	*	*	A	C	T
Seahorse2	2	*	*	*	*	A	C	T
Seahorse3	2	*	*	*	*	A	C	T
Seahorse4	2	*	*	*	*	A	C	T
Seahorse5	2	*	*	*	*	A	C	T
Seadragon1	2	*	*	*	*	A	C	T
Seadragon2	2	*	*	*	*	A	C	T
Trumpetfish	2	*	*	*	*	A	C	T
Python1	2	*	*	*	*	A	C	T
Turtle	2	*	*	*	*	A	C	T
Biofilm1	2	*	*	*	*	A	C	T
Biofilm2	2	*	*	*	*	A	C	T
Biofilm3	2	*	*	*	*	A	C	T
Cow	2	*	*	*	*	A	C	T
H7	2	*	*	*	*	A	C	T
H8	2	*	*	*	*	A	C	T
H10	2	*	*	*	*	A	C	T
H11	2	*	*	*	*	A	C	T
H12	2	*	*	*	*	A	C	T
H13	2	*	*	*	*	A	C	T
H14	2	*	*	*	*	A	C	T
H15	2	*	*	*	*	A	C	T
H18	2	*	*	*	*	A	C	T
H17	3	T	*	A	C	A	C	T
H19	3	T	*	A	C	A	C	T
H20	3	T	*	A	C	A	C	T

Supplementary Table 3.1. Sequevar type and nucleotide variation among *M. chelonae* isolates at the whole 16S rRNA sequence.

[T] Denotes type strain

* Denotes nucleotide homology between isolates

Chapter 4

DESCRIPTION OF *MYCOBACTERIUM SUSANAE* SP. NOV., A RAPIDLY GROWING MYCOBACTERIUM IDENTIFIED IN SYNGNATHIDS[1]

[1]Fogelson, S.B., Al Camus, A., Lorenz, W.W., Phillips, A., Bartlett, P., and Sanchez, S. To be submitted to *Journal of Systematic and Evolutionary Microbiology*

Abstract

Two closely related isolates, 27335 and 24999, of rapidly growing, non-pigmented, environmental mycobacteria were cultured from two clinically ill fish of the Family Syngnathidae. Whole genome sequencing of these two isolates revealed low sequence homology to documented mycobacteria within public databases such as NCBI. Evaluation of targeted housekeeping genes including 16S, ITS, *rpoB*, and *hsp65* related the two bacteria to *Mycobacterium senegalense* CK2 M4421 and *Mycobacterium farcinogenes* DSM 43637. Phenotypic, biochemical, and DNA-DNA hybridization testing demonstrate that *Mycobacterium susanae* is a new species separated from other documented rapidly-growing mycobacteria and we propose the formal recognition of *Mycobacterium susanae* sp. nov. Strain 27335 is the type strain.

Rapidly growing mycobacteria are ubiquitous in the environment and have the capacity to cause disease in a variety of hosts. Human hosts are uncommonly affected by atypical mycobacteriosis and disease is often seen as a sequela to immunosuppressive conditions such as cystic fibrosis and AIDs (1, 2). Rare cases of atypical mycobacteriosis in immune competent individuals have also been identified (3, 4). In contrast to humans, aquatic animals such as fish commonly succumb to fatal disease caused by non-tuberculous (NTM) mycobacteria. *Mycobacterium marinum*, *Mycobacterium fortuitum*, and *Mycobacterium chelonae* are the most readily cultured isolates from fish (5). However, other species of mycobacteria have been documented to cause disease. Within the last 15 years, reports of NTM mycobacteriosis in humans as well as fish have increased in number (6-8). This escalation may be the result of increased surveillance, the advancement of reliable identification methods, increased prevalence of disease or a combination of the former. In addition, a distinct connection between the ability of mycobacteria to form disinfectant resistant surface biofilms in places such as public water supply lines and aquaculture life support systems and the distribution of disease in humans and fish has been described (9-13). The ability of these organisms to inhabit a wide variety of environments, as well as persist within them post-disinfection lends to the epidemiology of NTM mycobacteriosis. Furthermore, the ability of these environmental bacteria to cause disease in animals highlights the importance of evaluating humans, animals, and the environment together to fully comprehend the dynamics of NTM mycobacteria.

One family of fish that appear to have a unique susceptibility to mycobacteriosis are the Syngnathidae (seahorses, pipefish, weedy seadragons and leafy seadragons)

(14, 15). *M. chelonae* is readily cultured from members of the *Syngnathidae* and the pathologic presentation of disease appears to be different in these fish when compared to other vertebrates infected with *Mycobacterium* spp. (15). In a previous study, syngnathids were evaluated to determine if disease susceptibility and their unique pathologic presentation was due to a differential host immune response or bacterial strain differences. Mycobacterial isolates from biofilms, fish, reptiles, mammals, and humans were evaluated by whole genome sequencing and core genome analysis, as well as by housekeeping genes, to taxonomically classify several strains of *M. chelonae* (Fogelson et al., under review). Through this analysis, two isolates from syngnathid fish were observed that were not closely related to *M. chelonae*. When the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information website (NCBI) failed to identify the isolates as known species, a polyphasic approach using macroscopic and microscopic evaluation, biochemical testing, antimicrobial susceptibility testing, whole genome assembly, targeted gene extraction and analysis, and in-silico DNA-DNA hybridization was used to more fully characterize them.

The two isolates (27335 and 24999) were identified from clinically ill syngnathid fish from separate geographic (South Carolina and Georgia) and environmental enclosures. The fish, a pipefish (*Syngnathoides biaculeatus*) and a lined seahorse (*Hippocampus erectus*), had gross and histologic lesions consistent with mycobacteriosis and cultures were performed on affected tissues. Fresh ovarian tissue from the pipefish and skeletal muscle from the seahorse were inoculated onto Middlebrook 7H11 media. Ziehl-Neelsen stains confirmed the growing colonies as acid-

fast positive. Matrix-assisted laser desorption/ionization Time-of-Flight (MALDI-TOF) (Vitek M5, Biomerieux, St. Louis, MO) identified only a *Mycobacterium* spp.

Biochemical testing defined pertinent characteristics of the new species. Specific traits tested, included growth at 35-37°C on Lowenstein Jensen media, Middlebrook 7H11 and 5% sheep's blood agar, Arylsulfatase production, Tween 80 hydrolysis, single carbon source usage (mannitol, i-myo-inositol, sorbitol, D-Trehalose, or L-Rhamnose), catalase, oxidase. The results of the Tween 80 hydrolysis test were read at 5 and 10 days as described by standard protocols for the biochemical testing of mycobacteria (16). For carbon source utilization testing was performed using a GEN III MicroPlate (Hayward, CA) according to the manufacturer's instructions.

DNA was extracted from mycobacteria grown on Middlebrook 7H11 media using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA USA) following the manufacturers protocol. Approximately 15-28 ng/µL of DNA was submitted from each sample to Georgia Genomics Facility (Core Facility, The University of Georgia, Athens, GA) for library preparation, placement of Illumina TruSeq adaptors on the templates, and seeding of the DNA library onto a glass slide to perform MiSeq. An Illumina MiSeq PE300 sequencer (Illumina Inc., San Diego, CA, USA) was used to perform whole genome sequencing.

Antimicrobial susceptibility testing was performed two times per isolate using a Sensititre RAPMYCO panel (Trek Diagnostic Systems Limited, West Sussex UK) following the Clinical and Laboratory Standards (CLSI) recommendations. Sensititre RAPMYCO panel uses a standard-ordered broth microdilution panel for susceptibility testing of rapidly growing mycobacteria against amikacin, cefoxitin, ciprofloxacin,

clarithromycin, doxycycline, imipenem, linezolid, trimethoprim-sulfamethoxazole, tobramycin (17, 18). Established breakpoints were used from the CSLI document M24-A2 (19). The drug susceptibility data of isolates are shown in Table 4.1. Both isolates were 100% susceptible to clarithromycin, amikacin, and ciprofloxacin.

Isolate 27335 and 24999 Illumina reads were evaluated by FastQC and quality trimmed using Trimmomatic to trim off Illumina adapters and filter reads at the leading and trailing ends (20). The de novo assembler, SPAdes 3.8.0, was run using default parameters to assemble the reads (21). Once paired-end reads were assembled, Quality Assessment Tool for Genome Assemblies (Quast) was performed on the output to visualize assembly metrics for each dataset (22). Isolates were annotated using Rapid Annotation Subsystem Technology (RAST) (23). Custom BLAST databases made on each isolate and reads were mapped back to the assembly file to identify reads shorter than 1,000 bp with low coverage, repeats, or that lacked annotation. Ultimately, reads under 1,000 bp were excluded from the final assembly.

Final draft assembly of isolate 27335 produced 74 contigs with a total length of 6,340,198 bp, N50 of 191,633 bp, G+C content of 66.17%, and 6,183 protein coding regions (CDS). Assembly of isolate 24999 produced 92 contigs with a total length of 6,331,478 bp, N50 of 160,739 bp, G+C content of 66.19%, and 6,156 CDS. Of the CDS for the 27335 and 24999, 1,830 and 1,777 were hypothetical proteins. Eighty-five and 84 RNA genes (of which 78 and 77 were tRNAs) were detected in isolates 27335 and 24999, respectively. This whole-genome shotgun project was deposited in GenBank under the Sequence Read Archives: SRS1746189 and SRS1746185

Phylogenetic analysis of several housekeeping genes was performed to infer the taxonomic position of the isolates. Genes evaluated included; 16S rRNA, 16S-23S rRNA internal transcribed spacer (ITS), the 65 kD heat shock protein (*hsp65*), and polymerase beta subunit (*rpoB*). Extraction of genes was performed by importing the draft genome annotated file into Geneious 8.1.8 where the annotated gene could be visualized and then previously documented primers were used to extract partial or whole gene sequences for analysis (24-28) (supplementary Table 4.1). Pairwise phylogenetic analysis of the two isolates at the 16S, ITS, *hsp65*, and *rpoB* sequence revealed 100% identity to each other at these four loci. As with other previously described rapidly growing mycobacteria, BLASTn of the 16S rRNA alone could not identify these isolates to species level as several species including *Mycobacterium fortuitum* strain CT6 NZ_CP011269 and *Mycobacterium* sp. VKM AC-1817D NZ_CP009914 NZ_CP011269 had 99% identity to the isolates. The top hits observed using BLASTn on isolates 27335 or 24999 *hsp65* included; *Mycobacterium conceptionense* CIP 108544 AY859678.1 (96%), *Mycobacterium senegalense* strain RGTB192 HM454228.1 (95%), *Mycobacterium peregrinum* strain 03/423 EU156064.1 (95%), *Mycobacterium fortuitum* strain ATCC 13756 DQ866789.1 (95%), *Mycobacterium fortuitum* strain CT6 NZ_CP011269 (94%), *Mycobacterium* sp. VKM AC-1817D NZ_CP009914 (95%), and *Mycobacterium vulneris NZ_CCBG010000001 (*95%). When the isolates were evaluated at *rpoB, Mycobacterium conceptionense* CIP 108544 AY859695.1 (97%), *Mycobacterium porcinum* strain CIP 105392 AY262737.1 (96%), *Mycobacterium fortuitum* strain CT6 NZ_CP011269 (96%), *Mycobacterium* sp. VKM AC-1817D NZ_CP009914 NZ_CP011269 (96%) were closest in identity.

The ITS fragments extracted using primer Sp1 and Sp2 of both isolates were 245 bp in length and had only 89% identity to *M. senegalense* strain RGTB192 HM454228.1.

Phylogenies of the two isolates was estimated using multisequence alignment against the top BLAST hits. Using the default settings in MUSCLE program with a maximum of 10 Iterations, a percentage identity between sequences was achieved (29). RAxML (version 7.2.8) then used to estimate phylogenies and produce phylogenetic comparison matrices (30) . Phylogenetic trees were constructed for 16S, *hsp65, rpoB,* and concatenated sequences of 16S, *hsp65,* and *rpoB* by employing a GTR Gamma rapid bootstrapping and search for best scoring Maximum Likelihood model with 1000 bootstrap replications (Figure 4.1).

DNA-DNA Hybridization (DDH) was performed in-silico using formula 2 in the Genome-to-Genome Distance Calculator provided by Meier-Kolthoff et al. (31, 32) and DDH values were tested against eight genomes from Genbank (Table 4.2). Both draft and complete genomes used in this analysis were chosen based on the top BLAST hits of *hsp65* and *rpoB* for the two isolates. When compared to each other, the probability of DDH being greater than 70% was between 97-98.7%, supporting the two isolates as the same species and subspecies. Alternatively, the probability that the two isolates are the same as the top BLAST hits (8 *Mycobacterium* spp. isolates, Table 4.2) was no greater than 1.2%. *M. senegalense* NZ LDPU01000001 and *M. farcinogenes* NZ HG964481 had the highest DDH estimate at 36.9% (CI 34.3-39.3%) and 37.0% (CI 34.6-39.5%), respectively.

In summary, the phenotypic and genotypic characteristics of isolates 27335 and 24999 support their designation as a new species. Furthermore, they are not part of any currently described grouping or complex.

Description of *Mycobacterium susanae* sp. nov.

Mycobacterium susanae (of or pertaining to the first name of the investigators who first identified these bacteria). Cells are acid-fast bacilli that grow on conventional 7H11 solid agar media, Lowenstein Jensen, and 5% sheep's blood agar. Observable growth occurs at 3-5 days of incubation between 30-37°C. Both isolates did not grow in mannitol, myo-inositol, sorbitol, D-Trehalose, or L-Rhamnose. Tween 80 hydrolysis and oxidase reaction were negative while Arylsulfatase production and the catalase reaction were positive.

Both isolates are susceptible to clarithromycin, amikacin, and ciprofloxacin and variably susceptible to linezolid, doxycycline, moxifloxacin, and trimethoprim/sulfamethoxide (see Table 4.1). Whole genome sequencing of the isolates confirms they are the same species and DNA-DNA hybridization supports that they are also the same subspecies. Sequencing of the 441 bp fragment of- *hsp65* or the partial *rpoB* can distinguish this new species from other rapidly-growing mycobacterial species. *Mycobacterium susanae* has a 720 bp *rpoB* fragment length similar to *Mycobacterium fortuitum, M. peregrinum, M. senegalense, and M. farcinogenes* but has no more than 96.8% identity to any of these isolates.

Acknowledgements

The authors would like to thank the Nocardia/Mycobacteria Research Laboratory (Tyler, TX) for their technical support.

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Table 4.1. Antimicrobial drug susceptibility data for 27335 and 24999 isolates. (S) susceptible, (I) intermediate, (R) resistant.

	MIC (μg ml^{-1})			
	27335 (Pipefish, *Syngnathoides biaculeatus*)		**24999 (Seahorse, *Hippocampus erectus*)**	
Drug	**replicate 1**	**replicate 2**	**replicate 1**	**replicate 2**
Clarithromycin	0.5 (S)	0.25 (S)	0.06 (S)	0.06 (S)
Tobramycin	8 (R)	8 (R)	4 (I)	4 (I)
Amikacin	1 (S)	16 (S)	1 (S)	1 (S)
Linezolid	1 (S)	16 (I)	1 (S)	1 (S)
Doxycylcline	0.12 (S)	>16 (R)	0.12 (S)	0.12 (S)
Ciprofloxacin	0.25 (S)	1	0.25 (S)	0.25 (S)
Moxifloxicin	0.25 (S)	4 (R)	0.25 (S)	0.25 (S)
Trimethoprim/Sulfamethoxide	1/19 (S)	4/76 (R)	1/19 (S)	4/76 (R)
Imipenem	8 (I)	32 (R)	4 (S)	32 (R)
Cefoxitin	64 (I)	>128 (R)	16 (S)	16 (S)
Cefepime	>32	>32	>32	>32
Ceftnaxone	>64	>64	>64	>64
Tigecycline	0.25	0.25	0.12	0.03
Minocycline	>8	>8	1	1
Amoxicillin/davulanic acid 2:1	>64/32	>64/32	≤32/16	32/16

Table 4.2. DNA-DNA hybridization values of two new isolates compared to eight Genbank strains.

DNA-DNA hybridization

Bacterial species	**Strain**	**Genbank accession**	**DNA-DNA relatedness (%)**		**DNA-DNA relatedness (%)**	
			DDH	**Model C.I.**	**DDH**	**Model C.I.**
M. abscessus subsp. *abscessus*	ATCC 19977	NZ_CP010397	19.5	[17.3 - 21.8%]	19.3	[17.2 - 21.7%]
M.farcinogenes	DSM 43637	NZ_CCAY000000000.1	37	[34.6 - 39.5%]	36.8	[34.3 - 39.3%]
M.fortuitum	CT6	NZ_CP011269.1	30	[27.6 - 32.5%]	30.5	[28.2 - 33%]
M.peregrinum	CSUR P2098	NZ_CYSH00000000.1	31.4	[29 - 33.9%]	31.4	[29 - 33.9%]
M.senegalense	CK2 M4421	LDPU01000001	36.9	[34.5 - 39.5%]	36.9	[34.4 - 39.4%]
M.septicum	DSM 44393	NZ__CBMO0000000000	33	[30.6 - 35.5%]	33	[30.6 - 35.5%]
M.sp	VKM Ac-1817D	NZ_CP009914.1	30	[27.6 - 32.5%]	30.5	[28.1 - 33%]
M.vulneris	DSM 45247	NZ_CCBG000000000.1	33.6	[31.2 - 36.1%]	34.5	[32 - 37%]
Unknown (Seahorse)	24999	new species	93.8	[92 - 95.2%]	100	[100 - 100%]
Unknown (Pipefish)	27335	new species	100	[100 - 100%]	93.8	[92 - 95.2%]

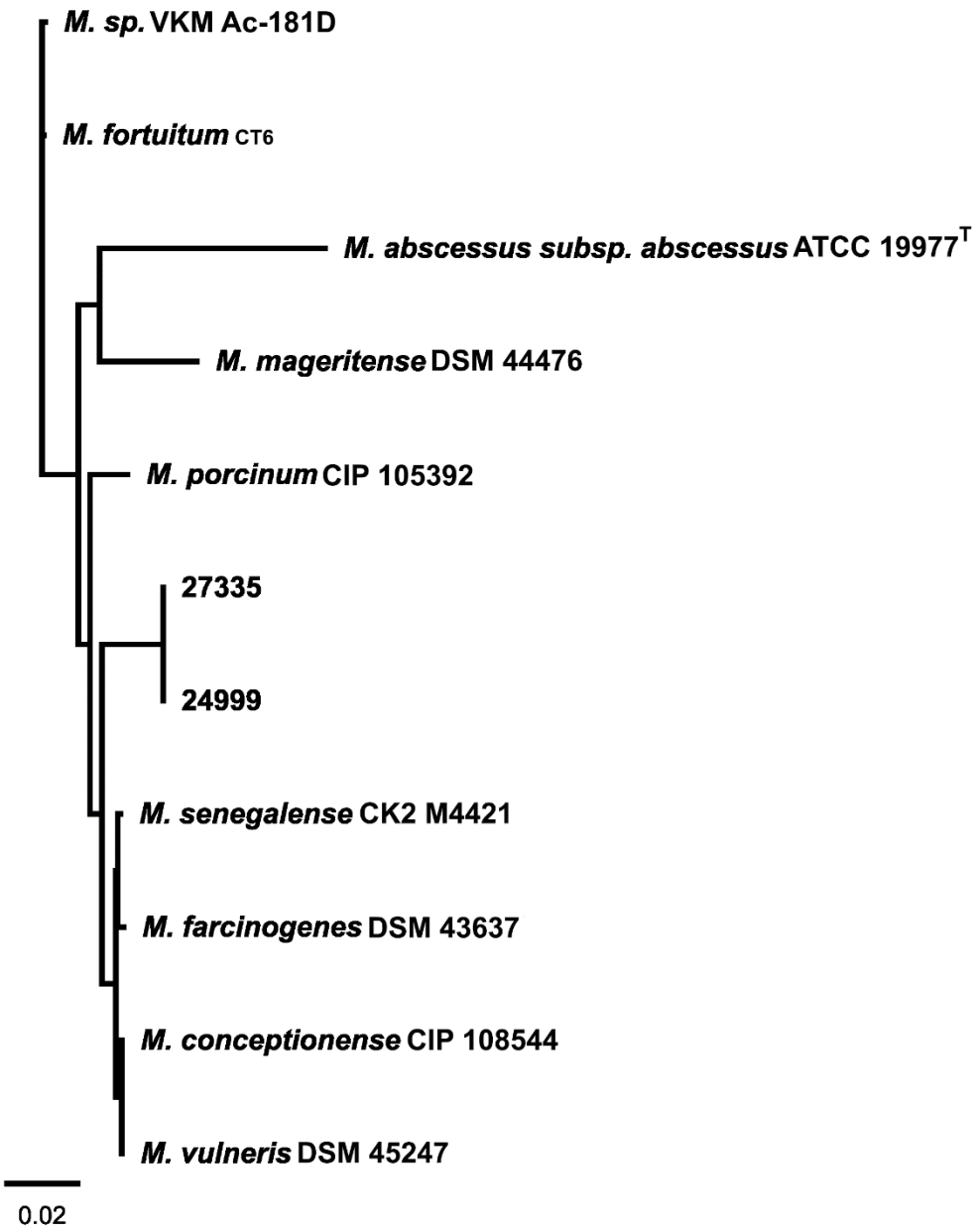

Figure 4.1. Maximum likelihood phylogenetic reproduction of the concatenated 16S, *hsp65*, and *rpoB* sequences from 24999 and 27335 compared to GenBank accessions (Supplementary Table 4.2). Branch support is denoted at the nodes in terms of 1000 bootstrap iterations. Bar represents 0.02 nucleotide substitutions per nucleotide position. ^{T} Denotes type strain

Supplementary Table 4.1. Primers used within this study to perform genotypic characterization of isolates.

Gene	Primer	Sequence 5'-3'	Reference
16S RNA	FD1	AGAGTTTGATCATGGCTCAG	Adekambi et al., 2004
16S RNA	RP2	ACGGCTACCTTGTTACGACTT	Adekambi et al., 2004
hsp65	Tb11	ACCAACGATGGTGTGTCCAT	Telenti et al., 1993
hsp65	Tb12	CTTGTCGAACCGCATACCCT	Telenti et al., 1993
rpoB	MycoF	GGCAAGGTCACCCCGAAGGG	Adekambi et al., 2003
rpoB	MycoR	AGCGGCTGCTGGGTGATCATC	Adekambi et al., 2003

Supplementary Table 4.2 GenBank accession information for isolates used in the 16S, *hsp65*, and *rpoB* phylogenetic tree reconstruction.

Species	Strain	Genbank accession	File type
M. abscessus	ATCC 19977	NZ_CP010397	Whole genome
M. conceptionense	CIP 108544	AY859684	16S rRNA
M. conceptionense	CIP 108544	AY859678.1	*hsp65*
M. conceptionense	CIP 108544	AY859695.1	*rpoB*
M.farcinogenes	DSM 43637	NZ_CCAY000000000.1	Whole genome
M.fortuitum	CT6	NZ_CP011269.1	Whole genome
M. mageritense	DSM 44476	NZ_CCBF000000000.1	Whole genome
M.peregrinum	CSUR P2098	NZ_CYSH00000000.1	Whole genome
M. porcinum	CIP 105392	AY457077	16S rRNA
M. porcinum	CIP 105392	AY458068	*hsp65*
M. porcinum	CIP 105392	AY262737	*rpoB*
M.senegalense	CK2 M4421	LDPU01000001	Whole genome
M.septicum	DSM 44393	NZ__CBMO000000000.1	Whole genome
M.sp	VKM Ac-1817D	NZ_CP009914.1	Whole genome
M.vulneris	DSM 45247	NZ_CCBG000000000.1	Whole genome

Chapter 5

PATHOLOGIC FEATURES OF MYCOBACTERIOSIS IN NATURALLY INFECTED SYNGNATHIDAE AND NOVEL TRANSCRIPTOME ASSEMBLY IN ASSOCIATION WITH DISEASE[1]

[1]Fogelson. S.B., Fast, M.D., Leary, J., and Camus. A.C. To be submitted to *Journal of Fish Diseases*

Abstract

Syngnathidae (seahorses, seadragons, and pipefish) suffer significant losses from nontuberculous mycobacteria (NTM). However, they produce markedly different lesions in response to the disease, notably infrequent granuloma formation, compared to other teleost species. The present study evaluated histological lesions in 270 syngnathid fish naturally infected by NTM to determine characteristic tissue changes. In addition, RNA-seq of the head kidney was performed to investigate the transcriptome of eight lined seahorses *Hippocampus erectus,* including two infected and six non-infected fish. Assembled and annotated putative transcripts serve to enrich the database for this species, as well as provide baseline data for understanding the pathogenesis of mycobacteriosis in seahorses. Microscopic lesions variably consisted of random foci of coagulative necrosis in multiple organs, containing high numbers of free bacteria and large aggregates or sheets of macrophages with cytoplasm laden with acid-fast bacilli. Granulomas were identified in only eight seahorses and some fish produced no appreciable inflammatory response. Putative components of the innate immune system (IL-1β, IL-6, TNF, NOS, TLR1, MH Class1, NF-κβ, TGF-β, MyD88) were identified in the RNA-seq dataset. However, a homolog for a key component in the TH1 adaptive immune response, IFN-γ, was not identified and may underlie the unique pathologic presentation.

Introduction

Mycobacteriosis is an important disease of captive and wild fish (1). The etiologic agents of mycobacteriosis are classified within a group of gram-positive, acid-fast, nontuberculous (NTM) bacteria that are ubiquitous in the environment. The most commonly identified NTM species in clinically ill fish include *Mycobacterium marinum*, *Mycobacterium* fortuitum, and *Mycobacterium chelonae* (2). In recent years, additional species of NTM have also been identified as significant fish pathogens, including *Mycobacterium shottsii* (3) and *Mycobacterium pseudoshottsii* (4) in striped bass *Morone saxatilis* (Walbaum) from Cheseapeake Bay, *Mycobacterium gordonae* from captive goldfish *Carrasius auratus* L. (5), and *Mycobacterium peregrinum* (6) and *Mycobacterium haemophilum* (7) in zebrafish *Danio rerio* (Hamilton)

Control of mycobacteriosis in fish and culture facilities is problematic and certain NTM pose a zoonotic risk to humans (8-10). NTM are highly resistant to disinfection and form biofilms that promote persistence under adverse environmental conditions (11, 12). In addition, antimicrobial susceptibilities range widely among NTM isolates, compounding difficulties associated with treatment unless proper species identification is achieved (13).

While disease caused by NTM occurs infrequently in immune compromised humans, many fish species are readily susceptible and most infections culminate in mortality. Stressors, including poor water quality, toxicants, handling, and high stocking densities, have been associated with increased disease prevalence (14, 15) and a link between genetic susceptibility and disease has also been suggested (16). Although all fish species are considered susceptible to mycobacteriosis, members of the families

Anabantidae (bettas and gouramis), Characidae (tetras and piranhas), Cyprinidae (danios, goldfish, and barbs), Cichlidae (cichlids), and Syngnathidae (seahorses, seadragons, and pipefish) appear to have increased susceptibility (1, 17-20). However, reports may be biased, as these families contain common aquarium species and may be overrepresented in datasets.

Mycobacteriosis is a disease of special concern in captive syngnathids (21, 22), where the most common species isolated is *M. chelonae* (18, 23, 24) . In addition, members of the Syngnathidae appear to have an atypical pathologic presentation when compared to other teleost species. However, a comprehensive evaluation across members of the family has not been performed. Microscopic lesions in most teleosts are characterized by discrete granulomas containing the mycobacteria. In contrast, granuloma formation is uncommon in syngnathids, making them an interesting model for study. At present, it is unclear whether these atypical lesions are a product of an ineffective or altered cell-mediated immune response, pathogen virulence, or a combination of the two.

Innate and adaptive immune responses are highly conserved in teleost fish and include many soluble and cellular components found in higher vertebrates (25). As part of the innate immune system, macrophages play a key role during NTM infections, using pattern recognition receptors as a first line of defense against infection and in the mobilization of the host immune system. Genes and gene products also commonly associated with responses to mycobacterial infection have also been identified in teleosts, including the up regulation of toll-like receptors. Specifically, TLR1 has been

reported in zebrafish infected with *M. marinum* and has been linked to the recognition of mycobacterial ligands (26, 27). Although the immune response of fish to NTM, such as *M. marinum*, has been researched in common laboratory species, host-pathogen interactions during infection remain poorly understood (28).

Activation of the adaptive immune system should occur following infection with mycobacteria, leading to interferon-gamma (IFN-γ) production by T helper 1 (Th1) CD4+ T-lymphocytes, recruitment of CD8+ cytotoxic T-lymphocytes, and induction of other pro-inflammatory cytokines in an effort to control dissemination. However, mycobacteria produce many virulence factors that aid in evasion of the host immune system and persistence within tissues (29). One important pathway that can be disrupted by mycobacteria is the Th1 host immune response. (30). Lack of a Th1 response can lead to uncontrolled proliferation of bacteria within tissues and dissemination throughout the body.

Although little information is available regarding immune responses to pathogens in syngnathids, rare reports provide preliminary evidence of the presence of a common innate immune system. For example, seahorses exposed to heat-killed *Vibrio* sp. up-regulate genes involved with the innate immune response to gram-negative bacteria (31). The histologic presentation of syngnathids with mycobacteriosis also suggests the presence of an innate immune response, but the capacity for an adaptive or cell-mediated response remains to be described. The transcriptome of the male brood pouch (32) demonstrated reproductive related gene homology between the potbellied

seahorse *Hippocampus abdominalis*, other teleosts, mammals and reptiles, suggesting transcriptomic analysis could also reveal similarities among immune related genes.

Two hundred and seventy syngnathid submissions from over 2000 fish cases evaluated by the University of Georgia Aquatic Pathology Service over a 10-year period were reviewed to assess common gross and histologic features of mycobacteriosis in this specific fish family. In addition, the head kidney of nine *Hippocampus erectus* were used to perform shotgun RNA sequencing (RNA-seq) to search for immune related genes. The transcriptome of *H. erectus* without mycobacteriosis was also compared to naturally infected individuals to observe whether differential expression of specific immune relevant genes occurs during infection and to describe the gene ontology during disease. This study contributes a significant amount of transcriptome data to enhance the database of previously described genes in syngnathids and provides information into the pathogenesis of mycobacterial disease in this group of fishes.

Materials and Methods

Histologic examination. A retrospective study of syngnathids, including seahorses (n=152), seadragons (n=65), trumpetfish (n=1), and pipefish (n=52) submitted to the University of Georgia Aquatic Pathology Service from November 2006 to June 2016 was performed. Gross and histologic descriptions in all animals were reviewed to assess characteristic changes associated with mycobacteriosis. Photomicrographs of lesions were taken with an Olympus BX41microscope and DP71 camera (Olympus America Inc., Center Valley, PA).

RNA isolation and *de novo* assembly. The anterior kidneys of nine *H. erectus* from multiple aquarium collections were harvested for total RNA extraction. The animals were diagnosed with mycobacteriosis (n=3) or determined to be uninfected (n=6) based on gross lesions, histopathology and culture findings. Anterior kidneys from infected fish were collected aseptically, placed immediately into 1.0 ml of TRIzol ® Reagent (Invitrogen, USA), homogenized using a nuclease free sterile mortar and pestle, then frozen at -20°C until processed further. Fresh uninfected anterior kidneys were preserved in a -80°C freezer until extraction could be performed.

Extraction of total RNA was performed using a Qiagen RNeasy mini extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Approximately 1 µg of total RNA was submitted to the University of Georgia Genomics Facility for paired end 75 high output flow cell next generation sequencing on a NextSeq 500 (Illumina, Inc, San Diego, CA). cDNA Libraries were constructed using Kapa Biosystems RNA library preparation chemistry and indexing primers with ligation of adapters for sequencing. Illumina RNA-NextSeq data was groomed to readable fastq files and assembled by Trinity (33). The assembled transcripts were evaluated using post-assembly transcriptome analysis tools, including RSEM and Trinotate (Grabherr et al., 2011), to assess quality statistics of the assembly, estimate abundance, gene length, and annotate the transcriptome. Differential expression analysis was performed using Trinity and edgeR (33). Transcripts associated with response to mycobacterial infection were evaluated in relation to previously described homologous transcripts in other vertebrates from the UniProtKB/Swiss-Prot database. To characterize the gene

ontology, Blast2GO Pro was used on the differential expression transcripts with a $p < 0.05$ and greater than two-fold change in expression.

cDNA synthesis and qPCR. RNA, isolated as above, was converted to cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Equivalent amounts of total RNA (1096 ng), measured by NanoDrop (Thermo Fisher), were primed with oligo d(t) per manufacturer's instructions. cDNA was frozen at -20°C prior to qPCR analysis.

Comparative quantification of genes of interest (GOI) transcripts for eight seahorses were assessed by qPCR on a Bio-Rad iQ5 thermal cycler and iQ5 Optical System Software (Bio-Rad Laboratories Hercules, CA, USA). qPCR mixes consisted of 150 nM of each primer, 200 µM dNTP's in Bio-Rad iQ SYBR Green Supermix, with 1 ul of cDNA and adjusted to a final volume of 20 µl with RNase free water. A 2-step protocol of 95°C for 10 s and 58°C for 30 s for 35 cycles was used. Melt curves were generated to assess specificity of amplification. Preliminary assay development measured efficiencies between 91 and 102% for individual primer sets (R^2 >95%) (Table 5.1). Amplicons were between 100-135 bp. Sanger sequencing was performed on all amplicons to verify their identity. Reactions were run in triplicate for each GOI/fish combination. Normalized gene expression values were calculated by the MNRQ method as modified in the Bio-Rad iQ5 System Software. Reference genes used for normalization included heat shock protein 90 (HSP90), hypoxanthine phosphoribosyl transferase (HPRT) and β-tubulin. One uninfected sample, S17, was arbitrarily chosen as the control against which to reference gene expression.

qPCR verification of actin, β2 microglobulin, cyclooxygenase 2 (COX2), MHC Class II, NADPH oxidase 2 (NOX2), recombination activating gene 1 (RAG1), eosinophil peroxidase (EPER), human endogenous retrovirus long terminal repeat sequence associated protein B7 family (HERV LTR AP), interferon regulatory factor 4 (IRF4), interleukin 1β (IL-1β), interleukin 1β receptor (IL-1β receptor), and toll-like receptor 1 (TLR1) was performed. Primer sequences are shown in Table 5.1. Output of the qPCRs were imported into GraphPad Prism 7.0 software, where a Pearson correlation was run on the Fragments Per Kilobase Million (FPKM) recovered from the transcriptome dataset versus the mean qPCR normalized fold expression findings to correlate the de novo transcriptome data to the qPCR results. Computed mean normalized fold expression values were tested using a t-test and a two-stage linear Benjamini, Krieger, Yekulieli FDR procedure (34) to determine statistical significance.

Results

Gross, histopathology, and bacteriology findings. Ninety-two of the 270 syngnathid cases reviewed (34%) were diagnosed with mycobacteriosis by histopathology, culture or both (in rare cases culture positive animals were negative via histolopathology). Species evaluated included weedy seadragons *Phyllopteryx taeniolatus* (Lacepède), leafy seadragons *Phycodurus eques* (Günther) , lined seahorses *Hippocampus erectus* (Perry), longsnout seahorses *Hippocampus reidi* (Ginsburg), potbelly seahorses *H. abdominalis* (Lesson), White's seahorses *Hippocampus whitei* (Bleeker), spotted seahorses *Hippocampus kuda* (Bleeker), banded pipefish *Doryrhamphus dactyliophorus* (Bleeker), bay pipefish *Syngnathus*

leptorhynchus (Girard), gulf pipefish *Syngnathus scovelli* (Evermann and Kendall), green pipefish *Solenostomus cyanopterus* (Bleeker) , alligator pipefish *Syngnathoides biaculeatus* (Bloch), and blue line pipefish *Doryrhamphus excisus* (Kaup). In most cases, the age and sex of the animal was not provided with the submission. Species identification of 25 mycobacterial isolates was made by either *hsp65* or whole genome sequencing (WGS). Twenty isolates were identified as *M. chelonae*, two isolates cultured from pipefish were identified as *M. marinum*, and three isolates were only designated as *Mycobacterium* sp.

Gross and histologic lesions were similar, occurring indiscriminately in tissues, in all syngnathid fish. Commonly reported gross changes, observed in 27 fishes, included tan nodules protruding from the skin of the tail, presence of opaque coelomic fluid, and friable, pale tan areas of necrosis in a variety of organs (Figure 5.1A, C, E, F). The most frequently reported histologic lesions occurred in kidney (n=55), liver (n=44), gills (n=40), skeletal muscle (n=35), coelomic mesentery/adipose (n=32), and skin (n=31), with the majority of skin lesions associated with the tail (21/31). Histologic lesions were characteristically necrotizing and granulomatous. Variably sized, multifocal to coalescing areas of necrosis were infiltrated by high numbers of plump macrophages with cytoplasm laden with acid-fast bacilli (Figure 5.1D, H, I). Acid-fast bacteria were also often free within tissues as individualized bacteria or in large colonies. Lesions were expansile, with no fibrous encapsulation and multinucleated giant cells were never observed. In addition, blood vessels often contained bacteria, free in circulation and phagocytized by macrophages. In the gills, lamellar capillaries were often distended

and occluded by large masses of mycobacteria (Figure 5.1G). Frequently, little or no inflammatory response was associated with the presence of bacteria, particularly when present in vascular lumens. Low numbers of granulomas containing acid-fast positive bacilli were identified in only eight seahorses.

De novo assembly, differential expression and GO analysis. The transcriptomes of eight of nine *H. erectus* seahorses were assembled. One animal was excluded from the analysis due to poor quality of the de novo assembly and low transcript recovery. According to the Trinity contig statistics, assembly of transcriptomes from the two infected and six non-infected animals resulted in 318,395 putative transcripts and 201,199 putative genes. The average contig length was 1030 nucleotides with an N50 of 1894 nucleotides. Differential expression of infected fish versus non-infected fish resulted in 14,000 transcripts with a $p < 0.05$ and greater than two-fold change. Down regulated transcripts composed 71% of the dataset.

A Cloud Blast query of the putative transcripts against the UniProtKB/Swiss-Prot database resulted in 7,786 successfully annotated sequences. Three main GO designations from the annotated files were compiled. These included 7,293 cellular component genes, 7,137 molecular function genes, and 7,123 biological process genes. Numerous biosynthetic pathways and metabolic processes were equally represented in the dataset. A representation of the main components within each specific GO category are presented in Figures 5.2 through 5.4 and Supplementary Figure 5.1. In Figure 5.2, many intracellular components of the cell response in infected fish appear equally represented, but five categories (cytosol, protein complexes, extracellular exosome,

integral component of membrane, and nucleoplasm) were highly regulated at the cellular level. Intracellular components of the cell response differentially expressed at the molecular level between infected and non-infected fish, such as metal ion binding and enzymatic activity, are prevalent in the dataset (Figure 5.3). Within the biological processes category of the differentially expressed putative transcripts, an immune response category was identified, containing 777 sequences that related to response to IFN-γ, toll-like receptor signaling pathways, T cell receptor signaling pathways, and Fc-gamma receptor signaling (Figure 5.4). Only 29% of the putative immune genes were considered up-regulated. A list of the putative immune genes identified as responses associated with IFN-γ stimulation are referenced in Table 5.2.

qPCR. Potential immune-related transcripts in *H. erectus* were identified by BLAST analysis using query sequences from model transcriptome databases. Twelve genes from the RNA-seq dataset were evaluated for differential expression using qPCR. Pearson correlation analysis of the FPKM values from the RNA-seq dataset versus the qPCR normalized fold expression values did not produce a linear relationship globally between the two variables. However, results of the t-test performed on the qPCR values revealed significant differences between the mean values of 7/12 genes (actin, β2 microglobulin, COX2, EPER, HERV LTR AP B7 family, IL-1β, and IL-1β receptor) in both the infected and non-infected fish (Figure 5.5). β2 microglobulin, IL-1β, and IL-1β receptor were significantly differentially expressed and downregulated in the RNA-seq and qPCR normalized fold expression datasets.

Discussion

NTM are an important group of pathogens, causing disease in a wide variety of hosts, including hundreds of fresh, salt, and brackish water fish species (9, 19, 35-37). Commonly displayed in aquaria, seahorses, seadragons, and pipefish suffer significant losses, due primarily to *M. chelonae* (18, 22, 23). In a study by Fogelson et al. (in review), whole genome sequencing and analysis of 28 *M. chelonae* isolates revealed minimal sequence variation between *M. chelonae* strains causing disease in fish, reptiles, and mammals, including humans, further highlighting the significance of this agent.

While *M. chelonae* was also the most commonly identified isolate in this study, other NTM less frequently infect syngnathids and cause similar lesions (21). As observed here and in other studies, it is uncommon for syngnathids infected with *M. chelonae* and other NTM to produce discrete granulomas typical of infection in other teleost species, such as African cichlids *Oreochromis mossambicus* (Peters) and Atlantic salmon *Salmo salar* (L.) (17, 38). These observations suggest a genetic basis may underlie the florid nature of infections and lack of granuloma formation among the syngnathid fish.

Lesions in syngnathids involve large numbers of bacilli and are more consistent with acute, fulminating, septicemic infection, as opposed to the relatively low numbers of bacteria and chronic granuloma formation seen commonly in other teleosts. The atypical presentation in syngnathids could be related to frequent exposure to large numbers of bacteria and duration of infection, but an inability of these fish to mount an effective cell-mediated immune response is suspected. While environmental monitoring

for mycobacteria is not typically performed in aquaria, the ubiquitous nature of NTM and their common presence in aquatic biofilms suggests that syngnathids and other fish are regularly exposed to similar infective conditions (9, 39). However, dose dependent responses have been demonstrated in goldfish inoculated with *M. marinum*. High doses produced acute tissue necrosis, inflammation, systemic dissemination of bacteria and death in 17 days or less, while lower doses produced chronic granulomas and survival beyond 56 days (40). While similar challenges have not been performed in syngnathids, the consistency of lesions within this family of fish and their dissimilarity to those of other teleosts kept in similar conditions suggests an intrinsic component is involved.

To better understand the pathogenesis of mycobacteriosis in syngnathids and their associated immune response, the transcriptomes of two infected and six non-infected *H. erectus* were evaluated using RNA-seq. Annotation of the dataset revealed transcripts designated by Blastx as homologous to previously described putative vertebrate genes that were globally categorized into cellular components, molecular functions or biological processes (Figures 5.2 through 5.4 and Supplementary Figure 5.1). Analysis also identified putative components of the *H. erectus* innate and adaptive immune pathways (Supplementary table 5.2).

Only seven of 12 differentially regulated genes from the RNA-seq dataset had significantly different values when comparing their mean normalized fold expression for infected versus non-infected fish. However, disparity in age and life stage, genetic diversity, length of infection, underlying disease, environmental conditions, and the low

number of biological replicates could have created variability within the results and limiting the usefulness of this data (41). For instance, Whittington et al. (32) showed differences in the transcriptome of the seahorse brood pouch at a range of time points before, during, and after pregnancy. Similarly, environmental conditions, such as poor water quality, can also alter the physiologic transcriptome profile of syngnathids (42, 43). While greater biological replication would have decreased variability in results, the study was limited by the availability of these valuable fish specimens. It is possible that reads expressed in low number may under represent the relative abundance of a specific transcript in the RNA-seq dataset and produce non-concordant results.

Three of the 12 arbitrarily chosen differentially regulated genes from the RNA-seq dataset were correlated with their qPCR values. Lack of concordance may have resulted from aberrant assembly for some transcripts and nucleotide calling, leading to mismatches in the sequences.

When compared to the transcriptome of zebrafish experimentally infected with *M. marinum*, the *H. erectus* transcriptome contains many homologous putative genes associated with classical innate immunity (28). The early-phase of mycobacteriosis is known to elicit an innate immune response, with up-regulation of acute inflammatory cytokines, such as IL-1β, IL-6, and TNF (28, 44, 45). In addition, intracellular microbicidal products, such as NOS and other reactive oxygen species, are expected to be elevated. In mice models of mycobacterial infection, IL-1β is important in early-phase protective immunity by providing key downstream signals for macrophage activation, cytokine production, and chemotaxis of inflammatory cells (46). TNF is also

important as a chemotactic factor for inflammatory cells (47). In addition, binding of TNF receptors produces many different outcomes, including transcription of nuclear factor kappa beta (NF-κB), induction of apoptosis via caspase mediated cell death, and induction of the caspase independent cell death pathway, necroptosis (48). Furthermore, TNF can also elicit the downstream production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), which are instrumental in the intracellular killing of microbes (49). Although the immune components IL-1β, lL-6, TNF and NOS, were present in the transcriptome of *H. erectus,* all were down-regulated more than two fold in the differential analysis. Deficiency of one or more of these innate cytokines, can lead to uncontrolled bacterial proliferation, with little or no inflammatory response to infection, similar to that observed in syngnathids.

Other putative immune related genes found in *H. erectus* that play an integral role in the pathogenesis of mycobacteriosis were major histocompatibility class I (MHC-I), myeloid differentiation factor 88 (MYD88), NF-κβ, transforming growth factor beta (TGF-β), NADPH oxidase (NOX2), and complement C3. Among these, only MHC I was up-regulated, while the remainder were all down-regulated in the differential expression.

Consequences of decreased innate cytokine and microbicidal element expression during mycobacterial infection, include defective granuloma formation, enhanced mycobacterial growth, defective leukocyte migration to areas of infection, and decreased microbial killing (50). This could be responsible for the massive numbers of intracellular and extracellular mycobacteria observed in infected syngnathids. In addition, 75% of the Fc-gamma receptor signaling pathways were down-regulated.

Decreased phagocytic capability could also contribute significantly to uncontrolled extracellular mycobacterial proliferation (51).

IFN-γ enhances resistance and control of infection by intracellular bacterial pathogens, including mycobacteria (52). Although analysis of the full RNA-seq dataset did not identify a homolog to IFN-γ, pathway components associated both up- and down-stream of this cytokine were identified in the differential expression, suggesting a functional counterpart exists in *H. erectus* (Supplementary Table 5.2). Failure to identify an IFN-γ homolog may be due to absence of a transcript or to low sequence homology with other vertebrate sequences available in public databases (53, 54). Molecules involved in the IFN-γ signaling pathway, such as IL-12, IL-17, NOD2, interferon regulatory factor (IRF), and NF-κβ, were identified in the transcriptome. Differential expression revealed 74/95 putative genes within the IFN-γ activation pathway as down-regulated (Table 1). As a critical component in Th1 mediated activation of macrophages and killing of mycobacteria, IFN-γ is also essential in activating macrophages to differentiate into epithelioid cells that aggregate to form granulomas and/or fuse to produce multinucleated giant cells (55). In humans, increased susceptibility to mycobacterial infection has been linked to decreased expression of IFN-γ, resulting from immunosuppression, concurrent disease, or impaired functional activity due to receptor gene mutations (56). Without sequencing the *H. erectus* genome and/or testing the activity of gene products, it cannot be stated with certainty whether it has an IFN-γ-like molecule. It is also possible that an IFN-γ gene is present but expressed at undetectable levels, that a constituent in the pathway (e.g. a receptor)

is absent, or disruption of the signaling pathway is hindering transcription and its presence in the dataset.

Evaluation of the histologic changes in 92 syngnathid fish naturally infected with *M. chelonae* revealed necrosis, granulomatous inflammation, and high numbers of acid-fast positive bacilli as the predominant lesions. Lesions were often widely, although randomly, distributed among tissues. Difference in lesion morphology and tissue responses between syngnathid and non-syngnathid fish species infected with *M. chelonae* suggest a deficiency in the cell-mediated immune response of syngnathids. However, further investigation is needed to determine whether infective dose or the duration of infection following controlled bacterial challenge plays a role in the pathological presentation of disease in these fish. The transcriptome profiles of infected and uninfected *H. erectus* provide evidence that components of the innate and adaptive immune responses are present in syngnathids. However, a key element (IFN-γ) of the adaptive intracellular immune response to mycobacteria was not identified and could underlie the marked susceptibility and atypical pathologic lesions observed in this group of fish. The large transcriptome dataset can also serve as a baseline foundational source of information to advance future research into the pathogenesis of mycobacteriosis and possibly other intracellular pathogens within this family of fish.

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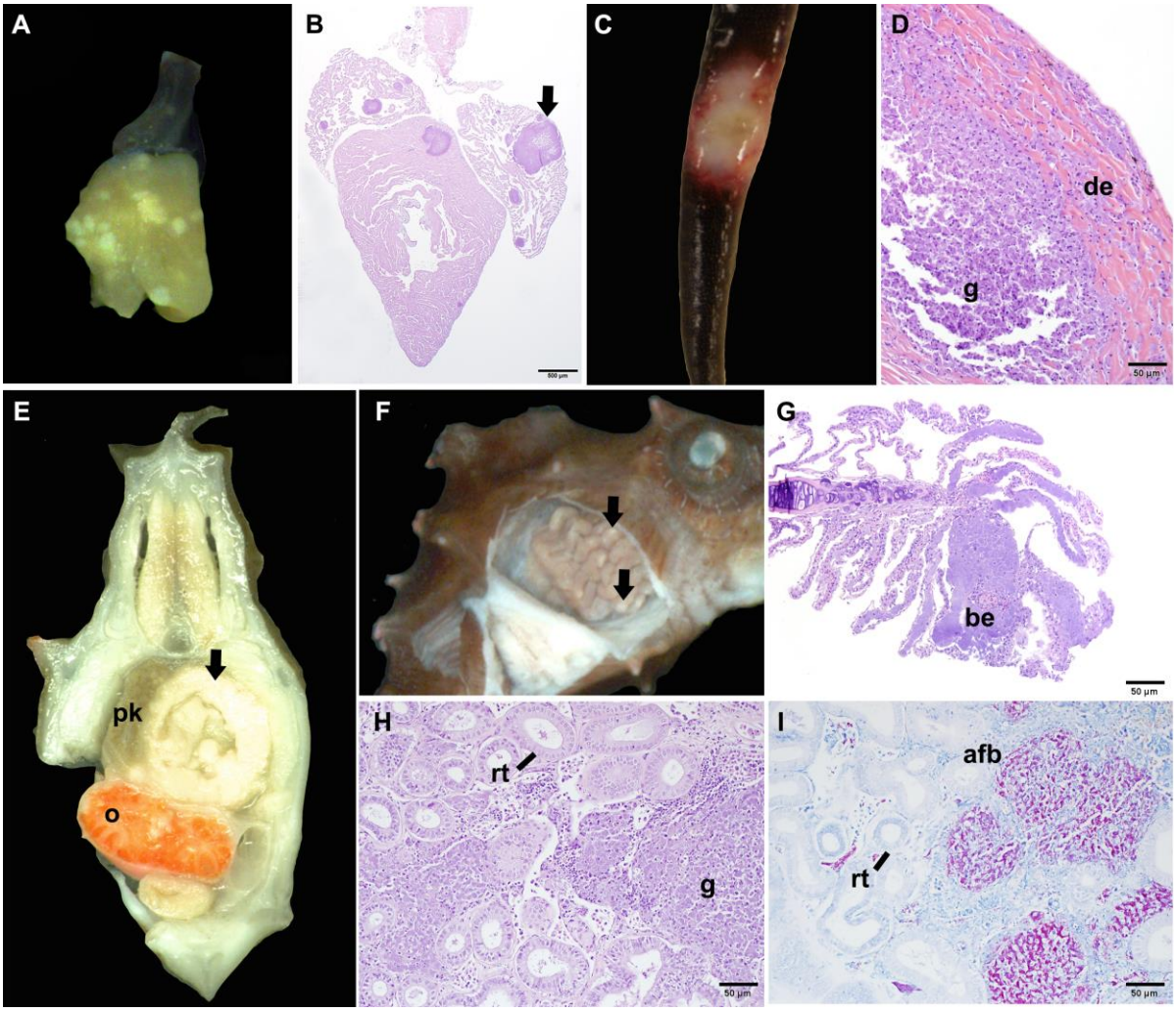


Figure 5.1. Characteristic gross and microscopic lesions of mycobacteriosis. A. Weedy seadragon *Phycodurus eques* heart with multifocal, raised white nodules elevating the epicardium. B. Photmicrograph of weedy seadragon heart with multiple bacterial colonies in the mycocardium (arrow). H&E, scale bar= 500µm. C. Lined seahorse *Hippocampus erectus* tail with focal ulceration and hemorrhage rimming tan necrotic tissue. D. Photomicrograph of lined seahorse skin with epidermal ulceration and infiltration of the dermis (de) and hypodermis by bacteria laden macrophages (g) and free bacilli. E. Lined seahorse body (transverse section) with necrosis of the posterior

kidney (pk) and ventral displacement of the ovary (o) by a large aggregation of tan, friable necrotic debris (arrow). F. Lined seahorse gills with pinpoint white nodules protruding from lamellae (arrows). G. Photomicrograph of a lined seahorse gill filament with lamellar capillaries dilated by bacilli (be) H&E,scale bar=50µm. H. Photomicrograph of weedy seadragon posterior kidney with sheets of macrophages laden with bacilli (G) effacing renal tubules (RT) and hematopoietic tissue. H&E, scale bar=50µm. I. Photomicrograph of weedy seadragon posterior kidney highlighting numerous acid-fast positive bacilli in the tissue and within macrophages. Ziehl-Neelsen, scale bar=50µm

Table 5.1 Primers used for qPCR analysis.

Gene	Primer name	Primer sequence	Amplicon size	Efficiency
HPRT1	Hprt_183F	CATTGTTGCCCTCTGTGTGC	125 bp	93.8%
	Hprt_307R	TGAGGCGAATGAAGTCCACC	125 bp	
HSP90	HSP90_634F	AATACGCCTGGGAGTCCTCT	117 bp	92.6%
	HSP90_750R	TGTACTCCGTCTGGTCGTCT	117 bp	
beta tubulin	btb_1204F	GCTGTTCAAGCGCATCTCTG	116 bp	90.0%
	btb_1319R	TGTTGCTCTCTGCCTCTGTG	116bp	
Actin	Beta actin_605F	CCCATCTACGAGGGTTACGC	117 bp	91.0%
	beta actin_721R	GGTGGTGAAGGAGTAGCCAC	117 bp	
beta 2 microglobulin	b2M_256F	AGTCGTTCAGGTGTACAGCC	113 bp	93.0%
	b2M_368R	CGTTTCTCAGCAGGTCCACT	113 bp	
COX2	COX 2_496R	ACAATCAAAGCAGTTGGCCA	117 bp	96.8%
	Cox 2_496R	CCGGAATTGACCTGGTGTGA	117 bp	
Eosinophil peroxidase	Eper_798F	TAAACTGCGAGGAGAGCTGC	113 bp	95.3%
	Eper_910R	CGATCTGAAGGAGGGGATGC	113 bp	
HERV LTR AP (B7 Family)	HERV_517F	TTCTCTGCTGCTCAAGGACG	100 bp	95.9%
	HERV_616R	GTGGAGGTTGACGAACGACT	100 bp	
IL-1β receptor	IL1R_506F	CACGTCAGGTTGCTTCAAGC	109 bp	92.3%
	IL1_613R	GTCACGCCTACTGTGAGGAC	109 bp	
MHC Class II alpha chain	ClassII_310F	GATCCTCCCTCCCATCCCAT	113 bp	94.5%
	ClassII_422R	CAGTGAACTTTGACAGGCGC	113 bp	
NOX2 (cytochrome b)	cytb_687F	ATTAGGATTCGCCGCCCTAC	114 bp	97.0%
	cytb_800R	TGGGGTGGAGTTACTAGGGG	114 bp	
IL-1β	IL1b_79F	ACCAACCTGTTCCTGTCGTG	135 bp	95.6%
	IL1b_213R	GTCGCGCTTGTAGAAGAGGA	135 bp	
RAG1	RAG1_1682F	TGTACCGGACTGTCAAAGCC	112 bp	102.0%
	RAG1_1793R	GGCTGCCATTCAAACTGGTG	112 bp	
IRF4	IRF4_794F	GTGTACCACCAAGAGTCCCG	106 bp	94.6%
	IRF4_899R	AGGGAAGTTGAGCCATTCCG	106 bp	

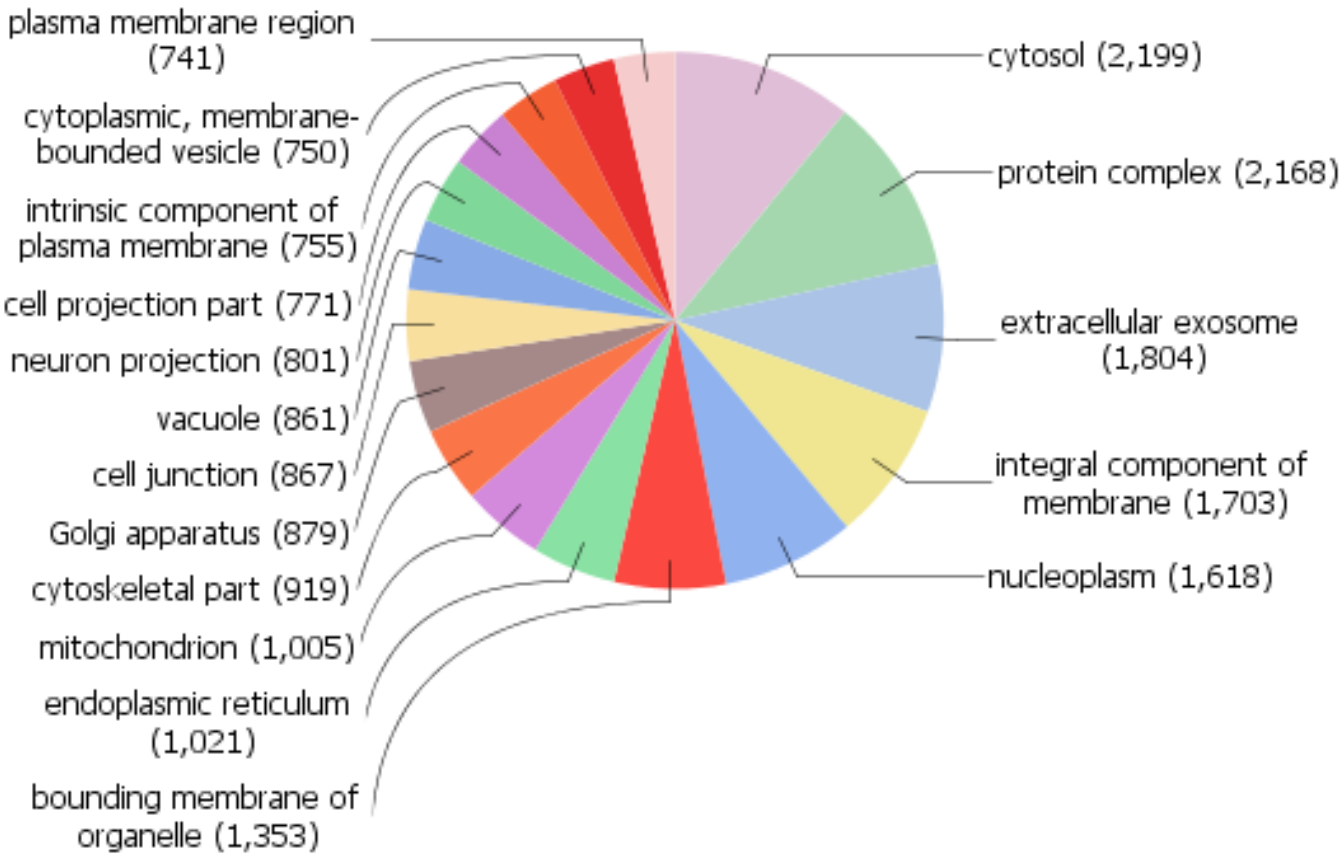


Figure 5.2. Blast2GO produced diagram of the differentially expressed transcripts that represent cellular processes.

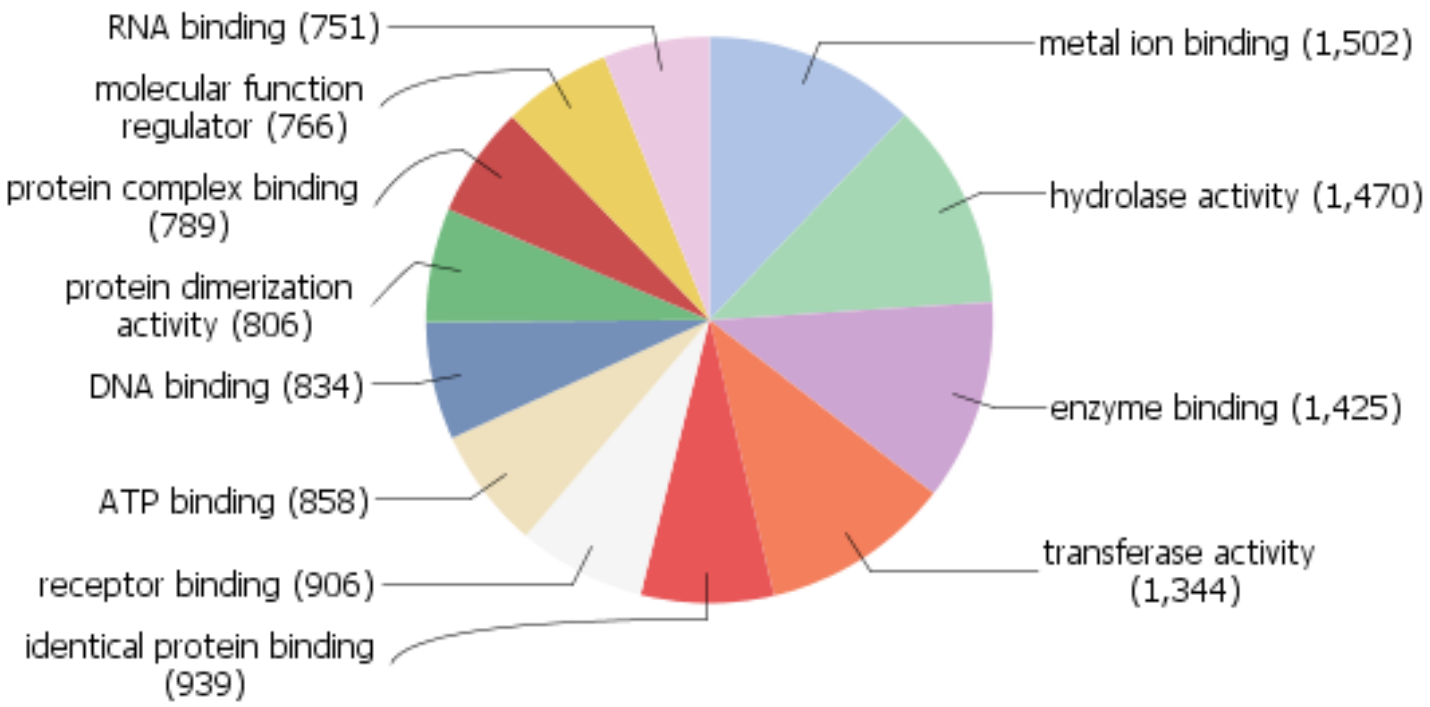


Figure 5.3. Blast2GO produced diagram of the differentially expressed transcripts that represent molecular function.

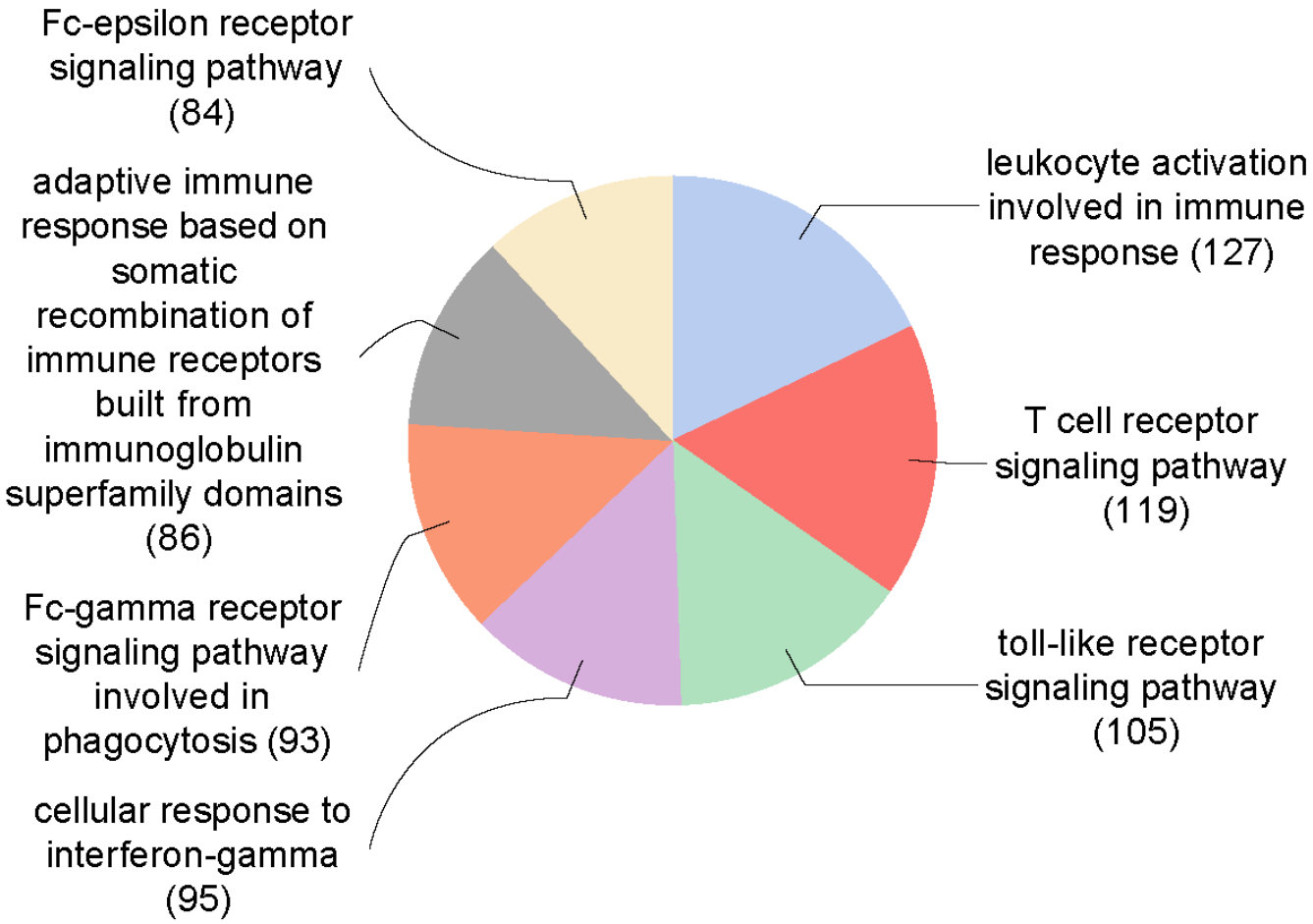


Figure 5.4. Blast2go produced representation of immune components observed within the biological processes category.

Table 5.2. Sequences identified in the GO produced by Blast2go related to the IFN-γ signaling pathway.

Immune pathway	UniProt gene designation	Regulation
Cellular response to IFN-γ	S26A6_MOUSE, S26A6_HUMAN, KPCD_CANFA , PTN2_HUMAN , LYN_MOUSE, S26A2_RAT, SOCS3_CHICK, TYK2_HUMAN , KPCT_MOUSE , AIF1_MOUSE , HEMO_DANRE , PTN1_MOUSE , JAK1_DANRE , AIF1L_MOUSE, GILT_HUMAN , GILT_PIG, STAT1_PIG , CSPG5_MOUSE, HNRPR_HUMAN, HNRPQ_MOUSE, NR1H3_BOVIN, TRIM1_HUMAN, SYEP_MOUSE , HCK_HUMAN, NR1H3_MOUSE, HCK_MACFA, DAPK3_HUMAN, KPCD_HUMAN , HNRPQ_HUMAN, HCK_MOUSE, HS90A_RABIT, IRF2_CHICK , SYEP_HUMAN	Down
	JAK2_MOUSE , NR1H3_BOVIN, STAT1_HUMAN, LCK_CHICK, PTN1_RAT, KCC2D_RAT, MRC1_HUMAN , C2TA_HUMAN , TEC_MOUSE, JAK2_PIG, IRG1_HUMAN , DNJA3_HUMAN, PTN2_RAT, KCC2G_HUMAN, STAT1_PIG, KPCD_RAT	Up

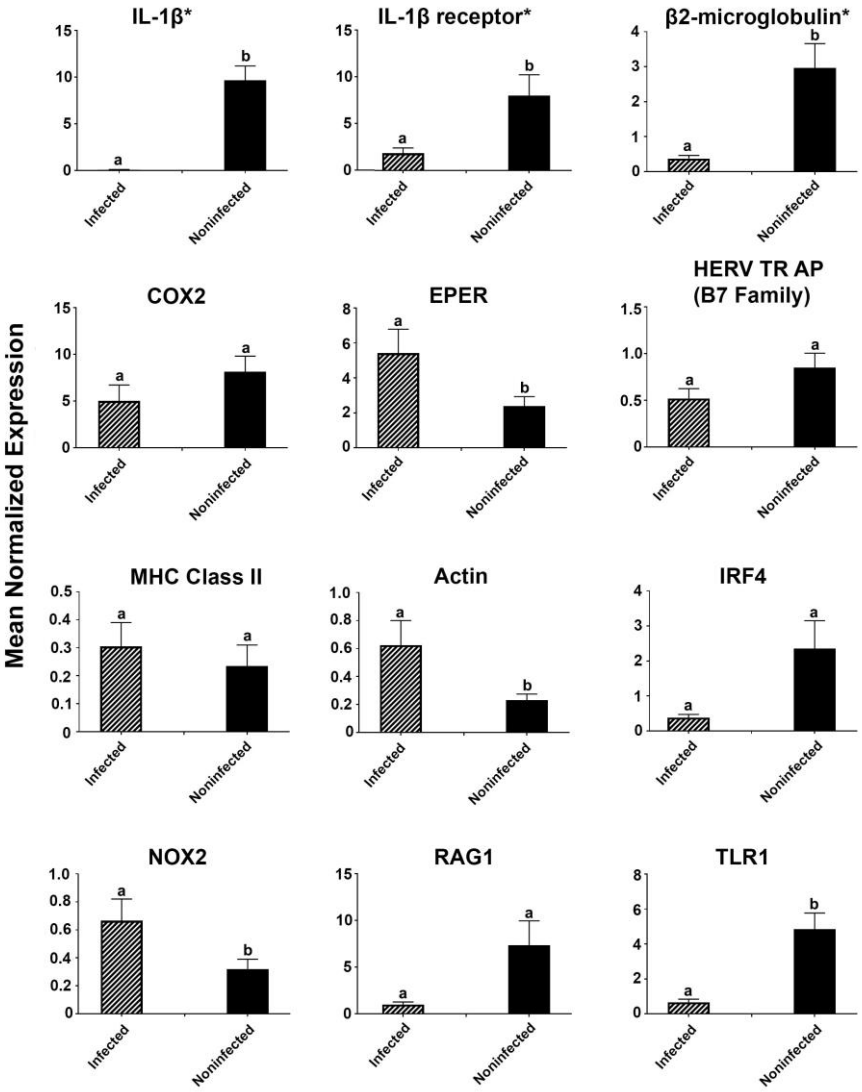
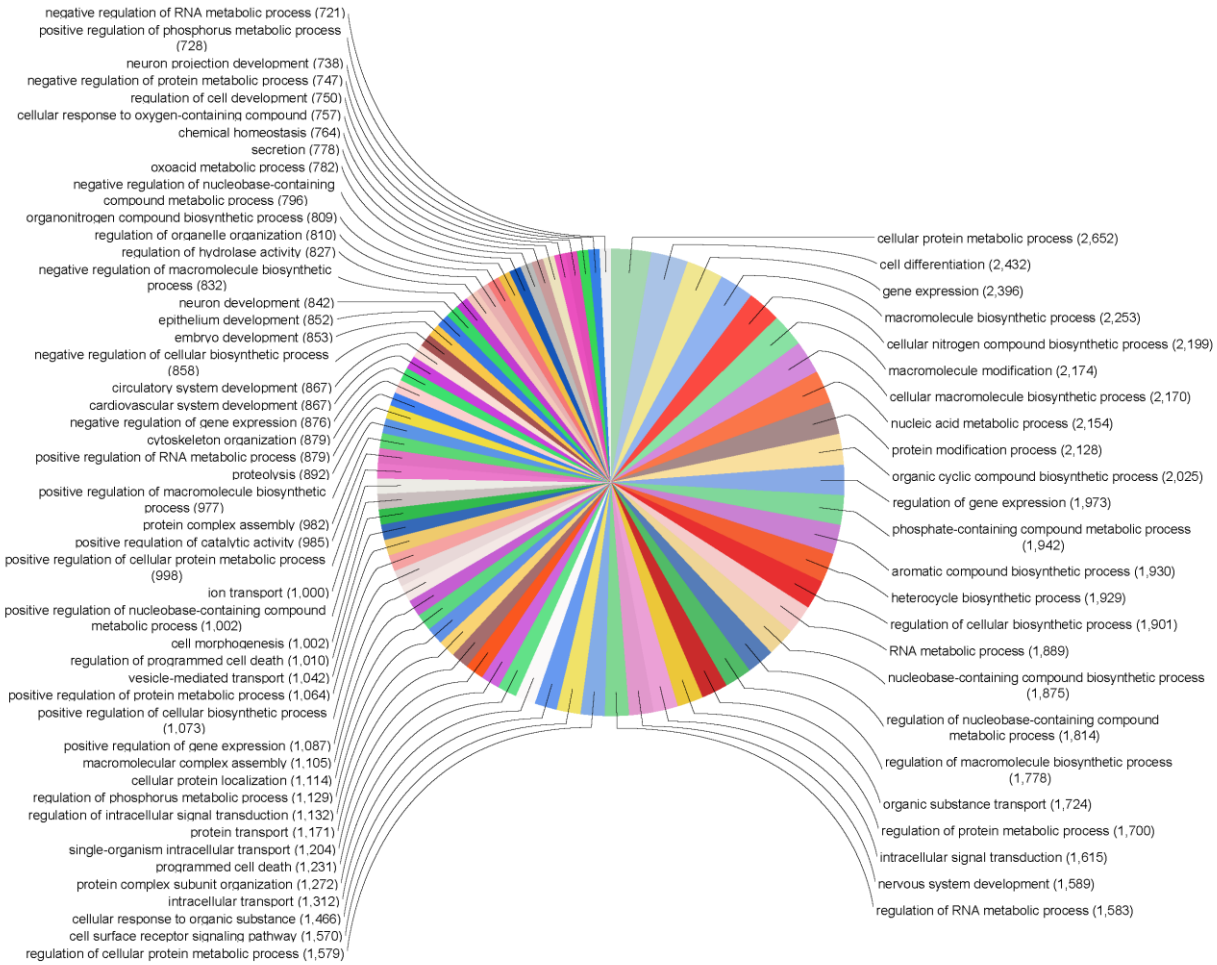


Figure 5.5. Mean qPCR normalized expression values of *H. erectus* naturally infected with *M. chelonae* (n=2) versus non-infected *H. erectus* (n=6). For each gene, significant difference in the mean normalized fold expression was calculated. Genes with a $p<0.01$ had significantly different means and have an “a” or “b” over the bar to denote significant difference between groups. Genes in the first row show concordance with RNA-seq data and have an asterisk (*) next to the name.

Supplementary Table 5.1. Summary of individual animal RNA-seq results.

Sample ID	Disease status	Read count	Putative transcript count
2	Infected	50,967,296	132,827
8	Infected	554,469,736	31,178
12	Infected	64,603,324	159,853
17	Uninfected	50,810,412	137,739
18	Uninfected	51,164,582	172,860
19	Uninfected	55,332,732	169,635
20	Uninfected	68,141,028	90,207
21	Uninfected	61,798,692	163,726
23	Uninfected	62,576,712	54,092



Supplementary Figure 5.1 Blast2GO produced diagram of the differentially expressed transcripts that represent biological processes.

Supplementary table 5.2. GO analysis of differentially responding putative immune genes in *H.erectus* infected with *M. chelonae*.

Immune pathway	UniProt gene designation	Regulation
Innate response	WWP1_MOUSE, PSME1_BOVIN, COL12_DANRE, MYD88_TAKRU, XIAP_XENTR, ABL1_HUMAN, PTN2_HUMAN, ATG9A_BOVIN, KSYK_RAT, BIRC2_HUMAN, CATK_BOVIN, S26A2_RAT, TAB2_DANRE, MALT1_HUMAN, RSAD2_SINCH, NEMO_MOUSE, KPCD_CANFA, CFAB_BOVIN, KPCT_MOUSE, AIF1_MOUSE, LGR4_DANRE, IKBA_MOUSE, HEMO_DANRE, PTN1_MOUSE, S26A6_HUMAN, H2AX_DANRE, S26A6_MOUSE, IPO7_HUMAN, PDK1_HUMAN, IL6_PAROL, NFKB1_HUMAN, PSB6_BOVIN, PRS7_HUMAN, PSMF1_MOUSE, PDK2_HUMAN, KPCD2_HUMAN, GRP3_HUMAN, YES_DANRE, TLR5_MOUSE, HAVR1_MOUSE, PLCG2_HUMAN, KAPCA_MOUSE, MAPK3_MOUSE, PK3CD_MOUSE, AIF1L_MOUSE, GCH1_HUMAN, PSB9_ORYLA, TNIP1_HUMAN, S14L1_MOUSE, KPCL_HUMAN, TBB1_GADMO, IKBA_HUMAN, UBE2K_BOVIN, PCBP3_HUMAN, SAMH1_DANRE, GILT_HUMAN, CAZA1_BOVIN, DMBT1_MOUSE, USP9X_HUMAN, PTN12_MOUSE, GILT_PIG, RN19B_DANRE, FLOT1_CARAU, FLOT1_PIG, PAK3_RAT, CY24B_MOUSE, DDX3X_MOUSE, NR1D1_HUMAN, NOD2_BOVIN, KPCL_RAT, PA2I_LATSE, PELI2_HUMAN, UROM_HUMAN, KS6A6_DANRE, CSPG5_MOUSE, IDE_HUMAN, UN13D_HUMAN, DYH10_HUMAN, ADAM8_MOUSE, TYK2_HUMAN, IRAK3_MOUSE, PSME3_CHICK, RPC4_BOVIN, PK3CB_RAT, HNRPR_HUMAN, IPO7_MOUSE, OTUL_MOUSE, HNRPQ_MOUSE, SOCS3_CHICK, KPCE_RAT, FAK1_RAT, PAK3_XENLA, DECR_RAT, TNIP2_HUMAN, CO8B_PAROL, CBP_HUMAN, TRIM1_HUMAN, LYN_MOUSE, CY24A_MOUSE, H2B1_DANRE, HCK_HUMAN, KAPCB_RAT, SAMH1_MOUSE, MYO1E_MOUSE, H2A_ONCMY, HMGB2_HUMAN, TNIP2_MOUSE, FLOT2_DANRE, NR1H3_MOUSE, HCK_MACFA, PAK2_RAT, NR1D2_HUMAN, VAMP2_RAT, SLAP1_RAT, ASM3A_BOVIN, SCAR3_MOUSE, SUSD6_MOUSE, OTU7A_HUMAN, DAPK3_HUMAN, SRC_DANRE, SYEP_MOUSE, CY24B_BISBI, ATG9A_HUMAN, PSD12_MOUSE, JAK1_DANRE, APLP2_HUMAN, CO3_ONCMY, KPCD_HUMAN, RHOG_HUMAN, UB2D3_BOVIN, PSB3_ONCMY, HNRPQ_HUMAN, LEG9_RAT, HCK_MOUSE, ADA12_HUMAN, ABL2_HUMAN, ARF6_HUMAN, HS90A_RABIT, NR1D2_MOUSE, RB27A_RAT, CSF11_TAKRU, KAPCB_HUMAN, PSD12_BOVIN, MYO1F_HUMAN, ANKH1_HUMAN, PTN11_CHICK, RASH_CHICK, IRA1A_ONCMY, LYST_MOUSE, AP1G1_MOUSE, H2B1A_RAT, NR1D1_MOUSE, CH60_RAT, PTN11_HUMAN, TF65_MOUSE, ITB2_BOVIN, ASM3B_HUMAN, PRS7_MOUSE, PSM4A_DANRE, HMGB2_MOUSE, DYHC1_MOUSE, LAMP1_HUMAN, RIPK2_MOUSE, TBB4B_HUMAN, PSMD1_CHICK, IRF2_CHICK, DDX3_XENLA, LYN_RAT, S14L1_HUMAN, CAV1_TAKRU, KAPCA_SHEEP, TBB7_CHICK, LEG9_BOVIN, A4_TETFL, SYEP_HUMAN,	Down
	DAB2P_HUMAN, TAB3_HUMAN, PSD11_HUMAN, PP2B3_DROME, ITB2_HUMAN, FES_HUMAN, JAK2_MOUSE, FAK2_HUMAN, COR1A_MOUSE, NR1H3_BOVIN, STAT1_HUMAN, CLM3_MOUSE, PTN1_RAT, PRDX1_CHICK, BCAP_HUMAN, TLR13_MOUSE, IRF8_CHICK, KCC2D_RAT, IFIH1_MOUSE, PUM1_XENLA, GFI1B_CHICK, BPI_HUMAN, SIN3A_MOUSE, M3K15_MOUSE, ANXA1_PONAB, CO3_CAVPO, SKP1_RAT, DDX3X_HUMAN, KS6A3_HUMAN, TNIP1_MOUSE, CO3_PIG, MERTK_RAT, DAB2P_MOUSE, PSD11_DROME, HMR1_RAT, PS11B_DANRE, C2TA_HUMAN, PSMD4_BOVIN, IPO8_HUMAN, CAR11_MOUSE, PS11A_DANRE, TEC_MOUSE, SIN3B_MOUSE, PSA3_MOUSE, SRPK1_HUMAN, A4_TAKRU, KPCD3_HUMAN, CYLD_HUMAN, CYLD_BOVIN, CP27A_RABIT, MIL_AVIMH, CDC42_BOVIN, MAPK3_HUMAN, LCK_CHICK, FCERG_MOUSE, UBE2N_RAT, CLC4E_HUMAN, PSD10_HUMAN, CD11B_HUMAN, PDPK1_RAT, OTUL_HUMAN, PUM1_CHICK, IRG1_HUMAN, DNJA3_HUMAN, PSMD5_BOVIN, PTN2_RAT, KS6AA_CHICK, SCRB2_RAT, CAZA1_XENLA, TBK1_MOUSE, PK3CD_HUMAN, MRC1_HUMAN, MYD88_ONCMY, KCC2G_HUMAN, JAK2_PIG, HUTU_MOUSE, SIN3A_HUMAN, M4K2_HUMAN, STAT1_PIG, AKIR2_HUMAN, LAMP1_BOVIN, FPS_AVISP, ZAP70_MOUSE, MUG2_RAT, PDK3_MOUSE, KPCD_RAT, PSA5_MOUSE,	Up
Adaptive response	UNG_MOUSE, KSYK_RAT, MALT1_HUMAN, RSAD2_SINCH, RFTN2_PONAB, KPCD_CANFA, KPCT_MOUSE, SLAP2_MOUSE, HEMO_DANRE, SKAP1_TAKRU, ORAI2_XENLA, MEF2C_HUMAN, NFKB1_HUMAN, NSD2_HUMAN, PK2L1_HUMAN, KPCD2_HUMAN, SHP2A_DANRE, HAVR1_MOUSE, PK3CD_MOUSE, MLH1_MOUSE, PLGF_HUMAN, BCL3_HUMAN, TRAF2_HUMAN, RN19B_DANRE, PO2F2_PIG, RN168_DANRE, NOD2_BOVIN, UN13D_HUMAN, PK3CB_RAT, SLAP1_MOUSE, KPCB_RAT, CO8B_PAROL, LYN_MOUSE, IL6RB_HUMAN, NEDD4_RAT, HCK_HUMAN, APLF_MOUSE, HCK_MACFA, RORA_HUMAN, GA45G_BOVIN, BCL6B_MOUSE, SUSD6_MOUSE, JAG1_RAT, EXO1_DANRE, BCL6_MOUSE, KLH24_RAT, KPCD_HUMAN, TRPM4_MOUSE, TRPM5_MOUSE, PO2F2_MOUSE, HSP7C_RAT, RB27A_RAT, DEN1B_MOUSE, PNPH_BOVIN, PKN1_BOVIN, KPCD2_MOUSE, RAG1_ONCMY, RASH_CHICK, DNLI4_HUMAN, LYST_MOUSE, CH60_RAT, JAM3_HUMAN, PTN11_HUMAN, CEAM5_HUMAN, RIPK2_MOUSE, GA45G_MOUSE, TRAF2_MOUSE, IRF2_CHICK, LYN_RAT, KPCB_DANRE, ZBTB2_HUMAN	Down
	THOC1_HUMAN, FZD5_XENLA, JAK2_MOUSE, RORB_HUMAN, ADA17_RAT, MYO1G_CHICK, ANXA1_PONAB, WASP_MOUSE, PKN2_HUMAN, TEC_MOUSE, JAG1B_DANRE, DLG1_HUMAN, NEDD4_HUMAN, FCERG_MOUSE, TCF7_MOUSE, ADA17_HUMAN, GNL1_PONAB, MSH2_BOVIN, LEF1_HUMAN, PK3CD_HUMAN, TFEB_HUMAN, GNL1_HUMAN, SWP70_HUMAN, JAK2_PIG, E2AK4_MOUSE, ZAP70_MOUSE, KPCD_RAT	Up
Leukocyte activation	UNG_MOUSE, ABL1_HUMAN, VAMP8_RAT, KSYK_RAT, UN13D_HUMAN, LYN_MOUSE, KIT_TAKRU, MYBA_CHICK, GATA2_RAT, SHE_MOUSE, PDK1_HUMAN, STXB1_RAT, NSD2_HUMAN, PDK2_HUMAN, HAVR1_MOUSE, FXP1B_DANRE, PK3CD_MOUSE, SBNO2_BOVIN, MLH1_MOUSE, KPCL_HUMAN, RABX5_HUMAN, BCL3_HUMAN, RAC1_RAT, KPCL_RAT, RN168_DANRE, RIPK2_MOUSE, CPLX2_RAT, PK3CB_RAT, MSH2_BOVIN, STXB3_MOUSE, ANXA3_RAT, KPCE_RAT, PLCH1_MOUSE, HCK_HUMAN, STXB2_CANFA, MYO1E_MOUSE, APLF_MOUSE, HCK_MACFA, RORA_HUMAN, GA45G_BOVIN, BCL6B_MOUSE, VAMP2_RAT, ATP7A_HUMAN, AA2BR_CHICK, EXO1_DANRE, BCL6_MOUSE, RHOG_HUMAN, ABL2_HUMAN, LYN_RAT, BCR_HUMAN, RB27A_RAT, MYO1F_HUMAN, P4K2A_DANRE, DNLI4_HUMAN, PLD2_HUMAN, AP1G1_MOUSE, CH60_RAT, SWP70_HUMAN, LAMP1_HUMAN, GA45G_MOUSE, 1433Z_BOMMO, 1433Z_CHICK, SNX4_HUMAN	Down
	PLSL_DANRE, SCO1_MOUSE, RORB_HUMAN, COR1A_MOUSE, FAK2_HUMAN, ANXA3_HUMAN, FES_HUMAN, ATP7A_MOUSE, THOC1_HUMAN, ITA1_RAT, ANXA1_PONAB, CL004_MOUSE, TEC_MOUSE, FCERG_MOUSE, CLC4E_HUMAN, TCF7_MOUSE, PDPK1_RAT, GATA2_CHICK, LEF1_HUMAN, PK3CD_HUMAN, LAMP1_BOVIN, E2AK4_MOUSE, FPS_AVISP, ZAP70_MOUSE, PDK3_MOUSE, RAC2_MOUSE	Up
Regulation of immune response	VAV3_MOUSE, WWP1_MOUSE, PSME1_BOVIN, COL12_DANRE, MYD88_TAKRU, XIAP_XENTR, UNG_MOUSE, ABL1_HUMAN, PTN2_HUMAN, CBL_HUMAN, KSYK_RAT, BIRC2_HUMAN, CATK_BOVIN, CFAB_BOVIN, SMAD3_CHICK, TAB2_DANRE, MYBA_CHICK, SAM11_HUMAN, SVEP1_HUMAN, CUED2_DANRE, FLT3_HUMAN, MALT1_HUMAN, RSAD2_SINCH, NEMO_MOUSE, RFTN2_PONAB, KPCD_CANFA, SPP2B_CHICK, ARC1A_HUMAN, KPCT_MOUSE, PK2L1_MOUSE, ACTB3_TAKRU, LGR4_DANRE, IKBA_MOUSE, SLAP2_MOUSE, CLUS_COTJA, HEMO_DANRE, P85A_PONAB, SKAP1_TAKRU, MYO10_BOVIN, CBL_MOUSE, PTN1_MOUSE, MEF2C_HUMAN, GATA2_RAT, SHE_MOUSE, PDK1_HUMAN, IL6_PAROL, PKHA1_HUMAN, PSB6_BOVIN, CO8A_RABIT, NSD2_HUMAN, PRS7_HUMAN, PSMF1_MOUSE, BAX_BOVIN, CO6_RAT, PDK2_HUMAN, PK2L1_HUMAN, MYO10_MOUSE, ITB1_HUMAN, KPCD2_HUMAN, HS90A_HUMAN, GRP3_HUMAN, YES_DANRE, TLR5_MOUSE, SHP2A_DANRE, HAVR1_MOUSE, PLCG2_HUMAN, KAPCA_MOUSE, MAPK3_MOUSE, PK3CD_MOUSE, PSB9_ORYLA, PTPRB_HUMAN, TNIP1_HUMAN, CO5_HUMAN, S14L1_MOUSE, KPCL_HUMAN, PDE4D_RAT, CO6_BOVIN, IKBA_HUMAN, EZRI_HUMAN, RABX5_HUMAN, UBE2K_BOVIN, SAMH1_DANRE, TRAF2_HUMAN, IL10_CHICK, DMBT1_MOUSE, STXB1_RAT, USP9X_HUMAN, PTN12_MOUSE, KIT_TAKRU, FLOT1_CARAU, FLOT1_PIG, RAC1_RAT, PAK3_RAT, CAR11_HUMAN, BAIP2_CRIGR, NR1D1_HUMAN, NOD2_BOVIN, CO6_HUMAN, KPCL_RAT, MK01_XENLA, CYFP2_PONAB, PA2I_LATSE, PELI2_HUMAN, CAR11_MOUSE, UROM_HUMAN, KS6A6_DANRE, STAP1_MOUSE, IDE_HUMAN, NFAT5_HUMAN, UN13D_HUMAN, ADAM8_MOUSE, HS90A_PIG, CD81_BOVIN, TYK2_HUMAN, IRAK3_MOUSE, PSME3_CHICK, PDE4B_RAT, ITB1_PIG, RPC4_BOVIN, PK3CB_RAT, ITPR1_MOUSE, OTUL_MOUSE, STXB3_MOUSE, SOCS3_CHICK, KPCE_RAT, PLCH1_MOUSE, FAK1_RAT, PAK3_XENLA, SLAP1_MOUSE, KPCB_RAT, TNIP2_HUMAN, CO8B_PAROL, PDE4B_HUMAN, CD81_MOUSE, PTPRS_HUMAN, CBP_HUMAN, LYN_MOUSE, CY24A_MOUSE, IL6RB_HUMAN, HCK_HUMAN, KAPCB_RAT, PLPL9_RAT, SAMH1_MOUSE, PDE4A_CAVPO, MYO1E_MOUSE, HMGB2_HUMAN, STXB2_CANFA, TNIP2_MOUSE, FLOT2_DANRE, NR1H3_MOUSE, APLF_MOUSE, HCK_MACFA, PAK2_RAT, NR1D2_HUMAN, VAV3_HUMAN, BCL6B_MOUSE, VAMP2_RAT, CD81_SAGOE, SLAP1_RAT, ASM3A_BOVIN, SCAR3_MOUSE, SUSD6_MOUSE, CO6_PONPY, OTU7A_HUMAN, PK3CA_RAT, NCKPL_HUMAN, SRC_DANRE, AA2BR_CHICK, BCL6_MOUSE, CO8G_HUMAN, PSD12_MOUSE, JAK1_DANRE, KLH24_RAT, CO3_ONCMY, KPCD_HUMAN, RHOG_HUMAN, CFAH_HUMAN, TRPM4_MOUSE, TRPM5_MOUSE, UB2D3_BOVIN, PSB3_ONCMY, LEG9_RAT, PTPRJ_HUMAN, HCK_MOUSE, ABL2_HUMAN, ARF6_HUMAN, BCR_HUMAN, HS90A_RABIT, HSP7C_RAT, NR1D2_MOUSE, PLD1_MOUSE, KAPCB_HUMAN, PDE4D_MOUSE, DEN1B_MOUSE, PNPH_BOVIN, MY1CA_XENLA, PKN1_BOVIN, PSD12_BOVIN, HS90A_BOVIN, MYO1F_HUMAN, ANKH1_HUMAN, KPCD2_MOUSE, PTN11_CHICK, RASH_CHICK, UBS3B_HUMAN, JUN_SERCA, PLD2_HUMAN, AP1G1_MOUSE, NR1D1_MOUSE, CH60_RAT, PTN11_HUMAN, ARP2A_DANRE, PDE4D_HUMAN, TF65_MOUSE, ITB2_BOVIN, SPP2B_HUMAN, ASM3B_HUMAN, MOES_BOVIN, CO7_PIG, CFAH_BOVIN, CEAM5_HUMAN, FKB1A_BOVIN, PRS7_MOUSE, PSM4A_DANRE, HMGB2_MOUSE, ARP3_MOUSE, LAMP1_HUMAN, FHR1_HUMAN, RIPK2_MOUSE, TRAF2_MOUSE, MYO1C_CHICK, PSMD1_CHICK, IRF2_CHICK, LYN_RAT, KPCB_DANRE, ELMO1_HUMAN, ARP3_TAKRU, STAP2_HUMAN, BCAR1_HUMAN, PAWR_MOUSE, S14L1_HUMAN, CAV1_TAKRU, KAPCA_SHEEP, SNX4_HUMAN, ZBTB2_HUMAN, LEG9_BOVIN,	Down
	ELF1_BOVIN, C209E_MOUSE, DAB2P_HUMAN, THOC1_HUMAN, TAB3_HUMAN, MYH16_CANFA, LAC_PIG, FZD5_XENLA, PSD11_HUMAN, ARC1B_BOVIN, ELMO2_HUMAN, PP2B3_DROME, OLFM_LITCT, FES_HUMAN, JAK2_MOUSE, FAK2_HUMAN, NR1H3_BOVIN, STAT1_HUMAN, CLM3_MOUSE, PTN1_RAT, BCAP_HUMAN, TLR13_MOUSE, CO1A1_CHICK, S39A6_MOUSE, IFIH1_MOUSE, PUM1_XENLA, GFI1B_CHICK, SCO1_MOUSE, ITA1_RAT, NFKB1_HUMAN, PHP14_MOUSE, FYB_MOUSE, BPI_HUMAN, SIN3A_MOUSE, M3K15_MOUSE, CAR14_MOUSE, ANXA1_PONAB, CO3_CAVPO, SKP1_RAT, WASP_MOUSE, KS6A3_HUMAN, TNIP1_MOUSE, CO3_PIG, LIMK1_MOUSE, VTNC_RABIT, DAB2P_MOUSE, PSD11_DROME, PS11B_DANRE, SOS1_HUMAN, C2TA_HUMAN, PSMD4_BOVIN, PKN2_HUMAN, PS11A_DANRE, TEC_MOUSE, SIN3B_MOUSE, PSA3_MOUSE, CRK_RAT, LIMK1_CHICK, CYLD_HUMAN, CYLD_BOVIN, MIL_AVIMH, WIPF2_HUMAN, CO2A1_HUMAN, CDC42_BOVIN, MAPK3_HUMAN, PRG4_HUMAN, LCK_CHICK, FCERG_MOUSE, UBE2N_RAT, CL004_MOUSE, CLC4E_HUMAN, PSD10_HUMAN, CD11B_HUMAN, PDPK1_RAT, OTUL_HUMAN, PUM1_CHICK, IRG1_HUMAN, DNJA3_HUMAN, GATA2_CHICK, SH2B1_RAT, SOS2_HUMAN, PSMD5_BOVIN, PTN2_RAT, KS6AA_CHICK, SCRB2_RAT, IGLL1_HUMAN, ITPR1_HUMAN, MP2K4_MOUSE, TBK1_MOUSE, PK3CD_HUMAN, CO1A1_HUMAN, FKB14_HUMAN, CASL_MOUSE, MYD88_ONCMY, JAK2_PIG, E2AK4_MOUSE, HUTU_MOUSE, SIN3A_HUMAN, NFAC3_HUMAN, STAT1_PIG, VAMP8_RAT, SPP2A_MOUSE, LAMP1_BOVIN, FPS_AVISP, ZAP70_MOUSE, MUG2_RAT, PDK3_MOUSE, KPCD_RAT, PSA5_MOUSE, RAC2_MOUSE, ITB2_HUMAN,	Up

Chapter 6

CONCLUSIONS

Mycobacteriosis is an important disease affecting a wide variety of animal species, especially captive fish. Syngnathids, in particular, are highly susceptible to mycobacteriosis and suffer significant losses due to infection. Furthermore, the reported pathologic presentation of mycobacteriosis in syngnathids is different from other teleost species. Increased susceptibility and atypical disease presentation make syngnathids an interesting model for study.

Of the numerous, ubiquitous, environmental, nontuberculous mycobacteria (NTM), *Mycobacterium chelonae* is the most frequently isolated mycobacterial species from clinically ill syngnathid fish. *M. chelonae* is a member of the *Mycobacterium* chelonae-*abscessus* complex (MCAC), which is composed of several closely related mycobacteria that are difficult to classify by traditional typing methods. Moreover, some members of the MCAC have zoonotic potential and immune compromised individuals are at risk for contracting infections. Inadequate identification of mycobacterial species presents numerous challenges for diagnosticians and clinicians. The most pertinent issue is the differential antimicrobial susceptibility among genetically similar isolates. Therefore, accurate, reliable species identification is crucial in order to formulate potential treatment regimens.

This research investigated multiple hypotheses intended to increase understanding of the dynamics of mycobacteriosis in syngnathid fish through next

generation sequencing of pathogenic mycobacteria, description of the concurrent pathologic changes associated with disease and examination of the host immune response to infection. The first hypotheses stated that strains of *M. chelonae* infecting syngnathids are genetically similar to those infecting other vertebrates and the second hypothesis stated that syngnathids have a different pathologic presentation of mycobacteriosis due to a limited cell-mediated immune response.

To fully investigate the hypotheses, whole genome sequencing was performed on reference strains from the MCAC and previously identified *Mycobacterium chelonae* clinical isolates from the aquatic environment, fish, reptiles, and mammals, in an effort to understand genetic variation among isolates, identify a reliable identification method for diagnostic use, and determine the host specificity of *M. chelonae* strains. Core genome phylogenies, targeted gene comparisons, and *in-vitro* antimicrobial susceptibilities were then determined for the clinical and reference isolates. In addition, the pathologic features of mycobacteriosis was described in numerous species of syngnathid fish and the transcriptome profile of healthy and diseased *Hippocampus erectus* was reported.

The study revealed interesting and relevant information regarding reliable identification methods for *M. chelonae*, current clinical strains, and the strains of mycobacteria infecting syngnathids. Whole genome sequencing with core genomic analysis was the most reliable method for identification of current *M. chelonae* isolates. Results of core genomic phylogenetic analysis placed closely related mycobacterial isolates into their appropriate groups and revealed certain previous identifications of clinical isolates to be inaccurate. Interestingly, reference sequences were identified as

being mislabeled in public databases. Similar strains of *M. chelonae* were identified from fish, reptiles, humans, and the environment. However, a direct epidemiologic link between strains was not observed in the evaluated data. Core genomic alignment and SNPs pattern of the complete 16S rRNA sequence of *M. chelonae* isolates clearly separated the currently used turtle type strain ATCC 35752^T from the clinical isolates and human reference strain ATCC 19237, providing evidence of two separate subspecies.

For diagnostic identification of *M. chelonae* isolates, several methods were employed with consistent results. Concatenation of the partial rpoB (752 bp) and complete hsp65 (1,626 bp) sequence produced the same sub-clusters as the core phylogeny. The partial rpoB and partial hsp65 sequences reliably identified isolates to species level when respective cut-offs of 98% and 98.4% identity to the *M. chelonae* type strain ATCC 35752^T were used, but the human reference strain ATCC19237 was clearly the type representative for current clinical strains. Although the majority of syngnathid mycobacterial isolates were *M. chelonae*, a new *Mycobacterium* sp. was isolated from two different syngnathids, a pipefish and a seahorse. This new species was proposed as *Mycobacterium susanae* sp. nov. Additionally, two isolates not isolated from fish were identified as *Mycobacterium saopaulense*, representing the first report of this species in the United States.

Lesions associated with mycobacteriosis in 92 syngnathids were characterized by random foci of coagulative necrosis in multiple organs, containing high numbers of free bacteria and large aggregates or sheets of macrophages with cytoplasm laden with acid-fast bacilli. Granulomas were identified in only eight seahorses and some fish

produced no appreciable inflammatory response. The presentation of disease in syngnathids is consistent with an innate immune response and putative components of the innate immune system (IL-1β, IL-6, TNF, NOS, TLR1, MH Class1, NF-κβ, TGF-β, MyD88) were identified in the RNA-seq dataset. Granuloma formation is considered typical for chronic mycobacteriosis in fish, but less than 1% of the evaluated syngnathids had granulomas associated with disease. It is possible that duration of disease and/or infective dose may play a role in the atypical presentation of mycobacteriosis. However, an altered or ineffective cell-mediated immune response is suspected due to the consistency of lesions across the family and varied environmental conditions. A homolog for a key component linking the innate and Th1 adaptive immune response, IFN-γ, could not be identified in the transcriptome and supports the hypothesis of an altered cell-mediated immune response. Deficiency or lack of an IFN-γ homolog could potentially explain the marked susceptibility and atypical pathologic lesions observed in this group of fish. The large transcriptome dataset produced from this research could serve as a foundational source of information to advance future research into the pathogenesis of mycobacteriosis within this family of fish.

In summary, syngnathid fish are susceptible to mycobacteriosis and the most common isolate affecting these fish is *M. chelonae.* However, other species of mycobacteria, such as the newly identified *M. susanae* sp. nov, can cause disease with similar lesions. Similar strains of mycobacteria cause disease in a variety of vertebrate hosts. *M. chelonae* is difficult to differentiate from other closely related strains within the MCAC and several isolates deposited within public databases are misidentified. Previous identification methods clearly misidentify isolates within the MCAC and care

should be taken when public databases are used for comparison of strains for identification. While whole genome sequencing and comparison is an ideal method for phylogenetic placement of isolates, partial *hsp65* and partial *rpoB* can be used to accurately identify *M. chelonae* to species level when specific cut-offs are employed. The proposed methods and associated break points will enable accurate identification of closely related species within the MCAC. In the future, these techniques will aid diagnosticians and clinicians to make educated decisions on patient care.

The underlying reason to the atypical syngnathid response to mycobacteriosis may be a deficiency or lack of an IFN-γ homolog. Controlled experiments are needed to further investigate the transcriptome of *H. erectus* and its response to pathogens. However, the dataset produced here can be used as a starting point to delve deeper into the syngnathid immune response to disease.