IDENTIFICATION OF NOVEL FLAVOBACTERIA FROM MICHIGAN AND ASSESSMENT OF THEIR IMPACTS ON FISH HEALTH

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ABSTRACT

IDENTIFICATION OF NOVEL FLAVOBACTERIA FROM MICHIGAN AND ASSESSMENT OF THEIR IMPACTS ON FISH HEALTH

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Flavobacteriosis poses a serious threat to wild and propagated fish stocks alike, accounting for more fish mortality in the State of Michigan, USA, and its associated hatcheries than all other pathogens combined. Although this consortium of fish diseases has primarily been attributed to *Flavobacterium psychrophilum, F. columnare,* and *F. branchiophilum*, herein I describe a diverse assemblage of *Flavobacterium* spp. and *Chryseobacterium* spp. recovered from diseased, as well as apparently healthy wild, feral, and famed fishes of Michigan. Among 254 fish-associated flavobacterial isolates recovered from 21 fish species during 2003-2010, 211 of these isolates were *Flavobacterium* spp., and 43 were *Chryseobacterium* spp. according to ribosomal RNA partial gene sequencing and phylogenetic analysis. Both *F. psychrophilum* and *F. columnare* were indeed associated with multiple fish epizootics, but the majority of isolates were either most similar to recently described *Flavobacterium* and *Chryseobacterium* spp. that have not been reported within North America, or they did not cluster with any described species. Many of these previously uncharacterized flavobacteria were recovered from systemically infected fish that showed overt signs of disease and were highly proteolytic to multiple substrates in protease assays. Polyphasic characterization, which included extensive physiological, morphological, and biochemical analyses, fatty acid profiling, and phylogenetic analyses using Bayesian and neighbor-joining methodologies, confirmed that there were at least eight clusters of isolates that belonged to the genera *Chryseobacterium* and *Flavobacterium,* which represented eight novel species. Experimental challenge studies to fulfill Koch's postulates for 16 representative *Flavobacterium* and *Chryseobacterium* spp. isolates in five

economically and ecologically important fishes of the Great Lakes demonstrated that the majority of these isolates caused pathological lesions in infected fish, and the bacteria spread to vital organs (i.e., brain, spleen, liver, and kidneys), which resulted in cumulative mortalities ≥ 80%. Gross pathological changes associated with experimental infection varied by isolate and host species, but were consistent with a bacterial septicemia. Median lethal dose experiments conducted with a *Chryseobacterium* sp. isolate that is proposed as a novel species, *C. aahli* sp. nov., suggested that this bacterium was only mildly pathogenic to fish under laboratory conditions. Similar experiments conducted with a *Flavobacterium* sp. also proposed as a novel species, *F. spartani* sp. nov., indicated that this bacterium was comparatively more pathogenic. Histopathological changes associated with experimental *F. spartani* sp. nov. infection in its original host, the Chinook salmon (*Oncorhynchus tshawytscha*), included severe proliferative branchitis, lymphocytic and histiocytic myositis, multifocal necrosis within the kidney and liver, lymphocytic hepatitis, renal tubular degeneration and necrosis, and multifocal edema within the granular cell layer of the cerebellar cortex and brainstem. The findings of this study underscore the complexity of etiologies associated with flavobacteriosis and suggest that negative impacts that multiple previously undescribed and/or novel flavobacteria and chryseobacteria can have on Michigan fish stocks.

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GENERAL INTRODUCTION AND OVERVIEW

Flavobacteriosis was first reported by Davis in 1922 and has since been recognized as a serious threat to wild and propagated fish stocks alike. Originally, these diseases were attributed to three bacteria within the family Flavobacteriaceae (Bernardet and Nakagawa 2006); namely, *Flavobacterium psychrophilum*, the etiological agent of bacterial coldwater disease and rainbow trout fry syndrome (Nematollahi et al. 2003; Starliper 2011); *F. columnare*, the causative agent of columnaris disease (Hawke and Thune 1992; Shotts and Starliper 1999); and *F. branchiophilum*, the putative agent of bacterial gill disease (Wakabayashi et al. 1989; Shotts and Starliper 1999). Others have reported additional *Flavobacterium* spp. associated with diseased fish, including *F. johnsoniae* (Christensen 1977), *F. succinicans* (Anderson and Ordal 1961), *F. hydatis* (Strohl and Tait 1978), as well as other uncharacterized yellow-pigmented bacteria (Austin and Austin 2007 and references therein). In acute flavobacteriosis, cumulative mortality upwards of 70% can occur among affected fish stocks, while survivors may suffer poor growth and spinal abnormalities (reviewed in Austin and Austin 2007). In subacute and chronic infections, flavobacteriosis elicits lingering mortalities that can lead to continuous economic losses (USDA 2003). Taxonomy and speciation of this family has undergone many revisions; therefore, throughout this review, the most currently recognized terminology of genera and species will be used.

With the recent advances in molecular biology and biotechnology, several novel genera within the family Flavobacteriaceae (e.g., *Chryseobacterium*, *Elizabethkingia*, *Tenacibaculum*, and *Ornithobacterium*) have emerged that encompass pathogens of fish, amphibians, reptiles, birds, and mammals, including humans (Bernardet and Nakagawa 2006). Moreover, the last decade has witnessed the emergence of multiple novel flavobacterial species that have been recovered

from diseased fishes worldwide (i.e., Bernardet et al. 2005; Flemming et al. 2007; Ilardi et al. 2009; Kämpfer et al. 2011; Kämpfer et al. 2012; Zamora et al. 2012a, b, c).

Recently, multiple wild fish kills in the Great Lakes Basin have been attributed to infectious agents, such as viral hemorrhagic septicemia virus (VHSv; Faisal et al. 2012), *Renibacterium salmoninarum* (Holey et al. 1998), and *Flavobacterium columnare* (Faisal and Loch unpublished). Indeed, as a group, flavobacteria have historically accounted for more fish mortality in the hatcheries of the State of Michigan than all other pathogens combined (Faisal and Hnath 2005; Faisal et al. 2011), though it was unclear which *Flavobacterium* spp. were involved. In this context, the Michigan Department of Natural Resources (MDNR) and the Aquatic Animal Health Laboratory at Michigan State University (MSU-AAHL) undertook extensive field and laboratory studies to identify pathogens threatening conservation efforts in wild fish stocks, as well as those reared within State Fish Hatcheries. During this multiyear study, I have identified flavobacteria and chryseobacteria as a major cause of fry and fingerling mortalities. Flavobacterial isolates were recovered from > 40 fish disease/mortality events that occurred in both wild and captive fish stocks. Some of the flavobacteria recovered from affected fishes exhibited characteristics typical of *F. psychrophilum* and *F. columnare* and were identified as such, but others did not match the descriptions of other characterized flavobacteria even though they were recovered from fishes with pathologies that mimicked those of flavobacteriosis. These uncharacterized flavobacteria from the genera *Flavobacterium* and *Chryseobacterium* form the core of this dissertation.

Concurrent with a perceptible increase in reports of novel/ uncharacterized flavobacteria and chryseobacteria among diseased fish is a complete lack of knowledge about their degree of pathogenicity. Indeed, very few studies have fulfilled Koch's postulates for emergent

flavobacterial pathogens. Information on pathogenesis and virulence is vital when assessing the potential impacts a pathogen may have in a population of susceptible hosts.

Study Overview and Objectives

The studies presented herein focused on identification and characterization of flavobacteria recovered from wild, feral, and farmed fish of Michigan that were isolated from 2003-2010. Once a more complete identification was reached and phylogenetic relationships were determined, representative isolates belonging to suspected fish-pathogenic clades were selected for polyphasic characterization in order to fully identify them, assess their relationship to other described flavobacterial species, and determine if any were novel flavobacterial taxa. Experiments were also designed to study the pathogenicity of these previously uncharacterized *Flavobacterium* and *Chryseobacterium* spp. under controlled laboratory conditions.

This dissertation consists of nine chapters. The first chapter summarizes the background information and available body of knowledge on the dissertation's topic, while the second chapter reports on the diverse assemblage of flavobacteria associated with diseased and apparently healthy fishes of Michigan. The third chapter summarizes polyphasic characterization studies conducted on Michigan fish-associated *Chryseobacterium* spp. and experiments testing their pathogenicity in fish under laboratory conditions, while the fourth chapter does the same for a number of previously uncharacterized *Flavobacterium* spp. isolated from Michigan fishes. Chapter five deals with the characterization of *Flavobacterium* spp. involved in a propoagated coho salmon mortality episode, while chapters six and seven describe two novel flavobacterial taxa, for which the names *Flavobacterium spartani* sp. nov. and *Chryseobacterium aahli* sp. nov. were proposed. Finally, chapter eight details future research directions and an overall summary, and chapter nine is a list of cited references.

CHAPTER ONE

REVIEW OF LITERATURE

I. History of Flavobacteriosis in Fish.

In the process of studying protozoan parasites at the U.S. Fisheries biological station in Fairport, Iowa, Davis (1922) observed multiple fish mortality events during the summers of 1917-1919 that he associated with an unidentified bacterium. The affected fish, which included buffalofish (*Ictiobus bubalus*), sunfish (*Leopomis* spp.), common carp (*Cyprinus carpio*), largemouth and smallmouth bass (*Micropterus salmoides* and *M. dolomieu*), crappie (*Pomixis* spp.), warmouth (*Lepomis gulcosus*), yellow perch (*Perca flavescens*), white bass (*Morone chrysops*), brook trout (*Salvelinus fontinalis*), bluntnose minnow (*Pimephalus notatus*), channel catfish (*Ictalurus punctatus*), and bullhead catfish (*Ameiurus* spp.), were cultured in aquaria and earthen ponds. Davis (1922) noted that affected fish displayed “dirty-white or yellowish areas” on the body, whereby lesions developed and caused death within 24 to 72 hrs. Fins (especially the caudal fin) were eroded and, in more severe cases, only “mere stubs” remained. There was also necrosis of the gills visible as white patches that spread rapidly, causing death. The author also observed mortalities in wild fishes of the Mississippi River associated with this bacterium. Although he was unable to isolate the bacterium in any of these outbreaks, he observed large numbers of long, slender, flexible rods associated with the necrotic lesions of the skin and gills of affected fish that formed “column-like masses”; thus, he named the bacterium *Bacillus columnaris*. Two decades later, Ordal and Rucker (1944) successfully isolated the yellow-pigmented bacterium and named it *Chondrococcus columnaris* due to its association with cartilage and what they characterized as the production of fruiting bodies and microcysts. However, Garnjobst (1945) showed that *C. columnaris* did not produce such structures and

reclassified the bacterium within the genus *Cytophaga*, as *Cytophaga columnaris*. While agreement on the negative impacts this bacterium had on fish stocks was well accepted, as evidenced by its inclusion in the list of notifiable fish diseases outlined in the British Diseases of Fish Act of 1937 (Austin and Austin 2007), the disagreement on its taxonomy continued when it was provisionally placed into the genus *Flexibacter* (Leadbetter 1974; Bernardet and Grimont 1989). Extensive molecular and phylogenetic studies by Bernardet et al. (1996) placed the bacterium in the genus *Flavobacterium* as *F. columnare*, where it has remained to the present day and is recognized as the etiologic agent of columnaris disease.

Another unidentified yellow-pigmented bacterium was associated with serious disease in rainbow trout (*Oncorhynchus mykiss*) fingerlings reared at the national fish hatchery in Leetown, West Virginia (Davis 1946). Although Davis was unable to isolate this bacterium, he observed huge numbers of non-motile bacterial rods in scrapings taken from deep ulcerations present on the caudal peduncle of affected fish. These bacteriay did not form the characteristic “columns” associated with *F. columnare*. Soon thereafter, Borg (1948) reported a similar pathological condition among diseased hatchery-reared juvenile coho salmon (*O. kisutch*) from Washington, In this case, a bacterium was successfully isolated from the kidneys and external lesions of systemically infected fish. Borg (1948, 1960) reproduced the disease in experimentally challenged fish, with signs that included ulcerations at the caudal peduncle that went so deep so as to almost detach the tail from the body. While this bacterium was initially placed in the order Myxobacterales and named *Cytophaga psychrophila* (Borg 1960; Pacha 1968), it was reclassified as *Flexibacter psychrophila* (Leadbetter 1974; Bernardet and Grimont 1989) and later as *Flavobacterium psychrophilum* (Bernardet et al. 1996). As its name implies, *F. psychrophilum* grows best at low temperatures (~15°C) and frequently causes disease when water temperatures are below 10°C. In North America, the terms low temperature disease

(Borg 1968) and bacterial coldwater disease (Holt 1987) are used to describe outbreaks associated with this bacterium, whereas outbreaks in Europe are commonly referred to as rainbow trout fry syndrome (Lorenzen et al. 1991, 1997).

A third yellow-pigmented bacterium also has a long history in association with freshwater fish diseases. Davis (1926, 1927) reported multiple disease outbreaks in fingerling brook trout and steelhead (*O. mykiss*) reared in Vermont and New York, which he attributed to an unknown bacterium that was associated with damage to the gills. He noted slow chronic mortalities that increased with time when temperatures began to rise. Davis (1926) noted that the bacteria formed "luxuriant growth over the surface of the gills" that coincided with increased mucus production, clubbing of the gill lamellae, and proliferation of gill epithelium causing fusion of the secondary lamellae. Otherwise, fish appeared normal until death. Other researchers observed similar disease outbreaks and isolated yellow pigmented bacteria from affected fish, but they were unable to reproduce the disease experimentally (Rucker et al. 1949, 1952; Bullock 1972). Wakabayashi (1980) successfully recovered a yellow pigmented bacterium from hatchery-reared salmonids from Japan and Oregon that was distinct from those used in the aforementioned studies of Rucker and Bullock and successfully reproduced the disease. This bacterium was classified as *Flavobacterium branchiophila* was given (Wakabayashi et al. 1989), which became *F. branchiophilum* (Bernardet et al. 1996). It is now widely believed that *F. branchiophilum* is a causative agent of bacterial gill disease (BGD; Bullock 1990); however, environmental parameters and other bacteria are also believed to play a role in some outbreaks of BGD.

As is quite clear from the aforementioned history of fish-pathogenic flavobacteria, their taxonomy has been quite tumultous. While the fish health literature is replete with reports of fish

diseases associated with unidentified or partially characterized yellow-pigmented bacteria referred to as *Cytophaga* spp. and *Cytophaga*-like bacteria, *Flexibacter* spp., *Flavobacterium*-like spp., and “myxobacteria” (e.g., Wood 1968; Bullock and McLaughlin 1970; Lien 1988; Kent et al. 1988; Pepin and Emery 1993; Frelier et al. 1994; Starliper 1992; Austin and Austin 2007), many now belong to the family Flavobacteriaceae.

II. The Family Flavobacteriaceae

The family Flavobacteriaceae (Phylum Bacteroidetes; Class Flavobacteriia; Order Flavobacteriales; Bernardet 2011) was first suggested by Jooste (1985). Despite being mentioned by Reichenbach (1989), the family was not validated until 1992 by Reichenbach and its formal description published by Bernardet et al. (1996). Included within the family Flavobacteriaceae at that time was the type genus, *Flavobacterium* (Bernardet et al. 1996), along with the genera *Chryseobacterium* (Holmes et al. 1984a; Vandamme et al. 1994), *Bergeyella* (Holmes et al. 1986a; Vandamme et al. 1994), *Empedobacter* (Holmes et al. 1978; Vandamme et al. 1994), *Capnocytophaga* (Holt and Kinder 1989; Vandamme et al. 1996), *Ornithobacterium* (Vandamme et al. 1994), *Weeksella* (Holmes et al. 1986b), *Riemerella* (Segers et al. 1993; Vancanneyt et al. 1999), *Myroides* (Holmes et al. 1977; Vancanneyt et al. 1996), and *Tenacibaculum* (Bernardet et al. 1996; Suzuki et al. 2001). Bernardet et al (2002) published minimal standards for describing new taxa within the family Flavobacteriaceae, which in addition to the genera mentioned above, also included *Coenonia* (Vandamme et al. 1999), *Psychroserpens* and *Gelidibacter* (Bowman et al. 1997), *Polaribacter* (Gosink et al. 1998), *Psychroflexus* (Bowman et al. 1998), *Salegentibacter* (McCammon and Bowman 2000), *Cellulophaga* (Johansen et al. 1999), and *Zobellia* (Barbeyron et al. 2001), along with two generically misclassified taxa (Bernardet et al. 2002). In subsequent years, the family has

rapidly expanded from < 20 genera to > 100 described and candidate genera (Bernardet 2011; Kim et al. 2012).

Characteristics of this family according to Bernardet (2011) are as follows: Nonspore-forming short to long filamentous rods that stain Gram negative, are motile via gliding or non-motile, and are non-flagellated with rare exceptions (i.e., *Formosa agariphila*). Colony shape ranges from round - rhizoid, flat - convex, and the bacteria can adhere to agar (i.e., *Flavobacterium columnare*). Although some colonies do not pigment, most contain a non-diffusable yellowish to orange pigment due to the presence of carotenoid and/or flexirubin. Growth is typically aerobic, but some genera exhibit micoaerophillic to anaerobic growth. Nitrates are not typically reduced, whereas oxidase and catalase activities are common. Most genera contain species that degrade organic substrates, including proteins (e.g., casein, gelatin, etc.), simple and complex carbohydrates (e.g., starch, esculin, pectin, chitin, carboxymethylcellose), and lipids (e.g., Tween). Many species are halophilic and mesophilic, while others are halotolerant and psychrophilic. Branched saturated, branched monounsaturated, and branched hydroxy C_{15} to C_{17} fatty acids are frequently present in large amounts. The term flavobacteria will be used throughout this literature review to encompass members of the family Flavobacteriaceae.

Flavobacteria commonly reside in extremely diverse habitats, ranging from fresh and marine aquatic environments, soils, foods, beverages and their processing plants, as well as human and veterinary hospitals (reviewed in Bernardet and Nakagawa 2006). Many flavobacteria are pathogenic to a multitude of organisms, including plants (Bernardet and Bowman 2006), invertebrates (Li et al. 2010), amphibians (Xie et al. 2009), reptiles (Hernandez-Divers et al. 2009), birds (Segers et al. 1993), and mammals (Haburjak and Schubert 1997), including

humans (Benedetti et al. 2011). Because the research presented herein focused on the genera *Flavobacterium* and *Chryseobacterum*, the remainder of this literature review will be comprised of information pertaining to them specifically.

III. The Genus *Flavobacterium*

History of the genus. The genus *Flavobacterium* was described in the first edition of Bergey's Manual of Determinative Bacteriology (Bergey et al. 1923), at which time it consisted of 46 species that included Gram negative, Gram positive, Gram variable, and flagellated bacteria (McMeekin and Shewan 1978). By the publication of the eighth edition of Bergey's Manual (Buchanan and Gibbons 1974), the majority of the flagellated and Gram positive strains were systematically removed, leaving only 12 species within the genus (McMeekin and Shewan 1978). This taxonomic upheaval continued, whereby only 7 species remained in 1984 (Holmes et al. 1984b); then, the extensive work conducted by Bernardet et al. (1996) resulted in the type species, *F. aquatile* (Frankland and Frankland 1889), being the only original member to be retained within the genus *Flavobacterium*. Other species assimilated into the genus *Flavobacterium* at this time included *F. psychrophilum, F. branchiophilum, F. columnare, F. hydatis, F. succinicans, F. johnsoniae, F. flevense, F. pectinovorum,* and *F. saccharophilum* (Bernardet et al. 1996). With much of the taxonomy settled, minimal standards for describing novel flavobacteria were published (Bernardet et al. 2002), at which point the number of *Flavobacterium* spp. was 15. Over the next 4 years, 11 more *Flavobacterium* spp. were described (Bernardet and Bowman 2006), bringing the total to 26, and the number of formally described *Flavobacterium* spp. has rapidly expanded to include almost 90 species (Bernardet and Bowman 2011; Kim et al. 2012).

Characteristics of the genus Flavobacterium (Bernardet and Bowman 2011). Members of the genus *Flavobacterium* are Gram negative rods that range from 0.3-0.5 μm in diameter and from 1.0-40.0 μm in length. All species are non-motile or display gliding motility (the presence of flagella has not been reported). It should be noted that the degree by which flavobacteria move by gliding is optimal in media with low nutrient and high moisture contents. Colonies contain non-diffusible, non-fluorescent, flexirubin and/or carotenoid –type pigments, giving them a pale to bright yellow appearance. Optimal growth occurs from 20-30 °C for most species, though growth is better at 15-20°C for the more psychrophilic species. The majority of *Flavobacterium* spp. grow on nutrient and trypticase soy agars (TSA) without the need for growth factors, though many of the fish pathogenic species (i.e., *F. psychrophilum, F. columnare, F. branchiophilum*) are not able to grow on TSA. Some species are able to oxidize carbohydrates, but strong proteolytic activity is almost universally present. The predominant fatty acids present within members of the genus are *iso*-$C_{15:0}$, $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*, $C_{15:0}$, *iso*-$C_{17:0}$ 3-OH, *iso*-$C_{15:0}$ 3-OH, $C_{15:1}$ ω6*c*, *iso*-$C_{16:0}$ 3-OH, *iso*-$C_{15:1}$ G, *iso*-$C_{15:0}$ 2-OH, and *anteiso*-$C_{15:0}$. The type species is *F. aquatile* (Frankland and Frankland 1889).

Culture and identification. *Flavobatecterium* spp. vary in the ease that they are cultured on microbiological media. For example, many of the species that inhabit soil and freshwater grow readily on agar plates made from commercially available media (e.g., nutrient agar and TSA), as is also the case for the marine species (e.g., marine agar; reviewed in Bernardet and Bowman 2006). However, a portion of the freshwater fish-pathogenic species (see *Flavobacterium* spp. as pathogens below) are fastidious and require specialized culture media, such as cytophaga agar (Anacker and Ordal 1955), Shieh's medium (Shieh 1980), Hsu-Shotts medium (Bullock et al. 1986), tryptone yeast extract salts medium (TYES; Holt 1987), and medium # 2 (Starliper et

al. 2007), to name a few. Some of these media and their derivatives were made more selective by incorporation of polymyxin-B, neomycin, tobramycin, and/or vancomycin to avoid overgrowth by less fastidious bacteria that may also be present in an inoculum, especially from external lesions of fish. Optimal incubation temperatures vary widely and depend upon the species, but most of the fish-pathogenic species grow best from 15-25 °C (Bernardet and Bowman 2006).

The morphological, physiological, and biochemical characteristics that help to differente *Flavobacterium* from other genera within the family Flavobacteriaceae were extensively reviewed by Bernardet (2011), and differential characters among *Flavobacterium* spp. are presented in Bernardet and Bowman (2011). In addition to bacterial culture and subsequent identification via phenotypic tests (Austin and Austin 2007), many other means for detection and identification were developed. Whole-cell agglutination (Pacha 1968; Morrison et al. 1981), fluorescent antibody tests (FAT; Panangala et al. 2006; Lindstrom et al. 2009), enzyme-linked immunosorbent assays (MacPhee et al. 1995; Lindstrom et al. 2009), *in situ* hybridization (Liu et al. 2001), loop-mediated isothermal amplification (LAMP; Yeh et al. 2006; Fujiwara-Nagata and Eguchi 2009), polymerase chain reaction (PCR; Toyama et al. 1994, 1996; Bader and Shotts 1998; Triyanto et al. 1999; Wiklund et al. 2000; Bader et al. 2003; Crumlish et al. 2007), immunomagnetic separation in conjunction with flow cytometry (Hibi et al. 2008), quantitative PCR (Orieux et al. 2011), and DNA array-based multiplex assay (Lievens et al. 2011) are used to detect and identify *F. psychrophilum, F. columnare,* and/or *F. branchiophilum*. It is noteworthy, however, that few diagnostic reagents specific for the lesser-known fish associated flavobacteria exist, which makes their identification more difficult and laborious.

Ecology. Although usually considered psychrophilic or psychrotolerant, some flavobacteria are also able to grow at temperatures of 37°C (i.e., *F. granuli, F. columnare, F. suncheonse,* and *F.*

succinicans), or even 40- 45 °C (*F. defluvi, F. indicum, F. croceum*; Bernardet and Bowman 2011). However, the vast majority are recovered from cool, cold, or even polar habitats (reviewed in Bernardet and Bowman 2011). *Flavobacterium* spp. reside in diverse habitats, including freshwater streams, lakes, and sediments (e.g., Qu et al. 2009; Lee et al. 2010), deep wells (Holmes et al. 1984a), glaciers and arctic ice (e.g., Xu et al. 2011; Dong et al. 2012), plants and plant material (e.g., Kim et al. 2011; Liu et al. 2011), soils (e.g., Lim et al. 2011), freshwater shrimp ponds (Sheu et al. 2011), marine sediments (e.g., Fu et al. 2011), seawater (e.g., Yoon et al. 2011), wastewater treatment systems (e.g., Ryu et al. 2007; Zhang et al. 2010), on marine algae (e.g., Miyashita et al. 2010), and even within air-conditioning units (Bernardet and Bowman 2006).

Flavobacterium spp. have also been detected intracellularly in amoebae (Horn et al. 2001) and from the guts of earthworms (*Aporrectodea caliginosa*; Horn et al. 2005), butterflies (Xiang et al. 2006), mosquitos (Campbell et al. 2004), nematodes (Park et al. 2011), leeches (Schulz and Faisal 2010), and in association with corals (e.g., Kannapiran and Ravindran 2012), marine sponges (Imhoff and Stöhr 2003), and marine mammals, such as beaked whales (*Ziphius cavirostris*; Vela et al. 2007). Caution should be exercised when referring to the primary literature documenting *Flavobacterium* spp. from many other animals, including humans, because many taxa originally belonging to the genus *Flavobacterium* were subsequently moved to other genera (i.e., "*Flavobacterium breve*", now *Empedobacter brevis*, Vandamme et al. 1994; "*Flavobacterium meningoseptica*", now *Elizibethkingia meningosepticum*, Kim et al. 2005a). By far and away, the most plentiful reports of *Flavobacterium* spp. in associated with animals are those that document their presence on fish and fish products. For example, *Flavobacterium* spp. have been detected within the intestines (Huber et al. 2004; Hu et al. 2007), on the gills (Farkas 1985), fins (Anderson and Ordal 1961), in the mucus (Bernardet and

Bowman 2006), from eggs (Brown et al. 1997) and reproductive fluids (Rangdale 1996), and from internal organs of cool, cold, and warm-water fishes (reviewed in Shotts and Starliper 1999, Bernardet and Bowman 2006, and Austin and Austin 2007). Indeed, while some *Flavobacterium* spp. are commensal (Shewan 1961; Pacha and Porter 1968; Trust 1975), a number are major pathogens of freshwater and marine fishes.

Flavobacterium spp. as pathogens. A few reports have documented *Flavobacterium* spp., such as *F. johnsoniae*, with diseases in plants (Liao and Wells 1986; Lelliott and Stead 1987; Bernardet and Bowman 2006). Flavobacterial infections were also occasionally reported in amphibians (Davis 1922; Bullock et al. 1971; Brown et al. 1997) and occasionally the bacteria were associated with disease in humans. For instance, an outbreak of respiratory disease in a group of workers from a textile plant occurred in the 1980's and was attributed to a *Flavobacterium* sp. strain that proliferated within an air-conditioning unit in the facility (Flaherty et al. 1984; Liebert et al. 1984; Bernardet and Bowman 2006). Most recently, *F. lindanotolerans* was recovered from the ascites of a child in China that died of fatal pulmonary edema and hemorrhage (Tian et al. 2011).

Flavobacteriosis in fish. Since the initial report of Davis (1922), multiple members of the genus *Flavobacterium* have been recognized as serious fish pathogens worldwide; namely, *F. columnare,* (Davis 1922; Ordal and Rucker 1944; Bullock et al. 1986); *F. psychrophilum* (Davis 1946; Wood 1974; Cipriano and Holt 2005; Starliper 2011) and rainbow trout fry syndrome (Lorenzen et al. 1991, 1997); and *F. branchiophilum* (Wakabayashi 1980; Wakabayashi et al. 1989; Bullock 1990). Extensive reviews have been written on fish-pathogenic *Flavobacterium* spp. (e.g., Shotts and Starliper 1999; Bernardet and Bowman 2006; Austin and Austin 2007), which highlight the predominance of *F. psychrophilum, F. columnare,* and *F. branchiophilum* in

fish health literature. The three aforementioned reviews described the impacts that the "big three" fish-pathogenic *Flavobacterium* spp. have on fish stocks worldwide and additional publications provided even greater depth concerning the impacts of *F. psychrophilum* (e.g., Holt 1987; Nematollahi et al. 2003; Cipriano and Holt 2005; Starliper 2011; Barnes and Brown 2011), *F. columnare* (e.g., Amend et al. 1983; Bullock et al. 1986; Kuntuu 2010), and *F. branchiophilum* (e.g., Sniezko 1981; Bullock 1990; Wakabayshi et al. 1989; Ferguson et al. 1991; Ostland et al. 1994; Good et al. 2008). Thus, while the importance of *F. psychrophilum, F. columnare,* and *F. branchiophilum* as etiologic agents of fish disease is undeniable, the research presented herein focuses on the "less well-known" freshwater fish-associated flavobacteria and the rest of the literature review will concentrate on those bacteria.

"Other" Flavobacterium spp. associated with diseased fishes. *Flavobacterium johnsoniae* is occasionally associated with disease in fish. Christensen (1977) first mentioned *F. johnsoniae* in association with diseased fish, followed by the report of Carson et al. (1993) of *F. johnsoniae* causing shallow ulcerative lesions on the skins, fins, and jaws of juvenile aquacultured barramundi (*Lates calcarifer*) in Australia. In an attempt to fulfill Koch's postulates, Soltani et al. (1994) utilized an isolate of *F. johnsoniae* from the disease outbreak in barramundi and challenged various fishes via bath exposure. Juvenile barramundi were the only studied species that were susceptible to infection with *F. johnsoniae*, and infections ensued only after fish were stressed thermally (Soltani et al. 1994). Rintamäki-Kinnunen et al. (1997) also found an *F. johnsoniae*-like bacterium associated with external lesions on the gills, jaws, skin, and fins in multiple aquacultured salmonids in Finland. Most recently, *F. johnsoniae* and *F. johnsoniae*-like isolates were associated with disease in aquacultured longfin eels (*Anguilla mossambica*), rainbow trout, and koi (*Cyprinus carpio*) in South Africa (Flemming et al. 2007), in cultured

Russian sturgeon (*Acipenser gueldenstaedtii*) in Turkey (Karatas et al. 2010), and in farmed rainbow trout in Korea (Suebsing and Kim 2012).

Another *Flavobacterium* sp. implicated as a putative agent of fish disease is *F. hydatis* (formerly *Cytophaga aquatilis*; Strohl and Tait 1978; Bernardet et al. 1996). This bacterium was first isolated from the gills of propagated salmonids displaying "signs of bacterial gill disease" in Michigan by Strohl and Tait (1978) but its pathogenicity was not assessed. Others have also recovered a similar bacterium from the gills of fish suffering from bacterial gill disease epizootics (Bernardet and Bowman 2006; Austin and Austin 2007), thus suggesting a role as an opportunistic fish pathogen. Similarly, *F. succinicans* was originally isolated from the water and eroded caudal fin of a Chinook salmon fingerling at the University of Washington School of Fisheries (Seattle, Washington) and from a "lesion" on an adult Chinook salmon collected from the Brownlee Dam on the Snake River in Hells Canyon, Idaho (Anderson and Ordal 1960). However, the original author suggested that this bacterium was not a fish pathogen but rather a commensal, and further studies were not conducted.

Most recently, a number of novel *Flavobacterium* spp. were isolated from diseased fish in Europe and South America, including *F. chilense* and *F. araucananum* (Kämpfer et al. 2012), as well as *F. oncorhynchi* (Zamora et al. 2012a). *Flavobacterium chilense* was originally recovered from an external lesion of a farmed rainbow trout in a mixed culture with *F. psychrophilum*, while *F. araucananum* was recovered from the kidneys and external lesions of farmed Atlantic salmon (*Salmo salar*) in a mixed culture with *F. psychrophilum* (Kämpfer et al. 2012), both of which were cultured in Chile. The original isolations of *F. oncorhynchi* took place in Spain, whereby the bacterium was recovered from the livers and gills of farmed rainbow trout with clinical signs suggestive of an *F. psychrophilum* infection.

In addition to the aforementioned fish-associated *Flavobacterium* spp., other partially characterized and/or unidentified *Flavobacterium* spp. have periodically been reported from diseased fishes. Indeed, in their review, Shotts and Starliper (1999) make brief mention of "other poorly defined *Flavobacterium*-like organisms" that have been implicated as facultative fish pathogens, while a similar statement that implicates partially characterized yellow-pigmented bacteria as agents of fish disease is made by Austin and Austin (2007). For example, Holliman et al. (1991) documented a disease outbreak in captive-reared rainbow trout (*Oncorhynchus mykiss*) supplied with unfiltered lake water in Windermere, England, that was attributed to an unidentified "*Cytophaga*-like" bacterium, which generated a 16% mortality over 10-days and mortality abated after administration of oxytetracycline. On cytophaga agar, nearly pure bacterial growth was cultured from external lesions. Holliman et al. (1991) found that they possessed the characters of "*Cytophaga*-like bacteria" that were distinct from *F. aquatile, F. psychrophilum, F. columnare* and indicated that this bacterium had never been previously reported in association with fish disease. Additionally, the authors confirmed pathogenicity via immersion, intraperitoneal, and intramuscular routes of transmission in rainbow trout and Atlantic salmon.

Similarly, Bowman and Nowak (2004) detected a *Flavobacterium* sp. from the gills of net-penned Atlantic salmon in Tasmania that concurrently suffered from amoebic gill disease. Their isolate was most similar to *F. frigidarium* according to 16S rDNA sequences. It was not clear, however, what role this bacterium had played in the disease process. Indeed, a number of unidentified "*Cytophaga*-like bacteria" were also reported in association with disease outbreaks in marine fishes (e.g., Kent et al. 1988; Pepin and Emery 1993; Frelier et al. 1994).

IV. The Genus *Chryseobacterium*

History of the genus. The genus *Chryseobacterium* was originally created by Vandamme et al. (1994) for six bacterial taxa that, at that time, were classified as members of the genus *Flavobacterium; F. balustinum, F. indologenes, F. gleum, F. meningosepticum, F. indoltheticum,* and *F. scophthalmum*. In keeping with the taxonomic upheaval that many of the taxa within the family Flavobacteriaceae endured, an additional genus, *Elizabethkingia*, was subsequently created for two species within the genus *Chryseobacterium*; namely, *C. meningosepticum* and *C. miricola* (Kim et al. 2005a). Since then, more taxonomic clarity has been achieved through the widespread use of improved molecular techniques and guidelines for description of novel chryseobacterial species (Bernardet et al. 2002). By 2006, the genus *Chryseobacterium* had expanded to 10 species, along with one unvalidated species (e.g., *C. proteolyticum*; Bernardet et al. 2006), but the genus contained > 60 species when this review was written.

Characteristics of the genus Chryseobacterium (Bernardet et al. 2011). Members of the genus *Chryseobacterium* are non-motile, Gram negative, straight rods that are usually 1-3 µm in length and ~ 0.5 µm in width. None of the species within this genus have flagella, nor do they display gliding motility or swarming growth. Colonies range from pale to a bright golden yellow color due to the presence of a non-diffusible flexirubin-type pigment. *Chryseobacterium* spp. grow well on commercial media (i.e. TSA, nutrient agar, etc.) at 4°C - 42°C and at salinities of up to 5%, depending upon the species. Catalase and oxidase activities are present, isolates are strongly proteolytic, and most are resistant to numerous antibiotics. The predominant fatty acids contained by members of this genus are *iso*-$C_{15:0}$, *iso*-$C_{17:1}$ ω9*c*, *iso*-$C_{17:0}$ 3-OH, and

iso-$C_{15:0}$ 2-OH/$C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*. The type species is *Chryseobacterium gleum* (Holmes et al. 1984a).

Culture and identification. *Chryseobacterium* spp. are readily cultivable using commercially available media, such as TSA, brain heart infusion, marine, blood, nutrient, and Mueller Hinton agars (reviewed in Bernardet et al. 2006, 2011). The ideal incubation temperature for chryseobacteria is from 20-30°C (Bernardet et al. 2006), though some species grow below 5°C and above 37°C (Bernardet et al. 2011). The phenotypic and biochemical characteristics that differentiate *Chryseobacterium* spp. from other closely related members of the family Flavobacteriaceae were reviewed by Bernardet (2011), while differential characters among the various *Chryseobacterium* spp. were presented in Bernardet et al. (2011). Presumptive identification of a *Chryseobacterium* sp. is often based upon phenotypic characters (e.g., Gram negative, non-motile rods that produce bright yellow colonies due to the presence of flexirubin-type pigments; possess oxidase and catalase activities; produce a *Chryseobacterium* spp. profile on commercial galleries; Bernardet et al. 2005), after which a definitive identification is based upon polyphasic characterization, including biochemical, morphological, and physiological characterization, fatty acid profiling, and sequence/phylogenetic analyses (Bernardet et al. 2002). Additional techniques that have most recently been utilized to identify *Chryseobacterium* spp. include matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; Fernández-Olmos et al. 2012) and PCR amplification of the 16S and internal transcribed spacer (ITS) rDNA and subsequent sequence analysis (Daroy et al. 2011).

Ecology. Akin to the genus *Flavobacterium*, members of the genus *Chryseobacterium* inhabit diverse habitats. For example, chryseobacteria were recovered from soils (e.g., Benmalek et al. 2010; Im et al. 2011; Li and Zhu 2012), plant roots (e.g., Park et al. 2006), flowers (Luis et al.

2004), decaying plant material (Behrendt et al. 2008), and maple sap (Lagacé et al. 2004). Interestingly, some plant-associated *Chryseobacterium* strains inhibited plant-pathogenic fungi (Park et al. 2006). *Chryseobacterium* spp.were also recovered from freshwater creeks (Strahan et al. 2011), lakes (Joung and Joh 2011), their sediments (Kim et al. 2005b), water cooling systems (Park et al. 2008), drinking water (Gallego et al. 2006), lactic acid beverages (Shimomura et al. 2005), beer bottling plants (Herzog et al. 2008), bioreactor sludge (Quan et al. 2007), polluted soil (Zhou et al. 2007), marine sediment (Campbell and Williams 1951), and permafrost (Loveland-Curtze et al. 2010). In contrast to *Flavobacterium* spp., multiple studies that examined the bacterial assemblages of glaciers and Antarctic ice have not detected *Chryseobacterium* spp. (Bernardet et al. 2006 and references therein).

Chryseobacterium spp. are associated with a multitude of animals. For instance, chryseobacteria were detected from the midgut of mosquitos (*Culicoides sonorensis*, Campbell et al. 2004; *Culex quinquefasciatus,* Kämpfer et al. 2010a), within cockroach guts (*Periplaneta americana*, Dugas et al. 2001), millipede feces (*Arthrosphaera magna*, Kämpfer et al 2010b), and penguin guano (*Pygoscelis adeliae*, Zdanowski et al. 2004), gut homogenates of freshwater copepods (*Eudiaptomus gracilis*, Homonnay et al. 2012), bird feathers (Riffel et al. 2003), cow's milk (e.g., Hugo et al. 2003; Hantsis-Zacharov and Halpern 2007; Hantsis-Zacharov et al. 2008a, b), and from raw meats and chicken (Garcia-López et al. 1998; de Beer et al. 2005; Olofsson et al. 2007). Chryseobacteria were recovered from the mucus of apparently healthy fish (Lijnen et al. 2000; Bernardet et al. 2006) and thus, they may be commensalistic. However, *Chryseobacterium balustinum* was first isolated from the scales of halibut (*Hippoglossus hippoglossus*) freshly harvested from the Pacific Ocean (Harrison 1929) where it was considered as a spoilage organism (Austin and Austin 2007). In this context, Engelbrecht et al. (1996) recovered multiple *Chryseobacterium* spp. from marine fish caught in South Africa that

produced H_2S and proteolyzed multiple substrates (e.g., casein, gelatin). Engelbrecht et al. (1996) reported pungent and stale odors in muscle extracts, suggesting involvement in fish spoilage. Similarly, *C. piscium*, which was first isolated from fish in the south Atlantic Ocean of South Africa (de Beer et al. 2006), was suggested to be a spoilage organism due to the presence of urease and phenylalanine deaminase activities. However, Gonzàlez et al. (2000) suggested that chryseobacteria were not an important cause of spoilage in fish, because they comprised less than 1% of the bacterial communities of the fish that they sampled.

Chryseobacterium spp. as pathogens. As was mentioned for *Flavobacterium* spp., caution should be exercised when referring to the literature regarding chryseobacteria as pathogens, because *Chryseobacterium meningosepticum*, which caused numerous infections, was reclassified to the genus *Elizabethkingia* (Kim et al. 2005a). Nevertheless, *Chryseobacterium* spp. were recovered from multiple diseased organisms. For example, *C. indologenes* is pathogenic to the soft tick (*Ornithodoros moubata*, Burešová et al. 2006), whereas other chryseobacteria were recovered from diseased turtles (Hernandez-Divers et al. 2009) and frogs (Olson et al. 1992; Mauel et al. 2002). In addition, multiple *Chryseobacterium* spp. were recovered from human clinical sources (Holmes et al. 1984a; Yabuuchi et al. 1983; Vaneechoutte et al. 2007; Kämpfer et al 2009a; Yassin et al. 2010). For example, *Chryseobacterium indologenes* has caused bacteremia in humans on numerous occasions (Hsueh et al. 1996; Lin et al. 2010; Chou et al. 2011) with multiple clinical manifestations, such as hospital-acquired pneumonia, periotonitis, surgical wound infections (Hseueh et al. 1996), and cellulitis (Green and Nolan 2001). Chryseobacteria were also isolated from burn wounds (Azzopardi et al. 2011), eye infections (Daroy et al. 2011), and pneumonia in newborns (Calderón et al. 2011). Such infections are nosocomial in nature and frequently associated with indwelling devices (e.g., catheters, tracheal tubes, ventilators; Bernardet et al. 2011).

Chryseobacterium infections in fish. *Chryseobacterium balustinum* was first isolated from the scales of halibut (*Hippoglossus hippoglossus*) freshly harvested from the Pacific Ocean (Harrison 1929). However, these isolates are no longer available in culture collections (Bernardet et al. 2006) and the current type strain was isolated from the heart blood of a freshwater dace (*Leuciscus leuciscus*) that had signs of septicemia (Brisou et al. 1959). When this isolate was experimentally injected into multiple fish species, it caused mortality (Brisou et al. 1959), thus suggesting its pathogenic potential (Bernardet et al. 2006).

Mudarris and Austin (1989) reported a disease outbreak in farmed turbot (*Scophthalmus maximus*) in Scotland associated with a novel *Chryseobacterium* sp. that they later named *C. scophthalmum* (Mudarris et al. 1994; Vandamme et al. 1994). They recovered "dense pure cultures" of this bacterium from the gills and viscera of fish that exhibited hyperplasia of the gills, hemorrhage of the eyes, skin, and jaw, necrosis and hemorrhage of the brain, stomach, intestine, liver, and kidney, and ascites within the peritoneum (Mudarris and Austin 1989). They also recovered the same bacterium from apparently healthy adult and juvenile wild turbot. The same authors then experimentally assessed the pathogenicity of *C. scophthalmum* in juvenile turbot in seawater and rainbow trout in freshwater using both immersion exposure and intraperitoneal injection and found similar disease signs as what was seen in naturally infected fish. The authors recovered the same bacterium from infected fish, and fulfilled Koch's postulates. Moreover, injection of bacterial cell homogenates resulted in the same disease signs, but injections of either lipopolysaccharide (LPS) or cell supernatant did not produce any lesions (Mudarris and Austin 1989). The same authors later examined the histopathological changes associated with the natural and experimental *C. scophthalmum* infections (Mudarris and Austin 1992) and reported swelling, necrosis, and edema within the gills, as well as epithelial hyperplasia of the secondary lamellae and proliferation of the interlamellar cells that

"filled up the spaces between the secondary lamellae." Degeneration and necrosis of the renal tubular epithelial cells, dilation and necrosis of the glomeruli, necrosis of interstitial renal tissue, focal necrosis of hepatic cells, edema of the bile ducts, and necrosis of the mucosal epithelial cells of the intestine were also noted. Pathological changes were not observed in either the heart or spleen.

More recently, Bernardt et al. (2005) performed polyphasic characterization on 52 *Chryseobacterium* spp. isolates recovered from diseased fish in Belgium, Finland, France, Spain, Taiwan, Singapore, and Cambodia. While the majority of these isolates could not be ascribed to a definitive species, 14 clusters were defined and 3 of these were believed to represent novel *Chryseobacterium* spp (Bernardet et al. 2005). In addition, two of the isolates recovered from external lesions of farmed Atlantic salmon in Finland were identified as *C. joostei*, which was originally isolated from raw cow's milk in South Africa (Hugo et al. 2003); however, experiments investigating the pathogenicity of these strains were not reported. The authors did suggest that many of the chryseobacteria they studied represented facultative fish pathogens, because many were recovered from recently imported fish that were stressed (Bernardet et al. 2005).

Most recently, a number of novel *Chryseobacterium* spp. were recovered from fish, some of which were diseased. For example, *C. piscicola* was isolated from diseased Atlantic salmon in Finland (Ilardi et al. 2009) and later indicated as moderately virulent in experimental challenges (Ilardi et al. 2010). Likewise, *C. chaponense* is a recently described fish-associated species from Chile (Kämpfer et al. 2011), while *C. viscerum* and *C. oncorhynchi* were just isolated from the livers and gills of diseased rainbow trout (*O. mykiss*) in Spain (Zamora et al. 2012b, c).

***Antimicrobial resistance in Chryseobacterium* spp.** *Chryseobacterium* spp. are inherently resistant to a wide spectrum of antibiotics, including tetracyclines, erythromycin, linezolid, polymyxins, aminoglycosides, chloramphenicol, and many β-lactams, while also being intermediately sensitive to vancomycin and clindamycin and vary in their sensitivity to trimethoprim-sulfamethoxazole (Fraser and Jorgensen 1997; Kirby et al. 2004; Bernardet et al. 2006; Chou et al. 2011). Michel et al. (2005) found that among the 65 isolates they obtained from aquatic habitats, 89% were resistant to polymyxin-B, 97% were resistant to ampicillin, 62% were resistant to erythromycin, and 54% were resistant to oxytetracycline, while 21.5% and 41.5% were resistant and moderately resistant to florfenicol. Additionally, 69% of the isolates were sensitive to trimethoprim-sulfamethoxazole.

V. The Challenges of Working with Members of the Family Flavobacteriaceae

Research on flavobacteria is essential due to their role as important etiological agents of disease and their importance in microbial ecology. However, researchers working with these organisms are faced with significant challenges. First, many flavobacteria, especially those that are pathogenic to fish, are fastidious and grow only on nutrient poor media (Bernardet and Bowman 2006) that, under many conditions, must be supplemented with a variety of antibiotics to prevent overgrowth by other less fastidious bacteria. The ratio and brand of ingredients incorporated into these media, as well as osmotic conditions, can affect the ability to cultivate some flavobacteria (Lorenzen 1993; Cipriano and Teska 1994; Michel et al. 1999). Even when an ideal culture medium for a particular situation is used, the slow generation time of some species and variety in preferred incubation conditions (e.g., temperature, aerobic vs. microaerophillic atmosphere, etc.) can impede successful culture of all organisms.

A second challenge, which was alluded to above, is the rapid pace at which members of the family Flavobacteriaceae are discovered and described. Indeed, one must constantly search the literature to include species that must be incorporated into any identification scheme and to stay abreast with numerous changes in nomenclature (i.e., Kämpfer et al. 2009a, b). A third challenge, especially when working with fish-associated flavobacteria, is their ubiquity in aquatic habitiats and on/in the skin, gills, mucus, and intestines of fish (Bernardet and Bowman 2006). While some of the flavobacteria that are present on apparently healthy fish have also been implicated as facultative fish pathogens (i.e., *F. hydatis*, *F. succinicans*, *C. scophthalmum*; Anderson and Ordal 1960; Strohl and Tait 1978; Mudarris and Austin 1989), the role that many flavobacteria play in fish health is not well understood. It may not be clear if external flavobacteria are transient inhabitants of fish or if they are normal constituents of their bacterial flora and which of the newly described species are truly pathogens. Indeed, some of the bacterial flora that are present in the skin, gills, and intestine of fish inhibited known fish pathogens (Smith and Davey 1993; Spanggaard et al. 2001), while some chryseobacterial strains present on the skin of salamanders (*Hemidactylum scutatum*) exhibit antifungal activity (Lauer et al. 2008) and thus may play a mutualistic role with their host.

Fourth, there is a lack of specific diagnostic reagents available to detect and identify many fish-associated flavobacteria outside of those that are commonly associated with fish disease. For example, the majority of recent reports of novel or less common fish-associated flavobacteria have based their identification on extensive polyphasic characterization as outlined by Bernardet et al. (2002). These methods work well for a definitive identification and allow for a consistent comparison amongst the described flavobacteria, but they necessitate culture of the organism and the technical capabilities to perform an array of biochemical and molecular

assays, some of which are specialized (e.g., DNA-DNA hybridization, fatty acid methyl ester analyses, etc.).

Finally, another impediment to flavobacterial research, especially fish-pathogenic flavobacteria, are the problems associated with experimental challenge models to study their pathogenicity. Despite the fact that *F. psychrophilum* causes economically depressing disease outbreaks in fish all over the world and has been studied extensively, a truly reliable experimental model to assess its pathogenicity still does not exist. For example, experimental infections conducted by numerous authors utilizing immersion challenges have yielded highly variable results and highlight that bath infections are difficult to control, standardize, and reproduce (Rangdale 1995; Madsen and Dalsgaard 1999; Decostere et al. 2000; Garcia et al. 2000; Madetoja et al. 2000; Liu et al. 2001; Busch et al. 2003). However, Madsen and Dalsgaard (1999) studied the reproducibility of *F. psychrophilum* challenge methods and showed that, for the most part, intraperitoneal injection of the bacterium was reproducible, though some parameters (i.e., isolate used, number of fish within the tank, origin of fish, weight of fish, etc.) could introduce variability. Thus, recent research aimed at elucidating the virulence mechanisms and pathogenesis of *F. psychrophilum*, as well ways to prevent and treat the diseases it causes, now use intraperitoneal (Korkea-aho et al. 2011; Nagai and Nakai 2011; Castillo et al. 2012), subcutaneous (Plant et al. 2011), or intramuscular injection (Hadidi et al. 2008; Silverstein et al. 2009; Leeds et al. 2010), even though it is recognized that these methods bypass important aspects of the innate immune system.

CHAPTER TWO

DIVERSITY OF FISH-ASSOCIATED FLAVOBACTERIA OF MICHIGAN

INTRODUCTION

Over the past two decades, the taxonomy of the family Flavobacteriaceae (Reichenbach 1992) was amended on multiple occasions (Bernardet et al. 1996; Bernardet et al. 2002) and advances in molecular techniques have rapidly expanded the number of genera and species within the family (Bernardet et al. 2011). Bacteria within this family occupy a wide range of ecological niches (Bernardet and Nakagawa 2006) and are associated with disease in an array of organisms, including invertebrates (Li et al. 2010), amphibians (Xie et al. 2009), reptiles (Hernandez-Divers et al. 2009), birds (Segers et al. 1993), and mammals (Haburjak and Schubert 1997), including humans (Benedetti et al. 2011). Serious diseases that pose threats to wild and propagated fishes are caused by multiple genera within the family Flavobacteriaceae, such as *Flavobacterium* spp. (Starliper 2011), *Tenacibaculum* spp. (Suzuki et al. 2001), and *Chryseobacterium* spp. (Muddarris and Austin 1989).

Most flavobacteriosis outbreaks in freshwater fishes are attributed to three *Flavobacterium* spp.; namely, *F. psychrophilum, F. columnare,* and *F. branchiophilum* (Austin and Austin 2007). In addition, a number of other fish-associated *Flavobacterium* spp. have been reported in association with fish disease, such as *F. johnsoniae* (Suebsing and Kim 2012), *F. succinicans* (Anderson and Ordal 1961), *F. hydatis* (Strohl and Tait 1978), as well as a number of uncharacterized yellow-pigmented bacteria (Austin and Austin 2007). Most recently, a number of novel *Flavobacterium* spp. were isolated from diseased fish in Europe and South America,

including *F. oncorhynchi* (Zamora et al. 2012a), *F. chilense*, and *F. araucananum* (Kämpfer et al. 2012).

Similarly, the genus *Chryseobacterium* (Vandamme et al. 1994), contains a number of fish-pathogenic species that have recently emerged as a serious problem in Europe and Asia (Michel et al. 2005; Bernardet et al. 2005). Indeed, numerous novel fish-associated *Chryseobacterium* spp. have been recently described, such as *C. piscium* (de Beer et al. 2006), *C. piscicola* (Ilardi et al. 2009), *C. arothri* (syn *C. hominis*; Kämpfer et al. 2009c) *C. chaponense* (Kämpfer et al. 2011), and *C. viscerum* (Zamora et al. 2012b). Currently there are no published reports of *Chryseobacterium*-caused diseases in fish in the continental USA.

In Michigan and its associated state fish hatcheries, *Flavobacterium* spp. have been associated with more fish mortality than all other fish pathogens combined (Faisal and Hnath 2005; Faisal et al. 2011). Flavobacterial isolates ranged from the most common three *Flavobacterium* spp. to other *Flavobacterium* and *Chryseobacterium* spp. whose identity remains to be determined. The present manuscript details the diversity of *Flavobacterium* and *Chryseobacterium* spp. that were recovered during detailed fish health surveys performed by Michigan State University-Aquatic Animal Health Laboratory (MSU-AAHL) personnel during surveillance and disease epizootics from the state of Michigan during 2003-2010. This study represents the first to detail the fish-associated flavobacterial diversity within the Laurentian Great Lakes.

MATERIALS AND METHODS

Fish and bacterial isolation. From 2003-2010, fish (21 different species, Supp. Table 2.1) were submitted to the MSU-AAHL for routine disease surveillance and diagnostic purposes. Fish were either delivered to the laboratory alive and then euthanized with an overdose of

tricaine methane sulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA) or were euthanized by Michigan Department of Natural Resources (MDNR) personnel and sampled on site. Tissues for bacterial culture were collected as described in Loch et al. (2011) with the exception that 1 µl loops were used for fish ≤6 cm in length and 10 µl loops were used for fish ≥6 cm in length. Kidney and/or gill tissues for bacterial isolation were collected during fish health surveillance, while other tissues, including gills, fins, swim bladder fluid, and/or external ulcers were also bacteriologically analyzed when disease signs were observed. Collected samples were inoculated directly onto Hsu-Shotts medium (HSM; Bullock et al. 1986) and cytophaga agar (CA; Anacker and Ordal 1955), both of which were supplemented with neomycin sulfate at 4 mg l^{-1} of medium, and plates were incubated at 22°C for up to 7 days and 15°C for up to 14 days, respectively. Bacterial growth was then recorded, and individual colonies were sub-cultured for phenotypic and molecular analyses. For cryopreservation, an individual colony was inoculated into HSM or Cytophaga broth, incubated for 3-5 days, 20% glycerol (V/V) added, and frozen at -80°C.

Characterization of recovered isolates. Bacteria that grew on neomycin-supplemented HSM or CA were visually inspected for non-diffusible yellow pigment, and 24 to 48 -hr old cultures on HSM (at 22°C) and 48 to 96 -hr old cultures on CA (at 15°C) were assayed via the Gram reaction and/or the string test (AFS-FHS 2010). Representative isolates were tested for their ability to degrade hemoglobin [0.1% w/v using HSM as the basal medium as modified from Shotts et al. (1985); n=118], to degrade casein (5% w/v; n=91) and elastin (0.5%; n=116) as modified from Shotts et al. (1985) using HSM as the basal medium, and gelatin (n=123) as detailed in Whitman (2004). Enzymatic assays were incubated at the temperature at which bacteria were initially isolated and results were read up to 7-days post-inoculation. Gram negative rods that grew on HSM and/or CA and possessed a non-diffusible yellow pigment

(n=254) were suspected to be members of the family Flavobacteriaceae and were then subjected to molecular analyses.

16S rRNA gene amplification. The DNA from each of the 254 isolates was extracted using a Qiagen DNeasy Tissue Extraction kit (QIAGEN Sciences, Valencia, CA, USA) according to the manufacturer's protocol. Quantification of extracted DNA was performed using the Quant-iT™ DS DNA assay kit in conjunction with a Qubit® flourometer (Life Technologies, Grand Island, NY, USA). Amplification of partial 16S rRNA gene was conducted via the polymerase chain reaction (PCR) using the universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG -3') and 1387R [Marchesi et al. (1998); 5'-GGG CGG WGT GTA CAA GGC-3'; numbering is based on 16S rRNA gene of *Escherichia coli*, Brosius et al. (1978)]. The 50- µl PCR reaction for each sample contained a final concentration of 200- nM for each primer, 25- µl of 2× Go-Taq Green master mix (Promega, Madison, WI, USA), and 20- ng of DNA template, with DNase-free water comprising the remainder of the reaction mixture. The DNA amplification was carried out in a Mastercycler® Pro Thermalcycler (Eppendorf, Hauppauge, NY, USA) with an initial denaturation step at 95°C for 5- min, followed by 32 cycles of amplification, which included denaturation at 95°C for 30- sec, annealing at 58°C for 30- sec, and elongation at 72°C for 60- sec. A final extension step was performed at 72°C for 7- min. Amplicons were combined with SYBR® Green gel stain (Cambrex Bio Science Rockland Inc., Rockland, ME, USA), run on a 1.5% agarose gel at 50 V for 30- min, and then visualized under UV exposure. A 1-kb ladder (Roche Applied Science, Indianapolis, IN, USA) was used as a molecular marker.

Phylogenetic analyses. Amplicons were purified using the QIAquick Purification kit (Qiagen) according to the manufacturer's protocol except that the same 35- µl of elution buffer preheated

to 56 °C was used for both elution steps. Amplified DNA was then sequenced at the Genomics Technology Support Facility of Michigan State University using the 27F primer. Generated sequences were initially analyzed using the nucleotide Basic Local Alignment Search Tool (BLASTN) software from the National Center for Biotechnology Information (NCBI, USA). Sequences for all formally described and "candidate" *Flavobacterium* and *Chryseobacterium* spp., as well as *Elizabethkingia miricola, E. meningosepticum, Capnocytophaga ochracea* (outgroups), were downloaded from NCBI and the EzTaxon-e database (Kim et al. 2012) and the percent 16S rDNA similarity between the 254 Michigan isolates and the closest type strains determined using the alignment function in BLAST. Type strains were also aligned with the 254 isolates recovered from Michigan fishes using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 4.0) to assess phylogentic relationships. Neighbor-joining (NJ) analysis was then performed (Saitou and Nei 1987) in MEGA, with evolutionary distances being calculated using the Maximum Composite Likelihood method (Tamura et al. 2004). Topology robustness was evaluated by bootstrap analysis based upon 10,000 resamplings of the sequences, and a total of 711 characters were examined. Alignment gaps and/or missing data were deleted only in pair-wise sequence comparisons, and the tree was rooted with *Capnocytophaga ochracea* as the outgroup. Only bootstrap values ≥70 were displayed on the resultant dendrograms and were interpreted as strong support for the topology present at that respective node.

RESULTS

Bacterial cultures yielding Gram negative yellow pigmented bacteria on HSM and/or CA were obtained from 21 fish species, including channel catfish *Ictalurus punctatus*, coho salmon *Oncorhynchus kisutch*, Chinook salmon *O. tshawytscha*, rainbow trout *O. mykiss*, steelhead *O. mykiss*, bluegill *Lepomis macrochirus*, sea lamprey *Petromyzon marinus*, walleye *Sanders*

vitreus, lake whitefish *Coregonus clupeaformis*, lake herring *C. artedi*, brown trout *Salmo trutta*, Atlantic salmon *S. salar*, brook trout *Salvelinus fontinalis*, lake trout *S. namaycush*, mottled sculpin *Cottus bairdii*, northern brook lamprey *Ichthyomyzon fossor*, yellow perch *Perca flavescens*, smallmouth bass *Micropterus dolomieu*, largemouth bass *Micropterus salmoides*, muskellunge *Esox masquinongy*, and northern pike *E. lucius* during 101 sampling events that took place between 2003-2010. Information on each of the isolates, including fish species, site, purpose of sampling, date, and organ are detailed in Supp. Table 2.1. Of the 254 Gram negative yellow-pigmented flavobacterial isolates analyzed in this study, 211 were identified as *Flavobacterium* spp. (retrieved from 88 sampling events and 21 fish species) and 43 as *Chryseobacterium* (retrieved from 26 sampling events and 12 fish species, Supp. Table 2.1) according to partial 16S rRNA gene and BLASTN analysis.

The 211 *Flavobacterium* spp. were 96.5-100 % similar to 21 of the 83 formally described and "candidate" *Flavobacterium* spp. Among them, 123 were recovered from wild and feral Michigan fishes while 88 were recovered from fish reared within hatcheries. The majority of the isolates were retrieved during routine health surveys (n=155) while 56 isolates were associated with mortality episodes. Organs of recovery included the kidneys (n=92), gills (n=88), brain (n=16), fins (n=9), fluid within the swimbladder lumen (n=4), and ulcers of the skin/muscle (n=2; Supp. Table 2.1).

Forty-three of the yellow-pigmented bacteria recovered from Michigan fishes were most similar to members of the genus *Chryseobacterium*, ranging from 96.7-99.9% similarity with described and *Candidatus Chryseobacterium* spp. The isolates were recovered from wild/feral fish (n=17) and fish reared within hatcheries (n=26) during health surveys (n=27) and from mortality episodes (n=16). Organs of recovery included the gills (n=21), kidneys (n=11), fins (n=7), skin/muscle ulcers (n=2), and brains (n=2) of infected fish (Supp. Table 2.1).

Michigan *Flavobacterium* spp. were most similar to *F. anhuiense* (n=2), *F. aquidurense* (n=15), *F. araucananum* (n=19), *F. chilense* (n=2), *F. chungangense* (n=10), *F. chungbukense* (n=2), *F. columnare* (n=15), *F. degerlachei* (n=2), *F. frigidimaris* (n=12), *F. glacei* (n=2), *F. hercynium* (n=33), *F. hibernum* (n=2), *F. hydatis* (n=3), *F. oncorhynchi* (n=20), *F. pectinovorum* (n=28), *F. psychrolimnae* (n=1), *F. psychrophilum* (n=19), *F. reichenbachii* (n=3), *F. resistans* (n=2), *F. succinicans* (n=16), and *F. tiangeerense* (n=3). Phylogenetic analysis of Michigan fish-associated *Flavobacterium* spp. placed them into 32 distinct clusters (bootstrap value ≥ 70; Fig. 2.1, Supp. Table 2.1); however, the topology of 32 isolates was unresolved (bootstrap value <70). On the other hand, Michigan *Chryseobacterium* spp. were most similar to *C. aquaticum* (n=1), *C. chaponense* (n=2), *C. ginsenosidimutans* (n=6), *C. greenlandense* (n=2), *C. indologenes* (n=1), *C. indoltheticum* (n=6), *C. piscicola* (n=2), *C. piscium* (n=5), *C. scophthalmum* (n=1), *C. shigense* (n=1), *C. viscerum* (n=14), and *C. vrystaatense* (n=2). Phylogenetic analysis of the 43 Michigan fish-associated *Chryseobacterium* spp. yielded 10 distinct clusters (bootstrap value ≥ 70; Fig. 2.2, Supp. Table 2.1), while the topology for 16 isolates could not be resolved (bootstrap value <70). Disease signs in fish infected with bacteria belonging to each Cluster can be found in Table 2.1; however, it must be noted that the observed pathological changes cannot be solely attributed to the flavobacteria that were recovered since these fish were naturally infected and other fish pathogens may have also contributed to the observed lesions.

The % 16S rRNA gene similarity of the 33 Michigan isolates most similar to *F. hercynium* ranged from 97.0 – 98.8% (Supp. Table 2.1). Phylogenetic analysis yielded 5 distinct clusters (Fig. 2.1); Cluster I (6 isolates; Supp. Fig. 2.1), Cluster II (13 isolates; Supp. Fig. 2.1), Cluster III (6 isolates; Supp. Fig. 2.2), Cluster IV (4 isolates; Supp. Fig. 2.3), and Cluster V (3 isolates; Supp. Fig. 2.4), while the topology of 3 isolates (T65, S53, T132) was unresolved (Fig. 2.1).

Within Cluster I, isolates S113 and S114, both of which were recovered from the necrotic fins of hatchery-reared brown trout fingerlings undergoing a mortality episode, formed a well-supported group that was distinct from the other 4 isolates (Supp. Fig. 2.1). It is also of interest that isolate T129 was most similar to *F. succinicans* by % 16S rDNA and yet also fell within Cluster I. Cluster II was comprised of the largest number of isolates most similar to *F. hercynium*, within which distinct sub-clusters were present (Supp. Fig. 2.1). For example, isolates T101 and T102, which were recovered from the kidneys of Chinook salmon and brook trout fingerlings raised at two different Michigan hatcheries, were quite distinct from the other members of Cluster II (bootstrap= 99). With the exception of S148, all of the isolates belonging to Cluster II were recovered from wild and hatchery-reared salmonids (Supp. Table 2.1). Five of the six isolates belonging to Cluster III were recovered from mortality events involving hatchery-reared salmonid fingerlings and the remaining isolate originated from the kidney of a feral Chinook salmon returning to the Swan River Weir (Presque Isle County, Lake Huron watershed). The isolates comprising Cluster IV, which included isolate S15 that was most similar to *F. chungangense* by 16s rDNA % similarity (Supp. Fig. 2.3), were all recovered from the kidneys and swimbladders of spawning brown and steelhead trout. Cluster V isolates all originated from hatchery-reared brown trout fingerlings (Supp. Table 2.1), with 1 of the 3 isolates (S86) being associated with mortalities. Enzymatic activities for isolates within Clusters I-V varied (Table 2.2). Cluster I was uniformly negative for gelatinase, but varied in hemoglobin hydrolysis, caseinase and elastase activities. Cluster II was variable for all four proteases. Results for protease activities for Clusters III-V can be found in Table 2.2.

The % 16S rRNA gene similarity of the 28 Michigan isolates most similar to *F. pectinovorum* ranged from 97.1- 98.4% (Supp. Table 2.1). Phylogenetic analysis yielded 3 distinct clusters (Fig. 2.1) that contained 3 isolates (Cluster VI; Supp. Fig. 2.2), 11 isolates (Cluster VII; Supp. Fig. 2.2), and 4 isolates (Cluster VIII; Supp. Fig. 2.1), while the topology of 12 isolates was

unresolved (Fig. 2.1 and Supp. Fig. 2.2). Interestingly, S31, S37, and S164, which were most similar to *F. aquidurense, F. aquidurense,* and *F. frigidimaris*, according to 16S rDNA % similarity respectively, also fell within Cluster VII (Supp. Fig. 2.2). Eight isolates (S40, S29, S35, S34, S38, S37, S31, and S41) belonging to Cluster VII were all recovered from the brains of hatchery-reared coho salmon fry undergoing a single mortality episode (Faisal et al. 2011). Isolates belonging to Cluster VIII were recovered exclusively from hatchery-reared brook and rainbow trout, while the other 2 clusters were recovered from both wild and hatchery reared fishes (Supp. Table 2.1). Among the 3 isolates belonging to Cluster VII that were assayed for proteolytic activity, all degraded casein, hemoglobin, and elastin but did not proteolyze gelatin, while the 3 tested isolates within Cluster VIII proteolyzed elastin and casein, but varied in gelatinase activity (1/3 positive) and hemoglobin hydrolysis (1/3 positive; Table 2.2).

The isolates (n=20) recovered from Michigan that were most similar to newly described *F. oncorhynchi* ranged from 97.4-100 in percent 16S rDNA similarity. Phylogenetic analysis yielded 2 distinct clusters (Fig. 2.1); Cluster IX, which contained 8 isolates (Supp. Fig. 2.4), and Cluster X, which contained 12 isolates (Supp. Fig. 2.2). Isolates belonging to Cluster IX were nearly identical to the *F. oncorhynchi* reference sequence (99.3-100% similar), originated from 6 different fish species, and were predominantly recovered from the gills, though 3 isolates were also recovered from kidneys (Supp. Table 2.1). Despite the high % similarity, there was evidence of a distinct sub-clade within Cluster IX (Supp. Fig. 2.4). Three of the eight isolates were recovered from two mortality events in hatchery-reared brown trout and brook trout fingerlings raised at two Michigan hatcheries. Cluster X formed a distinct clade (Supp. Fig. 2.2). Similarly to Cluster IX, the majority of the isolates within Cluster X were recovered from gills (9/12), and the remainder from kidneys (Supp. Table 2.1). Cluster X isolates were recovered from hatchery-reared salmonids, with the exception of T103, which originated from a wild sea

lamprey (Supp. Table 2.1). Proteolytic activities varied in both clusters (Table 2.2), but none of the isolates degraded hemoglobin.

The % 16S rRNA gene similarity of the 19 Michigan isolates most similar to the *F. araucananum* reference strain ranged from 96.9 - 98.8%. When analyzed phylogenetically, two clusters were evident (Fig. 2.1); Cluster XI (n=9; Supp. Fig. 2.5) and Cluster XII (n=9; Supp. Fig. 2.3), and two isolates (T157 and S162) were unresolved (Fig. 2.1). Isolates falling into Cluster XI were recovered from 9 different species of wild/feral (n=6) and hatchery-reared (n=3) fishes (Supp. Table 2.1). Isolate T17, which was most similar to *F. aquidurense* by 16S rDNA similarity, was one of nine isolates in this cluster. All 3 isolates that originated from hatchery-reared fishes were associated with mortality events in chinook salmon fingerlings, northern pike fingerlings, and rainbow trout fingerlings (Supp. Table 2.1). Among these, isolate S21 was recovered from deep necrotic ulcers present on the dorsum of a rainbow trout (Fig. 2.3a). *Flavobacterium* spp. isolates that belonged to Cluster XII were primarily recovered from gills of wild fishes sampled during fish health surveillance (Supp. Table 2.1), though isolate S43 was recovered from the brain of a hatchery-reared coho salmon fry during a mortality event (Faisal et al. 2011). Interestingly, the majority of the wild fish from which the isolates belonging to Cluster XII were recovered from the creek supplying water to the hatchery where the coho salmon outbreak occurred (represented by isolates S126, S130, S149), as well as within the hatchery effluent pond (represented by isolates S163, S161, S166; data not shown), despite being sampled 4 years apart. Within Cluster XI, all tested isolates were positive for caseinase and elastase activity, but varied in gelatinase and hemoglobin hydrolysis activities (Table 2.2). The 7 tested isolates within Cluster XII were all positive for caseinase and hemoglobin hydrolysis, and were gelatinase negative, but varied in elastase activity (Table 2.2).

Nineteen isolates examined in this study were most similar to *F. psychrophilum* according to % 16S rDNA similarity (98.6 – 99.9%). Phylogenetic analysis performed on the isolates recovered from Michigan fishes yielded two well-supported clusters within the species (Fig. 2.1); Cluster XIIIa (Supp. Fig. 2.6) was comprised of 15 isolates recovered predominantly from feral spawning (n=13) *Oncorhynchus* spp. (Supp. Table 2.1), while Cluster XIIIb (Supp. Fig. 2.6) consisted of the *F. psychrophilum* reference strain, 1 *F. psychrophilum* isolate recovered from feral spawning Chinook salmon, and 3 *F. psychrophilum* isolates recovered from hatchery-reared Atlantic salmon fingerlings (Supp. Table 2.1). All 19 Michigan *F. psychrophilum* isolates were recovered from the kidneys of infected fishes.

Isolates most similar to *F. succincans* (n=16) were the next most numerous group and ranged from 96.6 – 98.0% 16S rDNA similarity with that of the reference strain. Upon phylogenetic analysis, 3 well-supported clusters were evident (Fig. 2.1); Cluster XIV (n=2; Supp. Fig. 2.5), Cluster XV (n=10; Supp. Fig. 2.7), and Cluster XVI (n=3; Supp. Fig. 2.7), while isolate T129 fell into Cluster I (described above; Supp. Fig. 2.1). Interestingly, isolates belonging to these three clades were exclusively recovered from wild fishes (Supp. Table 2.1) and were rarely associated with disease. The two *Flavobacterum* spp. isolates comprising Cluster XIV, which was quite distinct from the Clusters XV and XVI, were both recovered from the kidneys of lake whitefish collected from Naubinway, Lake Michigan. While Cluster XV and Cluster XVI were close relatives, phylogenetic analysis provided evidence for their divergence (bootstrap=77; Supp. Fig. 2.7). Cluster XV isolates were recovered from both coldwater and warmwater fishes, and were predominantly isolated from gills (7/10). Cluster XVI isolates originated from the gills of brown trout yearlings residing in Cherry creek sampled during 2008 and 2010 (Supp. Table 2.1). Representative isolates belonging to Cluster XV did not degrade gelatin or hemoglobin and were variable for caseinase and elastase activities (Table 2.2).

Fifteen isolates most similar to *F. columnare* (16S rDNA similarity of 98.7 – 100%) were also analyzed in this study. Among these, 14/15 were nearly identical to the *F. columnare* reference sequence (99.7-100% similar), while isolate S81 was more distinct at 98.7% similarity. Phylogenetic analysis also reflected this difference (Fig. 2.1), whereby the *F. columnare* reference sequence and 14 of the Michigan *F. columnare* isolates formed a distinct and homogenous cluster (Cluster XVII; Supp. Fig. 2.8), while *F. columnare* strain S81 formed a distinct branch outside of this Cluster, termed Cluster XVIIa (Supp. Fig. 2.8). Four Michigan *F. columnare* isolates were recovered from three mortality events involving hatchery-reared yellow perch (S19), hatchery-reared muskellunge fingerlings (T89 and T90), and wild smallmouth bass from Lake St. Clair (T79; Supp. Table 2.1). The remaining 13 *F. columnare* isolates were recovered from the kidneys of feral coho and Chinook salmon returning to the Platte River Weir (Benzie County, Lake Michigan watershed), the Little Manistee River Weir (Manistee County, Lake Michigan watershed), and the Swan River Weir (Presque Isle County, Lake Huron watershed). Representative isolates (n=4) belonging to Cluster XVII uniformly degraded gelatin, casein, and elastin, but only 1 of 4 isolates hydrolyzed hemoglobin (Table 2.2).

Fifteen isolates examined in this study were most similar to *F. aquidurense* according to % 16S rDNA similarity (97.3 – 98.2%). Among these, 2 isolates (S31 and S37) fell into Cluster VII (described above; Supp. Fig. 2.2), one isolate (T17) fell into Cluster XI (Supp. Fig. 2.5), one isolate (S30) fell into Cluster XX (described below; Supp. Fig. 2.9), one isolate was unresolved (S107; Fig. 2.1), and the remaining 10 isolates formed Cluster XVIII (Supp. Fig. 2.9). Isolates belonging to Cluser XVIII, which displayed varying degrees of genetic heterogeneity (Fig. 2.12), were recovered from the kidneys of feral Chinook salmon (n=6) during 2005 and 2007 (Supp. Table 2.1), as well as from the kidneys of hatchery-reared salmonid fingerlings (n=3) and wild larval sea lamprey (n=1). Among these, isolate T16 was associated with a mortality event in cultured Chinook salmon fingerlings. Protease assays performed on representative Cluster

XVIII isolates found that this group uniformly degraded casein and elastin, but varied in gelatin and hemoglobin degradation (Table 2.2).

Isolates most similar to *F. frigidimaris* (n=12) ranged in % 16S rDNA similarity to the *F. frigidimaris* reference strain from 97.4 – 100%. Phylogenetic analysis revealed the formation of Cluster XIX, which contained 4 isolates and the *F. frigidimaris* reference sequence (Fig. 2.1 and Supp. Fig. 2.9), along with Cluster XX (Supp. Fig. 2.9), which contained isolates S5 and S30 (most similar to *F. aquidurense* according to 16S rDNA %), while the remaining 6 isolates were unresolved (Fig. 2.1, Supp. Table 2.1). However, among the unresolved isolates, 5 fell close to *F. frigidimaris* despite having a bootstrap value < 70, while 1 isolate fell close to *F. hercynium* in a similar fashion (Fig. 2.1). In addition, isolate S164 fell into cluster VII (Supp. Fig. 2.2). Isolates belonging to Cluster XIX were recovered from kidneys, fins (Fig. 2.3b), and brains of hatchery-reared salmonid fingerlings undergoing mortality (Supp. Table 2.1). Isolates within Cluster XIX did not degrade gelatin or elastin and varied in caseinase activity and hemoglobin hydrolysis (Table 2.2). One isolate belonging to Cluster XX was positive for degradation of gelatin, hemoglobin, and elastin (Table 2.2).

The % 16S rRNA gene similarity of the 10 Michigan isolates most similar to the *F. chungangense* reference strain ranged from 96.5 - 98.0%. When phylogenetically analyzed, 2 clusters were evident (Fig. 2.1); Cluster XXI (n=3; Supp. Fig. 2.2) and Cluster XXII (n=5; Supp. Fig. 2.3), while isolate S129 was unresolved (Fig. 2.1) and isolate S15 fell into Cluster IV (Supp. Fig. 2.3), as previously described. Isolates in Cluster XXI were recovered from the gills of wild fish, while those from Cluster XXII were isolated from the gills and fins (Fig. 2.3c) of wild and hatchery-reared salmonids (Supp. Table 2.1), and only some of those were associated with mortality events. In addition, isolate T27 of Cluster XXII was recovered from the kidneys of wild northern brook lamprey. Cluster XXI isolates did not degrade gelatin, but degraded casein, and

varied in hemoglobin hydrolysis and elastase activities (Table 2.2). Representative Cluster XXII isolates degraded hemoglobin and elastin and did not degrade gelatin (Table 2.2).

The three Michigan isolates most similar (97.1%) to the *F. reichenbachii* reference sequence formed Cluster XXIII (Fig. 2.1), which shared a most recent common ancestor with the *F. reichenbachii* reference sequence (Supp. Fig. 2.5). All three of these isolates were recovered from wild/feral salmonids. Isolates most similar to *F. tiangeerense* (96.5 – 98.7%) varied in their phylogenetic topology, whereby isolates T56 and S160 were unresolved and isolate T105 formed Cluster XXIV with the *F. tiangeerense* reference sequence (Supp. Fig. 2.6). *Flavobacterium hydatis*-like isolates (n=3; 97.7-98.9%) fell into two clusters (XXV and XXVI; Supp. Fig. 2.1), while 1 isolate (T159) was unresolved. Cluster XXV was comprised of isolate S54, as well as isolate S118 (closest to *F. hibernum* by % 16S rDNA similarity), both of which were recovered from the gills and kidneys of wild fishes (Supp. Table 2.1), while Cluster XXVI contained the *F. hydatis* reference sequence and isolate S171 (Fig. 2.1). Isolates belonging to Clusters XXV and XXVI were positive for casein, hemoglobin, and elastin degradation, but did not degrade gelatin (Table 2.2).

The two isolates most similar to *F. anhuiense* (97.4 – 98.0%) were recovered from the kidneys of hatchery-reared channel catfish (Supp. Table 2.1) and formed Cluster XXVII, which was distinct from that of the *F. anhuiense* and *F. ginsenosidimutans* (Supp. Fig. 2.4). These isolates degraded gelatin, casein, and elastin, but did not hydrolyze hemoglobin (Table 2.2). Both of the *Flavobacterium chilense*-like isolates (98.3–98.4 % 16S rDNA similarity) were recovered from kidneys of wild lake whitefish collected from Lake Michigan. Phylogenetically, these isolates appeared distinct from the *F. chilense* reference sequence and formed Cluster XXVIII (Supp. Fig. 2.4). The two isolates most similar to *F. chungbukense* (97.5-97.9%), which were recovered from gills of wild sculpin and brown trout (Supp. Table 2.1), were both unresolved

phylogenetically (Fig. 2.1). The two *Flavobacterium degerlachei*- like isolates (96.7-96.9%) formed Cluster XXIX (Supp. Fig. 2.5), and were both recovered from a hatchery mortality event among brown trout fingerlings (Supp. Table 2.1). Isolates most similar to *F. glacei* (n=2; 98.5-98.6%) formed Cluster XXX (Supp. Fig. 2.1), whereby one isolate (S42) was recovered from the brain of a moribund coho salmon fry in a hatchery stock undergoing a mortality episode (Faisal et al. 2011) and the other was recovered from the kidney of a wild walleye (Supp. Table 2.1). The two isolates most similar to *F. hibernum* (97.7%) were distinct in that isolate S118 belonged to Cluster XXV (described above; Supp. Fig. 2.1), while isolate S140 was unresolved, but shared a most recent ancestry with *F. hibernum* (Supp. Fig. 2.5). *F. resistans*-like isolates (n=2; 97.1-97.3%), which were recovered from the kidneys of wild largemouth bass, formed Cluster XXXI, and shared a most recent ancestry with *F. resistans* (Supp. Fig. 2.4). Isolate S2, which was most similar to *F. psychrolimnae* (99.6%), formed Cluster XXXII with the *F. psychrolimnae* reference strain (Supp. Fig. 2.6) and was originally recovered from the kidney of a wild walleye.

Among the Michigan isolates within the genus *Chryseobacterium*, 14 were most similar to the recently described *C. viscerum*, ranging from 98.8-99.7% 16S rDNA similarity (Supp. Table 2.1). Phylogenetic analysis yielded the formation of 1 cluster (Cluster XXXIII; Supp. Fig. 2.10) that contained isolates T86, T87, and T88, but the remaining 11 isolates were unresolved although they were close in proximity to the *C. viscerum* reference sequence (Unresolved Group 2; Supp. Fig. 2.10). Isolates within Cluster XXXIII were recovered from the gills and kidneys of hatchery-reared muskellunge fingerlings undergoing a single mortality event (Supp. Table 2.1) that were also infected with *F. columnare* and all uniformly degraded gelatin, casein, hemoglobin, and elastin (Table 2.2). The 11 unresolved *C. viscerum* isolates were recovered from gills of wild sculpin, brook trout, and brown trout (Supp. Table 2.1) residing in 4 different Michigan creeks. All of these isolates displayed gelatinase, caseinase, and elastase activities, as well as hemoglobin hydrolysis (Table 2.2).

Six Michigan isolates were most similar to *C. ginsenosidimutans* (97.7-98.4%), all of which comprised Cluster XXXIV (Supp. Fig. 2.11). However, some genetic heterogeneity was observed within this cluster, whereby isolates T107 and T130 diverged from their most recent common ancestor and that of isolates T62, S104, T68, and S110 (Supp. Fig. 2.11). The six isolates within this cluster were recovered from hatchery-reared fish, and four of the six were recovered from four different morbidity/mortality events in brook and brown trout fingerlings (Supp. Table 2.1) and were recovered from infected gills, as well as necrotic and hemorrhagic fins (Fig. 2.3e). Moreover, isolates T68 and T130 were recovered from the kidneys of infected salmonid fingerlings (Supp. Table 2.1). Protease assays found that 2/6 were positive for gelatinase and 4/6 were positive for hemoglobin hydrolysis, but all tested isolates were positive for elastase and caseinase activities (Supp. Table 2.1). Among the six isolates most similar to *C. indoltheticum* (97.4-99.1% 16S rDNA similarity), three formed Cluster XXXV (Supp. Fig. 2.11) and three were unresolved despite clustering near *C. indoltheticum* and Cluster XXXV (Supp. Fig. 2.11). The isolates of Cluster XXXV were recovered from gills and brains of hatchery-reared steelhead fingerlings during a single mortality event (Supp. Table 2.1), while isolate S63 was recovered from a necrotic ulcer in the musculature of hatchery-reared coho salmon fingerlings and isolate T72 was recovered from the kidney of hatchery-reared lake herring with septicemia (Fig. 2.3d).

The % 16S rRNA gene similarity of the five Michigan isolates most similar to *C. piscium* ranged from 98.3- 98.4% (Supp. Table 2.1). Phylogentic analysis placed the five isolates, along with one isolate (S56) most similar to *C. scophthalmum,* into two clusters (Fig. 2.2). Cluster XXXVI contained isolate T24, along with *C. balustinum, C. piscium, C. scophthalmum* (Supp. Fig. 2.11), while Cluster XXXVII contained the other five isolates (Supp. Fig. 2.11). Isolate T24 of Cluster XXXVI was recovered from the gills of a hatchery-reared steelhead trout fingerling (Supp. Table

2.1) with signs similar to bacterial gill disease and it degraded gelatin, casein, hemoglobin, and elastin (Table 2.2), while three of the isolates within Cluster XXXVII were also recovered from hatchery mortality events in lake herring and brown trout fingerlings. Representative Cluster XXXVII isolates degraded casein and elastin, but were variable in gelatinase activity and hemoglobin degradation (Table 2.2).

The two isolates most similar to *C. chaponense* (99.1%) formed Cluster XXXVIII, which also included the *C. chaponense* reference sequence (Supp. Fig. 2.12). Isolate T115 was recovered from the kidneys of feral Chinook salmon, while isolate T60 was recovered from the kidney of a hatchery-reared rainbow trout fingerling (Supp. Table 2.1). *Chryseobacterium greenlandense*-like isolates (n=2; 98.1% similarity) comprised Cluster XXXIX (Supp. Fig. 2.11) and were recovered from the kidneys of wild walleye (S4) and from dermal ulcers on feral steelhead (S25, Fig. 2.3f.) The two isolates most similar to *C. piscicola* (96.7-99.7%) were isolate T63, which formed Cluster XL with the *C. piscicola* reference sequence (Supp. Fig. 2.11), and T85, which was unresolved, but shared a most recent ancestry with Cluster XL (Supp. Fig. 2.11). The two isolates most similar to *C. vrystaatense* (99.5-99.9%) fell into Cluster XLI, along with the *C. vrystaatense* reference sequence (Supp. Fig. 2.10). These isolates were recovered from fins of wild sea lamprey and gills of wild mottled sculpin (Supp. Table 2.1). The remaining three isolates from Michigan fishes were most similar to *C. aquaticum* (S105; 99.7%), *C. indoltheticum* (S7; 97.4%), and *C. shigense* (S108; 98.6%). Isolate S105 fell into Cluster XLII with *C. aquaticum* (Supp. Fig. 2.11), isolate S108 shared a most recent ancestry with *C. shigense* (Supp. Fig. 2.10), while isolate S7 was unresolved (Supp. Fig. 2.11).

DISCUSSION

As expected, *F. psychrophilum* and *F. columnare* were associated with serious losses in an array of wild, feral, and aquacultured fish stocks on Michigan. However, this study clearly demonstrated the multitude of other *Flavobacterium* and *Chryseobacterium* spp. that are also associated with diseased fishes, as evidenced by the formation of 42 distinct clusters upon phylogenetic analysis of 254 Michigan flavobacterial isolates (Figs. 2.1 and 2.2). Among the Michigan isolates identified as described *Flavobacterium* spp., *F. columnare* was recovered from multiple mass mortality events involving wild and cultured fishes of Michigan during which thousands of fish died (Records of the MSU-AAHL). In addition, this bacterium was isolated from numerous feral salmonid fish stocks returning to spawn in Michigan's gamete collecting facilities from 2006-2010, where the prevalence of systemic columnaris disease can exceed 50% in some locations on an annual basis (Records of the MSU-AAHL). While the majority of the *F. columnare* isolates examined in this study were homogeneous and nearly identical to the *F. columnare* reference sequence (accession number AB078047.1), isolate S81, which was recovered from a feral adult coho salmon returning to spawn at the Platte River weir in 2006, was distinct. It is well established that there are at least three *F. columnare* genomovars (Triyanto and Wakabayashi 1999) and isolates belonging to distinct genomovars differ in pathogenicity (Shoemaker et al. 2008). While a comprehensive examination of intraspecies genetic heterogeneity for Michigan *F. columnare* isolates was not undertaken in this study, 16S rDNA sequencing results suggested that more than one *F. columnare* genomovar was present within Michigan salmonids. Because of this, further studies investigating the genetic profiles of Michigan *F. columnare* isolates according to the methods of Arias et al. (2004) are underway.

Flavobacterium psychrophilum isolates were also recovered in this study. Phylogenetic analysis demonstrated 2 distinct and well-supported *F. psychrophilum* genotypes; Cluster XIIIa was

comprised of *F. psychrophilum* isolates recovered from *Oncorhynchus* spp. returning to the Little Manistee River Weir (Lake Michigan watershed) and Swan River Weir (Lake Huron watershed), as well as from hatchery-reared brown trout, while Cluster XIIIb was comprised of isolates recovered from hatchery-reared Atlantic salmon and 1 isolate from Chinook salmon returning to the Swan River weir (Lake Huron watershed). Numerous studies have demonstrated the genetic heterogeneity of *F. psychrophilum* by various molecular methods (i.e., Madsen and Dalsgaard 2000; Izumi et al. 2003; Soule et al. 2005; Ramsrud et al. 2007; Chen et al. 2008; Del Cerro et al. 2010) and Charkroun et al. (1998) found strong correlations between *F. psychrophilum* ribotype and host of origin, which may explain the predominance of isolates recovered from Atlantic salmon within Cluster XIIIb.

It is also noteworthy that *F. branchiophilum,* the purported agent of bacterial gill disease (Wakabayashi et al. 1989), was not recovered throughout the course of this study despite the fact that a number of the examined fishes displayed disease signs that are often associated with bacterial gill disease (i.e., gill clubbing, gill pallor, etc.; Table 2.1). Indeed, the original *F. branchiophilum* isolates that the species description was based upon were recovered on cytophaga medium (Wakabayashi et al. 1989), which was one of the media types utilized in this study. Thus, based on this study, it appears that multiple flavobacterial species can be associated with what would often be diagnosed as bacterial gill disease. However, it is also possible that *F. branchiophilum* may have been present on some fish at low intensities, which could result in their overgrowth by other flavobacteria. As such, what role these “less well-known” flavobacteria may play in bacterial gill disease deserves to be further investigated.

While *F. columnare* and *F. psychrophilum* were indeed recovered in this study, they comprised only ~ 26% of the flavobacteria recovered from the internal organs of diseased and/or systemically infected fishes. Other described *Flavobacterium* spp. that were identified in

association with Michigan fishes included the Cluster IX isolates that were recovered from three hatcheries and four creeks/lakes, which also contained the *F. oncorhynchi* reference sequence. *Flavobacterium oncorhynchi* was recently described in diseased juvenile rainbow trout in Spain (Zamora et al. 2012a) and, for the first time, this study reports its presence in North America. While the type strain was originally recovered from the liver of a trout exhibiting signs of an *F. psychrophilum* infection (Zamora et al. 2012a), disease signs in this study included congestion of the fins, unilateral exophthalmia, hepatic, splenic, and renal pallor, and occasionally necrosis and epithelial hyperplasia of the gills. This study also provided evidence for the wide host range of *F. oncorhynchi*, as it was recovered from four genera within the family Salmonidae (i.e., *Salmo, Salvelinus, Oncorhynchus,* and *Coregonus*), as well as from an important prey species, the mottled sculpin, and from the invasive fish-parasitic sea lamprey. Previous studies in our laboratory have highlighted the potential for sea lamprey to possibly vector important fish pathogens, such as *A. salmonicida* (Faisal et al. 2007) and *F. psychrophilum* (Elsayed et al. 2006).

Four Michigan isolates (Cluster XIX) were definitively identified as *F. frigidimaris*, a species that was originally isolated from arctic sea water (Nogi et al. 2005). While we are unaware of any other published reports of this bacterium associated with fish, a sequence within GenBank that is 99% similar to the *F. frigidimaris* type strain (accession number HE612101.1) indicates that similar bacteria were recovered from kidneys of rainbow trout in Spain. One Michigan isolate was also identified as *F. psychrolimnae*, a bacterium that was first isolated from microbial mats in antarctic lakes (Van Trappen et al. 2005). Though sequences given the title *F. psychrolimnae* by depositors indicated that this bacterium was associated with the gastrointestinal tract of fish, closer inspection shows that these sequences are distinct from the *F. psychrolimnae* reference sequence (data not shown). *Flavobacterium* sp. isolate S171 was 98.9% similar to *Flavobacterium hydatis* and formed a well-supported cluster with the *F. hydatis* reference strain.

Flavobacterium hydatis was first isolated from the gills of diseased salmonids being reared at the Platte River State Fish Hatchery in Michigan by Strohl and Tait in 1978 and > 40 years later, this same bacterium was recovered from the gills of a brook trout inhabiting Kinney Creek that feeds the same hatchery. However, signs of disease were not observed in the fish from which isolate S171 was recovered. *Flavobacterium* isolateT105 was also identified as *F. tiangeerense*, which was originally isolated from a glacier in China (Xin et al. 2009). Again, no published reports have linked this bacterium to fish and a search within Genbank indicates similar sequences are predominantly associated with glaciers.

A number of described *Chryseobacterium* spp. were identified among Michigan fishes, some of which were associated with disease. For instance, 11 isolates (Unresolved Group 2) were 99.1 – 99.7% similar to *C. viscerum*, a species just described among septicemic rainbow trout in Spain (Zamora et al. 2012b). While specific signs in fish infected with *C. viscerum* were not reported in their original description (Zamora et al. 2012b), infected fish within this study showed mild melanosis, mild unilateral exophthalmia, hepatic pallor, and congestion of the kidney and liver, though the role that other fish pathogens may have played in the development of these disease signs was not assessed. It is worth noting that all 11 isolates were recovered from the gills of infected fish, and 6 of the fish infected with this bacterium were apparently healthy. Thus, experimental challenges verifying the pathogenicity of this bacterium would be prudent, as it may have an opportunistic relationship in fish. Two isolates identified as *C. vrystaatense* (Cluster XLI) were recovered from the eroded fins of a sea lamprey and from the gills of mottled sculpin in this study. Although *Chryseobacterium vrystaatense* was originally recovered from raw chicken in a South African processing plant (de Beer et al. 2006), searches within Genbank indicated that a similar bacterium was recovered from aquaculture systems in South Africa (accession number EU598811). Thus, the role that this bacterium may play in association with fish warrants attention. *Chryseobacterium* isolate T63 (Cluster XL) was identified as *C.*

piscicola, a species described in association with ulcerative skin and muscle lesions in Atlantic salmon in Chile (Ilardi et al. 2009) and subsequently identified in diseased Atlantic salmon from Finland (Ilardi et al. 2010). In this study, *C. piscicola* was recovered from severely eroded and necrotic fins of hatchery-reared brown trout, indicative of either a facultative or secondary pathogenic nature. Indeed, Ilardi and colleagues (2010) assessed the ability of *C. piscicola* to cause disease under laboratory conditions and found it to be moderately virulent to salmon. Once again, we are unaware of any other reports of this bacterium associated with diseased fish in North America.

Yet another *Chryseobacterium* sp. recovered from Michigan fish was *C. aquaticum*, a bacterium that was originally described from a Korean water reservoir (Kim et al. 2008) It was also recovered from Siberian sturgeon (*Acipenser baeri*) fry in France (sequence number AY468465; Bernardet et al. 2005). Interestingly, a strain of *C. aquaticum* was shown to produce a novel antifungal protease (Pragash et al. 2009), possibly indicating a mutualisitic relationship between this bacterium and its host. Two Michigan fish-associated isolates (Cluster XXXVIII) were also identified as *C. chaponense*, which was recently reported in farmed Atlantic salmon in Chile co-infected with *F. psychrophilum* (Kämpfer et al. 2011) and from skin ulcers in rainbow trout in France (sequence number AY468464; Bernardet et al. 2005). In the original description by Kämpfer et al. (2011), *C. chaponense* was recovered from external lesions, fins, and gills of infected fish, while in this study, both isolates were recovered from the kidneys of systemically infected Chinook salmon and rainbow trout. Disease signs in these fish included mildly swollen and friable spleens in salmon, whereas rainbow trout were apparently healthy. Nevertheless, to our knowledge this is the first report of this bacterium systemically infecting fish in North America and thereby illustrates its widespread presence in North and South America, as well as Europe.

In addition to recovering the seven aforementioned *Flavobacterium* spp. and five *Chryseobacterium* spp., the vast majority of isolates recovered from Michigan fishes did not cluster with any formally described *Flavobacterium* and *Chryseobacterium* spp. despite clearly belonging to the two genera. In fact, the majority of isolates in this study (n=170) were <98.7% similar to described members of the family Flavobacteriaceae, a value that can be seen between distinct *Flavobacterium* spp. (Bernardet and Bowman 2006 and references therein). However, in order to definitively delineate novel flavobacterial taxa, polyphasic characterization must be carried out as recommended by Bernardet et al. (2002). As such, ongoing studies in the authors' laboratory elucidating this diverse assemblage of fish-associated flavobacteria are underway. Still, it is clear from this study that not only are many of the clusters likely novel bacterial species, but some are likely pathogenic to fish. For example, Cluster XVIII was comprised of *Flavobacterium* sp. isolates recovered exclusively from systemically infected fish from 2005 through 2010 and was also associated with a large mortality event in Chinook salmon fingerlings at Thompson State Fish Hatchery in 2005. Similarly, *Flavobacterium* sp. isolates within Cluster XI were recovered from kidneys, gills, and ulcers of infected fish, some of which had signs that mimicked those typical of bacterial cold water disease (i.e., isolate S21 recovered from the ulcer in Fig. 8a). Within the genus *Chryseobacterium*, Cluster XXXVII isolates were associated with morbidity and mortality in aquacultured lake herring fingerlings and were also recovered from kidneys and brains of systemically infected steelhead and walleye fry, respectively. Moreover, Cluster XXXIV isolates were recovered from multiple mortality events that occurred at 3 different hatcheries during 3 different years that likely represent a novel taxon. Many of the other clusters may also represent novel taxa. Clearly, there is a dire need to discern what role these previously uncharacterized flavobacteria play in the health and diseases of fish.

Flavobacterium and *Chryseobacterium* spp. hyrdolyze an array of substrates (Bernardet and Nakagawa 2006), which was also observed among *in vitro* protease assays conducted in this study. However, this study suggested that Michigan fish-associated *Chryseobacterium* spp. are even more proteolytic than their *Flavobacterium* spp. counterparts, as evidenced by the fact that a higher percentage of chryseobacteria proteolyzed all four the substrates examined in this study. Indeed, *Flavobacterium* spp. isolates that degraded all four substances were rare, while this was common in the Michigan chryseobacteria. Gelatin (a derivative of collagen), elastin, and chondroitin sulfate are important components of the host extracellular matrix in connective tissue, skin and blood vessels, and cartilage (Alberts et al. 2002), while hemoglobin is the oxygen-carrying molecule of erythrocytes. As such, it is possible that a bacterium possessing the ability to degrade these substances could enhance their invasiveness in a host, which was suggested for other bacterial fish pathogens (Pacha 1968; Paniagua et al. 1990; reviewed in Austin and Austin 2007). Thus, studies elucidating what role, if any, these proteases play in the pathogenesis of these organisms are warranted. In conclusion, this study illustrates the diversity of flavobacteria that are associated with both diseased and apparently healthy fishes of the Laurentian Great Lakes and can serve as a platform for numerous studies to understand the role that these uncharacterized flavobacteria play in the health of Great Lakes fishes.

ACKNOWLEDGEMENTS

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Table 2.1. Gross signs of disease observed among fish infected within the 42 clusters of flavobacteria in this study. Clinical signs were not observed in fish infected with flavobacteria of Clusters XVI, XXI, XXIV, XXV, XXVI, XXX, XXXI, XXXII, XXXVI, and XLI. It should also be noted that the reported disease signs cannot be solely attributed to the flavobacteria that were recovered from infected fish, as the role of other fish pathogens in disease progression was not investigated in this study.

Cluster	Disease signs in infected fish
I	Melanosis, abdominal distension, fin erosion, hemorrhagic enteritis, renal and hepatic pallor, generalized visceral edema
II	Melanosis, gill pallor, external focal ecchymotic hemorrhage, fin erosion/necrosis, hepatic, splenic, and renal pallor, generalized visceral edema, fluid within swimbladder lumen, friable and congested kidney *
III	Melanosis, gill clubbing, mild gill pallor, fin erosion, unilateral exophthalmia, renal and hepatic pallor, splenomegaly, renal and hepatic congestion *
IV	Unilateral exophthalmia, fluid within swimbladder lumen, friable spleen, renal and hepatic congestion *
V	Erosion, necrosis, and hemorrhage fins, clubbed gills, melanosis, splenomegaly, renal and hepatic pallor *
VI	Enophthalmia, dermal ulceration, splenomegaly
VII	Erratic swimming/spinning, mottled liver *
VIII	Melanosis, gill pallor, fin erosion, hepatic, splenic, and renal pallor
IX	Melanosis, lardosis, unilateral exophthalmia, renal and hepatic pallor, congested and swollen kidney
X	Gill pallor, lamellar erosion, unilateral exophthalmia, congestion at base of fins, hemorrhagic fins, hepatic and splenic pallor, congested and swollen kidney *
XI	Enophthalmia, deep muscular ulceration, gill pallor, splenomegaly, swollen, pale, and mottled liver *
XII	Melanosis, erratic swimming/spinning, fluid within swimbladder lumen, splenomegaly, hepatic and splenic pallor, mottled liver *
XIII	Muscle ulceration, fin erosion, gill pallor, splenomegaly, swollen liver, spleen and kidney, hepatic and renal pallor, mottled liver, congested liver and kidney, multifocal ecchymotic hemorrhage liver, excessive amount of ovarian fluid in gravid spawning females, edematous kidney *
XIV	Hemorrhagic fins, external ecchymotic hemorrhage, clubbed gills, splenomegaly
XV	Fin erosion, external hemorrhage, hepatic pallor, congested liver and kidney, splenomegaly *
XVII	Periocular hemorrhage, gill necrosis, external petechial hemorrhage, fin erosion, muscular ulceration, hepatic, splenic, and renal pallor, mottled liver, hepatomegaly, swollen kidney, friable and swollen spleen, splenomegaly *
XVIII	Unilateral exophthalmia, congestion base of fins, gill pallor and necrosis, muscular ulceration, erosion of dermis overlying jaw, erythema isthmus, splenomegaly, hepatic pallor, friable kidney *
XIX	Melanosis, enophthalmia, fin erosion, congestion base of fins, friable kidney
XX	Erratic swimming/spinning
XXII	Melanosis, gill pallor, fin erosion, hepatic, splenic, and renal pallor *
XXIII	Gill pallor *
XXVII	Petechial hemorrhage ventrum, gill pallor, congestion base of fins
XXVIII	Unilateral exophthalmia, splenomegaly, friable congested kidney, pale mottled liver
XXIX	Fin erosion, hepatic and renal pallor, friable and/or swollen kidney
XXXIII	Dermal erosion, fin erosion, swollen spleen, hepatic/renal pallor, visceral edema

Table 2.1 (cont'd)

XXXIV	Fin erosion, melanosis, lardosis, muscle ulceration, bilateral exophthalmia, hepatic, splenic, and renal pallor, swollen spleen
XXXV	Erratic swimming behavior, irregular opercular movement,
XXXVII	Visceral edema, hepatic pallor, fluid within swimbladder lumen, petechial hemorrhage base of fins
XXXVIII	Swollen friable spleen *
XXXIX	Fin erosion, multifocal dermal ulceration, swollen spleen, congested liver and kidney
XL	Gill pallor, unilateral exophthalmia, fin erosion, hemorrhagic nares, swollen spleen
XLII	Enophthalmia, melanosis, fin erosion, splenic pallor, swollen kidney

*, a portion of the infected fish did not present with any clinical signs of disease.

Table 2.2. Percent of Michigan fish-associated *Flavobacterium* spp. and *Chryseobacterium* spp. isolates exhibiting gelatinase, caesinase, and elastase activities, as well as the ability to degrade hemoglobin. Clusters VI, XIII, XIV, XVI, XXIII, XXIV, XXXV were not tested for protease activity.

Flavobacterial Cluster	Gelatin	Casein	Hemoglobin	Elastin
Michigan *Flavobacterium* spp.				
I	0% (4)	75% (4)	25% (4)	33% (3)
II	17% (12)	88% (8)	50% (12)	83% (12)
III	75% (4)	ND	100% (1)	100% (1)
IV	100% (4)	100% (1)	75% (4)	100% (4)
V	33% (3)	100% (1)	0% (1)	100% (1)
VII	0% (3)	100% (3)	100% (3)	100% (3)
VIII	33% (3)	100% (3)	33% (3)	100% (3)
IX	25% (8)	75% (4)	38% (8)	50% (8)
X	27% (11)	83% (6)	0% (10)	50% (10)
XI	38% (8)	100% (4)	88% (8)	100% (8)
XII	0% (7)	100% (7)	100% (7)	88% (8)
XV	0% (4)	50% (4)	0% (4)	75% (4)
XVII	100% (5)	100% (4)	25% (4)	100% (4)
XVIII	67% (6)	100% (4)	67% (6)	100% (6)
XIX	0% (4)	67% (3)	75% (4)	0% (4)
XX	0% (1)	ND	100% (1)	0% (1)
XXI	0% (3)	100% (3)	75% (4)	75% (4)
XXII	0% (2)	100% (1)	100% (2)	100% (2)
XXV	0% (1)	100% (1)	100% (1)	100% (1)
XXVI	0% (1)	100% (1)	100% (1)	100% (1)
XXVII	100% (2)	100% (2)	0% (2)	100% (2)
XXVIII	0% (2)	ND	100% (2)	100% (2)

Table 2.2 (cont'd)

Flavobacterial Cluster	Gelatin	Casein	Hemoglobin	Elastin
XXIX	0% (1)	100% (1)	0% (1)	0% (1)
XXX	0% (1)	ND	0% (1)	0% (1)
XXXI	100% (2)	100% (1)	100% (1)	0% (1)
XXXII	0% (1)	ND	0% (1)	0% (1)
Unres. Group 1	11% (9)	100% (9)	89% (9)	100% (9)
Total	**30% (112)**	**91% (75)**	**56% (105)**	**76% (105)**
Michigan *Chryseobacterium* spp.				
XXXIII	100% (3)	100% (3)	100% (3)	100% (3)
XXXIV	33% (6)	100% (4)	67% (6)	100% (6)
XXXVI	100% (1)	100% (1)	100% (1)	100% (1)
XXXVII	75% (4)	100% (4)	50% (4)	100% (4)
XXXVIII	100% (1)	100% (1)	0% (1)	0% (1)
XXXIX	50% (2)	ND	0% (1)	100% (1)
XL	0% (1)	0% (1)	0% (1)	100% (1)
XLI	100% (2)	100% (1)	100% (2)	100% (2)
XLII	0% (1)	ND	100% (1)	100% (1)
Unres. Group 2	100% (11)	100% (11)	100% (11)	100% (11)
Total	**72% (32)**	**96% (26)**	**71% (31)**	**97% (31)**

Figure 2.1. Dendrogram generated using the neighbor-joining method in MEGA4 that depicts the phylogenetic relationship between Michigan fish-associated *Flavobacterium* spp. and other described and candidate *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

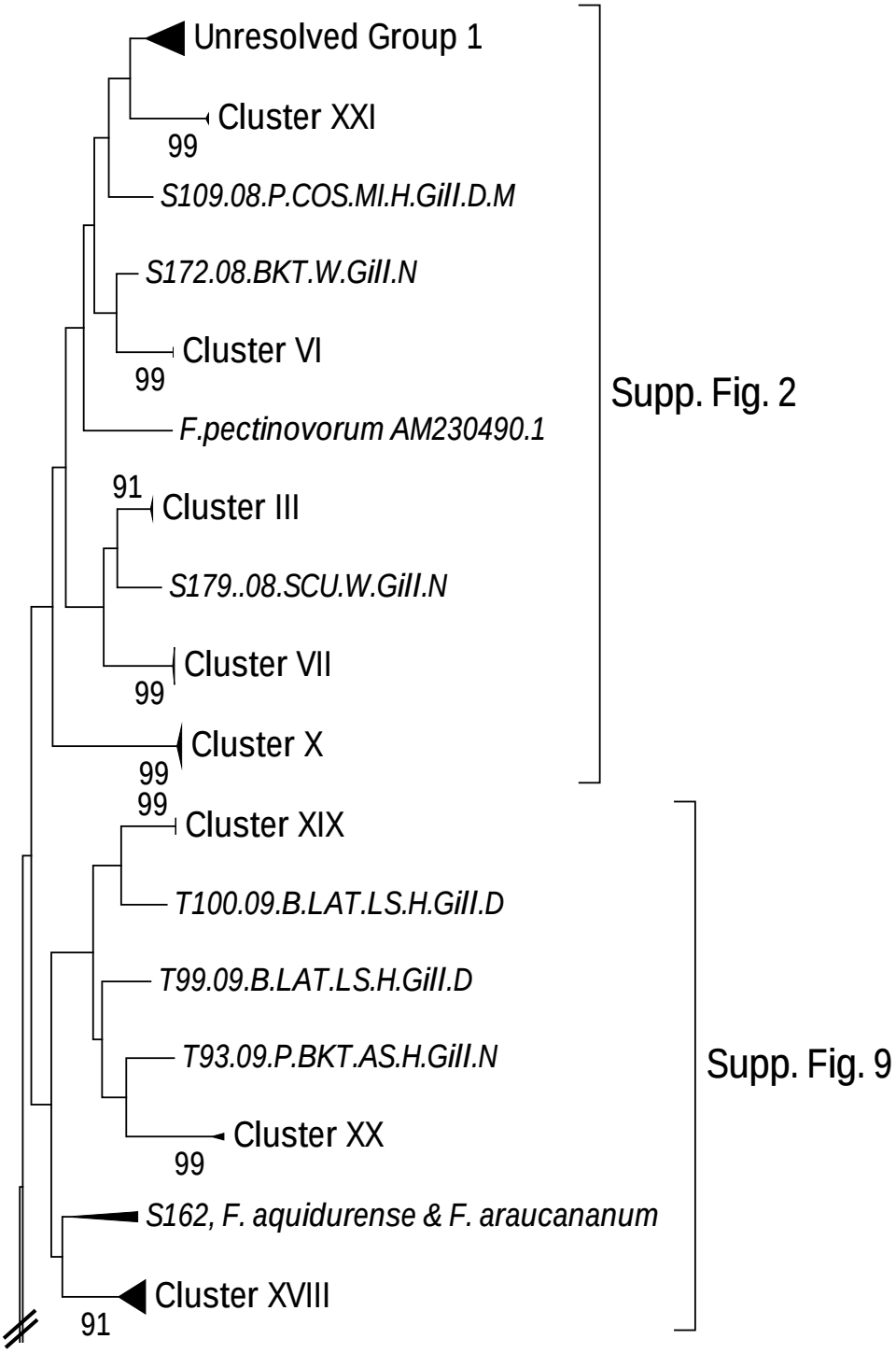
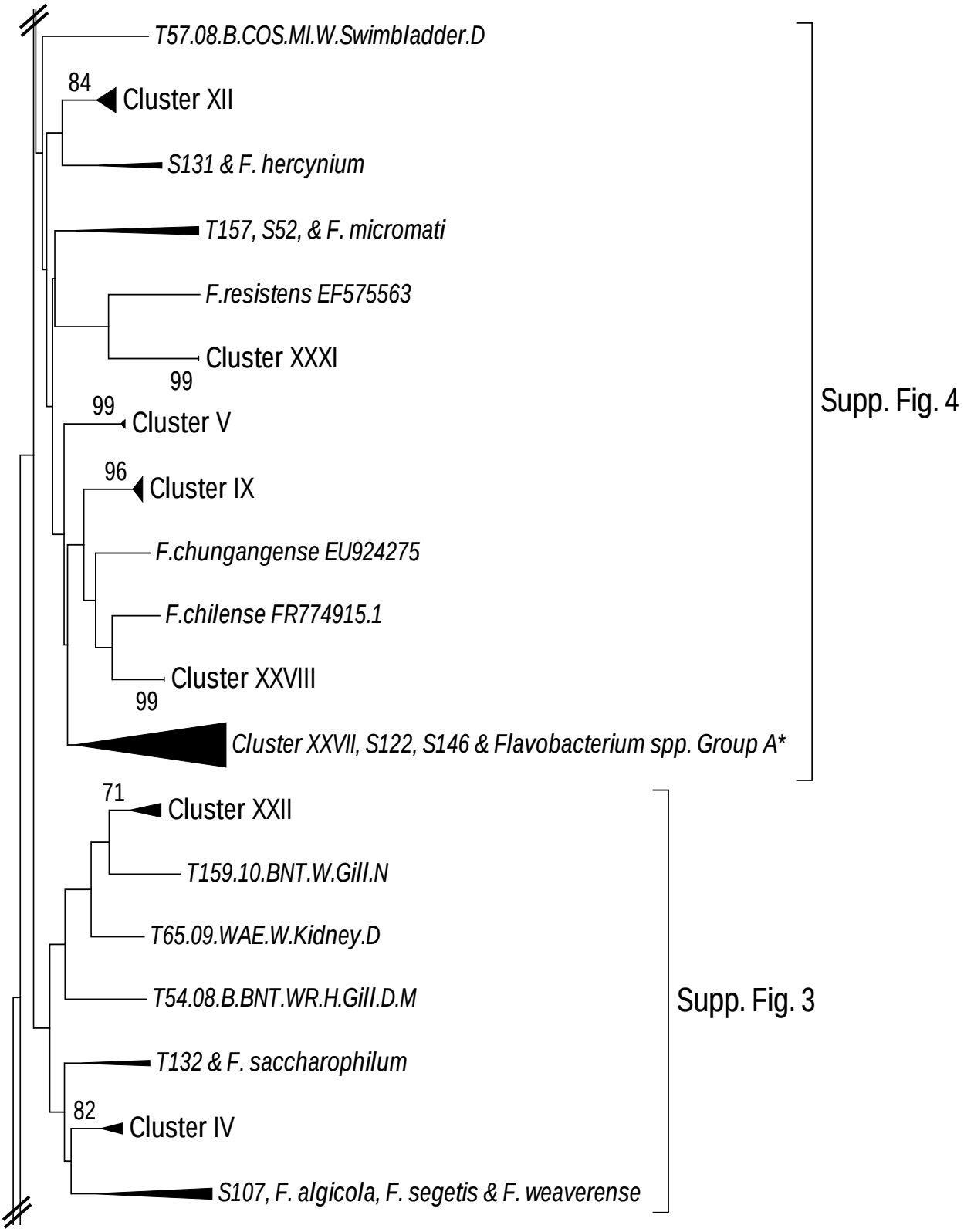


Figure 2.1. (cont'd)



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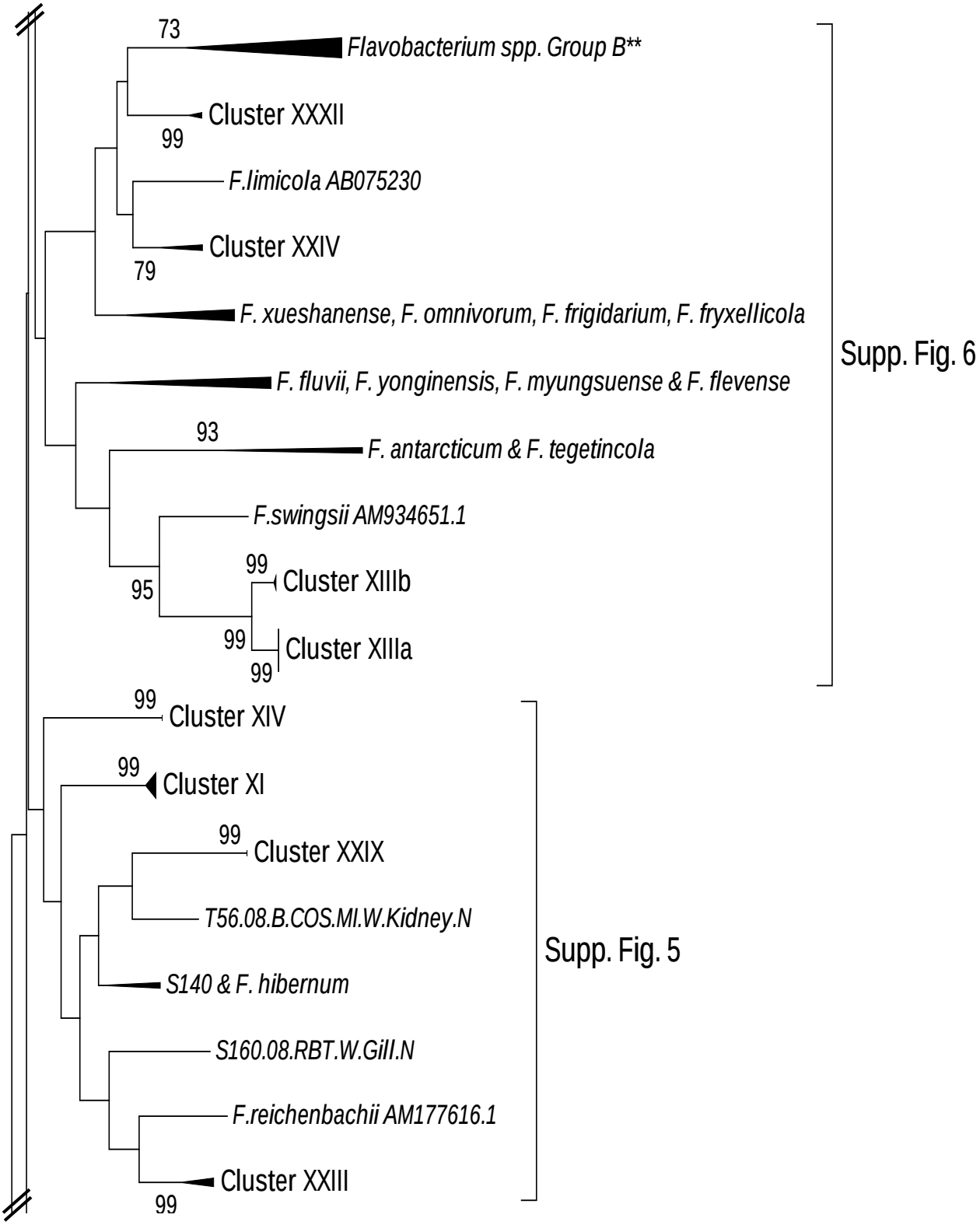

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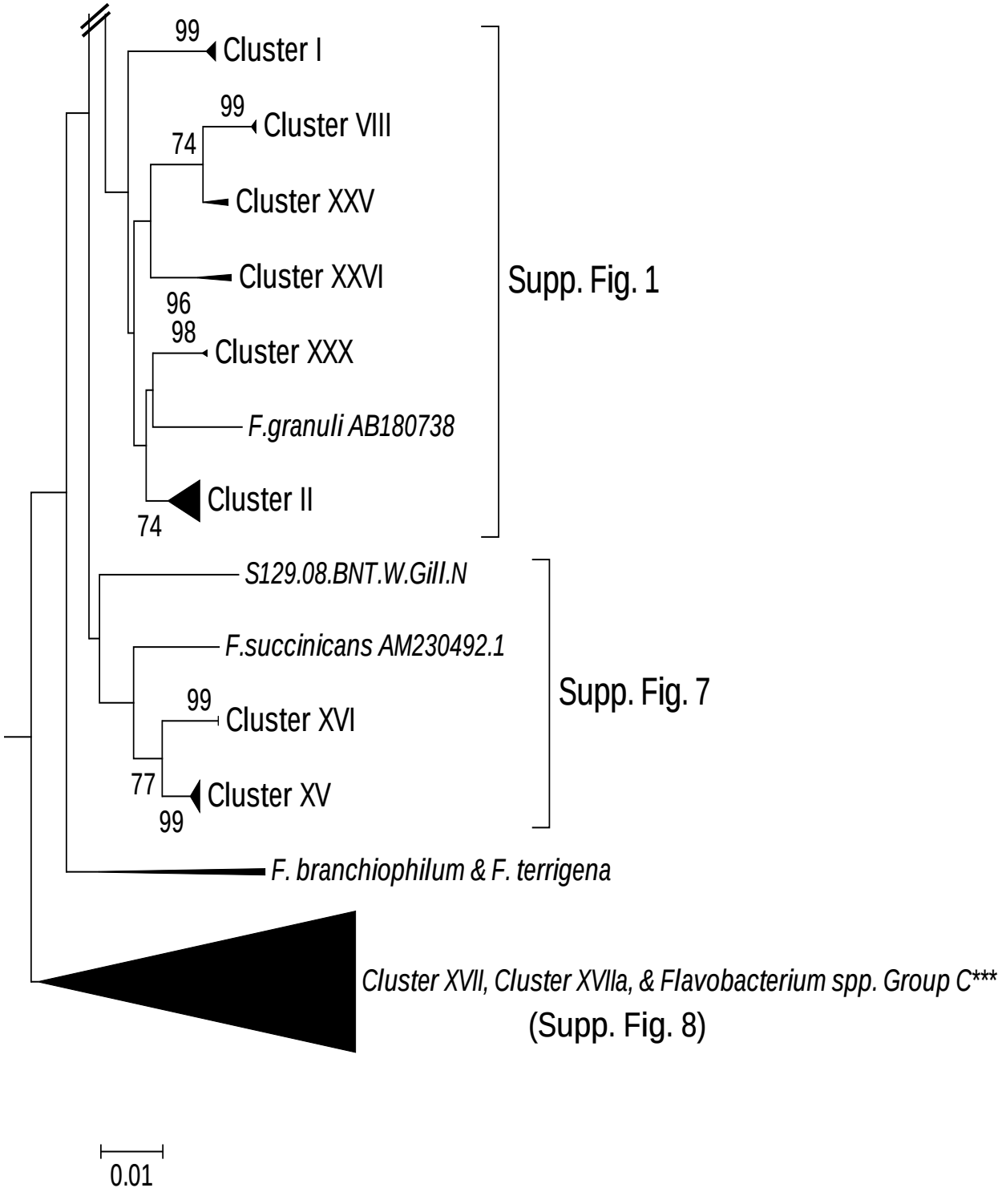
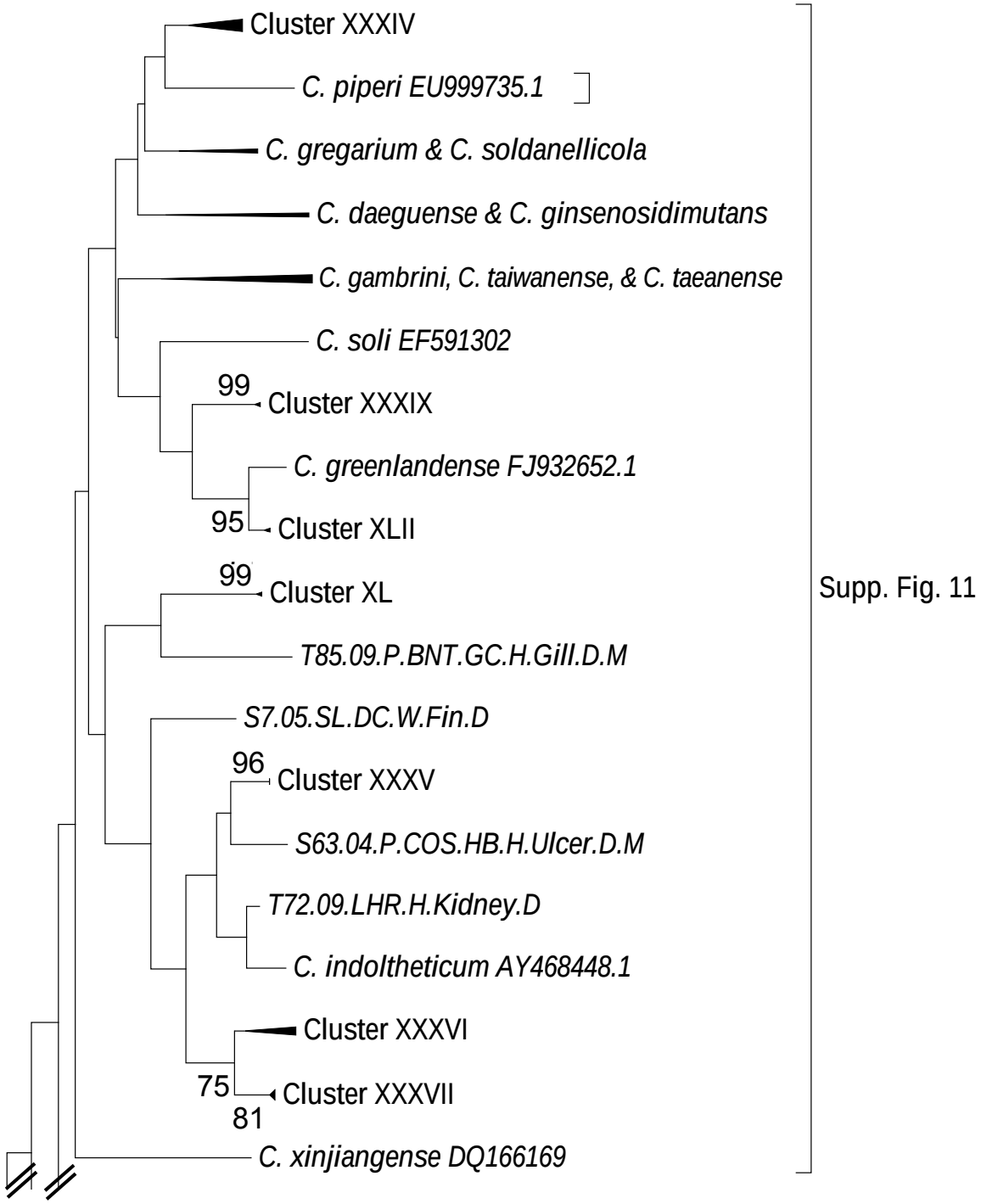


Figure 2.1. (cont'd)

*,***Flavobacterium* spp. Group A** *includes F. chungbukense, F. glaciei, F. daejeonense, F. glycines, F. defluvii, F. johnsoniae, F. compostarboris, F. banpakuense, F. denitrificans, F. phragmitis, F. anhuiense* and, *F. ginsenosidimutans;* **, ***Flavobacterium* spp. Group B** includes *F. degerlachei, F. gillisiae, F. frigoris, F. sinopsychrotolerans, F. urumqiense, F. xinjiangense,* and *F. xanthum*; ***, ***Flavobacterium* spp. Group C** includes *F. chunnamense, F. koreense, F. cheonanse, F. macrobrachii, F. soli, F. aquatile, F. cheniae, F. cucumis, F. sasangense, F. cauense, F. saliperosum, F. ceti, F. ummariense, F. suncheonense, F. dongtanese, F. haoranii, F. gelidilacus, F. poni, F. caeni, F. lindanitolerans, F. filum, F. beibuense, F. rakeshii, F. rivuli, F. subsaxonicum, F. croceum, F. indicum,* and *F. terrae.*

Figure 2.2. Dendrogram generated using the neighbor-joining method in MEGA4 that depicts the phylogenetic relationship between Michigan fish-associated *Chryseobacterium* spp. and other described and candidate *Chryseobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. *Elizabethkingia meningosepticum* and *E. miricola* served as the outgroup.



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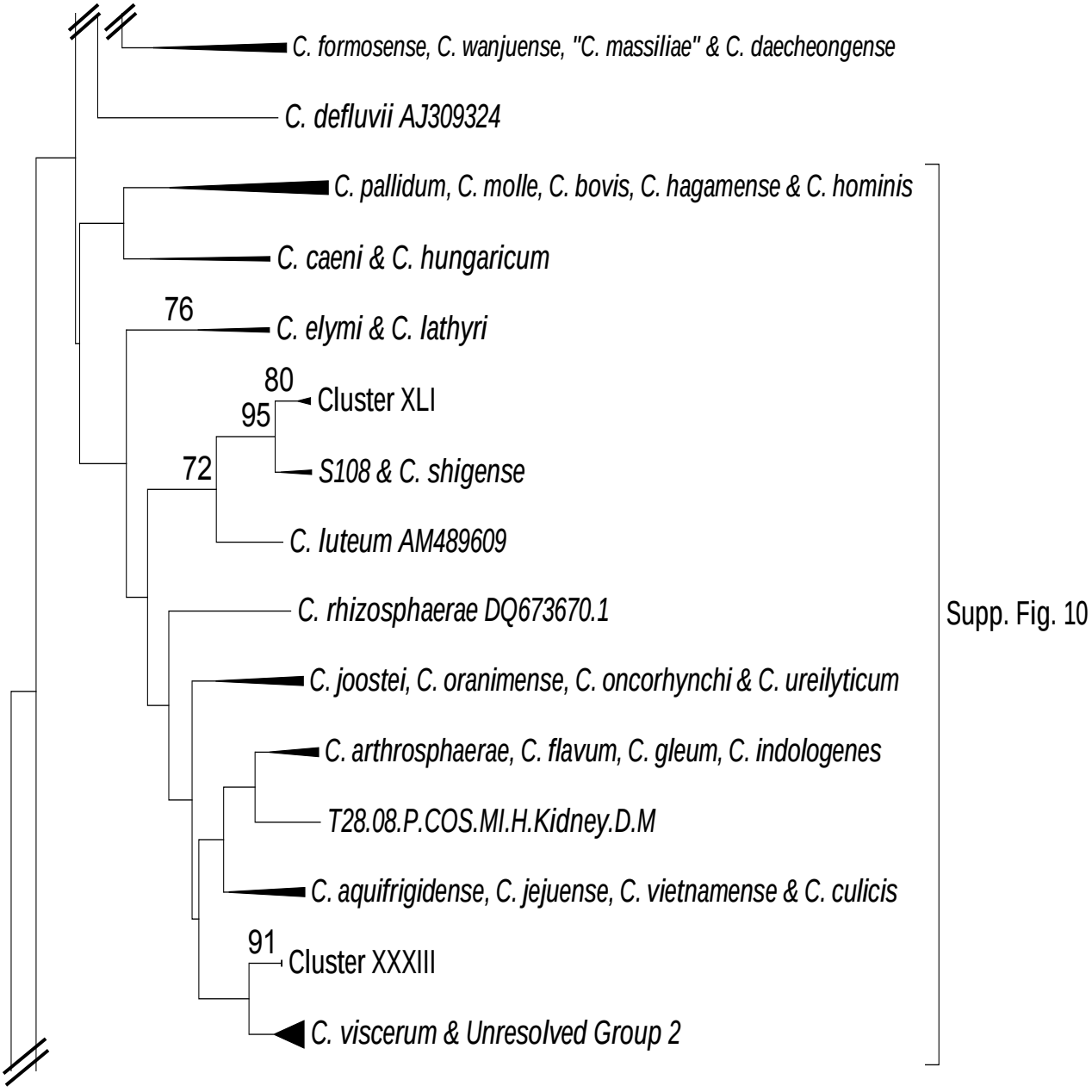

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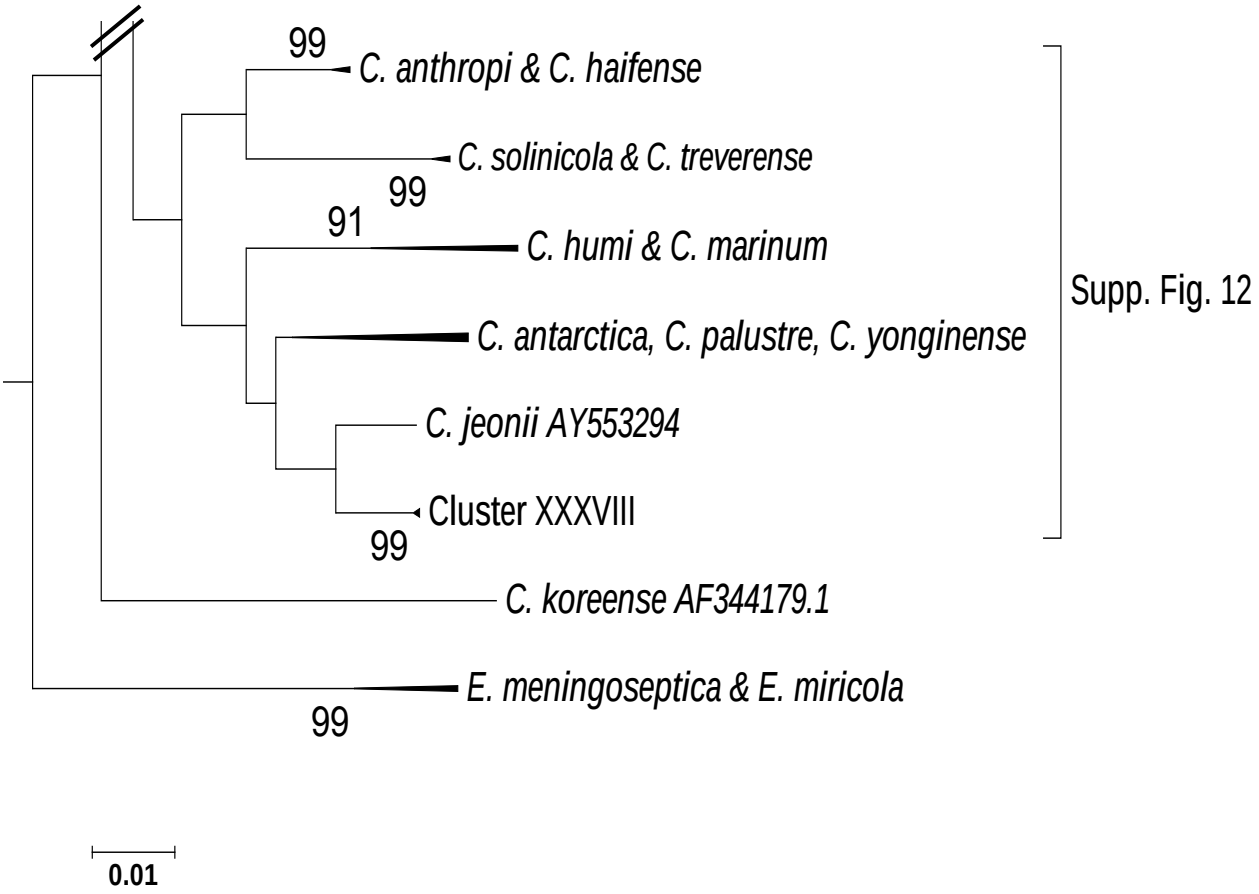
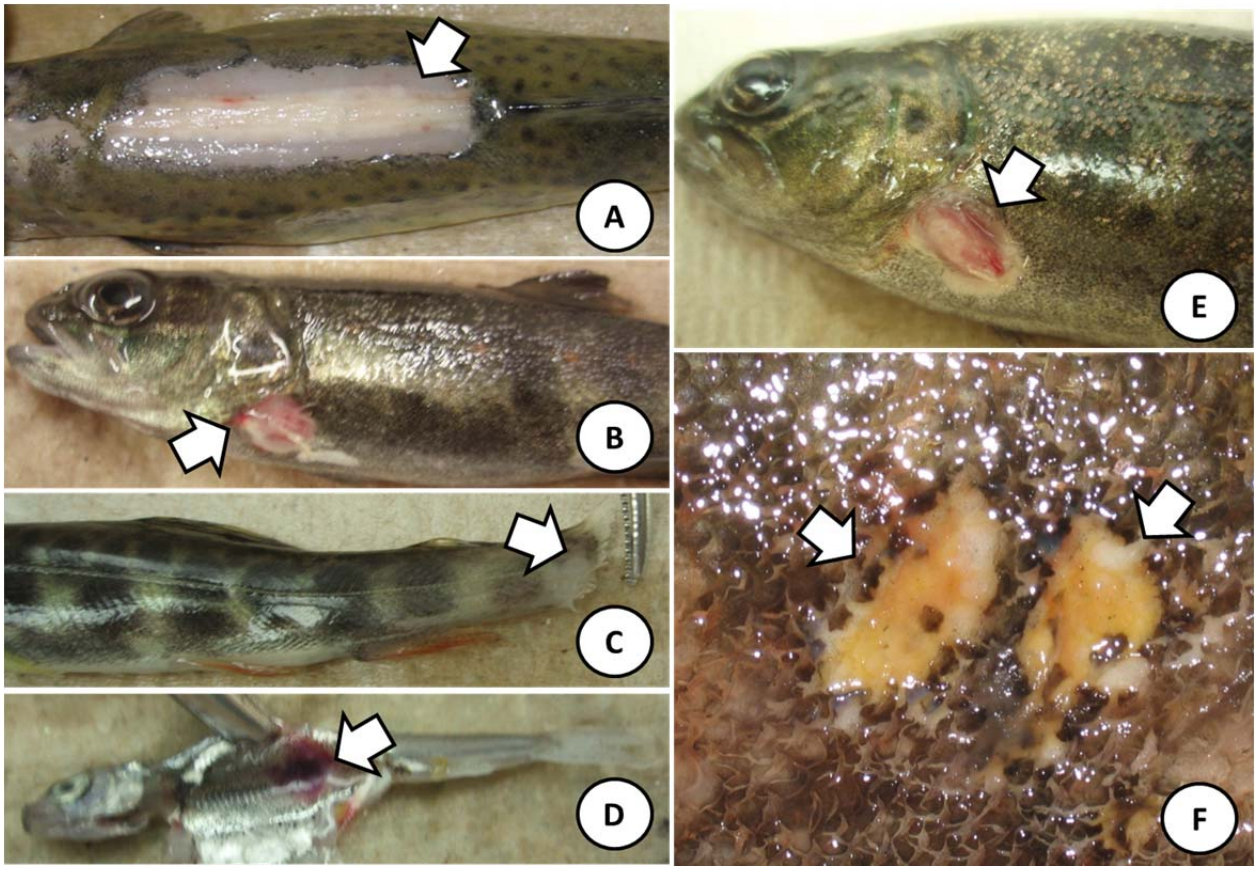
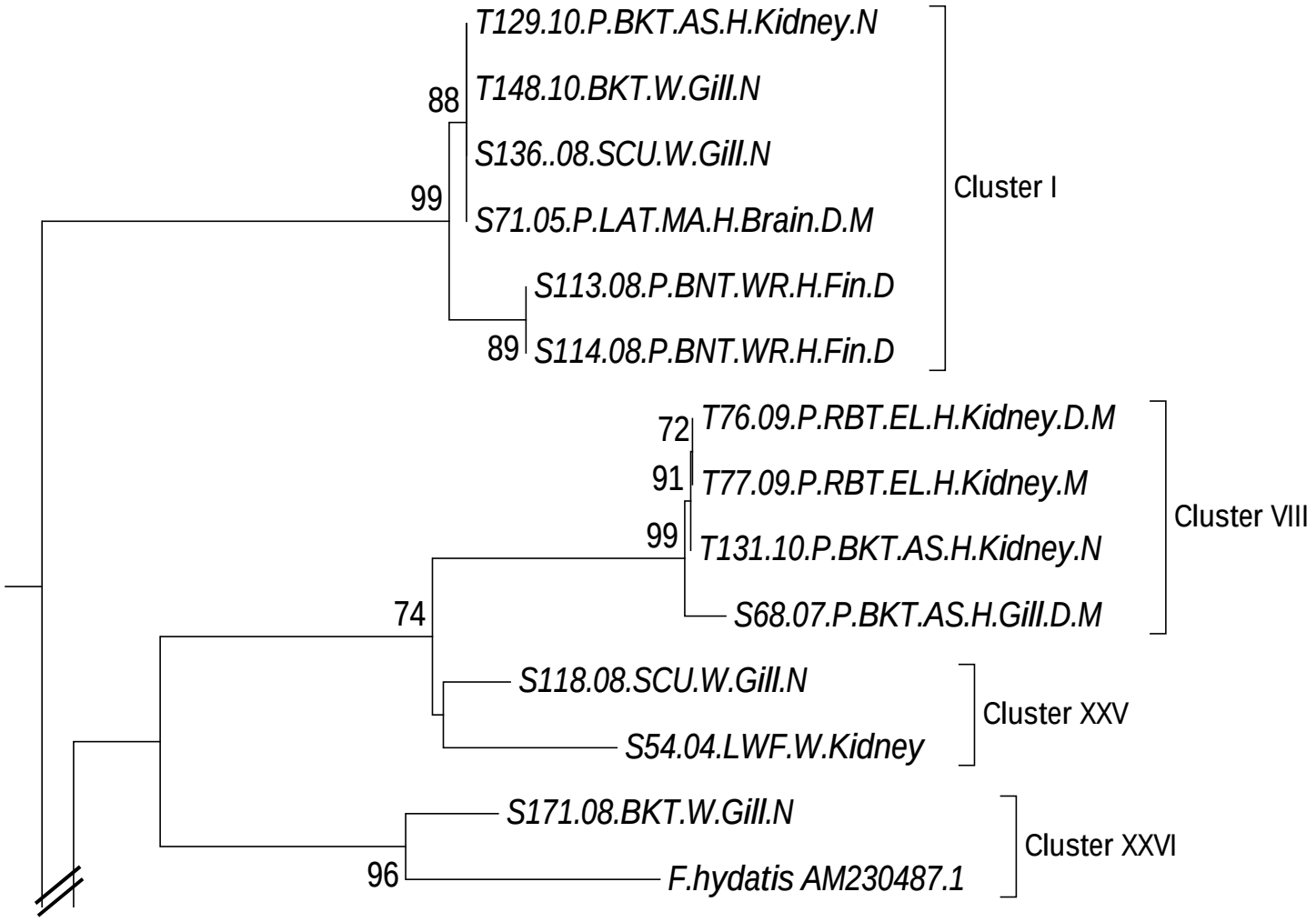


Figure 2.3. Gross lesions present in Michigan fishes infected with *Flavobacterium* and *Chryseobacterium* spp. A) Necrotic ulceration (arrow) present on the dorsum of a rainbow trout from which *Flavobacterium* sp. S21 was recovered. Note the complete erosion of the dorsal fin and penetration into the underlying musculature. B) Severe necrosis and hemorrhage of the left pectoral fin (arrow) of a brown trout fingerling from which *Flavobacterium* sp. isolates belonging to Cluster XIX were recovered. C) Erosion and necrosis of the caudal fin and caudal peduncle (arrow) of a brook trout fingerling from which *Flavobacterium* sp. isolates belonging to Cluster XXII were recovered. D) Severe hemorrhage of the kidney and surrounding in the muscle (arrow) of a lake herring fingerling from which *Chryseobacterium* sp. T72 was recovered. E) Left pectoral fin of a yearling brown trout from which *Chryseobacterium* sp. strain T62 was recovered. Note severe necrosis and hemorrhage of the fin, with concurrent exposure of the eroded fin rays (arrow). F) Multifocal dermal ulcerations (arrows) present on the trunk of a feral spawning steelhead trout from which *Chryseobacterium* sp. S25 was recovered. **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.**

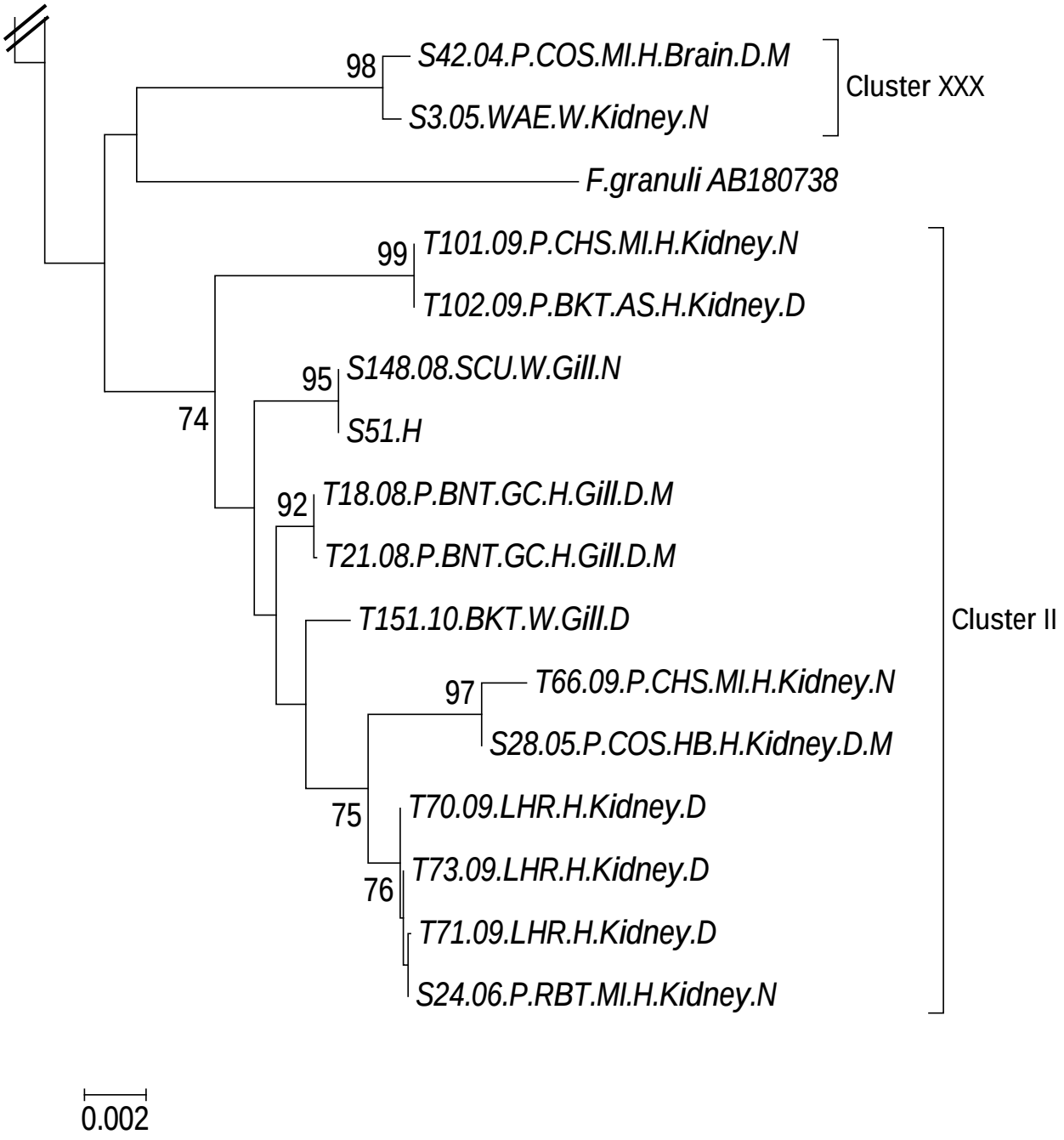


APPENDIX A

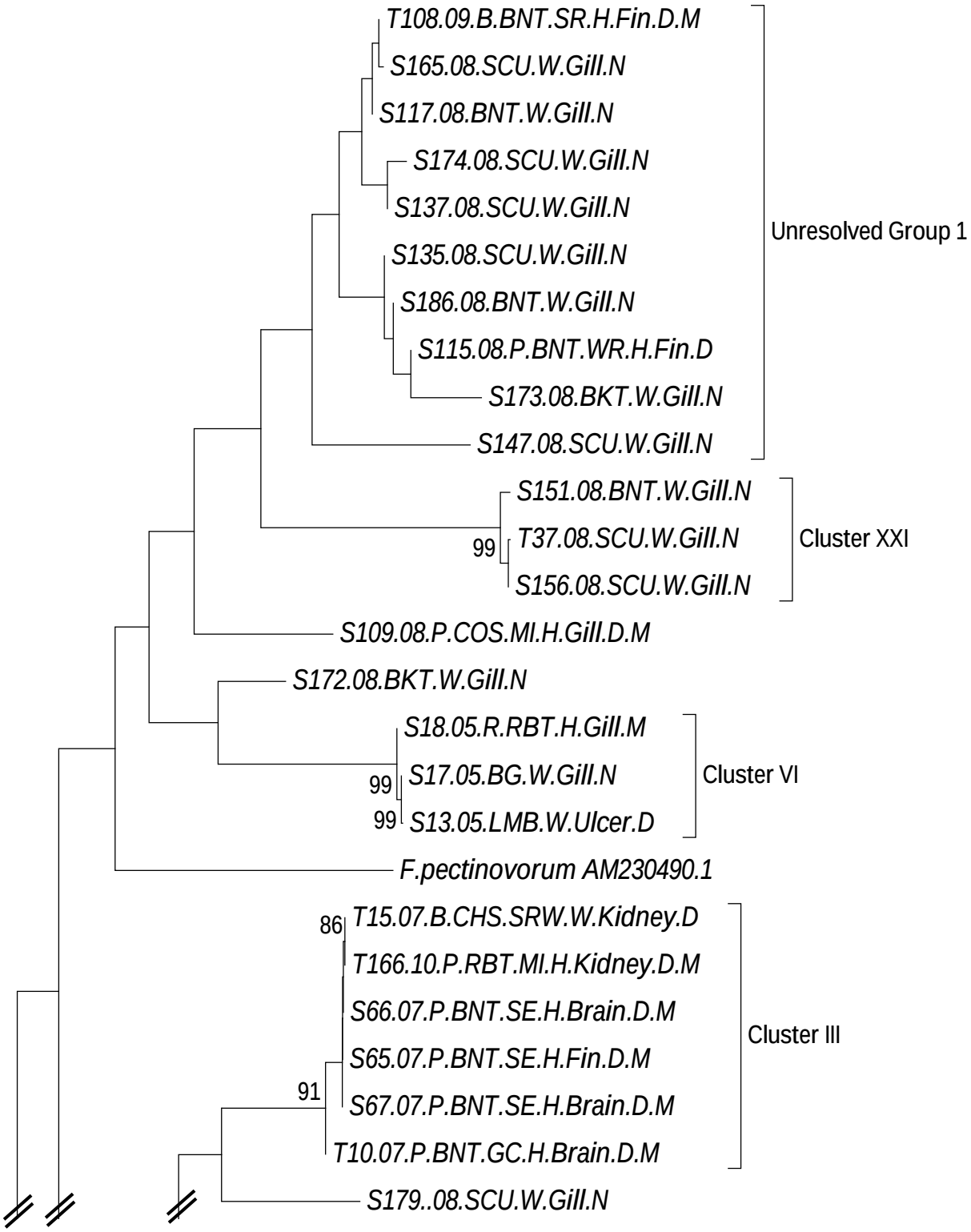
Supplementary Figure 2.1. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 6 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



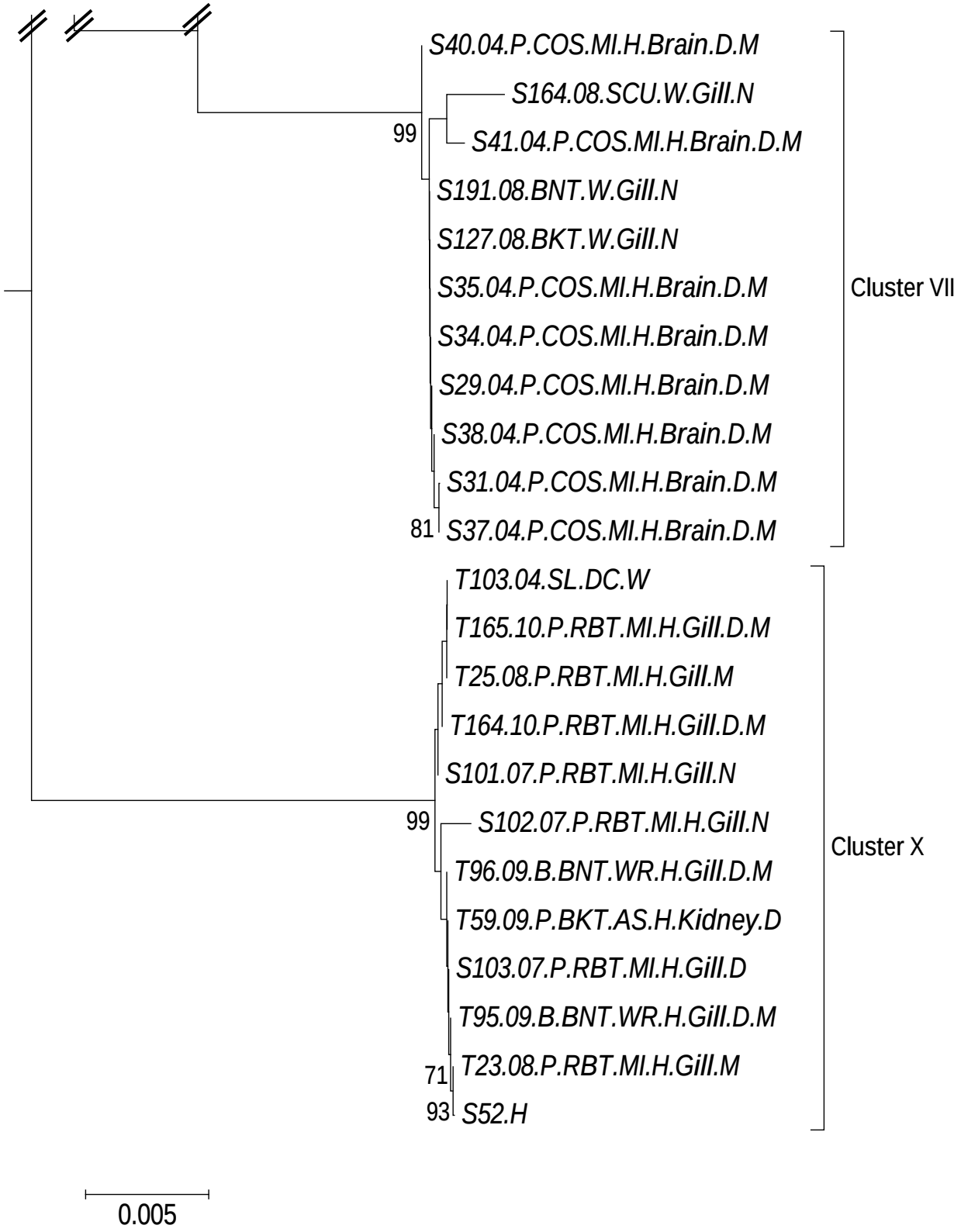
Supplementary Figure 2.1 (cont'd)



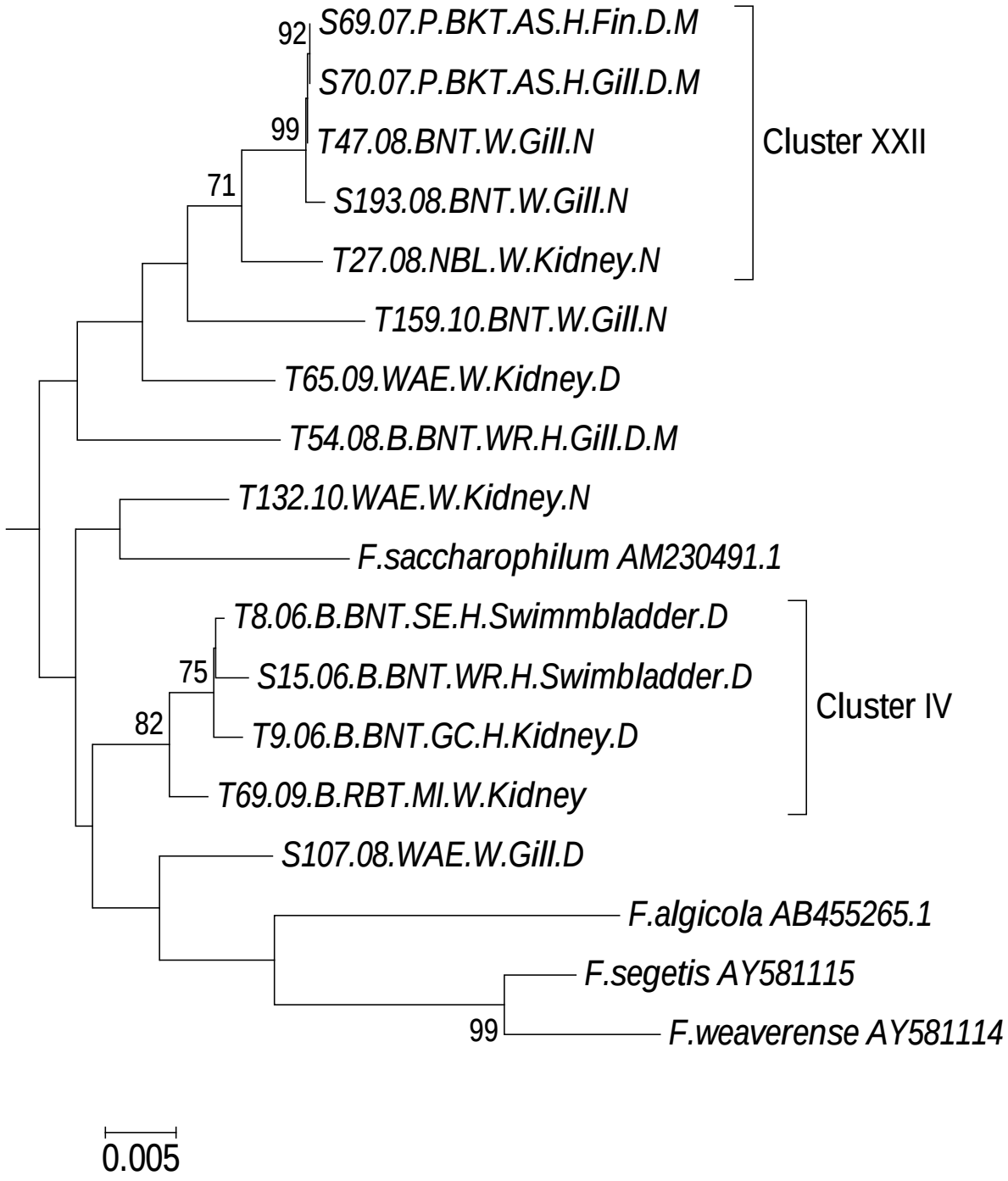
Supplementary Figure 2.2. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 5 clusters and 1 unresolved group of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



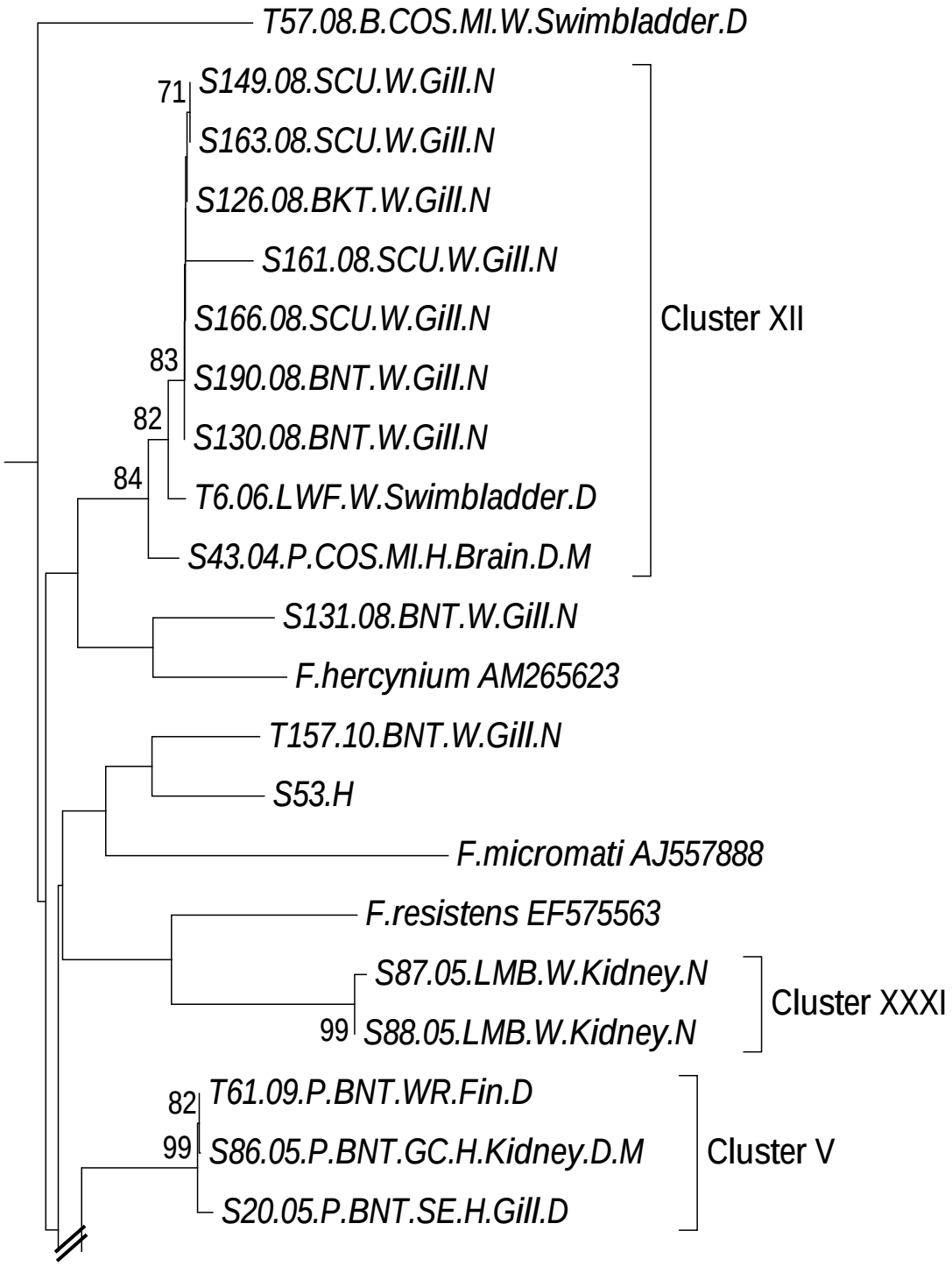
Supplementary Figure 2.2 (cont'd)



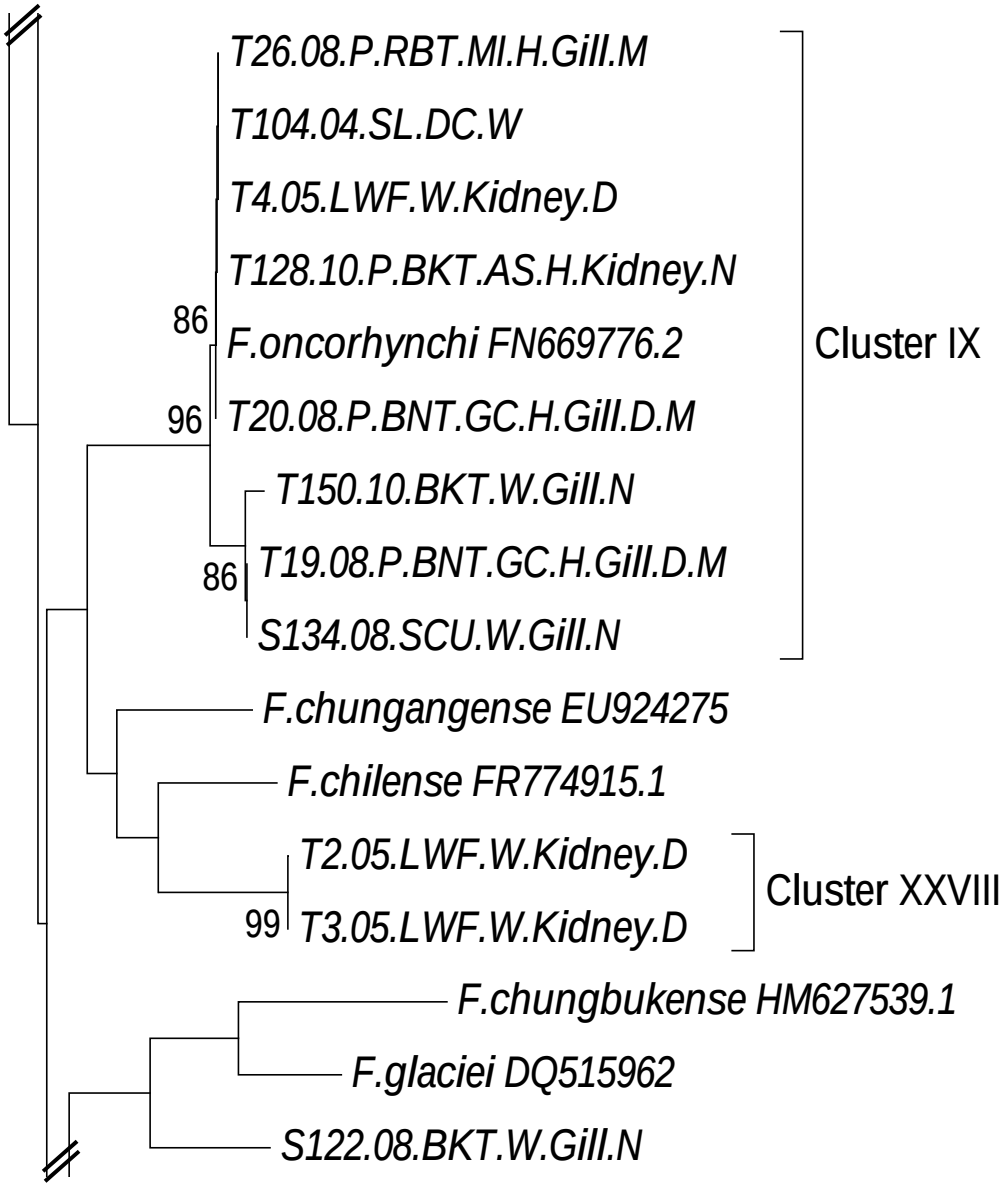
Supplementary Figure 2.3. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 2 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



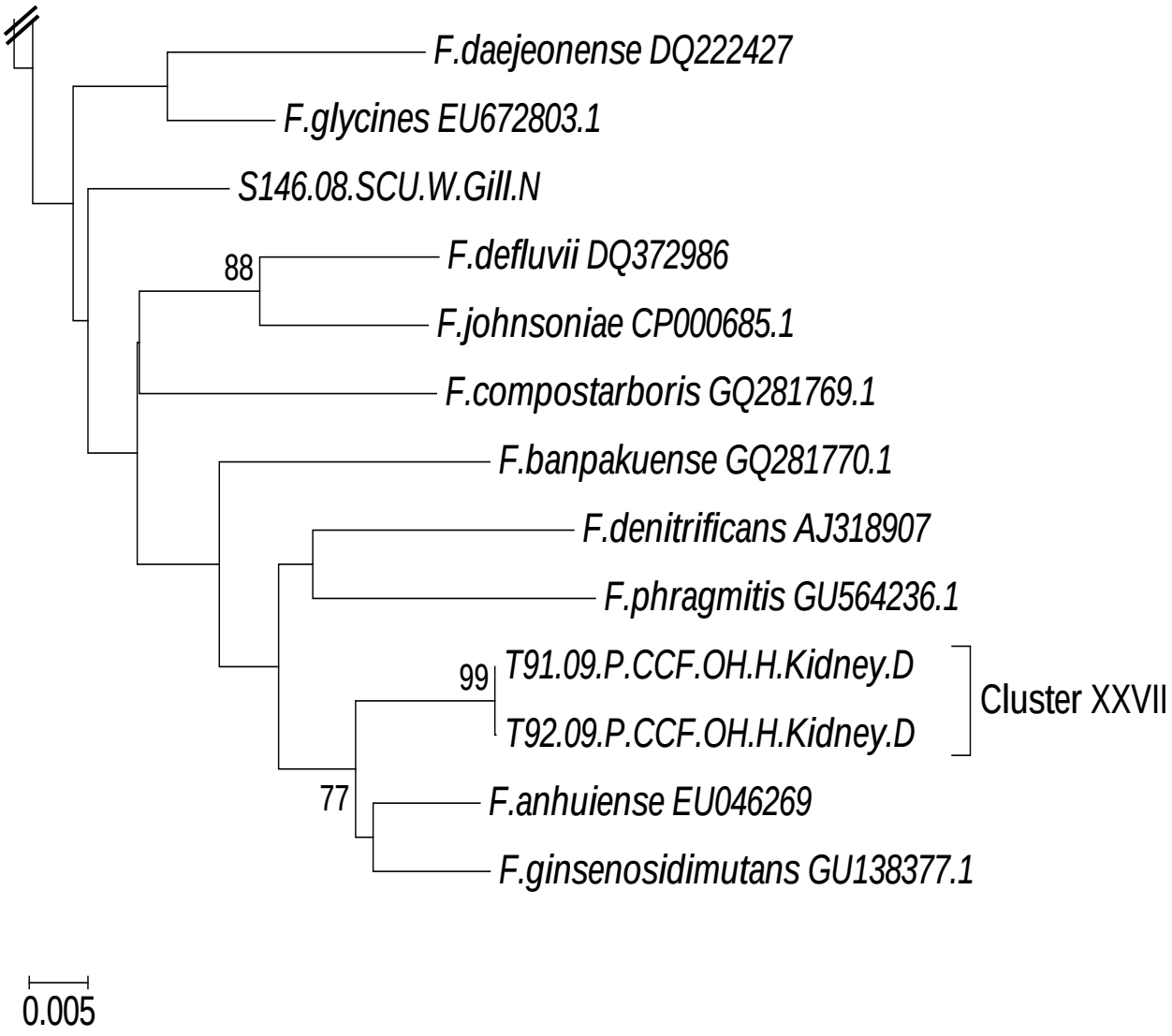
Supplementary Figure 2.4. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 6 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



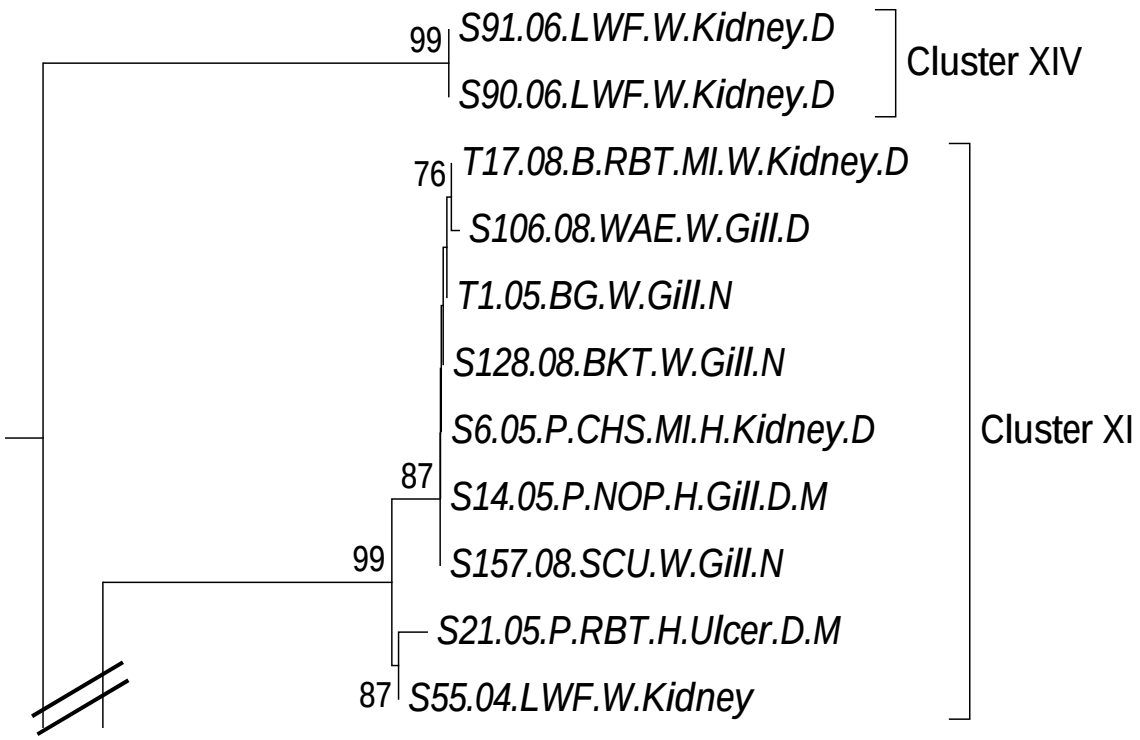
Supplementary Figure 2.4 (cont'd)



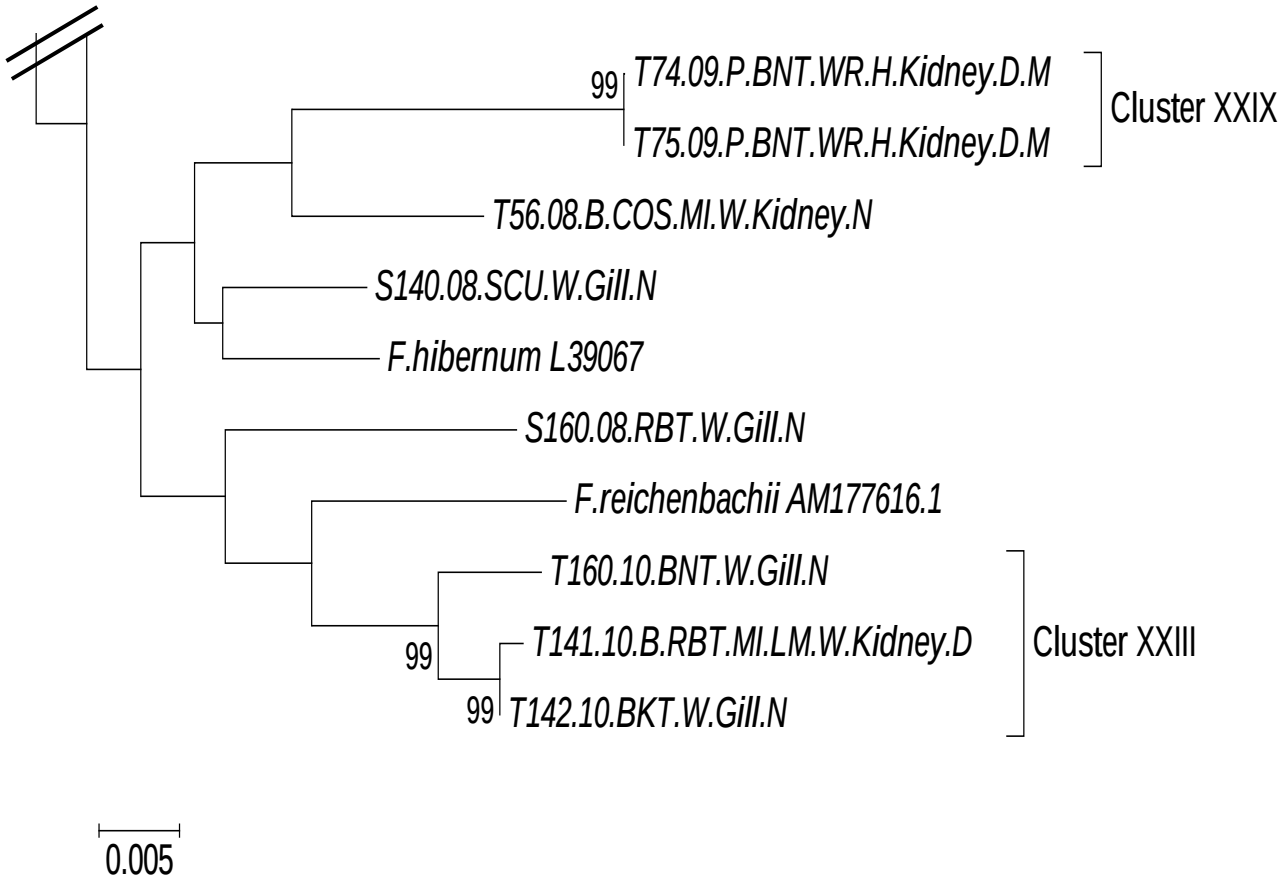
Supplementary Figure 2.4 (cont'd)



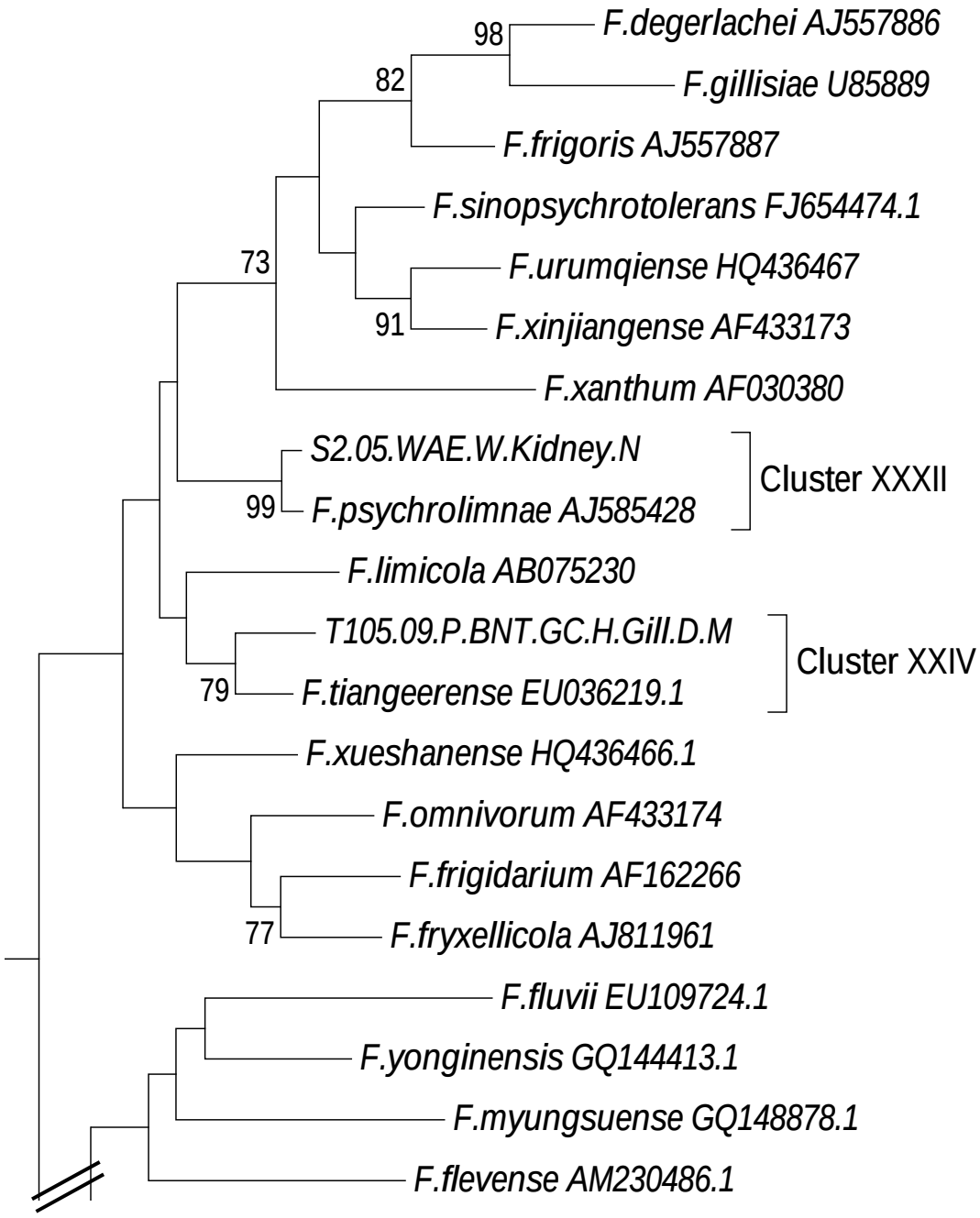
Supplementary Figure 2.5. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 4 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



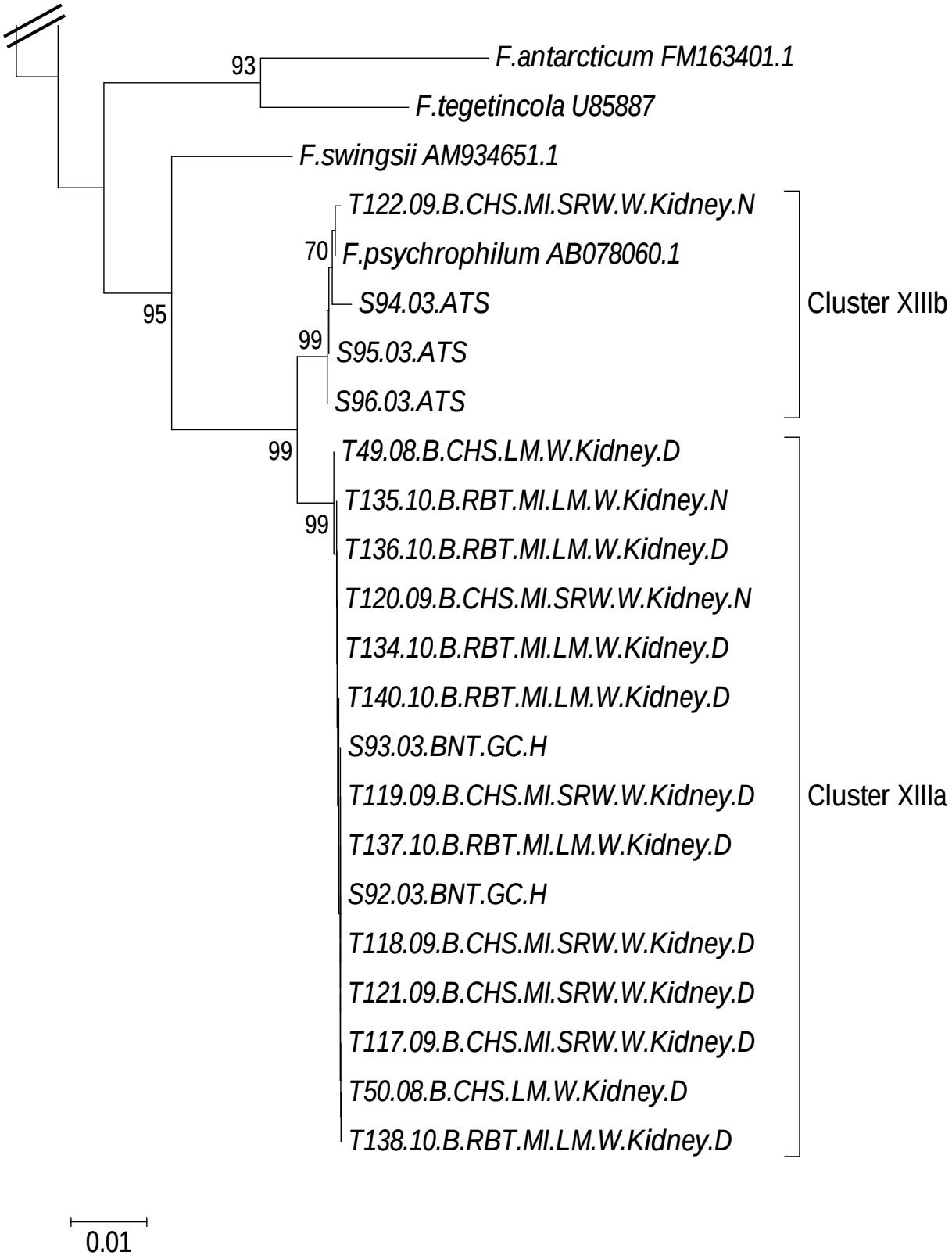
Supplementary Figure 2.5 (cont'd)



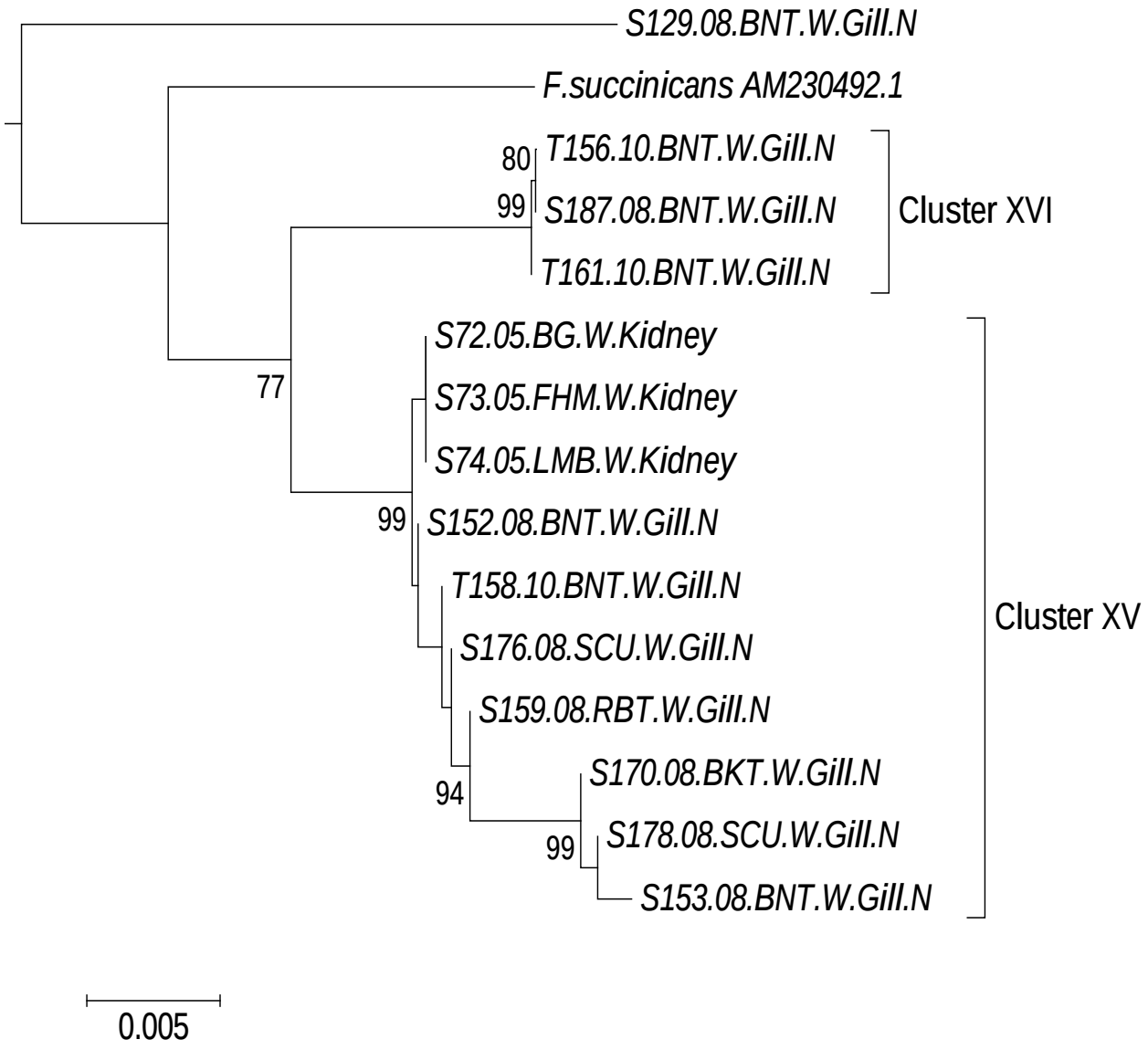
Supplementary Figure 2.6. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between four clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



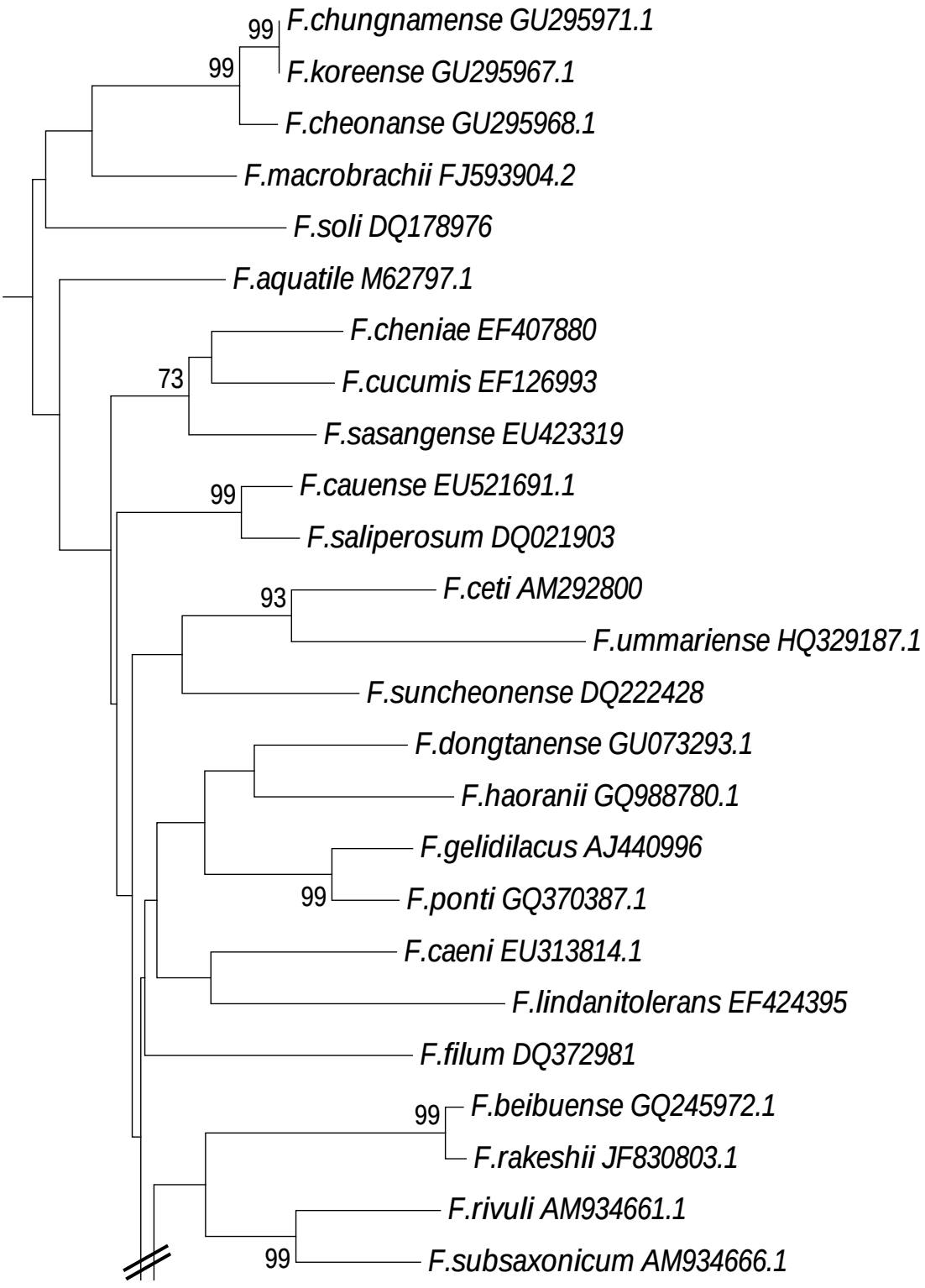
Supplementary Figure 2.6 (cont'd)



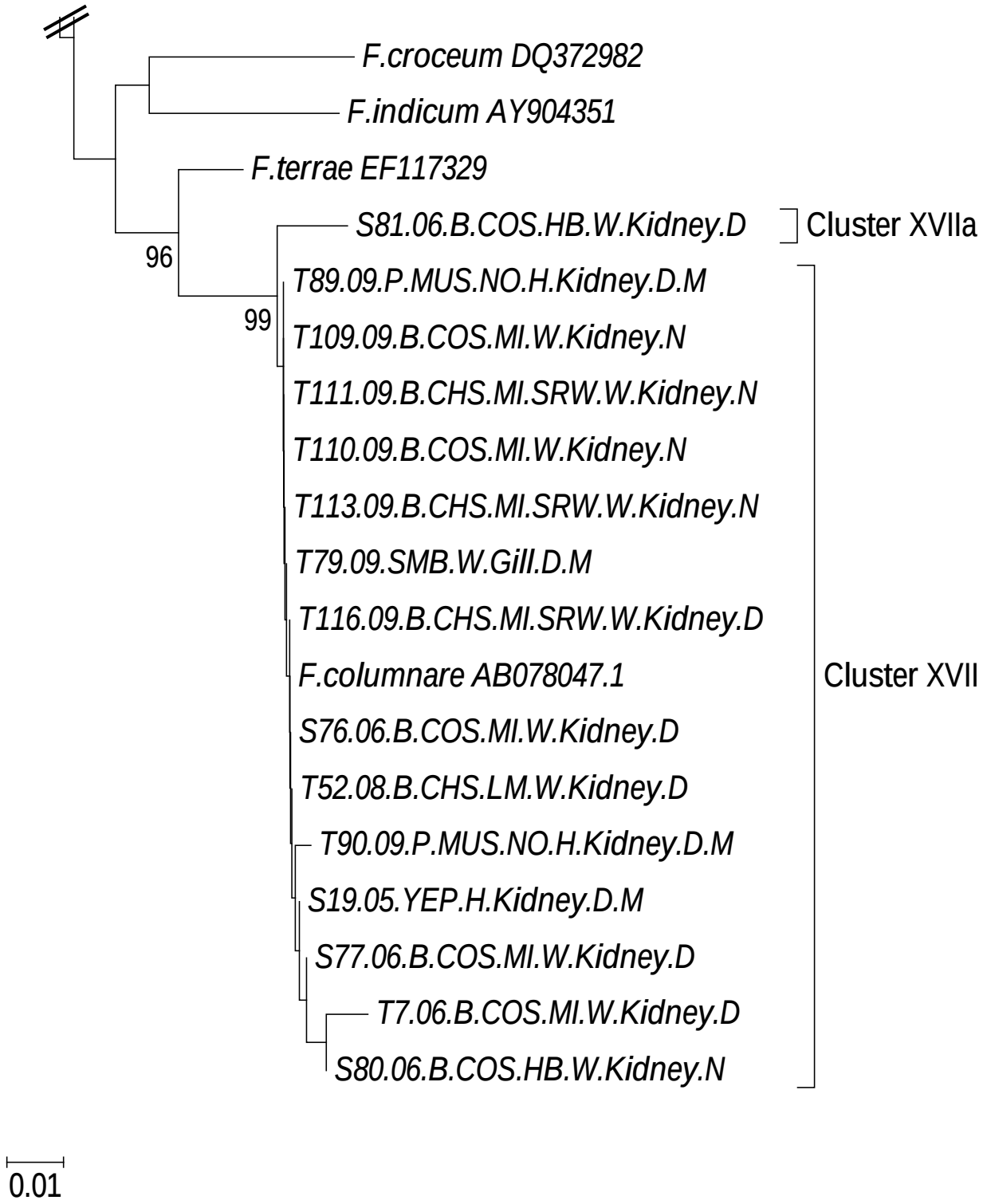
Supplementary Figure 2.7. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between two clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



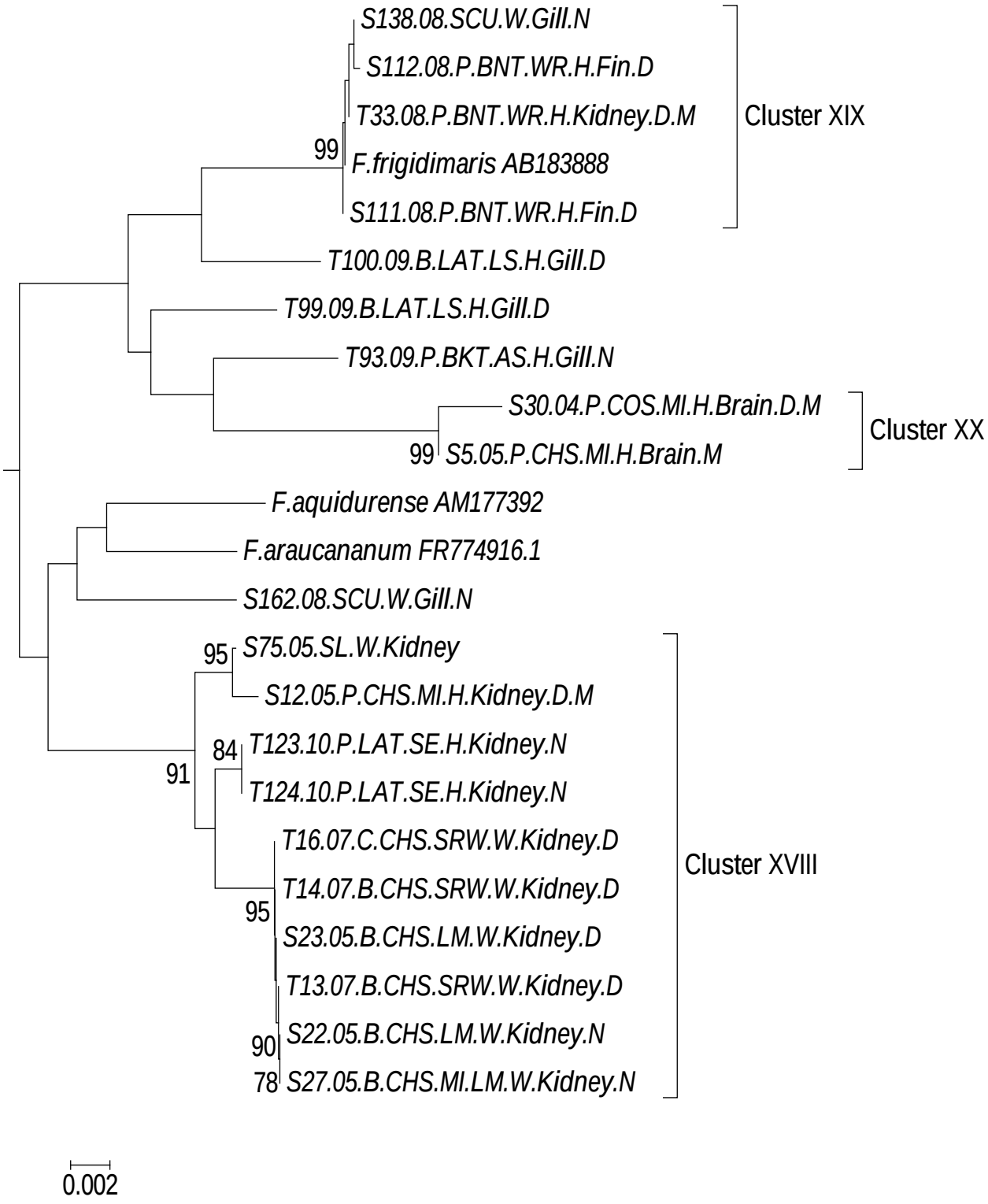
Supplementary Figure 2.8. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between Michigan fish-associated *F. columnare*. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



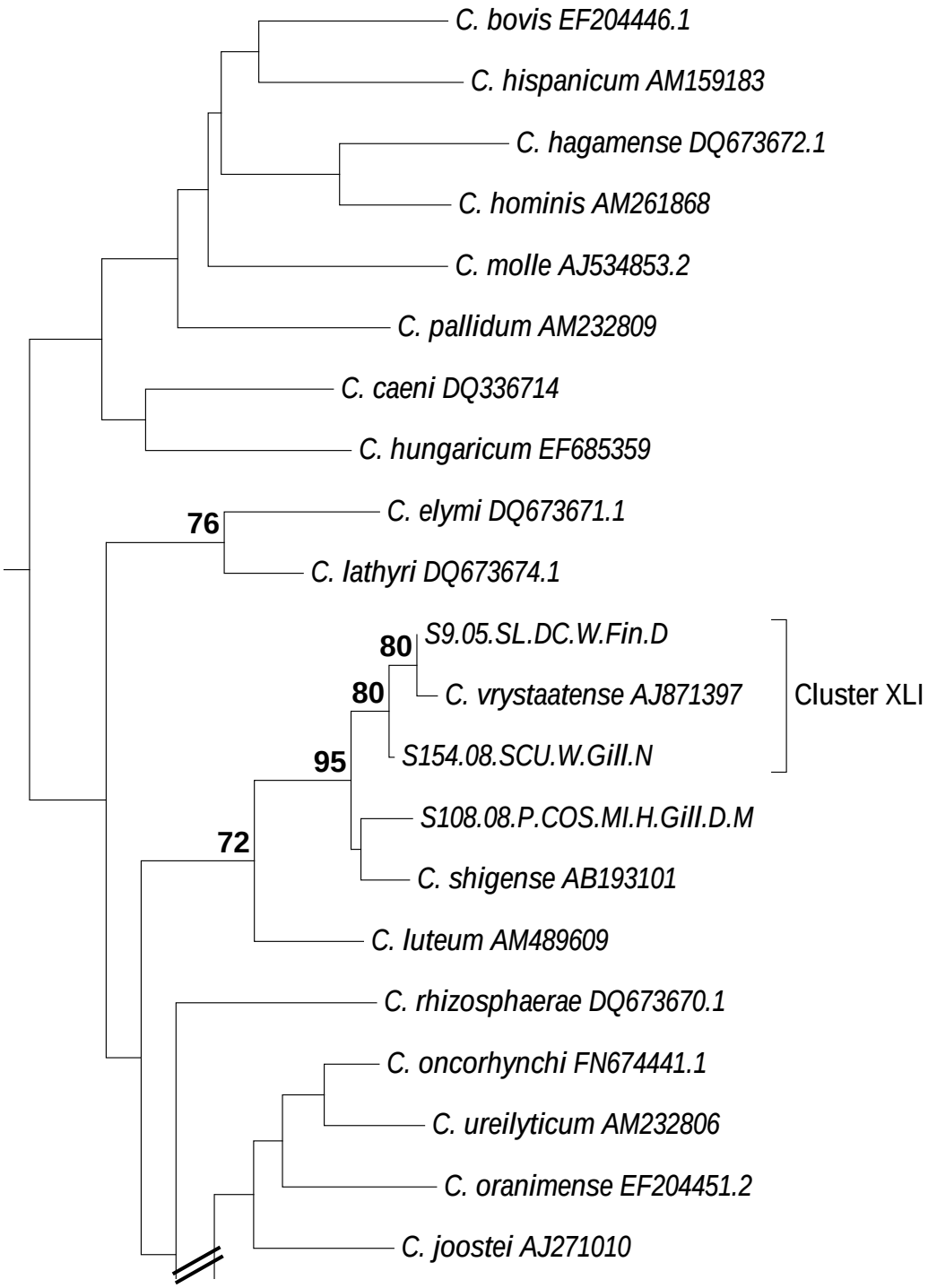
Supplementary Figure 2.8 (cont'd)



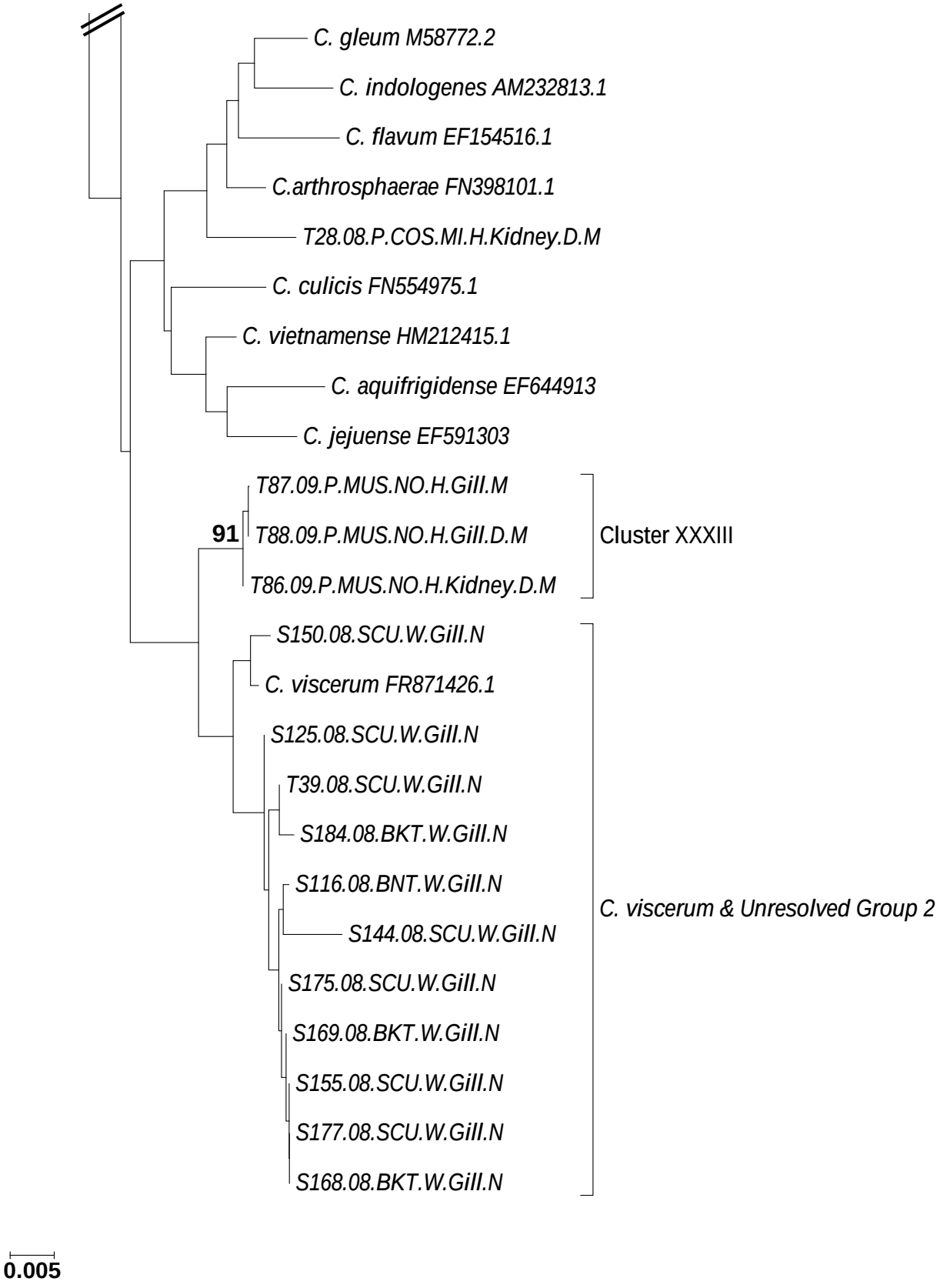
Supplementary Figure 2.9. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between three clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



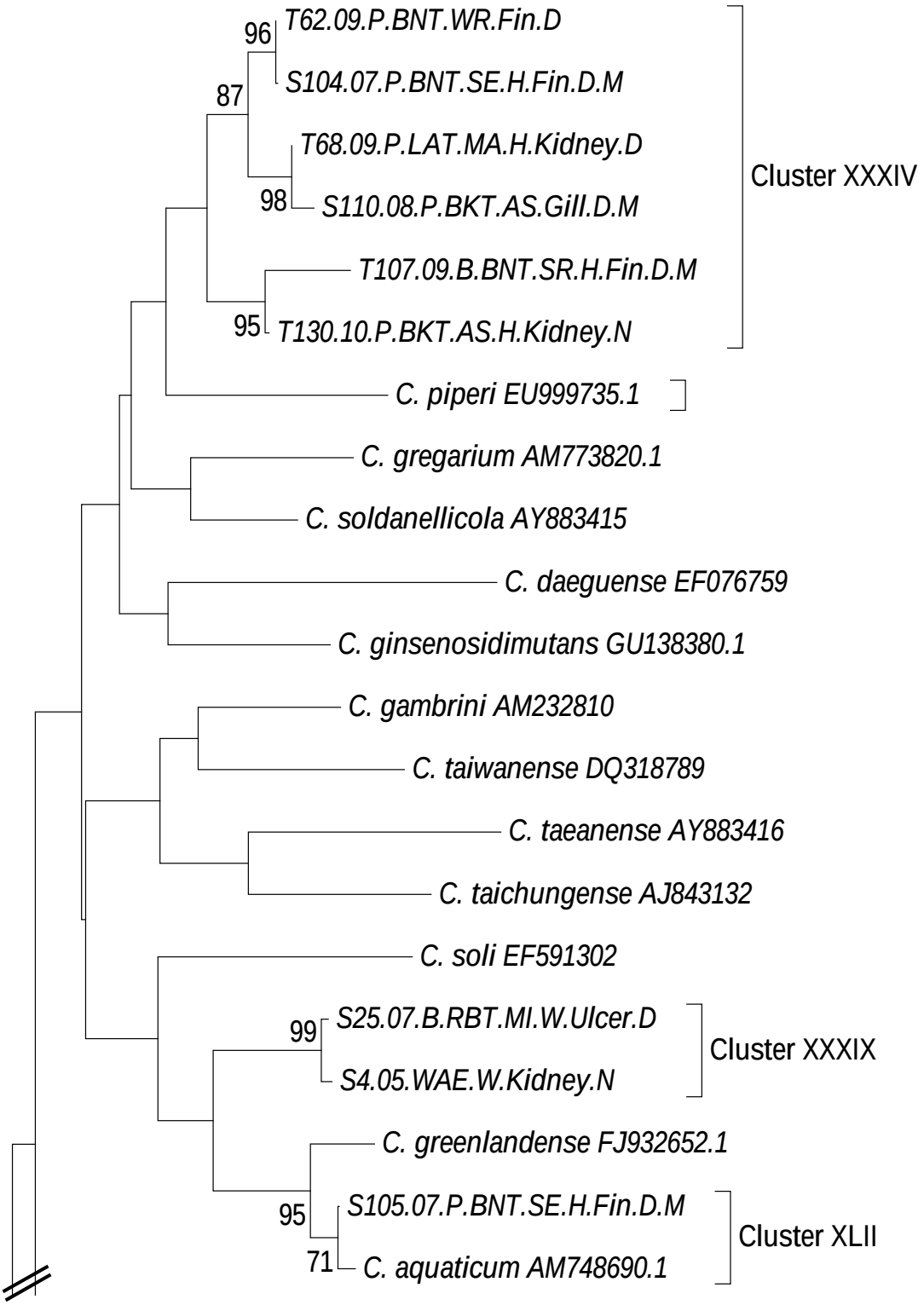
Supplementary Figure 2.10. Subtree of the dendrogram presented in Figure 2 depicting the phylogenetic relationship between two clusters of Michigan fish-associated *Chryseobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



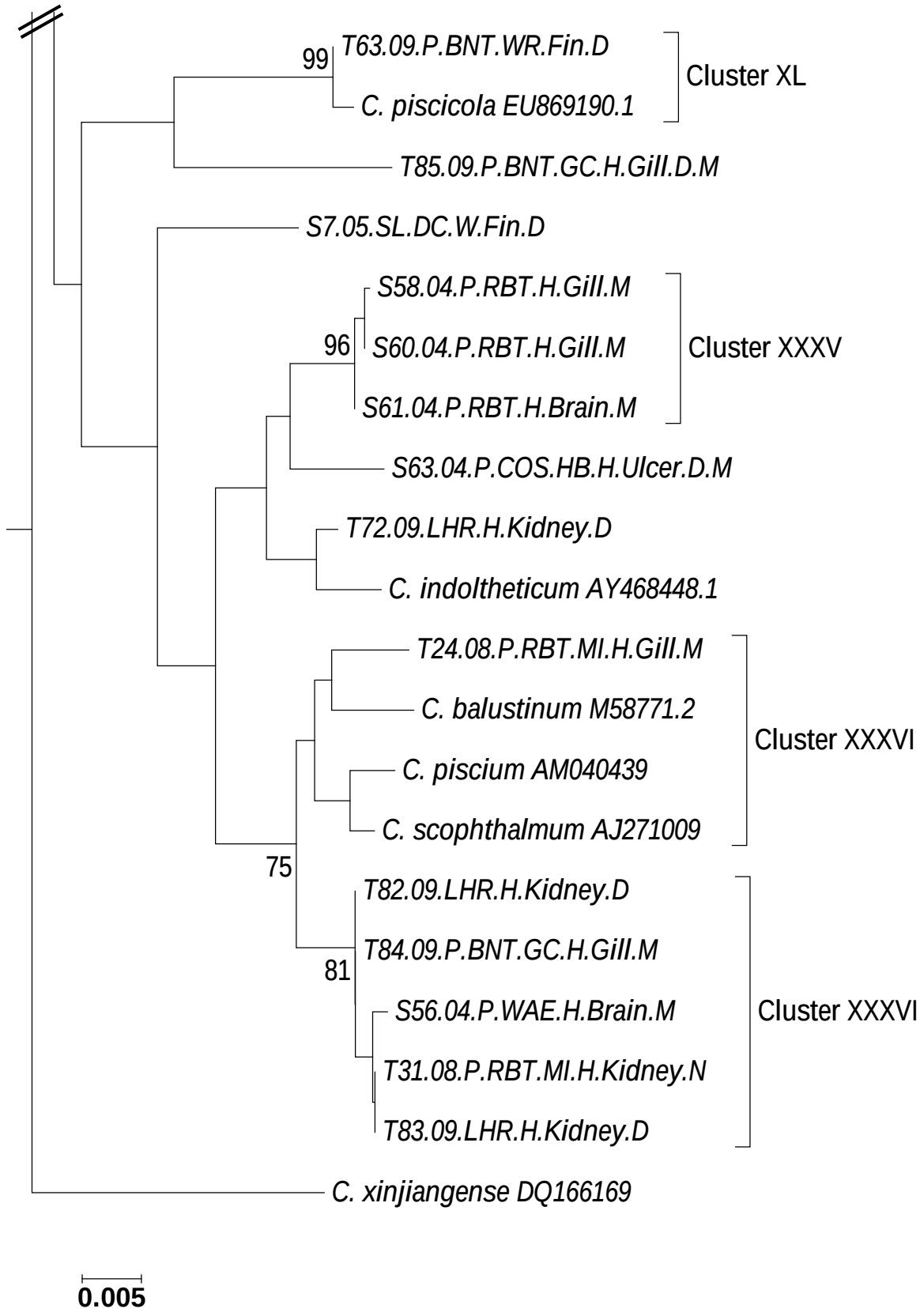
Supplementary Figure 2.10 (cont'd)



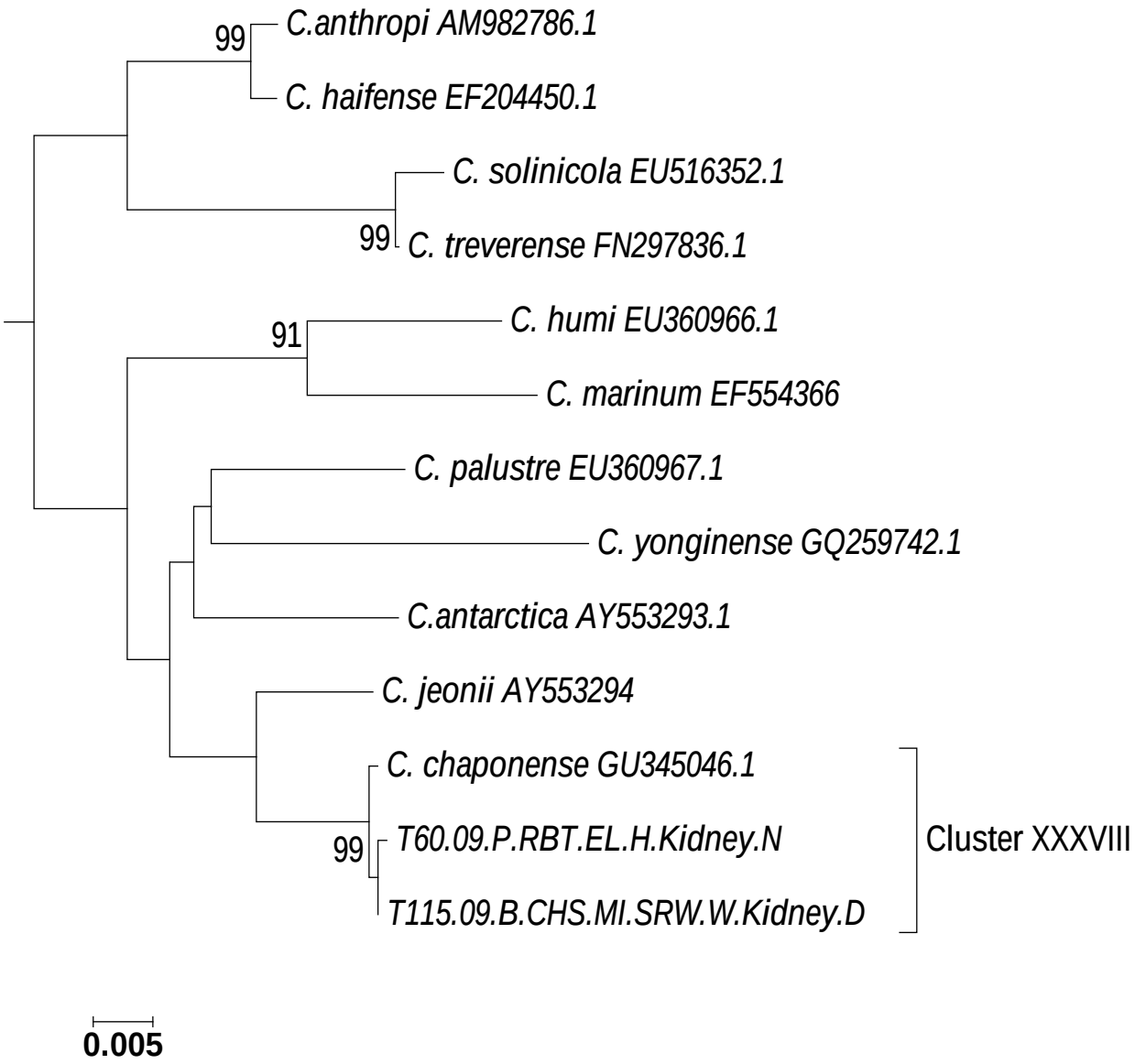
Supplementary Figure 2.11. Subtree of the dendrogram presented in Figure 2 depicting the phylogenetic relationship between seven clusters of Michigan fish-associated *Chryseobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



Supplementary Figure 2.11 (cont'd)



Supplementary Figure 2.12. Subtree of the dendrogram presented in Figure 2 depicting the phylogenetic relationship between one cluster of Michigan fish-associated *Chryseobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



Supplementary Table 2.1. Information on each of the 255 flavobacterial isolates examined in this study, including phylogenetic cluster, % 16S rDNA similarity to its closest relative, host of recovery, month/year of recovert, organ from which the bacterium was originally isolated, locale from which the infected host was recovered, and GenBank accession number. M, mortality event; S, Fish health surveillance; Unres., unresolved; OSFH, Oden State Fish Hatchery (Alanson, MI); Marquette State Fish Hatchery (Marquette, MI); WLSFH, Wolf Lake State Fish Hatchery (Mattawan, MI); TSFH, Thompson State Fish Hatchery (Manistique, MI); HSFH, Harrietta State Fish Hatchery, (Harrietta, MI); PRSFH, Platte River State Fish Hatchery, Beluah, MI; PRW, Platte River Weir, Beluah, MI; SRW, Swan River Weir, Rogers City, MI; LMRW, Little Manistee River Weir, Manistee, MI.

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Cluster I	S113	*F. hercynium (97.0%)*	Hatchery-reared brown trout fingerlings	M	08/2008	Fins	OSFH	JX287661
Cluster I	S114	*F. hercynium (97.0%)*	Hatchery-reared brown trout fingerlings	M	08/2008	Fins	OSFH	JX287663
Cluster I	T148	*F. hercynium (97.2%)*	Wild brook trout yearlings	S	06/2010	Gills	Cherry Creek	JX287618
Cluster I	S71	*F. hercynium (97.2%)*	Hatchery-reared lake trout fingerlings	M	03/2005	Brain	MSFH	JX287726
Cluster I	S136	*F. hercynium (97.2%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287665
Cluster I	T129	*F. succinicans (97.1%)*	Hatchery-reared brook trout fingerlings	S	03/2010	Kidney	MSFH	JX287607
Cluster II	T151	*F. hercynium (97.6%)*	Wild brook trout yearlings	S	06/2010	Gills	Cherry Creek	JX287620
Cluster II	T102	*F. hercynium (97.6%)*	Hatchery-reared brook trout fingerlings	S	02/2009	Kidney	MSFH	JX287588
Cluster II	T101	*F. hercynium (97.6%)*	Hatchery-reared	S	03/2009	Kidney	TSFH	JX287587

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			Chinook salmon fingerlings					
Cluster II	S148	*F. hercynium (97.9%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287645
Cluster II	S51	*F. hercynium (97.9%)*	Hatchery-reared brook trout fingerlings	S	Unknown	Kidney	MSFH	JX287715
Cluster II	T21	*F. hercynium (97.5%)*	Hatchery-reared brown trout fingerlings	M	05/2008	Gills	TSFH	JX287551
Cluster II	T18	*F. hercynium (97.6%)*	Hatchery-reared brown trout fingerlings	M	05/2008	Gills	TSFH	JX287548
Cluster II	T66	*F. hercynium (97.0%)*	Hatchery-reared Chinook salmon fingerlings	S	03/2009	Kidney	WLSFH	JX287568
Cluster II	S28	*F. hercynium (97.3%)*	Feral spawning coho salmon	S	11/2005	Kidney	PRW	JX287706
Cluster II	T71	*F. hercynium (97.0%)*	Hatchery-reared lake herring fingerlings	M	05/2009	Kidney	WLSFH	JX287571
Cluster II	T73	*F. hercynium (97.1%)*	Hatchery-reared lake herring fingerlings	S	05/2009	Kidney	WLSFH	JX287572
Cluster II	S24	*F. hercynium (97.2%)*	Hatchery-reared steelhead trout fingerlings	M	07/2006	Kidney	WLSFH	JX287690
Cluster II	T70	*F. hercynium (97.2%)*	Hatchery-reared lake herring fingerlings	M	05/2009	Kidney	WLSFH	JX287570
Cluster III	T10	*F. hercynium (97.3%)*	Hatchery-reared	M	05/2007	Brain	TSFH	JX287542

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			brown trout fingerlings					
Cluster III	S66	*F. hercynium (97.4%)*	Hatchery-reared brown trout fingerlings	M	06/2007	Brain	HSFH	JX287721
Cluster III	S67	*F. hercynium (97.4%)*	Hatchery-reared brown trout fingerlings	M	06/2007	Brain	HSFH	JX287722
Cluster III	S65	*F. hercynium (97.4%)*	Hatchery-reared brown trout fingerlings	M	06/2007	Fins	HSFH	JX287720
Cluster III	T15	*F. hercynium (97.0%)*	Feral spawning Chinook salmon	S	10/2007	Kidney	SRW	JX287545
Cluster III	T166	*F. hercynium (97.2%)*	Hatchery-reared steelhead trout fingerlings	M	06/2010	Kidney	WLSFH	JX287629
Cluster IV	S15	*F. chungangense (98.0%)*	Spawning hatchery-reared brown trout broodstock	S	11/2006	Swim bladder	OSFH	JX287691
Cluster IV	T9	*F. hercynium (97.4%)*	Spawning hatchery-reared brown trout	S	11/2006	Kidney	OSFH	JX287541
Cluster IV	T8	*F. hercynium (97.6%)*	Spawning hatchery-reared brown trout broodstock	S	11/2006	Swim bladder	OSFH	JX287540
Cluster IV	T69	*F. hercynium (98.0%)*	Feral spawning steelhead trout	S	04/2009	Kidney	LMRW	JX287569
Cluster V	S20	*F. hercynium (98.6%)*	Hatchery-reared brown trout	S	08/2005	Gills	OSFH	JX287707

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			fingerlings					
Cluster V	T61	*F. hercynium (98.6%)*	Hatchery-reared brown trout fingerlings	S	02/2009	Fins	HSFH	JX287566
Cluster V	S86	*F. hercynium (98.8%)*	Hatchery-reared brown trout fingerlings	M	07/2005	Kidney	TSFH	JX287734
Cluster VI	S17	*F. pectinovorum (97.5%)*	Wild bluegills	S	06/2005	Gills	Gourdneck Lake	JX287688
Cluster VI	S18	*F. pectinovorum (97.6%)*	Hatchery-reared rainbow trout fingerlings	M	06/2005	Gills	Harrietta Hills Aquaculture Facility	JX287713
Cluster VI	S13	*F. pectinovorum (97.9%)*	Wild largemouth bass	S	06/2005	Ulcer	Pine Lake	JX287699
Cluster VII	S31	*F. aquidurense (98.1%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287693
Cluster VII	S37	*F. aquidurense (98.2%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287705
Cluster VII	S164	*F. frigidimaris (97.4%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Pond	JX287667
Cluster VII	S41	*F. pectinovorum (97.5%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287692
Cluster VII	S40	*F. pectinovorum (97.5%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287696
Cluster VII	S191	*F. pectinovorum (97.5%)*	Wild brown trout yearlings	S	09/2008	Gills	Cherry Creek	JX287636
Cluster VII	S29	*F. pectinovorum (97.7%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287702
Cluster VII	S38	*F. pectinovorum (97.7%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287701
Cluster VII	S35	*F. pectinovorum (97.7%)*	Hatchery-reared	M	02/2004	Brain	PRSFH	JX287712

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			coho salmon fry					
Cluster VII	S34	*F. pectinovorum (97.7%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287687
Cluster VII	S127	*F. pectinovorum (97.7%)*	Wild brook trout yearlings	S	09/2008	Gills	Brundage Creek	JX287632
Cluster VIII	T77	*F. pectinovorum (97.1%)*	Hatchery-reared rainbow trout fingerlings	M	06/2009	Kidney	HSFH	JX287576
Cluster VIII	T76	*F. pectinovorum (97.3%)*	Hatchery-reared rainbow trout fingerlings	M	06/2009	Kidney	HSFH	JX287575
Cluster VIII	S68	*F. pectinovorum (97.4%)*	Hatchery-reared brook trout fingerlings	M	07/2007	Gills	MSFH	JX287723
Cluster VIII	T131	*F. pectinovorum (97.5%)*	Hatchery-reared brook trout fingerlings	S	03/2010	Kidney	MSFH	JX287608
Cluster IX	T20	*F. oncorhynchi (100%)*	Hatchery-reared brown trout fingerlings	M	05/2008	Gills	TSFH	JX287550
Cluster IX	T128	*F. oncorhynchi (100%)*	Hatchery-reared brook trout fingerlings	S	03/2010	Kidney	MSFH	JX287606
Cluster IX	T150	*F. oncorhynchi (99.3%)*	Wild brook trout fingerlings	S	06/2010	Gills	Cherry Creek	JX287619
Cluster IX	T19	*F. oncorhynchi (99.6%)*	Hatchery-reared brown trout fingerlings	M	05/2008	Gills	TSFH	JX287549
Cluster IX	T26	*F. oncorhynchi (99.7%)*	Hatchery-reared steelhead trout fingerlings	M	06/2008	Gills	WLSFH	JX287554

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Cluster IX	S134	*F. oncorhynchi (99.7%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287634
Cluster IX	T104	*F. oncorhynchi (99.7%)*	Wild adult sea lamprey	S	05/2004	Kidney	Duffins Creek	JX287590
Cluster IX	T4	*F. oncorhynchi (99.9%)*	Wild adult lake whitefish	S	11/2005	Kidney	Little Bay de Noc	JX287537
Cluster X	S102	*F. oncorhynchi (97.4%)*	Hatchery-reared steelhead trout fingerlings	S	07/2007	Gills	WLSFH	JX287639
Cluster X	T164	*F. oncorhynchi (97.4%)*	Hatchery-reared steelhead trout fingerlings	M	06/2010	Gills	WLSFH	JX287627
Cluster X	T25	*F. oncorhynchi (97.6%)*	Hatchery-reared steelhead trout fingerlings	M	06/2008	Gills	WLSFH	JX287553
Cluster X	T165	*F. oncorhynchi (97.6%)*	Hatchery-reared steelhead trout fingerlings	M	06/2010	Gills	WLSFH	JX287628
Cluster X	T103	*F. oncorhynchi (97.6%)*	Wild adult sea lamprey	S	05/2004	Kidney	Duffins Creek	JX287589
Cluster X	S101	*F. oncorhynchi (97.7%)*	Hatchery-reared steelhead trout fingerlings	S	07/2007	Gills	WLSFH	JX287643
Cluster X	T23	*F. oncorhynchi (97.7%)*	Hatchery-reared steelhead trout fingerlings	M	06/2008	Gills	WLSFH	JX287552
Cluster X	T96	*F. oncorhynchi (97.7%)*	Hatchery-reared brown trout broodstock	M	08/2009	Gills	OSFH	JX287584
Cluster X	S103	*F. oncorhynchi (97.8%)*	Hatchery-reared steelhead trout	S	07/2007	Gills	WLSFH	JX287637

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			fingerlings					
Cluster X	T59	*F. oncorhynchi (97.8%)*	Hatchery-reared brook trout fingerlings	S	01/2009	Kidney	MSFH	JX287565
Cluster X	T95	*F. oncorhynchi (97.8%)*	Hatchery-reared brown trout broodstock	M	08/2009	Gills	OSFH	JX287583
Cluster X	S52	*F. oncorhynchi (97.8%)*	Hatchery-reared brook trout fingerlings	S	Unknown	Kidney	MSFH	JX287716
Cluster XI	T17	*F. aquidurense (97.5%)*	Feral spawning steelhead trout	S	04/2008	Kidney	LMRW	JX287547
Cluster XI	T1	*F. araucananum (96.9%)*	Wild bluegill	S	06/2005	Gills	Gourdneck Lake	JX287533
Cluster XI	S157	*F. araucananum (97.0%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287685
Cluster XI	S21	*F. araucananum (97.0%)*	Hatchery-reared rainbow trout fingerlings	M	08/2005	Ulcer	Harietta Hills Aquaculture Facility	JX287703
Cluster XI	S106	*F. araucananum (97.1%)*	Wild spawning walleye	S	04/2008	Gills	Little Bay de Noc, Lake Michigan	JX287654
Cluster XI	S6	*F. araucananum (97.1%)*	Hatchery-reared Chinook salmon fingerlings	M	04/2005	Kidney	PRSFH	JX287695
Cluster XI	S14	*F. araucananum (97.1%)*	Hatchery-reared northern pike fingerlings	M	06/2008	Gills	WLSFH	JX287714
Cluster XI	S128	*F. araucananum (97.1%)*	Wild brook trout yearlings	S	09/2008	Gills	Brundage Creek	JX287631
Cluster XI	S55	*F. araucananum (97.1%)*	Wild adult lake	S	Unknown	Kidney	Naubinway,	JX287719

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			whitefish				Lake Michigan	
Cluster XII	S163	*F. araucananum (98,8%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Pond	JX287657
Cluster XII	T6	*F. araucananum (98.3%)*	Wild adult lake whitefish	S	05/2006	Swim bladder	Detour Village, Lake Huron	JX287538
Cluster XII	S161	*F. araucananum (98.4%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Pond	JX287648
Cluster XII	S166	*F. araucananum (98.6%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Pond	JX287684
Cluster XII	S43	*F. araucananum (98.7%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287686
Cluster XII	S126	*F. araucananum (98.7%)*	Wild brook trout yearlings	S	09/2008	Gills	Brundage Creek	JX287635
Cluster XII	S130	*F. araucananum (98.7%)*	Wild brown trout yearlings	S	09/2008	Gills	Brundage Creek	JX287652
Cluster XII	S190	*F. araucananum (98.7%)*	Wild brown trout yearlings	S	09/2008	Gills	Cherry Creek	JX287641
Cluster XII	S149	*F. araucananum (98.8%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287640
Cluster XIIIa	T49	*F. psychrophilum (98.6%)*	Feral spawning Chinook salmon broodstock	S	09/2008	Kidney	LMRW	JX287559
Cluster XIIIa	T50	*F. psychrophilum (98.7%)*	Feral spawning Chinook salmon broodstock	S	09/2008	Kidney	LMRW	JX287560
Cluster XIIIa	T140	*F. psychrophilum (98.8%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287615
Cluster XIIIa	T120	*F. psychrophilum (99.0%)*	Feral spawning	S	10/2009	Kidney	SRW	JX287601

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			Chinook salmon					
Cluster XIIIa	T135	*F. psychrophilum (99.0%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287611
Cluster XIIIa	T136	*F. psychrophilum (99.0%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287612
Cluster XIIIa	T134	*F. psychrophilum (99.0%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287610
Cluster XIIIa	T137	*F. psychrophilum (99.1%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287613
Cluster XIIIa	S92	*F. psychrophilum (99.1%)*	Hatchery-reared brown trout fingerlings	S	10/2003	Kidney	N/A	JX287739
Cluster XIIIa	S93	*F. psychrophilum (99.1%)*	Hatchery-reared brown trout fingerlings	S	10/2003	Kidney	N/A	JX287740
Cluster XIIIa	T121	*F. psychrophilum (99.1%)*	Feral spawning Chinook salmon broodstock	S	10/2009	Kidney	SRW	JX287602
Cluster XIIIa	T117	*F. psychrophilum (99.1%)*	Feral spawning Chinook salmon broodstock	S	10/2009	Kidney	SRW	JX287598
Cluster XIIIa	T118	*F. psychrophilum (99.1%)*	Feral spawning Chinook salmon broodstock	S	10/2009	Kidney	SRW	JX287599
Cluster XIIIa	T138	*F. psychrophilum (99.1%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287614
Cluster XIIIa	T119	*F. psychrophilum (99.1%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	SRW	JX287600
Cluster XIIIb	S94	*F. psychrophilum (99.5%)*	Hatchery reared Atlantic salmon fingerlings	S	10/2003	Kidney	N/A	JX287741

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Cluster XIIIb	S95	*F. psychrophilum (99.9%)*	Hatchery reared Atlantic salmon fingerlings	S	10/2003	Kidney	N/A	JX287742
Cluster XIIIb	S96	*F. psychrophilum (99.9%)*	Hatchery reared Atlantic salmon fingerlings	S	10/2003	Kidney	N/A	JX287743
Cluster XIIIb	t122	*F. psychrophilum (99.9%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	SRW	JX287603
Cluster XIV	S90	*F. succinicans (96.6%)*	Wild adult lake whitefish	S	06/2006	Kidney	Naubinway, Lake Michigan	JX287738
Cluster XIV	S91	*F. succinicans (96.6%)*	Wild adult lake whitefish	S	06/2006	Kidney	Naubinway, Lake Michigan	JX287737
Cluster XV	S153	*F. succinicans (97.4%)*	Wild brown trout yearlings	S	09/2008	Gills	Brundage creek	JX287680
Cluster XV	S159	*F. succinicans (97.7%)*	Wild rainbow trout yearlings	S	09/2008	Gills	Brundage Creek	JX287650
Cluster XV	S170	*F. succinicans (97.7%)*	Wild brook trout yearlings	S	09/2008	Gills	Kinney Creek	JX287677
Cluster XV	S178	*F. succinicans (97.7%)*	Wild mottled sculpin	S	09/2008	Gills	Kinney Creek	JX287678
Cluster XV	S152	*F. succinicans (97.7%)*	Wild brown trout yearlings	S	09/2008	Gills	Brundage Creek	JX287679
Cluster XV	S176	*F. succinicans (97.8%)*	Wild mottled sculpin	S	09/2008	Gills	Kinney Creek	JX287681
Cluster XV	T158	*F. succinicans (97.8%)*	Wild brown trout yearlings	S	06/2010	Gills	Cherry Creek	JX287623
Cluster XV	S72	*F. succinicans (98.0%)*	Wild largemouth bass	S	05/2005	Kidney	Big Lake	JX287727
Cluster XV	S74	*F. succinicans (98.0%)*	Wild largemouth	S	05/2005	Kidney	Big Lake	JX287729

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			bass					
Cluster XV	S73	*F. succinicans (98.0%)*	Wild largemouth bass	S	05/2005	Kidney	Big Lake	JX287728
Cluster XVI	T161	*F. succinicans (97.7%)*	Wild brown trout yearlings	S	06/2010	Gills	Cherry Creek	JX287626
Cluster XVI	S187	*F. succinicans (97.8%)*	Wild brown trout yearlings	S	09/2008	Gills	Cherry Creek	JX287682
Cluster XVI	T156	*F. succinicans (97.8%)*	Wild brown trout yearlings	S	06/2010	Gills	Cherry Creek	JX287621
Cluster XVII	S19	*F. columnare (100%)*	Hatchery-reared yellow perch	M	07/2005	Gills	Stoney Creek Hatchery	JX287710
Cluster XVII	S77	*F. columnare (100%)*	Feral spawning coho salmon	S	10/2006	Kidney	PRW	JX287731
Cluster XVII	S76	*F. columnare (100%)*	Feral spawning coho salmon	S	10/2006	Kidney	PRW	JX287730
Cluster XVII	S80	*F. columnare (100%)*	Feral spawning coho salmon	S	10/2006	Kidney	PRW	JX287732
Cluster XVII	T116	*F. columnare (100%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	SRW	JX287597
Cluster XVII	T7	*F. columnare (99.7%)*	Feral spawning coho salmon	S	10/2006	Kidney	PRW	JX287539
Cluster XVII	T52	*F. columnare (99.7%)*	Feral spawning Chinook salmon	S	09/2008	Kidney	LMRW	JX287561
Cluster XVII	T89	*F. columnare (99.7%)*	Hatchery-reared muskellunge fingerlings	M	07/2009	Kidney	WLSFH	JX287578
Cluster XVII	T90	*F. columnare (99.7%)*	Hatchery-reared muskellunge	M	07/2009	Kidney	WLSFH	JX287579
Cluster XVII	T79	*F. columnare (99.9%)*	Wild spawning smallmouth bass	M	06/2009	Gills	Lake St. Clair	JX287577
Cluster XVII	T111	*F. columnare (99.9%)*	Feral spawning	S	10/2009	Kidney	SRW	JX287595

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			Chinook salmon					
Cluster XVII	T113	*F. columnare (99.9%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	SRW	JX287596
Cluster XVII	T109	*F. columnare (99.9%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	PRW	JX287593
Cluster XVII	T110	*F. columnare (99.9%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	PRW	JX287594
Cluster XVIIa	S81	*F. columnare (98.7%)*	Feral spawning coho salmon	S	10/2006	Kidney	PRW	JX287733
Cluster XVIII	T13	*F. aquidurense (97.6%)*	Feral spawning Chinook salmon	S	10/2007	Kidney	SRW	JX287543
Cluster XVIII	S12	*F. aquidurense (97.7%)*	Hatchery-reared Chinook salmon fingerlings	M	05/2005	Gills	TSFH	JX287704
Cluster XVIII	S22	*F. aquidurense (97.8%)*	Feral adult spawning Chinook salmon	S	09/2005	Kidney	LMRW	JX287698
Cluster XVIII	S23	*F. aquidurense (97.8%)*	Feral adult spawning Chinook salmon	S	09/2005	Kidney	LMRW	JX287694
Cluster XVIII	S27	*F. aquidurense (97.8%)*	Feral adult spawning Chinook salmon	S	09/2005	Kidney	LMRW	JX287709
Cluster XVIII	T14	*F. aquidurense (97.8%)*	Feral spawning Chinook salmon	S	10/2007	Kidney	SRW	JX287544
Cluster XVIII	T16	*F. aquidurense (97.8%)*	Feral spawning Chinook salmon broodstock	S	10/2007	Kidney	SRW	JX287546
Cluster XVIII	S75	*F. aquidurense (98.0%)*	Wild larval sea lamprey (*Petromyzon*	S	10/2005	Kidney	Covert Creek	JX287534

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			marinus)					
Cluster XVIII	T124	*F. aquidurense (98.0%)*	Hatchery-reared lake trout fingerlings	S	02/2010	Kidney	MSFH	JX287605
Cluster XVIII	T123	*F. aquidurense (98.0%)*	Hatchery-reared lake trout fingerlings	S	02/2010	Kidney	MSFH	JX287604
Cluster XIX	S112	*F. frigidimaris (100%)*	Hatchery reared brown trout fingerlings	M	08/2008	Fins	OSFH	JX287660
Cluster XIX	S111	*F. frigidimaris (100%)*	Hatchery reared brown trout fingerlings	M	08/2008	Fins	OSFH	JX287662
Cluster XIX	S138	*F. frigidimaris (100%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287659
Cluster XIX	T33	*F. frigidimaris (99.7%)*	Hatchery reared brown trout fingerlings	M	08/2008	Kidney	OSFH	JX287556
Cluster XX	S30	*F. aquidurense (97.3%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287697
Cluster XX	S5	*F. frigidimaris (97.7%)*	Hatchery-reared Chinook salmon fingerlings	M	04/2005	Brain	PRSFH	JX287700
Cluster XXI	S151	*F. chungangense (96.5%)*	Wild brown trout yearlings	S	09/2008	Gills	Brundage Creek	JX287638
Cluster XXI	T37	*F. chungangense (96.6%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287557
Cluster XXI	S156	*F. chungangense (96.8%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287669
Cluster XXII	T27	*F. chungangense (97.0%)*	Wild northern brook lamprey	S	06/2008	Kidney	Sault St. Marie	JX287555

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Cluster XXII	S193	*F. chungangense (97.7%)*	Surveillance, gills of wild brown trout	S	09/2008	Gills	Cherry Creek	JX287683
Cluster XXII	T47	*F. chungangense (97.8%)*	Wild brown trout yearlings	S	09/2008	Gills	Cherry Creek	JX287558
Cluster XXII	S70	*F. chungangense (98.0%)*	Hatchery-reared brook trout fingerlings	M	07/2007	Gills	MSFH	JX287725
Cluster XXII	S69	*F. chungangense (98.0%)*	Hatchery-reared brook trout fingerlings	M	07/2007	Fins	MSFH	JX287724
Cluster XXIII	T141	*F. reichenbachii (97.1%)*	Feral spawning steelhead trout	S	04/2010	Kidney	LMRW	JX287616
Cluster XXIII	T142	*F. reichenbachii (97.1%)*	Wild brook trout yearlings	S	06/2010	Gills	Cherry Creek	JX287617
Cluster XXIII	T160	*F. reichenbachii (97.1%)*	Wild brown trout yearlings	S	06/2010	Gills	Cherry Creek	JX287625
Cluster XXIV	T105	*F. tiangeerense (98.7%)*	Hatchery-reared brown trout fingerlings	M	08/2009	Gills	TSFH	JX287591
Cluster XXV	S118	*F. hibernum (97.7%)*	Wild mottled sculpin	S	09/2008	Gills	Stanley Creek	JX287668
Cluster XXV	S54	*F. hydatis (97.7%)*	Wild adult lake whitefish	S		Kidney	Naubinway, Lake Michigan	JX287718
Cluster XXVI	S171	*F. hydatis (98.9%)*	Wild brook trout yearlings	S	09/2008	Gills	Kinney Creek	JX287664
Cluster XXVII	T92	*F. anhuiense (97.4%)*	Hatchery-reared channel catfish yearlings	S	07/2009	Kidney	St. Mary's SFH, Ohio	JX287581
Cluster XXVII	T91	*F. anhuiense (98.0%)*	Hatchery-reared channel catfish	S	07/2009	Kidney	St. Mary's SFH, Ohio	JX287580

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			yearlings					
Cluster XXVIII	T2	*F. chilense (98.3%)*	Wild adult lake whitefish	S	11/2005	Kidney	Little Bay de Noc, Lake Michigan	JX287535
Cluster XXVIII	T3	*F. chilense (98.4%)*	Wild adult lake whitefish	S	11/2005	Kidney	Little Bay de Noc, Lake Michigan	JX287536
Cluster XXIX	T74	*F. degerlachei (96.7%)*	Hatchery-reared brown trout fingerlings	M	06/2009	Kidney	HSFH	JX287573
Cluster XXIX	T75	*F. degerlachei (96.9%)*	Hatchery-reared brown trout fingerlings	M	06/2009	Kidney	HSFH	JX287574
Cluster XXX	S42	*F. glacei (98.5%)*	Hatchery-reared coho salmon fry	M	02/2004	Brain	PRSFH	JX287689
Cluster XXX	S3	*F. glacei (98.6%)*	Wild spawning walleye	S	04/2005	Kidney	Tittabawassee River	JX287708
Cluster XXXI	S87	*F. resistens (97.1%)*	Wild largemouth bass	S	08/2005	Kidney	Big Bass Lake	JX287735
Cluster XXXI	S88	*F. resistens (97.3%)*	Wild largemouth bass	S	08/2005	Kidney	Big Bass Lake	JX287736
Cluster XXXII	S2	*F. psychrolimnae (99.6%)*	Wild spawning walleye	S	04/2005	Kidney	Tittabawassee River	JX287711
Cluster XXXIII	T86	*C. viscerum (98.8%)*	Hatchery-reared muskellunge fingerlings	M	07/2009	Kidney	WLSFH	JX287757
Cluster XXXIII	T88	*C. viscerum (99.0%)*	Hatchery-reared muskellunge fingerlings	M	07/2009	Gills	WLSFH	JX287759
Cluster XXXIII	T87	*C. viscerum (99.1%)*	Hatchery-reared muskellunge	M	07/2009	Gills	WLSFH	JX287758

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			fingerlings					
Cluster XXXIV	T62	*C. ginsenosidimutans (97.7%)*	Hatchery-reared brown trout fingerlings	S	02/2009	Fins	HSFH	JX287749
Cluster XXXIV	S110	*C. ginsenosidimutans (97.8%)*	Hatchery-reared brook trout fingerlings	M	08/2008	Gills	MSFH	JX287776
Cluster XXXIV	S104	*C. ginsenosidimutans (97.9%)*	Hatchery-reared brown trout fingerlings	M	07/2007	Fins	HSFH	JX287777
Cluster XXXIV	T107	*C. ginsenosidimutans (98.0%)*	Spawning hatchery-reared brown trout broodstock	M	09/2009	Fins	OSFH	JX287760
Cluster XXXIV	T68	*C. ginsenosidimutans (98.1%)*	Hatchery-reared lake trout fingerlings	S	02/2009	Kidney	MSFH	JX287751
Cluster XXXIV	T130	*C. ginsenosidimutans (98.4%)*	Hatchery-reared brook trout fingerlings	S	03/2010	Kidney	MSFH	JX287762
Cluster XXXV	S58	*C. indoltheticum (98.7%)*	Hatchery-reared steelhead trout fingerlings	M	07/2004	Gills	TSFH	JX287783
Cluster XXXV	S60	*C. indoltheticum (98.8%)*	Hatchery-reared steelhead trout fingerlings	M	07/2004	Gills	TSFH	JX287784
Cluster XXXV	S61	*C. indoltheticum (99.0%)*	Hatchery-reared steelhead trout fingerlings	M	07/2004	Brain	TSFH	JX287785
Cluster XXXVI	T24	*C. piscium (98.3%)*	Hatchery-reared steelhead trout	M	06/2008	Gills	WLSFH	JX287744

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			fingerlings					
Cluster XXXVII	T82	*C. piscium (98.3%)*	Hatchery-reared lake herring fingerlings	M	05/2009	Kidney	WLSFH	JX287753
Cluster XXXVII	T84	*C. piscium (98.4%)*	Hatchery-reared brown trout fingerlings	M	06/2009	Gills	TSFH	JX287755
Cluster XXXVII	T83	*C. piscium (98.4%)*	Hatchery-reared lake herring fingerlings	M	05/2009	Kidney	WLSFH	JX287754
Cluster XXXVII	T31	*C. piscium (98.4%)*	Hatchery-reared steelhead trout fingerlings	S	07/2008	Kidney	WLSFH	JX287746
Cluster XXXVII	S56	*C. scophthalmum (98.6%)*	Hatchery-reared walleye fry	S	06/2004	Brain	Camp Dearborn	JX287782
Cluster XXXVIII	T115	*C. chaponense (99.1%)*	Feral spawning Chinook salmon	S	10/2009	Kidney	SRW	JX287761
Cluster XXXVIII	T60	*C. chaponense (99.1%)*	Hatchery-reared rainbow trout fingerlings	S	02/2009	Kidney	OSFH	JX287748
Cluster XXXIX	S4	*C. greenlandense (98.0%)*	Wild spawning walleye	S	04/2005	Kidney	Newaygo	JX287780
Cluster XXXIX	S25	*C. greenlandense (98.1%)*	Feral spawning steelhead trout	S	04/2007	Ulcer	LMRW	JX287778
XL	T63	*C. piscicola (99.7%)*	Hatchery-reared brown trout fingerlings	S	02/2009	Fins	HSFH	JX287750
Cluster XLI	S154	*C. vrystaatense (99.5%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287775
Cluster XLI	S9	*C. vrystaatense (99.9%)*	Wild sea lamprey	S	05/2005	Fins	Duffins Creek	JX287781
Cluster XLII	S105	*C. aquaticum (99.7%)*	Hatchery-reared	M	07/2007	Fins	HSFH	JX287772

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			brown trout fingerlings					
Unres. Group 1	T108	*F. pectinovorum (97.4%)*	Hatchery-reared brown trout yearlings	M	09/2009	Fins	OSFH	JX287592
Unres. Group 1	S117	*F. pectinovorum (97.5%)*	Wild brown trout yearlings	S	09/2008	Gills	Stanley Creek	JX287630
Unres. Group 1	S173	*F. pectinovorum (97.5%)*	Wild brook trout fingerlings	S	09/2008	Gills	Kinney Creek	JX287674
Unres. Group 1	S147	*F. pectinovorum (97.5%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287671
Unres. Group 1	S165	*F. pectinovorum (97.6%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Pond	JX287670
Unres. Group 1	S115	*F. pectinovorum (97.7%)*	Hatchery-reared brown trout fingerlings	M	08/2008	Fins	OSFH	JX287673
Unres. Group 1	S174	*F. pectinovorum (97.7%)*	Wild scuplin spp.	S	09/2008	Gills	Kinney Creek	JX287633
Unres. Group 1	S137	*F. pectinovorum (97.7%)*	Wild scuplin spp.	S	09/2008	Gills	Brundage Creek	JX287649
Unres. Group 1	S135	*F. pectinovorum (97.7%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287675
Unres. Group 1	S186	*F. pectinovorum (97.7%)*	Wild brown trout yearlings	S	09/2008	Gills	Cherry Creek	JX287672
Unres. Group 2	S168	*C. viscerum (99.1%)*	Wild brook trout yearlings	S	09/2008	Gills	Kinney Creek	JX287764
Unres. Group 2	S177	*C. viscerum (99.3%)*	Wild mottled sculpin	S	09/2008	Gills	Kinney Creek	JX287763
Unres. Group 2	S116	*C. viscerum (99.4%)*	Wild brown trout yearlings	S	09/2008	Gills	Stanley Creek	JX287768

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Unres. Group 2	S184	*C. viscerum (99.4%)*	Wild brook trout yearlings	S	09/2008	Gills	Cherry Creek	JX287773
Unres. Group 2	T39	*C. viscerum (99.6%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287747
Unres. Group 2	S150	*C. viscerum (99.7%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287765
Unres. Group 2	S125	*C. viscerum (99.7%)*	Wild mottled sculpin	S	09/2008	Gills	Stanley Creek	JX287771
Unres. Group 2	S144	*C. viscerum (99.7%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287770
Unres. Group 2	S155	*C. viscerum (99.7%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287767
Unres. Group 2	S169	*C. viscerum (99.7%)*	Wild brook trout yearlings	S	09/2008	Gills	Kinney Creek	JX287769
Unres. Group 2	S175	*C. viscerum (99.7%)*	Wild brown trout yearlings	S	09/2008	Gills	Kinney Creek	JX287766
Unres.	T28	*C. indologenes (98.1%)*	Hatchery-reared coho salmon fingerlings	M	07/2008	Kidney	PRSFH	JX287745
Unres.	S7	*C. indoltheticum (97.4%)*	Wild sea lamprey	S	05/2005	Fins	Duffins Creek	JX287779
Unres.	S63	*C. indoltheticum (99.0%)*	Hatchery-reared coho salmon fingerlings	M	08/2004	Ulcer	PRSFH	JX287786
Unres.	T72	*C. indoltheticum (99.1%)*	Hatchery-reared lake herring fingerlings	M	05/2009	Kidney	WLSFH	JX287752
Unres.	T85	*C. piscicola (96.7%)*	Hatchery-reared brown trout fingerlings	M	06/2009	Gills	TSFH	JX287756
Unres.	S107	*F. aquidurense (98.1%)*	Wild spawning walleye	S	04/2008	Gills	Little Bay de Noc	JX287655

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Unres.	T157	*F. aracananum (98.0%)*	Wild brown trout yearlings	S	06/2010	Gills	Cherry Creek	JX287622
Unres.	S162	*F. araucananum (98.1%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Pond	JX287647
Unres.	S129	*F. chungangense (97.5%)*	Wild brown trout yearlings	S	09/2008	Gills	Brundage Creek	JX287676
Unres.	S122	*F. chungbukense (97.5%)*	Wild brook trout yearlings	S	09/2008	Gills	Brundage Creek	JX287653
Unres.	S146	*F. chungbukense (97.9%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287651
Unres.	S179	*F. frigidimaris (98.1%)*	Wild mottled sculpin	S	09/2008	Gills	Kinney Creek	JX287666
Unres.	T93	*F. frigidimaris (98.3%)*	Hatchery-reared brook trout fingerlings	S	08/2009	Gills	MSFH	JX287582
Unres.	T100	*F. frigidimaris (98.7%)*	Hatchery-reared lake trout broodstock	S	08/2009	Gills	MSFH	JX287586
Unres.	T99	*F. frigidimaris (98.9%)*	Hatchery-reared lake trout broodstock	S	08/2009	Gills	MSFH	JX287585
Unres.	T54	*F. frigidmaris (97.4%)*	Spawning hatchery-reared brown trout broodstock	M	10/2008	Gills	OSFH	JX287562
Unres.	S131	*F. frigidmaris (98.8%)*	Wild brown trout yearlings	S	09/2008	Gills	Brundage Creek	JX287658
Unres.	T65	*F. hercynium (97.0%)*	Wild spawning walleye	S	04/2009	Kidney	Little Bay de Noc	JX287567

Supplementary Table 2.1 (cont'd)

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Unres.	S53	*F. hercynium (98.3%)*	Hatchery-reared brook trout fingerlings	S	Unknown	Kidney	MSFH	JX287717
Unres.	T132	*F. hercynium (98.2%)*	Wild spawning walleye	S	03/2010	Kidney	Muskegon River	JX287609
Unres.	S140	*F. hibernum (97.7%)*	Wild mottled sculpin	S	09/2008	Gills	Brundage Creek	JX287646
Unres.	T159	*F. hydatis (97.9%)*	Wild brown trout yearlings	S	06/2010	Gills	Cherry Creek	JX287624
Unres.	T57	*F. pectinovorum (97.3%)*	Feral spawning coho salmon	S	10/2008	Swim bladder	PRW	JX287564
Unres.	S109	*F. pectinovorum (98.1%)*	Hatchery-reared coho salmon fingerlings	M	07/2008	Gills	PRSFH	JX287642
Unres.	S172	*F. pectinovorum (98.4%)*	Wild brook trout fingerlings	S	09/2008	Gills	Kinney Creek	JX287644
Unres.	S160	*F. tiangeerense (96.5%)*	Wild rainbow trout yearlings	S	09/2008	Gills	Brundage Creek	JX287656
Unres.	T56	*F. tiangeerense (97.4%)*	Feral spawning coho salmon	S	10/2008	Kidney	PRW	JX287563
Unres.	S108	*C. shigense (98.6%)*	Hatchery-reared coho salmon fingerlings	M	07/2008	Gills	PRSFH	JX287774

CHAPTER THREE

EMERGENCE OF *CHRYSEOBACTERIUM* SPP. INFECTIONS IN MICHIGAN FISHES

INTRODUCTION

Members of the genus *Chryseobacterium* (Family Flavobacteriaceae) have emerged as serious fish pathogens on multiple continents (Bernardet et al. 2005). During the last three years alone, numerous novel *Chryseobacterium* spp., including *C. piscicola* (Ilardi et al. 2009), *C. chaponense (*Kämpfer et al*.* 2011), *C. viscerum* (Zamora et al. 2012a), and *C. oncorhynchi (*Zamora et al. 2012b) were described and recovered from systemically infected fishes exhibiting clinical disease signs worldwide, mirroring the rapid expansion of the genus (Kim et al. 2012). Concomitant with the emergence of fish-associated chryseobacteriosis has been increased concerns because *Chryseobacterium* spp. can cause rare yet fatal disease in humans (Bernardet et al. 2006), suggesting the zoonotic potential of these emerging pathogens. In addition, the constitutive resistance to a wide spectrum of antibiotics that is hallmark of the genus *Chryseobacterium* renders chemotherapy difficult (Bernardet et al. 2006).

Despite the increasing number of chryseobacterial disease outbreaks, reports documenting their presence in North America are almost nonexistent. Most recently, we recovered several *Chryseobacterium* spp. from diseased fishes of Michigan, USA. While a portion of the isolates were identified as *C. viscerum, C. vrystaatense, C. chaponense, C. piscicola,* and *C. aquaticum*, the majority did not match a described species and could be novel species. Therefore, the present study was initiated in order to 1) more fully characterize these novel *Chryseobacterium* spp., 2) perform in-depth polyphasic characterization on a representatives of the isolated

chryseobacteria and, 3) verify the pathogenicity of the fully characterized Michigan's *Chryseobacterium* spp. under controlled laboratory conditions.

MATERIALS AND METHODS

16S rRNA gene amplification and phylogenetic analysis. Nineteen Michigan fish-associated *Chryseobacterium* spp. isolates, which were maintained in Hsu-Shotts broth (Bullock et al. 1986) supplemented with 20% (v/v) glycerol at -80 °C, were analyzed in this study (Table 3.1). Extraction of genomic DNA and amplification of the 16S rRNA gene as detailed in Loch et al. (2011). Resultant sequences were then compared to all formally described and "candidate" *Chryseobacterium* spp. downloaded from the National Center for Biotechnology Information (NCBI, USA) and the EzTaxon-e databases (Kim et al. 2012) using the alignment tool available in the nucleotide Basic Local Alignment Search Tool (BLASTN) software. Sequence alignment and neighbor-joining analysis was performed using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 5.0), whereby evolutionary distances were calculated by the Maximum Composite Likelihood method and topology robustness was evaluated by bootstrap analysis (n=10,000 resamplings). Bootstrap (BS) values ≥70 were displayed on the resultant dendrogram.

Polyphasic characterization of representative *Chryseobacterium* spp. isolates. Seven *Chryseobacterium* spp. isolates (T86, T28, T72, T83, T31, T24, and T115) were selected based upon their association with systemic infections/mortality episodes, associations with gross pathological changes in infected hosts, and/or genetic distinctness from other *Chryseobacterium* spp. Assays for polyphasic characterization were those recommended by Bernardet et al. (2002) and included the following:

Morphological, physiological, and biochemical characterization. Isolates cultured for 24 – 48 hours at 22 °C were used during these analyses and all reagents were purchased from Remel Inc. (Lenexa, Kansas, USA) unless noted otherwise. Isolates were assayed for the Gram reaction, catalase (3% H_2O_2) and cytochrome oxidase (Pathotec test strips) activities, and the presence of a flexirubin-type pigment (3% KOH) and cell wall-associated galactosamine glycans (0.01% w/v congo red solution; Bernardet et al. 2002). Motility was assessed in sulfur-indole-motility deeps (SIM) and gliding motility via the hanging-drop technique (Bernardet et al. 2002). Additional characterization included colony morphology on cytophaga agar (CA), growth on cetrimide and nutrient agars (Sigma-Aldrich Corp., St. Louis, MO), marine agar (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ), trypticase soy agar (TSA), and MaConkey agar; growth on HSM at a pH of 5.0-10.0 in increments of 0.5; growth at 4°C, 15°C, 22°C, 37°C, and 42°C; growth on HSM at salinities ranging from 0%-5.0% in 1% increments; acid/gas from glucose and acid from sucrose (1% final concentration, phenol red broth base); triple sugar iron (TSI) reaction; hydrolysis of esculin (bile esculin agar); use of citrate as a sole carbon source (Simmon's citrate); production of indole and/or hydrogen sulfide on sulfur indole motility medium (SIM); lysis of hemoglobin (0.1% w/v) and degradation of collagen (0.1% w/v), casein (5% w/v), and elastin (0.5%) as modified from Shotts et al. (1985) using HSM as the basal medium; activity for gelatinase (Whitman 2004), phenylalanine deaminase (Sigma), and DNase; activity for alginase (5% w/v alginic acid, Sigma, in HSM), pectinase (5% w/v pectin from apple, Sigma, overlay), chitinase (5% w/v chitin from crab shells, Sigma), and carboxymethylcellulase (0.15% w/v, Sigma, overlay; all modified from Reichenbach 2006 with HSM as basal medium); activity for chondroitin sulfatase C (0.2% w/v chondroitin sulfate sodium salt from shark cartilage, Sigma, HSM basal medium) and amylase (as modified from Lin et al. 1988 using HSM as basal medium); degradation of Tween 20 and Tween 80 (1% v/v, Sigma); brown pigment production from L-Tyrosine [0.5% w/v, Sigma; modified from Pacha and Porter

(1968) using HSM as basal medium]; and degradation of agar on TSA. When HSM was used as the basal medium, gelatin or neomycin were not added. Commercially available identification galleries (i.e., API 20E, API 20NE, API ZYM, and API 50CH; BioMerieux, Inc., Durham, NC) were inoculated according to the manufacturers protocol; however, tests were incubated at 22°C and read from 24-hrs post inoculation up until 7- days, with the exception of the API ZYM, which was read at 72hrs.

Antibiotic susceptibility testing. Chryseobacterium spp. isolates were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method. Cultures grown on HSM (24-48 hr) were resuspended in sterile 0.85% saline and adjusted to an optical density (OD) of 0.5 at 600-nm in a Biowave CO8000 Cell Density Meter (WPA Inc., Cambridge, UK). Bacterial suspension (1 ml) was inoculated onto dilute Mueller-Hinton agar (Hawke and Thune 1992) without 5% calf serum in duplicate. Antibiotic-imbibed disks were placed onto the medium and plates were incubated at 22°C for 24 to 48 -hrs, at which time the zones of inhibition were measured. Antibiotics included polymyxin-B (300 iu), oxytetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), erythromycin (15 µg), ampicillin (10 µg), florfenicol (30 µg), penicillin G (10 iu), and the vibriostatic agent 0/129 (2,4-diamino,6,7-di-isopropyl pteridine;10 µg).

Fatty acid profiling. Fatty acid methyl esters (FAME) analysis was performed as described by Sasser (1990) and Bernardet et al. (2005).

Phylogenetic analyses based on near complete 16S rDNA.. PCR amplification was conducted using the universal primers 8F (5’ AGTTGATCCTGGCTCAG 3’) and 1492R (5’ ACCTTGTTACGACTT 3’; Sacchi et al. 2002) and phylogenetically analyzed as described above. However, primers 8F, 1492R, 518F (5’ TACCAGGGTATCTAATCC 3’), 800R (5’ CCAGCAGCCGCGGTAATACG 3’), and 1205F (5’ AATCATCACGGCCCTTACGC 3’) were

used for sequencing. In addition, Bayesian analysis was conducted in MrBayes 3.1.2 using the General Time Reversible (GTR). The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Results were visualized in FigTree v1.3.1.

Experimental challenge studies. Prior to determining the median lethal dose, pilot studies assessing the pathogenicity of 8 Michigan *Chryseobacterum* spp. isolates were conducted in accordance with the Michigan State University Institutional Animal Care and Use Committee.

Fish. One month post hatch Chinook salmon (*Oncorhynchus tshawytscha*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and coho salmon (*O. kisutch*) were obtained and held for a minimum of 2- months before use in experimental challenges. Fish were fed *ad lib* and maintained in aerated flow-through tanks (~400L; 12-hr photoperiod) with dechlorinated pathogen-free water at a temperature of 10 ºC$\pm$1 ºC. In addition, ~ 1.5 yr old muskellunge (*Esox masquinongy*) maintained in the authors' laboratory for >1 year were maintained in the same fashion, but were fed live, disease-free fathead minnows (*Pimephales promelas*). All tanks were cleaned daily. Prior to the experiment, subsets of all fish species were analyzed for the presence of any pathogenic bacteria, viruses, and parasites.

Determination of growth kinetics. One 48- hr old colony forming unit (cfu) from each chryseobacterial isolate was inoculated into 40 -ml Hsu-Shotts broth supplemented with 5% (v/v) horse serum and 0.02% (v/v) mineral solution of Lewin and Lounsberry (Michel et al. 1999) and incubated statically at 22 ºC. Immediately after inoculation and at 8, 24, 48, 72, 96, 120, 144, and 168 -hr post inoculation, the bacterial suspension was gently vortexed and 2 ml removed for OD determination and colony enumeration via plate counts.

Pilot experimental challenge via intraperitoneal (IP) injection. Chinook salmon (mean weight 5.8 g, SD=1.7; mean length 8.6 cm, SD=0.9), brook trout (mean weight 4.3 g, SD=1.4; mean length 7.7 cm, SD= 0.9), and brown trout (mean weight 4.0 g, SD=1.3; mean length 7.5 cm, SD= 0.7) were anesthetized in carbonate-buffered tricaine methane sulphonate (MS-222; n=5 fish of a specific species per isolate) at a concentration of 100mg L^{-1} and then each fish was IP injected with 100 µl of the aforementioned bacterial suspension. Control fish (n=5) were inoculated with 100- µl of sterile PBS. Challenged fish were immediately placed in randomly assigned, aerated flow-through tanks (70-L) at a flow rate of 1.26 L/min (5 fish per isolate per tank) and monitored for 14-days, at which time survivors were euthanized. In addition, *Chryseobacterium* spp. T28 and T86 were utilized in experimental challenges in coho salmon (mean weight 6.8 g, SD=3.4; mean length 8.9 cm, SD=1.4), and muskellunge (mean weight 30.2 g, SD=6.0; mean length 19.7 cm, SD=1.0), respectively, which were the original host species of recovery. Challenged fish were checked twice daily for morbidity/mortality and fed daily. Gross examinations were performed and bacterial re-isolation was attempted from visceral organs and brain. Representative isolates were identified via gene sequencing and phylogenetic analysis.

Estimation of median lethal dose (LD_{50}) of T68 and T28. Based upon the distinctness of T68 and T28 in phenotypic characteristics, their LD_{50} were determined according to Reed and Muench (1938). Log_{10} serial dilutions of bacterial inocula in PBS and injected IP into brook trout (10 fish per group per tank; 4 groups per isolate; mean weight 35.4 g, SD= 12.0; mean length 15.8 cm, SD= 1.8) for T68 and coho salmon (mean weight 16.9 g, SD= 4.7; mean length 12.3 cm, SD= 1.3) for T28. These two fish species were chosen because they represent the two genera from which these two isolates were originally obtained (coho salmon for T28 and lake trout for T68). Negative control fish (n=10 each) were injected IP with 100- µl of sterile

PBS. Challenged and control fish were monitored for 28- days as described above. Mortalities were immediately necropsied and attempts were made to reioslated the bacteria using HSM and CA for 7 days. Tissues of infected and control fish were fixed in phosphate-buffered 10% formalin, embedded within paraffin, sectioned at 5 -µm, stained with hematoxylin and eosin (H and E), and observed under a light microscope.

RESULTS

According to 16S rRNA gene sequence analysis, the 19 Michigan isolates were most similar (97.3-99.6%) to nine described species of *Chryseobacterium* (Table 3.1). Among these, isolates T130, T115, and T60 were 99.4-99.6% similar to *C. chaponense*, T63 was 99.6% similar to *C. piscicola*, T39 was 99.2% similar to *C. viscerum*, and T83 and T31 were 99.0% similar to *C. piscium*. The remaining 12 isolates could not be definitively speciated (Table 3.1). Phylogenetic analysis resulted in 15 of the 19 Michigan isolates forming six well-defined clusters (BS=91-99), while isolates T39, T28, T72, and T85 were unresolved (Fig. 3.1). For example, isolates T86-T88, which were 98.9% similar to *C. viscerum* according to %16S rDNA, formed a well-supported cluster (BS= 99) that was distinct from *C. viscerum* (Fig. 3.1). Similarly, isolates T62, T107, T68, and T130 formed a cluster and were distinct from their closest *Chryseobacterium* spp. relatives. In contrast, isolates T115 and T60, and isolate T63 formed well-supported clusters with their most closely related *Chryseobacterium* spp.; *C. chaponense* and *C. piscicola*, respectively (Fig. 3.1). Lastly, isolate T24 formed a cluster with three other *Chryseobacterium* spp. frequently associated with fish (Fig. 3.1), though the topology within this cluster could not be resolved conclusively (i.e., formed a polytomy).

Polyphasic characterization analyses performed for *Chryseobacterium* spp. isolates for T86, T28, T72, T83 and T31, T24, and T115 were as follows:

Sequence analysis of the near complete 16S rRNA gene found isolate T86 to be most similar to *C. viscerum* (98.9%) and *C. ureilyticum* (98.7%), and T28 to be most similar to *C. jejuense* (98.4%) and *C. indologenes* (98.4%). Isolate T72 was most similar to *C. piscium* (98.9%) and *C. indoltheticum* (98.7%), while isolates T83 and T31 were most similar to *C. piscium* (99.0%) and *C. scophthalmum* (98.7%). Lastly, T24 was most similar to *C. piscium* (98.8%) and *C. scophthalmum* (98.2%), and isolate T115 was most similar to *C. chaponense* (99.6%) and *C. jeonii* (98.8%).

Phylogenetic analyses (based upon the near complete 16S rRNA gene sequence) using Bayesian and neighbor joining methodologies showed that the topologies of the resultant dendrograms were identical at some nodes (Fig. 3.2), but Bayesian analysis predicted well-supported relationships for the most of the analyzed taxa when neighbor-joining analysis did not. For instance, while both methodologies demonstrated that isolates T83/T31 and T86 were distinct from their closest relatives (posterior probabilities of 0.97 and 0.96, respectively) and T115 as being the same as *C. chaponense* (Fig. 3.2), only the Bayesian analysis resolved the relationships of T72 and T24 and showed that they were distinct from other closely related *Chyrseobacterium* spp (posterior probabilities of 0.87; Fig. 3.2). However, the relationship of T28 to its closest relatives was unresolvable according to both methods.

Biochemical, morphological, and physiological characterizations yielded the following results: On cytophaga agar, the majority of the isolates produced colonies that were convex with entire margins, semi-translucent, and a golden yellow ranging in size from 1.0-2.5 (T86), 1.0-4.0 (T28, T83, and T31), and ~0.75-2.5 (T72) mm in diameter. Isolate T24 produced colonies that were convex with entire margins, opaque, and a pale yellow color that ranged in size from 1.0-3.5 mm in diameter, while isolate T115 produced colonies that were semi-translucent, pale yellow in

color, were convex with entire margins, and ranged in size from ~0.75-2.0 mm in diameter. The seven Michigan *Chryseobacterium* spp. isolates were non-motile, Gram negative rods (1.0-3.0μm in length) that did not contain cell wall-associated galactosamine glycans, and were able to grow on nutrient, trypticase soy, Hsu-Shotts, and cytophaga agars. All isolates grew at a pH range from 5.5 to 8.5 and at temperatures from 4°C - 22°C (T86 and T28 grew weakly at 4°C), but not at ≥37°C. The isolates grew at salinities from 0 -1%, but not at 4 - 5%. None of the isolates produced acid from glucose or sucrose, and they produced an alkaline slant with no reaction in the butt on TSI. None of the isolates produced H_2S or were agarolytic, nor did they display alginase or chitinase activities. All isolates had catalase, cytochrome oxidase, and caseinase activities, were able to utilize citrate as a sole carbon source, and produced a brown pigment in the presence of tyrosine. Additionally, none of the isolates produced acetoin, ornithine decarboxylase, lysine decarboxylase, reduced nitrate, or produced acid mannitol, inositol, sorbitol, rhamnose, melibiose, amygdalin, and arabinose on the API 20E, nor did they ferment glucose or assimilate D-glucose, L-arabinose, D-mannitol, D-mannose, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid on the API 20NE. On the API ZYM, all isolates were positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase (weak activity by T72), acid phosphatase, and Napthol-AS-BI-phosphohydrolase activities, but were negative for lipase, α-galactosidase, β-galactosidase, β-glucoronidase, α-mannosidase, and α-fucosidase activities. On the API 50CH, none of the isolates produced acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol,

potassium gluconate, potassium 2- ketogluconate, or potassium 5-ketogluconate. The remaining results were variable amongst isolates and can be found in Table 2.

Chryseobacterium sp. T86 was distinct from *C. viscerum* (Zamora et al. 2012a) in a number of characteristics, such as an ability to grow on MacConkey agar, production of DNase, esterase, and cysteine arylamidase, lack of production of α –glucosidase, and an inability to assimilate D-glucose, D-mannose, and D-maltose. Similarly, T28 was distinct from its closest relative, *C. jejuense* (Weon et al. 2008), in that it was unable to grow on MacConkey agar or at 37°C, was able to grow at a pH of 9.5 and 10.0, was unable to hydrolyze Tween 80, produced indole, pectinase, and dnase, and an inability to assimilate D-glucose, D-mannose, and D-maltose. *I*solates T72, T83, T31, and T24 were also distinct from their closest relative, *C. piscium* (de Beer et al. 2006), in that they were able to grow at 4-5% salinity, were unable to reduce nitrate or assimilate D-mannose, did not produce acid from glucose, D-maltose, or gentibiose, did not show phenylalanine deaminase activity, but did produce a brown pigment in the presence of tyrosine. Lastly, T115 was also distinct from *C. chaponense* (Kämpfer et al. 2011) in that it was grew at 37°C, utilized citrate, hydrolyzed casein, produced a brown pigment from tyrosine, and did not assimilate D-glucose, D-maltose, or D-mannose.

Antiobiotic susceptibility tests demonstrated that isolates T86, T28, T72, T83, T31, and T24 were sensitive to trimethoprim-sulfamethoxazole (16-27mm) and the vibriostatic agent O129 (21.5-34mm), but resistant to polymyxin-B (0mm), penicillin G (0mm), florfenicol (0mm), ampicillin (0-9mm), erythromycin (0-13.5mm), and oxytetracycline (0-14mm). Isolate T115 was unique in that it was resistant to trimethoprim-sulfamethoxazole (0mm) and O129 (0mm) but sensitive to penicillin (13mm). However, similar to the other isolates, it was resistant to florfenicol (0mm), erythromycin (0mm), oxytetracycline (0mm), polymyxin-B (12mm), and ampicillin (0mm).

Fatty acid acid profiles of the seven Michigan isolates revealed that *iso*-$C_{15:0}$, *iso*-$C_{17:1}$ ω9*c*, *iso*-$C_{17:0}$ 3-OH, and *iso*-$C_{15:0}$ 2-OH/$C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c* were predominant (Table 3.3), which is typical of the genus *Chryseobacterium* (Bernardet et al. 2006). These isolates also contained a variety of other fatty acids (Table 3.3). When the fatty profiles of T86 and T28 were compared to their closest relatives, clear similarities and differences were apparent. For example, T86 was comprised of a similar percentage of *iso*-$C_{13:0}$, *iso*-$C_{15:0}$, *anteiso*-$C_{15:0}$, $C_{16:0}$, *iso*-$C_{17:1}$ ω9*c*, and *iso*-$C_{15:0}$ 3-OH when compared to *C. viscerum* (Zamora et al. 2012a), but had a larger percentage of *iso*-$C_{17:0}$ 3-OH (23.4% vs. 14.7%) and did not contain any *iso*-$C_{15:0}$ 2-OH in contrast to *C. viscerum*. Similarly, T28 showed many commonalities when compared to *C. jejuense* (Weon et al. 2008; e.g., *iso*-$C_{15:0}$, *iso*-$C_{15:0}$ 3-OH, *iso*-$C_{17:0}$, and *iso*-$C_{17:0}$ 3-OH) but differed in the percentage of *iso*-$C_{16:0}$ (0% vs. 3.6%) and *iso*-$C_{17:1}$ ω9*c* (23.7% vs. 12.1%) when compared to *C. jejuense*. While T83, T31, T24, and T72 were similar to one another in fatty acid profile (Table 3.3), slight differences were observed amongst them (i.e., *anteiso*-$C_{15:0}$, *iso*-$C_{17:0}$, $C_{17:0}$ 2-OH; Table 3.3). When these four Michigan isolates were compared to *C. piscium* (de Beer et al. 2006), they were similar in percentages of $C_{16:0}$, *iso*-$C_{15:0}$ 3-OH, *iso*-$C_{17:1}$ ω9*c*, but contained a higher percentage of *iso*-$C_{15:0}$ (34.4-41.6% vs. 29.0%) and *iso*-$C_{17:0}$ 3-OH (15.5-19.3% vs. 14.0%) and a lower percentage of *anteiso*-$C_{15:0}$ (1.2-3.0% vs. 6.0%). The fatty acid profile of T115 was similar to that of *C. chaponense* (Kämpfer et al. 2011) in many regards, including percentages of $C_{13:0}$, *iso*-$C_{15:0}$

3-OH, *iso*-$C_{17:1}$ ω9*c,* *iso*-$C_{17:0}$ 3-OH, and $C_{17:0}$ 2-OH, but was also distinct in that T115 had only trace amounts of *iso*-$C_{14:0}$ and *iso*-$C_{16:1}$ H, higher percentages of *iso*-$C_{15:0}$ (29.0 vs. 23.6%) and *anteiso*-$C_{15:0}$ (23.9 vs. 19.4%), a lower percentage of *iso*-$C_{16:0}$ 3-OH (1.1 vs. 5.3%), and no *iso*-$C_{16:0}$ (versus 3.2% in *C. chaponense*).

Growth kinetic experiments showed that the *Chryseobacterium* spp. isolates were in logarithmic to late logarithmic growth after 24-hrs of static incubation at 22 °C. Thus, 18-24- hr old broth cultures were selected for use in experimental challenges. All of the *Chryseobacterium* spp. isolates were subsequently passaged in Chinook salmon (n=3 fish/isolate), reisolated on enriched Hsu-Shotts agar from kidney cultures, identified via 16S rDNA sequencing as described above and cryopreseved at -80°C. No restricted or reportable pathogens, nor any flavobacteria, were detected in the kidneys of the uninfected fish utilized in these experiments.

Fish were IP injected with $6.5 \times 10^7 - 2.6 \times 10^8$ cfu of the chryseobacterial isolates during the pilot experimental challenges. Percent cumulative mortalities varied amongst the Michigan *Chryseobacterium* spp. isolates, whereby total cumulative mortality across all fish species was highest among fish infected with T28 (55%), followed by T72 (53.3%), T86 (40%), T24 (33.3%), T31 (21.4%), T68 (20%), T83 (13.3%), and T115 (0%). No mortalities occurred in any of the mock-challenged fish throughout the course of this study, nor were any bacterial isolates recovered from negative control fish. In most cases, mortalities occurred within 7-days of injection, but occasionally death occurred between 8-10 days post-injection. *Chryseobacterium* spp. isolates were recovered from the livers, spleens, kidneys, and brains of all fish that died prior to the end of the 14-day challenge period, with the exception of one T86-infected Chinook

salmon that yielded growth from the liver, spleen and kidneys only. In all cases, bacteria recovered from experimentally challenged fish were identified as the bacterial strain that was initially injected into the fish according to 16S rDNA sequencing and phylogenetic analysis.

Gross pathological signs in fish infected with T28 included unilateral exophthalmia with periocular hemorrhage (Fig. 3.3a), gill hemorrhage and pallor (Fig. 3.3b), hemorrhage and deep ulceration of the muscle (Fig. 3.3c), ascites accumulation, swollen/enlarged/pale spleen, edema and multifocal hemorrhage of the kidney, hemorrhage of the stomach and swim bladder, distension of the stomach due to accumulation of a clear fluid, and intracranial hemorrhage (Fig. 3.3d) sometimes accompanied with hydrocephalus. In fish infected with T72, bilateral exophthalmia, diffuse external petechial hemorrhage, multifocal dermal ulceration of the trunk (Fig. 3.3e), pale hemorrhagic gills, hemorrhage of the body wall/muscle, congested/edematous/pale livers, swollen spleens, red-tinged ascites, pale edematous swollen kidneys, fluid within stomach, swim bladder hemorrhage, and intracranial hemorrhage were observed. Gross pathology in fish challenged with T86 included gill pallor, flared opercula, multifocal to coalescing ulceration and hemorrhage on the trunk, muscular hemorrhage (Fig. 3.3f), swollen/congested/pale liver, a swollen enlarged spleen, a pale ventricle, renal pallor congestion and edema, a distended hemorrhagic stomach containing a clear fluid, hemorrhagic enteritis, swim bladder hemorrhage, and intracranial hemorrhage (Fig. 3.3g).

In fish infected with T24, disease signs included perinasal and intracranial hemorrhage (Fig. 3.3h), pale gills, perioral hemorrhage, deep muscular ulcerations, unilateral/bilateral exophthalmia, diffuse hemorrhages on the ventrum, ascites accumulation, liver pallor and congestion, pale/swollen/enlarged spleens, distension of the stomach with a clear fluid and hemorrhage, swim bladder hemorrhage, and pale/hemorrhagic/edematous kidneys. Gross disease signs in T68-infected fish included hemorrhage of the isthmus, mouth, and eye,

multifocal diffuse to coalescing hemorrhagic ulcerations on the trunk, pale gills, pale/swollen/enlarged livers, swollen/enlarged spleens, red-tinged ascites, pale/swollen/edematous/hemorrhagic kidneys, swim bladder hemorrhage, and distension of the stomach. Fish infected with T31 had pale gills, unilateral exophthalmia with periocular hemorrhage, hemorrhage and ulcerations surrounding the injection site, a pale/swollen liver, a pale/swollen/enlarged spleen, a hemorrhagic swim bladder, edematous/hemorrhagic/swollen kidneys, and perirenal hemorrhage. T83-infected fish had disease signs that included exophthalmia and ocular hemorrhage, perioral hemorrhage, pale gills, congested/swollen/enlarged livers, swollen/enlarged spleens, and congested/edematous kidneys, hemorrhagic swim bladders, and pale/edematous/congested kidneys. Other than the negative control group, fish infected with T115 had the least pronounced disease signs, which included melanosis, enlarged/swollen/congested/pale livers, swollen/enlarged spleens, and mottled kidneys. Lastly, mock challenged fish showed mild splenomegaly and hepatic/renal congestion.

In median lethal dose (LD_{50}) experiments with isolate T28, an inoculum containing 4.5×10^5 - 4.5×10^8 cfu was IP injected into 10 coho salmon fingerlings per dose, while 2.0×10^6 cfu - 2.0×10^9 cfu – of isolate T68 were injected into brook trout fingerlings. In the T28 challenges, 4 coho salmon in the highest dose died within 5-days post-infection, but no other mortalities occurred in this group, nor in any of the other experimental groups, throughout the rest of the 28-day challenge period. Median lethal dose experiments with T68, which is currently being proposed as a novel species (Loch and Faisal, in preparation), did not generate any mortality in the four groups of challenged brook trout. Likewise, no mortality occurred in either of the two negative control groups in the median lethal dose experiments. In order to calculate the LD_{50}

using the methodologies of Reed and Muench (1938), cumulative mortality must be above 50% in at least one treatment group and below 50% in another. As such, it was not possible to calculate the LD_{50} for T28 or T68. However, based upon our results, the median lethal dose for each of these chryseobacterial isolates using our current challenge model exceeds 4.5×10^8 and 2.0×10^9 cfu for T28 and T68, respectively.

Chryseobacterium sp. T28 was recovered from the kidneys of all mortalities that occurred in the LD_{50} experiments, and was also recovered from the kidneys of 50% of the fish receiving the highest infectious dose that survived until the end of the 28- day challenge period. The bacterium was not recovered from any fish surviving until the end of the challenge period in the three other infectious doses (0/30), nor from any of the negative control fish (0/10). In the T68 LD_{50} experiments, the bacterium was recovered from 30% of the fish in each of the two highest infectious doses at the end of the 28 day challenge period, but was not recovered from any fish in the two treatment groups receiving the two lowest infectious doses (0/19). Once again, the bacterium was not recovered from any negative control fish.

Histopathological assessment of tissues from brook trout fingerlings challenged with 10^6 cfu of T68 (lowest challenge dose) revealed no abnormalities in the gills, skin, muscle, heart, liver, pancreas, adipose tissue, or brain; however, mild congestion of the anterior kidney and posterior kidney, as well as mild hemosiderosis within the spleen (Fig. 3.4a and b) were noted. In brook trout challenged with 10^7 cfu of isolate T68, mild hyperplastic branchitis, congestion and hemosiderosis of the spleen, mild patchy edema within the anterior kidney (Fig. 3.4c and d),

mild multifocal necrosis within the interstitum of the posterior kidney, mild hemorrhage and degeneration of myofibers lining the peritoneum, mononuclear pancreatitis (Fig. 3.4e), and a mild mononuclear epicarditis were noted. In the next highest infectious dose, multifocal proliferative branchititis that at times progressed to secondary lamellar fusion, multifocal hemorrhage within the muscle, a mild mononuclear pancreatitis, splenic congestion and hemosiderosis, edema and congestion in the anterior kidney, and necrosis of the interstitial tissue within the posterior kidney were observed. Lastly, brook trout in the highest infectious dose showed a proliferative branchitis with concurrent loss of the secondary lamellae, splenic congestion and hemosiderosis, pancreatitis, focal monocytic hepatitis, mild peritonitis, a large number of leukocytes within the atrium of the heart (Fig. 3.4f), hemorrhage within the adipose tissue, congestion and patchy edema of the anterior kidney, and congestion of the posterior kidney. No histological abnormalities other than mild splenic congestion were observed in the negative control fish.

Histological changes in coho salmon challenged with T28 were also assessed. The four mortalities that occurred in the highest infectious dose within the first five days of infection exhibited epithelial hyperplasia of the secondary lamellae and interlamellar space that resulted in secondary lamellar fusion (Fig. 3.5a and b), monocytic infiltrate and mucus cell hyperplasia within primary lamellae consistent with branchitis (Fig. 3.5c), monocytic myositis, hemorrhage within the muscle, liver, adipose tissue (Fig. 3.5d), and ovaries (Fig. 3.5e), pancreatitis, edema within the liver and interstitial tissue of the anterior kidney, renal tubular degeneration, and splenic congestion. In coho salmon surviving until the end of the 28 day challenge period in the highest infectious dose, moderate to severe proliferative branchitis, pancreatitis, spongiosis in the white matter of the brain, focal edema in the liver, renal tubular degeneration, and splenic congestion were evident. In the group challenged with the next lowest infectious dose, histological changes were similar but also included mild degeneration of the renal tubular

epithelium, as well as hyperemia of the vessels and multifocal edema within the granular cell layer of the cerebellar cortex (Fig. 3.5f). In the groups challenged with the two lowest infectious doses, microscopic changes included mild proliferative branchitis, mild epicarditis, congestion and edema within the kidneys, and hepatic/splenic congestion. Other than splenic congestion, no histological abnormalities were observed in the negative control fish.

DISCUSSION

The increasing number of reports on fish pathogenic *Chryseobacterium* spp. worldwide suggests that that group of bacteria are either emerging or have been previously misdiagnosed as more familiar, closely-related *Flavobacterium* spp. The current study is the first to document the diversity of fish pathogenic chryseobacteria in North America. For example, this study is the first to report on the presence of *C. piscicola* (i.e., T63) in North America, which was first isolated from diseased Atlantic salmon in Finland (Ilardi et al. 2009). Likewise, *C. chaponense,* a recently described fish-associated species from Chile (Kämpfer et al. 2011), and *C. viscerum ,* which was just isolated from diseased rainbow trout (*O. mykiss*) in Spain (Zamora et al. 2012a), were both identified from fish in North America for the first time (Table 3.1). In all cases, the original isolations of these novel chryseobacteria were from farmed fishes, possibly indicating that the stressors associated with aquaculture situations are necessary for chryseobacteriosis to ensue; however, it may also be that the dissemination of fish and fish products for the ever-growing aquaculture industry has played a role in their multi-continental presence. Nonetheless, this study is also the first to report the presence of *C. chaponense* and *C. viscerum* not only in captive fish in North America, but also in wild fishes.

While gene sequencing and phylogenetic analysis allowed for the identification of a portion of the Michigan fish-associated *Chryseobacterium* spp., a polyphasic approach was necessary to

better resolve the identity of the remainder of the isolates. For example, *Chryseobacterium* spp. T115 was identified as *C. chaponense* based upon genetic analysis, which was further supported using the polyphasic approach. However, some physiological and biochemical differences existed between strain T115 and the *C. chaponense* type strain reported by Kämpfer et al. (2011), which possibly suggests some divergence of these strains. While *C. chaponense* has been reportedly associated with diseases in fish, results from our experimental challenges suggested that *C. chaponense* strain T115 was avirulent to multiple salmonid species and is probably not a major threat for fish health. Also of interest was the unique antibiotic susceptibility profile of *C. chaponense* T115. While antibiotic susceptibility tests were not reported by Kämpfer et al. (2011), isolate T115 was resistant to 7 of 8 antibiotics that were tested. It is well established that *Chryseobacterium* spp. involved in human infections are highly resistant to multiple antibiotics (Kirby et al. 2004), as are chryseobacteria recovered from aquatic animals (Michel et al. 2005), but *C. chaponense* was interesting in that it was also resistant to trimethoprim-sulfamethoxazole, which has been recommended as an efficacious treatment in human infections (Chou et al. 2011).

Polyphasic characterization also allowed for a more definitive identification of other Michigan *Chryseobacterium* spp. According to near complete analysis of the 16S rRNA gene, *Chryseobacterium* sp. T72, T83, T31, and T24 were 98.8-99.0% similar to *C. piscium*, which was first isolated from fish caught from the South Atlantic Ocean in South Africa (de Beer et al. 2006). However, 98.7-99.0% has been suggested by Stackebrandt and Ebers (2006) to be a threshold above which further analyses should be carried out to delineate bacterial species. Thus, phenotypic characterization and additional phylogenetic analyses were conducted and collectively suggested that these four chryseobacterial isolates are strains of *C. piscium*, though some biochemical and physiological discrepancies were noted. As such, we believe T83/T31 represent unusual strains of *C. piscium*. Similarly, isolates T72 and T24 were distinct from

many chryseobacteria according to Bayesian analyses; thus, we believe T72 and T24 also represent unique strains of *C. piscium*.

It is interesting to note that *C. piscium* was originally considered to be a spoilage organism (de Beer et al. 2006); however, the four *C. piscium* isolates in this study were originally recovered from diseased lake herring (*Coregouns artedi*; T83 and T72), diseased steelhead (*O. mykiss*; T24), and systemically infected steel head (T31) being raised in Michigan hatcheries. Indeed, the four *C. piscium* isolates generated 13-53% cumulative mortalities in our experimental challenges and showed invasive potential by their recovery from organs of the nervous, gastrointestinal, excretory, and immune systems. Furthermore, while the severity of disease signs varied somewhat by fish species and isolate, the Michigan *C. piscium* isolates produced signs typical of a bacterial septicemia (i.e., exophthalmia, hemorrhage, edema/ascites, etc.). Thus, the recovery of *C. piscium* from multiple disease events in Michigan hatcheries in conjunction with the findings of this study, suggested that *C. piscum* is pathogenic to salmonids.

While polyphasic characterization identified 5 of the 7 Michigan isolates as previously described C*hryseobacterum* spp., the results for the remaining two isolates suggested that they may represent novel *Chryseobacterium* spp. For example, the identity of T86 was ambiguous according to its % 16S rDNA similarity, falling within the 98.7-99.0% threshold of Stackebrandt and Ebers (2006) to ascertain that a bacterium isolate is a novel species, while neighbor joining and Bayesian phylogenetic analyses using the near complete 16S rDNA sequence supported T86 as distinct from *C. viscerum*. Indeed, T86 was phenotypically distinct from *C. viscerum* in a number of enzymatic, biochemical, and fatty acid characters. Experimental challenge studies with *Chryseobacterium* sp. T86 also demonstrated the pathogenic potential of this bacterium.

T28 was distinct from its closest relatives genetically, physiologically, and biochemically despite having an unresolvable topology in both neighbor joining and Bayesian phylogenetic analyses. Furthermore, it was relatively indistinguishable from *C. jejuense* and *C. indologenes* in fatty acid profile. Thus, we were unable to ascribe isolate T28 to a defined *Chryseobacterium* sp. As such, DNA DNA hybridization studies or other epidemiological tools are needed in order to more definitively identify this bacterium. Initial experimental challenge studies with *Chryseobacterium* sp. T28 yielded cumulative mortalities of 40-60% in different salmonid species, including coho salmon, its original host. Moreover, fish infected with this bacterium showed the most severe gross signs of disease in all of the chryseobacteria examined in this study. Indeed, *in vitro* experiments demonstrated the ability of this bacterium to proteolyze a number of substrates that are constituents of the host extracellular matrix (Alberts et al. 2002). Unfortunately, it was not possible to calculate the LD_{50} for this isolate, as only a 40% cumulative mortality was achieved in our highest infectious dose. However, the median lethal dose lies somewhere above 4.5 x 10^8 cfu using our current experimental model. In this context, the ability of T28 to cause extensive proliferative branchitis in experimentally infected fish suggest that T28 is facultatively pathogenic and may require other conditions for mortalities to ensue.

Median lethal dose experiments were also carried out for *Chryseobacterium* sp. T68, which is being proposed as a novel species. Once again, it was not possible to determine the LD_{50} for this isolate, because no fish died during LD_{50} experiments. Hence, we suspect that this bacterium is only mildly pathogenic, though poor environmental conditions may facilitate an epizootic. Histological changes in brook trout challenged with *Chryseobacterium* sp. T68 were also similar to those reported by Mudarris and Austin (1992), indicating that various chryseobacterial species may produce similar signs of disease in infected fish.

The seven fish-associated *Chryseobacterium* spp. isolates recovered from Michigan fishes exhibited a striking resistance to the vast majority of the antibiotics that were tested in this study. These findings mirror the problems associated with treatment of chryseobacterial infections in humans (Kirby et al. 2004; Chou et al. 2011) and are troubling in light of the fact that the Michigan chryseobacteria are resistant to three of the four major antimicrobials approved within the United States for use in aquaculture (http://www.fws.gov/fisheries/aadap/desk-reference_introduction.htm). Thus, finding an efficacious chemotherapeutant to treat emerging fish chryseobacteriosis is necessary.

In conclusion, this study is the first to document the presence of multiple fish-associated *Chryseobacterium* spp. previously reported from Europe, Africa, and Asia, in North America. Polyphasic characterization studies highlighted their diversity, demonstrated the presence of additional multiple taxa that likely represent novel *Chryseobacterium* spp., and for the first time, confirmed their pathogenicity in controlled laboratory studies. Clearly, further studies elucidating the epizootiology and pathogenesis of the diverse assemblage of fish-pathogenic *Chryseobacterium* spp. in Michigan are needed.

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Table 3.1. The Michigan *Chryseobacterium* spp. isolates that were analyzed using 16S rDNA sequencing and phylogenetic analyses in this study.

Isolate ID	Accession Number	Closest Relative (% 16S rDNA similarity)	Sequence Length (bp)
T86	JX287899	*C. viscerum (98.9%)*	1451
T88	JX287901	*C. viscerum (98.9%)*	1374
T87	JX287900	*C. viscerum (98.9%)*	1373
T62	JX287891	*C. gregarium (97.3%)*	1380
T107	JX287902	*C. ginsenosidimutans (97.5%)*	1361
T68	JX287893	*C. ginsenosidimutans (97.8%)*	1417
T130	JX287904	*C. chaponense (99.6%)*	1362
T24	JX287886	*C. piscium (98.8%)*	1440
T82	JX287895	*C. piscium (98.7%)*	1370
T84	JX287897	*C. piscium (98.7%)*	1373
T83	JX287896	*C. piscium (99.0%)*	1437
T31	JX287888	*C. piscium (99.0%)*	1439
T115	JX287903	*C. chaponense (99.6%)*	1446
T60	JX287890	*C. chaponense (99.4%)*	1377
T63	JX287892	*C. piscicola (99.6%)*	1371
T39	JX287889	*C. viscerum (99.2%)*	1383
T28	JX287887	*C. jejuense (98.4%)*	1388
T72	JX287894	*C. piscium (98.9%)*	1447
T85	JX287898	*C. aquaticum (97.6%)*	1369

Table 3.2. Characteristics that varied among the seven Michigan fish-associated *Chryseobacterium* spp. isolates that were examined in this study; +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported. The results for uniform characteristics were discussed in the text.

Characteristic	T86	T28	T72	T83	T31	T24	T115
Flexirubin-Type Pigment	+	+	+	+	+	+	-
Esculin Hydrolysis	+	+	+	+	+	+	-
Hemoglobin Lysis	+	+	+	+	+	+	-
Growth On:							
Marine Agar	+	+	+	+	+	+	-
MacConkey Agar	+	-	-	-	-	-	-
Cytophaga Agar	+	+	+	+	+	+	+
Cetrimide Agar	+	(+)	-	-	-	-	-
pH Growth Range:							
5.0	+	+	+	+	(+)	(+)	-
9.0	+	+	+	+	(+)	(+)	-
9.5	+	+	(+)	(+)	(+)	(+)	-
10.0	+	+	(+)	(+)	(+)	(+)	-
Salinity Tolerance:							
2%	+	+	+	+	+	+	-
3%	(+)	(+)	+	(+)	(+)	(+)	-
Esculin Hydrolysis	+	+	+	+	+	+	-
Hemoglobin Lysis	+	+	+	+	+	+	-
Production of:							
Indole	+	+	+	+	+	+	-
Phenylalanine Deaminase	-	-	-	-	-	-	+
Gelatinase	+	+	+	+	+	+	-
Pectinase	+	+	-	-	-	-	+
Collagenase	+	-	-	-	-	-	-
Dnase	+	+	(+)	+	+	+	-
Elastase	+	+	+	+	+	+	-
Degradation of:							
Chondroitin sulfate	+	+	-	-	-	-	-
Starch	+	+	-	-	-	-	-
Carboxymethyl Cellulose	-	-	-	-	-	-	(+)
Tween 20	+	+	+	+	+	+	-
Tween 80	+	-	-	+	+	+	+
API 20E:							
ONPG	-	+	-	-	-	-	-
Arginine dihydrolase	(+)	(+)	+	-	(+)	-	-
TDA	+	+	+	+	+	+	-
API 20NE:							
Urease	+	+	+	-	-	+	-
Para-NitroPhenyl-BD-Galactopyranoside	-	+	-	-	-	-	-
API ZYM:							
Trypsin	+	-	-	+	+	-	-
α-chymotrypan	(+)	+	-	-	(+)	-	+
α -glucosidase	-	+	-	-	-	-	+
β-glucosidase	-	-	+	-	-	+	-
N-acetyl- β-glucosaminidase	+	+	+	+	+	+	-

Table 3.3. Cellular fatty acid profiles (%) of seven Michigan fish-associated *Chryseobacterium* spp. isolates (T86, T28, T72, T83, T31, T24, T115) using the commercial Sherlock Microbial Identification System (MIDI, version 4.0; Microbial Identification System Inc., Newark, DE). Tr, trace amounts (<1%) detected; -, not detected.

Fatty Acid	T86	T28	T72	T83	T31	T24	T115
***iso*-$C_{13:0}$**	Tr	Tr	1.4	Tr	Tr	Tr	9.8
***anteiso*-$C_{13:0}$**	-	-	-	-	-	-	4.8
***iso*-$C_{14:0}$**	-	-	-	-	-	-	Tr
$C_{14:0}$	Tr	-	Tr	-	-	-	-
***iso*-$C_{15:1}$ F**	Tr	-	-	Tr	-	Tr	Tr
***anteiso*-C $_{15:1}$ A**	-	-	-	-	-	-	Tr
***iso*-$C_{15:0}$**	36.5	33.4	41.6	39.8	38.8	34.4	29.0
***anteiso*-$C_{15:0}$**	Tr	Tr	3.0	1.2	1.2	2.7	23.9
***iso*-$C_{16:0}$**	Tr	-	-	Tr	Tr	Tr	-
***iso*-$C_{16:1}$ H**	-	-	-	-	-	-	Tr
$C_{16:1}$ ω6*c* and/or C $_{16:1}$ ω7*c*	11.1	10.7	9.8	6.4	7.5	7.5	4.2
$C_{16:1}$ ω5*c*	-	-	-	-	-	-	Tr
$C_{16:0}$	1.6	1.2	1.2	1.2	1.1	1.6	-
***iso*-$C_{15:0}$ 3-OH**	2.9	3.2	3.0	3.5	3.5	3.0	2.4
$C_{15:0}$ 2-OH	-	-	Tr	Tr	Tr	Tr	2.1
iso*-$C_{17:1}$ ω9*c	17.6	23.7	18.5	21.8	22.5	23.4	8.7
***anteiso*-C $_{17:1}$ B/iso I**	-	Tr	Tr	-	Tr	-	Tr
***iso*-$C_{17:0}$**	2.0	1.5	Tr	1.5	1.0	1.5	Tr
***iso*-$C_{16:0}$ 3-OH**	Tr	1.1	Tr	Tr	Tr	1.0	1.1
$C_{16:0}$ 3-OH	1.4	1.1	1.7	1.3	1.3	1.5	Tr
$C_{18:1}$ ω9*c*	Tr	Tr	Tr	Tr	Tr	Tr	Tr
***iso*-$C_{17:0}$ 3-OH**	23.4	22.4	15.5	19.3	19.1	18.3	9.2
$C_{17:0}$ 2-OH	Tr	Tr	1.5	1.1	1.0	2.8	2.3

Figure 3.1. Dendrogram generated using the Neigbor-joining method in MEGA5 that depicts the phylogenetic relationship between 19 *Chryseobacterium* spp. strains recovered from Michigan fishes and their most closely related *Chryseobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. *Elizabethkingia meningosepticum* and *E. miricola* served as the outgroup, and a total of 1395 positions were included in the final data set.

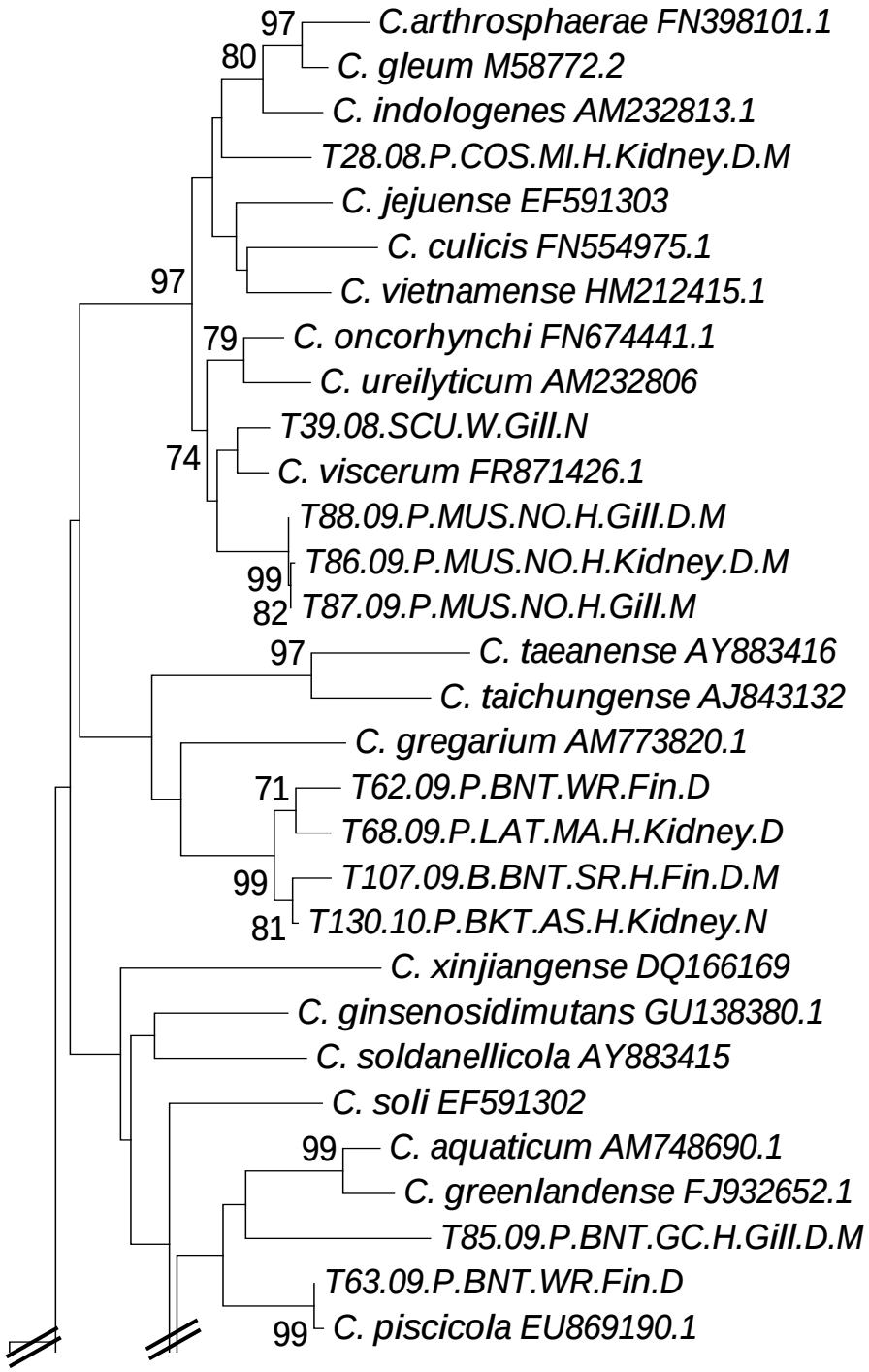


Figure 3.1 (cont'd)

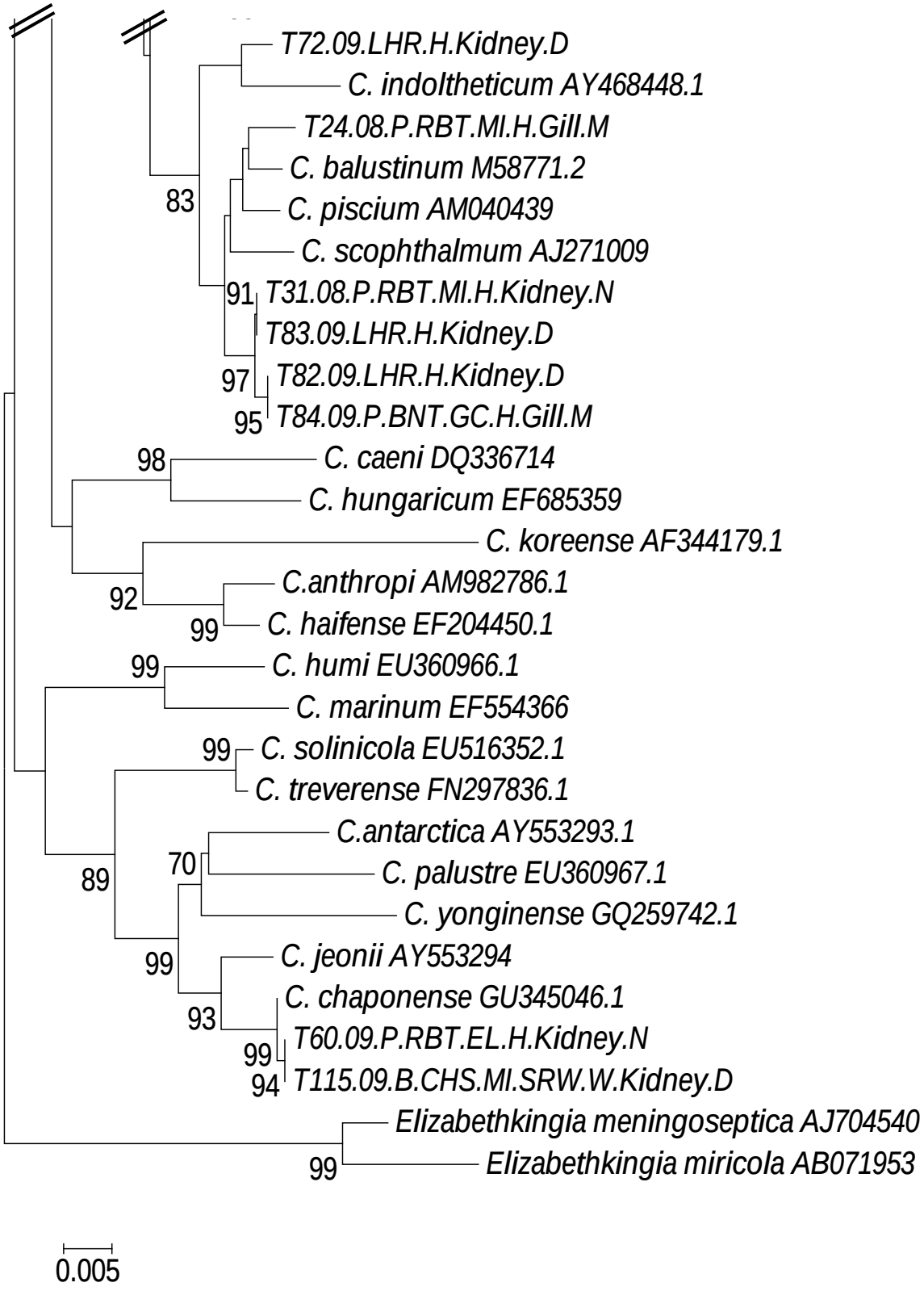


Figure 3.2. Dendrogram depicting the relationships of 7 Michigan fish-associated *Chryseobacterium* spp. (red rectangles) generated using Bayesian analysis in MrBayes 3.1.2. Filled circles are present when that node was also present in neighbor-joining analysis. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

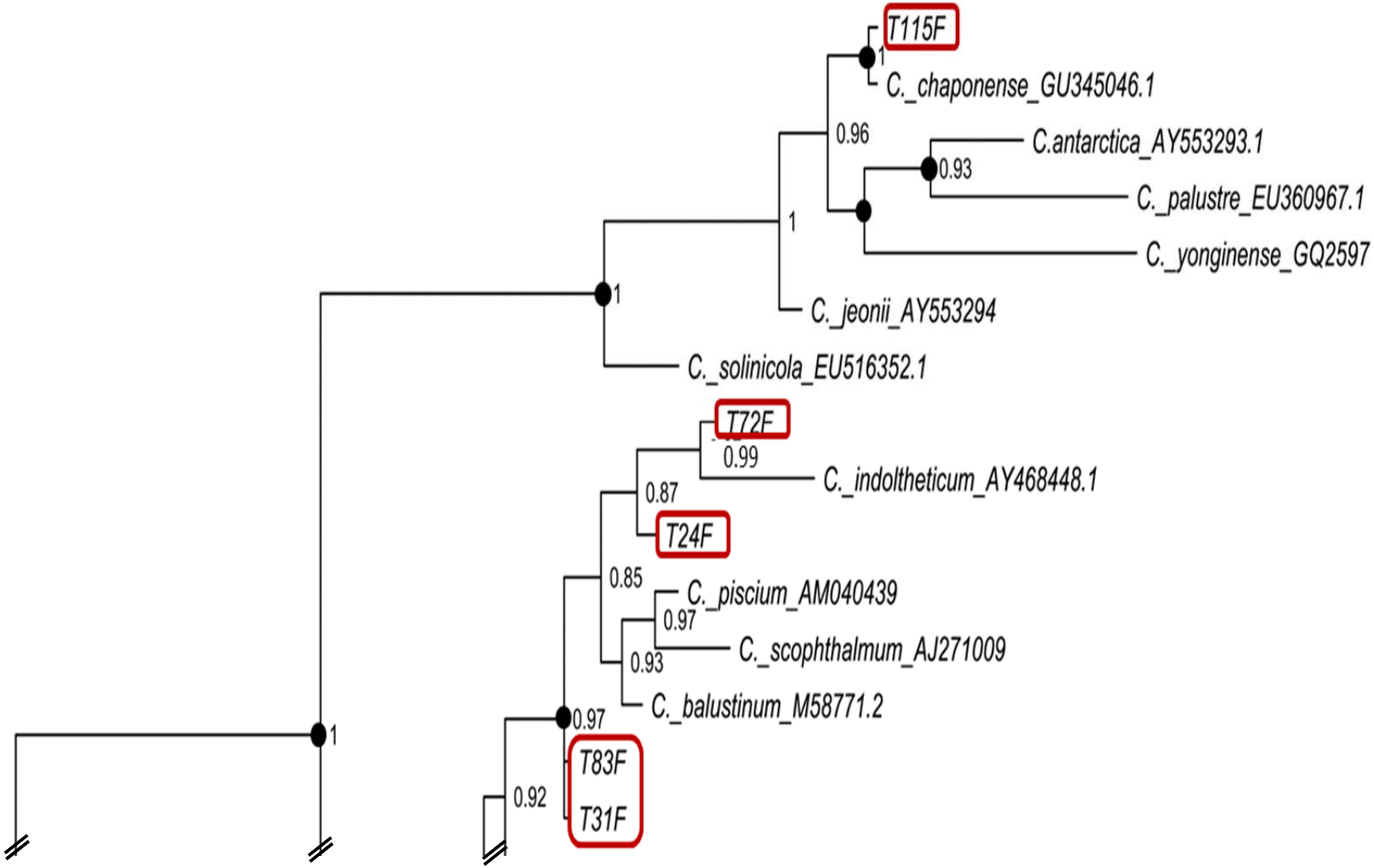


Figure 3.2 (cont'd)

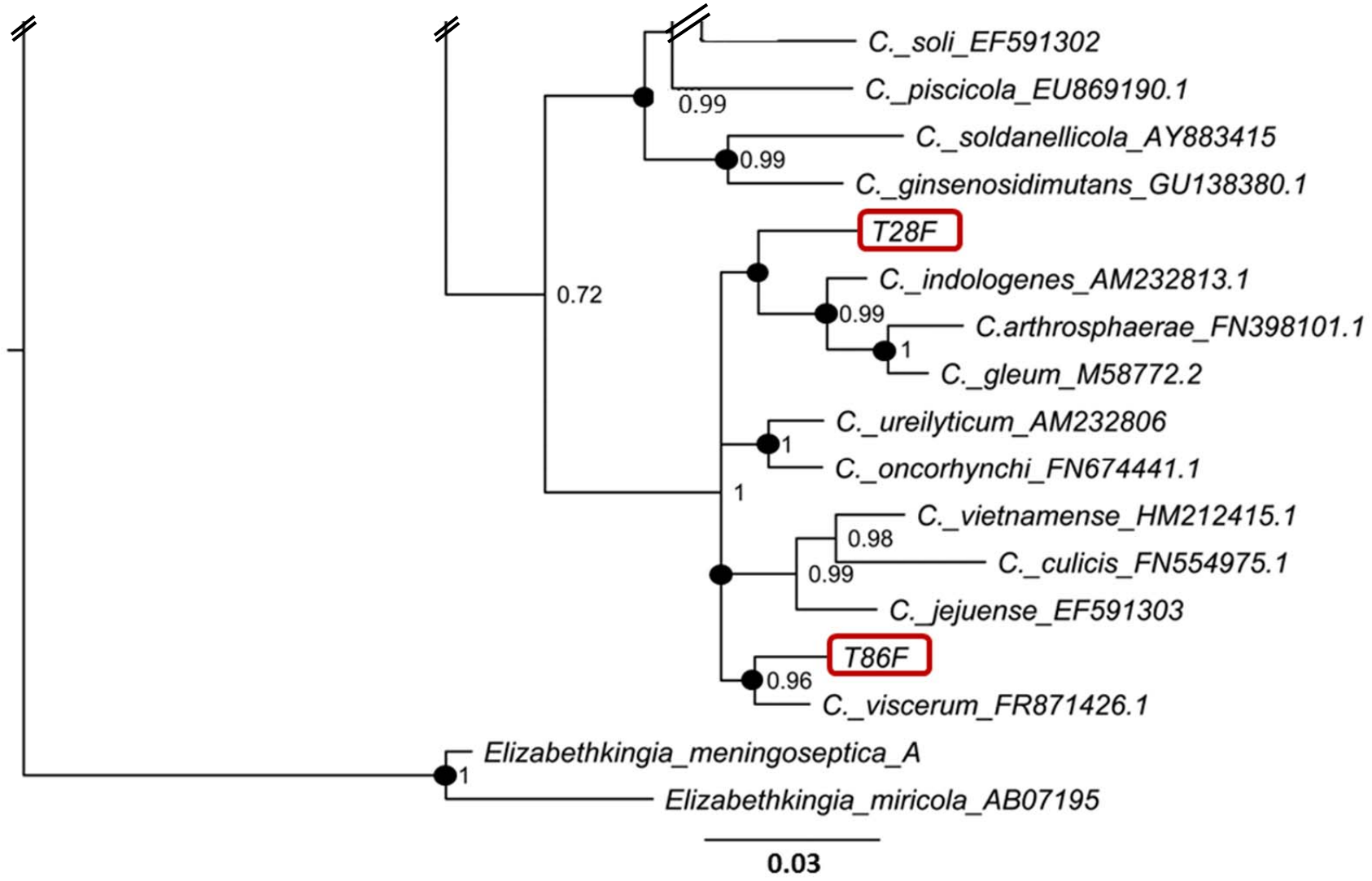


Figure 3.3. Gross lesions observed in fish intraperitoneally injected with Michigan *Chryseobacterium* spp. isolates. A) Unilateral exophthalmia and periocular hemorrhage (arrow) in a Chinook salmon fingerling infected with isolate T28. B) Pallor and multifocal hemorrhage (arrow) within the gills of a T28-infected brown trout fingerling. C) A T28-infected brown trout trout fingerling in dorsal recumbency a deep hemorrhagic ulceration (arrow) in the ventral musculature. D) Intracranial hemorrhage anterior to the optic lobes (arrow) in a T28-infected brown trout fingerling. E) Multifocal ulceration (arrows) present on the trunk and isthmus of a Chinook salmon fingerling infected with isolate T72. F) Severe ecchymotic hemorrhage (arrows) within the lateral muscle of a T86-infected Chinook salmon fingerling. G) Hemorrhage within the cranium (arrow) of a T86-infected muskellunge fingerling. H) Intracranial and perinasal hemorrhage (arrows) in a T24-infected brown trout fingerling.

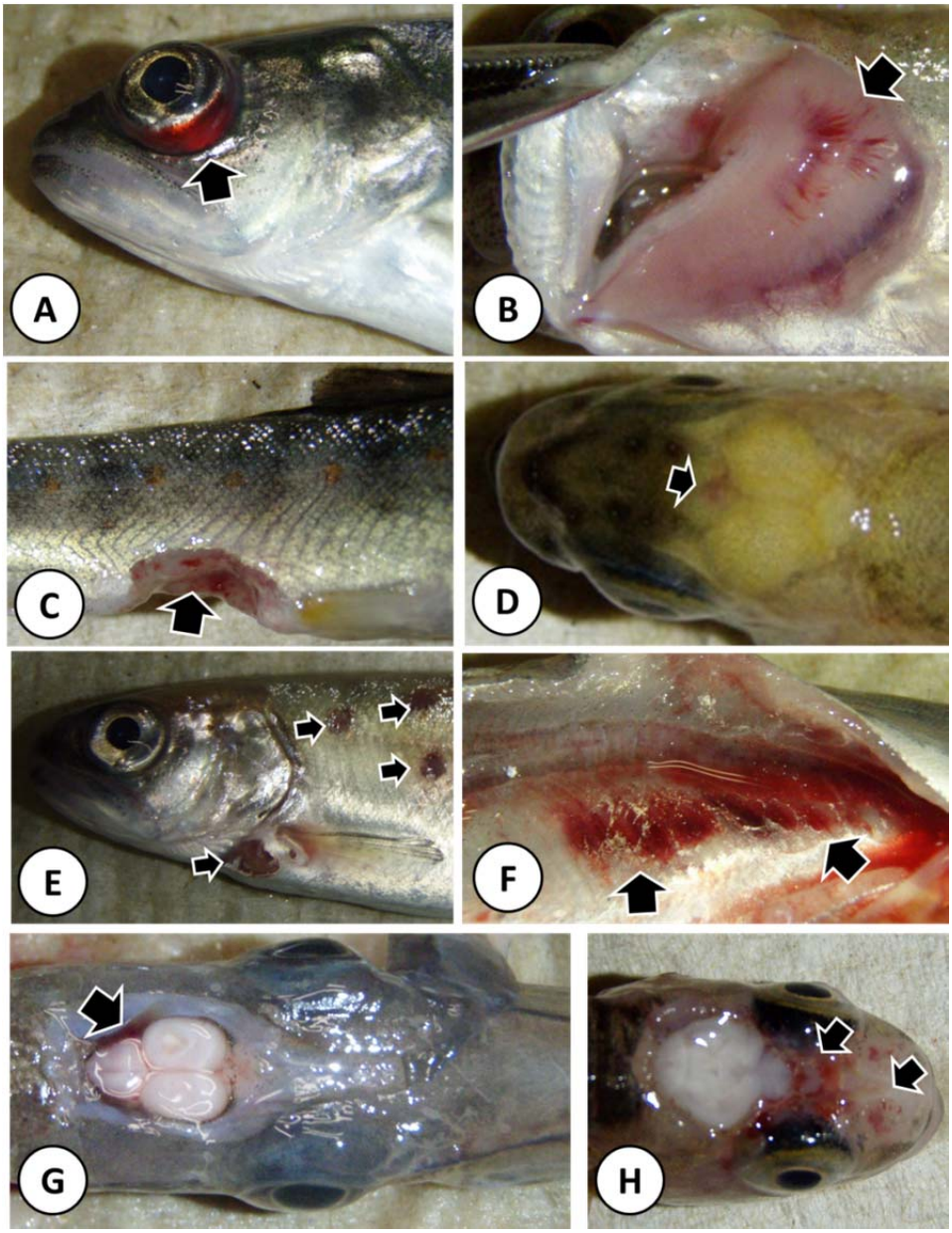


Figure 3.4. Hematoxylin and eosin (H&E) stained tissue sections from brook trout challenged (A, C, E, F) and mock challenged (B& D) with *Chryseobacterium* sp. T68. A) A spleen with hemosiderosis (arrows; 400x). B) A spleen from a mock-challenged brook trout (400x). C) Anterior kidney with focal edema (arrows; 200x) D) Anterior kidney of a mock-challenged brook trout (200x). E) Pancreatitis (arrows) in a challenged brook trout fingerling (200x). F) Atrium of the heart with a large number of leukocytes providing evidence for a peripheral leukocytosis (400x).

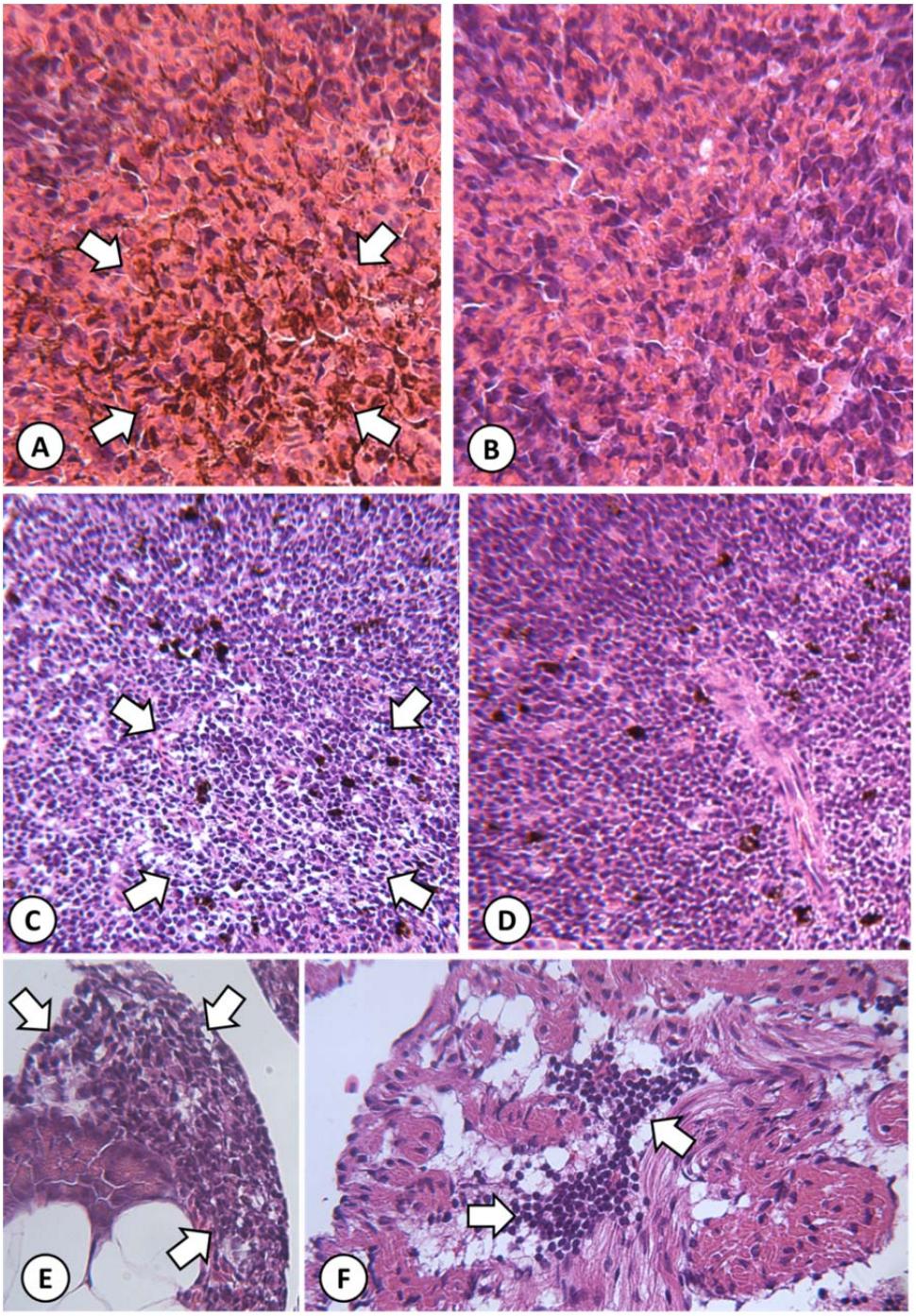
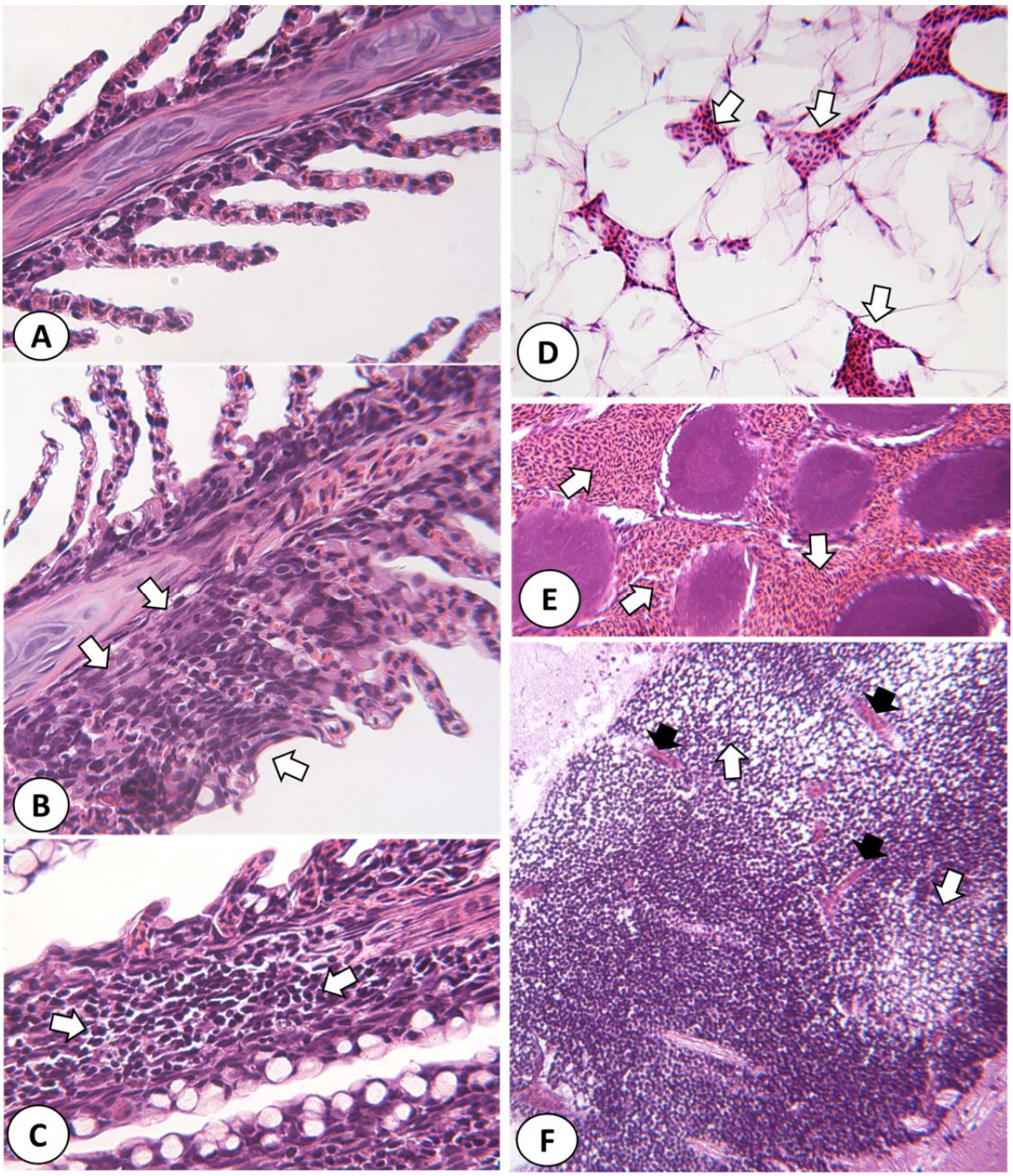


Figure 3.5. Hematoxylin and eosin stained tissue sections from coho salmon challenged (B-F) and mock challenged (A) with *Chryseobacterium* sp. T28. A) Gills showing normal secondary lamellae (400x). B) Gills showing epithelial hyperplasia of the secondary lamellae and interlamellar space (arrows; 400x). C) Primary lamella with marked monocytic infiltrate (arrows) and mucus cell hyperplasia (400x). D) Granular cell layer of the cerebellar cortex showing hyperemia of the vessels (black arrows) and multifocal edema (white arrows; 100x). E) Massive hemorrhage (arrows) within the ovaries (200x). F) Diffuse hemorrhage (arrows) within the adipose tissue (200x).



CHAPTER FOUR

DECIPHERING THE BIODIVERSITY OF FISH-PATHOGENIC *FLAVOBACTERIUM* SPP. RECOVERED FROM MICHIGAN, USA, USING A POLYPHASIC APPROACH

INTRODUCTION

Flavobacteriosis is a major disease of wild and cultured fishes worldwide that is historically attributed to three species within the genus *Flavobacterium* (Family Flavobacteriaceae); *F. psychrophilum, F. columnare,* and *F. branchiophilum* (reviewed in Shotts and Starliper 1999; Austin and Austin 2007). On occasion, other *Flavobacterium* spp. have also been recovered from diseased fishes, such as *F. johnsoniae* (Suebsing and Kim 2012), *F. succinicans* (Anderson and Ordal 1961), and *F. hydatis* (Strohl and Tait 1978). Outbreaks of flavobacteriosis manifest along a continuum of acute to chronic forms (Shotts and Starliper 1999; Austin and Austin 2007) and can be associated with considerable economic losses.

Since 2010, multiple novel *Flavobacterium* spp. have been reported from South America and Europe (i.e., *F. chilense* and *F. araucananum*, Kämpfer et al. 2012; *F. oncorhynchi,* Zamora et al 2012a). Most recently, we recovered numerous *Flavobacterium* spp. from diseased Michigan fishes. While a portion of the isolates were identified as *F. psychrophilum, F. columnare,* and *F. oncorhynchi*, the vast majority were distinct from the described *Flavobacterium* spp. and may be novel bacterial taxa. Thus, the present study was undertaken to 1) characterize these novel *Flavobacterium* spp. 2) perform in-depth polyphasic characterizations on representatives of the isolated *Flavobacterium* spp and, 3) to shed light on the potential pathogenicity of the fully characterized *Flavobacterium* spp. under controlled laboratory conditions.

MATERIALS AND METHODS

16S rRNA gene amplification and phylogenetic analysis. Ninety-nine Michigan fish-associated *Flavobacterium* spp. isolates, which were maintained in Hsu-Shotts broth (Bullock et al. 1986) or cytophaga broth (Anacker and Ordal 1955) supplemented with 20% (v/v) glycerol at -80 °C, were analyzed in this study. Extraction of genomic DNA and amplification of the 16S rRNA gene was performed as detailed in Loch et al. (2011). Resultant sequences were compared to all formally described and "candidate" *Flavobacterium* spp. that were downloaded from the National Center for Biotechnology Information (NCBI, USA) database and the EzTaxon-e database (Kim et al, 2012) using the alignment tool available in the nucleotide Basic Local Alignment Search Tool (BLASTN) software. Sequence alignment and neighbor-joining analysis was performed using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 5.0), whereby evolutionary distances were calculated by the Maximum Composite Likelihood method and topology robustness was evaluated by bootstrap analysis (n=10,000 resamplings). Bootstrap (BS) values ≥70 were displayed on the resultant dendrogram.

Polyphasic characterization of representative *Flavobacterium* spp. isolates. Six *Flavobacterium* spp. isolates (T91, T75, T18, S87, S21, and T76) were selected based upon their association with systemic infections/mortality episodes, association with gross pathological changes in infected hosts, and/or genetic distinctness from other *Flavobacterium* spp. Assays for polyphasic characterization were those recommended by Bernardet et al. (2002) and included the following:

Morphological, physiological, and biochemical characterization. Isolates cultured for 24 – 48 hours at 22 °C were used during these analyses and all reagents were purchased from Remel Inc. (Lenexa, Kansas, USA) unless noted otherwise. Isolates were assayed for the Gram

reaction, catalase (3% H_2O_2) and cytochrome oxidase (Pathotec test strips) activities, and the presence of a flexirubin-type pigment (3% KOH) and cell wall-associated galactosamine glycans (0.01% w/v congo red solution; Bernardet et al. 2002). Motility was assessed in sulfur-indole-motility deeps (SIM) and gliding motility via the hanging-drop technique (Bernardet et al. 2002). Additional characterization included colony morphology on cytophaga agar (CA), growth on cetrimide and nutrient agars (Sigma-Aldrich Corp., St. Louis, MO), marine agar (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ), trypticase soy agar (TSA), and MaConkey agar; growth on HSM at a pH of 5.0-10.0 in increments of 0.5; growth at 4°C, 15°C, 22°C, 37°C, and 42°C; growth on HSM at salinities ranging from 0%-5.0% in 1% increments; acid/gas from glucose and acid from sucrose (1% final concentration, phenol red broth base); triple sugar iron (TSI) reaction; hydrolysis of esculin (bile esculin agar); use of citrate as a sole carbon source (Simmon's citrate); production of indole and/or hydrogen sulfide on sulfur indole motility medium (SIM); lysis of hemoglobin (0.1% w/v) and degradation of collagen (0.1% w/v), casein (5% w/v), and elastin (0.5%) as modified from Shotts et al. (1985) using HSM as the basal medium; activity for gelatinase (Whitman 2004), phenylalanine deaminase (Sigma), and DNase; activity for alginase (5% w/v alginic acid, Sigma, in HSM), pectinase (5% w/v pectin from apple, Sigma, overlay), chitinase (5% w/v chitin from crab shells, Sigma), and carboxymethylcellulase (0.15% w/v, Sigma, overlay; all modified from Reichenbach 2006 with HSM as basal medium); activity for chondroitin sulfatase C (0.2% w/v chondroitin sulfate sodium salt from shark cartilage, Sigma, HSM basal medium) and amylase (as modified from Lin et al. 1988 using HSM as basal medium); degradation of Tween 20 and Tween 80 (1% v/v, Sigma); brown pigment production from L-Tyrosine [0.5% w/v, Sigma; modified from Pacha and Porter (1968) using HSM as basal medium]; and degradation of agar on TSA. When HSM was used as the basal medium, gelatin and neomycin were not added. Commercially available identification galleries (i.e., API 20E, API 20NE, API ZYM, and API 50CH; BioMerieux, Inc., Durham, NC) were inoculated according to the manufacturers protocol; however, tests were

incubated at 22°C and read from 24-hrs post inoculation up until 7- days, with the exception of the API ZYM, which was read at 72hrs.

Antibiotic susceptibility testing. Flavobacterium spp. isolates were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method. Cultures grown on HSM (24-48 hr) were resuspended in sterile 0.85% saline and adjusted to an optical density (OD) of 0.5 at 600-nm in a Biowave CO8000 Cell Density Meter (WPA Inc., Cambridge, UK). The bacterial suspension (1 ml) was inoculated onto dilute Mueller-Hinton agar (Hawke and Thune 1992) without 5% calf serum in duplicate. Antibiotic-imbibed disks were placed onto the medium and plates were incubated at 22°C for 24 to 48 -hrs, at which time the zones of inhibition were measured. Antibiotics included polymyxin-B (300 iu), oxytetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), erythromycin (15 µg), ampicillin (10 µg), florfenicol (30 µg), penicillin G (10 iu), and the vibriostatic agent 0/129 (2,4-diamino,6,7-di-isopropyl pteridine;10 µg).

Fatty acid profiling. Fatty acid methyl esters (FAME) analysis was performed as described by Sasser (1990) and Bernardet et al. (2005).

Phylogenetic analyses based on near complete 16S rDNA. PCR amplification was conducted using the universal primers 8F (5' AGTTGATCCTGGCTCAG 3') and 1492R (5' ACCTTGTTACGACTT 3'; Sacchi et al. 2002) and phylogenetically analyzed as described above. However, primers 8F, 1492R, 518F (5' TACCAGGGTATCTAATCC 3'), 800R (5' CCAGCAGCCGCGGTAATACG 3'), and 1205F (5' AATCATCACGGCCCTTACGC 3') were used for sequencing. In addition, Bayesian analysis was conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the General Time Reversible (GTR) model. The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Four independent analyses were

conducted, with the initial 25% of Markov Chain Monte Carlo (MCMC) samples being discarded as burnin and sampling occurring every 1000 generations. Results from Bayesian analyses were visualized in FigTree v1.3.1.

Experimental challenge studies. In order to assess the pathogenicity of the six Michigan *Flavobacterium* spp. isolates, the following experiments were conducted in accordance with the Michigan State University Institutional Animal Care and Use Committee:

Fish. One month post hatch Chinook salmon (*Oncorhynchus tshawytscha*), brook trout (*Salvelinus fontinalis*), and brown trout (*Salmo trutta*) were obtained and held for a minimum of 2- months before use in experimental challenges. Fish were fed *ad libitum* and maintained in aerated flow-through tanks (~400L; 12-hr photoperiod) with dechlorinated pathogen-free water at a temperature of 10 ºC$\pm$1 ºC. All tanks were cleaned daily. Prior to the experiment, subsets of all fish species were analyzed for the presence of any pathogenic bacteria, viruses, and parasites (AFS-FHS 2010).

Determination of growth kinetics. One 48- hr old cfu from each flavobacterial isolate was inoculated into 40- ml Hsu-Shotts broth supplemented with 5% (v/v) horse serum and 0.02% (v/v) mineral solution of Lewin and Lounsberry (Michel et al. 1999) and incubated statically at 22 ºC. Immediately after inoculation (Time 0) and at 8, 24, 48, 72, 96, 120, 144, and 168- hr post inoculation, the bacterial suspension was gently vortexed and 2 ml removed for optical density (OD) determination at 600- nm in a Biowave CO8000 Cell Density Meter and colony enumeration via plate counts.

Experimental challenge via intraperitoneal (IP) injection. Prior to experimental challenge, each *Flavobacterium* spp. isolate was passaged in Chinook salmon. Briefly, ~10^8 cfu were injected intraperitoneally (IP) into three Chinook salmon per isolate as described below. Fish were then euthanized 3 days post-infection and bacterial cultures taken from the kidneys on enriched Hsu-Shotts agar. Bacterial growth was then subcultured, purity verified, and freezer stocks were prepared. In addition, recovered isolates were identified via gene sequencing and phylogenetic analysis.

Chinook salmon (mean weight 14.0 g, SD=6.0; mean length 11.5 cm, SD=1.9), brook trout (mean weight 11.3 g, SD=4.2; mean length 10.9 cm, SD= 1.5), and brown trout (mean weight 3.1 g, SD=0.9; mean length 7.0 cm, SD= 1.5) were first anesthetized in carbonate-buffered tricaine methanosulphonate (MS-222; n=5 per isolate per fish species) at a concentration of 100mg L^{-1} and then IP injected with 100- µl of the aforementioned bacterial suspension. Control fish (n=5) were injected with 100- µl of sterile PBS. Challenged fish were immediately placed in randomly assigned, well aerated flow-through PVC tanks (70- L) receiving 1.26 L/min and monitored for 14-days, at which time survivors were euthanized. Challenged fish were checked twice daily for morbidity/mortality and fed daily. In the event that severe signs of morbidity were observed, the affected fish was euthanized with an overdose of MS-222 and immediately necropsied. Gross examinations were performed on all fish, and liver, spleen, kidney, and brain samples were collected and inoculated directly onto enriched Hsu-Shotts medium (incubated at 22 ºC) and CA (incubated at 15 ºC) plates and incubated for up to 7-d. Representative isolates recovered from challenged fish in each experiment were identified via gene sequencing and phylogenetic analysis as described previously to confirm their original identities.

RESULTS

According to 16S rRNA gene sequence analysis (sequence length ranging from 1337-1380 bp), 7 of the Michigan *Flavobacterium* spp. isolates were 99.3-99.9% similar to *F. oncorhynchi*, 14 were 99.1-99.7% similar to *F. psychrophilum*, 10 were 99.6-99.8% similar to *F. columnare*, 2 were 99.1-99.7% similar to *F. frigidimaris*, and 1 was 99.1% similar to *F. tiangeerense* (Table 4.1). The remaining 65 *Flavobacterium* spp. isolates could not be speciated conclusively (Table 4.1). Phylogenetic analysis of all Michigan isolates yielded 19 well-supported clusters (i.e., bootstrap value ≥70) comprised of 84 Michigan *Flavobacterium* spp., while15 taxa were unresolved (Fig. 4.1). Michigan *F. psychrophilum* isolates displayed a degree of genetic heterogeneity, as evidenced by two well-supported clusters (bootstrap value of 100); one that consisted of the *F. psychrophilum* reference sequence and isolate T122, and the other that contained the other Michigan *F. psychrophilum* isolates (Figs. 4.1 and 4.2). Michigan *F. columnare* isolates fell into one cluster, along with the *F. columnare* reference sequence (Figs. 4.1 and 4.3), while seven isolates that were identified as *F. oncorhynchi* also formed a well-supported cluster with the *F. oncorhynchi* reference sequence, though genetic heterogeneity was observed in the form of 3 distinct and well supported sub-clades (Figs. 4.1 and 44.5.4). Other Michigan isolates definitively identified as described *Flavobacterium* spp. include T33, which clustered with *F. frigidimaris*, and T105, which clustered with *F. tiangeerense* (Fig. 4.1). The remaining Michigan *Flavobacterium* spp. isolates formed well-supported clusters that were either distinct from the *Flavobacterium* spp. reference strains or yielded unresolved topologies (Fig. 4.1). For example, isolates T164, T25, T165, T103, T95, T59, T23, and T96 were distinct from, yet most closely related to, *F. oncorhynchi* (Figs. 4.1 and 4.4), while isolates T14, T16, T13, S12, T123, and T124 formed a cluster that was distinct (bootstrap value= 99) from all other *Flavobacterium* spp. (Fig. 4.1). Similarly, isolates T18, T21, T101, T102, T151, T71, T66, T70, and T73 clustered apart from all other *Flavobacterium* spp, though intra-clade heterogeneity

was also observed (Figs. 4.1 and 4.5). Isolates T131, T76, and T77, S21, T1, and T17 were also distinct from other flavobacteria (Fig. 4.1). In addition, isolates T74 and T75 shared a well-supported most recent common ancestor with *F. frigidarium* yet they were genetically distinct, as was also the case for isolates T91 and T92 *F. anhuiense* (Fig. 4.1). The remaining seven clusters, as well as the unresolved taxa, are displayed in Fig. 4.1.

Results from the polyphasic characterization analyses performed on isolates T91, T75, T18, S87, S21, and T76 were as follows:

Sequence analysis of the near complete 16S rRNA gene found isolate T91 to be most similar to *F. anhuiense* (98.2%) and *F. ginsenosidimutans* (97.8%), while T75 was most similar to *F. tiangeerense* (97.5%) and *F. frigidarium* (97.4%). Isolate T18 was most similar to *F. hydatis* (98.7%) and *F. oncorhynchi* (98.2%), while S87 was most similar to *F. resistens* (97.9%) and *F. oncorhynchi* (97.8%). Lastly, S21 was most similar to *F. aquidurense* (98.1%) and *F. frigidimaris* (98.0%), while T76 was most similar to *F. pectinovorum* (98.4%) and *F. hydatis* (98.1%).

Phylogenetic analyses (based upon the near complete 16S rRNA gene sequence) using Bayesian and neighbor joining methodologies showed that the topologies of the resultant dendrograms were identical at some nodes (depicted in Figs. 4.6 and 4.7 as nodes with a black circle), but Bayesian analysis was able to predict well-supported relationships for most of the Michigan fish-associated taxa when neighbor-joining analysis could not. For example, *Flavobacterium* spp. T75 and T91 fell into clades with bootstrap values < 70, but were supported as being distinct according to Bayesian analysis (posterior probabilities of 0.79 and 0.75 respectively; Fig. 4.6). Similarly, *Flavobacterium* sp. T18 was well-supported as being distinct (posterior probability of 0.92; Fig. 4.7), as was also the case for isolate S87 (posterior

probability of 0.95; Fig. 4.7). However, while *Flavobacterium* spp. T76 and S21were supported as sharing a most recent common ancestor with one another and with *F. tiangeerense* and *F. xueshanense*, their topology remained unresolved (Fig. 4.7).

When grown on CA, T91, T18, S87, S21, and T76 produced colonies that were yellow, semi-translucent, and nearly flat with irregular spreading margins, while T75 produced colonies were yellow, semi-translucent, and low convex with entire margins. All six isolates were Gram negative rods that were nonmotile in SIM deeps, but 5 of 6 were motile via gliding. Only T75 was unable to glide. All isolates possessed a flexirubin-type pigment and grew on TSA (T75 with weak growth), nutrient agar, and HSM, but did not grow on MacConkey and cetrimide agars. The six isolates grew at a pH from 5.5-9.0 (T75 grew weakly from 8.5-9.0 and T18 grew weakly at 9.0), from 4-22 °C but not at 42 °C, and at salinities of 0-1% but not at 3-5%. All six isolates produced an alkaline slant and no reaction in the butt in TSI without hydrogen sulfide or gas, hydrolyzed esculin (weakly for T18), and utilized citrate as a sole carbon source. None of the isolates produced cytochrome oxidase, indole, alginase, collagenase, chitinase, or carboxymethyl cellulase, nor did they degrade agar or Tween 80. However, all six isolates produced catalase, caseinase, pectinase, and amylase. On the API 20E, none of the isolates had arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, or tryptophan deaminase activities, nor did they produce acid from mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, or arabinose. On the API 20NE, the isolates degraded para-nitrophenyl-BD-galactopyranoside and assimilated D-mannose and D-glucose, while none were able to assimilate D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, or phenylacetic acid. On the API ZYM, all isolates were positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, Napthol-AS-BI-phosphohydrolase, α –glucosidase, and N-acetyl- β-glucosaminidase activities, but did not show lipase or α-mannosidase activities. On the API 50CH (using CHB/E medium), none of

the isolates produced acid from glycerol, erythritol, D-ribose, L-xylose, D-adonitol, Methyl-βD-Xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, D-melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2- ketogluconate, or potassium 5-ketogluconate. The remaining results that were variable amongst the six Michigan *Flavobacterium* spp. are presented in Table 4.2. Antibiotic sensitivity testing based upon the disk diffusion method revealed that all 6 Michigan flavobacterial isolates were sensitive to florfenicol, 5 of 6 were resistant to trimethoprim-sulfamethoxazole, polymyxin-B, P, and penicillin G, while sensitivities to the remaining antibiotics varied amongst the isolates (Table 4.3).

As displayed in Supp. Table 4.1, *Flavobacterium* sp. T91 was distinct from *F. anhuiense* (Liu et al. 2008) in a number of biochemical and physiological characteristics. Similarly, T75 was distinct from its closest relative, *F. tiangeerense* (Xin et al. 2009), in multiple characteristics (Supp. Table 4.2), as was also the case for T18 when compared to *F. hydatis* (Strohl and Tait 1978; Bernardet and Bowman 2011; Supp. Table 4.3). Likewise, S87 was phenotypically distinct from *F. resistens* (Ryu et al. 2008; Supp. Table 4.4), while S21 was also distinct from *F. aquidurense* (Cousin et al. 2007; Supp. Table 4.5). Lastly, *Flavobacterium* sp. T76 stood apart from *F. pectinovorum* (Dorey 1959; Bernardet and Bowman 2011) in a number of physiological and biochemical attributes (Supp. Table 4.6).

The major fatty acid constituents of *Flavobacterium* spp. include *iso*-$C_{15:0}$, $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*, $C_{15:0}$, *iso*-$C_{17:0}$ 3-OH, *iso*-$C_{15:0}$ 3-OH, $C_{15:1}$ ω6*c*, *iso*-$C_{16:0}$ 3-OH, *iso*-$C_{15:1}$ G, *iso*-$C_{15:0}$ 2-OH, and *anteiso*-$C_{15:0}$ (Bernardet and Bowman 2011), of which all except $C_{15:0}$ and *iso*-$C_{15:0}$ 2-OH were detected in the 6 Michigan *Flavobacterium* sp. isolates (Table 4.4). In

addition, the 6 isolates contained relatively large amounts of $C_{16:0}$, $C_{16:0}$ 3-OH, and *iso*-$C_{17:1}$ ω9*c*, but only trace amounts of $C_{18:1}$ ω9*c*, $C_{15:0}$ 2-OH, and *iso*-$C_{13:0}$. Additional fatty acids that were present only in a portion of the isolates are also presented in Table 4.4. As was the case for biochemical characterization analyses, when the fatty acid profiles of the six Michigan isolates were compared to their closest relatives, a number of discrepancies were apparent (Supp. Tables 4.1 – 4.6).

Growth kinetic experiments revealed that the six flavobacterial isolates were in a logarithmic to late-logarithmic growth phase at ~24 hrs post-inoculation at 22 °C. Hence, 18-24- hr broth cultures were selected for use in experimental challenges. Analysis performed on experimental fish prior to their use showed the absence of flavobacteria and other pathogens in their internal organs.

The percent cumulative mortality resulting from each of the experimental challenges, during which fish were IP injected with $3.7 \times 10^7 – 7.0 \times 10^8$ cfu, varied between 0 - 40% amongst the isolates. Cumulative mortality was highest in fish infected with isolate S21 (20%, 40%, and 20% in Chinook salmon, brook trout, and brown trout, respectively), followed by isolate T76 (40% in brown trout) and isolate T91 (20% in brown trout), whereby mortalities occurred between 1 - 4 d post-infection. No mortalities occurred in fish challenged with T18, T75, or S87, or in the control fish. Among fish mortalities, flavobacteria were recovered from the livers, spleens, kidneys, and brains of the infected fish. In fish surviving until 14 d PI, isolates T91, S21, and T76 were recovered from all four organs in a portion of the challenged fish, while isolate S87 was recovered from the livers, spleens, and kidneys only. However, T75 was recovered from one kidney of a challenged brown trout, and T18 was not recovered from any organs of the 15

challenged fish. In all cases, bacteria recovered from experimentally challenged fish were identified as the original bacterial strain utilized in the injection according to 16S rDNA sequencing and phylogenetic analysis. *Flavobacterium* spp. were not recovered from any control fish during the course of this study.

Gross pathology in fish challenged with S21 included unilateral exophthalmia and concurrent periocular hemorrhage (Fig. 4.8a), mottled external coloration, gill pallor, flared opercula, swelling/pallor/congestion/friability of the liver, swollen and enlarged spleen, swim bladder hemorrhage (Fig. 4.8b), swelling/edema/congestion/pallor of the kidney, and focal petechial hemorrhage within the brain. Pathological changes in fish challenged with T76 included gill pallor, mottled external coloration, hepatic congestion/hemorrhage, fibrinous adhesions from the liver to the body wall, swollen/enlarged spleens, and swollen/mottled/congested/edematous kidneys. In fish challenged with T91, disease signs included gill pallor, bilateral exophthalmia, muscular ulceration and ecchymotic hemorrhage, hepatic swelling/congestion/enlargement, swelling of the spleen, swim bladder edema, and renal congestion and edema. In T75-infected fish, signs of disease included, gill pallor, swollen, congested, hemorrhagic livers, swollen and enlarged spleens, and renal congestion. Pathological changes observed in fish challenged with T18 included gill pallor, pale and congested livers, swollen and enlarged spleens, and pale swollen congested edematous kidneys. Lastly, in fish challenged with S87, observed signs of disease included gill pallor, congested and pale livers, swollen and enlarged spleens (Fig. 4.8c), multifocal ecchymotic hemorrhage in the swim bladder, swollen, congested, pale kidneys, and intracranial hemorrhage (Fig. 4.8d).

DISCUSSION

The results from this study not only illustrate the diversity of *Flavobacterium* spp. associated with diseased fishes in Michigan, but also demonstrate that a large proportion likely represent novel flavobacterial taxa. For example, phylogenetic analysis demonstrated that Michigan *Flavobacterium* spp. isolates belonging to clusters XVIII, XXVII, X, IV, XXII, III, VIII, II, XVI, XI, XXIII, and XXIX formed well-supported clades that were distinct from all other described *Flavobacterium* spp. Furthermore, sequence analysis showed that isolates within these clades were ≤ 98.7% similar to all described *Flavobacterium* spp. sequences. Indeed, a degree of 16S rDNA sequence similarity up to 98.7% can occur between distinct *Flavobacterium* spp. (Bernardet and Bowman 2006), while the threshold for the delineation of a bacterial species is 98.7-99.0% (Stackebrandt and Ebers 2006). Hence, the 16S rDNA sequence and phylogenetic analyses of this study provided strong evidence that a large number of novel *Flavobacterium* spp. are associated with diseased fish in Michigan.

Indeed, polyphasic characterization showed that at least 5 out of 6 Michigan *Flavobacterium* spp. isolates represented novel flavobacterial taxa. For example, T91 (Cluster XXVII), T75 (Cluster XXIX), S87 (Cluster XXXI), S21 (Cluster XI), and T76 (Cluster VIII) had % 16S rDNA similarities below the 98.7-99.0% threshold recommended for delineation of a species (Stackebrandt and Ebers 2006), and isolate T18 was at the threshold (98.7%). In addition, five of the six formed well-supported (bootstrap values of 83-100) clades that were distinct from all other described *Flavobacterium* spp., while isolate S87 had an unresolved topology (Figs. 1 and 5). However, further phylogenetic analyses using Bayesian methodologies and a longer stretch of the 16S rRNA gene found that the six *Flavobacterium* spp. isolates were distinct from their closest relatives, though S21 and T76 formed a polytomy with one another (Fig. 7). Moreover, all six Michigan isolates were distinct from their closest relatives in multiple biochemical and

physiological characteristics, while also having cellular fatty acid profiles that placed them within the genus *Flavobacterium* (Bernardet and Bowman 2011) and yet were dissimilar from their closest relatives. According to the minimal standards for describing novel members of the family Flavobacteriaceae recommended by Bernardet et al. (2002), DNA-DNA hybridization experiments should also be performed when a taxon is ≥97.0% similar (according to Stackebrandt and Goebel 1994), whereby the proposed novel taxon and it's 2-3 closest relatives should have ≤ 70% reassociation similarity values. However, Stackebrandt and Ebers (2006) evaluated the major hybridization techniques that were performed on a large number of prokaryotes among many different phyla and found that DNA-DNA hybridization should only be performed when %16S rDNA similarity is ≥ 98.7%. Because the 97.0% threshold for performing DNA-DNA hybridization experiments recommended in (Bernardet et al. 2002) was based upon the recommendation of Stackebrandt and Goebel (1994), which was then revised by the original author (Stackebrandt and Ebers 2006), no DNA-DNA hybridization experiments were carried out in this study.

Another noteworthy finding of this study is the large number of Michigan *Flavobacterium* spp. isolates that were recovered from systemically infected fishes. While it is well known that some *Flavobacterium* spp., such as *F. psychrophilum*, *F. columnare*, and *F. branchiophilum,* negatively impact both wild and cultured fishes (Shotts and Starliper 1999; Austin and Austin 2007; Bernardet and Bowman 2006), the etiology of systemic disease caused by other *Flavobacterium* spp. is less well-known. Only on rare occasions have other *Flavobacterium* spp. been implicated to cause fish diseases, and most often these outbreaks were associated with external lesions rather than systemic bacterial septicemia. For instance, *F. succinicans* (Anderson ad Ordal 1961), *F. johnsoniae* (Christensen 1977; Carson et al, 1993; Rintamäki-Kinnunen 1997), *F. hydatis* (Strohl and Tait 1978), and other uncharacterized flavobacteria (Borg 1960; Anderson and Conroy 1969; Lien 1988; Holliman et al. 1991) were recovered from

the external lesions of diseased freshwater fish. However, reports of “less well-known” and novel *Flavobacterium* spp. being recovered from the internal organs of fish in Africa (Flemming et al. 2007), Asia (Suebsing and Kim 2012; Karatas et al. 2010), Europe (Zamora et al. 2012a), and South America (Kämpfer et al. 2012) are beginning to surface. Thus, the findings of this study, in conjunction with the findings of the aforementioned studies, illustrate that a much more diverse assemblage of flavobacteria are capable of systemically infecting fish. Hence, *F. psychrophilum, F. columnare,* and *F. branchiophilum* are not the only flavobacteria that are problematic for fish health. Unfortunately, without adequate baseline data documenting the presence of these novel/previously uncharacterized fish-associated flavobacteria, it is not possible to determine if the apparent increase in reports of systemically infected fish from around the world represent an emergence of these pathogens, whether the significant improvements in molecular techniques are only now giving us adequate resolution to better delineate members of this genus, or whether some flavobacteria have been occasionally misidentified as the more typical fish-pathogenic flavobacteria. Likewise, it is unknown if the novel *Flavobacterium* spp. isolates described in this study are naturally present within the Great Lakes basin, or if they were introduced into this region. Indeed, international trade has been incriminated as a source for multiple invasive species that have been introduced into the Great Lakes (Faisal, 2007), while the importation of salmonids (e.g., Chinook salmon, brown trout, etc.) from the Pacific Northwest and Europe into the Great Lakes to establish a sport fishery could be another potential source. Nevertheless, the current rapid increase in aquaculture to produce fish for both conservation and food production will necessitate an even better understanding of flavobacterial fish-pathogens, obligate and facultative alike.

Flavobacterium sp. S21, originally recovered from a mortality event in hatchery-reared rainbow trout fingerlings, produced cumulative mortalities ranging from 20-40% in three salmonid species and resulted in the most severe signs of disease among all 6 *Flavobacterium* spp.

isolates utilized in these experimental challenges. Gross disease signs in naturally infected fish included enophthalmia, deep muscular ulceration, gill pallor, splenomegaly, and a swollen/pale/mottled liver (data not shown), while signs observed in experimentally infected fish included gross changes to the eyes, gills, spleen, and liver, as well as hemorrhaging within the brain and deterioration of the kidney. The bacterium was readily recoverable, in a pure form, from multiple internal organs of infected fish, indicating that a widespread infection involving multiple tissue systems (i.e., gastrointestinal, nervous, excretory, hematopoietic) had ensued. This bacterium was also recovered from the kidneys of all challenged fish, including those that survived until the end of the challenge period. Thus, these results provide strong evidence in support of *Flavobacterium* sp. S21 being pathogenic to Great Lakes salmonids under laboratory conditions.

However, it must be stated that experimental infections conducted via IP injection do not reproduce a natural infection route. Still, experimental challenge models using immersion, oral/anal intubation, and cohabitation routes of exposure for the well-known fish-pathogenic *Flavobacterium* spp. are rife with reproducibility problems despite being extensively studied (Holt 1987; Rangdale 1995; Decostere et al. 2000; Madetoja et al. 2000). In contrast, using an IP route of exposure produced reproducible results for *F. psychrophilum* challenges (Madsen and Dalsgaard 1999), while supplementing the experimental inoculum with horse serum and trace elements also reduced experimental variability (Michel et al. 1999). It is also noteworthy that, despite the circumvention of portions of the immune system during our IP infections, no mortalities occurred in any challenge involving *Flavobacterium* spp. isolates T75, T18, or S87. While it is possible that our experimental conditions may not have reproduced what is necessary for disease to ensue with these isolates, the fact that T75 and S87 were recovered from a portion of the challenged fish at the end of the study suggests that the mere presence of 10^8 cfu of flavobacteria within the body cavity of a fish does not mean that a fish will die from it. In other

words, despite bypassing portions of the innate immune system, a large dose of a non-pathogenic bacterium will not necessarily kill the host, further suggesting that isolates S21, along with isolates T76 and T91, represent fish-pathogenic flavobacteria. It should also be noted that *Flavobacterium* sp. T91 (Cluster XXVII) was originally recovered from the kidneys of a channel catfish (*Ictalurus punctatus*), while *Flavobacterium* sp. S87 (Cluster XXXI) was recovered from the kidneys of a largemouth bass (*Micropterus salmoides*). As such, the results of this study cannot be used to predict how these two isolates would behave in their original host species and/or at warmer water temperatures.

Antibiotic susceptibility analysis showed that the 6 Michigan *Flavobacterium* spp. isolates were sensitive to florfenicol, an antibiotic currently approved by the United States Food and Drug Administration (FDA) to treat disease outbreaks associated with *F. columnare* infections in farmed channel catfish and disease outbreaks associated with *F. psychrophilum* in aquacultured salmonids (http://www.fws.gov/fisheries/aadap/desk-reference_introduction.htm). Four of the six flavobacteria were also sensitive to oxytetracycline, which is approved to treat *F. columnare* outbreaks in freshwater-reared rainbow trout and *F. psychrophilum* outbreaks in freshwater reared salmonids, while 5/6 isolates were resistant to trimethoprim-sulfamethoxazole, another antibiotic approved to treat some bacterial diseases of cultured fishes (http://www.fws.gov/fisheries/aadap/desk-reference_introduction.htm). In the event that disease outbreaks associated with any of these *Flavobacterium* spp. are associated with substantial losses in an aquaculture situation, it is imperative to have antibiotic sensitivity data.

In conclusion, the results of this study elucidate the heterogeneous assemblage of *Flavobacterium* spp. associated with diseased fish in Michigan and provide further evidence that *F. psychrophilum, F. columnare,* and *F. branchiophilum* are likely not the only flavobacteria capable of negatively impacting ecologically and economically important salmonid stocks of the

Great Lakes. Clearly, further studies aimed at characterizing all of the flavobacterial clusters highlighted in this study that likely comprise novel *Flavobacterium* spp., as well as studies further investigating what role(s) they may play as fish pathogens, commensals, or mutualists are imperative if we are to understand host-flavobacteria-environment interactions in wild and cultured fishes.

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Table 4.1. Ninety-nine Michigan *Flavobacterium* spp. isolates selected for 16S rDNA sequence and phylogenetic analyses in this study.

Isolate ID	Accsn. #	Closest Relative (% 16S rDNA similarity)	Isolate ID	Accsn. #	Closest Relative (% 16S rDNA similarity)
T148	JX287871	*F. glacei (97.9%)*	T118	JX287852	*F. psychrophilum (99.4%)*
T129	JX287860	*F. succinicans (98.0%)*	T138	JX287867	*F. psychrophilum (99.4%)*
T151	JX287873	*F. hercynium (98.6%)*	T119	JX287853	*F. psychrophilum (99.4%)*
T102	JX287841	*F. hercynium (98.6%)*	T122	JX287856	*F. psychrophilum (99.7%)*
T101	JX287840	*F. hercynium (98.6%)*	T158	JX287876	*F. succinicans (98.8%)*
T21	JX287804	*F. hydatis (98.5%)*	T161	JX287879	*F. succinicans (98.5%)*
T18	JX287801	*F. hydatis (98.7%)*	T156	JX287874	*F. succinicans (98.5%)*
T66	JX287821	*F. hercynium (98.4%)*	T116	JX287850	*F. columnare (99.8%)*
T71	JX287824	*F. hercynium (98.3%)*	T7	JX287792	*F. columnare (99.6%)*
T73	JX287825	*F. hercynium (98.4%)*	T52	JX287814	*F. columnare (99.6%)*
T70	JX287823	*F. hercynium (98.5%)*	T89	JX287831	*F. columnare (99.6%)*
T10	JX287795	*F. pectinovorum (98.4%)*	T90	JX287832	*F. columnare (99.6%)*
T15	JX287798	*F. pectinovorum (98.2%)*	T79	JX287830	*F. columnare (99.6%)*
T166	JX287882	*F. pectinovorum (98.4%)*	T111	JX287848	*F. columnare (99.7%)*
T9	JX287794	*F. frigidimaris (98.4%)*	T113	JX287849	*F. columnare (99.8%)*
T8	JX287793	*F. aquidurense (98.5%)*	T109	JX287846	*F. columnare (99.6%)*
T69	JX287822	*F. chilense (98.5%)*	T110	JX287847	*F. columnare (99.8%)*
T61	JX287819	*F. hercynium (98.7%)*	T13	JX287796	*F. aquidurense (98.3%)*
T77	JX287829	*F. pectinovorum (98.0%)*	S12	JX287884	*F. aquidurense (98.7%)*
T76	JX287828	*F. pectinovorum (98.4%)*	T14	JX287797	*F. aquidurense (98.4%)*
T131	JX287861	*F. pectinovorum (98.3%)*	T16	JX287799	*F. aquidurense (98.3%)*
T20	JX287803	*F. oncorhynchi (99.8%)*	T124	JX287858	*F. aquidurense (98.6%)*
T128	JX287859	*F. oncorhynchi (99.7%)*	T123	JX287857	*F. aquidurense (98.5%)*
T150	JX287872	*F. oncorhynchi (99.3%)*	T33	JX287809	*F. frigidimaris (99.7%)*
T19	JX287802	*F. oncorhynchi (99.4%)*	T37	JX287810	*F. aquidurense (97.5%)*
T26	JX287807	*F. oncorhynchi (99.6%)*	T27	JX287808	*F. chungangense (98.2%)*
T104	JX287843	*F. oncorhynchi (99.8%)*	T47	JX287811	*F. chungangense (98.4%)*
T4	JX287790	*F. oncorhynchi (99.9%)*	T141	JX287869	*F. limicola (97.9%)*
T164	JX287880	*F. oncorhynchi (98.3%)*	T142	JX287870	*F. limicola (97.8%)*
T25	JX287806	*F. oncorhynchi (98.4%)*	T160	JX287878	*F. limicola (98.1%)*
T165	JX287881	*F. oncorhynchi (98.4%)*	T105	JX287844	*F. tiangeerense (99.1%)*
T103	JX287842	*F. oncorhynchi (98.4%)*	T92	JX287834	*F. anhuiense (97.8%)*
T23	JX287805	*F. oncorhynchi (98.4%)*	T91	JX287833	*F. anhuiense (98.2%)*
T96	JX287837	*F. oncorhynchi (98.5%)*	T2	JX287788	*F. chilense (98.7%)*
T59	JX287818	*F. oncorhynchi (98.5%)*	T3	JX287789	*F. chilense (98.9%)*
T95	JX287836	*F. oncorhynchi (98.5%)*	T74	JX287826	*F. degerlachei (97.4%)*
T17	JX287800	*F. aquidurense (98.4%)*	T75	JX287827	*F. tiangeerense (97.5%)*
T1	JX287787	*F. frigidimaris (97.8%)*	S87	JX287883	*F. resistans (97.9%)*
S21	JX287885	*F. aquidurense (98.1%)*	T108	JX287845	*F. pectinovorum (98.5%)*
T6	JX287791	*F. hercynium (98.8%)*	T157	JX287875	*F. aquidurense (98.8%)*
T49	JX287812	*F. psychrophilum (99.1%)*	T93	JX287835	*F. frigidimaris (98.8%)*
T50	JX287813	*F. psychrophilum (99.2%)*	T100	JX287839	*F. frigidimaris (98.9%)*
T140	JX287868	*F. psychrophilum (99.3%)*	T99	JX287838	*F. frigidimaris (99.1%)*
T120	JX287854	*F. psychrophilum (99.3%)*	T54	JX287815	*F. frigidimaris (98.3%)*
T135	JX287864	*F. psychrophilum (99.3%)*	T65	JX287820	*F. hercynium (98.1%)*
T136	JX287865	*F. psychrophilum (99.3%)*	T132	JX287862	*F. hercynium (98.8%)*

Table 4.1 (cont'd)

Isolate ID	Accsn. #	Closest Relative (% 16S rDNA similarity)	Isolate ID	Accsn. #	Closest Relative (% 16S rDNA similarity)
T134	JX287863	*F. psychrophilum (99.3%)*	T159	JX287877	*F. hydatis (98.9%)*
T137	JX287866	*F. psychrophilum (99.4%)*	T57	JX287817	*F. pectinovorum (98.1%)*
T121	JX287855	*F. psychrophilum (99.3%)*	T56	JX287816	*F. tiangeerense (97.8%)*
T117	JX287851	*F. psychrophilum (99.3%)*			

Table 4.2. Characteristics that were variable among 6 Michigan fish-associated *Flavobacterium* spp. isolates examined in this study; +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported. The results for characteristics that were uniform amongst the 6 isolates are described in the text.

Characteristic	**T91**	**T75**	**T18**	**S87**	**S21**	**T76**
Cell size (µm)	2-10	1.5-3.0	1.0-2.0	1.0-2.5	2.0-4.0	2.0-4.0
Congo Red Absorption	(+)	-	-	-	+	+
Growth on Marine Agar	+	+	-	-	-	+
Growth at pH of 5.0 and 9.5-10.0	+	-	(+)	+	+	+
Growth at 37 °C	-	-	-	(+)	-	-
Growth at 2% Salinity	(+)	-	-	-	+	+
Nitrate Reduction	+	-	-	-	+	+
Degradation of:						
Chondroitin sulfate	+	+	(+)	-	-	-
Tween 20	+	-	-	+	+	+
Tyrosine	+	-	(+)	+	+	+
Hemoglobin	+	-	+	+	+	+
Production of:						
Phenylalanine Deaminase	(+)	-	-	-	+	+
Gelatinase	+	-	+	+	+	+
Dnase	-	NG	-	-	-	+
Elastase	-	-	-	-	+	+
Cystine arylamidase	-	+	-	+	+	+
Trypsin	-	-	-	-	(+)	-
α-chymotrypan	-	-	+	-	-	-
α-galactosidase	+	-	+	-	-	+
β-galactosidase	+	-	+	+	-	-
β-glucoronidase	-	-	+	-	-	-
β-glucosidase	+	+	-	+	-	-
α-fucosidase	-	+	+	-	+	-
Brown Pigment from Tyrosine	-	-	-	-	-	+
Acetoin	-	+	+	-	-	+
Assimilation of:						
L-Arabinose	+	-	+	+	-	-
N-acetyl-glucosamine	+	-	+	+	+	+
D-Maltose	+	-	+	+	+	+
Acid Production from:						
D-Arabinose	-	-	+	-	-	-
L-Arabinose and D-Xylose	+	-	(+)	+	-	-
D-Galactose	+	-	(+)	+	-	-

Table 4.2 (cont'd)

Characteristic	T91	T75	T18	S87	S21	T76
D-Glucose and D-Mannose	+	(+)	(+)	+	(+)	-
D-Fructose	-	-	(+)	+	-	-
N-AcetylGlucosamine	(+)	-	(+)	+	+	+
Amygdalin	(+)	-	(+)	+	+	+
Arbutin	-	-	-	+	-	+
Salicin	-	-	-	(+)	-	-
D-Cellobiose	+	+	+	+	-	-
D-Maltose	+	+	(+)	+	(+)	+
D-Lactose and D-Melibiose	-	-	(+)	-	-	-
D-Sucrose	-	-	(+)	+	-	-
D-Trehalose	-	(+)	-	-	(+)	+
Inulin and D-Raffinose	-	-	(+)	(+)	-	-
Glycogen	+	-	(+)	+	(+)	(+)
L-Fucose	-	-	(+)	-	-	-

Table 4.3. Antibiotic susceptibility results for 6 Michigan *Flavobacteirum* spp. isolates as determined via the Kirby-Bauer disk diffusion method. R, resistant; S, sensitive; number in parentheses is the mean diameter of the zone of inhibition in mm. PB, polymyxin-B (300 iu); SXT, trimethoprim-sulfamethoxazole (25 µg); P, penicillin G (10 iu); O129, (2,4-diamino,6,7-di-isopropyl pteridine;10 µg); FFC, florfenicol (30 µg); AMP, ampicillin (10 µg); E, erythromycin (15 µg); T, oxytetracycline (30 µg).

Isolate	SXT	PB	P	O129	FFC	AMP	E	T
T91	R (12.0)	R (9.5)	R (0)	R (0)	S (32.0)	R (11.5)	R (16.0)	S (23.0)
T75	R (11.0)	R (10.5)	R (0)	R (0)	S (33.0)	S (29.0)	S (31.0)	R (14.5)
T18	R (14.0)	R (9.5)	R (0)	R (0)	S (32.0)	S (13.0)	S (24.5)	S (30.0)
S87	R (14.0)	R (8.0)	S (13.0)	S (15.0)	S (25.0)	S (15.5)	R (17.5)	S (30.0)
S21	R (0)	R (11.5)	R (0)	R (0)	S (27.5)	R (0)	S (18.0)	S (27.0)
T76	S (18.0)	S (13.0)	R (0)	R (0)	S (24.0)	S (13.0)	S (24.5)	R (17.0)

Table 4.4. Cellular fatty acid profiles (%) of 6 Michigan fish-associated *Flavobacterium* spp. isolates (T91, T75, T18, S87, S21, T76) as determined using the commercial Sherlock Microbial Identification System (MIDI, version 4.0; Microbial Identification System Inc., Newark, DE). Tr, trace amounts (<1%) detected; -, not detected.

Fatty Acid	T91	T75	T18	S87	S21	T76
iso-$C_{15:0}$	29.1	19.2	29.0	24.2	27.1	23.2
$C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*	21.8	10.6	12.7	23.3	11.8	15.7
iso-$C_{17:0}$ 3-OH	7.0	10.9	14.5	9.2	14.7	12.3
$C_{16:0}$	8.7	4.9	3.6	10.2	4.8	10.0
iso-$C_{15:0}$ 3-OH	8.4	12.0	12.4	7.2	10.3	8.3
$C_{16:0}$ 3-OH	9.2	3.1	4.0	8.6	2.9	6.2
iso-$C_{17:1}$ ω9*c*	2.1	3.5	5.4	3.2	7.2	3.5
iso-$C_{15:1}$ G	2.6	3.3	1.2	1.1	2.9	3.2
iso-$C_{16:0}$ 3-OH	1.1	1.6	3.6	1.4	3.2	3.1
anteiso-$C_{15:0}$	2.2	6.1	4.0	3.7	2.0	3.1
iso-$C_{17:0}$	Tr	Tr	Tr	Tr	1.7	1.5
iso-$C_{16:0}$	Tr	1.1	Tr	Tr	1.8	1.4
$C_{17:0}$ 2-OH	Tr	-	1.2	Tr	Tr	1.2
$C_{14:0}$ 3-OH/ *iso*-$C_{16:1}$ I	1.3	Tr	Tr	1.5	Tr	1.2
$C_{16:0}$	1.7	Tr	Tr	1.3	Tr	1.1
$C_{17:1}$ ω6*c*	Tr	3.5	Tr	Tr	1.4	1.1
$C_{15:1}$ ω6*c*	Tr	7.9	1.5	Tr	1.8	1.0
$C_{18:1}$ ω9*c*	Tr	Tr	Tr	Tr	Tr	Tr
$C_{15:0}$ 2-OH	Tr	Tr	Tr	Tr	Tr	Tr
$C_{17:1}$ ω8*c*	Tr	1.3	Tr	Tr	Tr	Tr
iso-$C_{14:0}$	Tr	Tr	Tr	-	-	Tr
$C_{17:0}$ 3-OH	T	Tr	Tr	-	Tr	Tr
iso-$C_{14:0}$ 3-OH	-	Tr	Tr	-	Tr	Tr
iso-$C_{13:0}$	Tr	Tr	Tr	Tr	Tr	Tr
anteiso-$C_{17:0}$	-	Tr	Tr	Tr	Tr	Tr
$C_{17:0}$	-	Tr	-	-	-	Tr
iso-$C_{12:0}$	-	Tr	-	-	-	-
$C_{13:0}$	-	Tr	-	-	-	-
$C_{13:0}$ 3-OH /*iso*-$C_{15:1}$ H	-	Tr	Tr	Tr	-	-
$C_{16:1}$ H	-	Tr	-	-	Tr	-

Table 4.4 (cont'd)

Fatty Acid	T91	T75	T18	S87	S21	T76
$C_{16:1}$ ω5*c*	-	Tr	-	-	-	-
$C_{15:0}$ 3-OH	1.0	2.0	-	-	-	-
***anteiso*-$C_{17:0}$ B/*iso* I**	-	-	-	-	1.6	-

Figure 4.1. Dendrogram generated using the neigbor-joining method in MEGA5 that depicts the phylogenetic relationship between 99 *Flavobacterium* spp. isolates recovered from fishes and other members of the genus *Flavobacterium.* Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

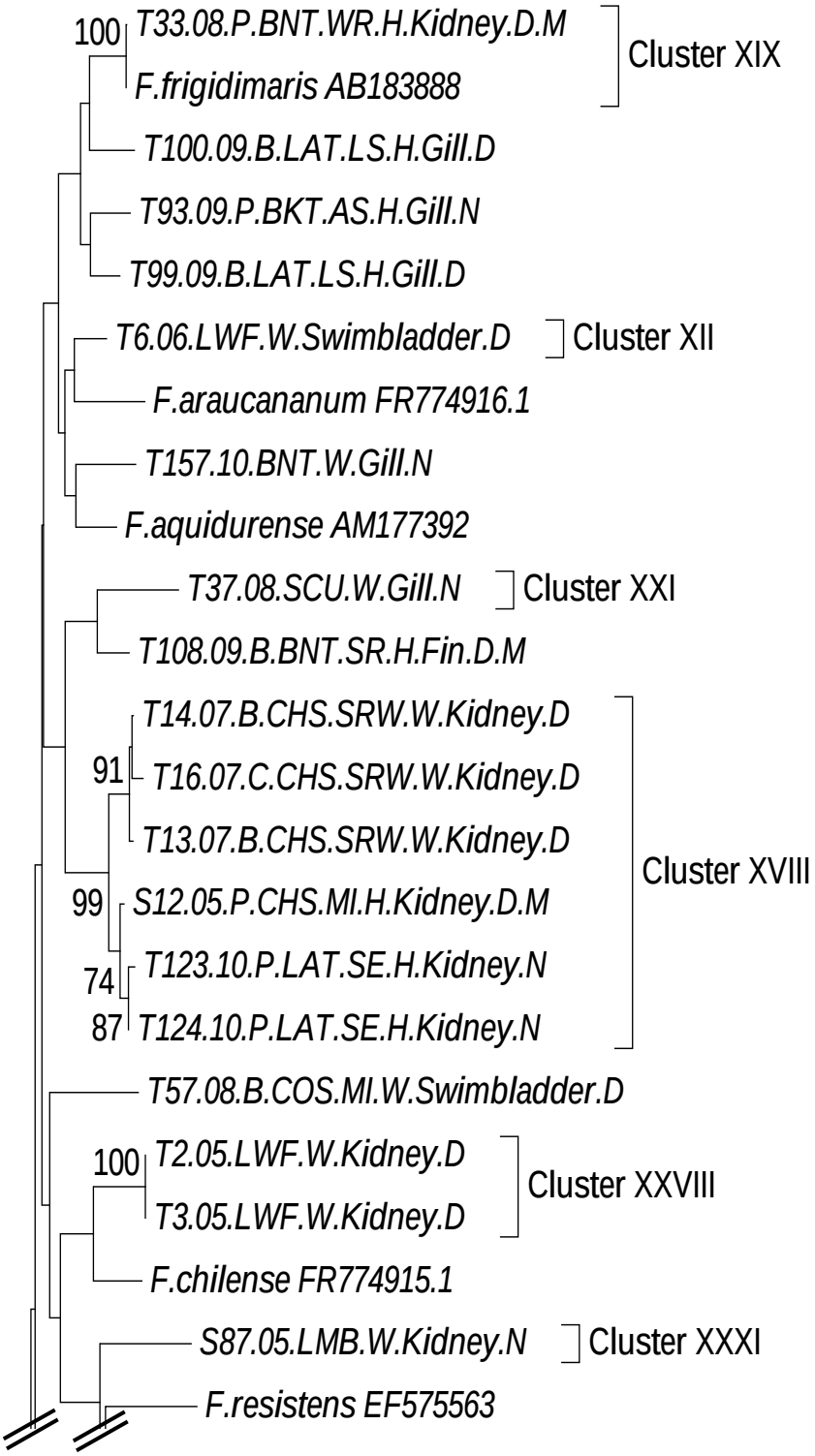


Figure 4.1 (cont'd)

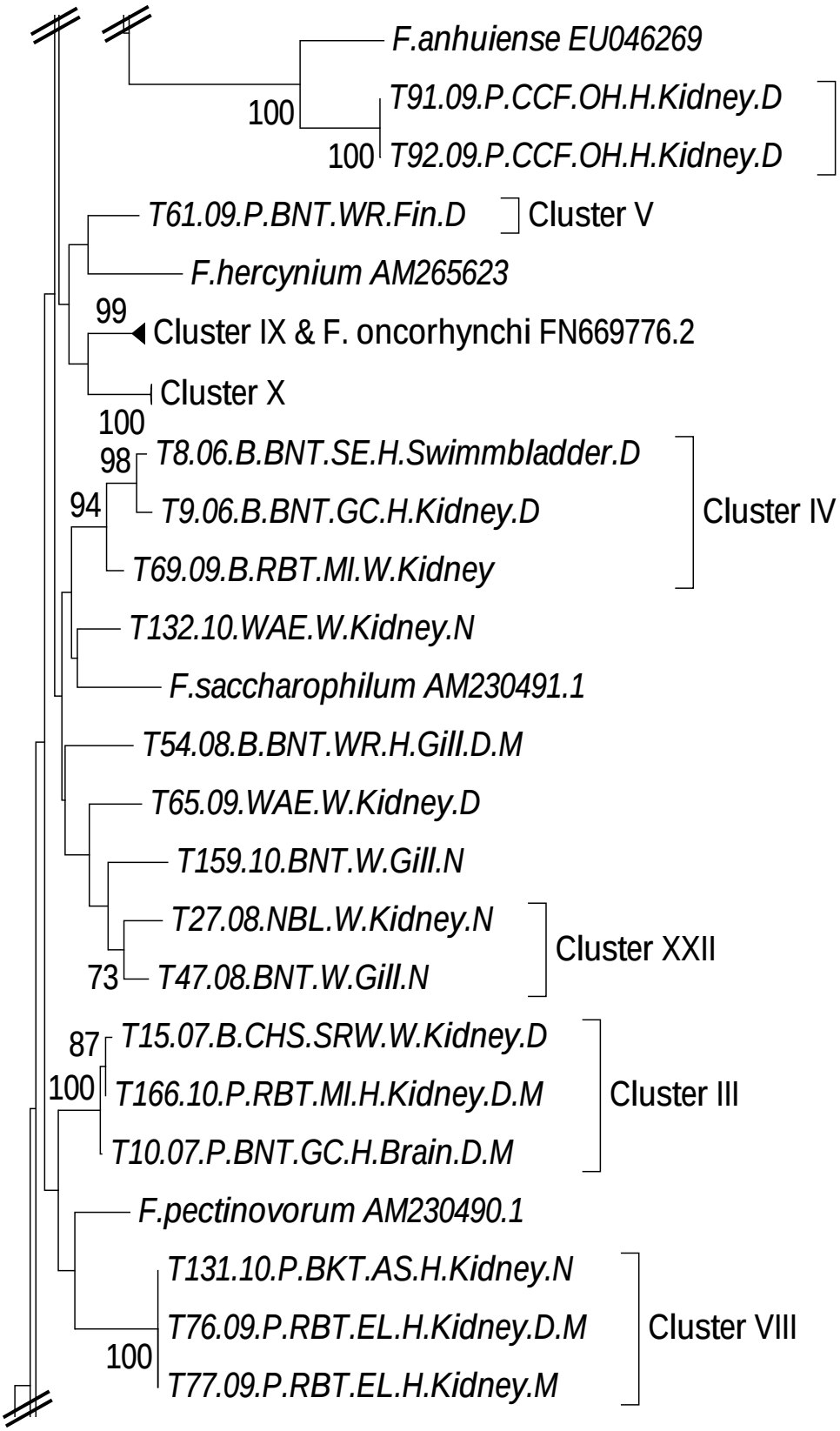


Figure 4.1 (cont'd)

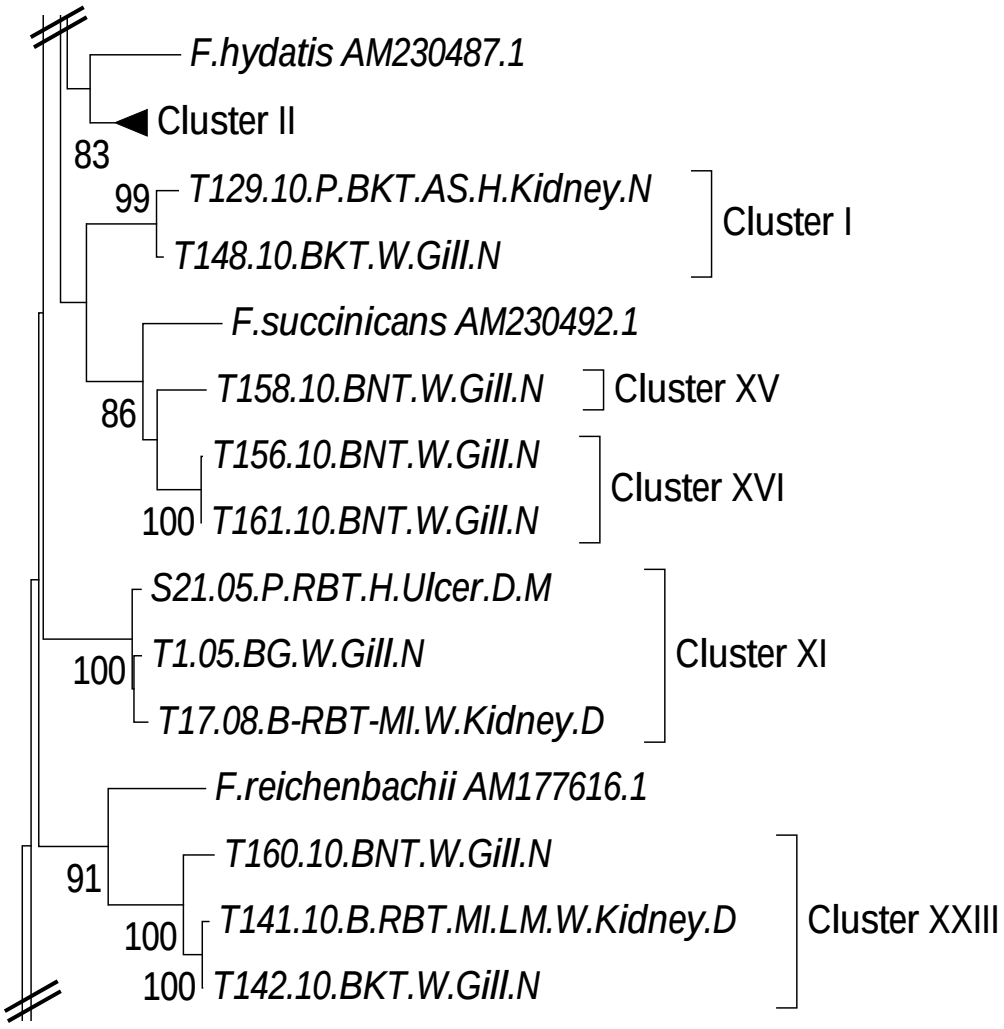


Figure 4.1 (cont'd)

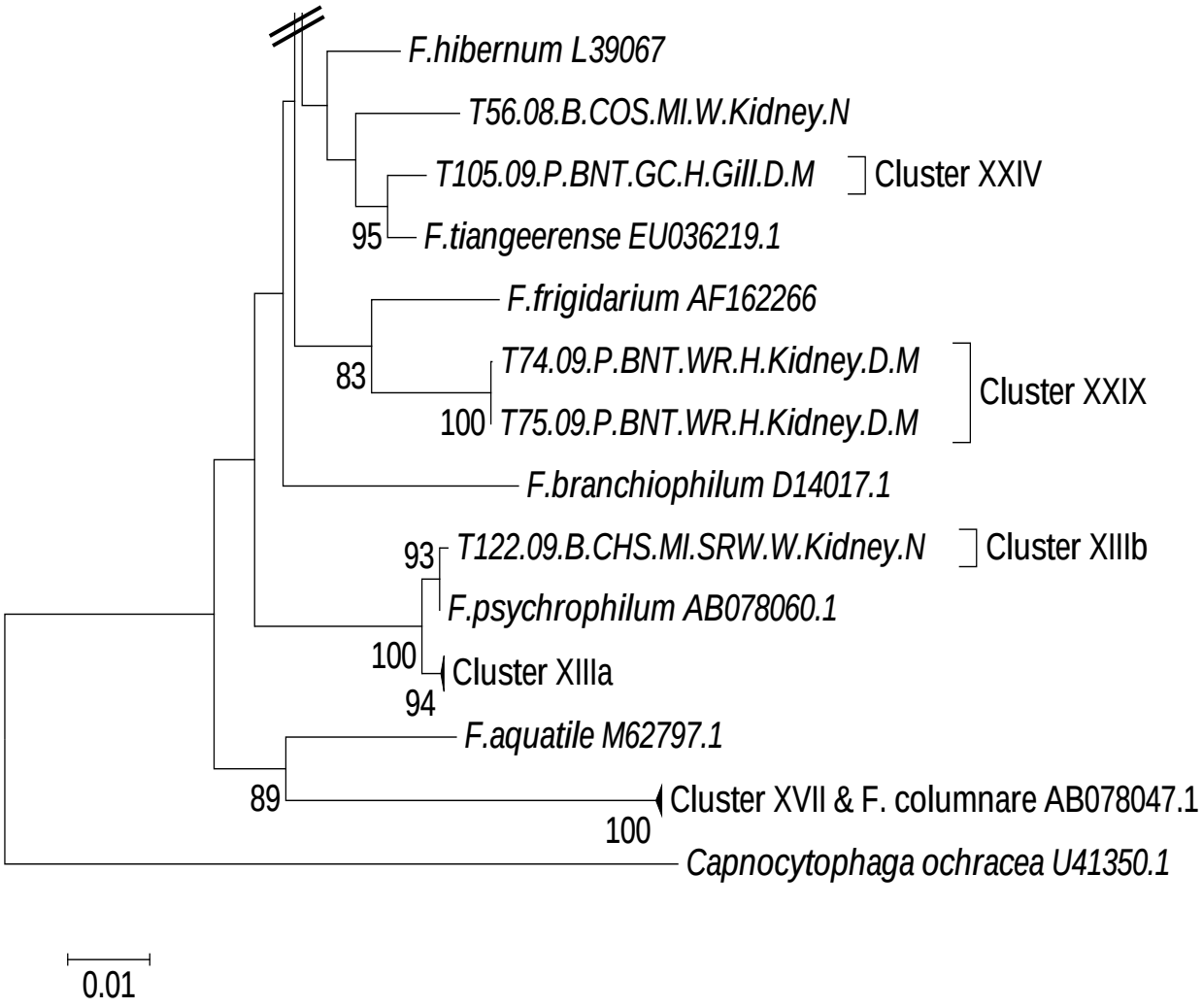


Figure 4.2. Subtree of dendrogram displayed in Fig. 1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 14 Michigan *F. psychrophilum* isolates recovered from fishes and the *F. psychrophilum* reference sequence. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

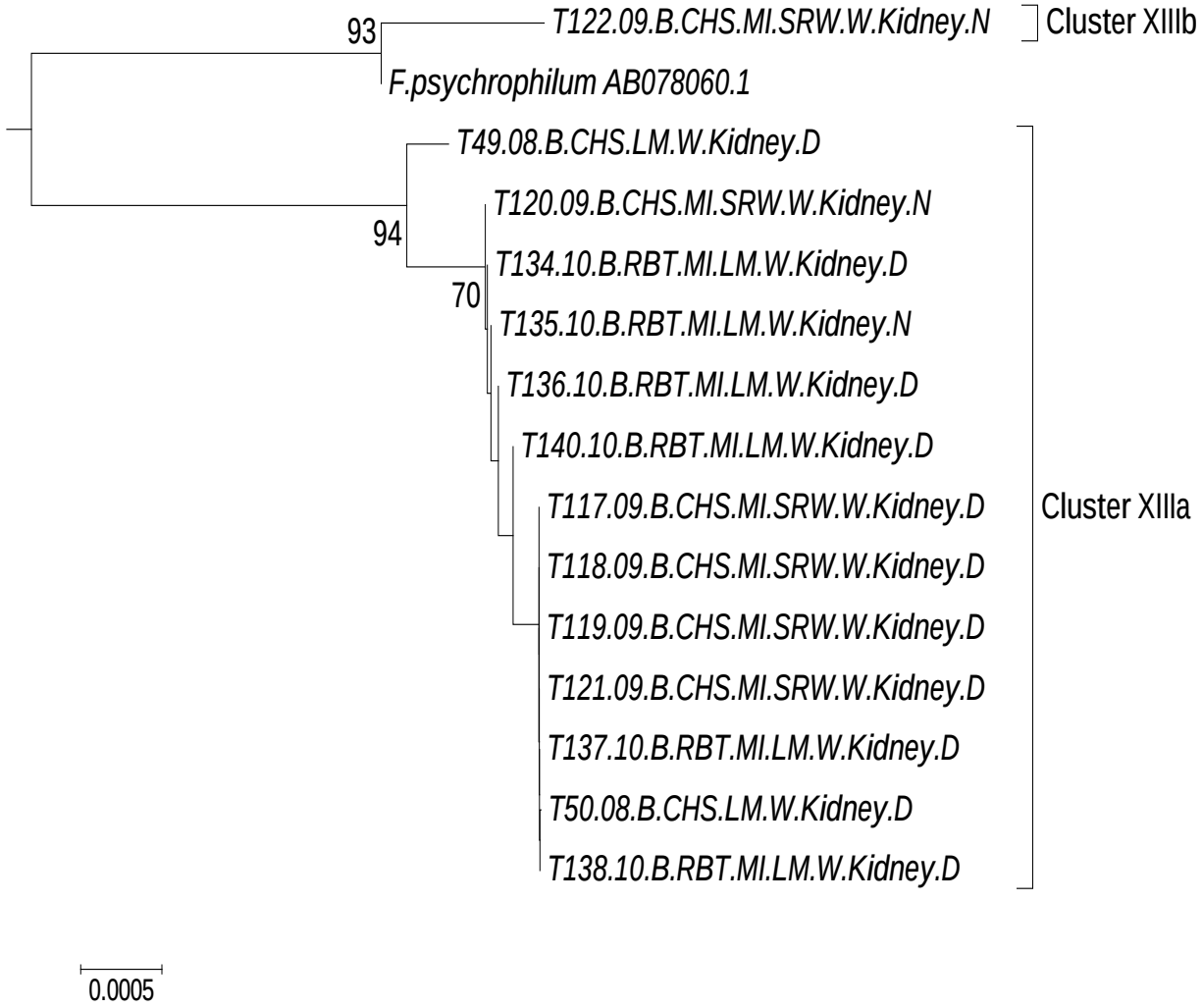


Figure 4.3. Subtree of dendrogram displayed in Fig. 1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 10 Michigan *F. columnare* isolates recovered from fishes and the *F. columnare* and *F. aquatile* reference sequences. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

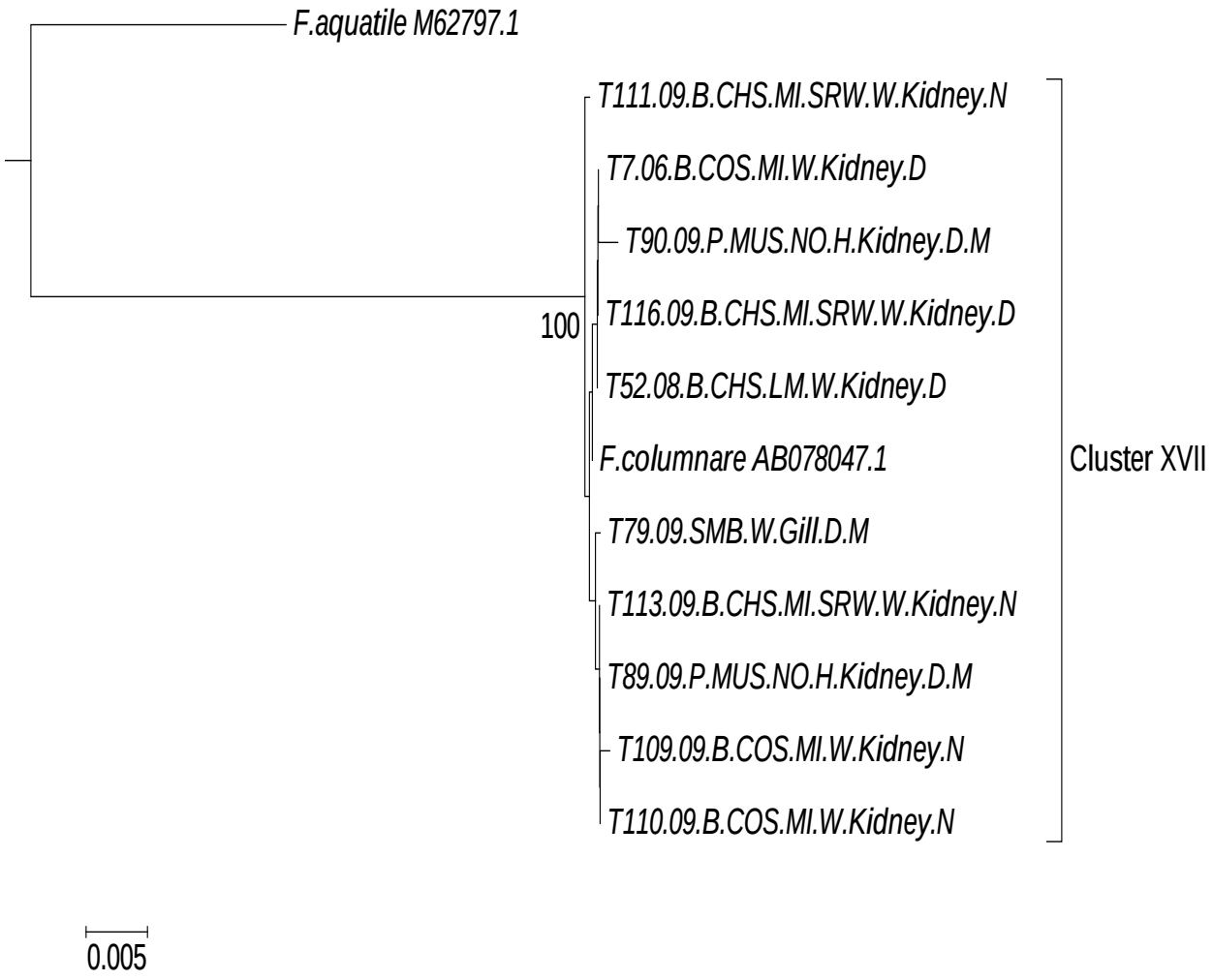


Figure 4.4. Subtree of dendrogram displayed in Fig. 1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 16 Michigan *Flavobacterium* spp. isolates recovered from fishes and the *F. hercynium* and *F. onchorynchi* reference sequences. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

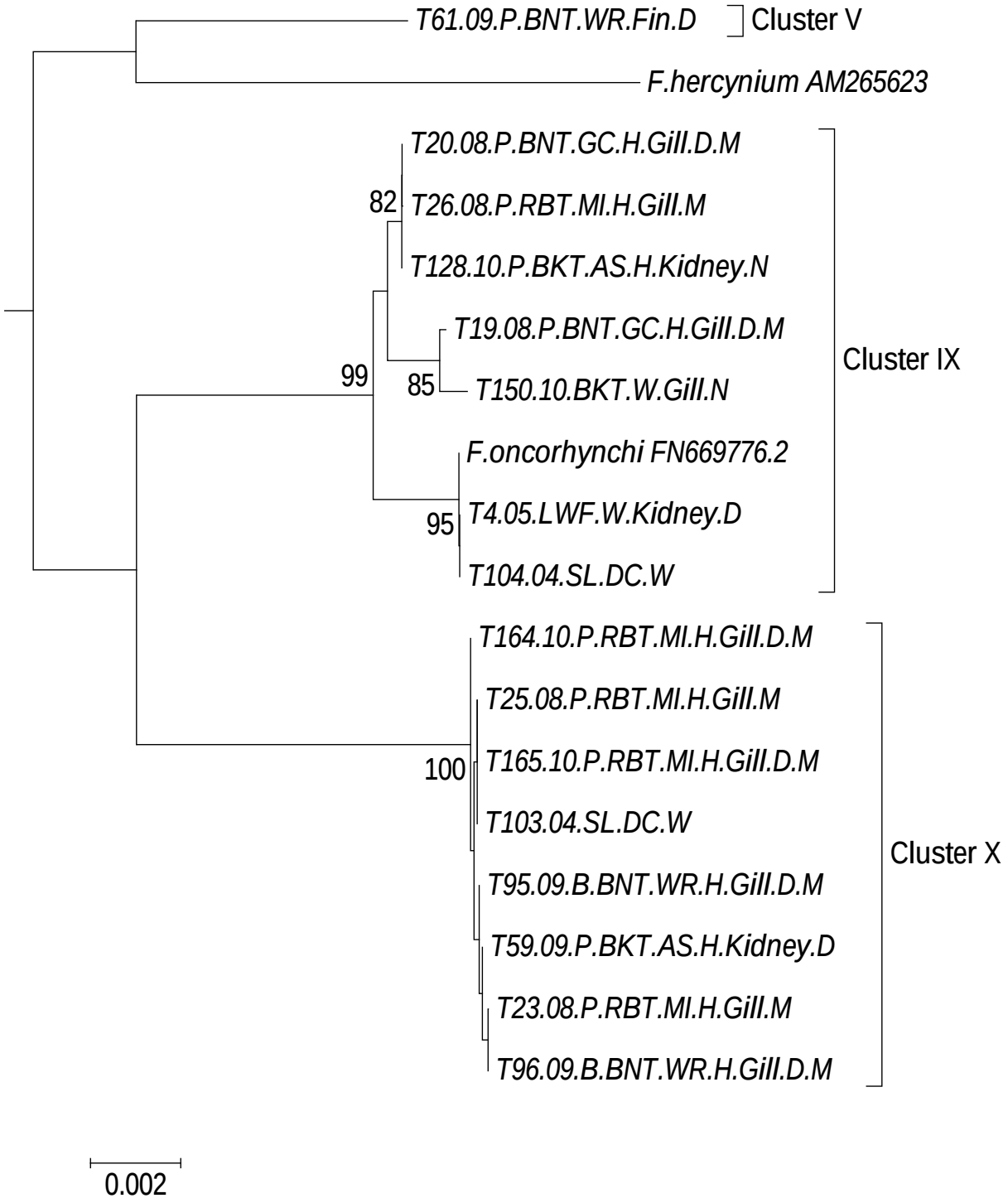


Figure 4.5. Subtree of dendrogram displayed in Fig. 1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 9 Michigan *Flavobacterium* spp. isolates recovered from fishes and the *F. hydatis* reference sequence. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

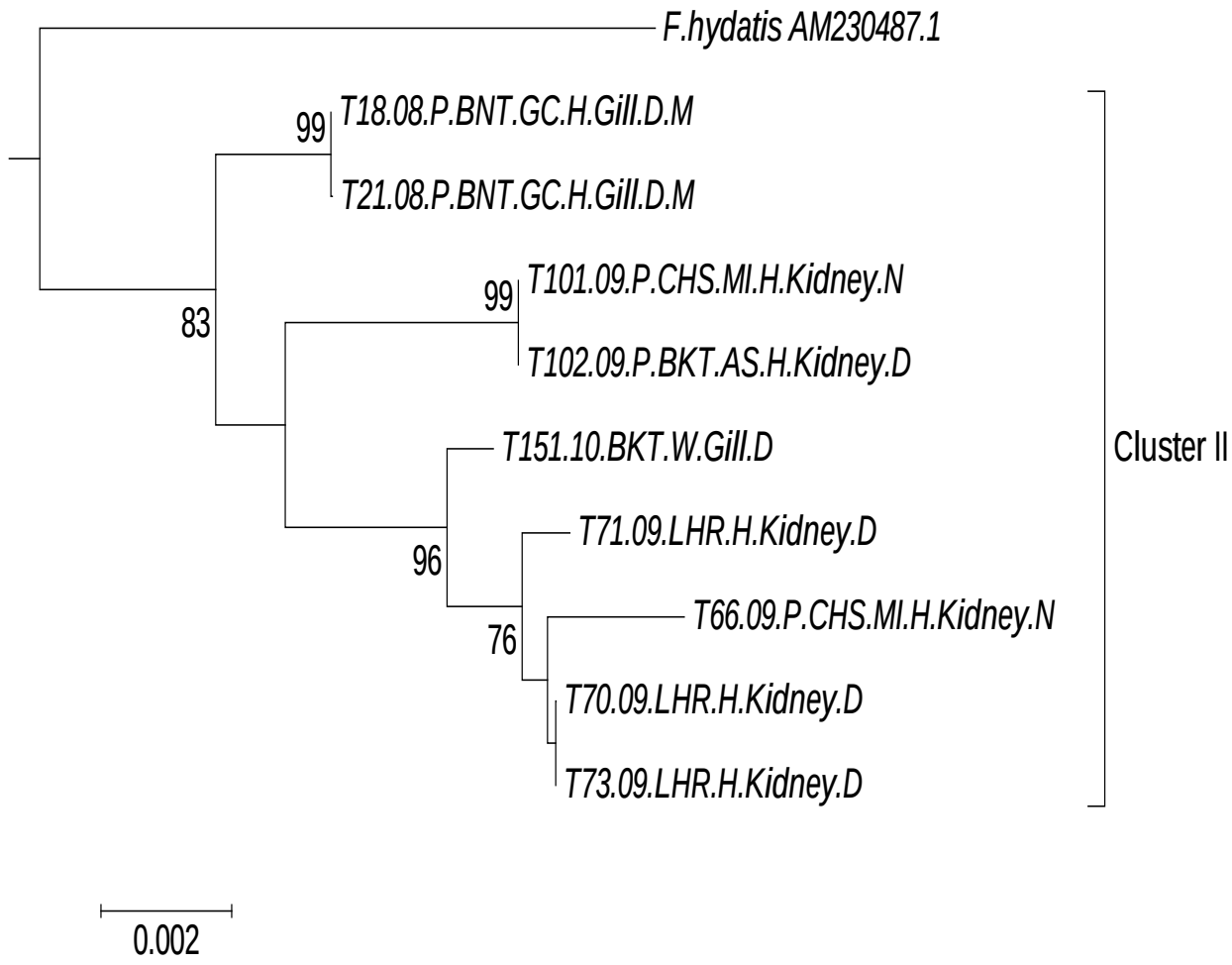


Figure 4.6. Cladogram depicting the relationships of *Chryseobacterium* spp. T91 and T75 (red rectangles) generated using Bayesian analysis with the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites Bayesian in MrBayes 3.1.2. The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Four independent analyses were conducted, both with 1 cold and 3 heated chains using the default heating parameter (temp=0.2). The initial 25% of Markov Chain Monte Carlo (MCMC) samples were discarded as burnin and sampling occurred every 1000 generations. Filled circles are present when that node was also present in the neighbor-joining analysis.

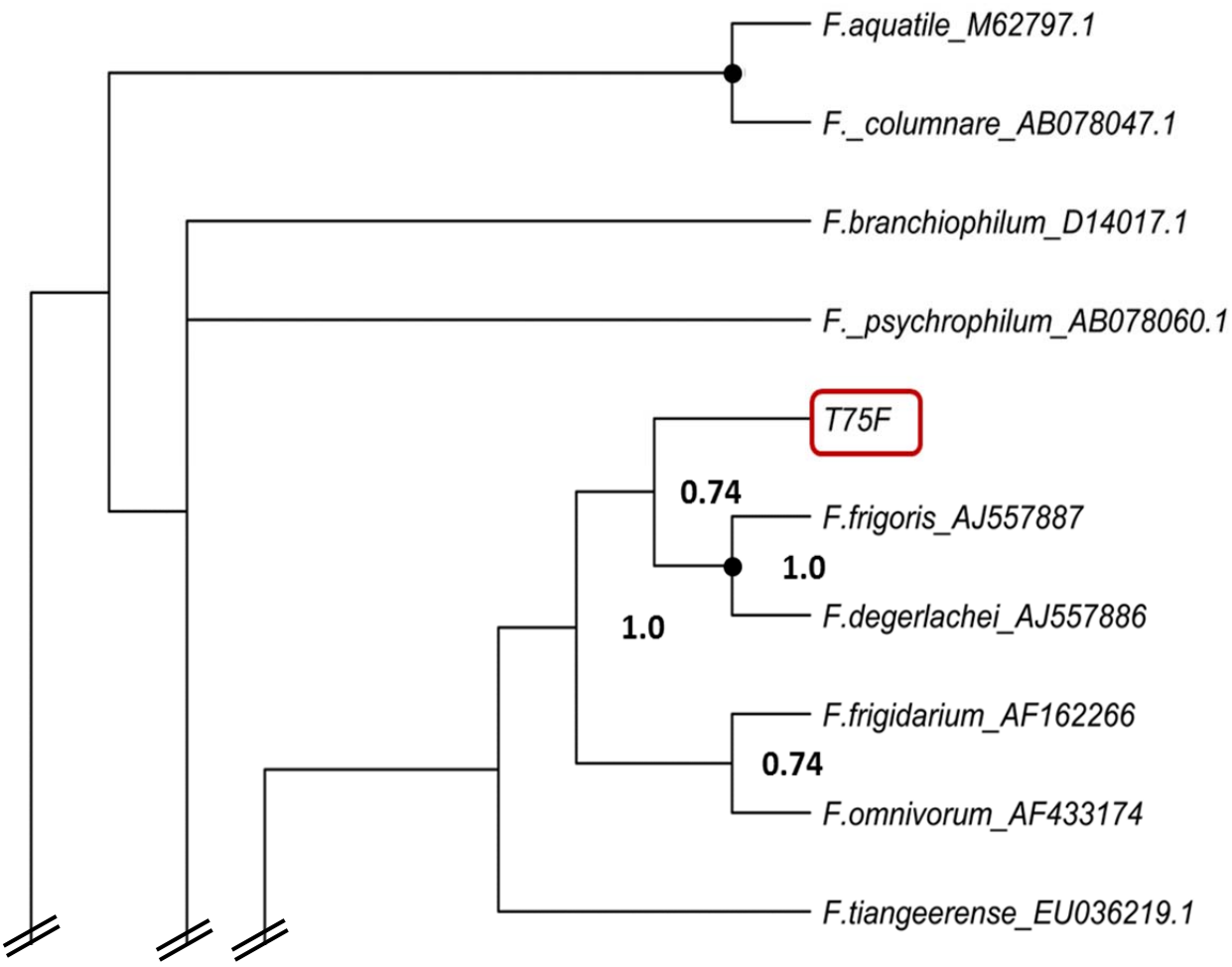


Figure 4.6 (cont'd)

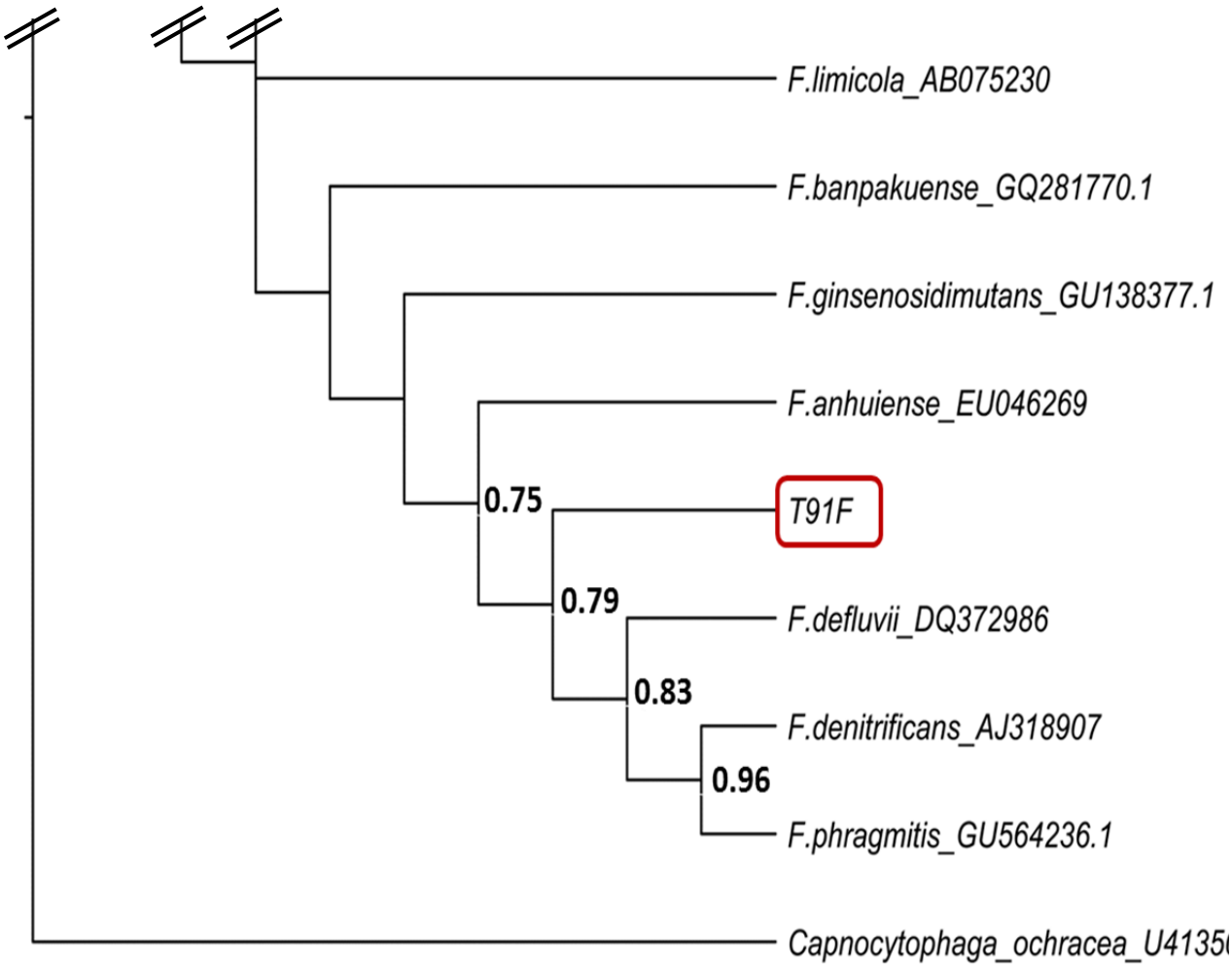


Figure 4.7. Cladogram depicting the relationships of *Chryseobacterium* spp. T18, T76, S21, and S87 (red rectangles) generated using Bayesian analysis with the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites Bayesian in MrBayes 3.1.2. The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Four independent analyses were conducted, both with 1 cold and 3 heated chains using the default heating parameter (temp=0.2). The initial 25% of Markov Chain Monte Carlo (MCMC) samples were discarded as burnin and sampling occurred every 1000 generations. Filled circles are present when that node was also present in the neighbor-joining analysis.

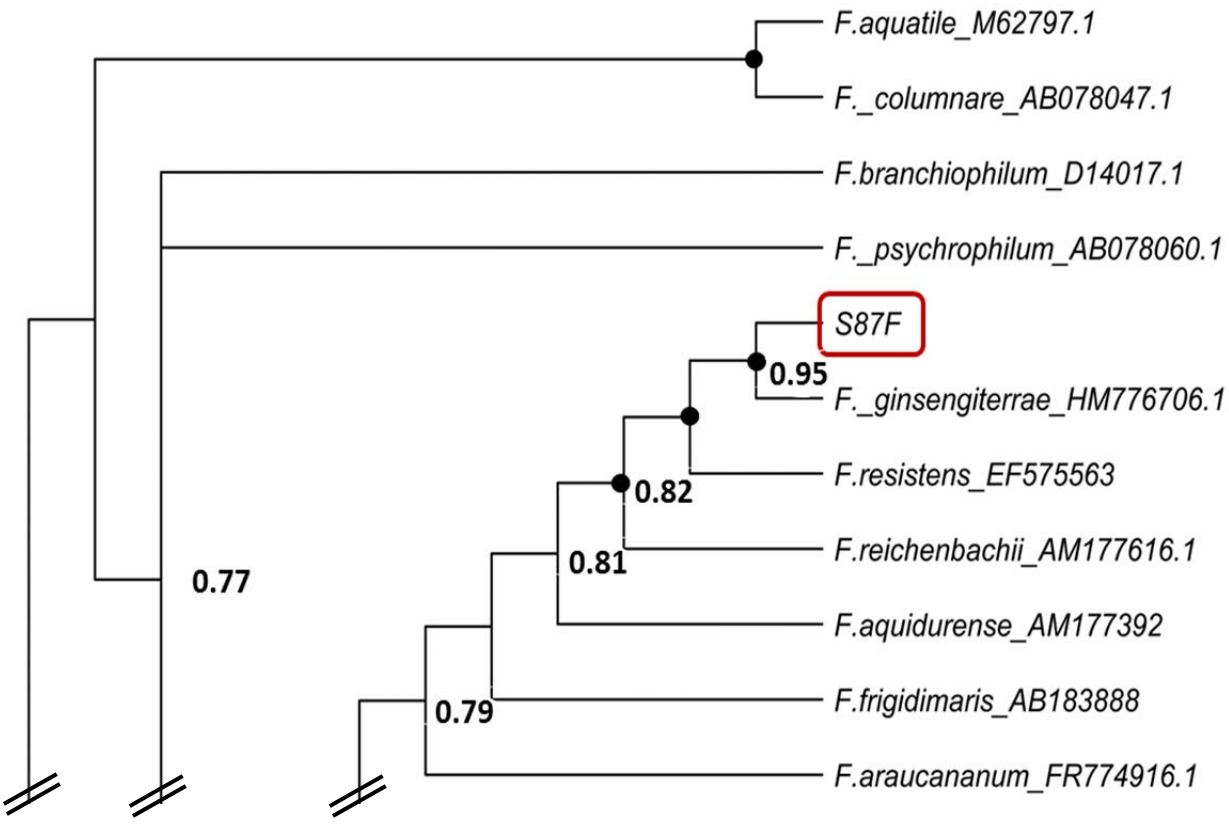


Figure 4.7 (cont'd)

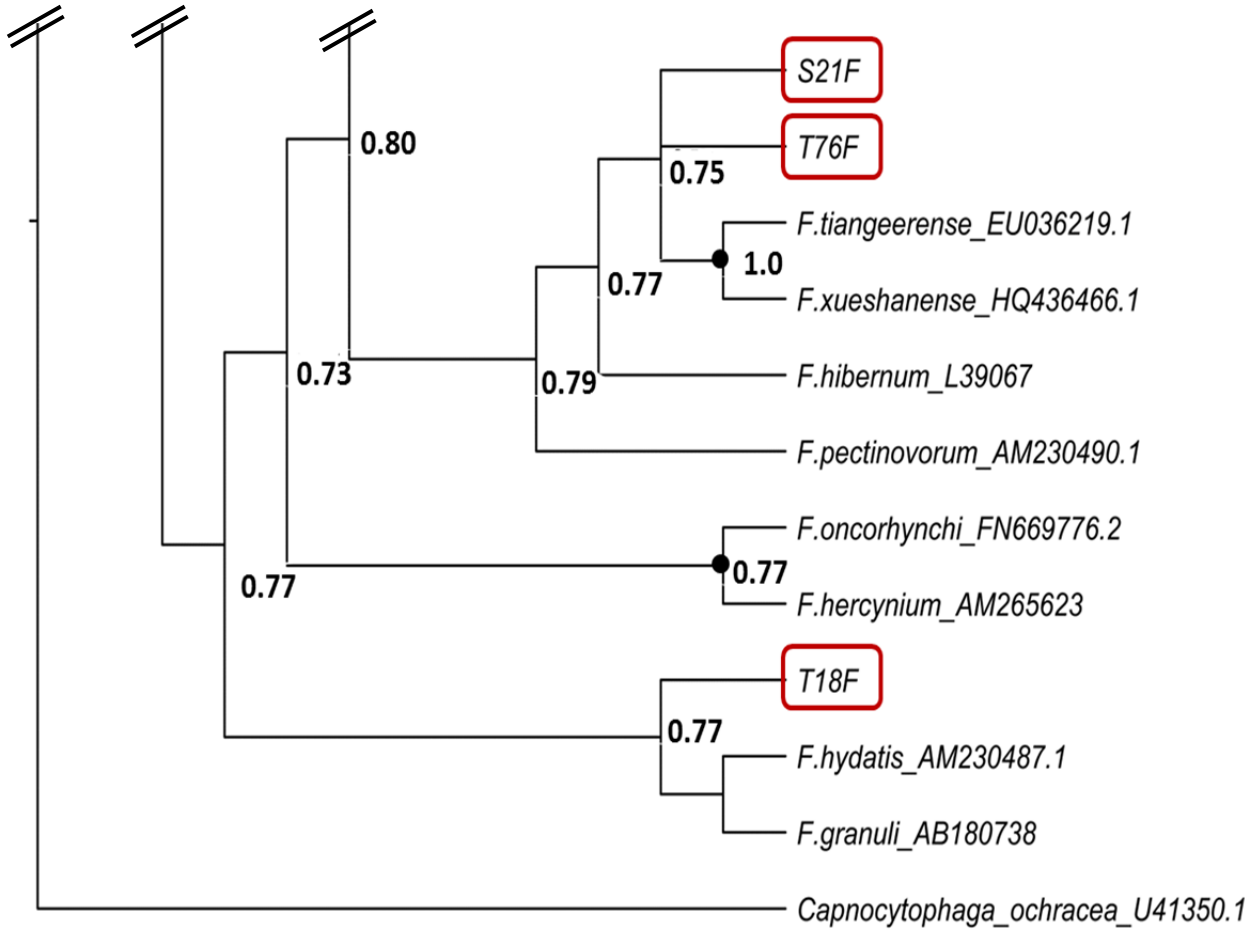
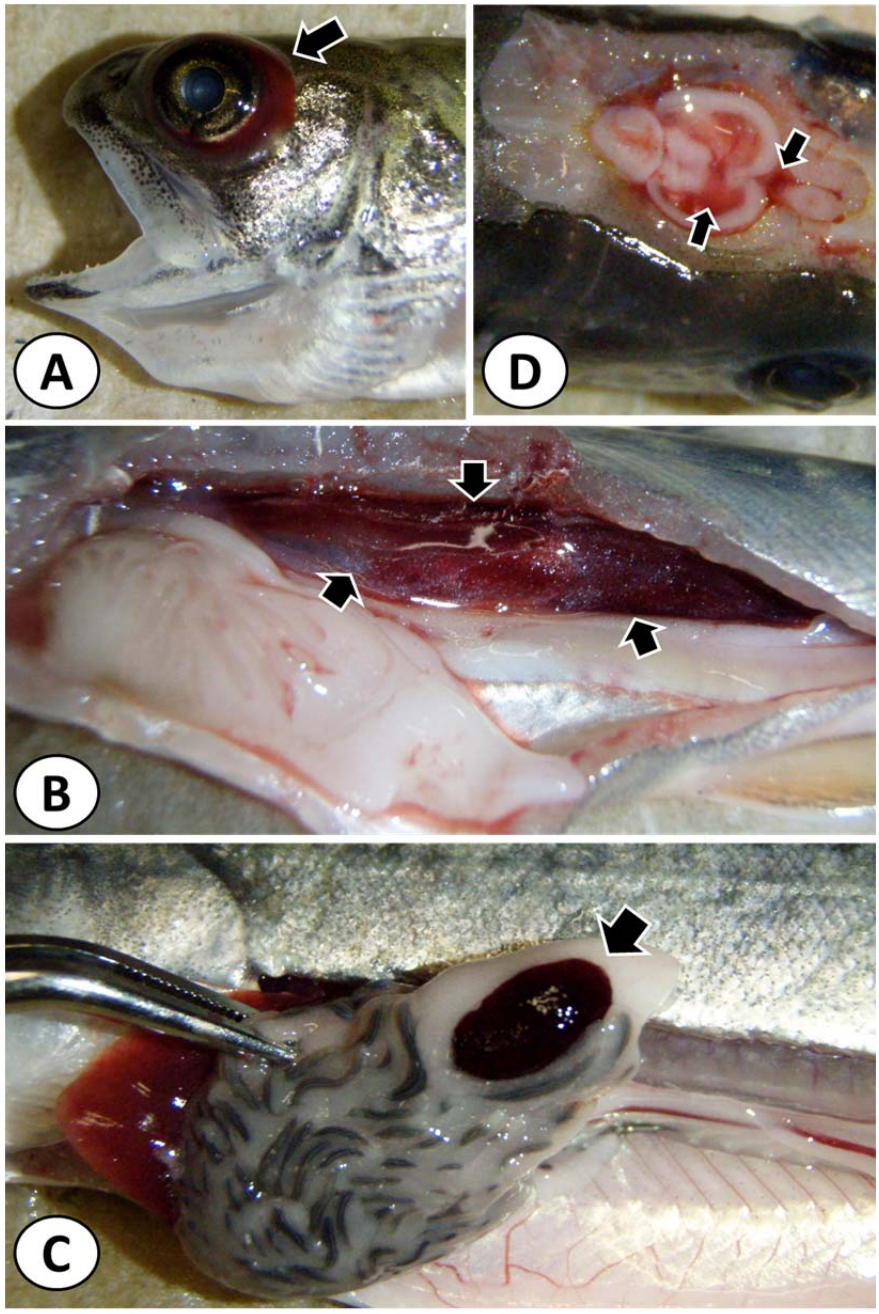


Figure 4.8. Gross lesions observed in fish intraperitoneally injected with Michigan *Flavobacterium* spp. isolates. A) Severe unilateral exophthalmia with diffuse periocular hemorrhage (arrow) in an S21-infected brook trout fingerling. B) Severe diffuse hemorrhage present within the swim bladder (arrows), along with a small amount of red-tinged ascites within the body cavity, of an S21-infected brook trout fingerling. C) Swollen spleen (arrow) in an S87-infected Chinook salmon fingerling. D) Multifocal hemorrhage and edema (arrows) within the brain of an infected brook trout fingerling.



APPENDIX B

Supplementary Table 4.1. Differential characteristics of *Flavobacterium* sp. T91 and related *Flavobacterium* spp. *F. anhuiense* (Liu et al. 2008); *F. ginsenosidimutans* (Yang et al. 2011). +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported; Tr, traces (<1%); ND, not detected; *, Summed feature 3 comprises $C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7*c* that could not be separated by GLC with the MIDI system.

Characteristic	**T91**	***F. anhuiense***	***F. ginsenosidimutans***
Growth on Cetrimide Agar	-	+	NR
Growth at 4 °C	+	-	-
Growth at 37 °C	-	+	+
Hydrolysis of Tween 20	+	-	NR
Citrate Utilization	+	-	-
Nitrate Reduction	+	-	-
Assimilation of D-Mannose	+	-	+
Production of:			
Gelatinase	+	-	NR
Pectinase	+	-	NR
Arginine dihydrolase	-	+	NR
Lysine decarboxylase	-	+	NR
Ornithine decarboxylase	-	+	NR
α-galactosidase	+	-	-
β-glucosidase	+	-	+
Production of Acid from:			
D-Galactose	+	-	NR
L-Rhamnose	-	+	NR
D-Cellobiose	+	-	NR
L-Fucose	-	+	NR
% Fatty Acid Content			
$C_{10:0}$	ND	ND	3.6
iso-$C_{15:1}$ G	2.6	2.2	5.2
$C_{15:0}$	ND	3.4	ND
$C_{15:1}$ ω6*c*	Tr	Tr	3.0
$C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*	21.8	*	14.6
$C_{16:0}$	8.7	11.3	4.7
$C_{15:0}$ 2-OH	Tr	*	ND
$C_{15:0}$ 3-OH	1.0	6.0	1.7
$C_{16:0}$ 3-OH	9.2	5.1	5.7
anteiso-$C_{19:0}$	ND	ND	2.1
Summed feature 3*		11.9	

Supplementary Table 4.2. Differential characteristics of *Flavobacterium* sp. strain T75 and related *Flavobacterium* spp. T75, results from this study; *F. tiangeerense* (Xin et al. 2009); *F. frigidarium* (Humphry et al. 2001). +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported; Tr, traces (<1%); ND, not detected.

Characteristic	**T75**	***F. tiangeerense***	***F. frigidarium***
Flexirubin Type Pigment	+	-	-
Utilization of Citrate	+	-	-
Growth at 1% Salinity	+	-	+
Production of:			
Cytochrome Oxidase	-	+	+
Pectinase	+	-	-
Amylase	+	-	-
Esterase	+	+	-
Lipase	-	+	-
Trypsin	-	+	-
α-chymotrypan	-	+	(+)
α -glucosidase	+	-	-
β-glucosidase	+	-	-
N-acetyl- β-glucosaminidase	+	-	-
α-fucosidase	+	-	-
Production of Acid from:			
D-Mannose	(+)	(+)	-
D-Maltose	+	-	-
% Fatty Acid Content:			
iso-$C_{14:0}$	Tr	3.1	3.7
$C_{15:0}$	ND	ND	5.0
iso-$C_{15:1}$ G	3.3	5.4	ND
iso-$C_{15:0}$	19.2	26.9	8.8
anteiso-$C_{15:0}$	6.1	2.8	15.1
iso-$C_{16:0}$	1.1	3.6	9.0
iso-$C_{16:1}$ H	Tr	3.2	ND
$C_{16:0}$	4.9	1.3	3.0
iso-$C_{15:0}$ 3-OH	12.0	10.7	ND
$C_{15:0}$ 3-OH	2.1	ND	ND
$C_{16:0}$ 3-OH	1.6	5.1	ND
iso-$C_{17:0}$ 3-OH	10.9	4.6	ND

Supplementary Table 4.3. Differential characteristics of *Flavobacterium* sp. strain T18 and related *Flavobacterium* spp. T18, results from this study; *F. hydatis* (Strohl and Tait 1978; Bernardet et al. 2011); *F. oncorhynchi* (Strain 631-08, Zamora et al. 2012a). +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported.

Characteristic	**T18**	***F. hydatis***	***F. oncorhynchi***
Gliding Motility	+	+	-
Growth at 4 °C	+	-	NR
Growth at 2% Salinity	-	+	NR
Utilization of Citrate	+	-	-
Nitrate Reduction	-	+	+
Production of:			
Cytochrome Oxidase	-	-	+
Gelatinase	+	+	-
Dnase	-	+	-
Chitinase	-	(+)	NR
Brown Pigment from Tyrosine	-	-	+
Degradation of:			
Carboxymethyl Cellulose	-	+	NR
Tween 80	-	+	NR
Acid production from:			
D-Cellobiose	+	-	NR
D-Trehalose	-	(+)	NR
% Fatty Acid Content:			
$C_{15:0}$	ND	10.0	15.7
iso-$C_{15:1}$ G	1.2	4.0	5.0
iso-$C_{15:0}$	29.0	18.0	25.5
anteiso-$C_{15:0}$	4.0	ND	1.9
$C_{15:1}$ ω6*c*	1.5	5.0	7.6
iso-$C_{15:0}$ 3-OH	12.4	9.0	5.8
$C_{15:0}$ 3-OH	ND	2.0	ND
$C_{17:1}$ ω6*c*	Tr	4.0	2.4
iso-$C_{16:0}$ 3-OH	3.6	7.0	1.5
iso-$C_{17:0}$ 3-OH	14.5	8.0	5.4

Supplementary Table 4.4. Differential characteristics of *Flavobacterium* sp. strain S87 and related *Flavobacterium* spp. S87, results from this study; *F. resistens* (Ryu et al. 2008); *F. oncorhynchi* (Strain 631-08, Zamora et al. 2012a). +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported; Tr, traces (<1%); ND, not detected. ***,** contains $C_{16:1}$ *ω7c* and/or *iso*-$C_{15:0}$ 2-OH; $, includes only $C_{16:1}$ ω7c.

Characteristic	**S87**	***F. resistens***	***F. oncorhynchi***
Growth at pH of 5.0, 5.5, and 10.0	+	-	NR
Growth at 4°C	+	-	NR
Growth at 2% Salinity	-	+	NR
Production of:			
Cytochrome Oxidase	-	+	+
Gelatinase	+	-	-
Brown Pigment from Tyrosine	-	-	+
Nitrate Reduction	-	-	+
Assimilation of:			
D-Glucose	+	-	NR
L-Arabinose	+	-	+
D-Mannose	+	-	+
N-acetyl-glucosamine	+	-	+
Acid production from:			
D-Galactose	+	-	NR
Inositol	-	+	NR
D-Mannitol	-	+	NR
D-Lactose	-	+	NR
D-Melibiose	-	+	NR
% Fatty Acid Content:			
$C_{15:0}$	ND	11.4	15.7
iso-$C_{15:1}$ G	1.1	2.4	5.0
iso-$C_{15:0}$	24.2	35.7	25.5
$C_{15:1}$ ω6*c*	Tr	6.0	7.6
$C_{16:1}$ ω6*c*/$C_{16:1}$ ω7*c*	23.3	5.8*	9.8$
$C_{16:0}$	10.2	1.7	2.9
$C_{17:1}$ ω6*c*	Tr	1.5	2.4
iso-$C_{17:0}$ 3-OH	9.2	5.6	5.4

Supplementary Table 4.5. Differential characteristics of *Flavobacterium* sp. strain S21 and related *Flavobacterium* spp. S21, results from this study; *F. aquidurense* (Cousin et al. 2007); *F. frigidimaris* (Nogi et al. 2005). +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported; *, reported only as assimilation of substrate; Tr, traces (<1%); ND, not detected.

Characteristic	**S21**	***F. aquidurense***	***F. frigidimaris***
Gliding Motility	+	-	+
Growth at pH of 5.0., 5.5, and 7.0-10.0	+	-	NR
Growth at 2% Salinity	+	-	+
Growth as 3% Salinity	-	-	+
Hydrolysis of Tween 20	+	-	NR
Nitrate Reduction	+	-	-
Production of:			
Cytochrome Oxidase	-	+	-
Gelatinase	+	-	+
Chitinase	-	NR	+
Esterase	+	-	NR
Esterase Lipase	+	-	NR
α-fucosidase	+	-	NR
Brown Pigment from Tyrosine	-	+	NR
Acid Production from:			
L-Arabinose	-	-*	+
D-Xylose	-	NR	+
D-Galactose	-	-*	+
D-Fructose	-	+*	+
D-Mannitol	-	-*	+
D-Sucrose	-	-*	+
D-Raffinose	-	-*	+
% Fatty Acid Content:			
iso-$C_{15:1}$ G	2.9	6.0	ND
iso-$C_{15:0}$	27.1	15.1	26.7
$C_{15:0}$	ND	7.1	10.2
$C_{15:1}$ ω6*c*	1.8	6.7	5.4
$C_{16:0}$	4.8	Tr	1.2
iso-$C_{15:0}$ 3-OH	10.3	8.3	7.6
iso-$C_{17:1}$ ω9*c*	7.2	8.2	ND
iso-$C_{17:1}$ ω7*c*	ND	ND	6.6
$C_{15:0}$ 3-OH	ND	2.8	1.9
$C_{17:1}$ ω6*c*	1.4	6.4	5.9
iso-$C_{17:0}$ 3-OH	14.7	11.6	6.2

Supplementary Table 4.6. Differential characteristics of *Flavobacterium* sp. strain T76 and related *Flavobacterium* spp. T76, results from this study; *F. pectinovorum* (Dorey 1959; Bernardet et al. 2011); *F. hydatis* (Strohl and Tait 1978; Bernardet et al. 2011). +, positive test result; (+), weak positive test result; -, negative test result; NR, no result reported; Tr, traces (<1%); ND, not detected. *, also comprised of 15:0 2OH; $^{\$}$, comprised of C15:0 iso 2-OH and/ C $_{16:1}$ ω6*c* and/or C $_{16:1}$ ω7*c*.

Characteristic	**T76**	***F. pectinovorum***	***F. hydatis***
Congo Red Absorption	+	-	-
Growth on Marine Agar	+	-	-
Growth at 4°C	+	NR	-
Growth at 2% Salinity	+	-	+
Utilization of Citrate	+	-	-
Production of:			
Phenylalanine Deaminase	+	NR	-
Alginase	-	+	-
Chitinase	-	+	(+)
Brown Pigment from Tyrosine	+	-	-
Degradation of:			
Carboxymethyl Cellulose	-	+	+
Tween 80	-	NR	+
Acid Production from:			
Arabinose	-	+	(+)
Xylose	-	+	(+)
Lactose	-	+	(+)
Sucrose	-	+	(+)
% Fatty Acid Content:			
$C_{15:0}$	ND	7.0	10.0
iso-$C_{15:1}$ G	3.2	8.0	4.0
anteiso-$C_{15:0}$	3.1	2.0	ND
$C_{15:1}$ ω6*c*	1.0	6.0	5.0
$C_{16:1}$ ω6*c*/$C_{16:1}$ ω7*c*	15.7	5.0$^{\$}$	13.0*
$C_{16:0}$	10.0	ND	1.0
$C_{15:0}$ 3-OH	ND	2.0	2.0
$C_{17:1}$ ω6*c*	1.1	5.0	4.0
iso-$C_{16:0}$ 3-OH	3.1	5.0	7.0
$C_{16:0}$ 3-OH	6.2	ND	5.0

CHAPTER FIVE

CHARACTERIZATION OF A NOVEL *FLAVOBACTERIUM* SPP. INVOLVED IN THE MORTALITY OF COHO SALMON (*ONCORHYNCHUS KISUTCH*) IN THEIR EARLY LIFE STAGES

INTRODUCTION

Fish in their early life stages are particularly prone to infections, toxic chemical insults, and dietary deficiencies (Faisal 2007). This is particularly true for salmonid species in the Laurentian Great Lakes basin that endure low first year survival year (Johnson and Pecor 1969; Holey et al. 1995). A subset of these early mortalities has been collectively called early mortality syndrome (EMS), referring to yolk-sac and swim-up fry mortalities associated with an erratic, spinning, swimming behavior (Marcquenski and Brown 1997). In Europe, an identical syndrome (designated M74) was noticed in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) from the Baltic Sea (reviewed in Vuori and Nikinmaa 2007). While the exact etiology of EMS and M74 was not fully elucidated, depressed thiamine deficiencies are suspected (Fisher et al. 1996; Amcoff et al. 1998; Fitzsimons et al. 1999). The mechanism(s) by which thiamine deficiency leads to EMS is currently unknown.

Members of the genus *Flavobacterium* (Family Flavobacteriaceae, Phylum Bacteroidetes) pose a serious threat to wild and propagated fish worldwide (reviewed in Shotts and Starliper 1999). *Flavobacterium* spp. have been retrieved from diverse ecological niches such as soils, sediments, freshwater and marine water ecosystems, microbial mats, sea ice, mosquito guts, and diseased fish (Bernardet and Bowman 2006). While many flavobacteria are harmless, some are opportunistic or obligate pathogens that cause infections and disease in a wide variety of organisms, including invertebrates, fish, and humans (Bernardet and Bowman 2006). In

addition to the well-known fish pathogenic flavobacteria, such as *F. columnare*, *F. psychrophilum*, and *F. branchiophilum*, a number of novel fish-pathogenic flavobacteria have recently emerged (Bernardet et al. 2005; Flemming et al. 2007). Some flavobacteria (e.g*., F. psychrophilum*) have also been connected to early life stage mortalities of rainbow trout, *Oncorhynchus mykiss* (Nematollahi et al. 2003; Bebak et al. 2007). To this end, we report on the phenotypic, genotypic, and phylogenetic characteristics of multiple *Flavobacterium* spp. isolated from diseased coho salmon yolk sac and swim up fry with signs similar to EMS.

MATERIALS AND METHODS

Fish and sampling. Four-week old coho salmon in the late yolk sac to early swim up stages of development were collected from the Platte River State Fish Hatchery (Beulah, MI). The sampled fish were suffering from mortality that was associated with erratic, spiral swimming behavior. All of the moribund fish were isolated in a single raceway, where mortality reached 30% within one week. Originally, gametes were collected from feral coho salmon that returned to spawn at the Platte River Weir (Beulah, Michigan, Lake Michigan watershed) during the fall of 2003. As a prophylaxis against EMS, all coho salmon yolk sac fry at the state hatchery were treated with 750-ppm thiamine hydrochloride according to established protocols (Hornung et al. 1998; Koski et al. 1999; Wooster et al. 2000; Ketola et al. 2008).

Sixty moribund and 60 apparently healthy fish (from another raceway) were shipped alive to the Aquatic Animal Health Laboratory at Michigan State University, East Lansing, MI, USA. Fish were euthanized with an overdose of tricaine methane sulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA) and then were subjected to thorough gross examination, as well as virology, bacteriology, and parasitology analyses according to the American Fishery Society (AFS-FHS 2010) and the World Animal Health Organization (OIE) Aquatic Manual (OIE 2006).

Bacteriological analysis. Samples were aseptically collected from brains and kidneys after first disinfecting the outside of the fish with 70% ethanol. Due to the extremely small fish size, sterile inoculating needles (27 gauge) were used to collect brain and kidney tissues. All materials and reagents were purchased from Remel Inc. (Lenexa, KS) unless otherwise specified. Tissue samples were inoculated into Hsu-Shotts (Bullock et al. 1986) and trypticase soy broth and incubated at 22 ºC for 48 hrs. Any broth cultures displaying turbidity were then sub-cultured onto Hsu-Shotts and trypticase soy agars (TSA) and incubated for 24-48 hrs at 22 ºC. Tissue samples were concurrently inoculated into cytophaga broth and incubated at 15 ºC for 7-days, at which point any broth cultures with turbidity were sub-cultured onto Cytophaga agar. For long-term storage, pure bacterial stock cultures were frozen at -80 ºC in the appropriate fluid medium supplemented with 20% glycerol.

Characterization of recovered isolates. From the bacterial growth obtained from the brains of moribund fry, 12 isolates were visually differentiated by morphology and these isolates were selected for subsequent characterization. Single colonies from the representative isolates were streaked onto Hsu-Shotts agar, purity was verified under a dissecting scope, and then identified based on their cultural and morphological characteristics. Additional biochemical testing was performed on three isolates (CS30, CS36, and CS37) that included cytochrome oxidase, catalase reaction (3% H_2O_2), acid and gas production from glucose, indole production, hydrogen sulfide production, citrate utilization, Triple Sugar Iron reaction, ONPG (*o*-nitrophenyl-β-D-galactopyranoside), lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, esculin hydrolysis, phenylalanine deaminase (BD Scientific, Franklin Lakes, NJ), urease, starch hydrolysis, growth on MacConkey agar, gelatinase, and caseinase and lipase in a Hsu-Shotts agar base. All tests were inoculated with 24 hr old cultures and read within seven days post-inoculation. Moreover, the presence of flexirubin was determined using the potassium hydroxide

test (Bernardet et al. 2002), and Congo red adsorption, which tests the presence of extracellular galactosamine glycan (Johnson and Chilton 1966), was also performed. The activities of 19 different enzymes were examined using the API-ZYM kit (BioMerieux, Inc., Durham, NC) following the manufacturer's protocol and were incubated at 22 °C for 24 hours.

Antibiotic susceptibility tests were performed on the 12 isolates using standard agar disk diffusion (Bauer et al. 1966) with both Mueller-Hinton and Hsu-Shotts agars. The assayed antibiotics and their respective concentrations were as follows: polymyxin-B (300 iu), oxytetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), erythromycin (15 µg), ampicillin (10 µg), novobiocin (30 µg), azithromycin (15 µg), florfenicol (30 µg), and the vibriostatic agent 0/129 (2,4-diamino,6,7-di-isopropyl pteridine;10 µg).

PCR amplification of 16S rDNA genes for sequencing. Genomic DNA was extracted from the 12 bacterial isolates using the DNeasy blood and tissue kit (Qiagen Inc., Germantown, MD) following the manufacturer's protocol. 16S rRNA genes were amplified using the universal bacterial primers 27F (5' – AGA GTT TGA TCM TGG CTC AG – 3') and 1387R (5' – GGG CGG WGT GTA CAA GGC – 3'). Each PCR mixture of 100- µl contained 4- µl template DNA (20ng μl^{-1}), 0.2- µM of each primer, 0.25- mM of each deoxynucleoside triphosphate, and 2.5- U of *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) in a final concentration of 10mM Tris-HCl, 50mM KCl, and 1.5mM $MgCl_2$. PCR was performed under the following cycle conditions: an initial denaturation step at 94 °C for 5- min and 30 cycles of denaturation at 94 °C for 30-s, annealing at 58 °C for 30-s, and extension at 72 °C for 110-s. A final extension step at 72 °C for 7- min was performed. The PCR product was purified using QIAquick PCR purification kit (Qiagen) according to the manufacture's protocol. The purified PCR products were sequenced at the Research Technology Support Facility at Michigan State University. Partial sequences of

the rDNA genes were obtained using the 27F primer. The sequences have been deposited at National Institutes of Health GenBank under the following accession numbers: FJ479755 for CS29, FJ479756 for CS30, FJ479757 for CS31, FJ479758, for CS 34, FJ479759 for CS35, FJ479760 for CS36, FJ479761 for CS37, FJ479762 for CS38, FJ479763 for CS40, FJ479764 for CS41, FJ479765 for CS42, and FJ479766 for CS43).

Phylogenetic analysis. The phylogenetic relationships were analyzed using MEGA version 4.0 (Tamura et al. 2007). Nucleotide sequences of the 12 isolates were exported to and aligned in the Ribosomal Database Project (RDP; Cole et al. 2007) together with 25 publicly available strains in RDP. The aligned sequences were retrieved in MEGA and the phylogenetic relationships were inferred by the Neighbor-Joining algorithm (Saitou and Nei 1987) with the Kimura-2-parameter model (Kimura 1980) based on 676 bases of 16S rRNA sequences. The robustness of the topology was assessed by performing 1,000 bootstrap replicates.

Experimental challenge. An experiment was performed to assess the pathogenicity of *Flavobacterium* sp. strain CS36 that represented the dominant isolate recovered from the brains of moribund fry. Initially, 300 three-month post-hatch coho salmon fry (5.4 cm, 1.8 g average length/weight) from a lot without any previous disease signs/mortality events were obtained from the Platte River State Fish Hatchery. Prior to the infection study, 10 random coho salmon were sampled to assess *Flavobacterium* spp. infection status. The fish were allowed to acclimate to the new laboratory conditions until regular feeding was resumed. Fish were kept in flow-through PVC tanks at a flow rate of 1.26 L/min with the water temperature kept at 10 ºC$\pm$1 ºC.

The *Flavobacterium* sp. strain CS36 was revived in Hsu-Shotts broth (22 ºC for 48 -hrs), streaked onto solid Hsu-Shotts agar media, and a single colony was inoculated into a 500- ml of Hsu-Shotts broth, and incubated at 22 ºC for 72- hrs at 60 rpms on Daigger stir plates (Daigger

and Company, Vernon Hills, Illinois). Broth cultures were then centrifuged at 3500 rpm, rinsed in fresh Hsu-Shotts broth three times, pellets were resuspended in 0.85% sterile saline, serially diluted onto solid media for colony counts, and then immediately used for experimental infections.

Two experiments were carried out simultaneously. In both experiments, fish were exposed to three successive handling events (Flemming et al. 2007), which entailed netting the fish to be infected for 10-seconds and then releasing them back into their tank, immediately prior to challenge with the CS36 strain. In the first experiment, six groups of 10 fish each were used for intraperitoneal (IP) injection at doses of $3.0x10^{7}$ cfu100μL^{-1} to $3.0x10^{2}$ cfu100 μL^{-1} via serial tenfold dilutions in sterile 0.85% saline. Each fish received the respective dose in 100- µL of 0.85% saline using a 22-gauge needle (Becton Dickinson, Franklin Lakes, NJ). The needle was inserted approximately 3-5 mm dorsal to the anal fin and directed cranially into the intra-peritoneal space. An additional group of 10 fish was IP injected with 0.85% saline only (100 -µl $fish^{-1}$) and served as a negative control. In the second experiment, a group of 30 fish was immersed in 5- L of aerated 0.85% saline containing $3x10^{7}$ cfu mL^{-1} of CS36 for 60- min. An additional group (10 fish) was mock challenged in 5- L of 0.85% sterile saline for 60- min as a negative control.

Clinical signs and mortality were recorded for 28- days post-challenge. Dead fish were processed immediately for reisolation and histopathology. At the end of the 28 day observation period, the experiment was terminated. Half of the surviving fish were preserved whole in 10% buffered formalin for histopathological examination and the remainder were processed for clinical examination and bacterial reisolation on both TSA and Hsu-Shotts agar.

Histopathological analyses. The microscopic tissue alterations in experimentally infected fish were assessed in hematoxylin and eosin stained tissue sections. Fish were preserved in 10% buffered formalin, embedded within paraffin, sectioned at 5- µm, and stained with hematoxylin and eosin as described by Prophet et al. (1992). In total, eight negative control fish (four injected with sterile saline and four immersed in sterile saline) and 11 infected fish (4 IP injected with $3.0x10^{7}$ cfu $100\mu L^{-1}$, 3 IP injected with $3.0x10^{6}$ cfu $100\mu L^{-1}$, and 4 immersion infected in $3.0x10^{7}$ cfu mL^{-1}) were examined for histopathological alterations.

RESULTS

Bacterial isolation and isolate characterization. At the hatchery, affected fish exhibited a convulsive, spiral swimming behavior and congregated at the water inflow. Within two weeks, percent cumulative mortality amounted to 30% in the affected raceway. Following 48 -hrs of incubation at 22 ^{o}C, bacterial growth was noticed from brain and/or kidney samples of almost every moribund or dead fish that was tested. Similarly, after 48- hrs of incubation at 15 ^{o}C in cytophaga broth, almost every culture was turbid. When sub-cultured onto Hsu-Shotts, trypticase soy, and cytophaga agars, bacterial growth was pure and profuse, yielding yellow colonies with irregular margins. Representative isolates from all three media types were Gram negative, gliding, filamentous rods with pointed ends (3.5 µm x 0.5 µm), that produced catalase, tested positive for flexirubin, were Congo red negative, and did not produce cytochrome oxidase. Based on these characteristics, the 12 bacterial isolates were tentatively identified as *Flavobacterium* spp. (designated CS29-31, 34-38, 40-43). No bacteria were recovered from the brains or internal organs of apparently healthy fish.

The phylogenetic affiliation of the12 isolates was determined by comparative 16S rRNA sequence analysis. All 12 sequences were grouped within the genus *Flavobacterium* (Fig. 5.1). Nine out of 12 sequences were grouped into a single clade (CS29, 31, 34-38, 40, 41) and were essentially identical over the 676 aligned bases (Fig. 5.1a). This clade shared a 99.4% similarity with the previously described YO60 flavobacterial strain (Flemming et al. 2007). Sequence similarity between this clade with another known *Flavobacterium* species, *F. pectinovorum*, was 98.4%. The remaining three isolates, CS30, CS42, CS43 were closest to the other previously described *Flavobacterium* spp. strains; Wuba46 (100%), EP125 (100%), and WB 4.3-15 (99.9%; Fig. 5.1b).

Further phenotypic characterization was performed on isolates CS30, CS36, and CS37 (Table 5.1). *Flavobacterium* spp. CS36 and CS37 demonstrated 100% similarity with one another, but strain CS30 differed from CS36 and CS37 in its ability to produce phenylalanine deaminase and lipase and its inability to utilize citrate. According to the API ZYM assay, CS30 was also dissimilar in that it produced a weak positive reaction for trypsin, and positive reactions for α-galactosidase and N-acetyl- β-glucosaminidase, but a negative reaction for β-galactosidase. However, by conventional tube tests, all three isolates were positive for β-galactosidase (Table 5.2). Phenotypic comparisons of *Flavobacterium* Y060 (Flemming et al. 2007) with CS 36 and CS 37 showed that all exhibited gliding motility and produced caseinase, but differed in their ability to hydrolyze gelatin. No other phenotypic characteristics were reported by Flemming and colleagues for the strain YO60. Moreover, phenotypic characteristics for *Flavobacterium* sp. Wuba46 are not available and thus could not be compared to CS30. Phenotypic discrepancies between CS30 and *F. hydatis* included the former's inability to utilize glucose and the production of phenylalanine deaminase. The isolates were resistant to polymyxin-B, the vibriostatic agent 0/129, ampicillin, and azithromycin, but were sensitive to oxytetracycline (20-23 mm),

trimethoprim-sulfamethoxazole (15-24mm), erythromycin (12-19 mm), novobiocin (23-25 mm), and florfenicol (25-29 mm).

Experimental infection. No *Flavobacterium* spp. or other bacterial pathogens, viruses, or parasites were detected in the 10 randomly sampled coho salmon prior to experimental challenge. In coho salmon IP injected with CS36, both morbidity and mortality were observed. Morbidity varied amongst groups, with the most severe signs, such as melanosis, tachybranchia, lethargy, caudal fin erosion and necrosis (Fig. 5.2a), erythema in the caudal fin (Fig. 5.2b), occurring in fish groups IP challenged with $3.0x10^7$ cfu and $3.0x10^6$ cfu (the two highest challenge doses). Mortalities peaked to 10% after 5 days of infection for the $3.0x10^7$ cfu group and at 10% on day 8 for the $3.0x10^6$ groups, respectively; no mortalities occurred in the remaining IP infected groups. Dead fish had mild bilateral exophthalmia, ascites, severe hepatic and renal pallor, mild splenomegaly, and fluid within the swim bladder. In the immersion group, lethargy was the primary behavioral sign and no mortalities were observed. In both experiments, signs of infection appeared as early as two days post-infection and progressed in some cases, while in others, signs of healing were observed by the eighth day post-infection. *Flavobacterium* spp. were re-isolated from the brain, kidneys, ascites, and swim bladder fluid of dead and moribund fish. No *Flavobacterium* spp. were recovered from the kidneys or brains of fish surviving until the end of the experiment, nor from any of the control fish.

Histopathology. Microscopic changes observed in dead and moribund fish, as well as survivors following the 28- day observation period, included renal melano-macrophage hyperplasia, degeneration of kidney tubules, edema in the renal interstitial tissues, and proteinaceous casts within tubular lumens (Fig. 5.3). Sagittal sections from the grossly affected caudal peduncles

exhibited heterophilic cellulitis and myodegenerative changes (Fig. 5.4a and 5.4b). In infected fish suffering from ascites, a proteinaceous exudate was apparent in the coelomic cavity (Fig. 5.4c). Fish in the control group did not exhibit any histopathological abnormalities.

DISCUSSION

Flavobacterium spp. isolated in this study were retrieved from the brains and kidneys of moribund coho salmon fry that were tentatively diagnosed as suffering from EMS by hatchery personnel. Findings of this study, however, suggested that these *Flavobacterium* spp. are likely the etiologic agent of this mortality episode. This conclusion is based on a number of findings. First, the affected coho salmon fry at the Platte River State Fish Hatchery were prophylactically treated with a thiamine hydrochloride bath, a method that is considered very effective at combating the development of EMS (Hornung et al. 1998; Koski et al. 1999; Wooster et al. 2000; Ketola et al. 2008). Second, analysis of total thiamine contents in eggs and eyed eggs of 12 representative families of the same coho salmon spawning run averaged 3.48 ± 0.61 nmol/g (D. Honeyfield, unpublished data), meaning that thiamine was available in the eggs for the developing embryos. Third, *Flavobacterium* spp. were isolated from the brain and kidneys of nearly every moribund fish, which indicated that that the infection was both systemic and widespread in affected fish, whereas no flavobacteria were recovered from the examined healthy fish. Last, experimental infection studies demonstrated that strain CS36, originally isolated from moribund coho salmon yolk sac fry, caused morbidity and mortality in coho salmon fry. This is not the first time in which a *Flavobacterium* sp. has been associated with fry mortality of an *Oncorhynchus* sp. *Flavobacterium psychrophilum* causes heavy losses in the early life stages of coho salmon and rainbow trout (*Oncorhynchus mykiss*), collectively referred to as rainbow trout fry syndrome (Nematollahi et al. 2003), can be difficult to control (Bebak et al.

2007). In this study, *F. psychrophilum* was not isolated from healthy or diseased fish; rather *Flavobacterium* spp., that have never before been reported as causative agents of early life stage mortalities in fish, were the likely etiologic agents.

Biochemical, morphological, and 16s rDNA sequence investigations identified the 12 representative isolates retrieved from the brains and kidneys of moribund coho salmon as *Flavobacterium* spp. Within the genus *Flavobacterium*, the coho salmon strains clustered with many fish pathogenic *Flavobacterium* spp. (such as YO60, *F. saccarophilum, F. hydatis, F. succinicans, F. branchiophilum, F. johnsoniae,* and *F. psychrophilum;* (Bernardet and Bowman 2006; Flemming et al. 2007) as well as a number of recently isolated environmental strains from Europe with unknown pathogenicity to fish (O'Sullivan et al. 2006; Brambilla et al. 2007; Cousin et al. 2007). These findings are interesting in that they suggest that multiple *Flavobacterium* spp. can act synergistically during disease outbreaks in fish and that coho salmon fry are vulnerable to other *Flavobacterium* spp. that have not previously been associated with such syndromes in salmonids.

Phylogenetic analysis, along with cultural, morphological, and biochemical characterizations, provided evidence that nine of the 12 isolates belonged to one phylotype that also dominated the coho salmon fry disease outbreak. This strain exhibited substantial sequence resemblance (99.4%) to the YO60 *Flavobacterium* sp. isolated from longfin eels (*Anguilla mosambica*) in South Africa that were pathogenic to fish experimentally (Flemming et al. 2007). It also had a striking resemblance to *Flavobacterium* sp. WB 2.1-87, a strain that was isolated from the freshwater creek, Westerhöfer Bach, Harz Mountains, Germany (Brambilla et al. 2007), and *F. pectinivorum* (98.4%), which was degraded complex biopolymers. Strain CS43 also clustered with two *Flavobacterium* sp. strains of the Westerhöfer Bach that were not pathogenic. Strain CS30 was also of interest despite its infrequent isolation from moribund coho salmon due to its

100% sequence similarity with *Flavobacterium* sp. strain Wuba46, which was isolated from a freshwater aquifer in Germany (GenBank AF336355), and due to its clustering with *F. hydatis*, which was first isolated and described from diseased fish in two Michigan hatcheries (Strohl and Tait 1978; Bernardet et al. 1996). There are no reports on the pathogenicity of the Wuba46 isolate in fish, or on the EP125-*Flavobacterium* sp., isolated by O'Sullivan et al. (2006) from epilithon samples from the surfaces of stones in the River Taff in Cardiff, UK. Both strains, however, resembled *F. johnsoniae*, which has been associated with fish disease (Bernardet and Bowman 2006).

The phylogenetic relationship between the four coho salmon fry phylotypes is <98%, suggesting that they are not identical strains of *Flavobacterium* as per the criteria set by (Bernardet et al. 2002) for the taxonomy of the family Flavobacteriaceae. The heterogeneity of coho salmon strains was further verified by extensive biochemical and enzyme analyses performed on CS30 and CS36, which demonstrated that the two strains are phenotypically different. Both coho salmon strains exhibited strong protease activity on casein and/or gelatin substrates, indicating the presence of extracellular proteases that may enhance the virulence and/or disease progression in infected fish (Nematollahi et al. 2003). Both coho salmon strains were also different in their biochemical reactions when compared to common fish pathogenic flavobacteria, such as *F. johnsoniae, F. succinicans*, *F. saccarophilum*, *F. psychrophilum*, *F. columnare*, and *F. branchiophilum* (Reichenbach 1989). The mixed flavobacterial infection observed in the coho salmon fry case with four phylogenetically distinct strains of the same genus is rarely observed in bacterial diseases of vertebrates and deserves further bacteriological and epidemiological study.

Experimental infection studies with *Flavobacterium* sp. CS36 clearly demonstrate that it can cause morbidity and mortality in coho salmon fry. However, the clinical signs in the

experimentally infected fish were not identical to those originally observed in moribund yolk sac and swim-up fry. In particular, the spinning swimming behavior was not present despite successful re-isolation of CS36 from the brains of experimental fish. Moreover, mortalities were less than experienced with the spontaneously affected fry and many of the survivors showed signs of recovery and bacterial clearance. These differences may be related to the fact that experimental fish were several months older than the spontaneously infected fish, as older fish have a more completely ossified skull that may hinder penetration of bacteria into the brain. Moreover, yolk sac fry are often reared in incubators that are densely packed, a matter that can stress fish and enhance pathogen transmission. On the other hand, it has been frequently reported that, even with the most pathogenic *Flavobacterium* spp., experimental infections are often much milder than natural epizootics (Madsen and Dalsgaard 1999; Decostere et al. 2000). The stress of hatchery culture with fluctuations in the environment are believed to cause more pronounced disease in spontaneously infected fish (Madsen and Dalsgaard 1999; Decostere 2002; Nematollahi et al. 2003).

The source of infection for the coho salmon fry is unknown. Prior to incubation, fertilized eggs received an iodophore bath, which is a very effective egg surface disinfectant known for its bactericidal effects (Russell and Hugo 1987; Salvesen and Vadstein 1995). Egg incubators in the Platte River State Fish Hatchery receive sand-filtered spring water and embryonated eggs and yolk sac fry receive multiple formalin baths. Therefore, the likelihood that *Flavobacterium* spp. were introduced to the incubators from external sources, though possible, is minimal. Another possibility is intra-ovum transmission, which has been demonstrated in *F. psychrophilum* infections by Brown et al. (1997) and Cipriano (2005). Regardless of the route through which the coho salmon fry were infected, our results clearly demonstrated the presence of four *Flavobacterium* spp. strains in Michigan fishes that are remarkably similar to those

previously recovered from South Africa and Europe. How these novel flavobacteria emerged in the Platte River Hatchery warrants further investigation.

Transcontinental spread of pathogens through the international trade of fish and their products is not uncommon in the globalization era (Faisal 2007). For example, until the mid-1980s, *F. psychrophilum* was known only in North America, where it was originally isolated from coho salmon and rainbow trout (Borg 1948). It was then found in France (Bernardet et al. 1988), Germany (von Weis 1987), and Japan (Wakabayashi et al. 1991), and has since been reported wherever salmonids are cultured (Nematollahi et al. 2003). Since *F. psychrophilum* strains worldwide have an extremely low level of diversity (Nicolas et al. 2008), the international trade of fish eggs has been implicated for its widespread geographic range. The genetic similarity between *Flavobacterium* strains retrieved from coho salmon fry in Michigan, long-fin eels in South Africa, and surface water in Europe paints a picture that is similar to the case of *F. psychrophilum*, yet in the opposite direction. Michigan water is the recipient of many intentional and non-intentional introductions of fish and shellfish. For example, Michigan imported brown trout (*Salmo trutta*) from Germany in 1830, coho salmon in 1969 (Coon 1999), and was invaded by the zebra mussel (*Dreissena polymorpha*), a native of the Caspian Sea in Eurasia, in the mid-1980s (Nalepa and Schloesser 1993). These non-native species could have brought *Flavobacterium* spp. with them; unfortunately, there are no baseline data on the diversity of *Flavobacterium* spp. present in fish or the aquatic environment in Michigan or the Laurentian Great Lakes basin prior to the settlement of invasive species. Prevention of the spread of these emerging flavobacteria to aquaculture facilities, other areas in the Great Lakes, or other aquatic ecosystems in North America should be an important managerial priority. Additionally, the findings of this study underscore the complexity of the etiology of mortalities in fish in their early life stages, a matter that requires caution in diagnosis so as to choose an effective control strategy.

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Table 5.1. Biochemical characteristics of representative *Flavobacterium* spp. strains CS30, CS36 and CS37 recovered from coho salmon. Tests were inoculated with 24 hr old subcultures and incubated at 22 ºC for up to 7 days. Results are presented as + for positive reaction; - for negative reaction; (+) for weak positive reactions; and •, results not reported. For the TSI reaction, results are expressed as K/N/-/- designating alkaline slant/nonreactive butt/no production of H_2S/ no production of gas. For comparative purposes, biochemical characteristics of both *Flavobacterium pectinivorum* and *F. hydatis* are included as reported by Reichenbach (1989) and Bernardet and Bowman (1996).

	CS30	CS36	CS37	*F. pectinovorum*	*F. hydatis*
Gliding Motility	+	+	+	+	+
Flexirubin Type Pigment	+	+	+	+	+
Congo Red Absorption	-	-	-	-	-
Growth at 25 ºC	+	+	+	+	+
Glucose Utilization	-	-	-	+	+
TSI Reaction	K/N/-/-	K/N/-/-	K/N/-/-	•	•
Lysine Decarboxylase	-	-	-	•	-
Ornithine Decarboxylase	-	-	-	•	-
Arginine Dihydrolase	-	-	-	•	•
Phenylalanine Deaminase	+	-	-	•	-
Esculin Hydrolysis	+	+	+	+	+
Cytochrome Oxidase	-	-	-	+	-
Catalase	+	+	+	+	+
Lipase	+	-	-	+	+
Gelatinase	+	+	+	+	+
Caseinase	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+
Urease	-	-	-	-	-
Agar Degradation	-	-	-	-	-
ONPG	+	+	+	+	+
Susceptibility to 0129	-	-	-	+	-
Simmons Citrate	-	(+)	(+)	•	•
Production of Indole	-	-	-	-	-
Production of H_2S	-	-	-	-	-

Table 5.2. Enzyme activities of *Flavobacterium* strains CS30, CS36, and CS37 recovered from the brains of moribund coho salmon and tested by the API-ZYM kit. Results are recorded following incubation at 22 ºC for 24 hrs and presented as + for positive reaction; - for negative reaction; and (+) for weak positive reactions.

Enzyme	CS30	CS36	CS37
Alkaline Phosphatase	+	+	+
Esterase	(+)	(+)	(+)
Esterase Lipase	+	+	+
Lipase	-	-	-
Leucine arylamidase	+	+	+
Valine arylamidase	+	+	+
Cystine arylamidase	+	+	+
Trypsin	(+)	-	-
α-chymotrypan	-	-	-
Acid Phosphatase	+	+	+
Napthol-AS-BI-phosphohydrolase	+	+	+
α-galactosidase	+	-	-
β-galactosidase	-	+	+
β-glucoronidase	-	-	-
α-glucosidase	+	+	+
β-glucosidase	-	-	-
N-acetyl- β-glucosaminidase	+	-	-
α-mannosidase	-	-	-
α-fucosidase	-	-	-

Figure 5.1. A) Phylogenetic relationships of the 12 *Flavobacterium* isolates (CS29-31, 34-38, 40-43), retrieved from moribund coho salmon yolk sac and swim-up fry, identified by constructing a Neighbor-Joining tree with Kimura's 2-parameter model based on 676bp region of 16S rRNA sequences. The sequences were aligned in RDP. *Cytophaga hutchinsonii* ATCC 33406 was used as an outgroup. Bootstrap values greater than 50 were shown in the figure. B) Similarity index table constructed based on sequence identity of 676bp. CS36 represents the clade of 9 isolates. The highest similarities of the 12 isolates are highlighted.

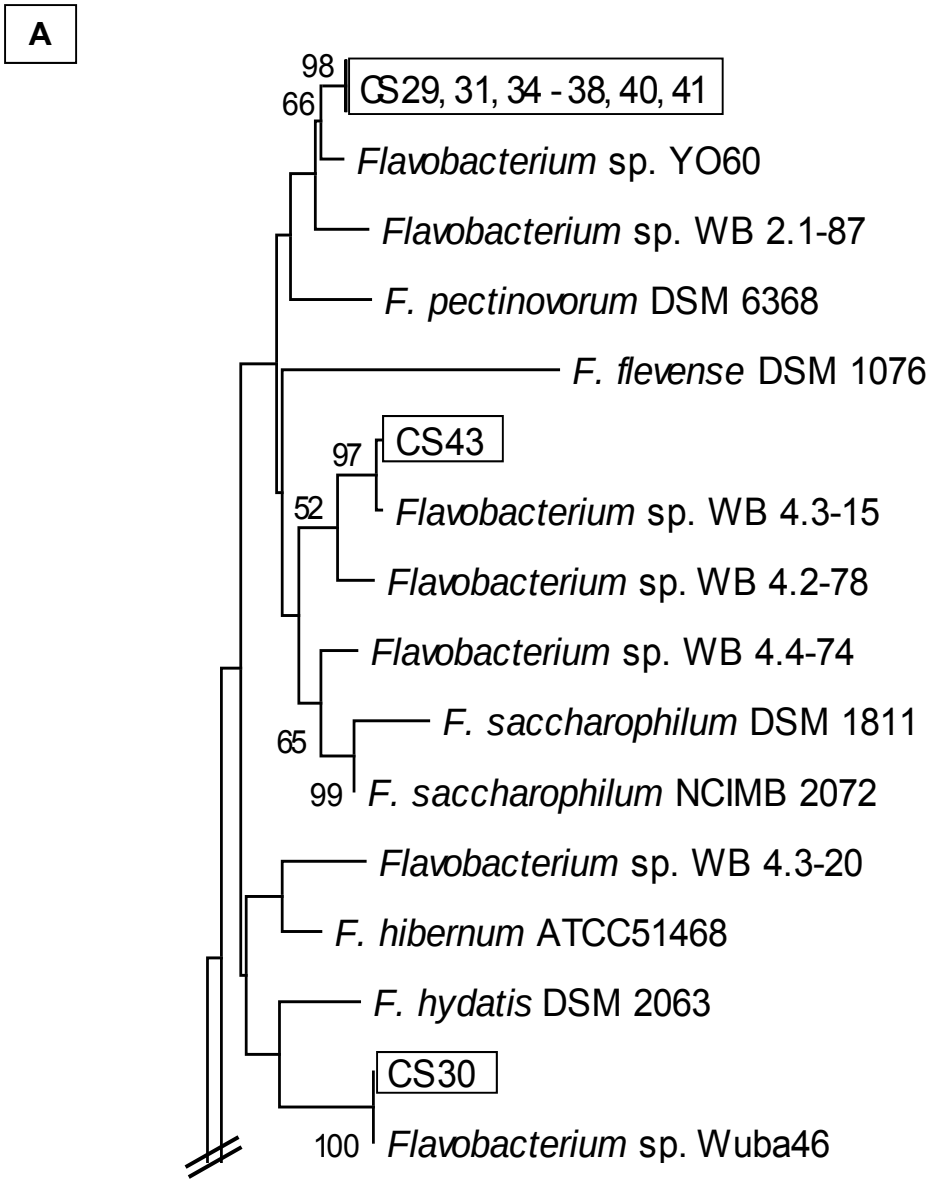


Figure 5.1 (cont'd)

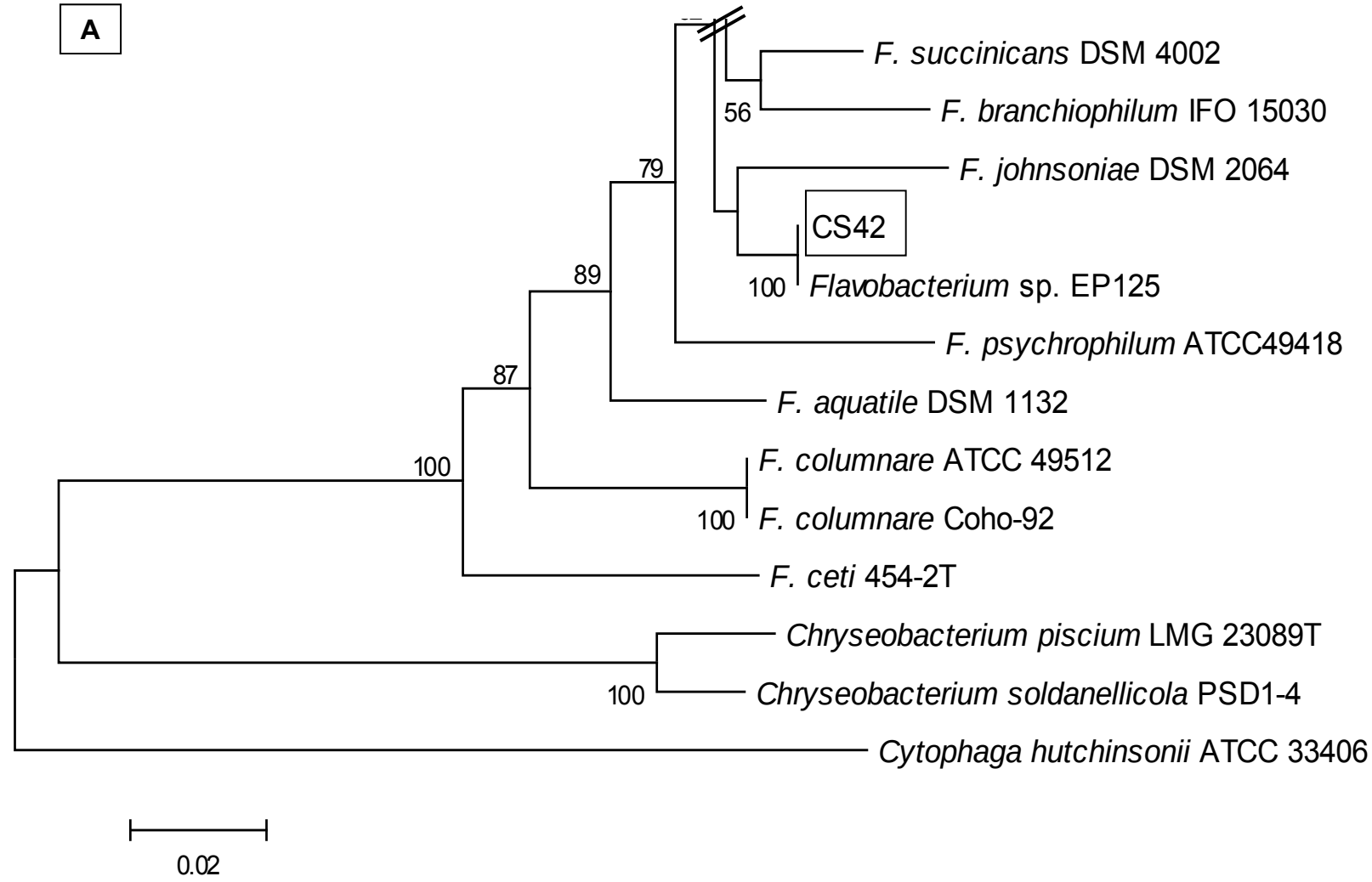


Figure 5.1 (cont'd)

B	CS36	CS43	CS30	CS42	YO60	**F.pecti*	WB4.3-15	Wuba46	EP125	*F.ceti*
CS36	1.000	---	---	---	---	---	---	---	---	---
CS43	0.978	1.000	---	---	---	---	---	---	---	---
CS30	0.970	0.975	1.000	---	---	---	---	---	---	---
CS42	0.970	0.970	0.975	1.000	---	---	---	---	---	---
YO60	**0.994**	0.979	0.972	0.970	1.000	---	---	---	---	---
**F. pecti*	0.984	0.979	0.964	0.963	0.982	1.000	---	---	---	---
WB4.315	0.979	**0.999**	0.973	0.969	0.978	0.981	1.000	---	---	---
Wuba46	0.970	0.975	**1.000**	0.975	0.972	0.964	0.973	1.000	---	---
EP125	0.970	0.970	0.975	**1.000**	0.970	0.963	0.969	0.975	1.000	---
F. ceti	0.908	0.904	0.907	0.904	0.910	0.904	0.904	0.907	0.904	1.000

Figure 5.2. Coho salmon (*Oncorhynchus kisutch*) fry (3 months old) intraperitoneally infected with the *Flavobacterium* sp. CS36 strain with A) extensive caudal fin erosion and necrosis that has progressed into the caudal peduncle and B) severe hemorrhage within caudal fin and caudal peduncle.

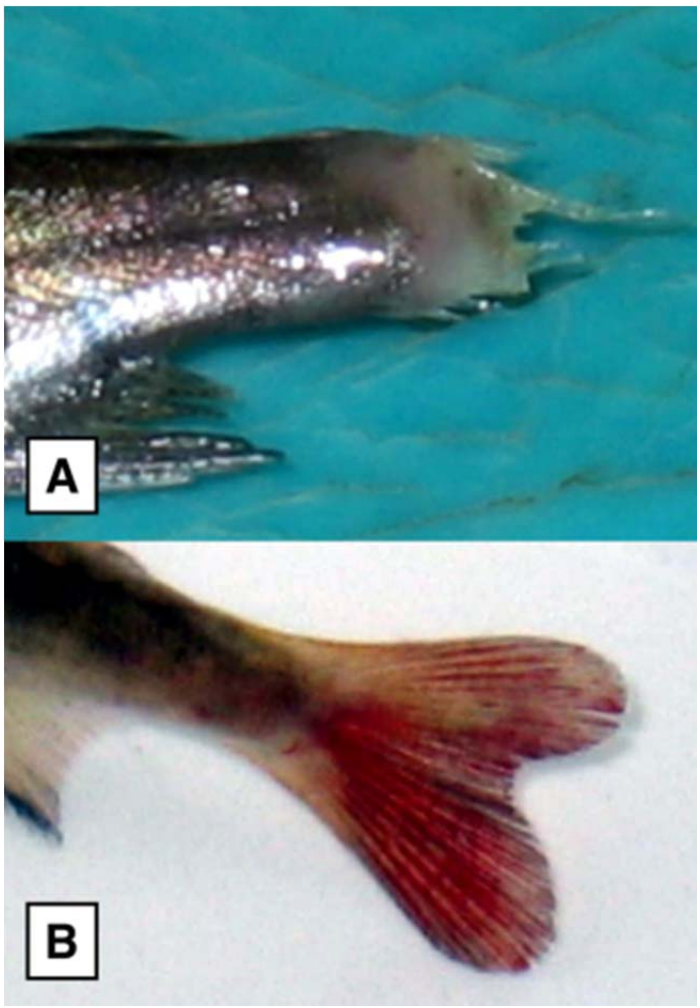


Figure 5.3. Sections of kidney tissues of 3-month coho salmon stained with H & E and magnified 400X showing: A) healthy kidney tissues of coho salmon from the negative control group, B) kidney tissues of an intraperitoneally infected fish with melanomacrophage hyperplasia, C) kidney tissues of an intraperitoneally injected fish with tubular degenerative changes and edema within the renal interstituim, D) kidney tissues of an intraperitoneally injected fish with renal tubular degeneration and proteinaceous casts in the tubular lumen (arrow).

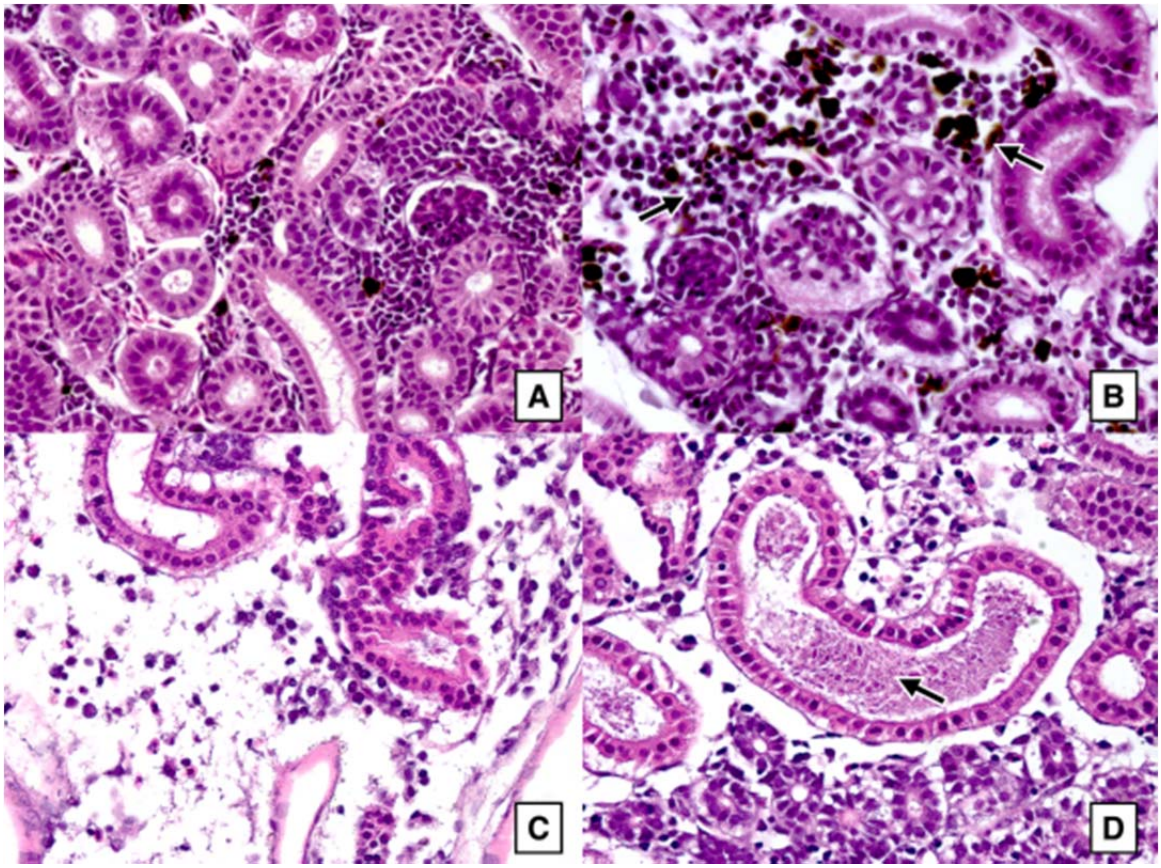
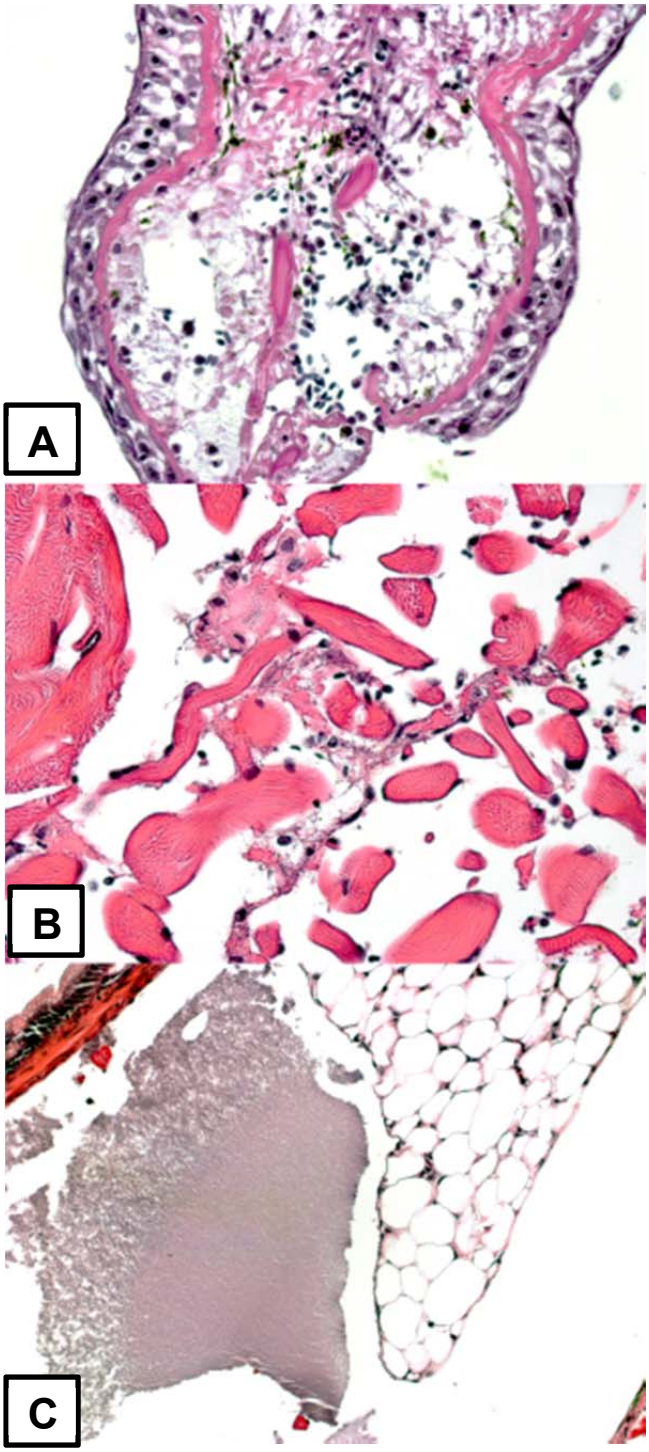


Figure 5.4. Sagittal section of a lesion on the caudal peduncle of a coho salmon that was intraperitoneally infected with the *Flavobacterium* sp. CS36 strain showing: A) heterophilic cellulitis (arrow), B) myodegeneration (arrows), and C) proteinaceous exudate within the coelom. The three H&E stained sections are shown at 400X magnification.



CHAPTER SIX

CHRYSEOBACTERIUM AAHLI SP. NOV., ISOLATED FROM LAKE TROUT (*SALVELINUS NAMAYCUSH*) AND BROWN TROUT (*SALMO TRUTTA*) IN MICHIGAN

INTRODUCTION

Members of the family Flavobacteriaceae (Reichenbach 1992; Bernardet et al. 1996), occupy an extremely wide range of ecological niches (reviewed in Jooste and Hugo 1999, Bernardet and Nakagawa 2006) and can be associated with disease in invertebrates (Li et al. 2010), amphibians (Xie et al. 2009), reptiles (Hernandez-Divers et al. 2009), birds (Segers et al. 1993), and mammals (Haburjak and Schubert 1997), including humans (Benedetti et al, 2011). In fish, serious diseases are caused by multiple species within the family Flavobacteriaceae, such as *Flavobacterium* spp. (Shotts and Starliper 1999; Starliper 2011), *Tenacibaculum* spp. (Suzuki et al. 2001), and *Chryseobacterium* spp. (Muddarris and Austin 1989), the latter of which have become an emerging problem across multiple continents (Bernardet et al. 2005). Since its original description by Vandamme et al. (1994), the genus *Chryseobacterium* has rapidly expanded to > 60 species. Concurrent with this rapid expansion are descriptions of numerous novel fish-associated *Chryseobacterium* spp., such as *C. piscium* (de Beer et al. 2006), *C. piscicola* (Ilardi et al. 2009), *C. arothri* (later heterotypic synonym of *C. hominis*; Kämpfer et al. 2009c) *C. chaponense* (Kämpfer et al. 2011), and *C. viscerum* (Zamora et al. 2012b). Within fish, *Chryseobacterium* spp. cause skin and muscle ulcerations (Bernardet et al. 2005; Ilardi et al. 2010; Kämpfer et al. 2011), gill hemorrhage and hyperplasia (Muddarris and Austin 1989; Muddarris et al. 1994), general signs of septicemia (Muddarris and Austin 1989; Muddarris et al. 1994; Bernardet et al. 2005; Zamora et al. 2012b). Some cause no overt signs of disease (de Beer et al. 2006; Cambell et al. 2008). Herein, we describe a novel fish-associated *Chryseobacterium* sp. recovered from diseased

salmonids in Michigan that is genotypically and phenotypically distinct from all other described *Chryseobacterium* spp.

MATERIALS AND METHODS AND RESULTS

Chryseobacterium sp. strain T62 was recovered from necrotic fins (Fig. 6.1) of a hatchery-reared yearling brown trout (*Salmo trutta;* Harrietta State Fish Hatchery, Wexford County, Michigan, USA) and *Chryseobacterium* sp. strain T68 was isolated from the kidney of a systemically infected yearling lake trout (*Salvelinus namaycush*; Marquette State Fish Hatchery, Marquette County, Michigan, USA). Tissues from affected organs were collected using sterile 1- µl disposable loops (Sigma), inoculated directly onto Hsu-Shotts Medium (HSM; Bullock et al. 1986), and incubated at 22 °C for 72- hrs, after which ~ 60 colony forming units (cfu) from the fin culture and 2 cfu from the kidney culture were observed. The semi-translucent colonies were golden yellow, ranged from 1.0-1.5 mm in diameter, and were low convex with entire margins. Both isolates were then sub-cultured onto HSM for purity and incubated for 24-48- hrs at 22 °C for initial morphological and phenotypic characterization. All reagents were purchased from Remel Inc. unless noted otherwise. Both isolates were Gram negative rods (1.5-2.0 µm in length) that had cytochrome oxidase (Pathotec test strips) and catalase (3 % H_2O_2) activity, contained a flexirubin-type pigment (using 3% KOH), and did not have cell wall-associated galactosamine glycans (0.01% Congo red solution, Bernardet et al. 2002). Both isolates were non-motile in Sulfur-Indole-Motility deeps (SIM) and did not glide according to the hanging-drop technique as described in Bernardet and Nakagawa (2006) under a light microscope. Isolates were cryopreserved at -80 °C in Hsu-Shotts broth supplemented with 20% glycerol.

In order to definitively determine the taxonomic position of the two bacterial strains, a polyphasic characterization was performed. Bacterial colonies from pure 48 -hr cultures on HSM were harvested and genomic DNA was extracted using a Qiagen DNeasy tissue kit (Qiagen Sciences) according to the manufacturer's protocol for Gram-negative bacteria. Quantification of extracted DNA was performed using the Quant-iT™ DS DNA assay kit in conjunction with a Qubit® flourometer (Invitrogen). Amplification of the near complete 16S rRNA gene was conducted via the polymerase chain reaction (PCR) using the universal primers 8F and 1492R (Sacchi et al. 2002; Table 6.1). The 50-µl PCR reaction for each sample contained a final concentration of 200- nM for each primer, 25- µl of 2x Go-Taq Green master mix (Promega), and 40- ng of DNA template, with DNase-free water comprising the remainder of the reaction mixture. The DNA amplification was carried out in a Mastercycler® Pro Thermalcycler (Eppendorf) with an initial denaturation step at 94°C for 2- min, followed by 35 cycles of amplification, which included denaturation at 94°C for 45- sec, annealing at 52°C for 45-sec, and elongation at 72°C for 90- sec. A final extension step was performed at 72°C for 5- min. Amplicons were combined with SYBR® Green gel stain (Cambrex Bio Science), run on a 1.5% agarose gel at 50 V for 40 min, and then visualized under UV exposure. A 1-kb plus ladder (Roche Applied Science) was used as a molecular marker.

Amplicon purification was conducted as described in Loch et al. (2011) and gene sequencing was carried out at the Genomics Technology Support Facility of Michigan State University using five primers (Table 6.1). Contigs were assembled in the BioEdit Sequence Alignment Editor (Hall 1999) using the contig assembly program (CAP). Generated sequences were initially analyzed using the nucleotide Basic Local Alignment Search Tool (BLASTN) software from the National Center for Biotechnology Information (NCBI, USA) to assess sequence similarity with other bacterial species contained within the nucleotide database of NCBI. Sequences for all

formally described *Chryseobacterium* spp. (n=61), as well as for *Candidatus "C. massiliase"*, *Candidatus "C. timonae"*, *Elizabethkingia miricola, E. meningosepticum,* and *Empedobacter brevis* (outgroups) were downloaded from NCBI and the EzTaxon-e database (Kim et al. 2012) and subsequently aligned with the sequences of strains T68 and T62. Neighbor-joining (NJ) analysis was then performed (Saitou and Nei 1987) using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 4.0), with evolutionary distances being calculated using the Maximum Composite Likelihood method (Tamura et al. 2004). Topology robustness was evaluated by bootstrap analysis based upon 1000 resamplings of the sequences. In order to confirm the phylogenetic validity of the initial NJ analysis, *Chryseobacterium* sp. T68/T62 and the 13 most closely related *Chryseobacterium* spp., along with eight *Chryseobacterium* spp. recovered from fish/fish products, the type species (*C. gleum*), Candidatus "*C. massiliae*", and members of the genus *Elizabethkingia* and *Empedobacter* (outgroup) were aligned as described previously and further analyzed using Bayesian and Maximum Parsimony (MP) analyses. Bayesian analysis was conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites. Default settings were used for the transition/transversion rate ratio (beta), topology (uniform), and prior probability distribution on branch lengths (unconstrained). The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Two independent analyses were conducted, both with 1 cold and 3 heated chains using the default heating parameter (temp=0.2). The initial 25% of Markov Chain Monte Carlo (MCMC) samples were discarded as burnin and sampling occurred every 100 generations. Maximum Parsimony analysis was conducted using a heuristic search of the tree space in PAUP 4.0 (Phylogenetic Analysis Using Parsimony, Swofford 2001) using the tree-bisection-reconnection as the branch-swapping algorithm. A total of 10,000 replicates were performed. Results from Bayesian and MP analyses were visualized in FigTree v1.3.1 (Rambaut 2009).

Strains T68 and T62 were nearly identical to one another (≥99%) across 1380 bp of the sequenced portion of the 16s rRNA gene. Initial searches using BLASTN demonstrated that bacterial strains T68 and T62 were members of the genus *Chryseobacterium* and T68 was most closely related to *C. ginsenosidimutans* (97.8% 16S rRNA gene sequence similarity%), *C. gregarium* (97.7%), *C. soldanellicola* (97.6%), *C. gambrini* (97.5%), *C. defluvii* (97.4%), and *C. piperi* (97.3%), *C. indoltheticum* (97.2%), *C. wanjuense* (97.1%), and *C. soli* (97.1%). T68 was also 97.7% similar to Candidatus *C. massiliae.* Sequence similarities were < 97% for all other recognized *Chryseobacterium* spp. present within the NCBI database. Interestingly, the most similar *Chryseobacterium* spp. strains recovered from diseased fish by Bernardet *et al.* (2005) were *Chryseobacterium* sp. JIP 13/00 (2) (97.4%) and *Chryseobacterium* sp. FRGDSA 4580/97 (96.8%), which were recovered from muscle lesions of neon tetras (*Paracheirodon innesi*) and siberian sturgeon (*Acipenser baeri*) fry, respectively. Neighbor-joining, MP, and Bayesian phylogenetic analyses yielded a similar topology (as indicated by filled circles when node was present in all three trees and a grey square when present in 2 of the 3 trees, Fig. 6.2) and demonstrated that *Chryseobacterium* sp. strains T68 and T62 formed an extremely well-supported cluster (bootstrap values of 100 and 97; posterior probability of 0.89, respectively) that was distinct from all other members of the genus *Chryseobacterium*.

Further morphological, physiological, and biochemical characterization was performed as recommended by Bernardet et al. (2002) and included: colony morphology on cytophaga agar (Anacker and Ordal 1955), growth on cetrimide and nutrient agars (Sigma), marine agar (Becton Dickinson Microbiology Systems), trypticase soy agar (TSA), TSA supplemented with 5% sheep erythrocytes, and MaConkey agar; growth on HSM at a pH of 5.0-10.0 in increments of 0.5; growth at 4°C, 15°C, 22°C, 37°C, and 42°C; growth on HSM at salinities ranging from 0%-5.0% in 1% increments; acid/gas from glucose and acid from sucrose (1% final concentration, phenol

red broth base); mixed acid fermentation and 2,3-butanediol production from glucose (methyl red - Voges-Proskauer test); triple sugar iron (TSI) reaction; hydrolysis of esculin (bile esculin agar); use of citrate as a sole carbon source (Simmon's citrate); production of indole and/or hydrogen sulfide on sulfur indole motility medium (SIM); lysis of hemoglobin (0.1% w/v) and degradation of collagen (0.1% w/v) and casein(5% w/v) and elastin (0.5%) as modified from Shotts et al. (1985) using HSM as the basal medium; activity for gelatinase (Whitman 2004), phenylalanine deaminase (Sigma), and DNase; activity for alginase (5% w/v alginic acid, Sigma, in HSM), pectinase (5% w/v pectin from apple, Sigma, overlay), chitinase (5% w/v chitin from crab shells, Sigma), and carboxymethylcellulase (0.15% w/v, Sigma, overlay; all modified from Reichenbach (2006) with HSM as basal medium); activity for chondroitin sulfatase C (0.2% w/v chondroitin sulfate sodium salt from shark cartilage, Sigma, HSM basal medium) and amylase (as modified from Lin et al. 1988 using HSM as basal medium); degradation of Tween 20 and Tween 80 (1% v/v, Sigma); brown pigment production from L-Tyrosine (0.5% w/v, Sigma; modified from Pacha and Porter (1968) using HSM as basal medium); and degradation of agar on TSA. When HSM was used as the basal medium in the morphological, physiological, and biochemical assays of this study, no gelatin or neomycin was added. Commercially available identification galleries (i.e., API 20E, API 20NE, API ZYM, and API 50CH; BioMerieux, Inc.) were inoculated according to the manufacturers protocol; however, tests were incubated at 22°C and read from 24- hrs post inoculation up until 7 days, with the exception of the API ZYM, which was read at 72- hrs.

For fatty acid profiling, *Chryseobacterium* sp. T68 was cultured on a medium containing 30- g of trypticase soy broth and 15- g of Bacto agar (Difco) per liter of distilled water for 24-hrs at 28 °C. Bacterial cells were then saponified, methylated to fatty acid methyl esters (FAMEs) and extracted according to the protocols of the commercial Sherlock Microbial Identification System (MIDI, version 4.0; Microbial Identification System Inc., Newark, DE). Separation of FAMEs was

conducted via gas chromatography on an Agilent 6890A series Gas Chromatograph with the 7683 autoinjector and autosampler tray module (Agilent Technologies, Inc.) using a fused silica capillary column (25mm x 0.2mm) with cross linked 5% phenylmethyl silicone. The carrier gas was H_2 and peak identification/integration was performed using the Agilent Chemstation and MIDI software (Agilent Technologies) and the Microbial Identification System database (Sasser 1990). The major fatty acid constituents of *Chryseobacterium* sp. T68 were *iso*-$C_{15:0}$, (30.9%), summed feature 3 ($C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*; 26.6%), *iso*-$C_{17:0}$ 3-OH (16.1%), $C_{16:0}$ (7.0%), $C_{16:0}$ 3-OH (5.1%), and smaller amounts of other fatty acids (Table 6.2). The predominant fatty acids typical of the genus *Chryseobacterium* are *iso*-$C_{15:0}$, *iso*-$C_{17:1}$ ω9*c*, *iso*-$C_{17:0}$ 3-OH, and summed feature 4 (*iso*- $C_{15:0}$ 2-OH and/or 16:1 ω7*t*; Segers et al. 1993; Bernardet et al. 2006). *Chryseobacterium* sp. T68 was quite distinct from the other most closely related *Chryseobacterium* spp. in the high percentage of $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c* (26.6%) compared to summed features containing the same fatty acid for *C. ginsenosidimutans* (9.5%; Im *et al.*, 2011), *C. gregarium* (0%; Behrendt et al. 2008), *C. soldanellicola* (9.7%; Park et al. 2006), *C. gambrini* (0%; Herzog et al. 2008), *C. defluvi* (9.4%; Kämpfer et al. 2003), and *C. piperi* (12.6%; Strahan et al. 2011). Interestingly, *Chryseobacterium* sp. T68 contained such a high percentage of $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c* that it was even higher than what is typical for members of the genus *Elizabethkingia* (17-19.6%; Kim et al. 2005). *Chryseobacterium* sp. T68 was also unique in that it contained a much smaller percentage of the hydroxy fatty acid *iso*-$C_{17:1}$ ω9*c* (1.4%) when compared to its closest *Chryseobacterium* relatives, including *C. ginsenosidimutans* (9.3%; Im *et al.*, 2011), *C. gregarium* (16.9%%; Behrendt et al. 2008), *C. soldanellicola* (14.6%; Park *et al.,* 2006), *C. gambrini* (6.7%; Herzog et al. 2008), *C. defluvi*

(4.8%; Kämpfer et al. 2003), and *C. piperi* (22.0%; Strahan et al. 2011). Other distinguishing characteristics of *Chryseobacterium* sp. T68 included the relatively large percentage of $C_{16:0}$ (7.0%) and $C_{16:0}$ 3OH (5.1%; Table 6.2). The biochemical characteristics of *Chryseobacterium* strains T68/T62 are described in the species description, while those characters that distinguish it from related *Chryseobacterium* spp. are listed in Table 6.3.

The results of the polyphasic characterization conducted in this study demonstrated that the two new isolates recovered from salmonids in Michigan indeed represent a novel *Chryseobacterium* sp., for which the name *Chryseobacterium aahlii* sp. nov. is proposed. Pathogenicity studies with *Chryseobacterium aahlii* sp. nov. demonstrated that it may be a facultative fish-pathogen in multiple Great Lakes salmonid species

Description of *Chryseobacterium aahli* sp. nov.

Chryseobacterium aahli (aah'li. N.L. gen. n. *aahli* of AAHL, in honor of the Aquatic Animal Health Laboratory of Michigan State University).

Cells are non-motile, non-gliding, Gram negative rods (1.5-2.0 µm in length) that do not contain cell wall-associated galactosamine glycans (do not absorb congo red). On cytophaga agar, colonies are semi-translucent, golden yellow in color due to the presence of a flexirubin-type pigment, range in size from 1.0-1.5 mm in diameter, and are low convex with entire margins. Growth occurs on nutrient, trypticase soy, Hsu-Shotts, cytophaga, and sheep's blood agars, but not on marine, MacConkey, or cetrimide agars at 22 °C. Grows well at a pH of 5.5-8.0, while weak/delayed growth occurrs at a pH of 5.0 and 8.5-10.0. Able to grow at 4 °C, 15°C, and 22°C, but not at 37 or 42°C. Can grow at a salinity from 0-2% (weakly at 2%), but not at 3-5%.

Does not produce indole or acid from glucose or sucrose in phenol red broth (1% final carbohydrate solution), and produces no reaction on triple sugar iron (TSI) slants without the production of H_2S or gas. Utilizes citrate as a sole carbon source. Produces catalase, cytochrome oxidase, gelatinase, caseinase, and elastase, but not alginase, pectinase, DNase, chitinase, lipase, phenylalanine deaminase, amylase, or carboxymethyl cellulose. Able to lyse hemoglobin and hydrolyze esculin, Tween 20, and Tween 80, but unable to degrade agar or chondroitin sulfate. Yields a brown pigment from tyrosine, and is variable in the production of collagenase. On the API 20E, negative for β- galactosidase, arginine dihydrolase, lysine and ornithine decarboxylase, urease, and tryptophan deaminase activities, and does not produce H_2S, indole, acetoin or acid from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose. Able to utilize citrate but does not reduce nitrate to nitrite or nitrogen gas. On the API 20NE, does not reduce nitrate, does not produce indole, arginine dihydrolase, or urease, and does not ferment glucose or utilize Para-NitroPhenyl-βD-Galactopyranoside, but does hydrolyze gelatin and esculin. Unable to assimilate D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, malic acid, trisodium citrate, or phenylacetic acid. Very weak assimilation of D-glucose, L-arabinose, and D-mannose. On the API ZYM, positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, α-chymotrypan, acid phosphatase, Napthol-AS-BI-phosphohydrolase, β-glucosidase, and N-acetyl- β-glucosaminidase activities, but negative for lipase, α-galactosidase, β-galactosidase, β-glucoronidase, α-mannosidase, and α-fucosidase. Variable in trypsin and α –glucosidase activities. On the API 50CH (using CHB/E medium), does not produce acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetyl-glucosamine, arbutin, D-cellobiose, D-maltose, D-lactose, D-melibiose, inulin, D-melezitose, D-

raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2- ketogluconate, and potassium 5-ketogluconate. Very weak acid production from D-glucose, D-trehalose, and gentibiose, and variable acid production from sucrose. One of the two strains gave a very weak positive result for acid production from D-fructose, D-mannose, L-rhamnose, amygdalin, and salicin. The fatty acid profile is primarily comprised of *iso*-$C_{15:0}$, (30.9%), summed feature 3 ($C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*; 26.6%), *iso*-$C_{17:0}$ 3-OH (16.1%), $C_{16:0}$ (7.0%), and $C_{16:0}$ 3-OH (5.1%),

The type strain is strain $T68^T$ (GenBank accession number - JX287893) isolated from the kidneys of a yearling lake trout (*Salvelinus namaycush*).

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Table 6.1. Primers used for amplification and sequencing of the 16S rRNA gene of *Chryseobacterium* sp. strains T68 and T62.

Primers for 16S rRNA gene amplification	
8F	5’ AGT TGA TCC TGG CTC AG 3’
1492R	5’ ACC TTG TTA CGA CTT 3’
Primers for 16S rRNA gene sequencing	
8F	5’ AGT TGA TCC TGG CTC AG 3’
518F	5’ TAC CAG GGT ATC TAA TCC 3’
1205F	5’ AAT CAT CAC GGC CCT TAC GC 3’
800 R	5’ CCA GCA GCC GCG GTA ATA CG 3’
1492R	5’ ACC TTG TTA CGA CTT 3’

Table 6.2. Cellular fatty acid profiles (%) of *Chryseobacterium* sp. strain T68, other closely related *Chryseobacterium* spp., and members of the genus *Elizabethkingia*. *Chryseobacterium* sp. strain T68 was grown on trypticase soy broth agar (TSBA) for 48h at 28°C in this study; 1, *C. ginsenosidimutans* grown on grown on nutrient agar for 48h at 27°C and fatty acids comprising less than 1% were not published (Im et al. 2011); 2, *C. gregarium* grown on TSA for 24h at 28°C (Behrendt et al. 2008); 3, *C. soldanellicola* grown on TSA for 48h at 30°C (Park et al. 2006); 4, *C. gambrini* grown on TSA for 24h at 30°C (Herzog et al. 2008); 5, *C. defluvi* ; 6, *C. piperi* grown on TSBA for 24h at 30°C (Strahan et al. 2011); 9, *C. scopthalmum* (ATCC 700039; this study); 7, *C. gleum*, 8, *C. balustinum,* 10, *Elizabethkingia meningoseptica,* and 11, *E. miricola* were grown on TSA for 24h at 28°C (Kim et al. 2005).

Table 6.2 (cont'd)

Fatty Acid	T68	1	2	3	4	5	6	7	8	9	10	11
iso-C13: 0	Tr	-	1.3	Tr	2.4	3.0	1.1	-	1.2	Tr	1.3	2.0
14:0	Tr	-	Tr	-	Tr	-	-	-	-	-	-	-
15:1 iso F	-	-	-	-	-	-	-	-	-	Tr	-	-
iso-C15: 0	30.9	50.3	35.1	41.8	57.4	56.3	36.6	35.6	36.8	39.0	43.9	46.4
anteiso-C15: 0	2.6	3.8	9.1	1.9	Tr	2.5	Tr	-	1.1	1.2	1.1	1.0
14:0 3OH/16:1 iso I	Tr	-	-	-	-	-	-	-	-	-	-	-
16:0 iso	Tr	-	Tr	-	-	-	-	-	-	-	-	-
16:1 w6c/16:1 w7c	26.6	-	-	†	-	†	‡	†	†	7.7	†	†
16:1 w5c	1.5	-	-	-	-	-	-	-	-	-	-	-
C16: 0	7.0	-	Tr	1.4	1.4	1.3	1.1	1.3	1.4	1.1	Tr	1.2
iso-C15:3-OH	2.5	5.2	2.8	2.7	2.7	2.6	4.2	2.5	2.7	3.3	2.8	3.0
15:0 2OH	Tr	-	Tr	†	-	†	-	†	†	Tr	†	†
iso-C15 : 0 2-OH	-	-	10.6	-	8.3	-	‡	-	-	-	-	-
iso-C17: 1 w9c	1.4	9.3	16.9	14.6	6.7	4.8	22.0	20.2	27.5	22.0	7.8	6.6
anteiso 17:1 B	-	-	-	-	-	-	-	-	-	Tr	-	-
iso-17: 0	Tr	-	Tr	Tr	2.3	2.1	Tr	1.5	1.0	1.0	Tr	Tr
iso-C17 : 1	-	-	Tr	-	-	-	-	-	-	-	-	-
iso-16: 0 3- OH	Tr	-	Tr	Tr	-	-	1.8	-	-	Tr	Tr	Tr
16:0 3OH	5.1	-	1.2	-	Tr	Tr	-	1.2	1.2	1.4	2.6	3.0
18:1 w9c	Tr	-	-	-	-	-	-	-	-	Tr	-	-
C18 : 1w5c	-	-	1.2	Tr	-	-	-	-	-	-	Tr	Tr
iso-C17: 0 3-OH	16.1	21.9	10.0	17.7	16.2	15.9	17.9	20.8	16.3	19.4	14.6	15.3
C17:0 2-OH	1.8	-	Tr	Tr	-	-	Tr	-	-	-	-	-
Unknown (ECL=13.556)	-	-	1.2	2.3	-	Tr	-	1.5	1.4	-	1.9	1.5
Unknown (ECL=16.582)	-	-	1.1	1.8	-	Tr	-	1.4	1.0		1.6	Tr
12:0 aldehyde	Tr	-	-	-	-	-	-	-	-	-	-	-
Summed feature 3							12.6 ‡					
Summed feature 4	-	9.5*		9.7†	-	9.4†	-	14.0†	8.4†		19.6†	17.0†

This study: Summed feature 3= **16:1 w6c/16:1 w7c.** Summed feature 4= 17:1 iso I and/or 17:1 anteiso B. ‡, summed feature 3 reported as 16:1 w6c/16:1 w7c and/or iso C15:0 2-OH. *, summed feature 4 reported as 16:1 w6c and/or C15:0 2-OH. †, summed feature 4 reported as iso-C15:0 2-OH and/or C16:1 w7*c/t.*

Table 6.3. Biochemical and physiological characteristics of *Chryseobacterium* sp. strains T68 and T62, other closely related *Chryseobacterium* spp., as well as members of the genus *Elizabethkingia*. Results are from: T68 and T62 (this study); 1, *C. ginsenosidimutans* (Im et al. 2011); 2, *C. gregarium* (Behrendt et al. 2008); 3, *C. soldanellicola* (Park et al. 2006; 4, *C. gambrini* (Herzog et al. 2008); 5, *C. defluvi* (Kim et al. 2005 and Kämpfer et al. 2003); 6, *C. piperi* (Strahan et al. 2011); 7, *C. scopthalmum* (ATCC 700039; this study); 8, *C. gleum* (Holmes et al. 1984a; Bernardet et al. 2006); 9, *C. balustinum* (Kim et al. 2005, Bernardet et al. 2006), 10, *Elizabethkingia meningoseptica* (Kim et al. 2005; Bernardet et al. 2006); 11, *E. miricola* (Kim et al. 2005).

Assay	T68	T62	1	2	3	4	5	6	7	8	9	10	11
Able to grow at:													
pH 5.0	(+)	(+)	-	NR	+	NR	NR	-	+	NR	NR	NR	NR
4-5 °C	+	+	-	(+)	+	-	-	-	+	-	(+)	-	-
37 °C	-	-	+	-	+	+	+	+	[+]	+	-	+	+
2% NaCl	(+)	(+)	-	NR	+	-	NR	+	+	NR	NR	NR	+
Production of:													
Indole	-	-	-	-	-	-	+	+	+	+	+	+	+
DNase	-	-	+	-	-	NR	NR	+	NR	v	+	+	+
Amylase	-	-	+	+	-	-	+	+	-	v	v	-	v
Arginine dihydrolase	-	-	+	-	-	+		-	-	NR	NR	NR	-
Urease	-	-	+	-	-	+	-	+	+	v	v	v	+
Esterase	+	+	-	-	+	NR	NR	(+)	+	-	NR	-	+
Cystine arylamidase	+	+	-	-	-	NR	NR	(+)	+	-	NR	v	+
Degradation of:													
Tween 80	+	+	NR	+	-	+	NR	+	+	v	+	-	+
Trypsin	+	[+]	-	-	-	NR	NR	-	+	-	NR	+	+
α-chymotrypan	+	+	-	-	-	NR	NR	-	+	-	NR	v	-
Assimilation of:													
D-Glucose	[+]	[+]	+	-	+	+	+	(+)	(+)	NR	+	+	+
L-Arabinose	[+]	[+]	+	-	+	+	-	-	-	NR	-	-	-
D-Mannose	[+]	[+]	+	-	+	+	+	(+)	(+)	NR	NR	NR	+
D-Maltose	-	-	+	-	+	+	+	(+)	-	NR	-	+	+
Acid from:													
Glucose	v *	v *	-	+	(+)	+	+	-	(+)	v	+	+	+
L-Arabinose	-	-	(+)	+	(+)	NR	-	NR	-	NR	-	-	-
D-Fructose	[+]	-	NR	-	+	NR	+	NR	-	v	+	+	+
D-Mannose	-	[+]	NR	+	+	NR		NR	-	NR	NR	NR	+
L-Rhamnose	[+]	-	NR	-	+	+	-	NR	-	-	NR	NR	-
Salicin	-	[+]	NR	+	-	NR	-	NR	-	v	-	-	-
D-Maltose	-	-	NR	+	+	NR	+	NR	-	v	-	+	+
D-Lactose	-	-	NR	+	-	+	-	-	-	-	-	+	+
D-Sucrose	(+)	-	-	+	-	+	-	NR	-	-	v	-	-

Table 6.3 (cont'd)

+, positive result; [+], very weak and/or delayed positive result; (+), weak positive result; -, negative result; v, variable result; NR, result not reported; *, negative in phenol red broth and on the API 20E, but very weakly positive on the API 50CH.

Figure 6.1. Left pectoral fin of a yearling brown trout (*Salmo trutta*) from which *Chryseobacterium* sp. strain T62 was recovered. Note severe necrosis and hemorrhage of the fin, with concurrent exposure of the eroded fin rays (arrow).

Figure 6.2. Dendrogram generated using the Neighbor-joining method in MEGA4 that depicts the phylogenetic relationship between *Chryseobacterium* sp. strains T68/T62, the 13 most closely related *Chryseobacterium* spp., along with 8 *Chryseobacterium* spp. recovered from fish/fish products, the type species (*C. gleum*), Candidatus "*C. massiliae*", and members of the genus *Elizabethkingia* and *Empedobacter* (outgroup). Bootstrap values >50% (expressed as percentages of 1000 replicates) are presented at the branch nodes. Filled circles are present when that node was also present in the maximum parsimony and Bayesian trees, while grey squares indicate that that node was present using 2 of the 3 methods. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

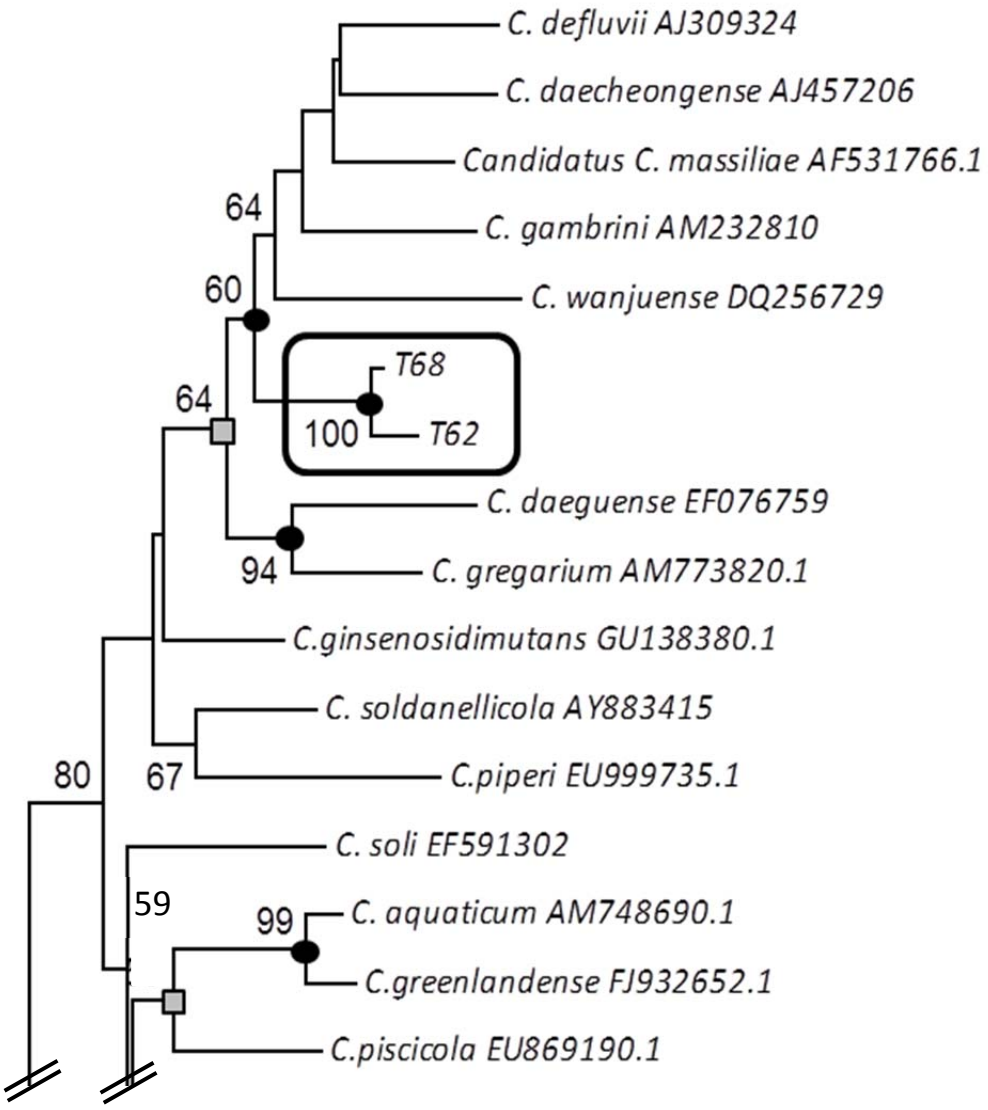
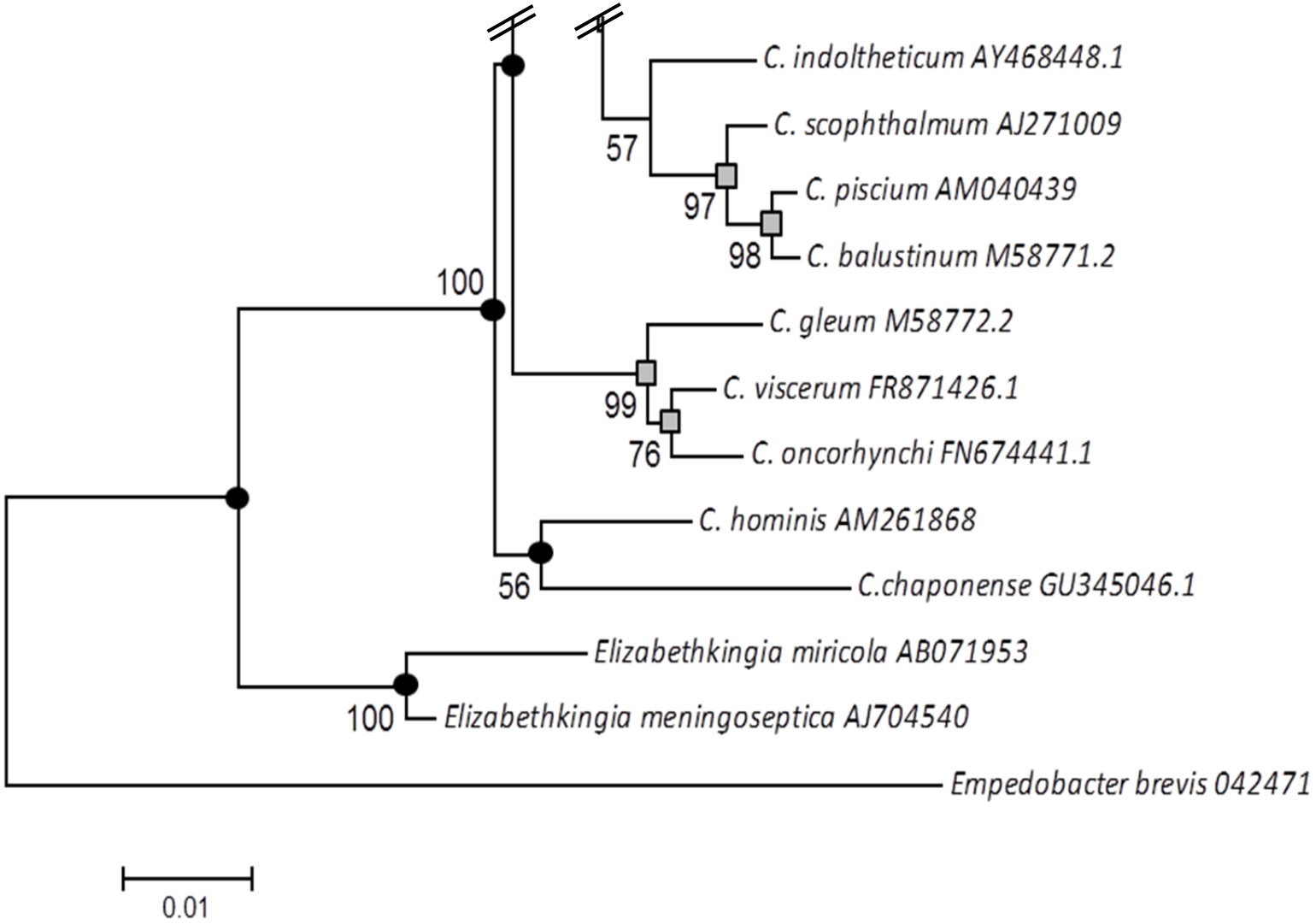


Figure 6.2 (cont'd)



CHAPTER SEVEN

FLAVOBACTERIUM SPARTANI SP. NOV., A NEWLY DESCRIBED PATHOGEN OF GREAT LAKES FISHES

INTRODUCTION

Flavobacteriosis poses a serious threat to wild and propagated fish stocks worldwide. Most flavobacteriosis outbreaks in freshwater fishes have been attributed to three *Flavobacterium* spp.; namely, *F. psychrophilum, F. columnare,* and *F. branchiophilum* (reviewed in Shotts and Starliper 1999; Austin and Austin 2007). On occasion, other fish-pathogenic flavobacteria have been reported in association with diseased fish, such as *F. johnsoniae* (Suebsing and Kim 2012), *F. succinicans* (Anderson and Ordal 1961), *F. hydatis* (Strohl and Tait 1978), and a number of uncharacterized yellow-pigmented bacteria (reviewed in Austin and Austin 2007). Recently, a number of novel *Flavobacterium* spp. were isolated from diseased fish in Europe and South America, including *F. chilense* and *F. araucananum* (Kämpfer et al. 2012), as well as *F. oncorhynchi* (Zamora et al. 2012a). Depending on the species, flavobacteriosis can cause acute, subacute, and chronic infections, characterized by gill damage (Wakabayashi et al. 1989), bacteremia (Starliper 2011), and deep necrotic ulcerations (Shotts and Starliper 1999).

Recent research in this laboratory highlighted the heterogeneous assemblage of *Flavobacterium* spp. associated with diseased fishes in Michigan. Herein, we describe a novel fish-pathogenic *Flavobacterium* sp. recovered from diseased salmonids in Michigan, USA.

MATERIALS AND METHODS AND RESULTS

Flavobacterium sp. strains T16 and S12, which belong to a cluster of *Flavobacterium* sp. isolates (n=10) suspected of comprising a novel bacterial species (Cluster XVIII, Chapter 2), were originally recovered from kidneys of feral spawning adult Chinook salmon (*Oncorhynchus tshawytscha*; Swan River Weir, Presque Isle County, Michigan, USA) and cultured Chinook salmon fingerlings suffering mortality (Thompson State Fish Hatchery, Schoolcraft County, Michigan, USA), respectively. Disease signs among systemically-infected feral Chinook salmon (4/60 infected) included mild to severe unilateral exophthalmia, muscular ulcerations, hepatic pallor, and friable kidneys. In hatchery-reared Chinook salmon fingerlings, large numbers of filamentous bacteria covering necrotic gill lamellae and hepatic pallor were observed. Tissues from affected organs were collected using sterile 10- µl disposable loops (Sigma-Aldrich Corp., St. Louis, MO) for the feral Chinook salmon, whereas 1- µl disposable loops were used for the smaller Chinook salmon fingerlings. Inocula were plated directly onto Hsu-Shotts Medium (HSM; Bullock et al. 1986), and incubated at 22 °C for 72- hrs. The resultant bacterial colonies grew nearly flat, had irregular spreading margins, were semi-translucent, and were dark yellow. Isolates were then sub-cultured onto HSM for purity and incubated for 24-48 -hrs at 22 °C for initial morphological and phenotypic characterization. All reagents were purchased from Remel Inc. (Lenexa, Kansas, USA) unless noted otherwise. Both isolates were Gram negative rods (3.0-5.0 µm in length) that had catalase (3 % H_2O_2) activity, contained a flexirubin-type pigment (using 3% KOH), but did not have cytochrome oxidase (Pathotec test strips) activity or cell wall-associated galactosamine glycans (0.01% Congo red solution, Bernardet et al. 2002). Both isolates were non-motile in sulfur-indole-motility deeps (SIM) but were motile via gliding according to the

hanging-drop technique described in Bernardet and Nakagawa (2006). Isolates were cryopreserved at -80 °C in Hsu-Shotts broth supplemented with 20% glycerol.

In order to definitively classify the two, apparently novel, bacterial strains, polyphasic characterizations were performed. Bacterial colonies from pure 48- hr cultures on HSM were harvested and genomic DNA was extracted using a Qiagen DNeasy tissue kit (Qiagen Sciences) according to the manufacturer's protocol. Quantification of extracted DNA was performed using the Quant-iT™ DS DNA assay kit in conjunction with a Qubit® flourometer (Invitrogen). Amplification of the near complete 16S rRNA gene was conducted via the polymerase chain reaction (PCR) using the universal primers 8F and 1492R (5' AGTTGATCCTGGCTCAG 3') and 1492R (5' ACCTTGTTACGACTT 3'; Sacchi et al. 2002). The 50- µl PCR reaction for each sample contained a final concentration of 200-nM for each primer, 25- µl of 2x Go-Taq Green master mix (Promega, Madison, WI,), and 40 ng of DNA template, with DNase-free water comprising the remainder of the reaction mixture. DNA amplification was carried out in a Mastercycler® Pro Thermalcycler (Eppendorf, Hauppauge, NY) with an initial denaturation step at 94°C for 2- min, followed by 35 cycles of amplification, which included denaturation at 94°C for 45- sec, annealing at 52°C for 45- sec, and elongation at 72°C for 90- sec. A final extension step was performed at 72°C for 5-min. Amplicons were combined with SYBR® Green gel stain (Cambrex Bio Science), run on a 1.5% agarose gel at 50 V for 40- min, and then visualized under UV exposure. A 1-kb plus ladder (Roche Applied Science) was used as a molecular marker.

Amplicon purification was conducted as described in Loch et al. (2011) and gene sequencing was carried out at the Genomics Technology Support Facility of Michigan State University using the following five primers: 8F, 1492R (see above), 518F (5' TACCAGGGTATCTAATCC

3'), 800R (5' CCAGCAGCCGCGGTAATACG 3'), and 1205F (5' AATCATCACGGCCCTTACGC 3'). Contigs were assembled in the BioEdit Sequence Alignment Editor (Hall 1999) using the contig assembly program (CAP). Generated sequences were initially analyzed using the nucleotide Basic Local Alignment Search Tool (BLASTN) software from the National Center for Biotechnology Information (NCBI, USA) to assess sequence similarity with other bacterial species contained within the nucleotide database of NCBI. Sequences for all formally described and candidate *Flavobacterium* spp., as well as *Capnocytophaga ochracea* (outgroup) were downloaded from NCBI and the EzTaxon-e database (Kim et al. 2012) and subsequently aligned with the sequences of strains T16 and S12. Neighbor-joining (NJ) analysis was then performed (Saitou and Nei 1987) using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 5.0), with evolutionary distances being calculated using the Maximum Composite Likelihood method (Tamura et al. 2004). Topology robustness was evaluated by bootstrap analysis based upon 1000 re-samplings of the sequences. In order to confirm the phylogenetic validity of the initial NJ analysis, *Flavobacterium* sp. T16/S12 and the 12 most closely related *Flavobacterium* spp., along with 5 *Flavobacterium* spp. also recovered from fish/fish products, the type species (*F. aquatile*), and *C. ochracea* (outgroup) were aligned as described above and further analyzed using Bayesian and Maximum Parsimony (MP) analyses. Bayesian analysis was conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites. Default settings were used for the transition/transversion rate ratio (beta), topology (uniform), and prior probability distribution on branch lengths (unconstrained). The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Two independent analyses were conducted, both with one cold and three heated chains using the default heating parameter (temp=0.2). The initial 25% of Markov Chain Monte Carlo (MCMC) samples were discarded as burnin and

sampling occurred every 100 generations. Maximum Parsimony analysis was conducted using a heuristic search of the tree space in PAUP 4.0 (Phylogenetic Analysis Using Parsimony, Swofford 2001) using the tree-bisection-reconnection as the branch-swapping algorithm. A total of 10,000 replicates were performed. Results from Bayesian and MP analyses were visualized in FigTree v1.3.1 (Rambaut 2009).

Strains T16 and S12 were nearly identical (≥99%) across 1383 bp of sequenced portion of the 16s rRNA gene. Initial searches using BLASTN demonstrated that these bacteria were members of the genus *Flavobacterium* and were most closely related to *F. aquidurense* (98.3%), *F. araucananum* (98.2%), and *F. frigidimaris* (98.1%), while similarity to the *Flavobacterium* type species, *F. aquatile,* was 98.3%. Interestingly, sequences available within GenBank are from similar strains (>99%) recovered from rainbow trout in Spain (accession number HE612100.1) and from aquaculture systems in South Africa (DQ778310.1 and DQ778309.1). Neighbor-joining, MP, and Bayesian phylogenetic analyses yielded a similar topology (as indicated by filled circles when a node had a posterior probability or bootstrap value >50 in all three trees and a grey square when supported in 2 of 3 trees, Fig. 7.1), which demonstrated that *Flavobacterium* sp. strains T16 and S12 formed an extremely well-supported clade (bootstrap values of 99 and 91; posterior probability of 100, respectively) that was distinct from the other members of the genus *Flavobacterium*.

Additionally, morphological, physiological, and biochemical characterizations were performed as recommended by Bernardet et al. (2002) and included: colony morphology on cytophaga agar (Anacker and Ordal 1955), growth on cetrimide and nutrient agars (Sigma), marine agar (Becton Dickinson Microbiology Systems), trypticase soy agar (TSA), TSA supplemented with 5% sheep erythrocytes, and MaConkey agar; growth on HSM at a pH of 5.0-10.0 in increments of 0.5 (adjusted using 1N hydrochloric acid and 1 N sodium hydroxide); growth at

4°C, 15°C, 22°C, 37°C, and 42°C; growth on HSM at salinities ranging from 0%-5.0% in 1% increments; acid/gas from glucose and acid from sucrose (1% final concentration, phenol red broth base); mixed acid fermentation and 2,3-butanediol production from glucose (methyl red - Voges-Proskauer test); triple sugar iron (TSI) reaction; hydrolysis of esculin (bile esculin agar); use of citrate as a sole carbon source (Simmon's citrate); production of indole and/or hydrogen sulfide on sulfur indole motility medium (SIM); lysis of hemoglobin (0.1% w/v) and degradation of collagen (0.1% w/v) and casein(5% w/v) and elastin (0.5%) as modified from Shotts et al. (1985) using HSM as the basal medium; activity for gelatinase (Whitman 2004), phenylalanine deaminase (Sigma), and DNase; activity for alginase (5% w/v alginic acid, Sigma, in HSM), pectinase (5% w/v pectin from apple, Sigma, overlay), chitinase (5% w/v chitin from crab shells, Sigma), and carboxymethylcellulase (0.15% w/v, Sigma, overlay; all modified from Reichenbach (2006) with HSM as basal medium); activity for chondroitin sulfatase C (0.2% w/v chondroitin sulfate sodium salt from shark cartilage, Sigma, HSM basal medium) and amylase (as modified from Lin et al. 1988 using HSM as basal medium); degradation of Tween 20 and Tween 80 (1% v/v, Sigma); brown pigment production from L-Tyrosine (0.5% w/v, Sigma; modified from Pacha and Porter (1968) using HSM as basal medium); and degradation of agar on TSA. When HSM was used as the basal medium in the morphological, physiological, and biochemical assays of this study, no gelatin or neomycin was added. Commercially available identification galleries (i.e., API 20E, API 20NE, API ZYM, and API 50CH; BioMerieux, Inc.) were inoculated according to the manufacturers protocol; however, tests were incubated at 22°C and read from 24- hrs post inoculation up until 7- days, with the exception of the API ZYM, which was read at 72- hrs.

For fatty acid profiling, *Flavobacterium* sp. T16 and S12 was cultured on a medium containing 30- g of trypticase soy broth and 15- g of Bacto agar (Difco) per liter of distilled water for 24- h at 28 °C. Bacterial cells were then saponified, methylated to fatty acid methyl esters (FAMEs)

and extracted according to the protocols of the commercial Sherlock Microbial Identification System (MIDI, version 4.0; Microbial Identification System Inc., Newark, DE). Separation of FAMEs was conducted via gas chromatography on an Agilent 6890A series Gas Chromatograph with the 7683 autoinjector and autosampler tray module (Agilent Technologies, Inc.) using a fused silica capillary column (25mm x 0.2mm) with cross linked 5% phenylmethyl silicone. The carrier gas was H_2 and peak identification/integration was performed using Agilent Chemstation and MIDI software (Agilent Technologies) and the Microbial Identification System database (Sasser 1990). The major fatty acid constituents of *Flavobacterium* sp. strains T16 and S12 were *iso*-$C_{15:0}$ (28.1-29.1%), C $_{16:1}$ ω6*c* and/or C $_{16:1}$ ω7*c* (18.4-21.4%), *iso*-$C_{17:0}$ 3-OH (8.2-8.7%), and *iso*-$C_{15:0}$ 3-OH (7.5-8.9%), while C $_{15:1}$ ω6*c* (2.1-3.4%), *iso*-C $_{16:0}$ 3-OH (1.6-1.8%), *iso*-C $_{15:1}$ G (1.2-2.1%), and *anteiso*-C $_{15:0}$ (1.1-1.4%) were also present in smaller quantities (Table 7.1). Interestingly, while the aforementioned fatty acids are typical of the genus *Flavobacterium* (Bernardet and Bowman 2011), two other fatty acids are also commonly seen within this genus; namely, $C_{15:0}$ and *iso*-$C_{15:0}$ 2-OH. However, *iso*-$C_{15:0}$ 2-OH was observed in only trace amounts (0.2%; Table 7.1) in strains T16 and S12, while the fatty acid $C_{15:0}$ was not detected, which has occasionally been reported for *F. indicum, F. frigoris,* and *F. suncheonse (*Bernardet and Bowman 2011). In addition, *Flavobacterium* sp. T16 and S12 were unique when compared to their closest relative, *F. aquideurense*, in that they contained larger percentages of *iso*-$C_{15:0}$, C $_{16:1}$ ω6*c* and/or C $_{16:1}$ ω7*c*, and C $_{16:0}$, and had smaller percentages of *iso*-C $_{15:1}$ G, C $_{15:1}$ ω6*c, iso*-$C_{17:1}$ ω9*c*, and C $_{17:1}$ ω6*c* (Table 7.1). Additional fatty acids distinguishing *Flavobacterium*

sp. T16 and S12 from *F. araucananum* and *F. frigidimaris* are provided in Table 7.1. The biochemical and physiological characteristics of *Flavobacterium* sp. strains T16 and S12 can be found in the species description below, while those characters that are unique for T16 and S12 when compared to their closest relative are listed in Table 7.2.

In order to assess the pathogenicity of *Flavobacterium* sp. T16 and S12, the following experiments were conducted in accordance with the Michigan State University Institutional Animal Care and Use Committee (AUF 12-10-218-00):

Three different genera/species of salmonids were obtained at ~1 month post hatch for the experimental challenges conducted within this study. Michigan strain Chinook salmon (*Oncorhynchus tshawytscha*), Assinica strain brook trout (*Salvelinus fontinalis*), and Gilchrist strain brown trout (*Salmo trutta*) were obtained from Wolf Lake State Fish Hatchery (SFH; Mattawan, MI), Marquette SFH (Marquette, MI), and Thompson SFH (Manistique, MI), respectively. Fish were fed a commercial diet *ad libitum* and maintained in well aerated flow-through PVC tanks (~400L; 12 hr photoperiod) with dechlorinated pathogen-free water at a temperature of 10 ºC$\pm$1 ºC for a minimum of 2 -months before use in experimental challenges. Tanks were cleaned daily. Thirty fish from each species were also sampled for the presence of flavobacteria, as well as other fish pathogenic bacteria, viruses, and parasites, according to the methodologies of the American Fisheries Society (AFS-FHS 2010) and World Animal Health Organization (OIE) Aquatic Manual (OIE 2006). No restricted or reportable pathogens, nor any flavobacteria, were detected in any of the uninfected Chinook salmon, brook trout, or brown trout utilized in this study.

Growth kinetic studies were initiated in order to determine when isolates T16 and S12 reached logarithmic phase of growth. One 48- hr old colony forming unit (cfu) from each isolate was

inoculated into 40- ml Hsu-Shotts broth supplemented with 5% (v/v) horse serum and 0.02% (v/v) mineral solution of Lewin and Lounsberry (Michel et al. 1999) and incubated statically at 22 ºC. Immediately after inoculation (Time 0) and at 8, 24, 48, 72, 96, 120, 144, and 168 hr post inoculation, the bacterial suspension was gently vortexed and 2- ml removed for OD determination and 100 µl for colony enumeration via plate counts. Optical density was recorded at 600 -nm in a Biowave CO8000 Cell Density Meter, while $\log_{(10)}$ serial dilutions in sterile PBS were plated on enriched Hsu-Shotts agar in duplicate and incubated at 22 ºC. Colonies were counted at 24 and 48 –hrs using a Quebec® Darkfield colony counter (Reichert, Inc., Buffalo, NY). Generated growth curves were used to predict the optical density that corresponded to 10^8 cfu 100 μl^{-1}. Both isolates attained logarithmic to late-logarithmic growth by 24- hrs post-inoculation at 22 °C in enriched Hsu-Shotts broth (Fig. 7.2); thus, 18-24 -hr cultures were used in experimental challenges. Both *Flavobacterium* sp. isolates were then passaged in Chinook salmon, reisolated on enriched Hsu-Shotts agar from kidney cultures, identity verified via 16S rDNA sequencing as described previously (data not shown), and cryo-preseved at -80°C.

An intraperitoneal (IP) injection was chosen because previous studies showed its reliability to reproduce infections with other fish-pathogenic flavobacteria (Madsen and Dalsgaard 1999). Chinook salmon (mean weight 14.2 g, SD=3.2; mean length 11.8 cm, SD=1.3), brook trout (mean weight 10.1 g, SD=3.3; mean length 10.5 cm, SD= 1.1), and brown trout (mean weight 3.4 g, SD=1.0; mean length 6.8 cm, SD= 0.7) were anesthetized in carbonate-buffered tricaine methanosulphonate (MS-222; n=5 per isolate per fish species) at a concentration of 100mg L^{-1} and then injected IP with 100- µl of a bacterial suspension containing $8.0 \times 10^7 – 4.5 \times 10^8$

cfu. Control fish (n=5) were injected with 100-µl of sterile PBS. Challenged fish were immediately placed in randomly assigned, well aerated flow-through PVC tanks (70- L) at a flow rate of 1.26 L/min using the same source water as described above. Fish were checked twice daily for morbidity/mortality, fed daily, and tanks were cleaned when fish waste/detritus was observed. Each experimental challenge lasted 14- days. If severe signs of morbidity were observed, the affected fish was euthanized with an overdose of MS-222 and immediately necropsied. Liver, spleen, kidney, and brain samples were collected and inoculated directly onto enriched HSM (at 22 ºC) and cytophaga agar (at 15 ºC) plates for up to 7- d. Representative isolates recovered from challenged fish in each experiment were identified via gene sequencing and phylogenetic analysis as described previously to confirm their original identities.

The cumulative mortalities for *Flavobacterium* sp. T16 -infected fish was 80% in Chinook salmon and brown trout fingerlings, and 40% in brook trout fingerlings, whereby all deaths occurred between 1 and 5-d postinfection. In fish infected with isolate S12, cumulative mortalitiesy were 20% in Chinook salmon and brook trout, and 60% in brown trout, with deaths occurring between 2 and 4- d post infection. In every case, isolate T16 was recovered from the livers, spleens, kidneys, and brains of dead fish, which was also the case for S12-infected fish. Isolate T16 was recovered from the spleen of one of the three brook trout that survived until the end of the 14- d period, and from the liver and spleen of the sole brown trout survivor, but it was not recovered from any of the organs of the lone Chinook salmon survivor. Isolate S12 was recovered from the brain of one of four Chinook salmon survivors, and from the spleen of one of four brook trout survivors, but it was not recovered from any organs of the two surviving brown trout fingerlings. In all cases, bacteria recovered from experimentally challenged fish were identified as the original bacterial strain that was injected into the fish

according to 16S rDNA sequencing and phylogenetic analysis. No bacteria were recovered from any control fish, nor was any mortality recorded in those fish.

Gross signs of disease were similar in Chinook salmon challenged with the two isolates, and included pale and swollen gills with multifocal hemorrhage, congestion at the base of the fins, shallow dermal ulceration, swollen/enlarged/friable spleens, petechial hemorrhage within the ventricle of the heart (Fig. 7.3a), petechial to echymotic hemorrhage within the body walls, muscle (Fig. 7.3b), and adipose tissue (Fig. 7.3c), enlarged pale liver, enlargement hemorrhagic enteritis, ascites accumulation, hemorrhage within the swim bladder, renal swelling, hemorrhage, and edema, hemorrhagic gonads, and focal to multifocal intracranial hemorrhage (Fig. 7.3d). Disease signs in brook trout were somewhat similar and included gill pallor, petechial hemorrhage within the fins, dorsal fin erosion (Fig. 7.3e), congestion at the base of the fins, splenic swelling and friability, hepatic pallor/friability/congestion, diffuse hemorrhage within the adipose tissue, hemorrhagic enteritis, renal pallor/edema/hemorrhage/swelling, multifocal intracranial hemorrhage, and swim bladder hemorrhage. Similar signs were also observed in brown trout; however, marked flaring of the opercula were also apparent.

Experiments to determine the median lethal dose (LD_{50}) of *Flavobacterium* sp. T16 were also undertaken. $Log_{(10)}$ serial dilutions of bacterial inocula in PBS were prepared as described previously and IP-injected into anesthetized Chinook salmon (mean weight 30.1 g $\pm$ 12.1 g; mean length 15.0 cm $\pm$ 2.1 cm), which were chosen because this was the host species from which isolates T16 and S12 were originally recovered. Bacteria- and mock-challenged fish (injected with 100 µl of bacterial suspension or sterile PBS) were monitored for 28 -d as described previously. Mortalities were immediately necropsied and kidney tissues streaked

directly onto enriched Hsu-Shotts medium (at 22 ºC) and cytophaga agar (at 15 ºC) plates and incubated for up to 7-d. In addition, gill, heart, liver, spleen, adipose tissue/pancreas, anterior and posterior kidney, brain, skin, and muscle samples from two fish at each dose (including control fish), as well as any mortalities, were preserved in phosphate-buffered 10% formalin, embedded within paraffin, sectioned at 5 -µm, stained with hematoxylin and eosin (H & E; Prophet et al. 1992), and observed by light microscopy. The median lethal dose of *Flavobacterium* sp. T16 was calculated (Reed and Muench 1938).

Seven groups of ten Chinook salmon were utilized to determine the LD_{50} for *Flavobacterium* sp. T16; six groups were IP injected with an inoculum ranging from 1.72×10^8 - 1.72×10^3 cfu, while the seventh group was the negative control group. A cumulative mortality of 90% occurred in the highest infectious dose (e.g., 1.72×10^8), while 10% mortality occurred in the group challenged with 1.72×10^7 cfu. All other groups had 0% cumulative mortality after the 28- d period except for the group challenged with 1.72×10^5 cfu, which was 10%. The LD_{50} for *Flavobacterium* T16 was 470×10^5 cfu.

Histopathological changes were also assessed in a portion of the experimentally challenged Chinook salmon within each group. Fish exposed to the lowest three infectious doses exhibited similar histological changes, which included a proliferative branchitis consisting of epithelial hyperplasia that resulted in focal fusion of the secondary lamellae, splenic congestion, multifocal degeneration of the myocardium, and multifocal necrosis of both the hepatocytes and interstitial cells of the posterior kidney. In the next highest dose, Chinook salmon also showed a proliferative branchitis, hepatocyte necrosis, and splenic congestion,

but also showed a focal lymphocytic hepatitis and a marked lymphocytic infiltrate within the atrium of the heart consistent with a peripheral leukocytosis. In Chinook salmon IP injected with 10^7 cfu of isolate T16, a focally extensive monocytic myositis, multifocal necrosis of the interstitial cells of the anterior kidney, and multifocal myocardial degeneration and necrosis were also evident in addition to the previously mentioned changes. However, histopathological changes were most severe in Chinook salmon exposed to 10^8 cfu and included a severe proliferative branchitis (Fig. 7.4a), massive hemorrhage within the muscle (Fig. 7.4b) where large numbers of bacterial rods were also observed (Fig. 7.4c), focally extensive monocytic myositis and peritonitis (Fig. 7.4d), patchy degeneration and necrosis within the liver along with occasional focal lymphocytic hepatitis (Fig. 7.4e), renal tubular degeneration and necrosis (Fig. 7.4f), splenic congestion with concurrent edema and capsulitis, necrosis of the interstitial tissue in the posterior kidney, edema and vasculitis within the anterior kidney, focal degeneration of the myocardium, and pancreatitis. In the brain, multifocal edema within the granular cell layer of the cerebellar cortex (Fig. 7.5a) was observed, while edema within the brain stem (Fig. 7.5b) was also apparent. No histological abnormalities other than splenic congestion were observed in negative control fish.

Interestingly, a portion of the gross and histopathological changes seen in T16/S12-infected Chinook salmon were quite similar to those reported in natural and experimental infections associated with the “well-known” fish-pathogenic flavobacteria (i.e., *F. psychrophilum* and *F. branchiophilum*). For example, Rangdale et al. (1999) observed gill pallor in rainbow trout (*O. mykiss*) infected with *F. psychrophilum*, while histologically they observed peritonitis, splenic edema, and pancreatitis, all of which were also observed in this study. Similarly, Nematollahi et al. (2003) reported gross signs of disease in *F. psychrophilum* infected fish that included anemia, gill hemorrhage, and renal, hepatic, and intestinal pallor dependent on the fish

species/age, while histological changes included necrotic myositis, necrotic scleritis, and cephalic osteochondritis, a portion of which were also observed in T16 infected fish. Moreover, Otis (1984) recorded widespread hemorrhage within the liver, heart, adipose tissue, intestine, swim bladder, and body wall in steelhead trout (*O. mykiss*) experimentally infected with *F. psychrophilum*, while Wood and Yasutake (1970) and Ostland et al. (1999) reported renal tubular degeneration and fusion of secondary lamellae of the gills, respectively, in *F. psychrophilum* infected fish. Indeed, the gill pathology observed in this study is also similar to what was reported in fish suffering from bacterial gill disease, caused by *F. branchiophilum* (Wakabayashi et al. 1989). For example, pale and swollen gills due to a proliferative hyperplasia of the gill epithelium that results in fusion of neighboring lamellae is hallmark of infections associated with *F. branchiophilum* (Bullock 1990; Ostland et al. 1995), which is in stark contrast to the widespread necrosis of the gills associated with columnaris disease, caused by *F. columnare* (reviewed in Shotts and Starliper 1999). Thus, a number of striking similarities for the gross signs of disease and histopathological changes are evident between *F. psychrophilum*, *F. branchiophilum,* and *Flavobacterium* sp. T16 /S12.

The results of the polyphasic characterizations conducted in this study demonstrate that the two new isolates recovered from Chinook salmon in Michigan indeed represented a novel *Flavobacterium* sp., for which the name *Flavobacterium spartani* sp. nov. is proposed. In addition, Koch's postulates have been fulfilled, demonstrating that this novel bacterium represents another *Flavobacterium* spp. that is pathogenic for Michigan fishes.

Description of *Flavobacterium spartani* sp. nov.

Flavobacterium spartani (spar'tan.i. N.L. gen. n. *spartani*, of Spartans, in honor of the mascot of Michigan State University).

Cells are non-motile, gliding, Gram-reaction-negative rods (3.0-5.0 μm in length) that do not contain cell wall-associated galactosamine glycans (do not absorb congo red). On cytophaga medium, colonies are dark yellow, semi-translucent, and nearly flat with irregular spreading margins. Growth occurs on nutrient, trypticase soy, Hsu-Shotts, cytophaga, and sheep's blood agar, but not on marine, cetrimide, or MacConkey agars at 22 °C. Grows well at a pH of 5.5-8.5, while weak/delayed growth occurs at a pH of 5.0 and 9.0-10.0. Able to grow at 4 °C, 15°C, and 22°C, but not at 37 or 42°C. Able to grow at salinities from 0-2% (weakly at 2%), but not at 3-5%. Does not produce indole or acid from glucose or sucrose in phenol red broth (1% final carbohydrate solution), and produces no reaction on triple sugar iron (TSI) slants without the production of H_2S or gas. Utilizes citrate as a sole carbon source.

Produces catalase, gelatinase, caseinase, pectinase, amylase, and elastase, but not cytochrome oxidase alginase, DNase, collagenase, urease, chitinase, lipase, or carboxymethyl cellulase. Variable in phenylalanine deaminase production (T16 is positive, S12 is negative). Able to hydrolyze esculin, lyse hemoglobin, and degrade Tween 20, but does not degrade chondroitin sulfate, agar, or Tween 80. Degrades tyrosine, which results in the production of a brown pigment. On the API 20E, negative for arginine dihydrolase, lysine and ornithine decarboxylase, urease, and tryptophan deaminase activities; does not produce H_2S, indole, or acid from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, or arabinose; Positive for ONPG, citrate utilization, and gelatinase, and variable in acetoin production (T16 is positive, S12 is negative). On the API 20 NE, does not produce indole, arginine dihydrolase, or urease; hydrolyzes esculin and gelatin and uses para-nitrophenyl-βD-galactopyranoside; assimilates D-glucose, D-mannose, N-acetyl-glucosamine, D-maltose, and trisodium citrate, but not L-arabinose, D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, or phenylacetic acid. On the API ZYM, positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cysteine

arylamidase, acid phosphatase, Napthol-AS-BI-phosphohydrolase, α –glucosidase, and N-acetyl- β-glucosaminidase activities, but negative for lipase, trypsin, β-glucoronidase, β-glucosidase, α-mannosidase, and α-fucosidase. Variable for β-galactosidase (T16 is a weak positive, S12 is negative), and weakly positive for α-chymotrypan and α-galactosidase activities. For the API 50CH (using CHB/E medium), does not produce acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, D-lactose, D-melibiose, D-sucrose, inulin, D-melezitose, D-raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2- ketogluconate, or potassium 5-ketogluconate; produces acid from D-galactose, D-glucose, D-cellobiose, D-maltose, D-trehalose, starch, glycogen, and gentibiose; variable acid production from D-mannose (T16 is a weak positive, S12 is negative). The main fatty acid constituents are *iso*-$C_{15:0}$ (28.1-29.1%), $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c* (18.4-21.4%), *iso*-$C_{17:0}$ 3-OH (8.2-8.7%), and *iso*-$C_{15:0}$ 3-OH (7.5-8.9%).

The type strain is strain T16 (GenBank accession number- JX287799) isolated from the kidneys of a feral adult Chinook salmon (*Oncorhynchus tshawytscha*) returning to the Swan River Weir (Presque Isle County, Michigan, USA) to spawn.

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Table 7.1. Cellular fatty acid profiles (%) of *Flavobacterium* sp. strains T16 and S12 and three most closely related *Flavobacterium* spp. Results for *Flavobacterium* sp. strains T16 and S12 are from this study, while results for *F. aquidurense* are from Cousin et al. (2007), *F. araucananum* are from Kämpfer et al. (2012), and *F. frigidimaris* are from Nogi et al. (2005). Fatty acids amounting to <1% of the total fatty acids in all strains are not shown. Tr, traces (<1%); ND, not detected; NR, not reported.

Fatty Acid	T16	S12	*F. aquidurense*	*F. araucananum*	*F. frigidimaris*
13:1 at 12-13	ND	ND	Tr	2.5	NR
14:0	1.34	1.49	Tr	Tr	NR
15:1 iso G	2.08	1.19	6.0± 0.2	2.3	NR
15:0 iso	29.1	28.2	15.1±0.1.1	24.5	26.7
15:0 anteiso	1.1	1.4	2.8±0.3	2.5	2.5
15:1 iso w10c	ND	ND	ND	ND	9.0
C15:0	ND	ND	7.1±0.7	4.6	10.2
15:1 w6c	3.4	2.2	6.7±0.5	5.6	5.4
16:1 w6c/16:1 w7c	18.4	21.4	11.8±09 ψ	12.8 φ	13.9
16:0	5.9	7.5	Tr	4.0	1.2
15:0 iso 3OH	8.9	7.5	8.3±0.5	7.5	7.6
17:1 iso w9c	3.7	3.5	8.2±0.3	2.9	NR
15:0 3OH	1.8	1.5	2.8±0.1	2.8	1.9
Iso 17:1w7c	ND	ND	ND	ND	6.6
17:1 w6c	1.1	Tr	6.4±0.7	2.2	5.9
16:0 iso 3OH	1.8	1.6	2.3±0.1	1.7	1.3
16:0 3OH	7.7	8.6	1.7±0.1	4.3	1.7
17:0 iso 3OH	8.7	8.2	11.6±0.7	6.6	6.2

Ψ, Comprised of C16:1w7c and/or iso C15:0 2-OH; φ, comprised of iso C15:0 2-OH and/or C16:1 *ω7c*.

Table 7.2. Biochemical and physiological characteristics of *Flavobacterium* sp. T16 and S12 and their 3 closest *Flavobacterium* spp. relatives. Results are from: T16 and S12 (this study); 1, *F. aquidurense* (Cousin et al. 2007); 2, *F. araucananum* (Kämpfer et al. 2012); 3, *F. frigidimaris* (Nogi et al. 2005).

Assay	**T16**	**S12**	**1**	**2**	**3**
Gliding Motility	+	+	-	+	+
Growth on marine agar	-	-	NR	-	+
pH growth range:					
5.5	+	+	-		NR
7.0	+	+	-	+	NR
7.5	+	+	-	+	NR
8.0	+	+	-	+	NR
8.5	+	+	-	+	NR
9.0-10.0	(+)	(+)	NR	-	NR
Growth at 4°C	+	+	-	+	+
Growth at 2% salinity	(+)	(+)	-	+	+
Growth at 3%salinity	-	-	-	+	+
Brown pigment from tyrosine	-	-	+	+	NR
Production of:					
Cytochrome oxidase	-	-	+	+	-
Gelatinase	+	+	-	+	+
Chitinase	-	-	NR	NR	+
Degradation of tween 20	+	+	-	NR	NR
Esterase	+	+	-	NR	NR
Esterase lipase	+	+	-	NR	NR
Acid production from:					
Glucose	v*	v*	NR	+	+
Mannitol	-	-	NR	NR	+
Sucrose	-	-	NR	NR	+
D-Xylose	-	-	NR	+	+
D-Cellobiose	+	(+)	-	+	+
D-Lactose	-	-	NR	+	-
D-Trehalose	+	(+)	-	+	+
D-Raffinose	-	-	-	-	+
Assimilation of:					
L-Arabinose	-	-	+	NR	NR
Trisodium citrate	+	+	-	NR	NR

+, positive result; [+], very weak and/or delayed positive result; (+), weak positive result; -, negative result; v, variable result; NR, result not reported; *, negative in phenol red broth and on the API 20E, but positive on the API 50CH.

Figure 7.1. Dendrogram generated using Bayesian analysis in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites. Default settings were used for the transition/transversion rate ratio (beta), topology (uniform), and prior probability distribution on branch lengths (unconstrained). The Markov chain was run for up to ten million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01%. Two independent analyses were conducted, both with 1 cold and 3 heated chains using the default heating parameter (temp=0.2). The initial 25% of Markov Chain Monte Carlo (MCMC) samples were discarded as burnin and sampling occurred every 100 generations. Filled circles are present when that node was also present in the maximum parsimony and neighbor-joining trees, while grey squares indicate that that node was present using 2 of the 3 methods. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

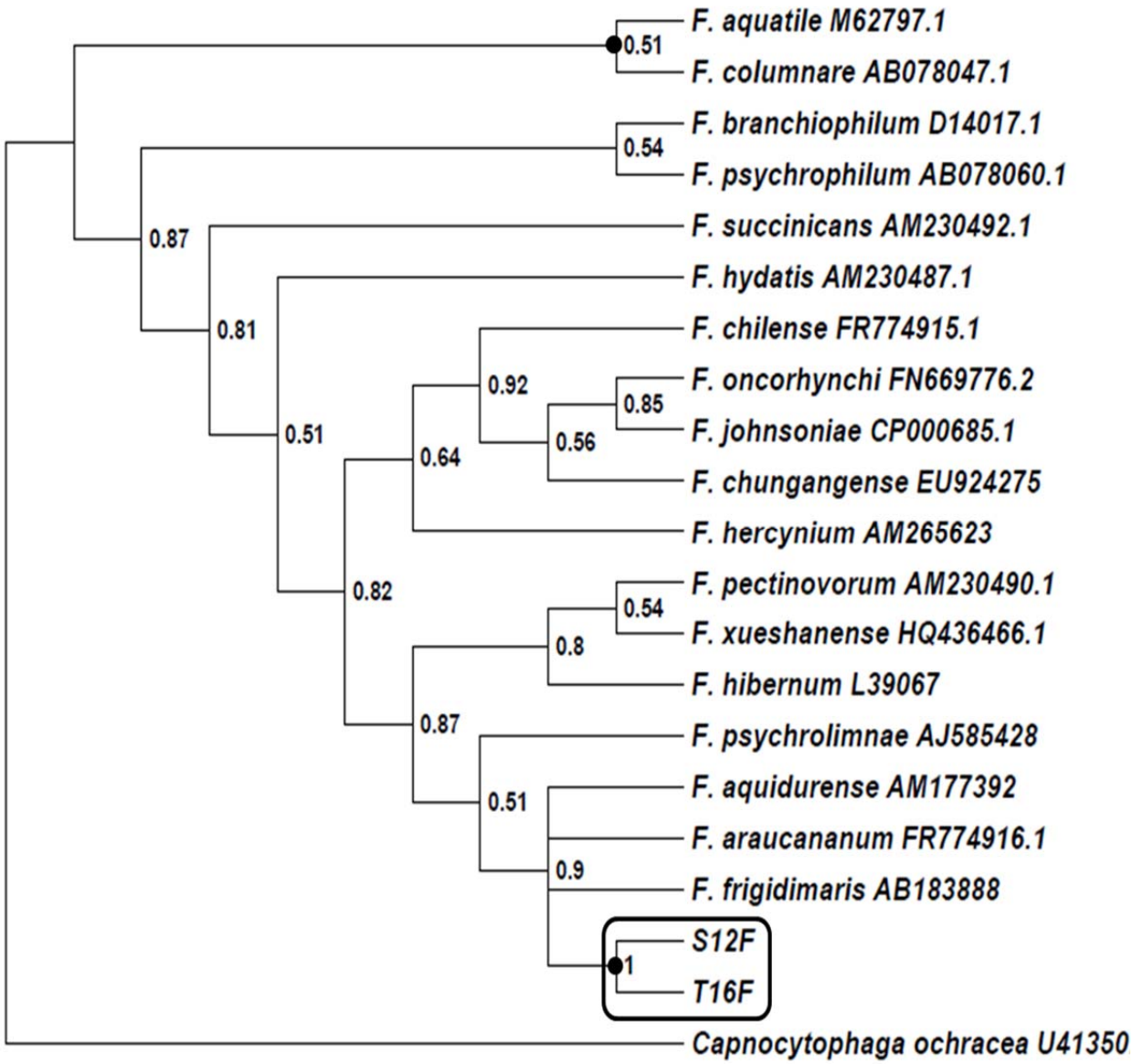


Figure 7.2. Growth kinetics for *Flavobacterium* sp. T16 and S12 as determined by 10 fold serial dilutions/plate counts and optical density (OD) readings taken at 600 nm (performed in duplicate). Isolates were inoculated into 40 ml of Hsu-Shotts broth supplemented with 5% (v/v) horse serum and 0.02% (v/v) mineral solution of Lewin and Lounsberry and incubated statically at 22 ºC. Error bars represent the standard deviation of the number of cfus recorded at each time point.

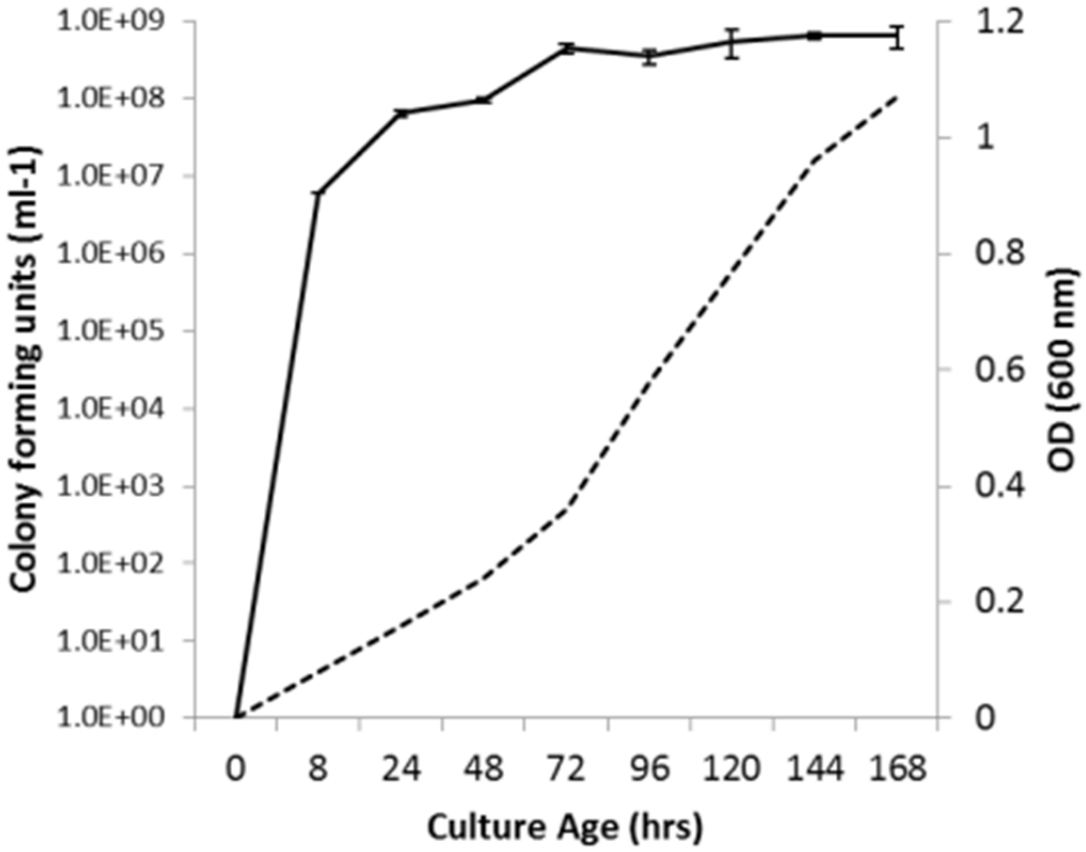


Figure 7.3. Gross lesions in fish intraperitoneally infected with *Flavobacterium* sp. T16 and S12. A) Petechial hemorrhage within the ventricle of the heart (arrow) of a Chinook salmon fingerling. Also note the hepatic pallor and red-tinged ascites within the pericardial and peritoneal cavities. B) Diffuse petechial hemorrhage within the trunk muscle of a Chinook salmon fingerling. C) Severe petechial and echymotic hemorrhage (arrows) within the adipose tissue of a Chinook salmon fingerling. D) Focal hemorrhage within the optic lobes of the brain (arrow) of a Chinook salmon fingerling. E) An eroded and necrotic dorsal fin (arrow) with a hemorrhagic base of a brook trout fingerling.

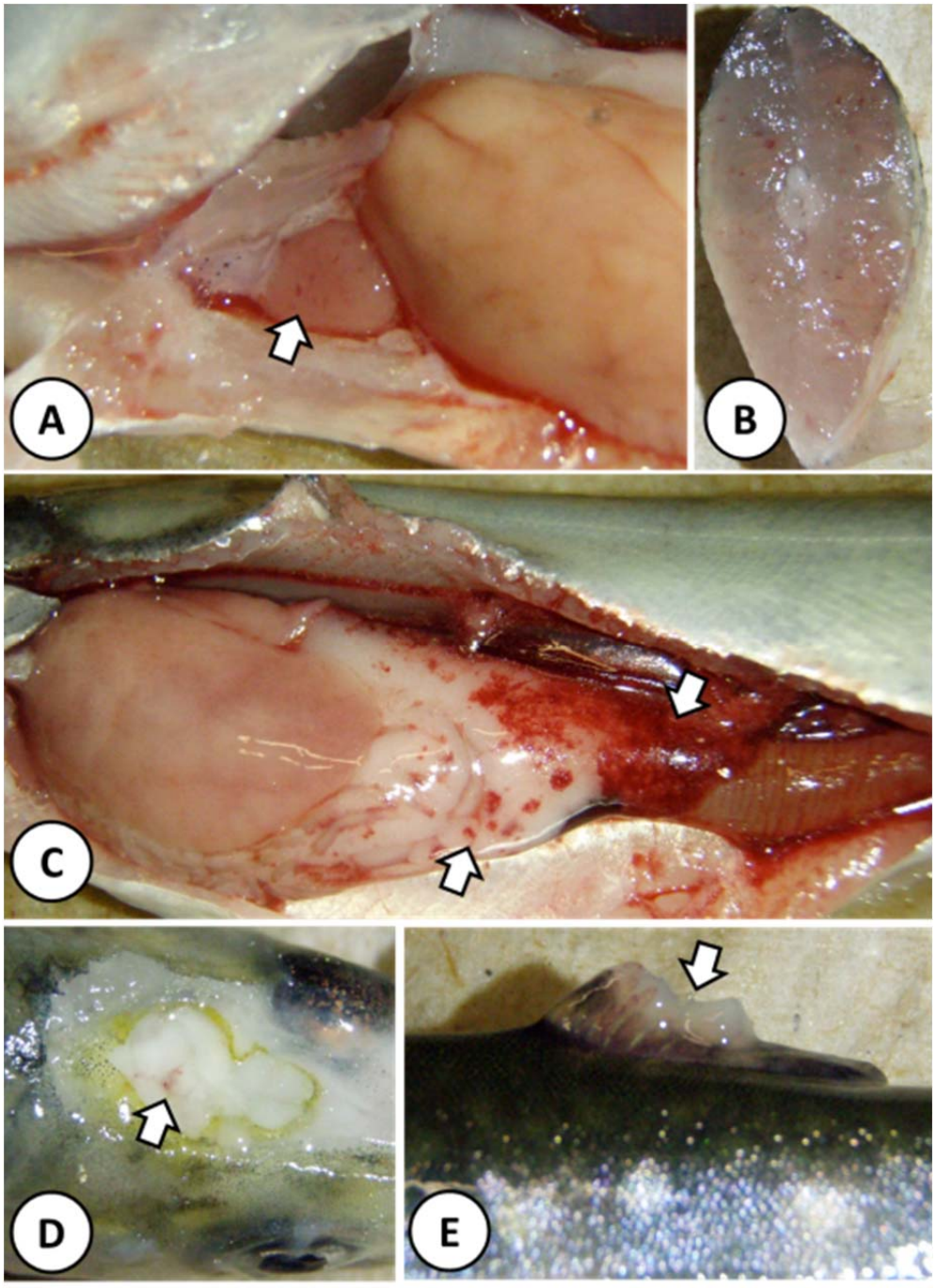


Figure 7.4. Hematoxylin and eosin (H & E) stained tissue sections from Chinook salmon intraperitoneally challenged with *Flavobacterium* sp. T16 and S12. A) Gills showing a proliferative branchitis consisting of epithelial hyperplasia of the secondary lamellae and interlamellar space resulting in secondary lamellar fusion (200x). B) Severe hemorrhage within the muscle, along with degeneration of the myofibers (400x). C) Lymphocytic and histiocytic myositis, along with the presence of a large number of bacterial rods (arrows; 400x). D) Focally extensive monocytic myositis at the peritoneal lining (200x); Normal muscle fibers are apparent in the upper left of the micrograph. E) Focal lymphocytic hepatitis (arrows) within the liver (400x). F) Focal renal tubular degeneration and necrosis (arrows) in the posterior kidney (400x).

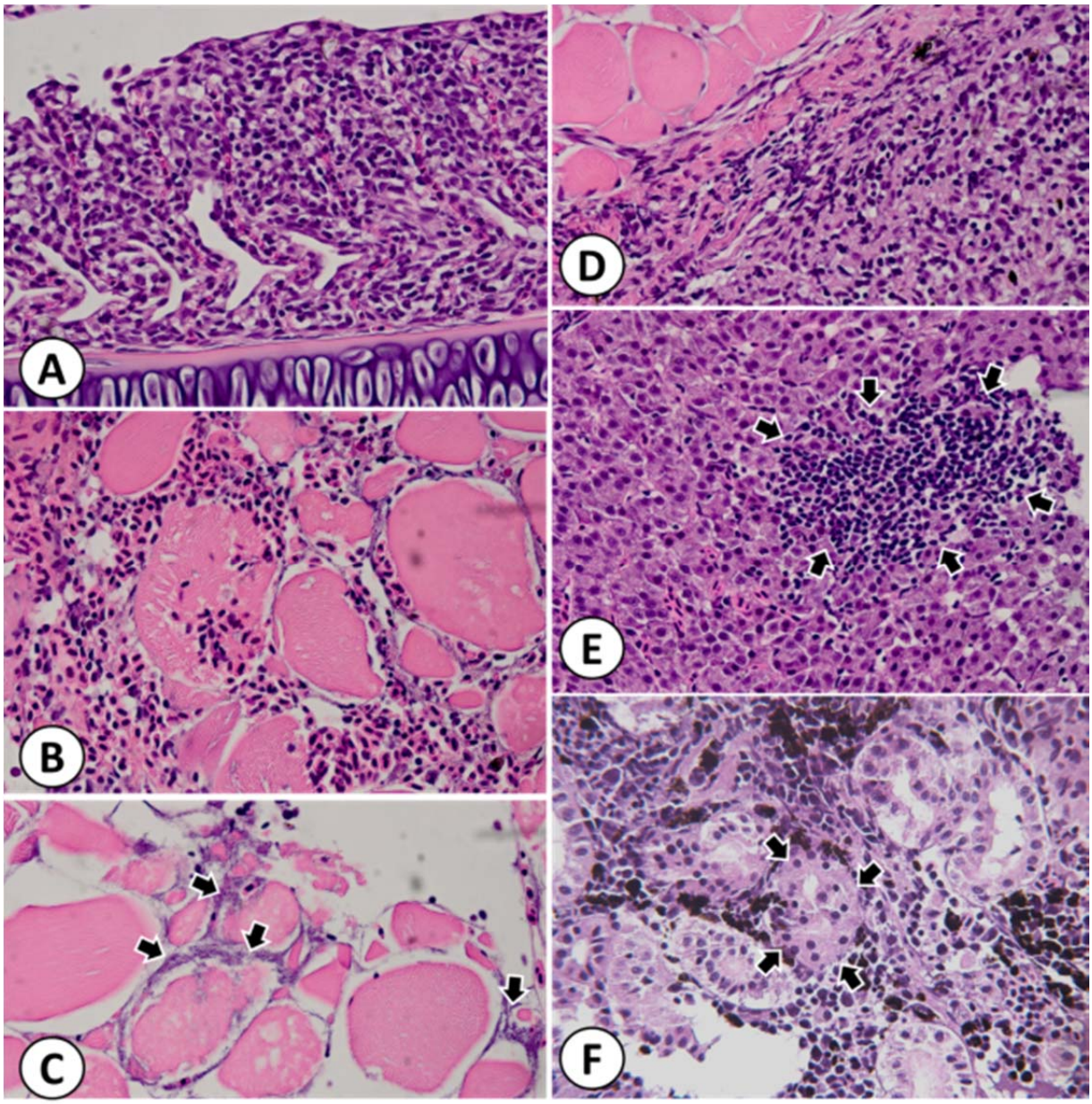
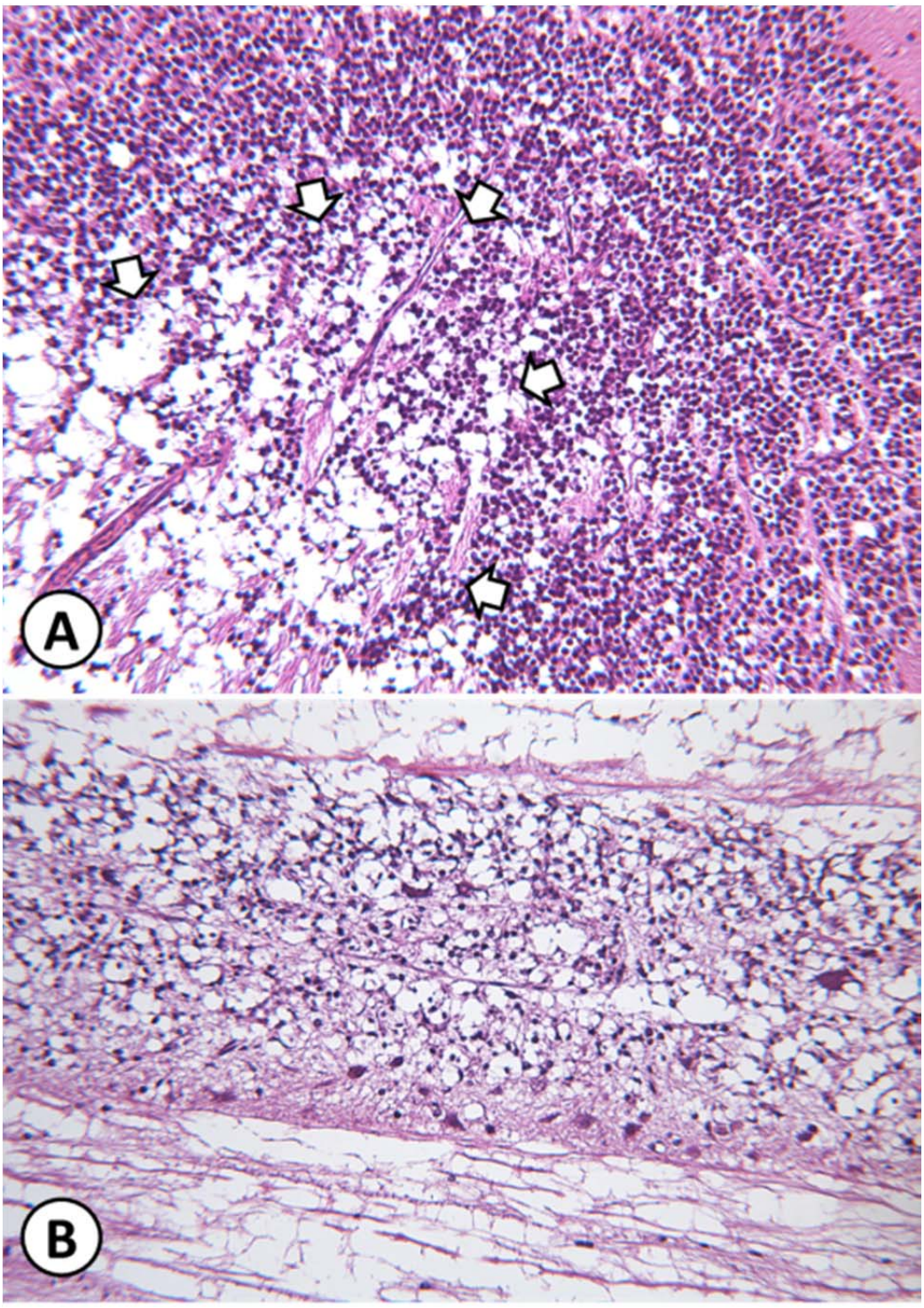


Figure 7.5. Hematoxylin and eosin (H & E) stained tissue sections from Chinook salmon intraperitoneally challenged with *Flavobacterium* sp. T16 and S12. A) Multifocal edema within the granular cell layer of the cerebellar cortex (arrows; 200x). B) Spongiosis and edema within the brain stem (200x).



CHAPTER EIGHT

CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS

Much of the research conducted during the last century conclusively showed that flavobacteriosis is a major threat to both cultured and wild fish worldwide. Indeed, three etiologies in particular have been implicated time and again for the role they play in this consortium of diseases; namely, *F. psychrophilum*, *F. columnare*, and *F. branchiophilum*. While these three flavobacterial fish-pathogens are undeniably important causes of epizootics that result in major economic losses on a global scale, research presented herein underscores that we do not have a comprehensive understanding of flavobacteriosis in fish. For example, I discovered and described two new *Flavobacterium* and *Chryseobacterium* spp. that were recovered from diseased fish with pathologies that mimicked those often seen in bacterial coldwater disease and bacterial gill disease epizootics. In addition, other phylogenetic clades were discovered that very likely represent novel flavobacterial taxa from diseased and systemically infected Michigan fishes. When studies aimed at fulfilling Koch's postulates for a number of these unusual flavobacteria were conducted, results conclusively showed that some were truly pathogenic to fish. The research conducted in this study provided additional evidence that more than one flavobacterial species is capable of contributing to diseases of the gills in fish. For the first time, the presence of a number of recently described putative fish-pathogenic *Flavobacterium* and *Chryseobacterium* spp., such as *F. oncorhynchi*, *C. chaponense, C. piscium, C. viscerum,* and *C. piscicola*, were described that had not been reported in North America. Even if infections with these "less well-known" flavobacteria are less frequent than those caused by the *F. psychrophilum, F. columnare,* and *F. branchiophilum*, it is imperative that etiologic agents are properly identified. It is my hope that this research will increase the collective awareness of the diversity of flavobacteria that are associated with fish

diseases and that these results provide a foundation upon which we can understand the role that multiple flavobacteria have in both the health and disease of fishes in the Great Lakes and beyond.

FUTURE RESEARCH DIRECTIONS

While the research presented in this dissertation allows for a better understanding of the flavobacteria that are associated with Michigan fishes, it also underscores that much more work is needed to fill numerous gaps of knowledge. For instance, the known presence of less-typical fish-pathogenic flavobacteria necessitates the development of diagnostic reagents that are specific and allow for a quick identification and differentiation from the other known fish-pathogenic flavobacteria, especially since many of the methods currently used for a definitive identification hinge upon isolation of the organism and extensive characterization using multiple methodologies. Indeed, at least three regions within the 16S rRNA gene that were sequenced in this study show promise for specific primers. Once these tools are available, it will facilitate further epizootiological studies of these organisms, such as potential reservoirs, presence in the water column and sediments, prevalence of infections in wild and cultured fishes, and mode of transmission, as at least one *Flavobacterium* sp. is known to be vertically transmitted from parent to progeny. Along similar lines, this laboratory is conducting an ongoing study using bacterial culture to study the trafficking of flavobacteria within closed and open water hatchery systems, and improved diagnostic techniques would greatly aid this process.

Further investigations that fully characterize the remaining clades that were described in this study are indeed warranted. Results strongly suggested that many of these organisms represent novel taxa within the genera *Chryseobacterium* and *Flavobacterium*. Concurrently, investigations of the flavobacterial communities associated with both healthy and diseased

fishes, cultured, wild, and feral alike, are direly needed so that fish health professionals can more easily distinguish those species that are commensalistic, or even mutualistic, from those that are pathogenic. In fact, some chryseobacteria and flavobacteria may be potential candidates for inclusion in future probiotic supplementation studies.

With the widespread availability of next generation sequencing techniques (Roche 454 pyro-sequencing, illumina sequencing, etc.), much could be learned about these "uncharacterized" flavobacteria by sequencing their whole genomes and comparing them to the ever growing list of genomes from other well-known fish-pathogenic bacteria, especially *F. psychrophilum, F. columnare,* and *F. branchiophilum*, which are now published and available. Indeed, this would help elucidate their means of pathogenesis. In this context, further studies to develop a more natural route of experimental infection (i.e., bath exposure, cohabitation, etc.) would also help to explain the *in vivo* pathogenesis of these organisms, while also allowing for further studies of histopathological changes and tissue predilections associated these infections. Natural experimental models would also greatly facilitate further studies aimed at learning more about the virulence factors employed by these less-typical flavobacterial fish pathogens.

In closing, Reichenbach et al. (1981) stated "It is hardly an exaggeration to say that at the present the taxonomy of the gliding bacteria is in a desolate state. Not that it had been in a better state before, but now we know enough about many of these bacteria to be reasonably sure that the current state of classification cannot be correct." Since then, the advancement of molecular techniques has afforded much more taxonomic clarity for members of the Family Flavobacteriaceae. With these tools now in hand, there is no doubt that a more comprehensive understanding of flavobacteriosis in fish is now realistically attainable.

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