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EMERGING *FLAVOBACTERIUM* SPP. IN THE GREAT LAKES BASIN: IDENTIFICATION AND ASSESSMENT OF THEIR IMPACTS ON FISH HEALTH

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Abstract
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 flavobacterial fish diseases are primarily attributed to *Flavobacterium psychrophilum, F. columnare,* and *F. branchiophilum*, herein we 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.

Managerial implications of the concluded study

The conducted studies prove that multitudes of flavobacteria are associated with diseased wild, feral, and cultured fish stocks of the Great Lakes. While the "well-known" flavobacterial fish pathogens (i.e., *F. psychrophilum, F. columnare*) continue to plague Great Lakes fish stocks, this study demonstrated that many other *Flavobacterium* spp., as well another closely related genus of bacteria, *Chryseobacterium,* are also associated with fish mortality events. Many of these bacteria have either never been reported from the Great Lakes or have never before been described (i.e., novel species). This is of major importance for fishery managers, as it means that the current method of diagnosing flavobacterial diseases in fish is vastly oversimplified and likely has implications for treatment outcomes in hatchery situations. Moreover, this study heightens the issue of the origin of these unusual flavobacteria; are they invasive pathogens brought here in todays globalization era, or have our traditional diagnostic and identification schemes misdiagnosed them? In this context, the results from this study will serve as a platform from which the role, source, and improved diagnosis of these unusual flavobacteria can be elucidated. In addition to *F. columnare* and *F. psychrophilum*, which continue to be negatively impact fish health in the Great Lakes, we have described the presence of over 60 distinct clusters of flavobacteria and chryseobacteria that were recovered from feral Chinook salmon, their eggs/reproductive fluids, their progeny at both the swim up fry and fingerling life stages, the waters that supply and run through hatchery systems, and tools used to clean hatchery rearing units. It is quite alarming that the majority of these taxonomic clusters are genetically distinct from all described flavobacteria and are novel bacterial species never before described. As a result, the impacts that these "less-typical" flavobacteria can have on the health of fish is not known. However, our studies have definitively shown that some of the flavobacterial isolates recovered under Objective III are nearly identical to those that were described under Objective I and proved to be pathogenic to Great Lakes salmonids under Objective II. The presence of such a multitude of fish-associated flavobacteria complicates the diagnosis and treatment of flavobacterial diseases and necessitates the revision of current disinfection protocols employed within Great Lakes fish hatcheries. Within hatcheries, special attention needs to be directed at ways to reduce or eliminate flavobacterial transmission, while also finding ways to lessen the likelihood of disease outbreaks. Our findings indicate that disinfecting hatchery pipelines and enclosures between rearing cycles is a viable way to reduce some flavobacterial reservoirs. Similarly, egg disinfection as currently adopted by the Great Lakes Fishery Commission/Fish Health Committee is not sufficient in eradicating all of the potentially pathogenic flavobacteria and chryseobacteria in and on eggs. Likewise, the persistence of flavobacteria and chryseobacteria on hatchery tools, as well as in hatchery water sources, dictates that improved eradication measures be adopted. This study has provided a more complete picture of the potentially problematic flavobacteria/chryseobacteria. It is now imperative to disentangle the truly pathogenic species from those that are innocuous and develop practical measures to combat them.

Findings of this study were presented at the following scientific conferences:

- Loch TP and Faisal M. Diversity of Fish-Associated Flavobacteria of the Laurentian Great Lakes. Presented at the 36th Annual Eastern Fish Health Workshop, Mt Pleasant, SC 2011.
- Loch TP and Faisal M. Bacterial diseases of fish in Michigan (focus on emergent flavobacteria). St. Sault Marie, Aug. 2011. Great Lakes Fish Health Committee Meeting.
- Loch TP and Faisal M. *Flavobacterium spartani* sp. nov., a newly described pathogen of Great Lakes fishes. Presented at the 37th Annual Eastern Fish Health Workshop, Lake Placid, NY 2012. Received the "**Best Student Presentation Award"**
- Van Vliet D, Loch TP, Diamanka A, Faisal M. Prevalence of bacterial coldwater disease within michigan state fish hatcheries from 2005 to 2011 Annual Fish Health Section Meeting of the American Fishery Society, July 31-Aug 2, 2012, Lacrosse, WI.
- Loch TP, Xu W, Morrison JC, and Faisal M. Exposure of *Flavobacterium spartani* sp. nov. to host factors is associated with differential gene expression. Annual Fish Health Section Meeting of the American Fishery Society, July 31-Aug 2, 2012, Lacrosse, WI.

Dissertation resulting from this GLFT-Funded study:

- Loch TP. 2012. Identification of Novel Flavobacteria from Michigan and Assessment of Their Impacts on Fish Health. Ph.D. Dissertation, Department of Pathobiology & Diagnostic Investigation, Michigan State University.

Publications in peer-reviewed periodicals:

- Faisal M, Loch TP, Fujimoto M, Woodiga S, Eissa A, Honeyfield D, Wolgamood M, Walker E, Marsh T. (2011): Characterization of Novel *Flavobacterium* spp. Involved in the Mortality of Coho Salmon (*Oncorhynchus kisutch*) in Their Early Life Stages. *Journal of Aquaculture Research and Development*. S2:1-8.
- Loch TP, M. Fujimoto, S. A. Woodiga, E. D. Walker, T. L. Marsh, Faisal M. Diversity of Fish-Associated Flavobacteria of Michigan. In press (to be published June, 2013), *Journal of Aquatic Animal Health.*
- Loch TP and Faisal M. Emergence of *Chryseobacterium* spp. infections in Michigan fishes. Submitted to *Veterinary Microbiology.*
- Loch TP and Faisal M. Deciphering the biodiversity of fish-pathogenic *Flavobacterium* spp. recovered from Michigan, USA, using a polyphasic approach. Submitted to *Systematic and Applied Microbiology*.
- Loch TP and Faisal M. *Flavobacterium spartani* sp. nov., a newly described pathogen of Great Lakes fishes, and emended descriptions of *Flavobacterium aquidurense* and *Flavobacterium araucananum.* Submitted to *International Journal of Systematic and Evolutionary Microbiology.*
- Loch TP and Faisal M. *Chryseobacterium aahli* sp. nov., isolated from lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*) in Michigan. In preparation.

Problem Statement and Background:
Since the first report in the early 1920s, flavobacteriosis has posed a serious threat to wild and propagated fish stocks. Traditionally, the disease has been attributed to three bacteria within a family now known as Flavobacteriaceae (Bernardet and Nakagawa 2006). As a group, *Flavobacterium* spp. have historically accounted for more fish mortality in the state of Michigan and its associated hatcheries than all other pathogens combined (Records of Michigan DNR Fish Health Laboratory).

For decades until present, three *Flavobacterium* spp. have dominated the literature; *F. columnare*, the causative agent of columnaris disease, *F. psychrophilum*, the etiological agent of bacterial coldwater disease and rainbow trout fry syndrome, both of which are serious diseases of salmonids, and *F. branchiophilum*, the agent of bacterial gill disease. Subsequent reports have linked a number of additional *Flavobacterium* spp. to external lesions in fish, such as *F. succinicans*, *F. johnsoniae*, *F. hydatis*, and a number of uncharacterized flavobacteria (Bernardet and Bowman 2006). It is mentionworthy, however, that these flavobacteria were predominantly isolated from external lesions, including the deep musculature, but were rarely isolated from internal organs.

With the recent advances in molecular biology and biotechnology, several novel genera within the family Flavobacteriaceae have emerged that encompass pathogens of fish, amphibians, reptiles, birds, mammals, and humans. Moreover, the last decade has witnessed the emergence of multiple novel flavobacterial species that have caused substantial damage to fish stocks worldwide (Michel et al. 2005; Bernardet et al. 2005; Flemming et al. 2007). In fish, Chryseobacterium infections were rarely reported, but now are responsible for numerous devastating diseases in Europe and Asia (Michel et al. 2005; Bernardet et al. 2005). This apparent increase has heightened concerns involving emerging flavobacteria and chryseobacteria, as many isolates are highly resistant to antibiotics (Michel et al. 2005) and are able to infect homeotherms, including humans (Bernardet et al. 2005). Currently, there are no published reports of Chryseobacterium-caused diseases in fish in the continental USA.

Recently, numerous wild fish kills have occurred within the Great Lakes Basin (GL), several of which were attributed to infectious agents, such as Viral Hemorrhagic Septicemia Virus, *Renibacterium salmoninarum*, and *Flavobacterium columnare*. 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 emerging and resurging pathogens threatening conservation efforts in wild fish stocks, as well as those reared within State Fish Hatcheries. The multiyear studies identified flavobacteria and chryseobacteria as a major cause of fry and fingerling mortalities and were recovered from over 40 disease/mortality events that occurred in both wild and captive fish stocks. When 144 Michigan flavobacteria isolates were analyzed by ribosomal RNA partial gene sequencing, numerous novel flavobacterial strains were identified, in addition to *F. columnare* and *F. psychrophilum*. Members of the genera *Flavobacterium* and *Chryseobacterium* were associated with deadly septicemias in brown trout, rainbow trout, coho salmon, and chinook salmon fry in Michigan State Fish Hatcheries and were also recovered from numerous diseased wild fishes. These bacterial strains, never before reported from North America, shared astounding similarities with strains found in Europe (Bernardet et al. 2005) and South Africa (Flemming et al. 2007), a matter that implies the emergence of these bacteria to the Great Lakes. Other novel flavobacteria isolated from Michigan were associated with mortality episodes in which disease signs and tissue alterations mimicked those associated with *F. columnare*, *F. psychrophilum*, and *F. branchiophilum*. Phylogenetic

analysis provided evidence that the recently isolated flavobacteria and chryseobacteria are very diverse and several are most likely novel.

This completed study spanned two years with the following objectives:

Objective 1: to finalize the characterization of novel GL flavobacteria recovered from diseased Great Lakes fishes.

Objective 2: to ascertain the pathogenicity of representative strains of novel flavobacteria as selected per the results of Objective 1.

Objective 3: to conduct epizootiological studies involving flavobacterial infections within production Chinook salmon reared within two Michigan State Fish Hatcheries and in feral broodstock returning to two gamete collecting stations in order to elucidate flavobacteria trafficking and disease dynamics.

Objective 4: Determination of potential virulence factors that are employed by a proven fish pathogenic Great Lakes flavobacterial strain in vivo using suppression subtractive hybridization.

Objective I- to finalize the characterization of novel flavobacteria involved in systemic and external infection of Great Lakes fishes.

We started this study with ~260 isolates from the family Flavobacteriaceae that were retrieved from 21 farmed and wild fish species. Of the 260 flavobacteria, we performed preliminary phenotypic testing, partial sequencing of the 16S small ribosomal RNA (16S rDNA), and phylogenetic analysis on 144 isolates. Analyses placed all of the 144 isolates in the genera F*lavobacterium* and *Chryseobacterium*. Moreover, we were able to speciate 5 isolates as *F. psychrophilum* , 6 as *F. columnare*, 1 as *C. aquaticum*, 2 as *C. vrystaatense*, 3 as *F. frigidimaris*, 6 as *F. johnsoniae*, and 1 as *F. succinicans* (all >99% homologous), while 120 flavobacterial isolates did not fall into any known species within the family.

Tasks to fulfill this objective were directed to fully characterize a multitude of "less-typical' potentially fish-pathogenic flavobacteria that were associated with an array of diseased fish/mortality events in wild, feral, and cultured fish stocks.

Methods used included:

Fish and bacterial isolation. From 2003-2010, fish (21 different species, Supp. Table 1.2.1) 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.

Bacterial characterization. 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.

Major findings & their discussion:

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 1.2.1. 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 1.2.1) 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 1.2.1).

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 1.2.1).

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. 1.2.1, Supp. Table 1.2.1); 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. 1.2.2,

Supp. Table 1.2.1), 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 1.2.1; 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 1.2.1). Phylogenetic analysis yielded 5 distinct clusters (Fig. 1.2.1); Cluster I (6 isolates; Supp. Fig. 1.2.1), Cluster II (13 isolates; Supp. Fig. 1.2.1), Cluster III (6 isolates; Supp. Fig. 1.2.2), Cluster IV (4 isolates; Supp. Fig. 1.2.3), and Cluster V (3 isolates; Supp. Fig. 1.2.4), while the topology of 3 isolates (T65, S53, T132) was unresolved (Fig. 1.2.1). 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. 1.2.1). 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. 1.2.1). 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 1.2.1). 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. 1.2.3), 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 1.2.1), with 1 of the 3 isolates (S86) being associated with mortalities. Enzymatic activities for isolates within Clusters I-V varied (Table 1.2.2). 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 1.2.2.

The % 16S rRNA gene similarity of the 28 Michigan isolates most similar to *F. pectinovorum* ranged from 97.1- 98.4% (Supp. Table 1.2.1). Phylogenetic analysis yielded 3 distinct clusters (Fig. 1.2.1) that contained 3 isolates (Cluster VI; Supp. Fig. 1.2.2), 11 isolates (Cluster VII; Supp. Fig. 1.2.2), and 4 isolates (Cluster VIII; Supp. Fig. 1.2.1), while the topology of 12 isolates was unresolved (Fig. 1.2.1 and Supp. Fig. 1.2.2). 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. 1.2.2). 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 1.2.1). 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 1.2.2).

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. 1.2.1); Cluster IX, which contained 8 isolates (Supp. Fig. 1.2.4), and Cluster X, which contained 12 isolates (Supp. Fig. 1.2.2). 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 1.2.1). Despite the high % similarity, there was evidence of a distinct sub-clade within Cluster IX (Supp. Fig. 1.2.4). 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. 1.2.2). 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 1.2.1). Cluster X isolates were recovered from hatchery-reared salmonids, with the exception of T103, which originated from a wild sea lamprey (Supp. Table 1.2.1). Proteolytic activities varied in both clusters (Table 1.2.2), 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. 1.2.1); Cluster XI (n=9; Supp. Fig. 1.2.5) and Cluster XII (n=9; Supp. Fig. 1.2.3), and two isolates (T157 and S162) were unresolved (Fig. 1.2.1). Isolates falling into Cluster XI were recovered from 9 different species of wild/feral (n=6) and hatchery-reared (n=3) fishes (Supp. Table 1.2.1). 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 1.2.1). Among these, isolate S21 was recovered from deep necrotic ulcers present on the dorsum of a rainbow trout (Fig. 1.2.3a). *Flavobacterium* spp. isolates that belonged to Cluster XII were primarily recovered from gills of wild fishes sampled during fish health surveillance (Supp. Table 1.2.1), 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 1.2.2). 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 1.2.2).

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. 1.2.1); Cluster XIIIa (Supp. Fig. 1.2.6) was comprised of 15 isolates recovered predominantly from feral spawning (n=13) *Oncorhynchus* spp. (Supp. Table 1.2.1), while Cluster XIIIb (Supp. Fig. 1.2.6) 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 1.2.1). 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. 1.2.1); Cluster XIV (n=2; Supp. Fig. 1.2.5), Cluster XV (n=10; Supp. Fig. 1.2.7), and Cluster XVI (n=3; Supp. Fig. 1.2.7), while isolate T129 fell into Cluster I (described above; Supp. Fig. 1.2.1). Interestingly, isolates belonging to these three clades were exclusively recovered from wild fishes (Supp. Table 1.2.1) 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. 1.2.7). 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 1.2.1). Representative isolates belonging to Cluster XV did not degrade gelatin or hemoglobin and were variable for caseinase and elastase activities (Table 1.2.2).

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. 1.2.1), whereby the *F. columnare* reference sequence and 14 of the Michigan *F. columnare* isolates formed a distinct and homogenous cluster (Cluster XVII; Supp. Fig. 1.2.8), while *F. columnare* strain S81 formed a distinct branch outside of this Cluster, termed Cluster XVIIa (Supp. Fig. 1.2.8). 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 1.2.1). 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 1.2.2).

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. 1.2.2), one isolate (T17) fell into Cluster XI (Supp. Fig. 1.2.5), one isolate (S30) fell into Cluster XX (described below; Supp. Fig. 1.2.9), one isolate was unresolved (S107; Fig. 1.2.1), and the remaining 10 isolates formed Cluster XVIII (Supp. Fig. 1.2.9). Isolates belonging to Cluser XVIII, which displayed varying degrees of genetic heterogeneity (Fig. 1.2.12), were recovered from the kidneys of feral Chinook salmon (n=6) during 2005 and 2007 (Supp. Table 1.2.1), 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 1.2.2).

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. 1.2.1 and Supp. Fig. 1.2.9), along with Cluster XX (Supp. Fig. 1.2.9), which contained isolates S5 and S30 (most similar to *F. aquidurense* according to 16S rDNA %), while the remaining 6 isolates were unresolved (Fig. 1.2.1, Supp. Table 1.2.1). 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. 1.2.1). In addition, isolate S164 fell into cluster VII (Supp. Fig. 1.2.2). Isolates belonging to Cluster XIX were recovered from kidneys, fins (Fig. 1.2.3b), and brains of hatchery-reared salmonid fingerlings undergoing mortality (Supp. Table 1.2.1). Isolates within Cluster XIX did not degrade gelatin or elastin and varied in caseinase activity and hemoglobin hydrolysis (Table 1.2.2). One isolate belonging to Cluster XX was positive for degradation of gelatin, hemoglobin, and elastin (Table 1.2.2).

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. 1.2.1); Cluster XXI (n=3; Supp. Fig. 1.2.2) and Cluster XXII (n=5; Supp. Fig. 1.2.3), while isolate S129 was unresolved (Fig. 1.2.1) and isolate S15 fell into Cluster IV (Supp. Fig. 1.2.3), 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. 1.2.3c) of wild and hatchery-reared salmonids (Supp. Table 1.2.1), 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 1.2.2). Representative Cluster XXII isolates degraded hemoglobin and elastin and did not degrade gelatin (Table 1.2.2).

The three Michigan isolates most similar (97.1%) to the *F. reichenbachii* reference sequence formed Cluster XXIII (Fig. 1.2.1), which shared a most recent common ancestor with the *F. reichenbachii* reference sequence (Supp. Fig. 1.2.5). 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. 1.2.6). *Flavobacterium hydatis*-like isolates (n=3; 97.7-98.9%) fell into two clusters (XXV and XXVI; Supp. Fig. 1.2.1), 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 1.2.1), while Cluster XXVI contained the *F. hydatis* reference sequence and isolate S171 (Fig. 1.2.1). Isolates belonging to Clusters XXV and XXVI were positive for casein, hemoglobin, and elastin degradation, but did not degrade gelatin (Table 1.2.2).

The two isolates most similar to *F. anhuiense* (97.4 – 98.0%) were recovered from the kidneys of hatchery-reared channel catfish (Supp. Table 1.2.1) and formed Cluster XXVII, which was distinct from that of the *F. anhuiense* and *F. ginsenosidimutans* (Supp. Fig. 1.2.4). These isolates degraded gelatin, casein, and elastin, but did not hydrolyze hemoglobin (Table 1.2.2). 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. 1.2.4). 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 1.2.1), were both unresolved phylogenetically (Fig. 1.2.1). The two *Flavobacterium degerlachei*- like isolates (96.7-96.9%) formed Cluster XXIX (Supp. Fig. 1.2.5), and were both recovered from a hatchery mortality event among brown trout fingerlings (Supp. Table 1.2.1). Isolates most similar to *F. glacei* (n=2; 98.5-98.6%) formed Cluster XXX (Supp. Fig. 1.2.1), 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 1.2.1). The two isolates most similar to *F. hibernum* (97.7%) were distinct in that isolate S118 belonged to Cluster XXV (described above; Supp. Fig. 1.2.1), while isolate S140 was unresolved, but shared a most recent ancestry with *F. hibernum* (Supp. Fig. 1.2.5). *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. 1.2.4). Isolate S2, which was most similar to *F. psychrolimnae* (99.6%), formed Cluster XXXII with the *F. psychrolimnae* reference strain (Supp. Fig. 1.2.6) 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 1.2.1). Phylogenetic analysis yielded the formation of 1 cluster (Cluster XXXIII; Supp. Fig. 1.2.10) 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. 1.2.10). Isolates within Cluster XXXIII were recovered from the gills and kidneys of hatchery-reared muskellunge fingerlings undergoing a single mortality event (Supp. Table 1.2.1) that were also infected with *F. columnare* and all uniformly degraded gelatin, casein, hemoglobin, and elastin (Table 1.2.2). The 11 unresolved *C. viscerum* isolates were recovered from gills of wild sculpin, brook trout, and brown trout (Supp. Table 1.2.1) residing in 4 different Michigan creeks. All of these isolates displayed gelatinase, caseinase, and elastase activities, as well as hemoglobin hydrolysis (Table 1.2.2).

Six Michigan isolates were most similar to *C. ginsenosidimutans* (97.7-98.4%), all of which comprised Cluster XXXIV (Supp. Fig. 1.2.11). 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. 1.2.11). 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 1.2.1) and were recovered from infected gills, as well as necrotic and hemorrhagic fins (Fig. 1.2.3e). Moreover, isolates T68 and T130 were recovered from the kidneys of infected salmonid fingerlings (Supp. Table 1.2.1). 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 1.2.1). Among the six isolates most similar to *C. indoltheticum* (97.4-99.1% 16S rDNA similarity), three formed Cluster XXXV (Supp. Fig. 1.2.11) and three were unresolved despite clustering near *C. indoltheticum* and Cluster XXXV (Supp. Fig. 1.2.11). The isolates of Cluster XXXV were recovered from gills and brains of hatchery-reared steelhead fingerlings during a single mortality event (Supp. Table 1.2.1), 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. 1.2.3d).

The % 16S rRNA gene similarity of the five Michigan isolates most similar to *C. piscium* ranged from 98.3- 98.4% (Supp. Table 1.2.1). Phylogentic analysis placed the five

isolates, along with one isolate (S56) most similar to *C. scophthalmum,* into two clusters (Fig. 1.2.2). Cluster XXXVI contained isolate T24, along with *C. balustinum, C. piscium, C. scophthalmum* (Supp. Fig. 1.2.11), while Cluster XXXVII contained the other five isolates (Supp. Fig. 1.2.11). Isolate T24 of Cluster XXXVI was recovered from the gills of a hatchery-reared steelhead trout fingerling (Supp. Table 1.2.1) with signs similar to bacterial gill disease and it degraded gelatin, casein, hemoglobin, and elastin (Table 1.2.2), 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 1.2.2).

The two isolates most similar to *C. chaponense* (99.1%) formed Cluster XXXVIII, which also included the *C. chaponense* reference sequence (Supp. Fig. 1.2.12). 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 1.2.1). *Chryseobacterium greenlandense*-like isolates (n=2; 98.1% similarity) comprised Cluster XXXIX (Supp. Fig. 1.2.11) and were recovered from the kidneys of wild walleye (S4) and from dermal ulcers on feral steelhead (S25, Fig. 1.2.3f.) 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. 1.2.11), and T85, which was unresolved, but shared a most recent ancestry with Cluster XL (Supp. Fig. 1.2.11). 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. 1.2.10). These isolates were recovered from fins of wild sea lamprey and gills of wild mottled sculpin (Supp. Table 1.2.1). 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. 1.2.11), isolate S108 shared a most recent ancestry with *C. shigense* (Supp. Fig. 1.2.10), while isolate S7 was unresolved (Supp. Fig. 1.2.11).

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. 1.2.1 and 1.2.2). 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 1.2.1). 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.

Table 1.2.1. 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. 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, mildly swollen spleen, hepatic and renal pallor, visceral edema
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 1.2.2. 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)
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 1.2.1. 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.

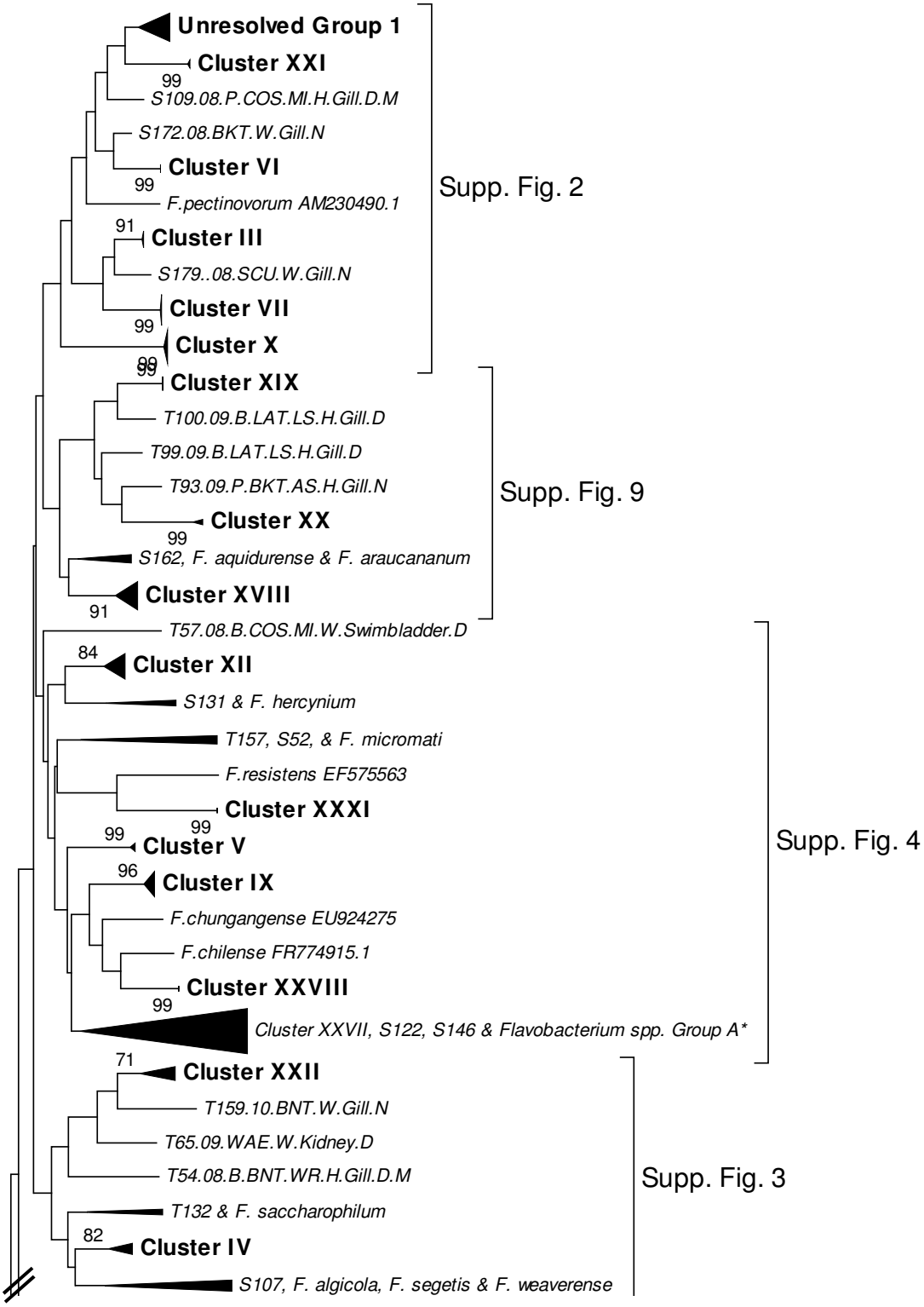
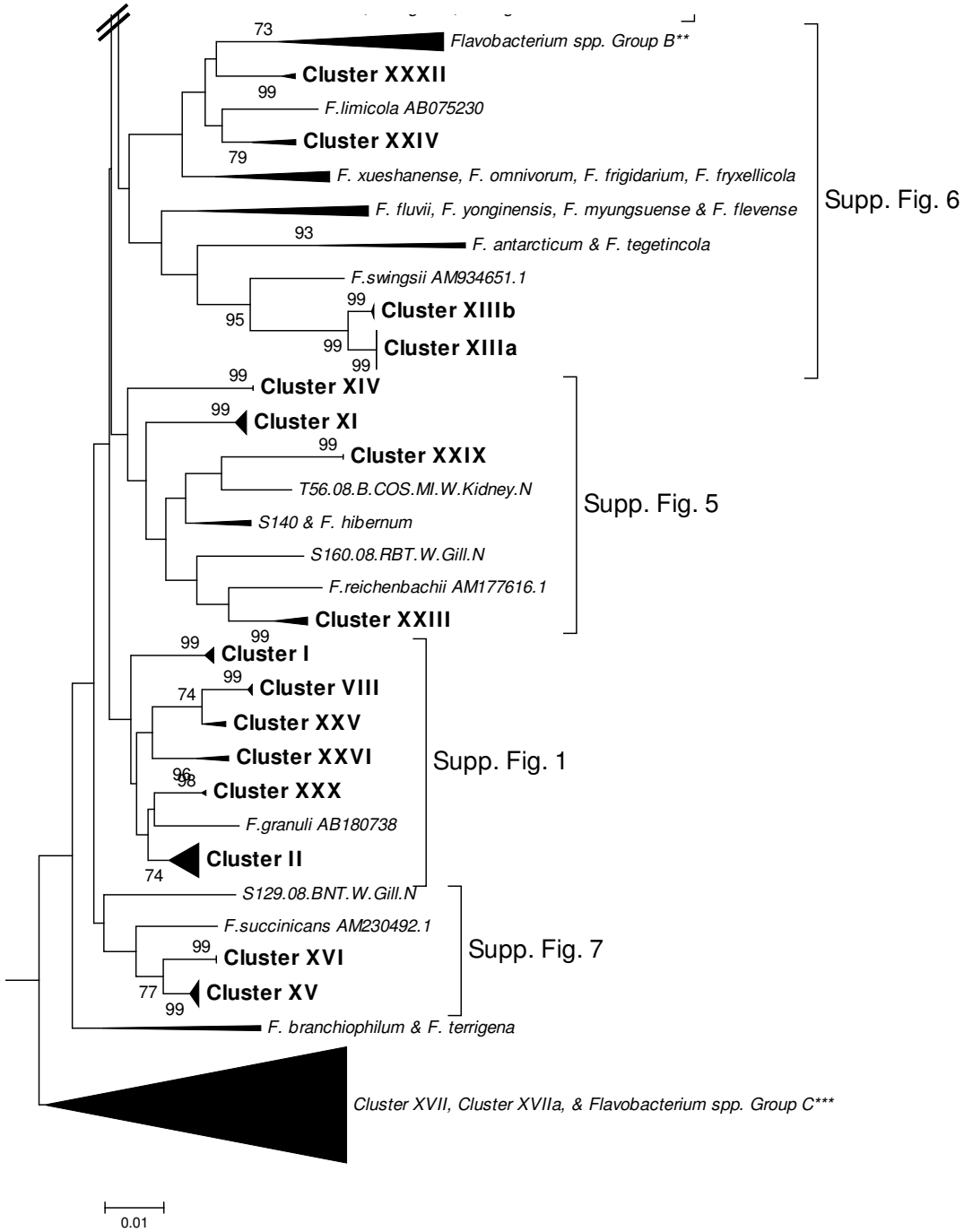


Figure 1.2.1. (cont'd)



*,***Flavobacterium* spp. Group A** *includes F. chu* (Supp. Fig. 8) *F. daejeonense, F. glycines, F. defluvii, F. johnsoniae, F. compostarboris, F. bar[illegible], 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 1.2.2. 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.

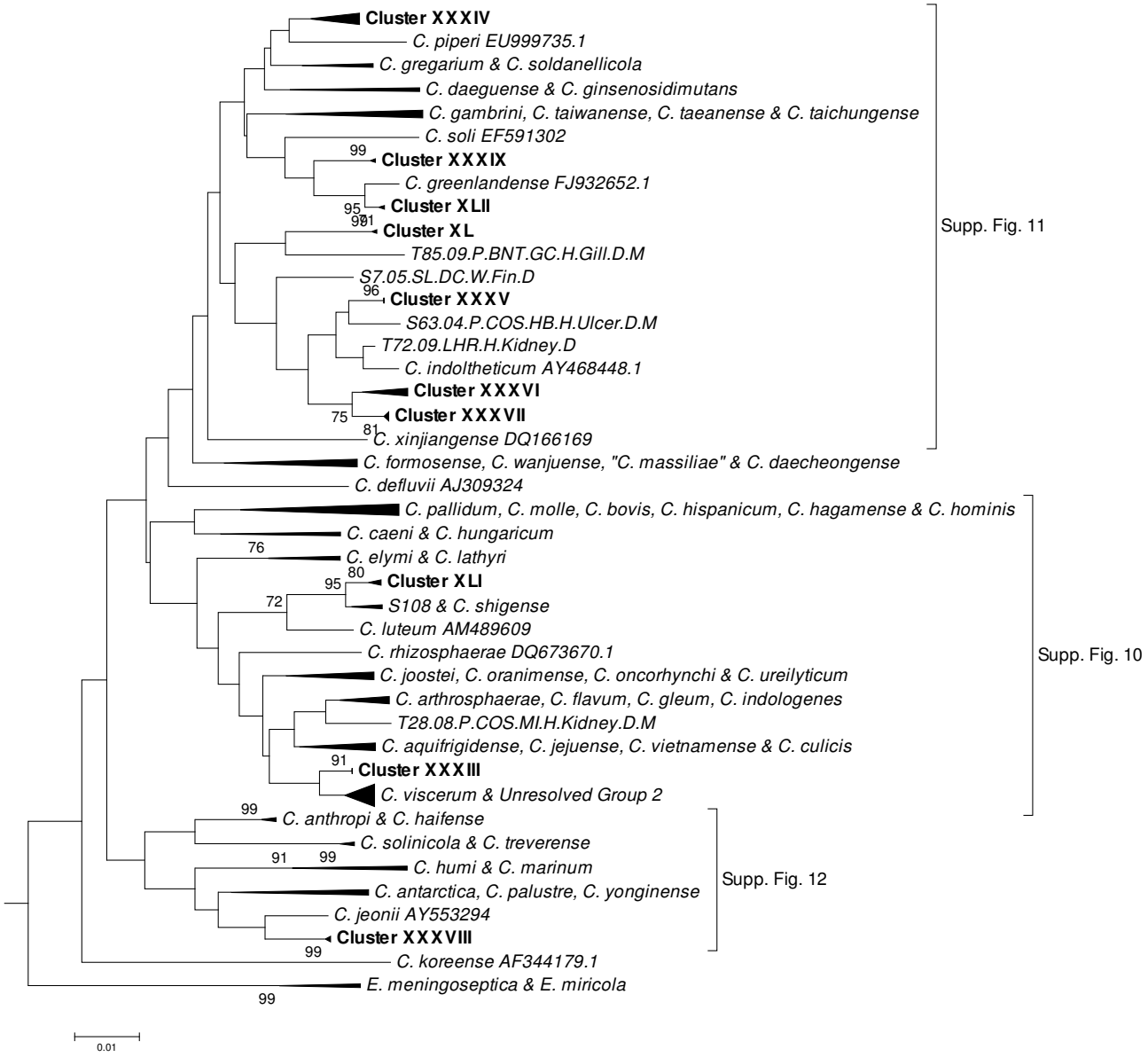
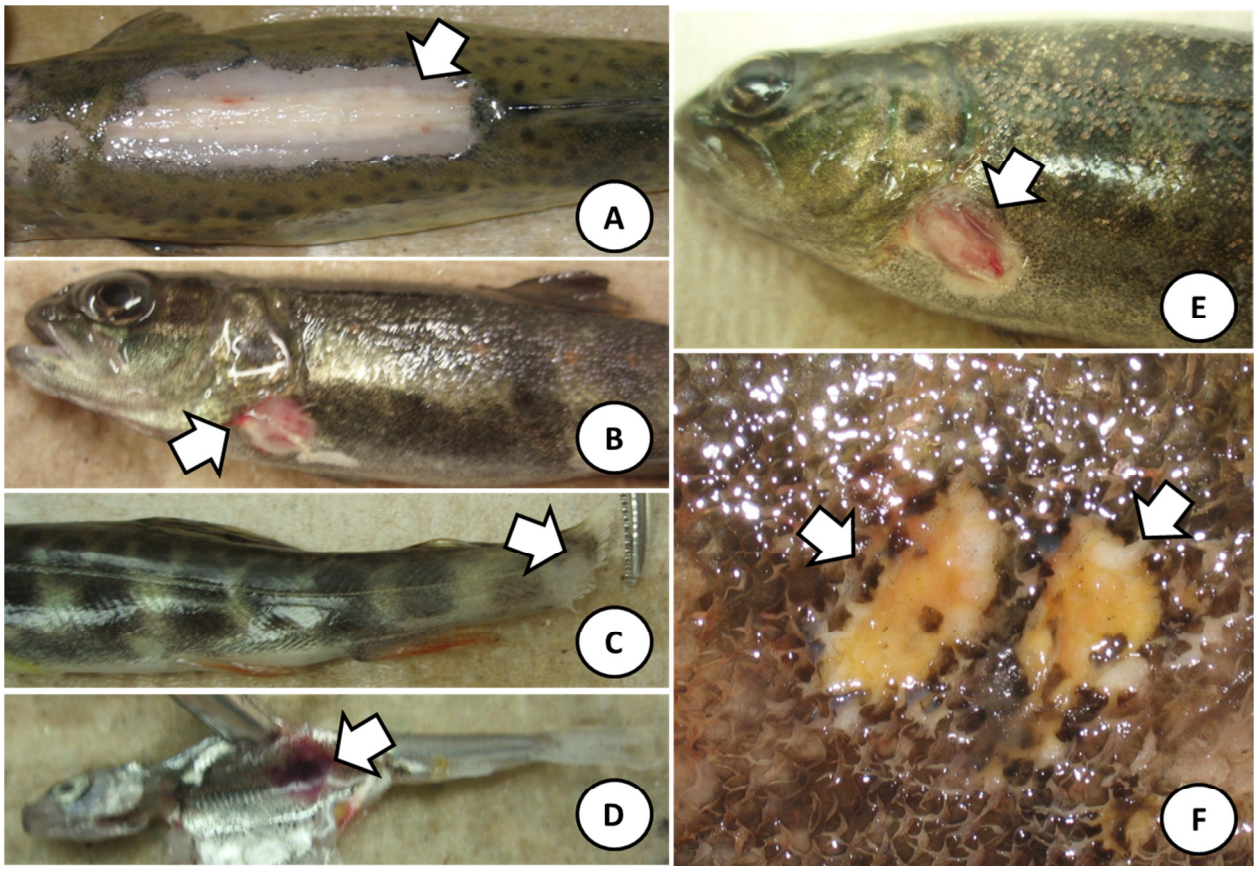


Figure 1.2.3. Gross lesions present in Michigan fishes infected with *Flavobacterium* and *Chryseobacterium* spp. A) Necrotic ulceration (arrow) present on the dorsum of a rainbow trout from which *Flavobacterium* sp. S21 was recovered. Note the complete erosion of the dorsal fin and penetration into 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.



Additional studies performed to fulfill Objective I: Polyphasic characterization of emerging chryseobacterial strains, combined with studies in fulfillment of Objective II- To ascertain the pathogenicity of representative strains of novel chryseobacteria as selected per the results of Objective 1.

As proposed, experimental infection trials were performed on the fully characterized *Flavobacterium* and *Chryseobacterium* spp. under Objective I, though we screened 16 isolates as opposed to the originally proposed 5-10 (in order to assess a wider diversity of flavobacterial strains). The original plan was to first screen the novel isolates for their pathogenicity in fish using an IP infection route, followed by experimental immersions. This approach was slightly modified since immersion infection does not yield consistent results. As an alternative approach, we followed the screening with determinination of the median lethal dose of strains using the IP route.

Methods used included:

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 ℃, were analyzed in this study (Table 2.3.1). 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 ℃ 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℃, 15℃, 22℃, 37℃, and 42℃; 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 methanosulphonate (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.

In addition, all of the aforementioned studies were also performed on *Chryseobacterium* sp. T68, which is currently being proposed as the novel species *C. aahli*. The manuscript describing these results can be found in Appendix D.

Major findings and their discussion:

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 2.3.1). 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 2.3.1). 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. 2.3.1). 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. 2.3.1). 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. 2.3.1). Lastly, isolate T24 formed a cluster with three other *Chryseobacterium* spp. frequently associated with fish (Fig. 2.3.1), 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. 2.3.2), 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. 2.3.2), 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. 2.3.2). 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℃ - 22℃ (T86 and T28 grew weakly at 4℃), but not at ≥37℃. 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 2.3.3), which is typical of the genus *Chryseobacterium* (Bernardet et al. 2006).

These isolates also contained a variety of other fatty acids (Table 2.3.3). 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 2.3.3), slight differences were observed amongst them (i.e., *anteiso*-$C_{15:0,}$ *iso*-$C_{17:0,}$ $C_{17:0}$ 2-OH; Table 2.3.3). 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 ℃. 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℃. 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 $6.5x\ 10^7 – 2.6 \times 10^8$ 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. 2.3.3a), gill hemorrhage and pallor (Fig. 2.3.3b), hemorrhage and deep ulceration of the muscle (Fig. 2.3.3c), 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. 2.3.3d) sometimes

accompanied with hydrocephalus. In fish infected with T72, bilateral exophthalmia, diffuse external petechial hemorrhage, multifocal dermal ulceration of the trunk (Fig. 2.3.3e), 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. 2.3.3f), 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. 2.3.3g).

In fish infected with T24, disease signs included perinasal and intracranial hemorrhage (Fig. 2.3.3h), 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. 2.3.4a 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. 2.3.4c and d), mild multifocal necrosis within the interstitum of the posterior kidney, mild hemorrhage and degeneration of myofibers lining the peritoneum, mononuclear pancreatitis (Fig. 2.3.4e), 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. 2.3.4f), 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. 2.3.5a and b), monocytic infiltrate and mucus cell hyperplasia within primary lamellae consistent with branchitis (Fig. 2.3.5c), monocytic myositis, hemorrhage within the muscle, liver, adipose tissue (Fig. 2.3.5d), and ovaries (Fig. 2.3.5e), 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. 2.3.5f). 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.

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 2.3.1). 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 4.5×10^8 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.

Table 2.3.1. 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 2.3.2. 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 2.3.3. 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 2.3.1. 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.

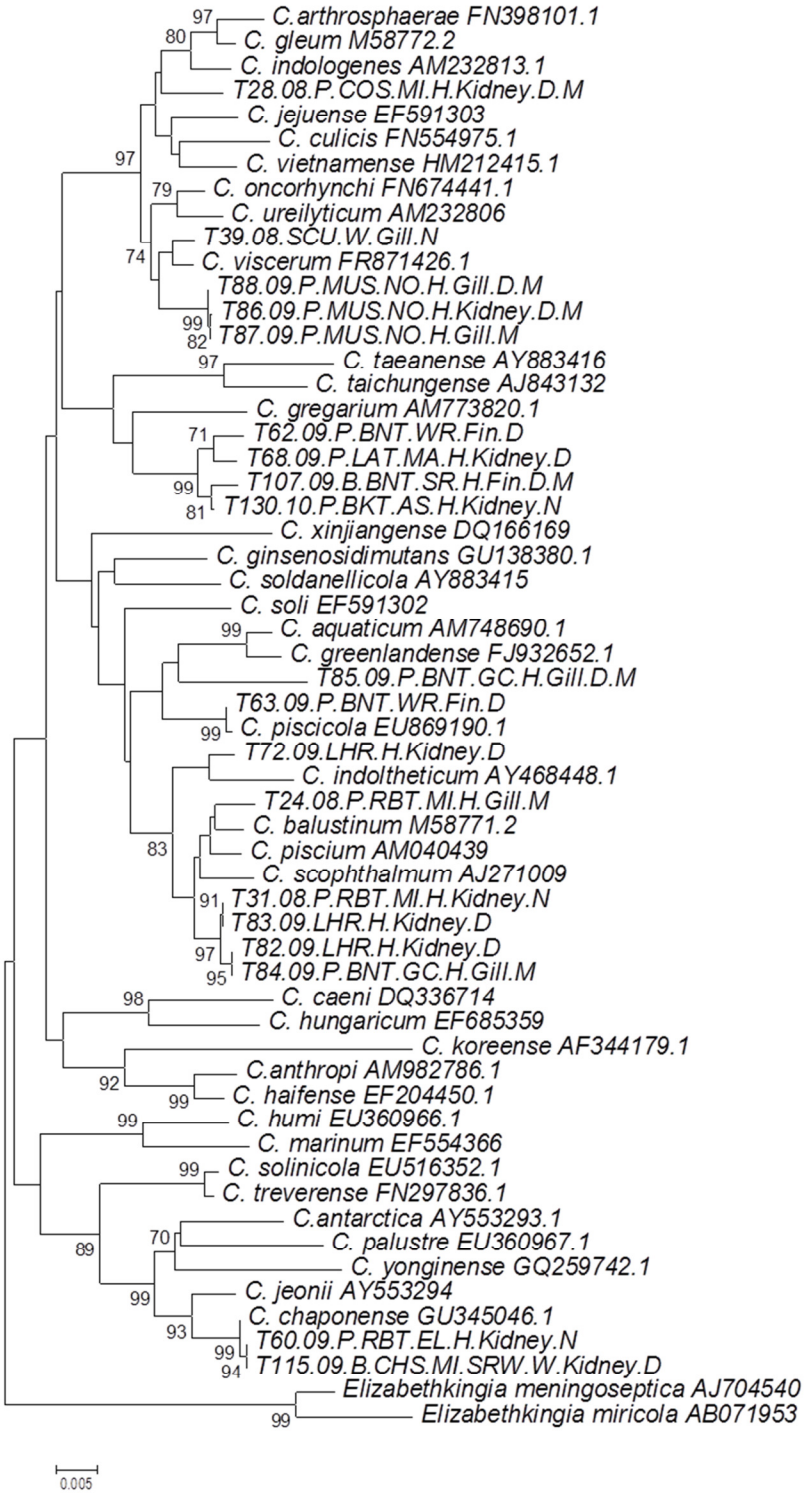


Figure 2.3.2. Dendrogram depicting the relationships of 7 Michigan fish-associated *Chryseobacterium* spp. (red rectangles) generated using Bayesian analysis in MrBayes 3.1.2. Filled circles are present when that node was also present in neighbor-joining analysis. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

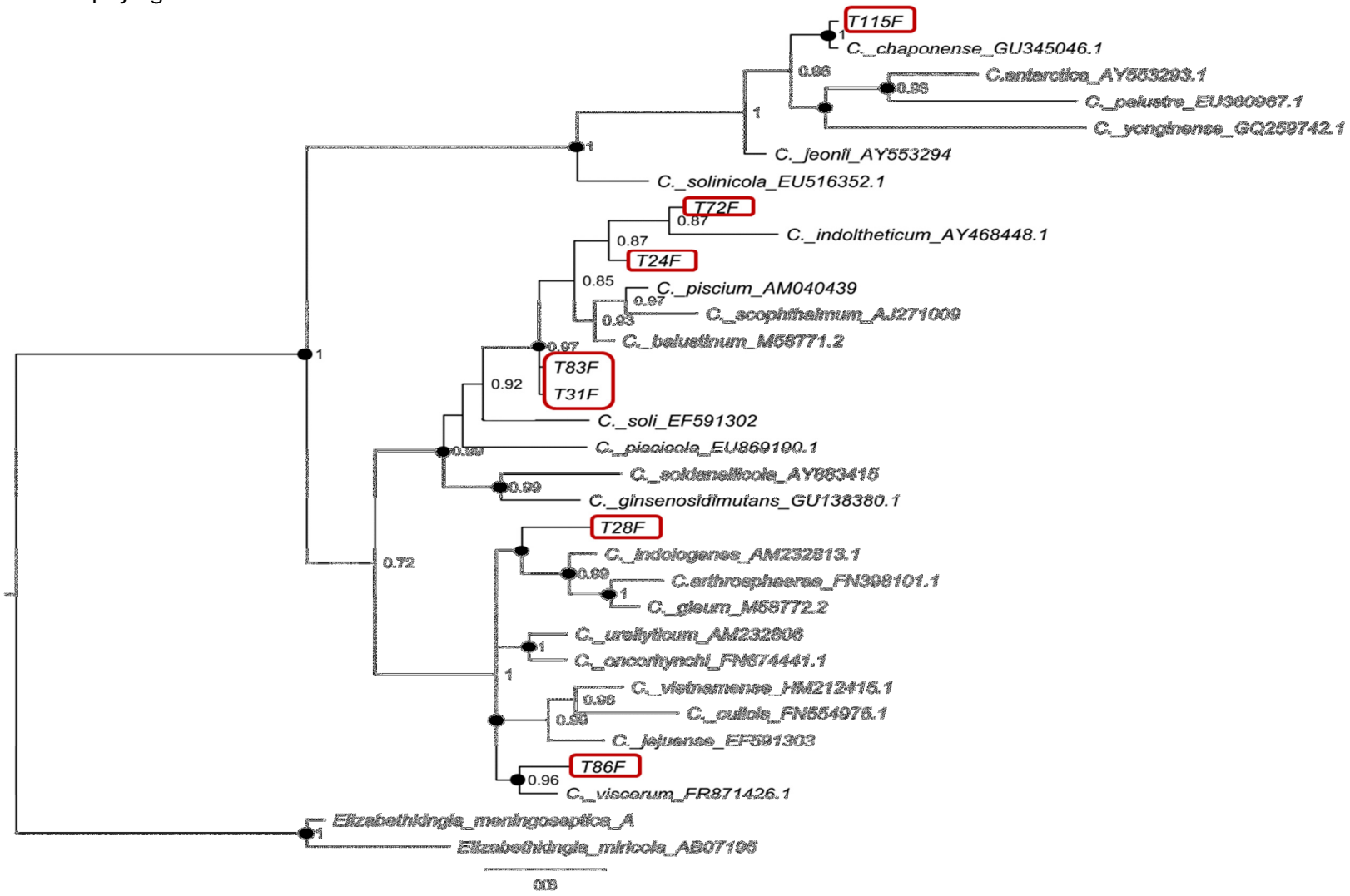


Figure 2.3.3. Gross lesions observed in fish intraperitoneally injected with Michigan *Chryseobacterium* spp. isolates. A) Unilateral exophthalmia and periocular hemorrhage (arrow) in a Chinook salmon fingerling infected with isolate T28. B) Pallor and multifocal hemorrhage (arrow) within the gills of 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.

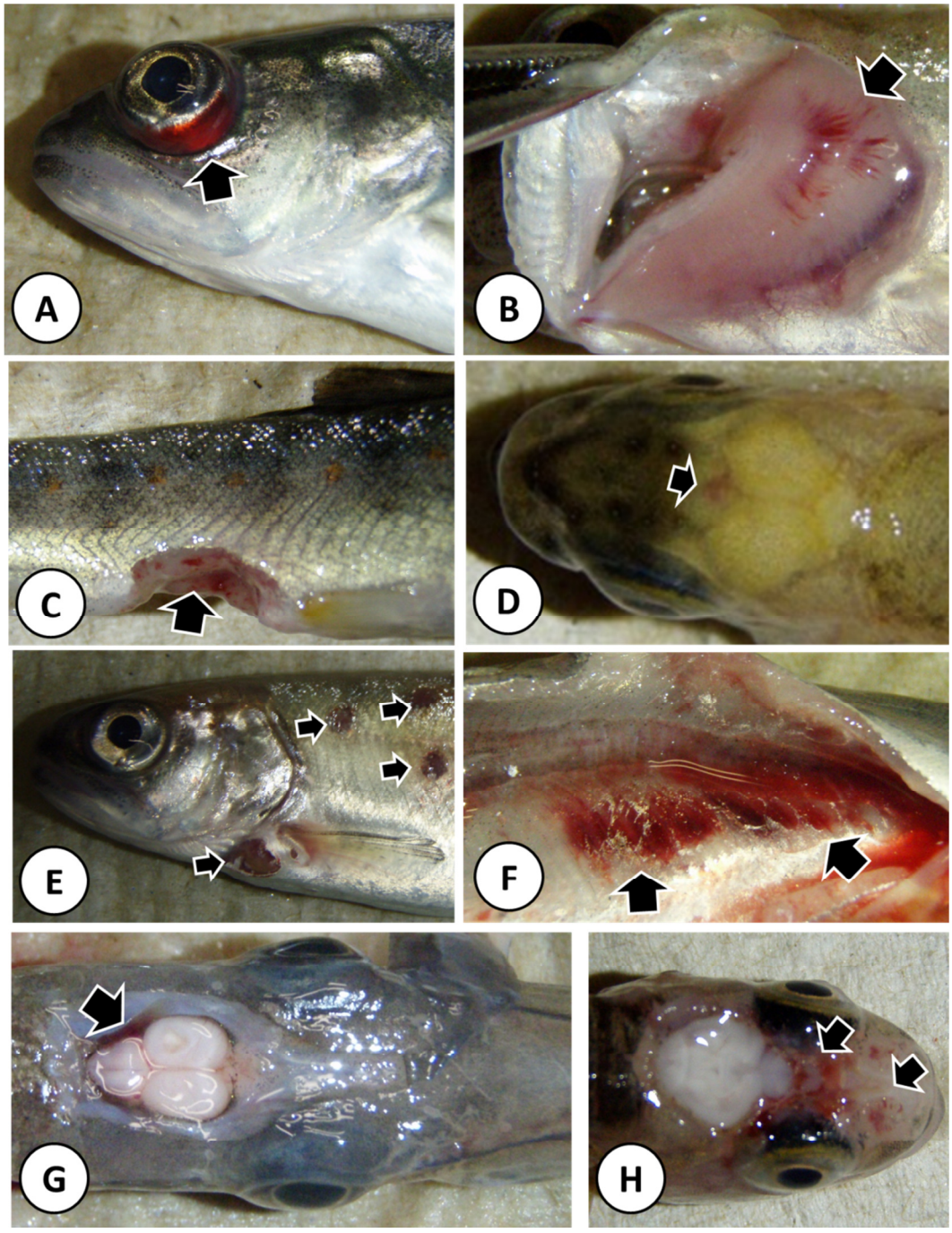


Figure 2.3.4. Hematoxylin and eosin (H&E) stained tissue sections from brook trout challenged (A, C, E, F) and mock challenged (B& D) with *Chryseobacterium* sp. T68. A) A spleen with hemosiderosis (arrows; 400x). B) A spleen from a mock-challenged brook trout (400x). C) Anterior kidney with focal edema (arrows; 200x) D) Anterior kidney of a a mock-challenged brook trout (200x). E) Pancreatitis (arrows) in a challenged brook trout fingeling (200x). F) Atrium of the heart with a large number of leukocytes providing evidence for a peripheral leukocytosis (400x).

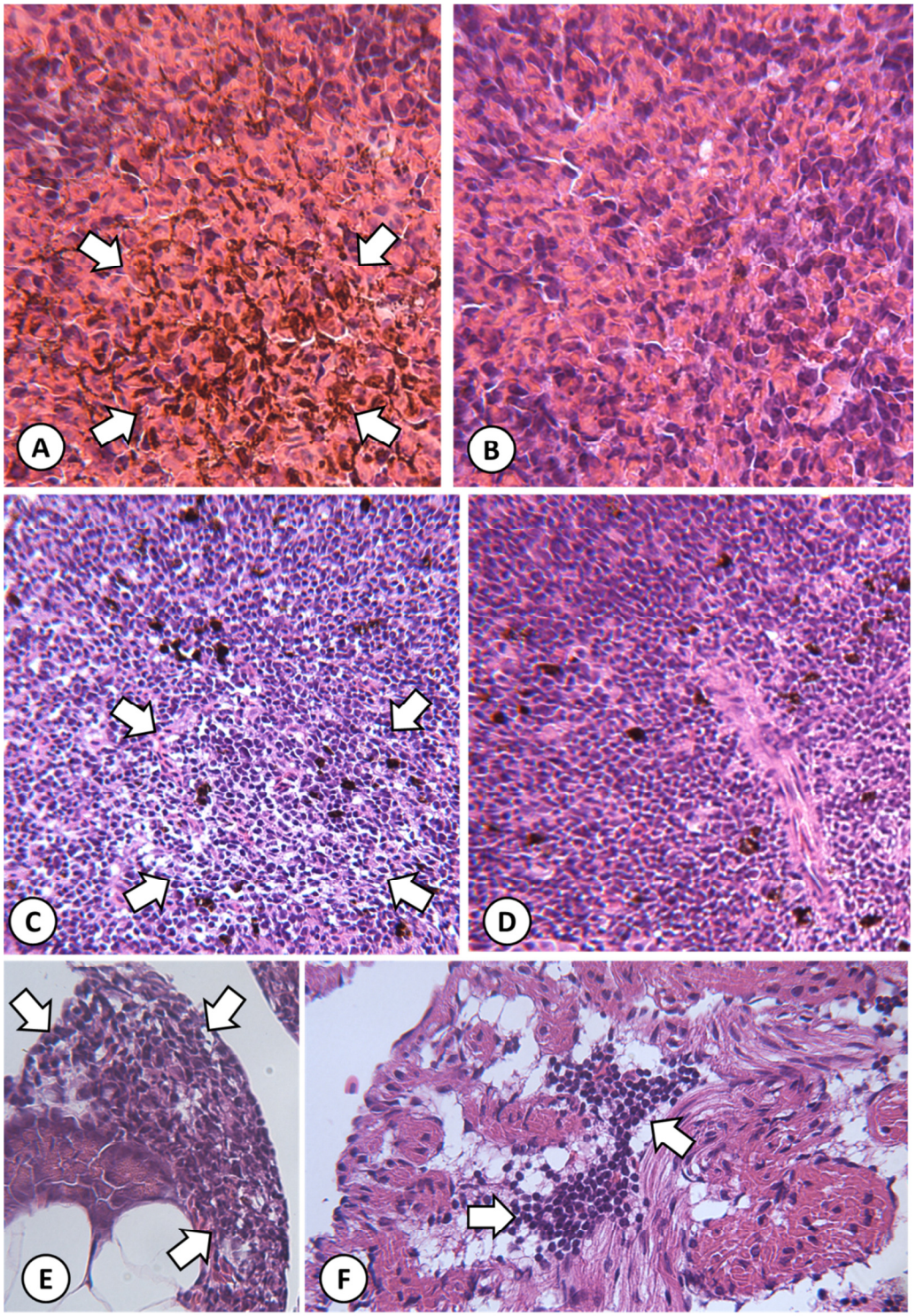
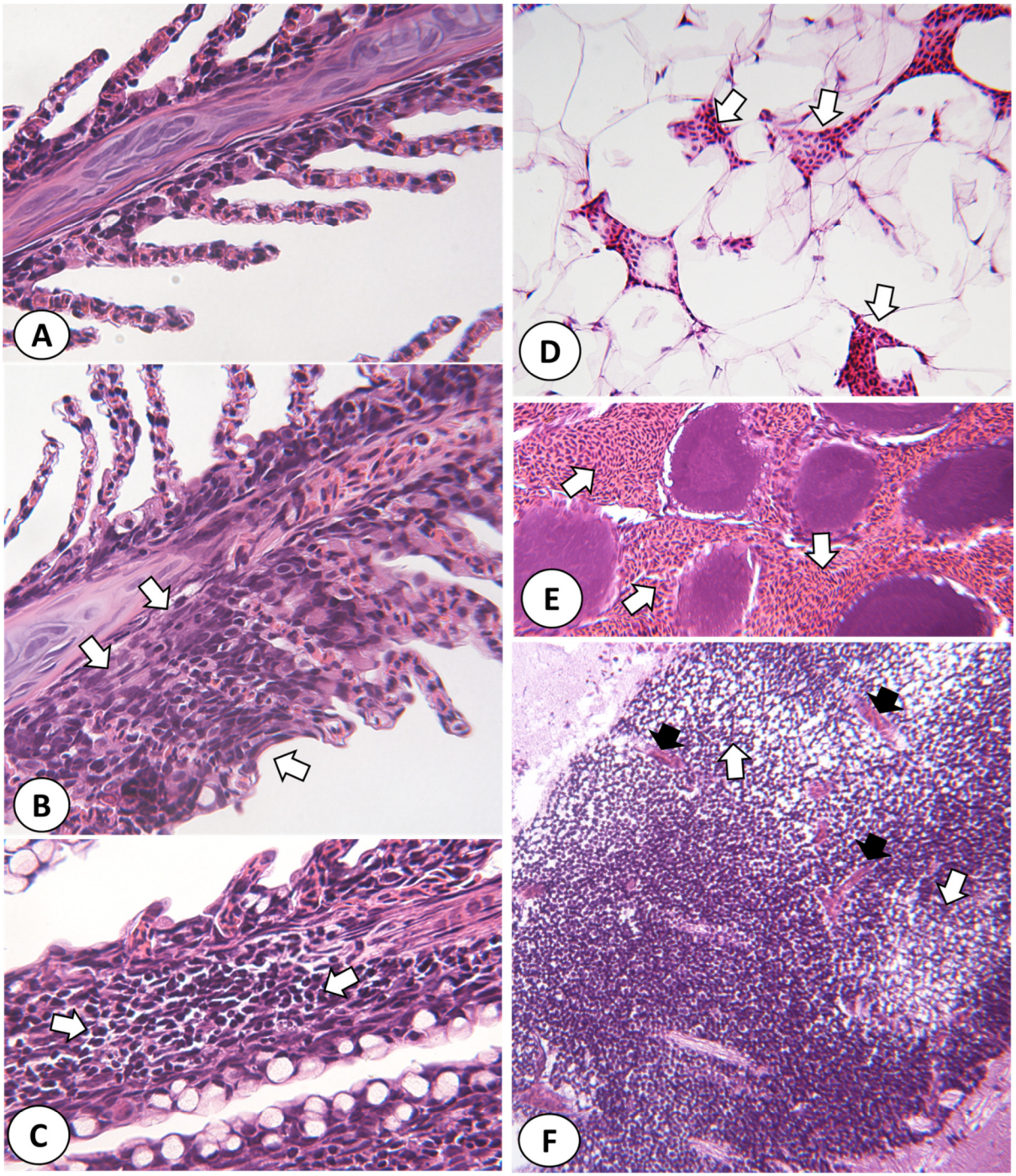


Figure 2.3.5. Hematoxylin and eosin stained tissue sections from coho salmon challenged (B-F) and mock challenged (A) with *Chryseobacterium* sp. T28. A) Gills showing normal secondary lamellae (400x). B) Gills showing epithelial hyperplasia of the secondary lamellae and interlamellar space (arrows; 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).



Additional studies performed to fulfill Objective I: Polyphasic characterization of emerging flavobacterial strains, combined with studies in fulfillment of Objective II- To ascertain the pathogenicity of representative strains of novel flavobacteria as selected per the results of Objective 1.

Methods used included:

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 ℃, were analyzed in this study as described above.

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 performed as described above. recommended by Bernardet et al. (2002) and included the following:

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

The aforementioned studies were also performed on *Flavobacterium* sp. T16 and S12, which are currently being proposed as the novel species, *F. spartani*. The manuscript describing these results (a portion of which has been submitted to IJSEM) can be found in Appendix D.

Major findings and their discussion:

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 3.4.1). The remaining 65 *Flavobacterium* spp. isolates could not be speciated conclusively (Table 3.4.1). 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. 3.4.1). 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. 3.4.1 and 3.4.2). Michigan *F. columnare* isolates fell into one cluster, along with the *F. columnare* reference sequence (Figs. 3.4.1 and 3.4.3), 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. 3.4.1 and 3.4.4). Other Michigan isolates definitively identified as described *Flavobacterium* spp. include T33, which clustered with *F. frigidimaris*, and T105, which clustered with *F. tiangeerense* (Fig. 3.4.1). 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. 3.4.1). For example, isolates T164, T25, T165, T103, T95, T59, T23, and T96 were distinct from, yet most closely related to, *F. oncorhynchi* (Figs. 3.4.1 and 3.4.4), while isolates T14, T16, T13, S12, T123, and T124 formed a cluster that was distinct (bootstrap value= 99) from all other *Flavobacterium* spp. (Fig. 3.4.1). 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. 3.4.1 and 3.4.5). Isolates T131, T76, and T77, S21, T1, and T17 were also distinct from other flavobacteria (Fig. 3.4.1). 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. 3.4.1). The remaining seven clusters, as well as the unresolved taxa, are displayed in Fig. 3.4.1.

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. 3.4.6 and 3.4.7 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. 3.4.6). Similarly, *Flavobacterium* sp. T18 was well-supported as being distinct (posterior probability of 0.92; Fig. 3.4.7), as was also the case for isolate S87 (posterior probability of 0.95; Fig. 3.4.7). 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. 3.4.7).

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 ℃ but not at 42 ℃, 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 3.4.2. 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 3.4.3).

As displayed in Supp. Table 3.4.1, *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 3.4.2), as was also the case for T18 when compared to *F. hydatis* (Strohl and Tait 1978; Bernardet and Bowman 2011; Supp. Table 3.4.3). Likewise, S87 was phenotypically distinct from *F. resistens* (Ryu et al. 2008; Supp. Table 3.4.4), while S21 was also distinct from *F. aquidurense* (Cousin et al. 2007; Supp. Table 3.4.5). 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 3.4.6).

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 3.4.4). 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 3.4.4. 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 ℃. 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. 3.4.8a), mottled external coloration, gill pallor, flared opercula, swelling/pallor/congestion/friability of the liver, swollen and enlarged spleen, swim bladder hemorrhage (Fig. 3.4.8b), 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. 3.4.8c), multifocal ecchymotic hemorrhage in the swim bladder, swollen, congested, pale kidneys, and intracranial hemorrhage (Fig. 3.4.8d).

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. 3.4.1 and 3.4.5). 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. 3.4.7). 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.

Table 3.4.1. 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%)*
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 3.4.2. 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 ℃	-	-	-	(+)	-	-
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	+	-	(+)	+	-	-
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 3.4.3. 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 3.4.4. 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	-
$C_{16:1}$ ω5*c*	-	Tr	-	-	-	-
$C_{15:0}$ 3-OH	1.0	2.0	-	-	-	-
***anteiso*-$C_{17:0}$ B/*iso* I**	-	-	-	-	1.6	-

Figure 3.4.1. 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.

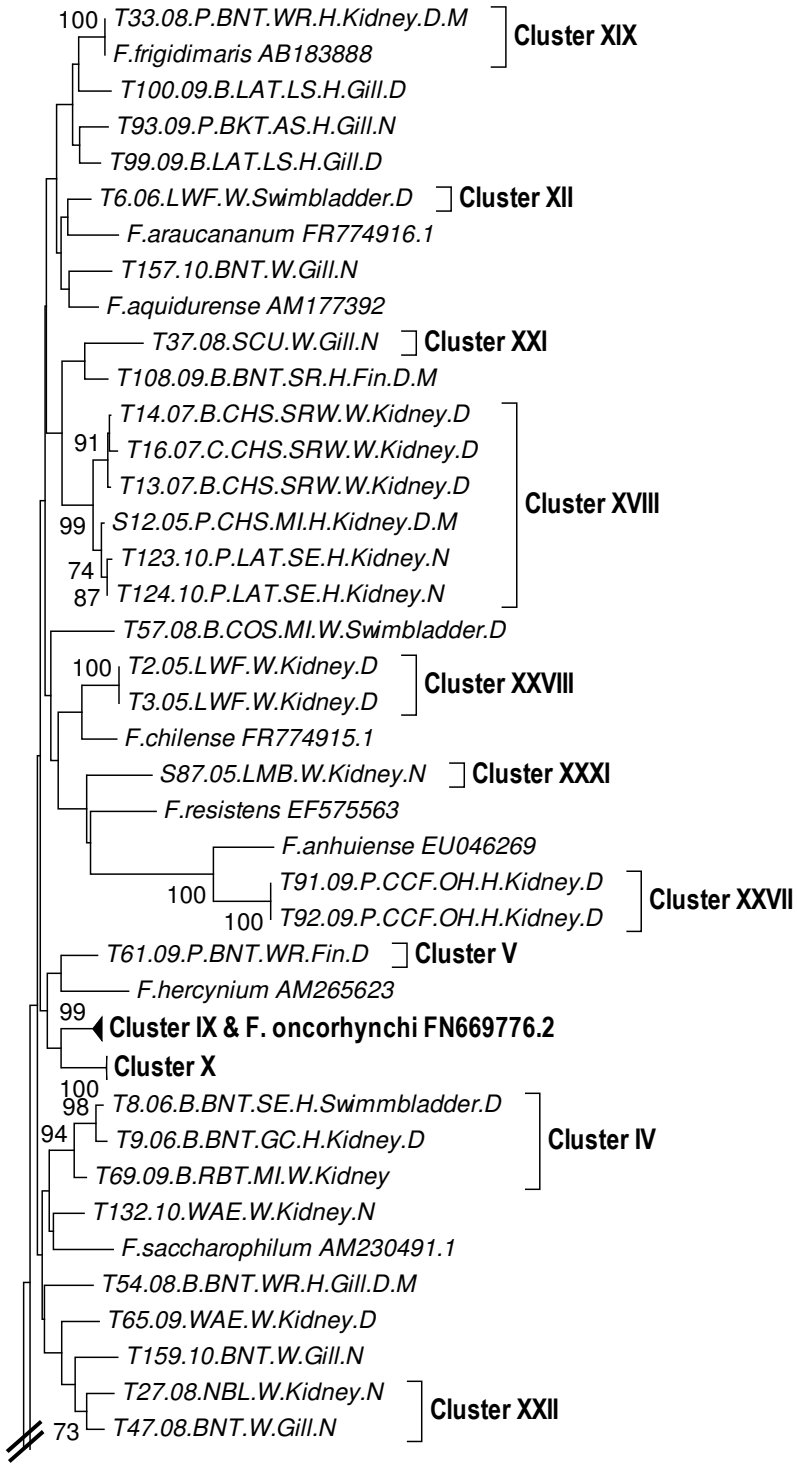


Figure 3.4.1 (cont'd)

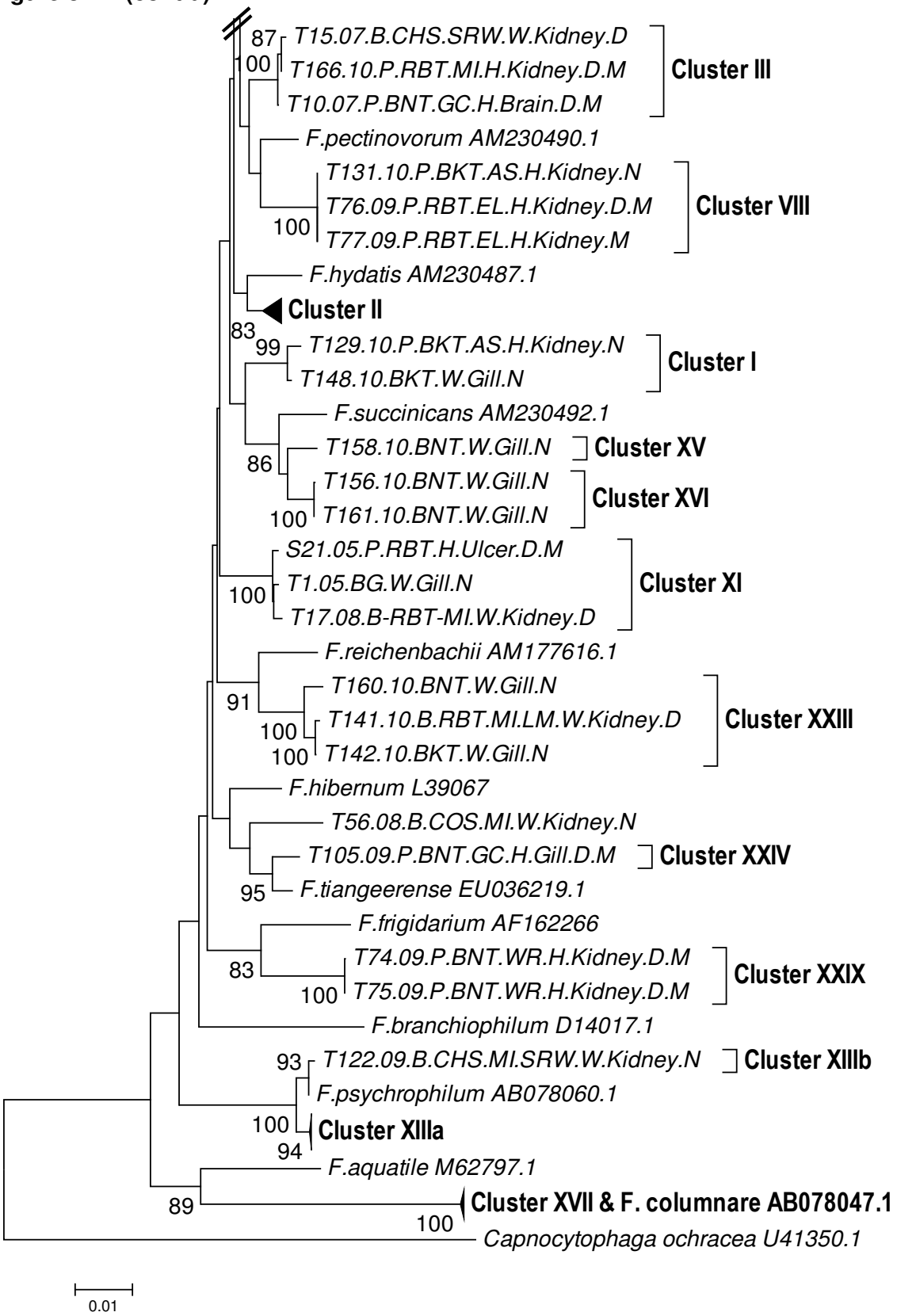


Figure 3.4.2. Subtree of dendrogram displayed in Fig. 3.4.1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 14 Michigan *F. psychrophilum* isolates recovered from fishes and the *F. psychrophilum* reference sequence. Bootstrap values >70% (expressed 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.

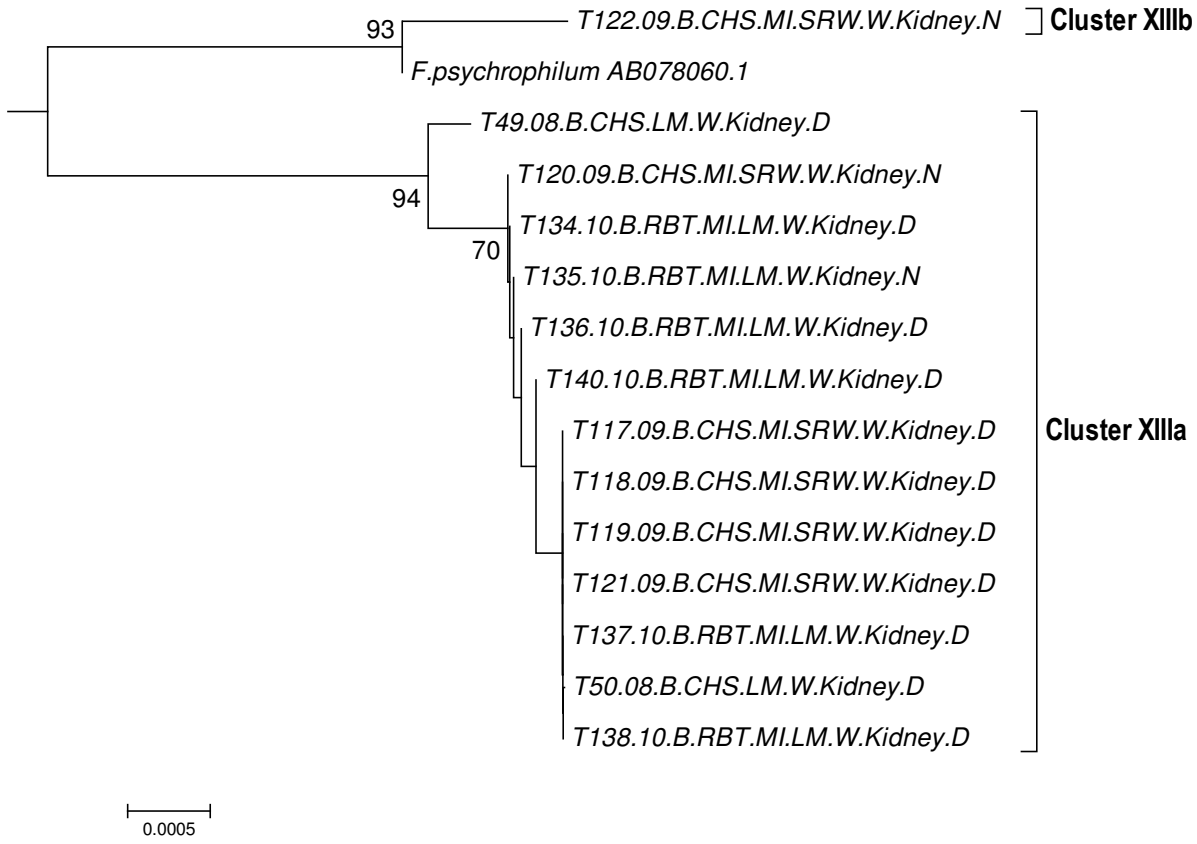


Figure 3.4.3. Subtree of dendrogram displayed in Fig. 3.4.1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 10 Michigan *F. columnare* isolates recovered from fishes and the *F. columnare* and *F. aquatile* reference sequences. Bootstrap values >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.

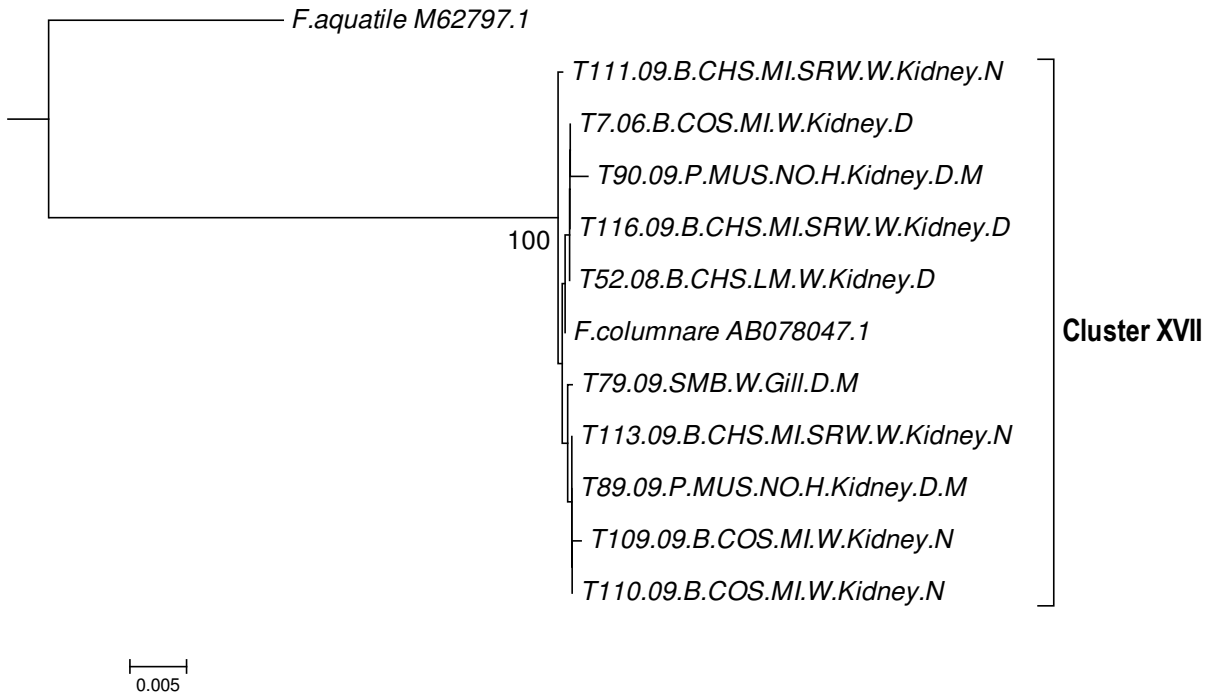


Figure 3.4.4. Subtree of dendrogram displayed in Fig. 3.4.1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 16 Michigan *Flavobacterium* spp. isolates recovered from fishes and the *F. hercynium* and *F. onchorynchi* reference sequences. Bootstrap 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.

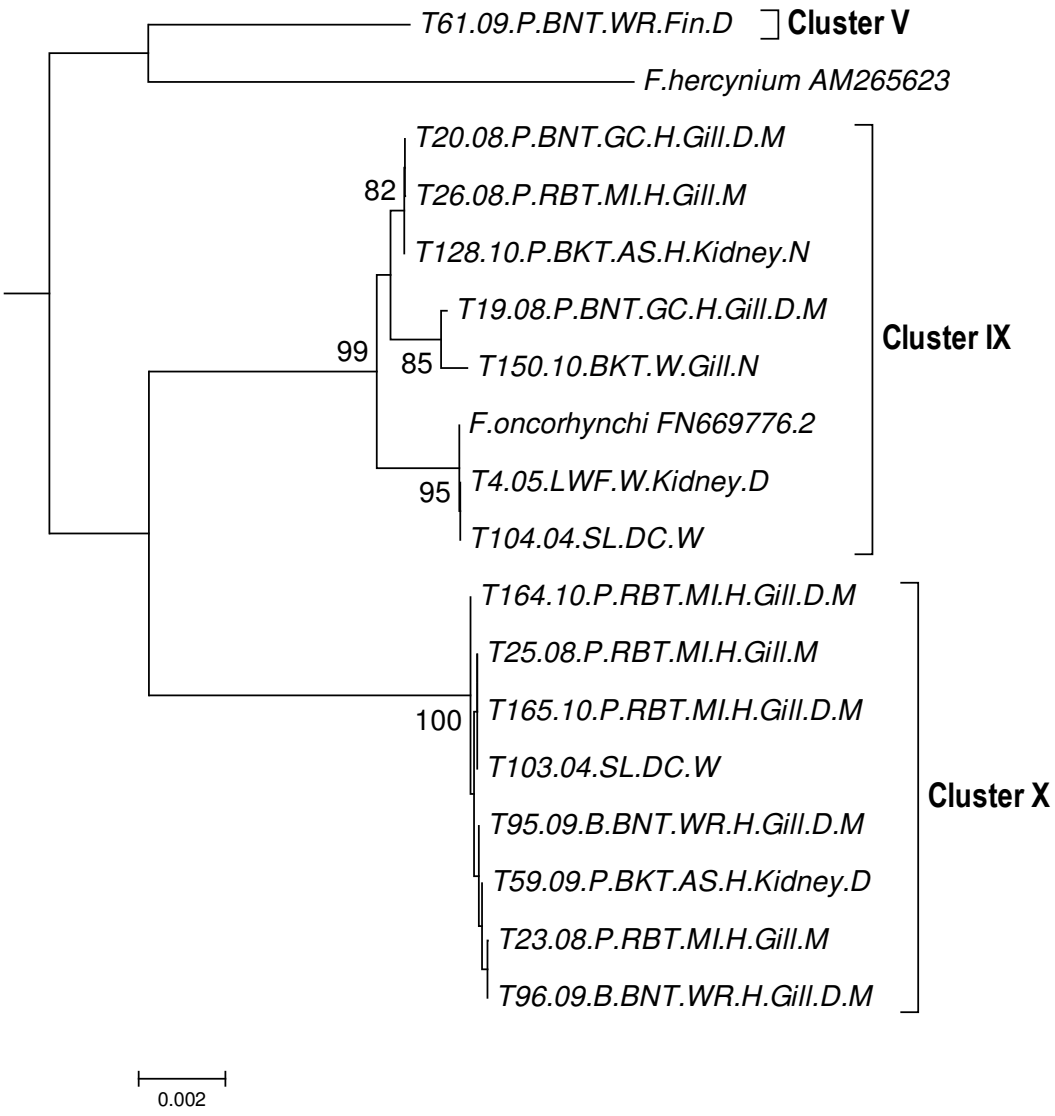


Figure 3.4.5. Subtree of dendrogram displayed in Fig. 3.4.1 that was generated using the neigbor-joining method in MEGA5 depicting the phylogenetic relationship between 9 Michigan *Flavobacterium* spp. isolates recovered from fishes and the *F. hydatis* reference sequence. Bootstrap values >70% (expressed 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.

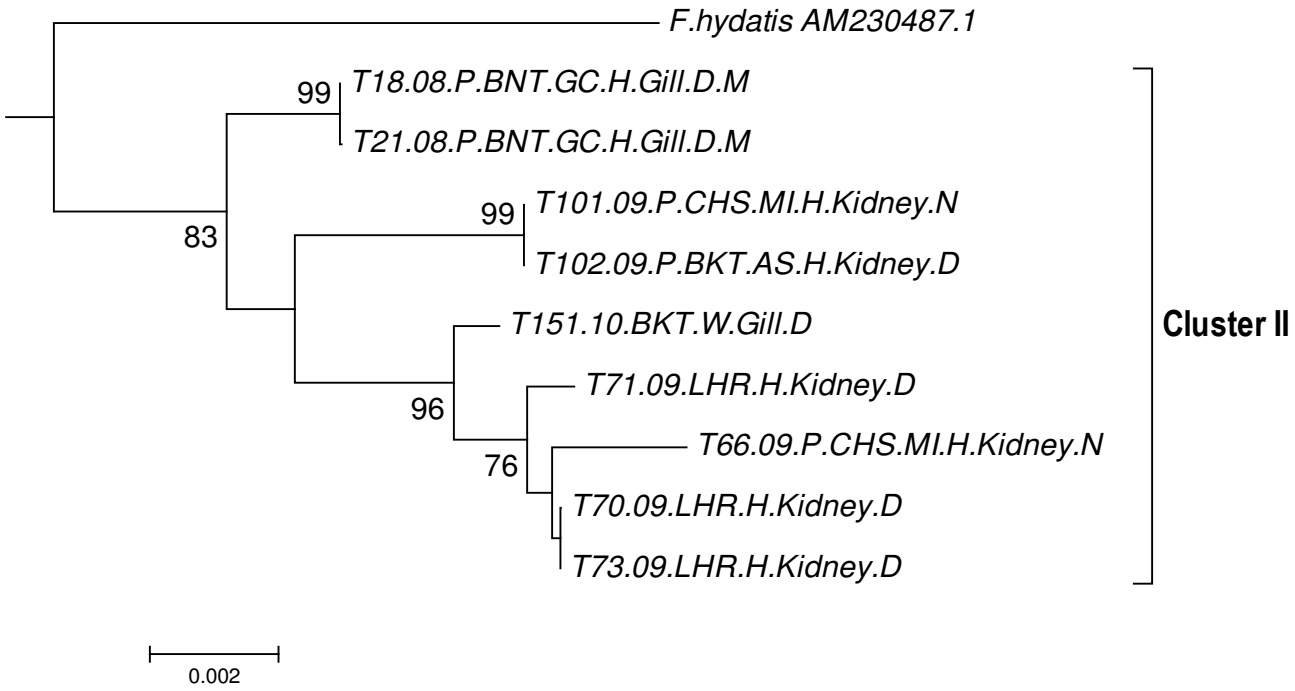


Figure 3.4.6. Cladogram depicting the relationships of *Chryseobacterium* spp. T91 and T75 (red rectangles) generated using Bayesian analysis with the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites Bayesian in MrBayes 3.1.2. The Markov chain 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.

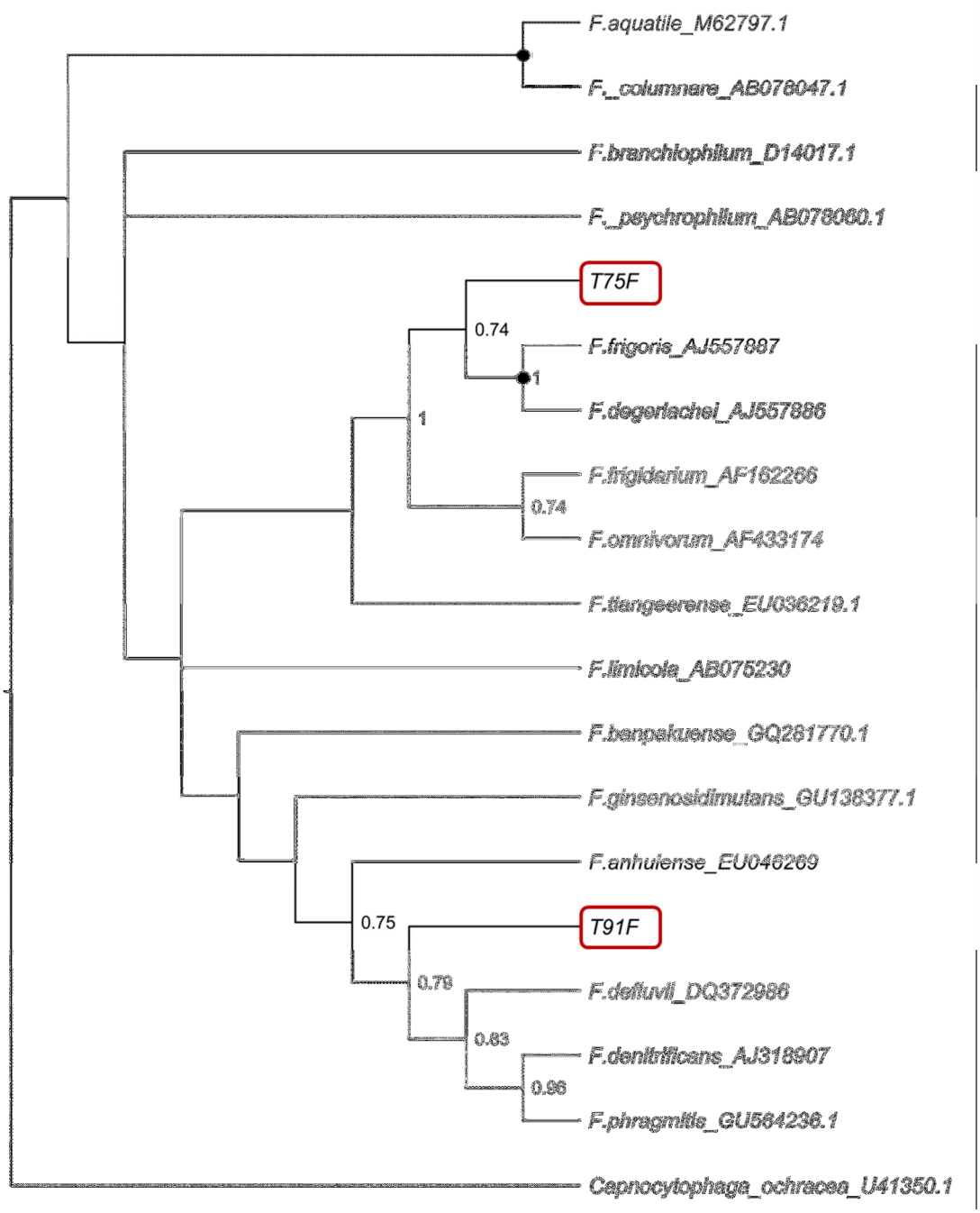


Figure 3.4.7. Cladogram depicting the relationships of *Chryseobacterium* spp. T18, T76, S21, and S87 (red rectangles) generated using Bayesian analysis with the General Time Reversible (GTR) model and gamma-shaped rate variation with a proportion of invariable sites Bayesian in MrBayes 3.1.2. The Markov 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.

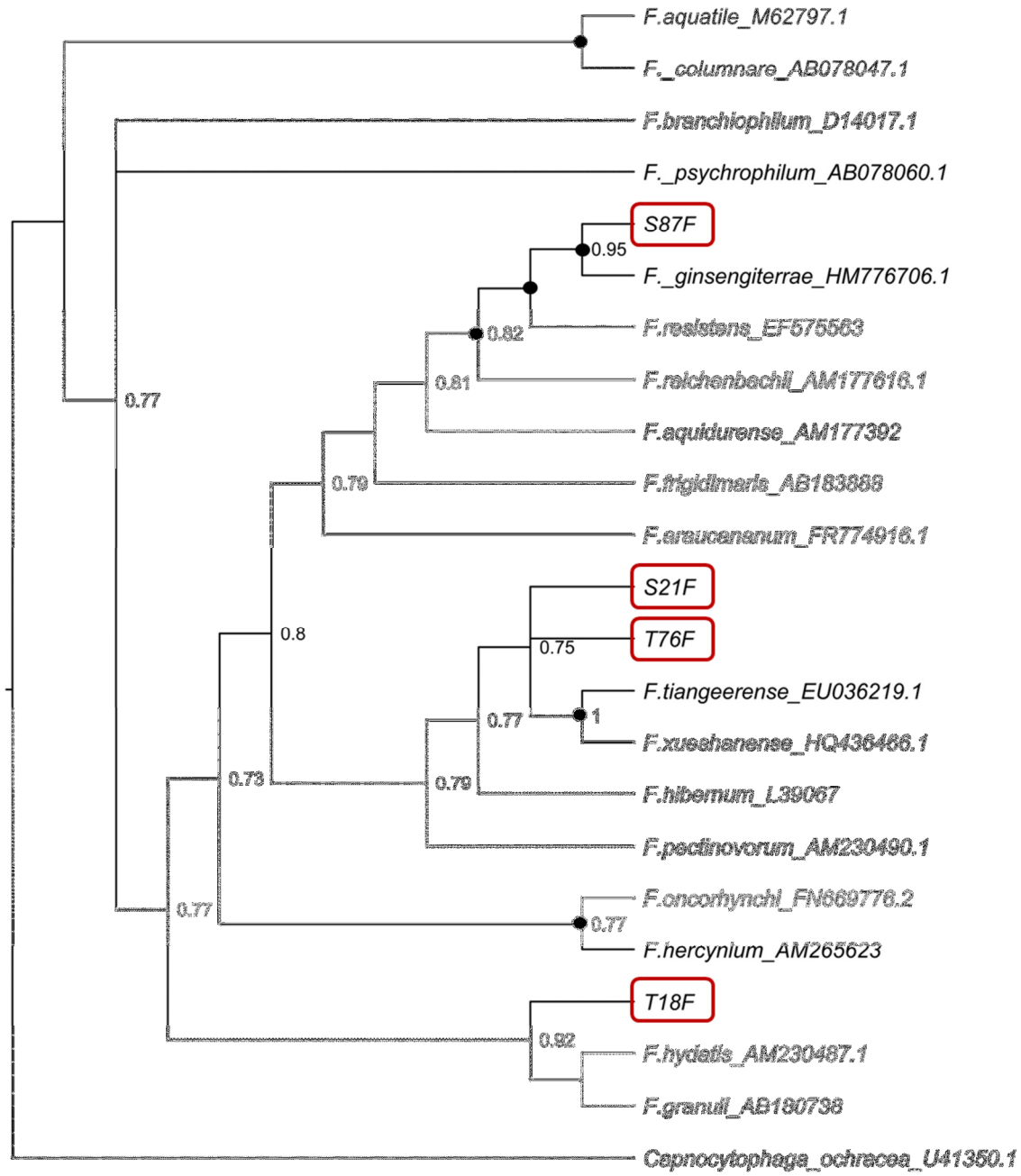
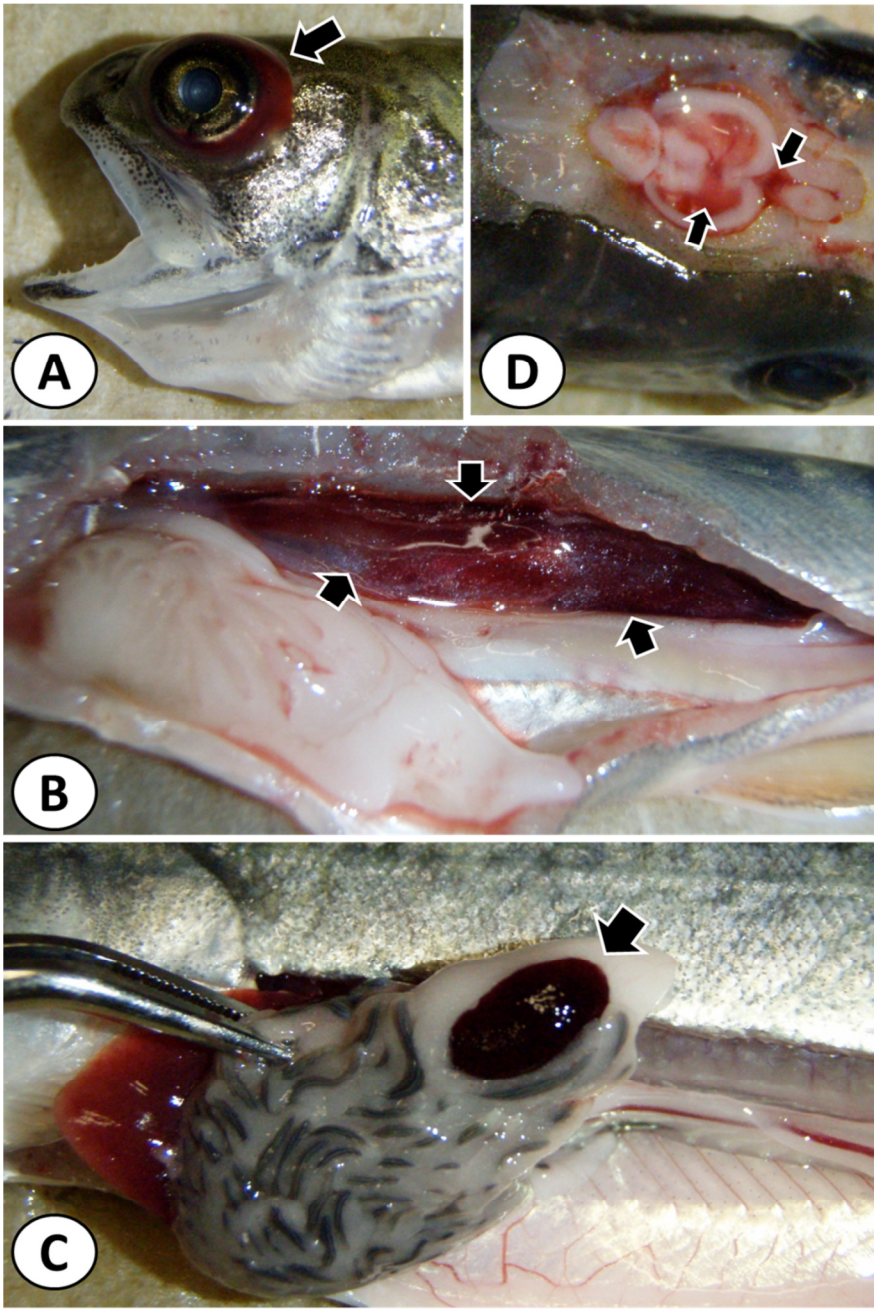


Figure 3.4.8. Gross lesions observed in fish intraperitoneally injected with Michigan *Flavobacterium* spp. isolates. A) Severe unilateral exophthalmia with diffuse periocular hemorrhage (arrow) in an S21-infected brook trout fingerling. B) Severe diffuse hemorrhage present within the swim bladder (arrows), 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.



Objective III- To conduct epizootiological studies involving flavobacterial infections within production Chinook salmon reared within Michigan State Fish Hatcheries and in feral broodstock returning to two gamete collecting stations in order to elucidate flavobacteria trafficking and disease dynamics.

There are 3 MDNR State Fish Hatcheries that currently raise Chinook salmon to be stocked in Michigan waters: Thompson (TSFH), Platte (PRSFH), and Wolf Lake (WLSFH). The source of gametes for production Chinook salmon is the feral Chinook salmon broodstock that return to the Little Manistee (LMRW) and Swan River Weirs (SRW). We proposed to assess the prevalence of flavobacterial infections in feral Chinook salmon broodstock (i.e., fish returning to LMRW and SRW to spawn) and their gametes/reproductive fluids, as well as three important fish life-stages during their tenure within TSFH and PRSFH: fertilized eggs, swim-up fry, and pre-stocking fingerlings. To address trafficking of flavobacteria within hatcheries, water samples were collected beginning at the source, through pipelines and head boxes, and as it passes through raceways, which will determine the stage at which the pathogens get into the water. This objective is crucial in determining the potential role that hatchery infrastructure and gamete sources may play in bacterial transmission.

In order to test the hypothesis that there are various sources (e.g., gametes, reproductive fluids, incoming water) that play a role in the initiation/outcome of hatchery derived flavobacterial infections. The main goal of performing this study was to direct the attention of hatchery managers to the foci of flavobacterial infections, thus allowing their future disinfection and control efforts to more efficaciously prevent flavobacterial transmission and, as a result, subsequent disease outbreaks.

Methods used include:

During the falls of 2010 and 2011, 60 feral spawning Chinook salmon returning to the LMRW (Lake Michigan watershed) and SRW (Lake Huron watershed) were sampled for flavobacterial isolation. Fish were euthanized by Michigan Department of Natural Resources (MDNR) personnel and sampled on site. Tissues for bacterial isolation were collected from the gills and kidneys using sterile disposable inoculating loops and were streaked directly onto HSM and CA plates. Brains cultures were taken by removing the mucus with a paper towel, surface disinfecting the head with 70% ethanol, and then carefully drilling through the cranium using a power drill (drill bit was ethanol-flame disinfected between each fish). A sterile disposable loop was then used to collect brain tissues, which were streaked directly onto HSM and CA plates. Spleen samples were collected asepctically (using new sterilized tools for each fish) and placed into whirl-paks and immediately placed on ice. Similarly, ovarian fluid and unfertilized eggs were collected directly from the body cavity of broodfish using sterile disposable transfer pipettes and forceps and placed on ice. Milt was collected midstream directly from the fish and immediately placed on ice. Once reaching the lab, undiluted reproductive fluids were first inoculated onto HSM and CA plates and then diluted in a 1:10 (v/v) phosphate buffered saline solution (PBS), serial 10- fold dilutions performed, and then 0.01 ml aliquots were plated onto HSM and CA plates (incubated at 22 ºC and 15 ºC, respectively). Spleens were weighed, diluted in 1:4 (w/v) sterile PBS, and then 0.01 ml aliquots were plated onto HSM and CA plates. Unfertilized eggs were disinfected in iodophore (50 ppm for 30 min; >4:1 ratio of iodophore to eggs) while being shaken at 100 rpm and individually placed in test tubes containing 3ml of cytophaga broth and 3ml of Hsu broth and incubated (15 and 22 ºC, 7d). Tubes with turbidity were sub-cultured onto HSM or CA plates for bacterial isolation, while those eggs that were surface sterile (i.e., no visible bacterial growth) were macerated with a sterile 16 gauge needle, further incubated for 7 days, and 0.01 ml aliquoted onto solid media.

Eyed Chinook salmon eggs, swim up fry, and fingerlings were collected from Platte River State Fish Hatchery (PRSFH; 2010), Thompson State Fish Hatchery (TSFH, 2010 & 2011), and Wolf Lake State Fish Hatchery (WLFSH; 2011). Upon arrival at the lab, a subset of the eggs (n=~60) were rinsed in sterile PBS 3 x and individual eggs were placed into 4 ml of Hsu broth (n=30; 22 ºC) and 4 ml of CA broth (n=30; 15ºC) and incubated for 7d. The other portion of eyed eggs was disinfected in iodophore (100 ppm solution; pH 7.5; >4:1 ratio iodophore to eggs) while being shaken at 100 rpm for 10 min. In the event that the iodine solution became lighter in color, the solution was decanted and fresh iodophore solution added. Eggs were then rinsed in sterile PBS 3x and placed into broth as previously described. Ten µl from all broth cultures with visible turbidity were plated onto the analogous solid media. Disinfected eggs without visible turbidity were ruptured and re-incubated as described above. Chinook salmon swim up fry (n=200) were collected just prior to being moved from egg incubation stacks to indoor tanks/raceways. Fry were euthanized in MS-222 as described above. One hundred fry were rinsed in sterile PBS 3x and then pools of 5 fish dipped 10x into 40 ml of Hsu broth (n=10 pools of 5) and CA broth (n=10 pools of 5). One hundred fry were disinfected in 100 ppm iodophore for 30 min while shaken at 100 rpm, rinsed 3x in sterile PBS, weighed and diluted 4:1 with sterile PBS, and then 5 fry (total of 20 pools) placed into sterile whirl-paks. The fry were then homogenized by rolling a disposable 25 ml stripette over the bag “like a rolling pin” and 10 µl inoculated into HSM (n=10) and CA (n=10) plates. Just prior to stocking, Chinook salmon fingerlings (n=180) were collected and driven back to the lab for bacterial culture. Fish were euthanized in MS-222 and 1 gill arch from each fish was immediately excised, placed in a whirl-pak (5 gill archs/pool), weighed and diluted with 4:1 sterile PBS, and 30 µl inoculated onto both HSM and CA plates. Fish were then surface disinfected with 70% ethanol and sampled for bacterial culture as described under Objective I, with the exception that the liver, spleen, kidney, and brain from every fish were cultured on both HSM and CA plates. Table 3.1 summarizes the samples that were collected for studies conducted under Objective III and includes the sampling location, date of collection, and type of sample that was collected.

During Chinook salmon fry and fingerling collection, water samples were concurrently collected from various sites in PRSFH (2011; Table 3.2; Figure 3.1), TSFH (2011 & 2012; Table 3.3; Figure 3.2), and WLSFH (2012; Table 3.4; Figure 3.3). Platte River State Fish Hatchery is supplied by water from Brundage Creek and Brundage Spring, while TSFH is supplied by water originating from a spring, shallow wells, and deep wells, and WLSFH is supplied by water from a spring and deep wells. For water sample collection, sterile 500 ml nalgene screw top bottles were plunged neck downward approximately 6 inches below the water surface. The bottle neck was then tilted upward and away from the hand to allow water to flow in. Water bottles were immediately placed on ice and driven back to the lab. For flavobacterial culture, water samples were diluted with sterile PBS (3 10- fold serial dilutions were made, along with 1 undiluted water sample) and vacuum filtered through a sterile 0.45 um filter using a bucchner funnel. The filter was then removed from funnel aseptically and placed face down on HSM and CA plates (this was repeated twice for each sample, 1 for CA and one for HSM). After incubation (≤2 weeks), all Objective III primary cultures were inspected for yellow-pigmented bacterial growth, representative morphologies sub-cultured, and then purified/cryopreserved/identified via 16s rDNA sequencing as described under Objective I, with the exception that *F. psychrophilum* and *F. columnare* suspects (as determined by colony morphology, Gram reaction, and the presence of a flexirubin-type pigment using 3%) were identified with the species-specific PCR assays of Toyama et al. (1994) and Welker et al. (2005), respectively.

Major findings and discussion:

Throughout the course of the studies conducted under Objective III, we recovered a number of *Flavobacterium* spp. and *Chryseobacterium* spp. that are suspected of being transmitted on eggs and/or within reproductive fluids, including *Flavobacterium* sp. O-3-1, *Flavobacterium* sp. O-3-9, *Flavobacterium* sp. O-3-10, *Flavobacterium* sp. O-3-12, *Flavobacterium* sp. O-3-13 (i.e., *F. spartani* sp. nov.), *Chryseobacterium* sp. Cluster O-3-51, *Chryseobacterium* sp. Group 3D (i.e., *C. aahli* sp. nov.), and *Chryseobacterium* sp. Group 3E, among others. This is a very significant finding, as it indicates that current disinfection protocols are not successfully eradicating all bacteria that are present on egg surfaces and in the reproductive fluids. Furthermore, some flavobacteria and chryseobacteria (see below) were recovered before and after iodophore disinfections that were conducted by the investigators under controlled laboratory conditions, further supporting that some bacteria are resisting disinfection.

Another important finding from a managerial perspective is that multiple *Flavobacterium* spp. (e.g., *Flavobacterium* sp. O-3-3, *Flavobacterium* sp. O-3-5, *Flavobacterium* sp. O-3-6, *Flavobacterium* sp. O-3-8, *Flavobacterium* sp. O-3-18), many of which were identified as likely being novel, are entering hatchery systems in the source water. While this may not be surprising for facilities that utilize surface waters, we also found some flavobacterial entering with ultra-violet irradiated spring water and deep well water (e.g., *Flavobacterium* sp. O-3-3). While the pathogenic significance of some these flavobacteria is unknown, their presence in water sources that are often considered "pristine" needs to be addressed in the form of further studies examining pathogenicity and ways to effectively eliminate influx into hatchery systems.

Another subset of flavobacterial that were recovered under Objective III appeared to have a tropism for Chinook salmon in there early life stages (e.g., *Flavobacterium* sp. O-3-4 and *Flavobacterium* sp. O-3-15) and were not recovered from water samples unless the water had first contacted Chinook salmon. Once again, these *Flavobacterium* spp. do not match any described species and one Cluster (O-3-4) closely matched *Flavobacterium* sp. T76, which was found to be mildly virulent under Objective II and is currently being proposed as a novel species. These findings further support our data from Objectives I and II that prove that many flavobacteria, not just *F. psychrophilum*, *F. branchiophilum*, and *F. columnare*, are pathogenic to fish. In the same context, isolates (e.g., *Flavobacterium* sp. Cluster O-3-13) that were nearly identical to the *F. spartani* sp. nov. were exclusively recovered from feral Chinook salmon brood stock, fingerlings, and unfertilized eggs. *Flavobacterium spartani* proved to be pathogenic to salmonids under Objective II and generated significant pathology in infected fish, including in the brain. The recovery of *F. spartani* from the brain of a feral Chinook salmon further solidifies this bacterium as an invasive fish-pathogenic bacterium.

The isolation of a multitude of *Flavobacterium* and *Chryseobacterium* spp., including *Flavobacterium* sp. O-3-11, *Flavobacterium* sp. O-3-16, *Flavobacterium* sp. O-3-19, *Chryseobacterium* sp. O-3-50 (i.e., *C. chaponense*), *Chryseobacterium* sp. Cluster O-3-52 (i.e., *C. piscicola*), *Chryseobacterium* sp. Cluster O-3-53, and *Chryseobacterium* sp. Group 3D, from hatchery nets, brushes, and pads indicate that hatchery tools can play an important role in flavobacterial transmission. These findings also demonstrate that some flavobacteria and chryseobacteria are resistant to the inhospitable conditions associated with the terrestrial environment, thus enhancing their risks as a biosecurity threat. As such, the disinfection of hatchery tools between uses (especially during disease outbreaks) and the avoidance of tools used in multiple rearing units are highly recommended.

Further information about the *Flavobacterium* and *Chryseobacterium* spp. that were recovered under Objective III are described below. In addition, Figures 3.4 and 3.5, along with Appendix 3-I, provide further information regarding their origin and relatedness to other flavobacteria.

***Flavobacterium psychrophilum*:** *Flavobacterium psychrophilum*. Etiological agent of bacterial cold water disease, was isolated from feral Chinook salmon returning to spawn at the LMRW and SRW in 2010 and 2011 (Table 3.5). In general, the prevalence of systemic *F. psychrophilum* infection was higher at the LMRW (48%-63%) when compared to SRW (7%-25%). Most isolates were recovered from the kidneys of infected fish (Table 3.5), but *F. psychrophilum* was also detected in the spleens, brain, and ovarian fluids (Table 3.5). Moreover, *F. psychrophilum* was also isolated from iodophore-disinfected unfertilized eggs from both LMRW and SRW, which is in keeping with the fact that this bacterium is transmitted vertically. *Flavobacterium psychrophilum* was not detected in any eyed eggs or Chinook salmon fry; however, this bacterium was isolated from the brains of TSFH Chinook salmon fingerlings in 2011, and from the gills of PRSFH fingerlings in 2011. Collectively, these findings further substantiate that *F. psychrophilum* is an important pathogen of Great Lakes salmonids, feral and cultured alike.

***Flavobacterium columnare*:** *Flavobacterium columnare*, causative agent of columnaris disease, was also recovered from feral Chinook salmon brood stock collected from the LMRW and SRW during the falls of 2010 and 2011 (Table 3.6). Contrary to *F. psychrophilum* infections, the prevalence of systemic *F. columnare* infections was higher in SRW brood stock (52%-70%) than at LMRW (8%-12%; Table 3.6). Similarly, the prevalence of *F. columnare* gill infections was higher at SRW (58%-93%) when compared to LMRW (2%-18%; Table 3.6). While all spleen, brain, milt, and ovarian fluid samples were negative for *F. columnare* at LMRW, this bacterium was detected in all four sample types at SRW (Table 3.6). In addition, *F. columnare* was recovered from the external surfaces of Chinook salmon swim up fry reared at WLSFH. In light of the fact that this bacterium was not recovered from any hatchery water sources but was detected in the parents of the fry, we feel it is highly likely that the iodine disinfection process was not completely effective in killing all of the *F. columnare* that was present. This is supported by 1) less than a 1:1 ratio of eggs to iodophore was being used (ratio of 4:1 has been suggested by some researchers) for disinfection, 2) eggs were not circulated during the disinfection process, and 3) *F. columnare* was never recovered from the source water at any point during this study. Egg disinfection procedures have subsequently been modified.

***Flavobacterium* sp. Cluster O-3-1:** *Flavobacterium* sp. isolates in Cluster O-3-1 (n=79) were extremely well-supported as being distinct from the currently described *Flavobacterium* spp (Figure 3.4; BS= 99) and were exclusively associated with early life stages of Chinook salmon in this study. For example, this bacterium was recovered from eyed eggs in both WLSFH & TSFH that were originally collected from both SRW and LMRW and was still present after eggs were surface disinfected with iodophore. Moreover, Cluster O-3-1 isolates were recovered from the external surfaces of Chinook salmon fry, as well as their internal organs. Interestingly, this bacterium was recovered from water samples that were associated with egg incubation stacks only. For instance, at TSFH, this bacterium was isolated from well water after it had flowed through the newly hatched swim up fry, and was also recovered from U.V.-treated spring water associated with egg/swim up fry incubation. Thus, this bacterium seems to be strongly associated with eggs and swim up fry, but is absent from the rest of the hatchery infrastructure, as well as from Chinook salmon fingerlings. An additional noteworthy finding is the fact that this bacterium was only recovered from the two hatcheries that

exclusively use well/spring water, and was not recovered from Chinook salmon eggs or fry that were reared at PRSFH, which uses primarily surface water. Thus, it is possible that *Flavobacterium* sp. O-3-1 is being outcompeted on Chinook salmon eggs and fry by other flavobacteria that are present in the surface water used by PRSFH (see results below). The absence of *Flavobacterium* sp. O-3-1 from Chinook salmon brood stock is also curious, as water culture results indicate that this bacterium is coming with the eggs. However, we did not sample the vent of Chinook salmon brood stock in this study and feel it is possible that this bacterium may be "seeded" onto the eggs when they leave the body cavity of the fish. This is supported by the absence of *Flavobacterium* sp. O-3-1 in the ovarian fluid and non-fertilized eggs of brood fish, which were collected directly from the body cavity of the fish. Alternatively, it is possible that this bacterium is only present in a limited number of brood fish, but rapidly proliferates in the presence of eggs/fry. The role of *Flavobacterium* sp. O-3-1 most definitely warrants further investigation and its description as a novel *Flavobacterium* sp. is being pursued.

***Flavobacterium* sp. Cluster O-3-2:** *Flavobacterium* sp. isolates in Cluster O-3-2 (n=34; BS=89; Figure 3.4) were 98.9-99.8% similar to *Flavobacterium* sp. Cluster II isolates from Objective I and were associated with multiple life stages of Chinook salmon, including brood stock, disinfected and non-disinfected eyed eggs reared at WLSFH and PRSFH, and non-disinfected fry at PRSFH. In addition, this bacterium was recovered from water samples taken prior to entering PRSFH, as well as throughout the hatchery infrastructure. For example, water samples from Brundage Creek, which is one of the primary water sources for PRSFH, along with the head box containing heated Brundage Spring water, were culture positive for *Flavobacterium* sp. belonging to Cluster O-3-2. Thus, this bacterium is entering PRSFH along with the source water, with the head box possibly acting as a reservoir. *Flavobacterium* sp. Cluster O-3-2 was also isolated from the water after passing through the egg stacks containing Chinook swim up fry and at the tail end of indoor fry tanks during Chinook swim up fry sampling. Similarly, when Chinook fingerlings were sampled in May, this bacterium was recovered from Brundage Creek, and in pooled reuse water containing Brundage Spring and Brundage Creek water that already passed through raceways containing fingerling Chinook salmon. As was the case with flavobacteria belonging to Cluster O-3-1, a portion of the Cluster O-3-2 isolates were recovered from eggs that were surface disinfected with iodophore. Although this bacterium was recovered from Chinook salmon brood stock at LMRW, it was isolated from the gills. Thus, despite the fact that *Flavobacterium* sp. O-3-2 was recovered from brood fish, eggs, and resultant fry, the presence of this bacterium in surface water and only on the gills of brood stock suggest that isolates belonging to Cluster O-3-2 are ubiquitous water borne flavobacteria that are not vertically transmitted. However, the isolation of this bacterium from disinfected and homogenized Chinook salmon fry suggest that it is capable of causing systemic infections in young salmonids, which is in agreement with that fact that many Cluster II isolates under Objective I were also recovered from the kidneys of systemically infected fish.

***Flavobacterium* sp. Cluster O-3-3:** *Flavobacterium* sp. isolates falling into Cluster O-3-3 (n=19) were robustly supported as being distinct from all currently described *Flavobacterium* spp. (BS=93; Figure 3.4) and were almost exclusively recovered from water samples, with only 1 isolate originating from the external surfaces of Chinook salmon swim up fry reared at WLSFH. When compared to the *Flavobacterium* spp. sequences from Objective I, Cluster O-3-3 isolates were 99.3-99.9% similar to isolates belonging to Cluster I. As was the case for isolates belonging to Cluster O-3-2, *Flavobacterium* sp. O-3-3 isolates are apparently entering the hatchery infrastructure

with source water. However, whereby O-3-2 isolates were present in creek and spring water, this study suggests that O-3-3 isolates may have a predilection for spring and well water. For example, this bacterium was recovered from deep well water after degassing in WLSFH during both sampling periods, and was also recovered from enclosed structures housing spring water. Similarly, *Flavobacterium* sp. O-3-3 was recovered from the spring water feeding the egg stacks, unheated spring water, and the heated spring water feeding indoor raceways, as well as at the tail of indoor fry tanks and in pooled spring water for reuse. A single isolate falling into this cluster was also recovered from U.V. treated spring water feeding the egg stacks at TSFH in 2011. Thus, in almost every case, *Flavobacterium* sp. O-3-3 was recovered from some form of spring or well water, though the lack of association with Chinook salmon seems to suggest that this bacterium is not a major cause for concern from a fish health perspective. Nevertheless, the presence of *Flavobacterium* sp. O-3-3 in water sources that are generally considered to be among the best in quality for cold water hatcheries should be further investigated, especially in light of the fact that similar isolates (i.e., *Flavobacterium* sp. Cluster I from Objective I) were recovered from systemically infected fish undergoing mortality events.

***Flavobacterium* sp. Cluster O-3-4:** *Flavobacterium* sp. isolates belonging to Cluster O-3-4 (n=18) were also well supported as distinct from other described *Flavobacterium* spp. (Figure 3.4; BS=99) and were 99.6-99.9% similar to *Flavobacterium* sp. T76 (Cluster VIII) of Objective I, which proved to be mildly virulent in experimental challenge studies of Objective II and is being described as a novel *Flavobacterium* sp. All isolates belonging to Cluster O-3-4 originated from WLSFH, with the exception of one isolate recovered from the gills of a spawning Chinook salmon collected from the LMRW. *Flavobacterium* sp. O-3-4 was recovered both externally and systemically from Chinook salmon fry despite the fact that the bacterium was never detected in any hatchery source water. However, isolates belonging to Cluster O-3-4 were recovered from water samples that were collected from hatchery structures that came in contact with fish (e.g., outflow from egg stacks, at the head and tail of indoor raceways containing Chinook salmon fry, and in 2nd pass water). Thus, although *Flavobacterium* sp. O-3-4 was not recovered from the reproductive fluids or eggs of Chinook salmon brood stock, its presence on the external surfaces of both parents and progeny and in the internal organs of swim up fry suggest pathogenic potential. In light of our findings, it is tempting to suggest that the bacterium can be transmitted from parent to progeny, albeit it not as a true form of vertical transmission, but rather as a process of "contamination" from parent to offspring. However, further studies to elucidate this process are necessary.

***Flavobacterium* sp. Cluster O-3-5:** Isolates belonging to *Flavobacterium* sp. Cluster O-3-5 (n=16), which also were robustly supported as unique from described *Flavobacterium* spp. (BS=95; Figure 3.4), primarily originated from TSFH water samples. However, there was not a discernible pattern in the water sources that contained this bacterium, which may suggest that multiple "hot-spots" or reservoirs are present in this hatchery. An isolate belonging to this cluster was also recovered from disinfected and homogenized Chinook salmon fry, indicating that this bacterium has the capability to systemically infect young fish. Interestingly, 3 Cluster O-3-5 isolates were also recovered from water exiting WLSFH at the final outdoor raceway series and in the discharge feeding one of the settling ponds. Thus, the role that this group of flavobacteria may play in the breakdown of organic matter associated with hatchery effluents should indeed be further studied. Moreover, throughout this study, many of the water samples collected from hatchery effluents were overgrown by fungus, but this bacterium was nevertheless able to grow in the presence of heavy fungal

overgrowth. As such, it is possible that anti-mycotic substances may be produced by members of *Flavobacterium* sp. Cluster O-3-5 and could represent a potential candidate for probiotic studies. When compared to the *Flavobacterium* spp. sequences from Objective I, Cluster O-3-5 were 99.5-99.9% similar to isolate T61 of Cluster V (under Objective I).

***Flavobacterium* sp. Cluster O-3-6:** Fourteen *Flavobacterium* sp. isolates fell into Cluster O-3-6 (BS=100; Figure 3.4), all of which originated from samples collected from PRSFH. These isolates were nearly identical to *Flavobacterium* sp. S21 (Cluster XI; currently being proposed as a novel *Flavobacterium* sp. under Objectives 1 & 2) according to partial 16S rDNA sequencing (99.3-99.8%)), which proved to be pathogenic to multiple salmonid species. Thus, the ubiquity of this flavobacterial cluster throughout the various PRSFH water sampling sites, including the source water (e.g., Brundage Creek and Brundage Spring water), along with its presence on the gills of Chinook salmon fingerlings, are important findings. This bacterium was also detected in multiple wild fishes, including sculpin and brook trout from Brundage Creek (described under Objective I). It is also noteworthy that this bacterium was not detected in any samples originating from hatcheries that exclusively use well and spring water. It is currently known if fish residing in Brundage creek serve as a reservoir for this novel bacterium, or rather if it is ubiquitous in Brundage Creek. The role that flavobacterial belonging to Clusters XI and O-3-6 play in natural disease outbreaks both in wild and farmed fishes should be investigated and targeted surveillance applied.

***Flavobacterium* sp. Cluster O-3-7:** *Flavobacterium* sp. isolates in Cluster O-3-7 (n=12) were robustly supported as being distinct from the currently described *Flavobacterium* spp. (Figure 3.4; BS= 100) and were exclusively isolated from water samples. When compared to the *Flavobacterium* spp. sequences from Objective I, isolates belonging to Cluster O-3-7 were 99.4-99.8% similar to Cluster XXIX isolates. Among Cluster XXIX isolates is *Flavobacterium* sp. T75, which is being described as a novel species but proved to be completely avirulent in our experimental challenge studies of Objective II. In TSFH, Cluster O-3-7 isolates were predominantly recovered from water samples taken from the latter portions of the hatchery infrastructure, including the hatchery outfall, at the tail ends of indoor raceways, and throughout the first and second pass water of the outdoor raceways. One isolate was also recovered from PRSFH water at the tail end of Chinook salmon fry tanks. At no point was this bacterium recovered from any life stages of feral or hatchery reared Chinook salmon. Thus, it seems likely that this bacterium is saprophytic in nature, with reservoirs existing on organic matter within and at the tail end of hatchery raceways.

***Flavobacterium* sp. Cluster O-3-8 and close relatives:** *Flavobacterium* sp. Cluster O-3-8 isolates (n=11; BS=95, Figure 3.4)) were all recovered from PRSFH and were 99.5-99.8% similar to *Flavobacterium* sp. isolates of Cluster XVI (recovered from the gills of apparently healthy wild brown trout; Objective I). The origins of Cluster O-3-8 isolates recovered from PRSFH were in keeping with our previous findings, in that none were recovered from systemically infected fish and were exclusively detected in the water column and on the gills of Chinook salmon fingerlings. Water sampling indicated that *Flavobacterium* sp. Cluster O-3-8 isolates were prevalent throughout the hatchery infrastructure, including in the head box containing spring water, in water feeding the egg stacks, at the head and tail of indoor Chinook salmon fry tanks, and at the clarifier just prior to hatchery discharge. *Flavobacterium* sp. O-3-8 thus appears to be entering the hatchery with spring water or, alternatively, may be present in reservoirs in the pipelines/head box of the hatchery. Interestingly, 3 closely related *Flavobacterium* sp. isolates (i.e., 973, 898, 905; Figure 3.4) were detected in the spring water feeding WLSFH, as well in the deep well water once it had reached the indoor hatchery

building. Another group of closely related flavobacterial isolates with an resolved topology (Unresolved 3A; Figure 3.4) were also exclusively recovered from water samples originating from WLSFH and PRSFH. The unresolved WLSFH isolates were recovered from the spring water feeding the hatchery and in the hatchery discharge water, while those from PRSFH were isolates from Brundage creek, unheated spring water, heated spring water in the headbox, at the head and tail of Chinook fry tanks/raceways, in pooled reuse water, and at the clarifier just prior to hatchery discharge. *Flavobacterium* sp. O-3-8 and the closely related isolates were never identified in any egg or swim up fry samples.

***Flavobacterium* sp. Cluster O-3-9:** Isolates falling into Cluster O-3-9 (n=9; BS=80; Figure 3.4) were 99.0-99.3% similar to the *F. frigidimaris*, yet phylogenetic analysis supports that these isolates are genetically distinct from the *F. frigidimaris* type strain. All isolates belonging to this cluster were recovered from Chinook salmon brood stock returning to the LMRW to spawn in the fall of 2011. Among these, 2 isolates were recovered from the gills, 5 isolates from the ovarian fluid, and 2 isolates from the spleens of infected fish. It should be noted that the spleens of fish are in intimate contact with the ovarian fluid of gravid Chinook salmon; thus, the detection of this bacterium in the spleen could be a result of this. Nevertheless, the isolation of this bacterium from the ovarian fluid of Chinook salmon is an interesting finding. Although *Flavobacterium* sp. Cluster O-3-9 was not recovered from any Chinook salmon fry or fingerlings, closely related flavobacteria (isolates 678 and 684; Figure 3.4) were isolated from disinfected eyed eggs being reared at PRSFH. Thus, the relationship of *Flavobaterium* sp. O-3-9 to isolates 678 and 684 and whether a form of vertical transmission is at play deserve further attention.

***Flavobacterium* sp. Cluster O-3-10:** *Flavobacterium* sp. O-3-10 isolates formed a robustly supported cluster (BS=98; Figure 3.4), were 99.1-99.7% similar to the *F. oncorhynchi* type strain sequence (which was also contained within the O-3-10 Cluster), and were 99.3-99.9% similar to the *F. oncorhynchi* isolates falling into Cluster IX (Objective I). Isolates within Cluster O-3-10 were recovered from the kidney of a feral Chinook salmon returning to spawn at the SRW in fall 2011, from disinfected eyed Chinook salmon eggs reared at PRSFH, and from the water at WLSFH after it had flowed through raceways containing Chinook salmon fry and fingerlings. Thus, in keeping with our findings of Objective I and those of the original description of *F. oncorhynchi*, this bacterium is definitively capable of causing systemic infections in salmonids and now has also been found in association with eyed eggs. While the mode by which eggs become colonized remains to be elucidated, the absence of this bacterium from all water samples unless it had first flowed through enclosures containing Chinook salmon suggests that fish and eggs may be the source/reservoir for *F. oncorhynchi*.

***Flavobacterium* sp. Cluster O-3-11 and close relatives:** Isolates falling into *Flavobacterium* sp. Cluster O-3-11 (n=9) were distinct from all described *Flavobacterium* spp. (BS=70; Figure 3.4), but were nearly identical (99.6-99.8%) to the *Flavobacterium* sp. isolates comprising Unresolved Group 1 (Objective I). The *Flavobacterium* sp. Cluster O-3-11 isolates originating from PRSFH were recovered from the spring water destined for reuse after it had flowed through Chinook salmon fry tanks, while isolates originating from WLSFH were recovered from deep well water once it reached indoor raceways, at the tail end of indoor Chinook salmon raceways, in 2nd pass water feeding outdoor raceways, and at the outdoor raceway outfall. This bacterium was also isolated from the external surfaces of Chinook salmon fry reared at WLSFH. In addition, 20 *Flavobacterium* sp. isolates with an unresolved topology despite being closely related to Cluster O-3-11 isolates (Figure 3.4) were recovered from multiple locations. For example, 3 isolates were recovered from the gills of feral Chinook salmon brood stock

at LMRW, 1 isolate was recovered from iodine disinfected eyed eggs being reared at TSFH, and the remainder originated from PRSFH. Among these, 9 isolates were recovered from iodine disinfected eyed eggs, 2 isolates were recovered the external surfaces of Chinook salmon fry, 1 isolate was recovered from an algae pad used to clean Chinook salmon raceways, and the remainder were recovered from water samples, including from heated spring water after flowing through the egg stacks containing Chinook salmon swim up fry, from the tail end of indoor Chinook salmon fry tanks, and from the head and tail ends of large indoor Chinook salmon fry raceways. Thus, *Flavobacterium* sp. Cluster O-3-11 and its close relatives are associated with the external surfaces of multiple life stages of Chinook salmon, including eyed eggs, swim up fry, and brood stock, but was never recovered from the internal organs of any fish. This was also the case with the nearly identical Unresolved Group 1 flavobacterial isolates from Objective I. It is also noteworthy that this bacterium appears resistant to the commonly employed iodine disinfection methods, though risk for disease causation currently appears minimal.

***Flavobacterium* sp. Cluster O-3-12:** Isolates within *Flavobacterium* sp. Cluster O-3-12 were extremely interesting in that they were only 95.7-96.0% similar to the *F. cucumis* type strain sequence, and were even more distantly related to all *Flavobacterium* spp. isolates described under Objective I. In addition to being taxonomically unique (BS=100; Figure 3.4), this bacterium was interesting because it was exclusively recovered from the ovarian fluid and non-disinfected unfertilized Chinook salmon eggs (SRW, 2011). *Flavobacterium* sp. Cluster O-3-12 was not recovered from any eyed eggs, fry, or fingerlings; however, Chinook salmon eggs from the SRW were not used for hatchery propagation purposes in the 2011-2012 rearing cycle and thus its presence in further life stages cannot be determined from the results of this study. Research on this novel *Flavobacterium* sp. will be conducted in the near future.

***Flavobacterium* sp. Cluster O-3-13:** *Flavobacterium* sp. Cluster O-3-13 isolates (n=5; Figure 3.4) were 99.5-99.8% similar to *F. spartani* sp. nov and other isolates belonging to Cluster XVIII (described as a novel species under Objective I). This newly described bacterium was isolated from the gills of feral Chinook salmon brood stock from LMRW, from the brain of a feral Chinook salmon from SRW, from the non-disinfected unfertilized eggs collected from SRW, and from the gills of fingerling Chinook salmon reared at TSFH. As was shown in the work done under Objective II, *F. spartani* is capable of eliciting systemic disease in multiple salmonid species and was recovered from multiple organ systems in experimentally challenged fish, including the brain. The recovery of this bacterium from the brain of a feral adult Chinook salmon offers further proof that *F. spartani* is an invasive fish pathogenic bacterium affecting both hatachery and wild/feral Great Lakes salmonids. Furthermore, the presence of this bacterium on Chinook salmon eggs could mean that *F. spartani* is vertically transmitted much in the same way as *F. psychrophilum*. Obviously, further studies would be required to support this notion.

***Flavobacterium* sp. Cluster O-3-14:** Six *Flavobacterium* sp. isolates fell into Cluster O-3-14 and were well-supported as being distinct from all described *Flavobacterium* spp. (BS=91; Figure 3.4). All isolates within this cluster were recovered from the water sources associated with TSFH & WLSFH. The five isolates originating from TSFH were recovered exclusively from the tail end of hatchery raceways (both indoor and outdoor) and at the hatchery outfall. Similarly, the single isolate originating from WLSFH was recovered from the hatchery outfall. These results collectively suggest that *Flavobacterium* sp. O-3-14 may be associated with the fish feces, detritus, and uneaten food that is present at the tail end of hatchery raceways (especially those that use baffles to enhance the flow of organics to the “back” of raceways. It should be

noted that some degree of genetic variability was present in this flavobacterial cluster, suggesting that it is comprised of multiple *Flavobacterium* species/strains.

***Flavobacterium* sp. Cluster O-3-15:** *Flavobacterium* sp. Cluster O-3-15 isolates (n=5) were 98.8-99.0% similar to the *F. saccharophilum* type strain sequence but taxonomically distinct (BS=95; Figure 3.4). Four isolates originated from PRSFH and were recovered from the external surfaces of Chinook salmon fry (n=3), as well from their internal organs (n=1). The remaining isolate was recovered from the surfaces of non-disinfected eyed eggs from TSFH. In light of the absence of this bacterium from all water samples, it appears that members of this cluster may have a tropism for Chinook salmon in their early life stages. However, 2 closely related and taxonomically unresolved isolates (#'s 225 and 104; Figure 3.4) were recovered from the tail end of an indoor raceway at PRSFH containing Chinook salmon fry and the head of an indoor raceway at TSFH containing Chinook salmon fingerlings, respectively. Nevertheless, both of these water samples were in intimate contact with Chinook salmon.

***Flavobacterium* sp. Cluster O-3-16:** Isolates falling into Cluster O-3-16 (n=6; BS=99; Figure 3.4) were 98.2-98.4% similar to the *F. oncorhynchi* type strain sequence but were nearly identical to *Flavobacterium* sp. T59 (99.7-99.9% similar; Cluster X; Objective I). Four isolates within this Cluster originated from WLSFH, whereby two were recovered from water at the tail end of an indoor Chinook salmon fingerling raceway and from the hatchery discharge (Pond 24), and two were recovered from hatchery nets. One isolate was also recovered from 2nd pass water at the tail end of an outdoor raceway at TSFH, and the remaining isolate was recovered from a brush at PRSFH used to clean hatchery raceways. The fact that *Flavobacterium* sp. O-3-16 was recovered from hatchery tools on multiple occasions at multiple hatcheries seems to suggest that this bacterium is capable of resisting desiccation and other environmental stressors that are associated with a "terrestrial-like" environment. It is also noteworthy that isolates belonging to Cluster X (Objective I), which are nearly identical to Cluster O-3-16 isolates, were found in association with multiple disease outbreaks/mortality events in hatchery-reared salmonids. While it is currently unknown if this bacterium is truly pathogenic, disinfection of hatchery tools between uses should be considered as a further way to reduce flavobacterial transmission.

***Flavobacterium* sp. Cluster O-3-17:** *Flavobacterium* sp. Cluster O-3-17 isolates (BS=99; Figure 3.4) were exclusively recovered from water samples collected from the discharge of WLSFH at Pond 24 during the time of fry and fingerling sample collection. This flavobacterial cluster was distinct from all described *Flavobacterium* spp. and may be saprophytic in nature due to its association with hatchery effluent. Because of the lack of association with any life stages of the Chinook salmon, this bacterium is unlikely to be a fish pathogen.

***Flavobacterium* sp. Cluster O-3-18:** Isolates belonging to *Flavobacterium* sp. Cluster O-3-18 (n=5; BS=95, Figure 3.4) were recovered from water samples collected at PRSFH and TSFH. Isolates from the former originated from pooled reuse Brundage spring water, pooled reuse Brundage Spring and Creek water, and at the hatchery discharge, while an isolate from the latter was recovered directly from the spring supplying the hatchery. Thus, reservoirs of this bacterium likely exist within those sites, but do not appear to be associated with any form of fish disease.

***Flavobacterium* sp. Cluster O-3-19:** Isolates belonging to *Flavobacterium* sp. Cluster O-3-19 (n=7; BS=77, Figure 3.4) were recovered from PRSFH and formed 3 sub-clusters. Four isolates were recovered from the external surfaces of Chinook salmon swim up fry, 2 from the internal organs of swim up fry, and 1 from a brush used to clean hatchery raceways. Thus, these groups of bacteria represent potential pathogens of Chinook salmon in their early life stages and are sufficiently resistant to environmental

conditions to persist on hatchery tools. The relationship that isolates belonging to *Flavobacterium* sp. Cluster O-3-19 have with the mortality events in young salmonids should be investigated further.

An additional 30 robustly supported clusters of *Flavobacterium* spp. were recovered in the course of this study (Figure 3.4; Table xxx).

***Chryseobacterium* sp. Cluster O-3-50:** Isolates belonging to *Chryseobacterium* sp. Cluster O-3-50 (n=33) were most similar to the *C. chaponense* type strain sequence (98.2-99.5%) and formed a well-supported cluster with it (Figure 3.5). Members of this clade were recovered from all three hatcheries that were sampling in this study. Among these, 26 *C. chaponense* isolates originated from TSFH, 25 of which were recovered from water samples coming from the hatchery effluent at the outfall (3/4 collection periods), from the head of indoor raceways containing Chinook salmon fry and fingerlings (2/4 collection periods), from the tail end of the same raceways (4/4 collection periods), from the head of outdoor raceways (1st pass water, 3/4 collections), from the tail of the same raceways (2/4 collections), and from the head and tail ends of the second series of outdoor raceways (2nd pass water, 2/4 collections). The remaining isolate was recovered from a brush used to clean the surfaces of hatchery raceways. The 5 isolates originating from WLSFH were recovered from water samples taken from the head and tail ends of indoor raceways housing fry and fingerling Chinook salmon, from the head of outdoor raceways (2nd pass water), and from the hatchery outfall (~4th pass water). The two PRSFH isolates were recovered from the tail end of a large indoor raceway containing Chinook salmon fingerlings and from the gills of Chinook salmon fingerlings. Thus, a commonality between hatcheries was that *C. chaponense* was never detected in water samples until water came in contact with Chinook salmon fry or fingerlings. It is also interesting that members of this cluster were never recovered from any eggs and recovered only once from Chinook salmon. This is curious in light of the apparent association of this bacterium with water containing fish. It is thus possible that bacterial numbers were too low on individual fish to be isolated but reach a detection threshold in water with high fish densities. Alternatively, it may be that some organic matter associated with fish (e.g., feces, fish food, dead fish, etc.) allowed this bacterium to proliferate to cultivable numbers. In Objectives I and II, we described the presence of *C. chaponese* in North America for the first time and were surprised by the widespread nature of this bacterium in the sampled hatcheries. Fortunately, this bacterium was avirulent to multiple salmonids in our experimental challenges despite being exposed to large doses of the bacterium.

***Chryseobacterium* sp. Cluster O-3-51:** *Chryseobacterium* sp. Cluster O-3-51 isolates (n=15) were taxonomically distinct from all described *Chryseobacterium* spp. (BS=81; Figure 3.5) and were exclusively recovered from feral Chinook salmon brood stock returning to the LMRW in 2010 and 2011. This bacterium was isolated from multiple organs if infected fish, including the kidney and brain, as well as from ovarian fluid, milt, and unfertilized, iodophore-disinfected eggs. Despite the association with reproductive fluids and unfertilized eggs, this bacterium was not recovered from any hatchery-reared eggs or Chinook salmon. In addition to being genetically distinct from all described *Chryseobacterium* spp., this bacterium was also vastly different from the *Chryseobacterium* spp. described under Objective I (97.5-97.9% 16s rDNA similarity). Thus, the isolates within Cluster O-3-51 likely comprise a novel *Chryseobacterium* sp., though what role this bacterium may play in fish health is currently unknown.

***Chryseobacterium* sp. Cluster O-3-52:** *Chryseobacterium* sp. Cluster O-3-52 (n=7) isolates were very similar to the *C. piscicola* type strain sequence (99.5-99.7%) and fell into a robustly supported cluster with the type strain (BS=99; Figure 3.5). This bacterium was recovered from the gills of Chinook salmon returning to the LMRW to spawn in

2011 and also from a PRSFH water sample originating from pooled reuse spring water. However, the majority of isolates were recovered from hatchery nets and brushes at PRSFH. Thus, this bacterium appears resistant to the environmental conditions associated with a terrestrial environment. The *C. piscicola* isolates recovered under Objective III were also highly similar to Cluster XL isolate T63 (Objective I), which was recovered from the fins of a hatchery-reared brown trout. *Chryseobacterium piscicola* was originally described from diseased Atlantic salmon in Finland and as a result, should be considered as a possible threat to fish health. The finding of *C. piscicola* on hatchery tools once again demonstrates that tool disinfection could be a viable way to reduce the chance of chryseobacteril transmission.

***Chryseobacterium* sp. Cluster O-3-53:** Isolates belonging to *Chryseobacterium* sp. Cluster O-3-53 (n=7) were most similar to, yet distinct from, *C. antarctica* (BS=99; Figure 3.5). All isolates belonging to this cluster were recovered from the water, hatchery tools, and Chinook salmon fingerlings at TSFH and specifically originated from apparently normal gills of fingerling Chinook salmon, from hatchery brushes and pads used to clean raceways, and from a water sample collected from the tail end of an indoor raceway containing Chinook salmon fry. Thus, *Chryseobacterium* sp. O-3-53 appears to be primarily associated with Chinook salmon fry and fingerlings, and also has the ability to survive on hatchery tools. The impact this bacterium has on fish is currently unknown.

***Chryseobacterium* sp. Unresolved Group 3D:** A large group (n=37) of closely related, yet unresolved *Chryseobacterium* sp. isolates (Unresolved Group 3D; Figure 3.5) that were highly similar (99.3-99.8%) to *C. aahli* sp. nov. (Cluster XXXIV; described under Objective 1) were recovered in this study. Among these, 30 isolates originated from TSFH, 21 of which were recovered from the surfaces of non-disinfected eyed Chinook salmon eggs in 2010 and 2011, 2 from the internal organs of Chinook salmon swim up fry in 2011 and 2012, and 7 from water collected after flowing through egg stacks housing swim up fry (2011 only). The 4 WLSFH isolates were recovered from the tail end of an indoor raceway (supplied with deep well water) containing Chinook salmon fry, from the head of outdoor raceways (2^{nd} pass water), and from the outdoor raceway hatchery outfall (~4^{th} pass). Three *Chryseobacterium* sp. Unresolved Group 3D isolates were also recovered from PRSFH; 2 from a raceway brush and 1 from the clarifier just prior to hatchery discharge. The absence of this bacterium from the source water of all three hatcheries, in conjunction with its occurrence only in association with Chinook salmon eggs, fry, the surrounding water, and tools used to clean their enclosures suggest that *C. aahli* has a tropism for Chinook salmon in their early life stages. Because *C. aahli* was also not detected in the brood stock or unfertilized eggs, the source of this bacterium is unknown. However, as suggested for *Flavobacterium* sp. O-3-1, it is possible that this bacterium may be "seeded" onto the eggs when they leave the body cavity of the fish (i.e., the bacterium is present near the vent of brood fish) or that a limited number of brood fish are infected with *C. aahli* (which were not sampled), which rapidly proliferates in the presence of eggs/fry. *Chryseobacterium aahli* was only mildly virulent in the experimental challenge studies conducted under Objective II, though it is possible that mortality is higher in salmonid fry.

***Chryseobacterium* sp. Unresolved Group 3E:** *Chryseobacterium* sp. isolates falling into Group 3E (n=22) were taxonomically unresolved (though the bootstrap value of 67 is just below the cutoff of 70; data not shown) but grouped with 3 *Chryseobacterium* spp.; *C. piscium, C. scophthalmum*, and *C. balustinum* (Figure 3.5). Interestingly, all three of these *Chryseobacterium* species were originally described in association with fish. Within this group, 17 isolates originated from WLSFH: 5 were recovered from the surfaces of non-disinfected eyed Chinook salmon eggs, 7 from the external surfaces of

Chinook salmon fry, 2 from the internal organs of Chinook salmon fry, and 3 from water collected from the tail end of indoor Chinook salmon fry and fingerling raceway and at the head of outdoor raceways receiving 2nd pass water. Four isolates were also recovered from the surfaces of non-disinfected eyed eggs being reared at TSFH. The final isolate was recovered from the liver of a Chinook salmon fingerling at PRSFH. Once again, this group of bacteria was not found in any of the source waters supplying the 3 hatcheries and seemed to have a predilection for Chinook salmon in their early life stages. Fortunately, iodophore disinfection of eyed eggs (100 ppm for 10 min) was effective at killing the bacterium, as no isolates were recovered from eyed eggs after disinfection. The absence of the bacterium in source water and the presence on non-disinfected eyed eggs has several potential explanations. First, there may be undiscovered bacterial reservoirs within the hatchery infrastructure that periodically "shed" bacteria when disturbed (i.e., biofilms on the walls of a raceway or within pipelines) but were not at the time of sampling. Secondly, the bacterium could have arrived to the hatchery on the eggs (seeded from parents as described previously) and were not destroyed by iodine disinfection for reasons previously described. Third, the bacterium could have been introduced to the hatcheries through a variety of breaches in biosecurity, though this seems highly unlikely given that this group of bacteria was found in all 3 hatcheries. We feel the first and second explanations are the most plausible in this instance. In this context, this group of chryseobacteria were 99.4-100% similar to the *Chryseobacterium* sp. isolates of Cluster XXXVII (Objective I), some of which were originally recovered from salmonids reared at TSFH and WLSFH and thus may be endemic in these facilities. A member of Cluster XXXVII (e.g., T31) proved to be moderately pathogenic in experimental challenges and generated disease signs commonly associated with a bacterial septicemia (Objective II).

Table 3.1. Samples collected for studies conducted under Objective III. Row colors are to identify sampling location and match those colors to denote sampling location in the dendrograms of Figures 3.4 and 3.5.

Sampling I.D.	Location	Sampling Period	Date Sampled	Collected Samples/Tissues
101007-1	SRW	Brood	10-7-10	Gills, kidney, spleen, brain, milt, ovarian fluid, unfertilized eggs
111012-1	SRW	Brood	10-12-11	
101012-1	LMRW	Brood	10-12-10	Gills, kidney, spleen, brain, milt, ovarian fluid, unfertilized eggs
111005-1	LMRW	Brood	10-5-11	
PLD/PLND	PRSFH	Eyed eggs	11-30-10	Eyed eggs
110104	PRSFH	Fry	01-04-11	Fry & H_2O
110512	PRSFH	Fingerlings	05-12-11	Fingerlings, tools, H_2O
TSD/TSND	TSFH	Eyed eggs	11-30-10	Eyed eggs
110118	TSFH	Fry	1-18-11	Fry & H_2O
110519	TSFH	Fingerlings	5-19-11	Fingerlings, tools, H_2O
111130-1	TSFH	Eyed eggs	11-30-11	Eyed eggs
120117-1	TSFH	Fry	1-17-12	Fry & H_2O
120515-1	TSFH	Fingerlings	5-15-12	Fingerlings, tools, H_2O
111130-2	WLSFH	Eyed eggs	11-30-11	Eyed eggs
120106-1	WLSFH	Fry	1-6-12	Fry & H_2O
120416-1	WLSFH	Fingerlings	4-16-12	Fingerlings, tools, H_2O

Table 3.2. Sites at Platte River State Fish Hatchery where water samples were collected for flavobacterial culture (January and May of 2011).

Sample I.D.	Water Collection Location	110104	110512
WS1	Brundage Creek	**X**	**X**
WS2	Upper Discharge (Hatchery Exit)	**X**	**X**
WS3	Hatchery Clarifier (Prior to Hatchery Exit)	**X**	**X**
WS4	Unheated Spring Water	**X**	**X**
WS5	Head box (Heated Spring Water)	**X**	**X***
WS6	Pre-Egg Stacks	**X**	**NA**
WS7	Post-Egg Stacks	**X**	**NA**
WS8	Head of Indoor Fry Tank (Unheated Spring)	**X**	**X**
WS9	Tail of Indoor Fry Tank (Unheated Spring)	**X**	**X**
WS10	Head of Large Fry Tank (Heated Spring)	**X**	**X***
WS11	Tail of Large Fry Tank (Heated Spring)	**X**	**X***
WS12	Reuse Pooled Spring Water (Pre-Coho)	**X**	**X**
WS13	Reuse Pooled Spring Water + Brundage Creek Water	**X**	Head outdoor raceway, Coho
WS14	Head of Large Fry Tank (#4; Heated Spring)	**X**	Tail outdoor raceway, Coho
WS15	Tail of Large Fry Tank (#4; Heated Spring)	**X**	**X**

*, Unheated during this sampling period

Table 3.3. Sites at Thompson State Fish Hatchery where water samples were collected for flavobacterial culture (January and May of 2011 and 2012).

Sample I.D.	Water Collection Location	110118	110519	120117	120515
WS1	Spring	**X**	**X**	**X**	**X**
WS2	Deep Well	**X**	**X**	**X**	**X**
WS3	Hatchery Outfall (Hatchery Exit)	**X**	**X**	**X**	**X**
WS4	Shallow Well (In Hatchery)	**X**	**X**	**X**	**X**
WS5	Spring Water (In Hatchery; pre-U.V.)	**X**	**X***	**NA**	**NA**
WS6	Spring Water (In hatchery; Post-U.V.)	**X**	**X***	**NA**	**NA**
WS7	Pre-Egg Stacks (Shallow Well)	**X**	**NA**	**X**	**Head box**
WS8	Post-Egg Stacks (Shallow Well)	**X**	**NA**	**X**	**Pre-U.V.**
WS9	Pre-Egg Stacks (Spring, U.V.-Tx.)	**X**	**NA**	**NA**	**NA**
WS10	Post-Egg Stacks (Spring, U.V.-Tx.)	**X**	**NA**	**NA**	**NA**
WS11	Head of Indoor Raceway (Deep Well & Spring)	**X**	**X**	**X**	**X**
WS12	Tail of Indoor Raceway (Deep Well & Spring)	**X**	**X**	**X**	**X**
WS13	Head of Outdoor Raceway (1st Pass)	**X**	**X**	**X**	**X**
WS14	Tail of Outdoor raceway (2nd Pass)	**X**	**X**	**X**	**X**
WS15	Head of Outdoor raceway (2nd Pass)	**X**	**X**	**X**	**X**
WS16	Tail of Outdoor raceway (3rd pass)	**X**	**X**	**X**	**X**

*, mixed with deep well water

Table 3.4. Sites at Wolf Lake State Fish Hatchery where water samples were collected for flavobacterial culture (January and May of 2012).

Sample I.D.	Water Collection Location	120106	120416
WS1	Pre-Egg Stacks	**X**	**NA**
WS2	Post-Egg Stacks	**X**	**NA**
WS3	Spring Water (Near Hatchery)	**X**	**X**
WS4	Deep Well (Mix of Wells 5 & 6)	**X**	**X***
WS5	Deep Well (Post Degassing)	**X**	**X***
WS6	Deep Well (In Hatchery, Pre-Fish)	**X**	**X***
WS7	Head of Indoor Raceway (Deep Well)	**X**	**X***
WS8	Tail of Indoor Raceway (Deep Well)	**X**	**X***
WS9	Head of Outdoor Raceway (1st Series, 2nd Pass)	**X**	**X***
WS10	Outdoor Raceway Outfall (4th Pass)	**X**	**X***
WS11	Spring Water (Head)	**X**	**X**
WS12	Deep Well (5)	**X**	**X#**
WS13	Pond 24 Discharge (Hatchery Exit)	**X**	**X**
WS14	Deep Well (6)	**X**	**X**

*, Mix of Deep Wells 6 & 7; #, Well 7.

Table 3.5. Prevalence of *Flavobacterium psychrophilum* in Chinook salmon returning to spawn at Michigan gamete collection stations during 2010-2011 as determined by bacterial culture on cytophaga agar. OF, ovarian fluid.

Location	Kidney	Spleen	Brain	Milt	OF
Swan River Weir					
2010	15/60	0/60	0/60	0/30	0/30
2011	4/60	0/60	1/60	0/30	1/30
Little Manistee River Weir					
2010	29/60	2/60	0/60	0/30	0/30
2011	38/60	0/60	7/60	0/30	2/30

Table 3.6. Prevalence of *Flavobacterium columnare* in Chinook salmon returning to spawn at Michigan gamete collection stations during 2010-2011.

Location	Kidney	Spleen	Brain	Gill	Milt	OF
Swan River Weir						
2010	31/60	8/60	2/60	56/60	1/30	2/30
2011	42/60	2/60	1/60	35/60	0/30	4/30
Little Manistee River Weir						
2010	5/60	2/60	0/60	1/60	0/30	0/30
2011	7/60	0/60	0/60	11/60	0/30	0/30

Figure 3.1. Sites of water collection for flavobacterial culture at the Platte River State Fish Hatchery (Beulah, MI). A, Brundage Creek water (WS1); B, unheated spring water (WS4); C, hatchery head-box (WS5); D, head of raceway R7 (WS8); E, tail of raceway R7 (WS9); F, head of raceway R3 (WS10); G, tail of raceway R3 (WS11); H, water reuse reservoir pre outdoor raceways (WS12); I, head of outdoor raceway C5 (WS13); J, tail of outdoor raceway C5 (WS14); K, upper hatchery discharge (WS2); L, post-clarifier hatchery discharge (WS3).

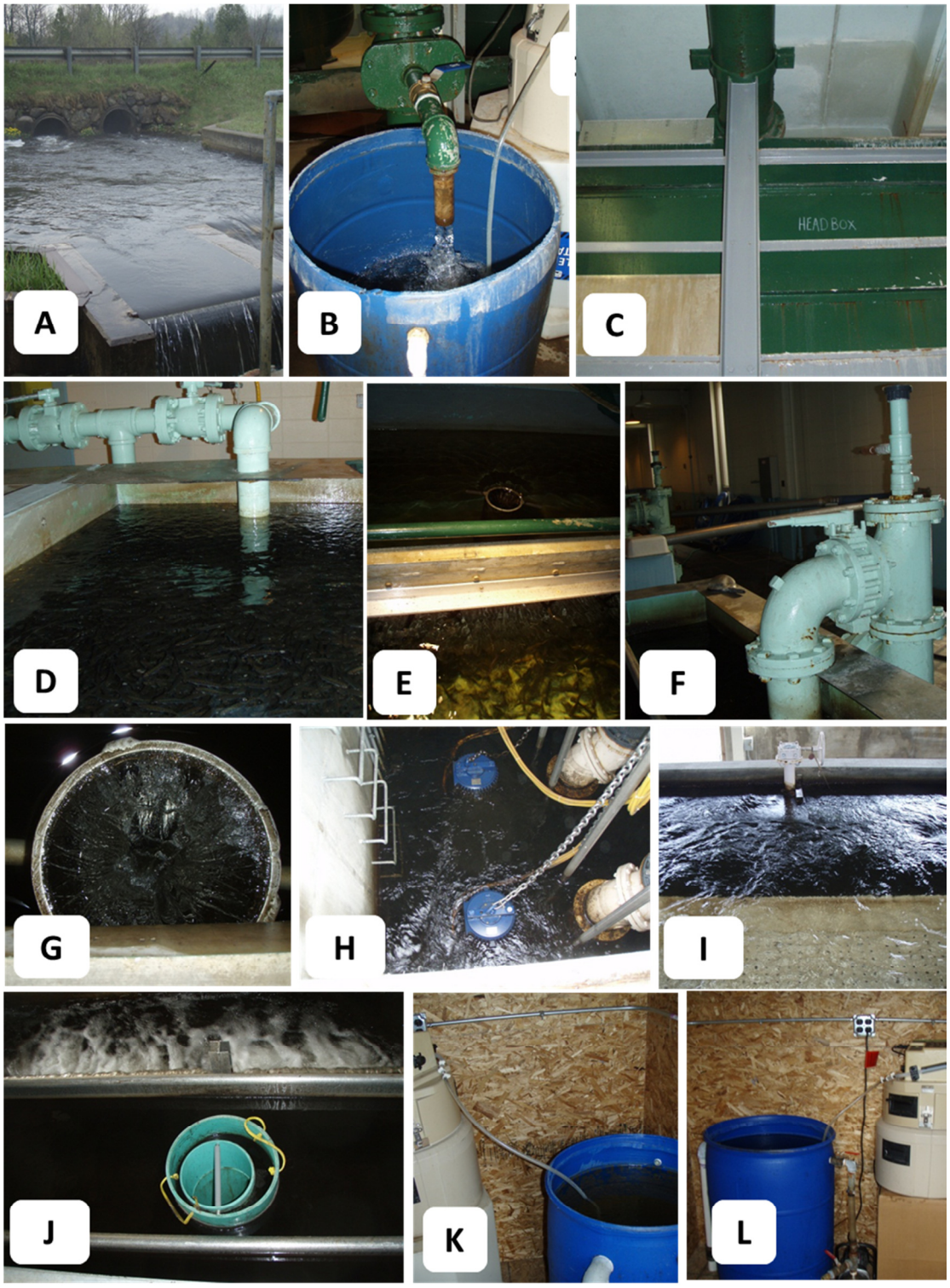
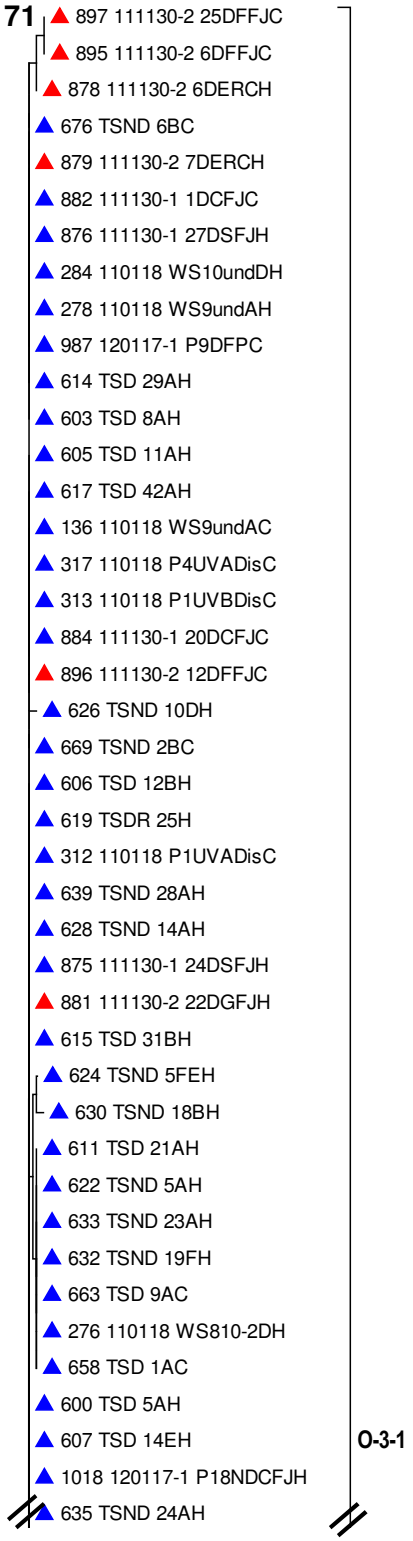


Figure 3.2. Sites of water collection for flavobacterial culture at the Thompson State Fish Hatchery (Manistique, MI). Letter within parentheses denote water sample number that corresponds to Table 3.3. A, spring (WS1); B, deep well (WS2); C, shallow well (in hatchery; WS 4); D, spring water, pre (WS 5) and post (WS 6) U.V. treatment; E, pre-egg stacks (WS7 & 9); F, post –egg stacks (WS8 & 10); G, head of indoor raceway containing Chinook salmon (WS11); H, tail end of indoor raceway containing Chinook salmon (WS12); I, head and tail of outdoor raceway (WS13 & 14); J, hatchery outfall (WS3).

Figure 3.3. Sites of water collection for flavobacterial culture at the Wolf Lake State Fish Hatchery (Mattawan, MI). Letter within parentheses denote water sample number that corresponds to Table 3.4. A, spring (WS11); B, deep well (WS12); deep well (WS14); D, spring (near hatchery, WS3); E, mix of deep wells (WS4); F, mix of deep wells (post-degassing; WS5); G, head of indoor raceway containing Chinook salmon (WS7); H, tail end of indoor raceway containing Chinook salmon (WS8); I, outdoor raceway outfall (4th pass; WS10); J, hatchery discharge (Pond 24; WS13).

Figure 3.4. Dendrogram generated using the neighbor-joining method in MEGA5 that depicts the phylogenetic relationship between Objective III *Flavobacterium* spp. and other described and candidate *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 1,000 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. Black triangles, *Flavobacterium* spp. reference sequences; Green triangles, PRSFH isolates; Blue triangles, TSFH isolates; Red triangles, WLSFH isolates; Yellow triangles, LMRW isolates; Purple triangles, SRW isolates.



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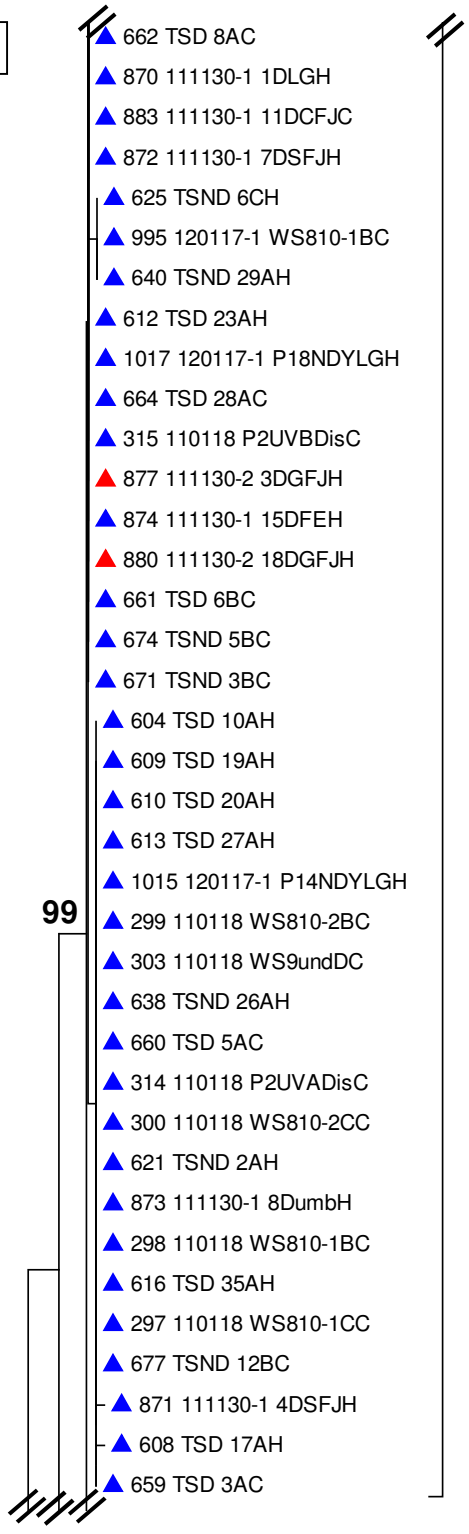

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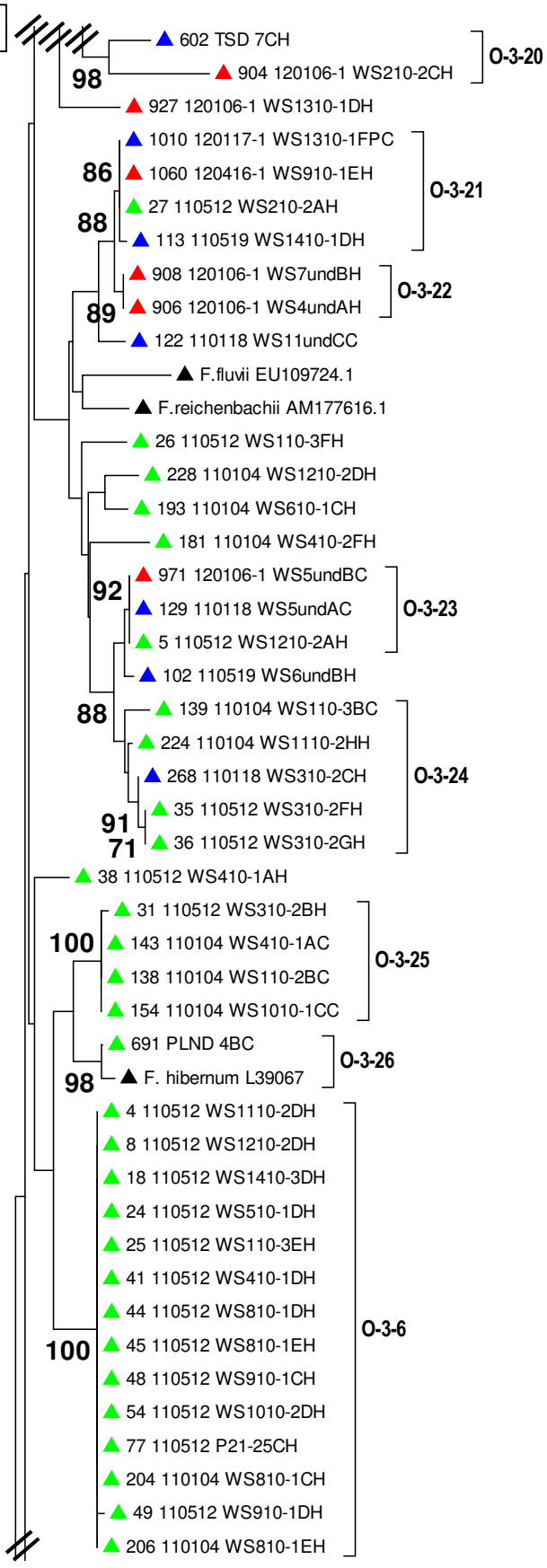

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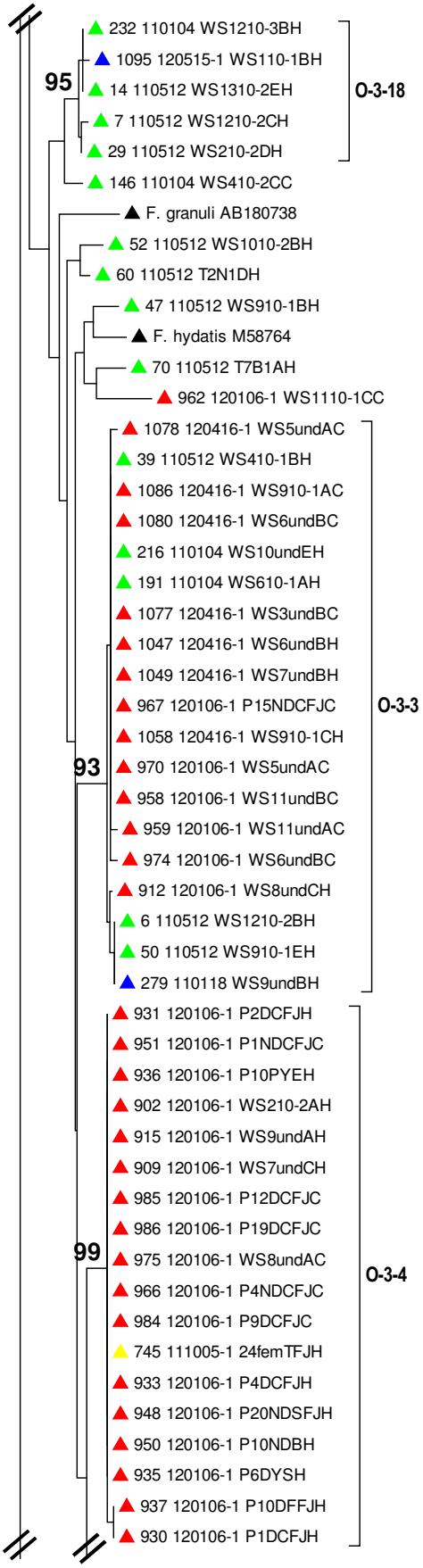

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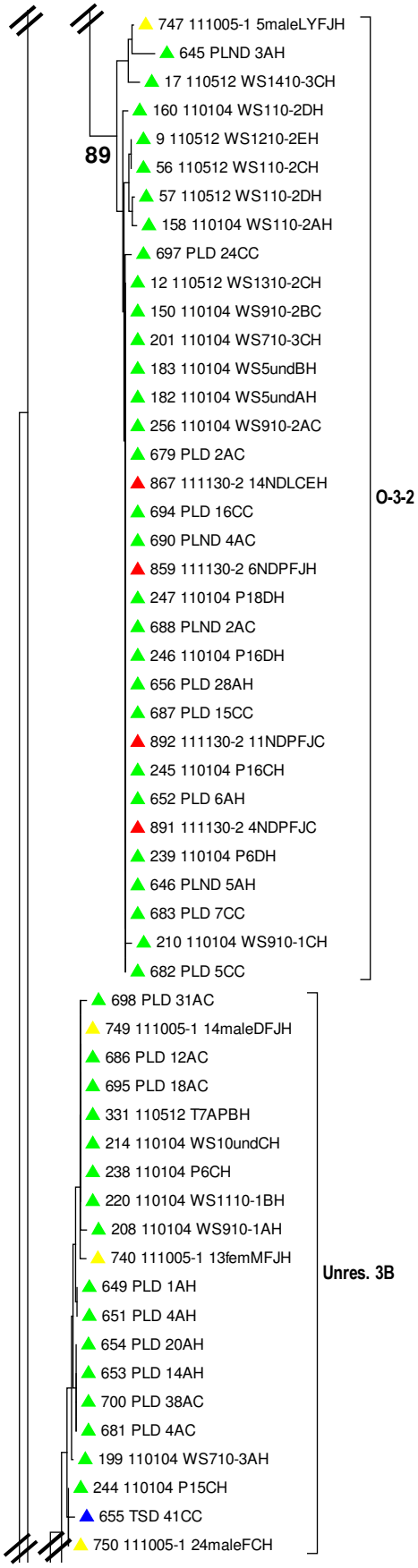

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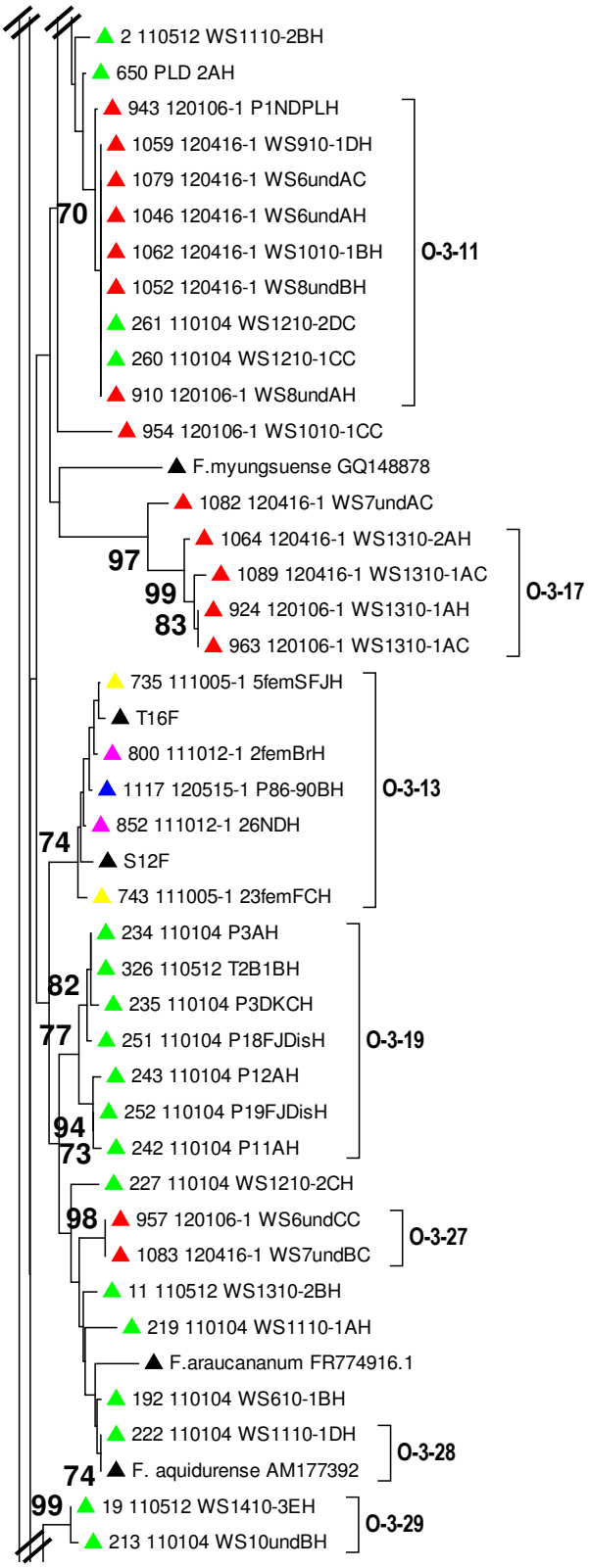

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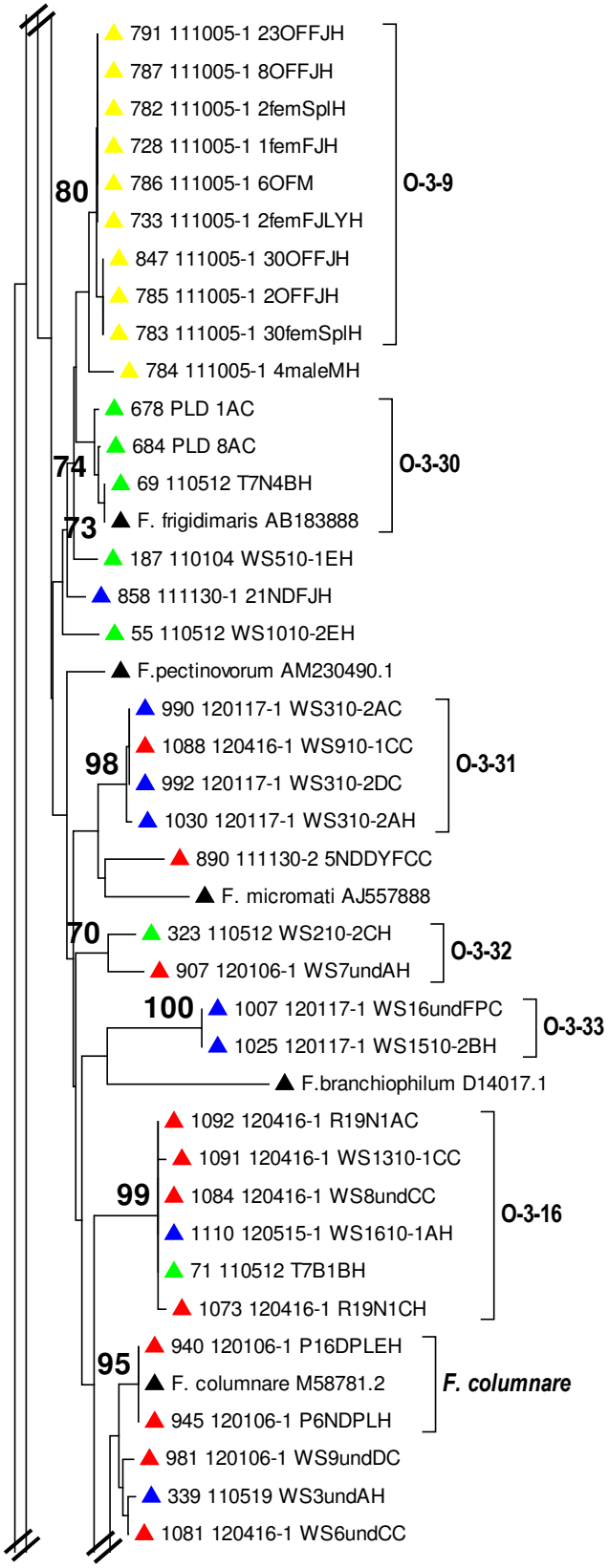

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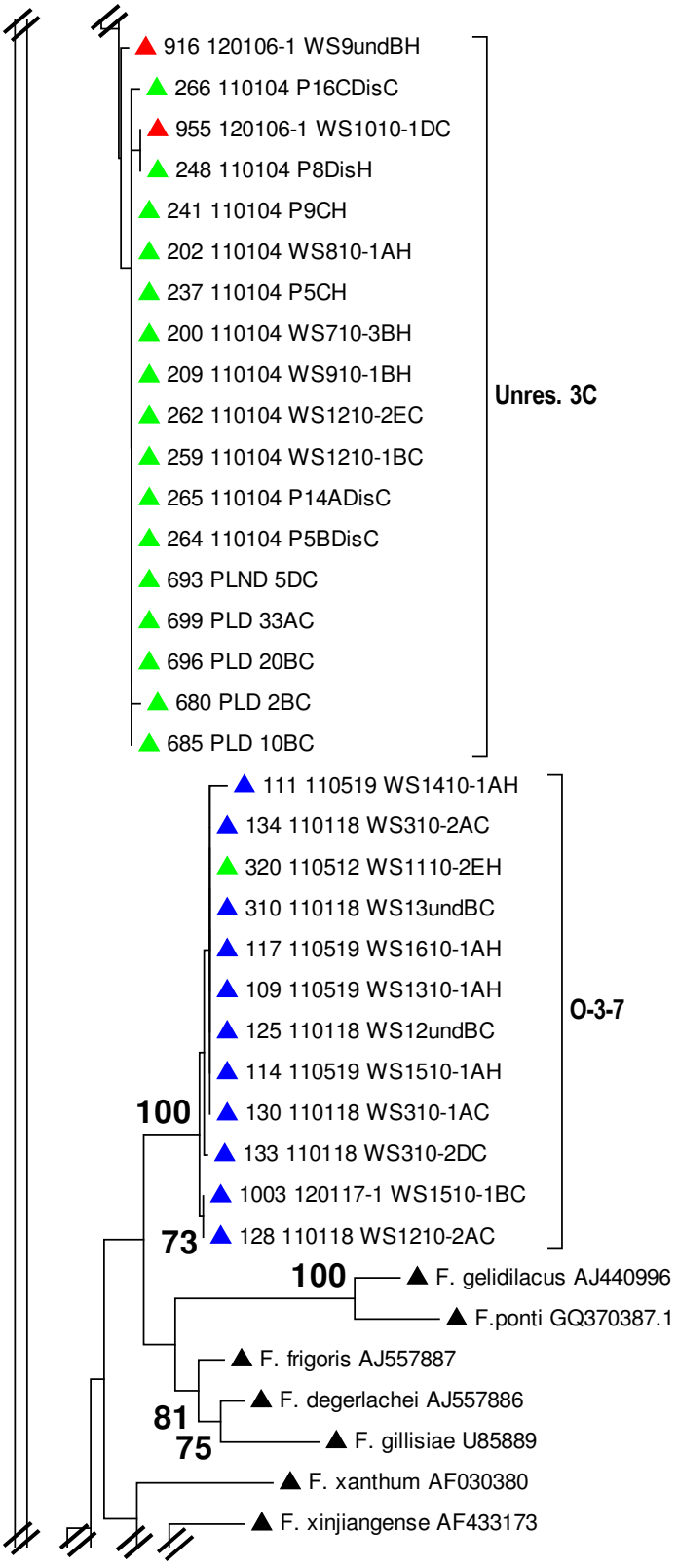

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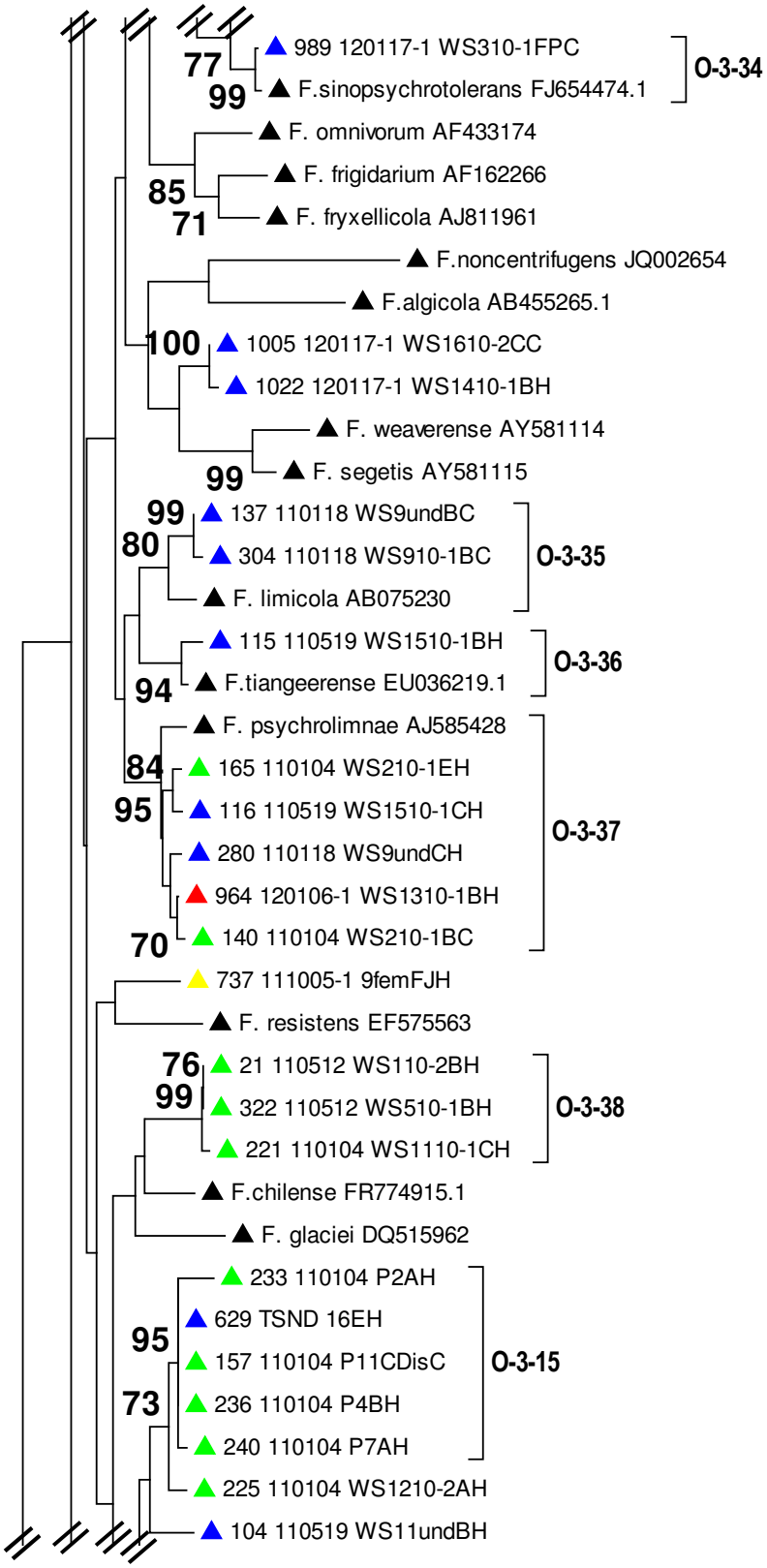

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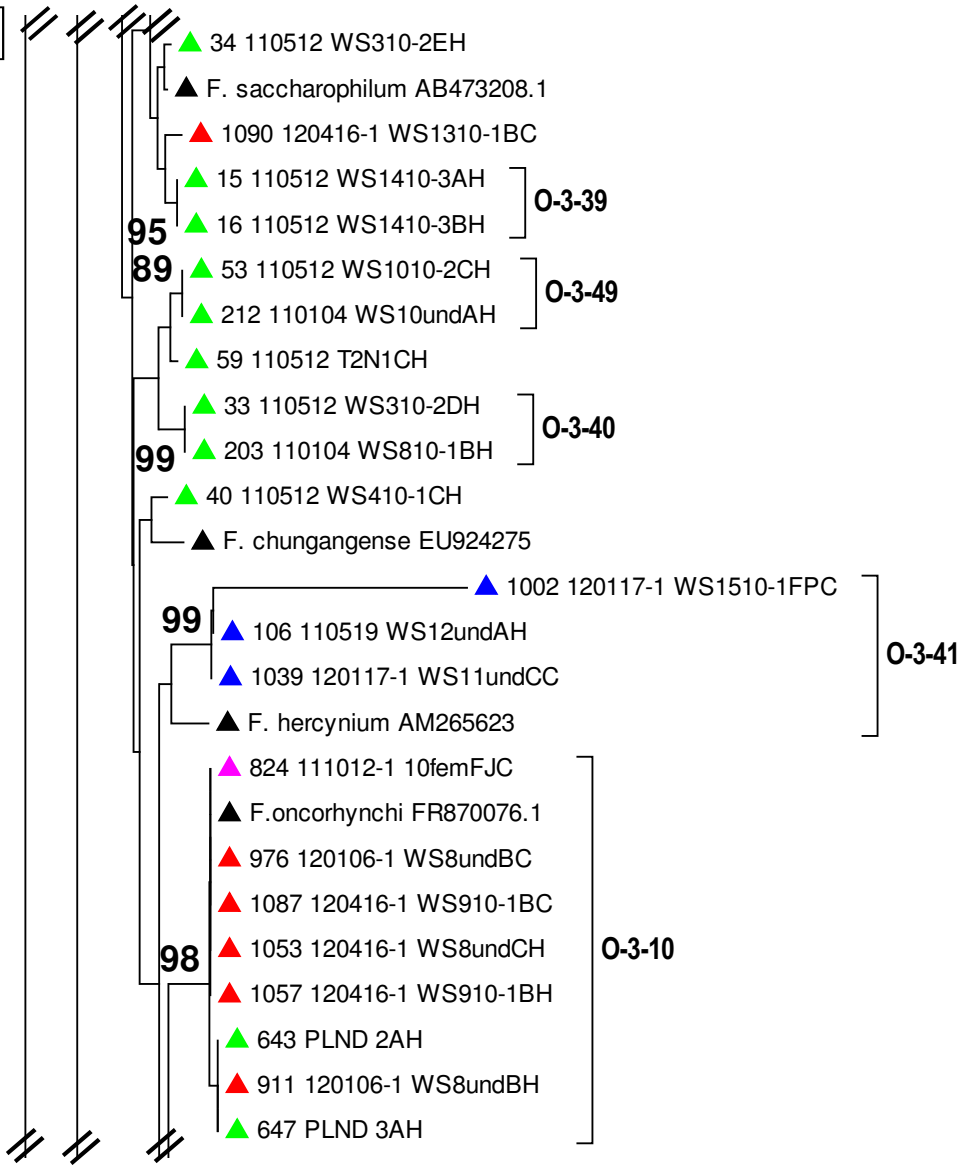

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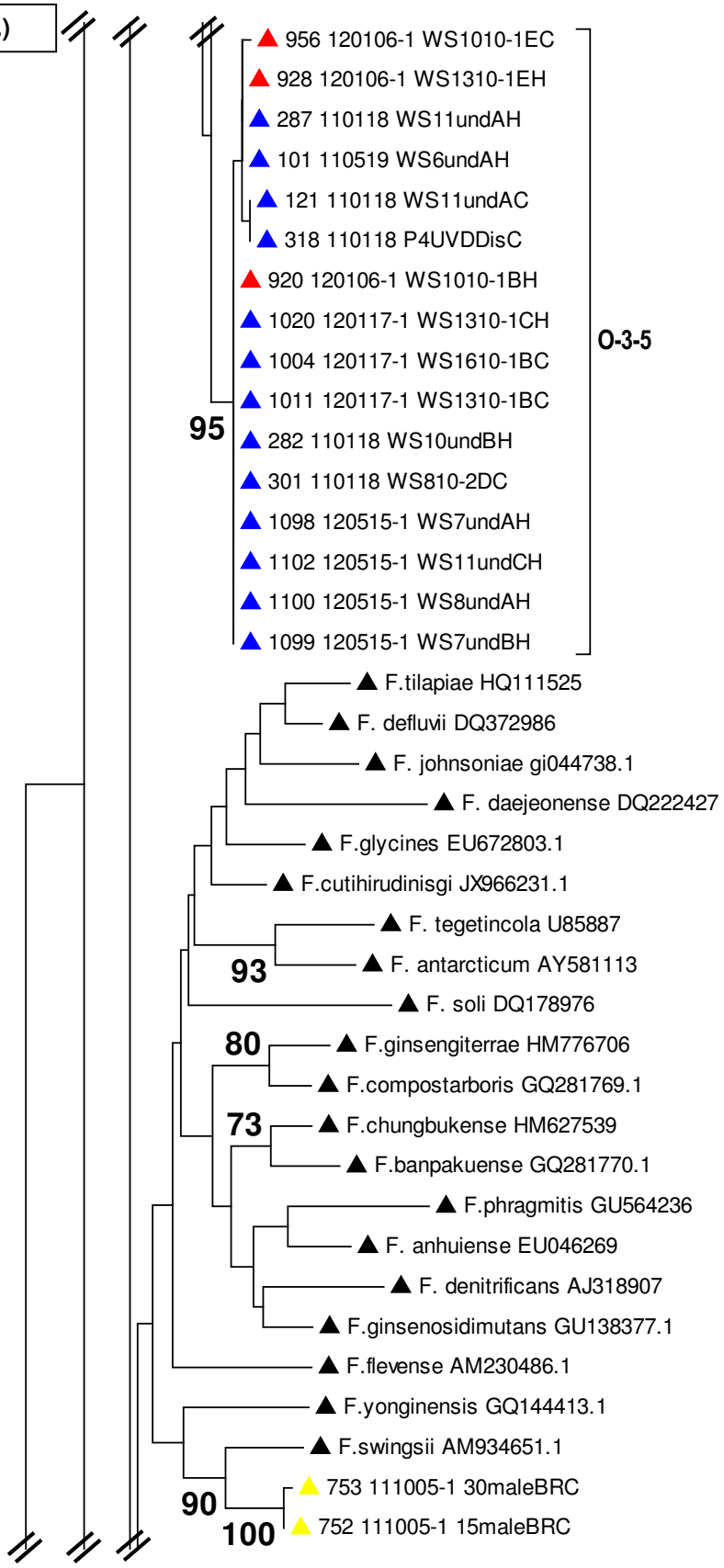

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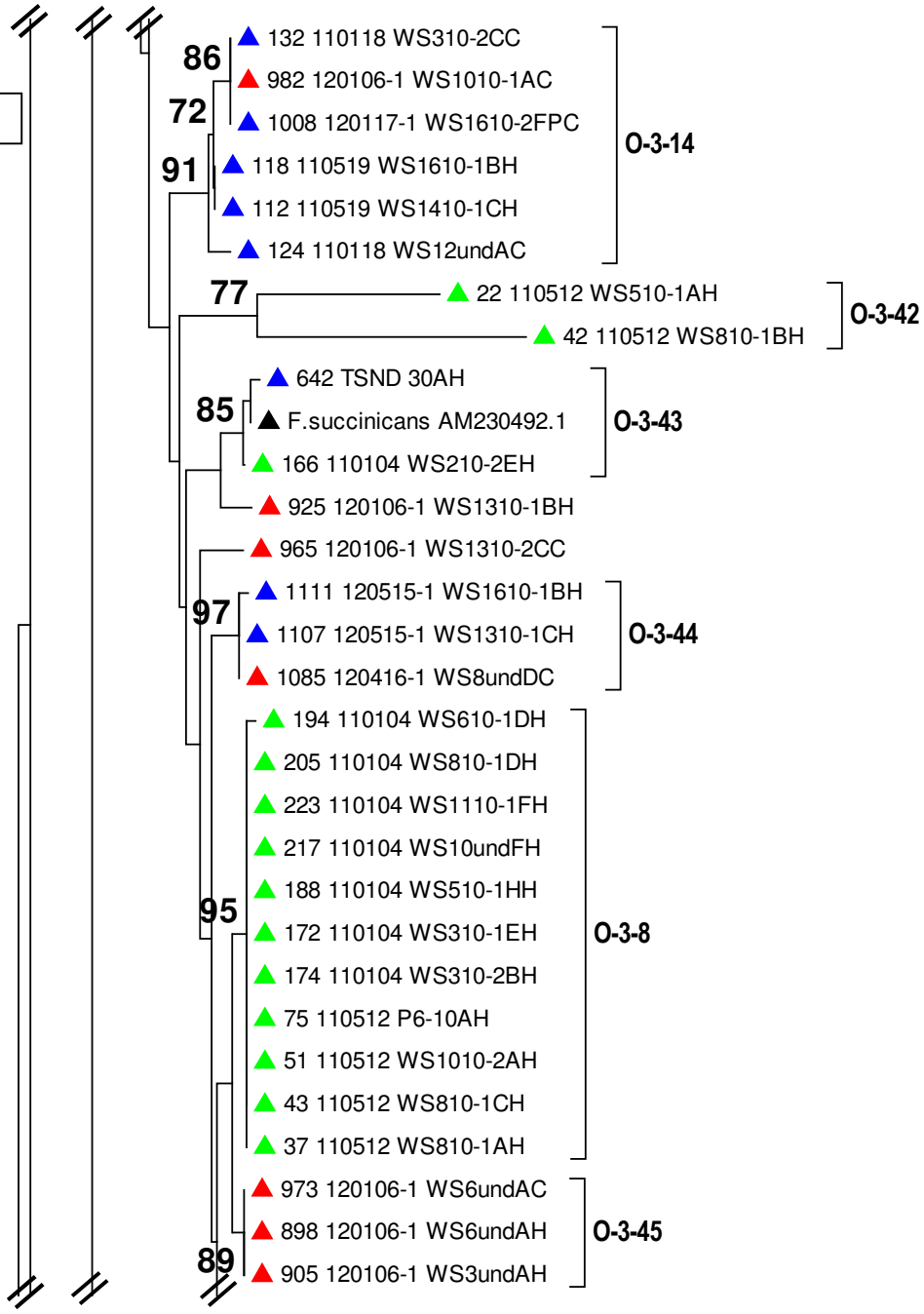

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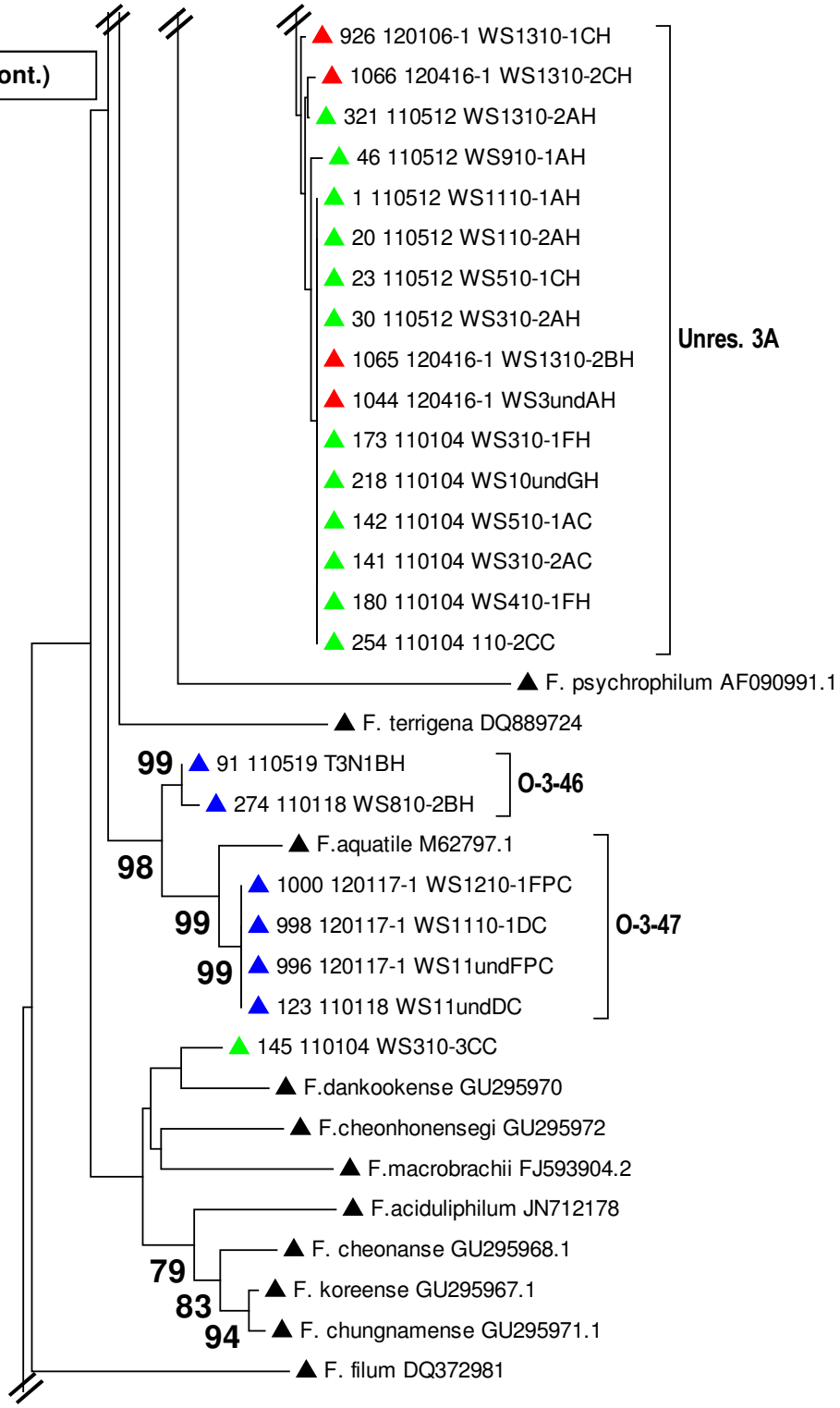

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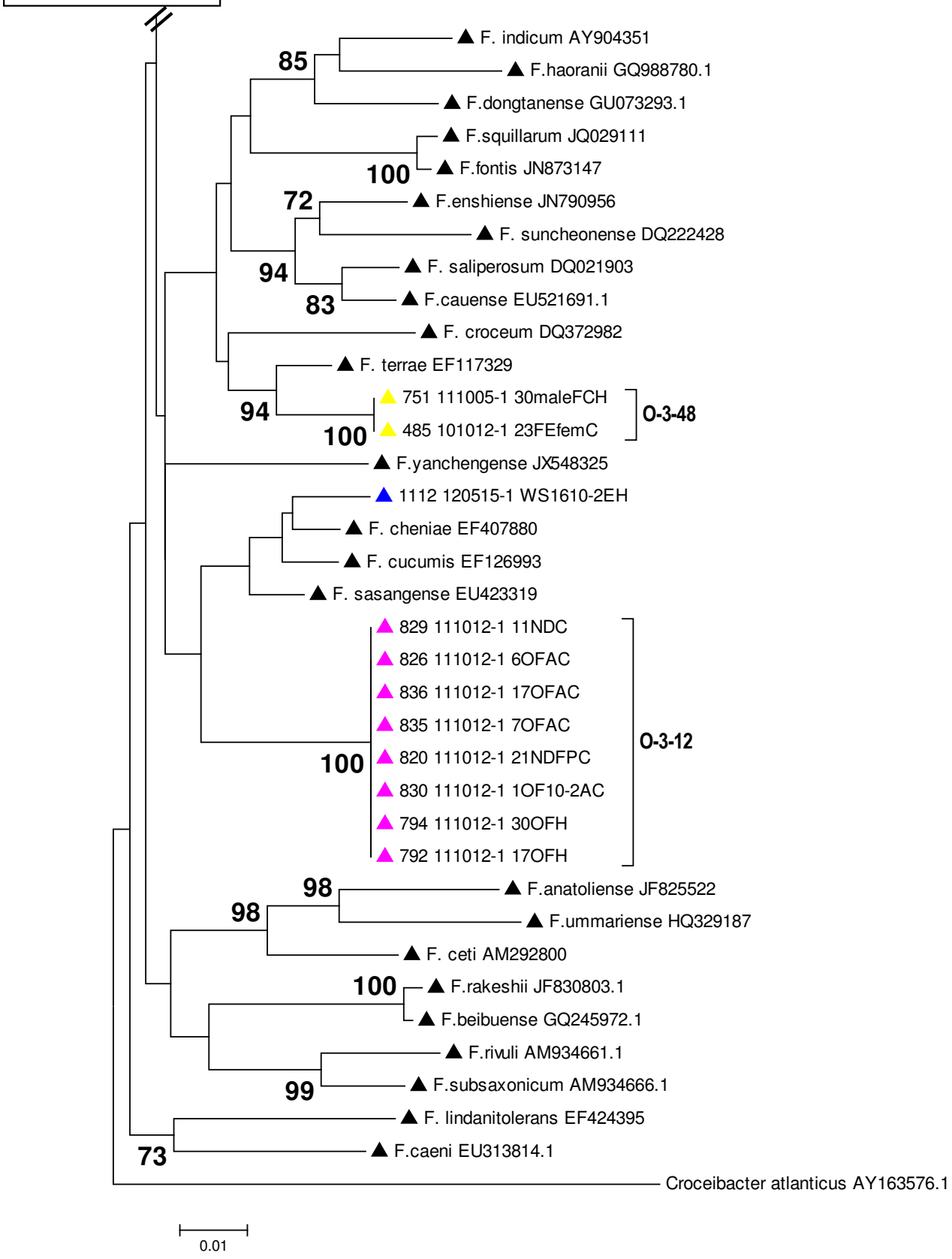
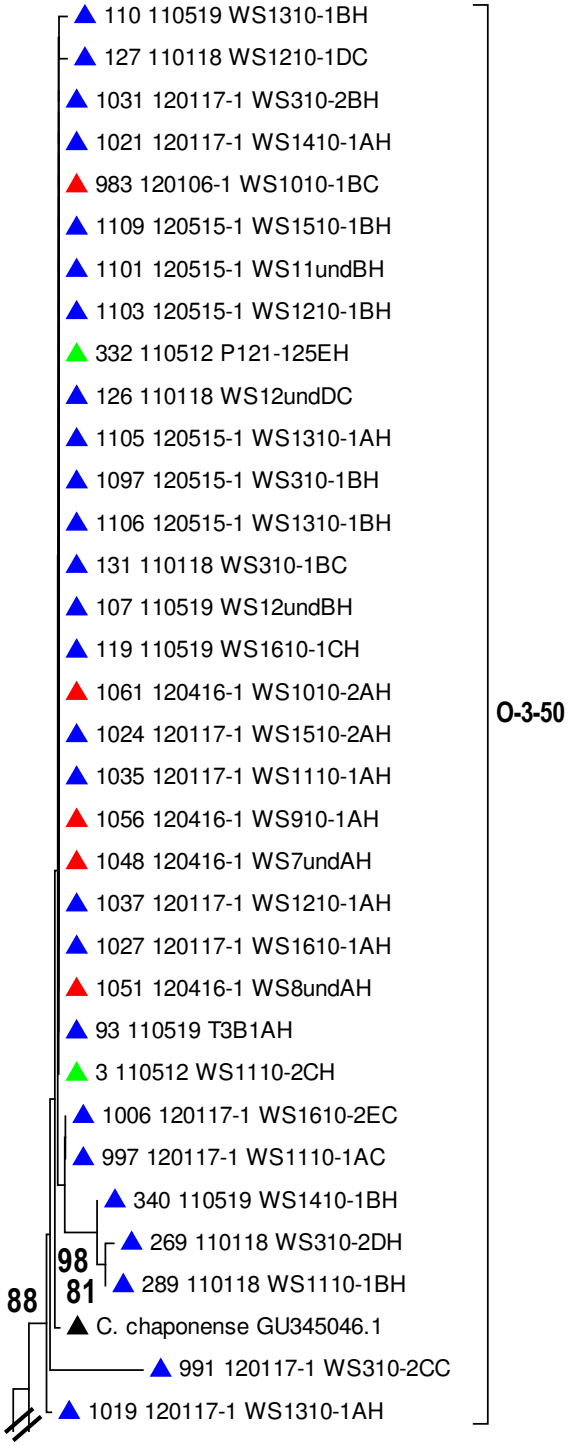


Figure 3.5. Dendrogram generated using the neighbor-joining method in MEGA5 that depicts the phylogenetic relationship between Objective III *Chryseobacterium* spp. and other described and candidate *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 1,000 replicates) are presented 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. Black triangles, *Chyseobacterium* spp. reference sequences; Green triangles, PRSFH isolates; Blue triangles, TSFH isolates; Red triangles, WLSFH isolates; Yellow triangles, LMRW isolates; Purple triangles, SRW isolates.



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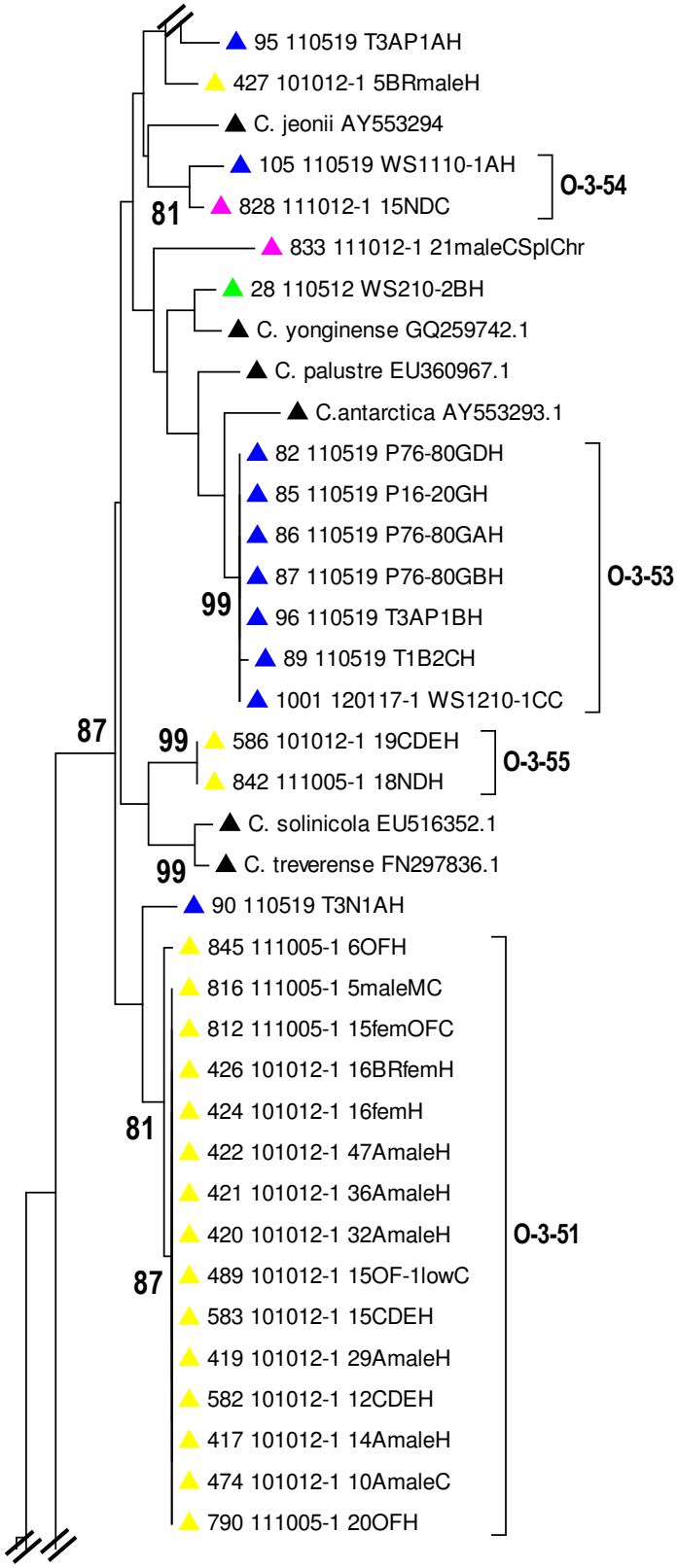

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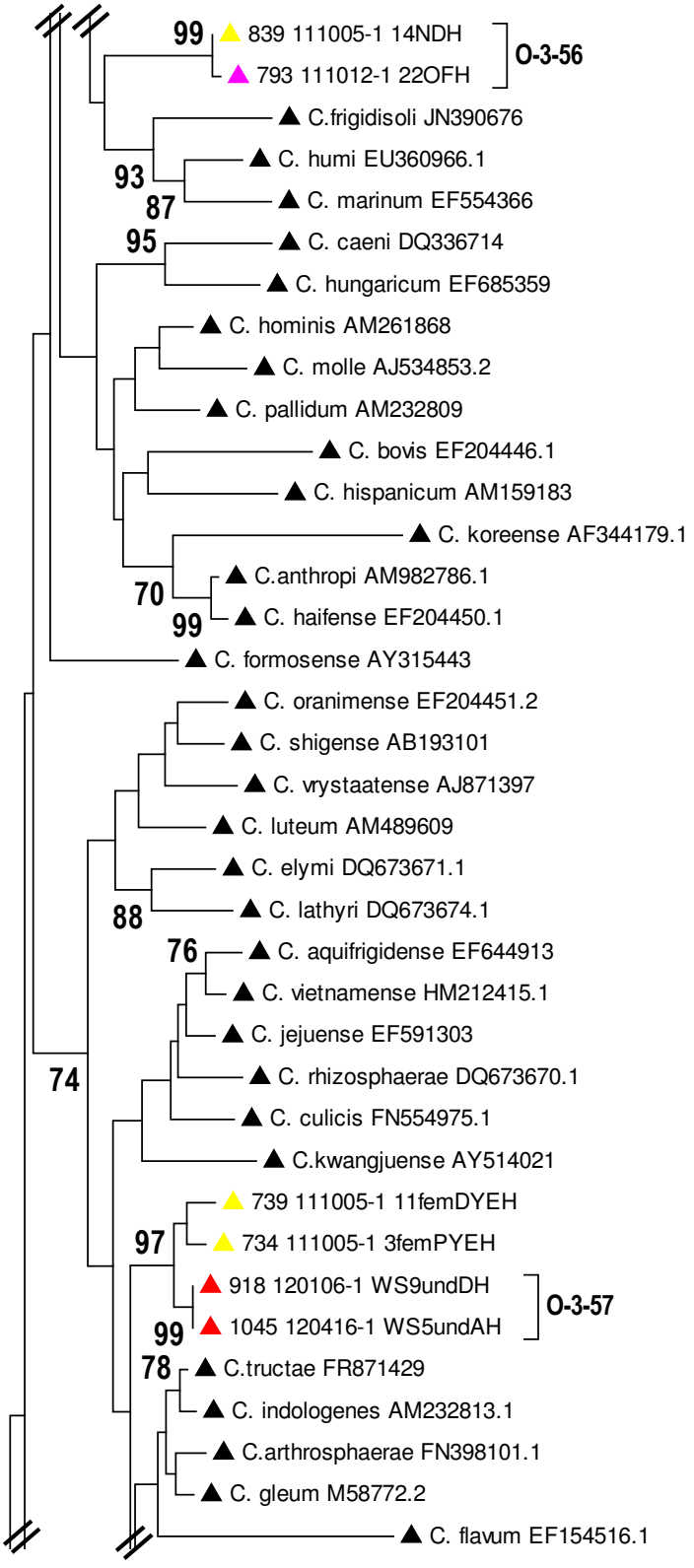

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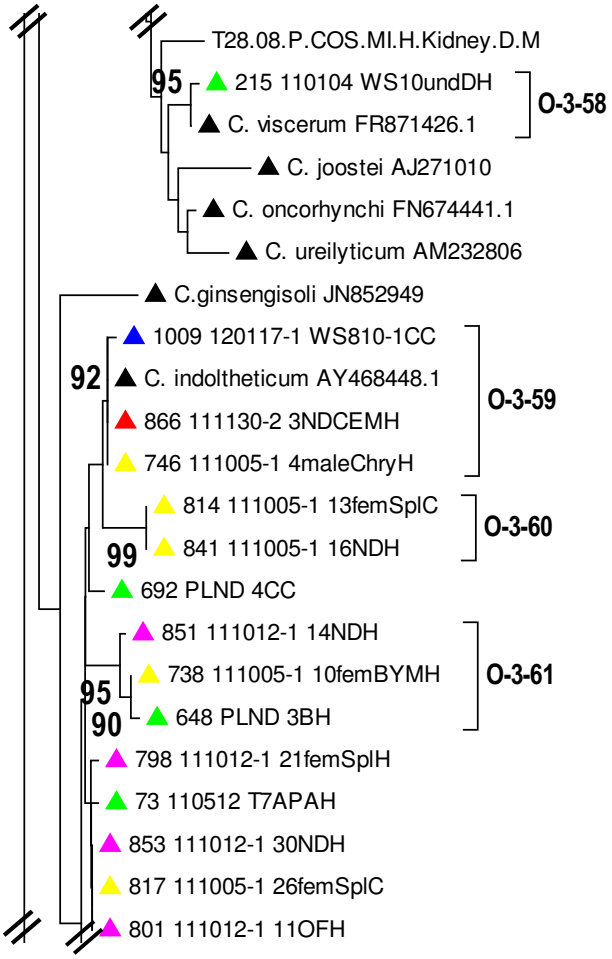

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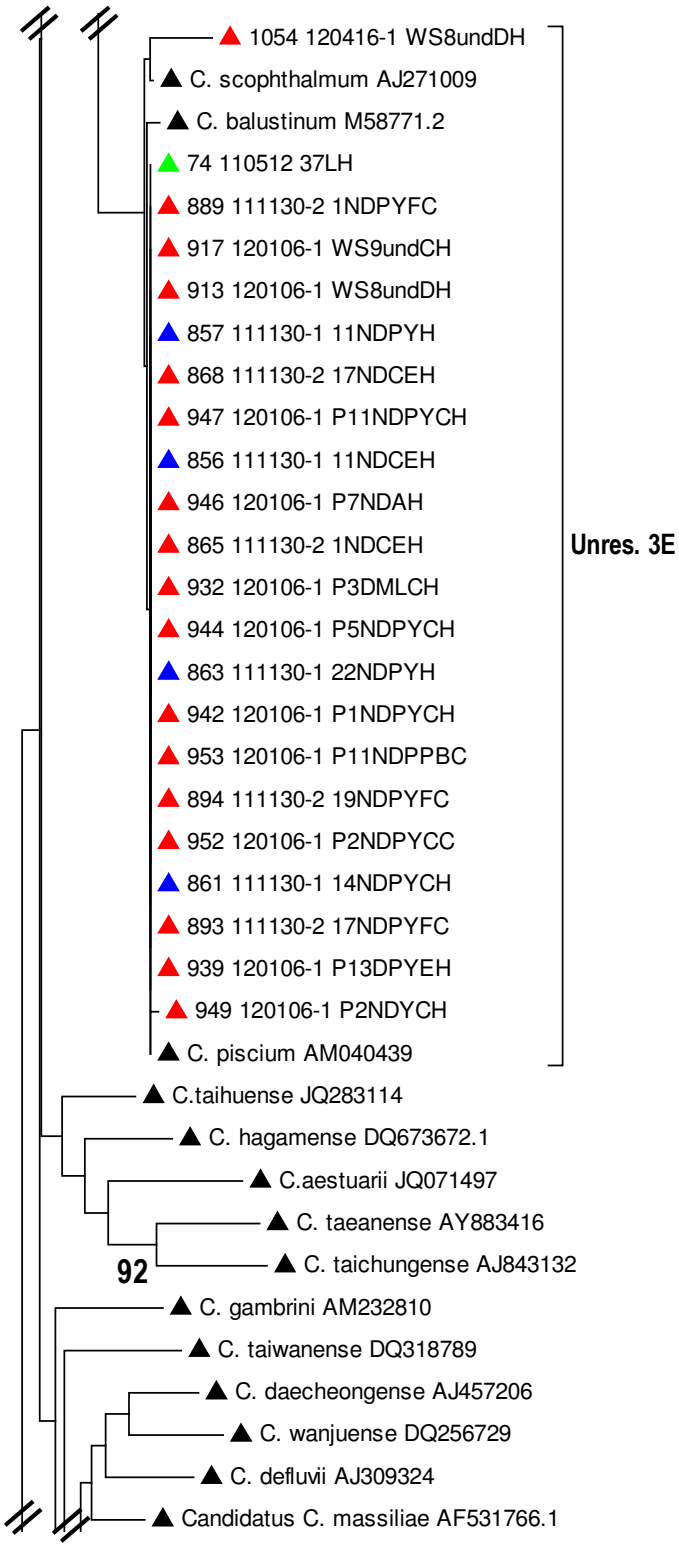

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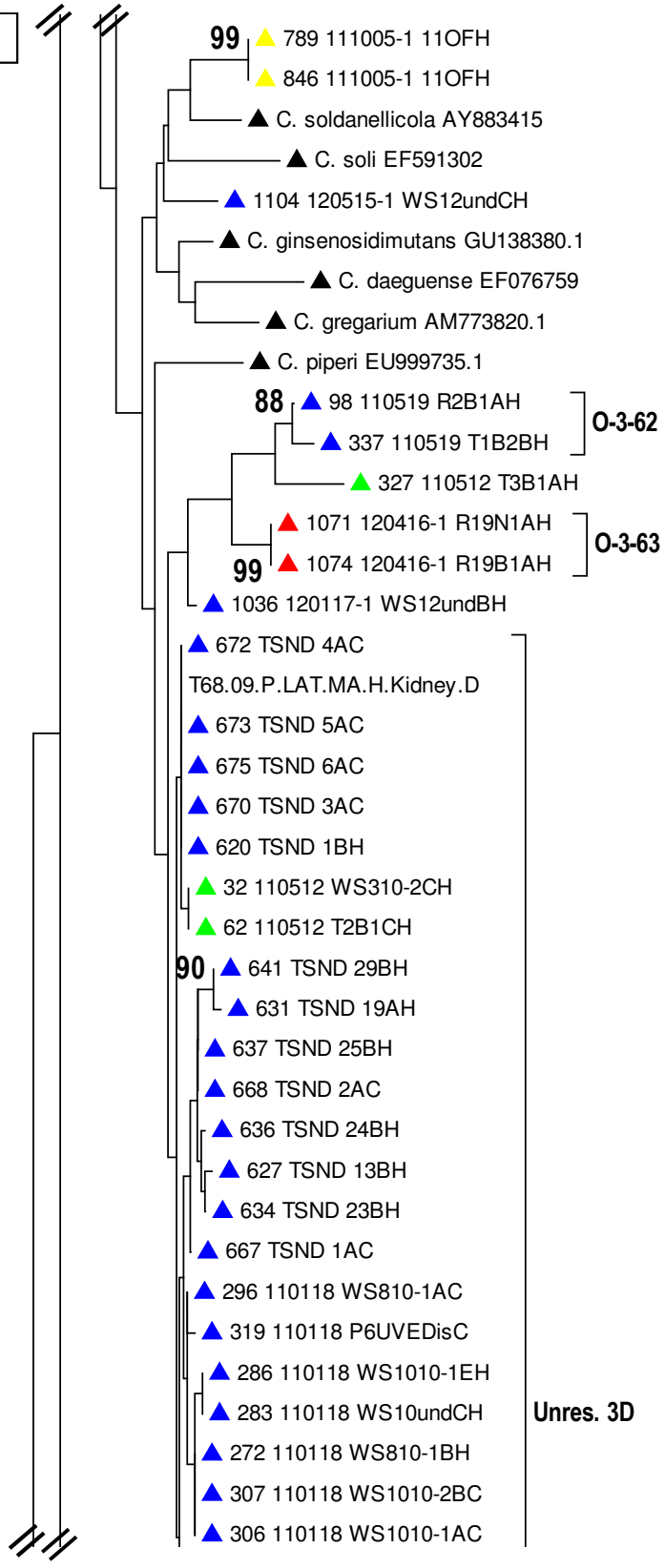

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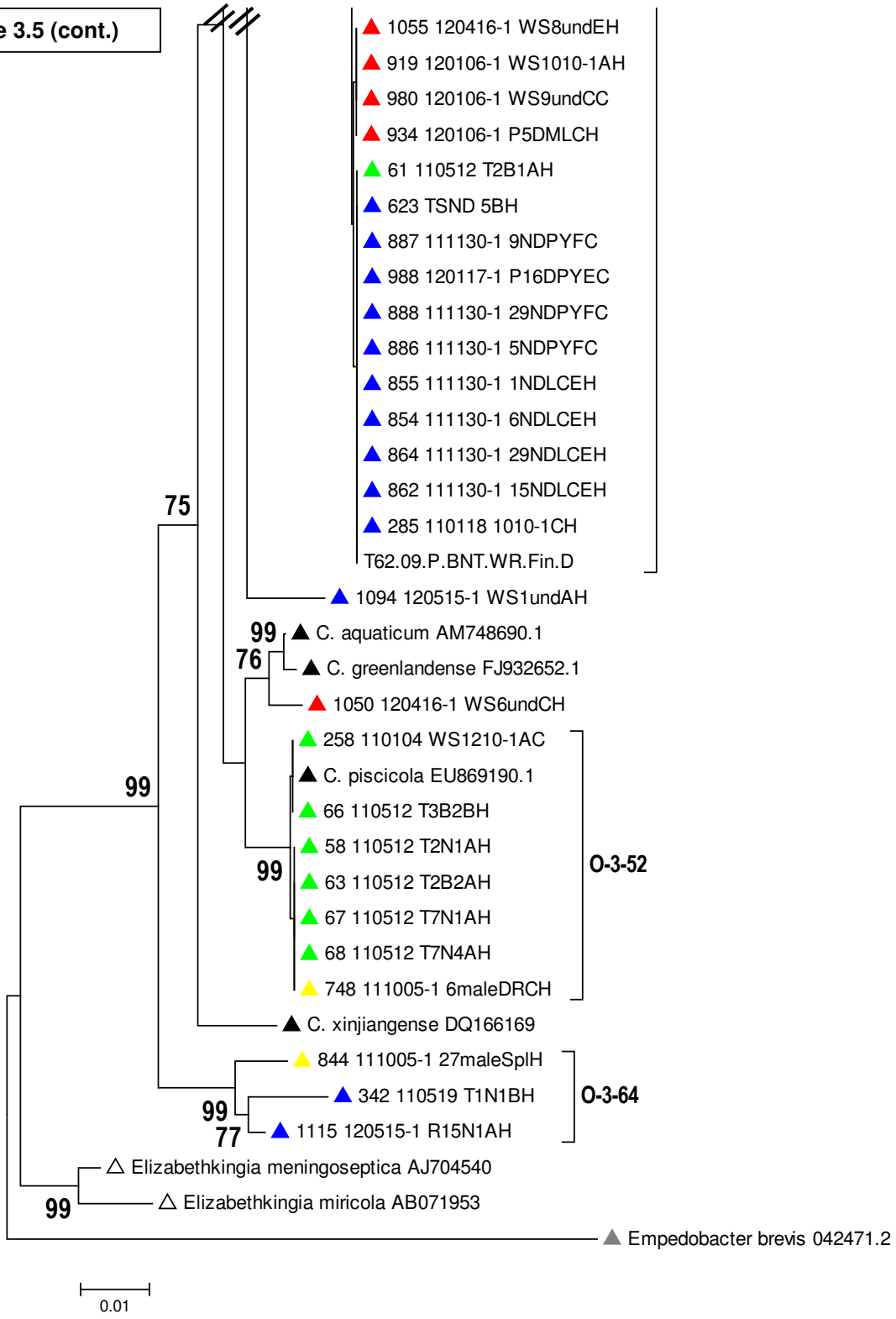


Objective IV: Determination of potential virulence factors that are employed by a proven fish pathogenic Great Lakes flavobacterial strain *in vivo* using the suppression subtractive hybridization assay.

This study was undertaken in order to identify genes that are associated with the *in vivo* virulence of a novel fish-pathogenic *Flavobacterium* sp., *F. spartani* (as determined under Objective I and II). Such information is critical to understand disease pathogenesis and develop a control strategy, including vaccine development.

MATERIALS & METHODS

Flavobacterium spartani T16 was revived in fresh CA broth, incubated for ~18 hrs at 15 ºC, cells harvested by centrifugation at 4300 x g for 15 min, washed once sterile PBS, and resuspended in sterile PBS to a concentration of 10^8 cfu/ml. Dialysis tubing chambers were prepared as described by Lafrentz et al. (2009) by cutting the tubing (12-14 kDa cutoff, 25mm flat width) into 20 cm lengths and soaking them in deionized water overnight at 4 ℃. Two consecutive square knots were tied in one end of each dialysis tube, placed in PBS, and autoclaved (121 ℃, 20 min). Then, 1.2ml of the bacterial suspension was added to each dialysis chamber (100 µl was saved for bacterial enumeration via colony counts/serial 10- fold dilutions) and the open end aseptically tied with two consecutive square knots (used to ligate vessels in surgical procedures). The new knotted area was then dipped in 70% ethanol, rinsed in sterile PBS, and chambers stored in PBS until implantation (~1 hr).

Adult lake trout (*Salvelinus namaycush*; mean length & weight of 55 cm, 1.6 kg) that were raised from eggs in the authors' laboratory were anesthetized, one at a time, in carbonate buffered MS-222 solution (100 mg/l MS-222 & 200 mg/l Na2CO3) for up to 10 min depending on the rate at which surgical anesthesia was reached. Anesthetic depth was assessed via observance of a loss of equilibrium, loss of muscle tone, and loss of reaction to stimuli. Specifically, loss of muscle tone was assessed by firmly grasping the caudal peduncle without any subsequent response by the fish (Harms 2003); reactivity to stimuli was assessed via the tail and eye reflexes; and loss of equilibrium was evidenced by a progression to lateral or dorsal recumbency. Fish were then placed in dorsal recumbency in a foam supportive holding tray and 100 mg L-1 solution of MS-222 flushed over the gills using a recirculating pump (Harms 2003; Figure 4.1A). The ventral surface of the fish was be gently wiped with a wet towel to remove the mucus over the incision site and then disinfected with 2% (w/v) chlorhexidine acetate surgical scrub. A clear plastic sterile drape (3M Steri-Drape) was placed over the fish in order to retain moisture around the fish and prevent moisture leakage that could compromise the surgical field (Figure 4.1B). A small ventral mid-line incision was made cranial to the pelvic fins and one dialysis tubing chamber containing the bacteria was gently fed through the incision. The incision site was immediately closed with a continuous Ford interlocking pattern using PDS II monofilament suture (3-0, 26mm 1/2c reverse cutting needle; Figure 4.1C), rinsed with sterile PBS, and fish immediately returned to the holding tank (11 ºC). A total of 6 lake trout underwent chamber implantation. In addition, 6 dialysis chambers containing bacteria were placed in 1000 ml of fresh sterile CA broth and incubated at 11 ºC; these served as *in vitro* grown bacteria, while those in the fish served as the *in vivo* group.

At day 1 and day 6 post-implantation, 3 fish were euthanized via MS-222 overdose (250 mg/l), bacterial chambers retrieved (Figure 4.1D) and washed in PBS, contents extracted using a sterile needle/syringe, CFUs enumerated via serial 10-fold dilutions, and immediately cryo-preserved in liquid nitrogen and maintained at -80 ºC until RNA extraction (Lafrentz et al.

2009), as was also done for the chambers incubated in media. Bacterial cultures were also taken from the coelom of euthanized lake trout. Chamber contents were also inoculated onto TSA and CA plates to rule out bacterial contamination.

Total RNA was extracted from cryo-preserved flavobacterial samples using the RNeasy Protect Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA extracts were then enriched for bacterial mRNA using a MICROB*Express* ™ kit according to the manufacturer's protocol, and then polyadenylated. Construction of the cDNA library was performed using the BD Clontech™ PCR-SelectTM cDNA Subtraction Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's protocols and as detailed in Xu and Faisal (2008, 2009, 2010). The cDNA from the *in vivo* flavobacteria served as the tester for the first hybridization, while the *in vitro* grown flavobacteria served as the driver. Expressed sequence tags (ESTs) that were present in the tester only were maintained and enriched, ligated to the pGEM®-T Easy Vector, transformed in DH5α competent cells, screened via PCR to ensure EST uptake, and finally sequenced (Xu and Faisal 2008). Resultant sequences were then searched in the GenBank database using BLAST and the putative function of each gene predicted based upon the function of the most similar gene(s).

Major findings and their discussion

Serial 10- fold dilutions and colony counts demonstrated that *F. spartani* grown under *in vivo* conditions proliferated to higher number than did those grown under *in vitro* conditions (Figure 4.2). Bacterial cultures from the dialysis chambers did not yield any bacterial growth other than *F. spartani* on TSA and CA; however, cultures from the coelomic cavity indicated that *F. spartani* managed to cross the dialysis membrane and invaded the fish, another indication of their potential pathogenicity.

A total of 196 clones were sequenced; among these (Figure 4.3), partial sequences most similar to the 23S rRNA gene of *F. johnsoniae* (accession # CP000685), *F. psychrophilum* (AM398681), and *F. branchiophilum* (FQ859183), the 16S rRNA gene of *F. johnsoniae* (AM988911), a transposase gene from *Listonella anguillarum* (AM402994), an open reading frame (ORF) for a hypothetical protein of unknown function from *Photobacterium damselae* subsp. *piscicida* (AJ749803), a gene encoding for a putative cytochrome C oxidase protein from *Photobacterium damselae* subsp. *piscicida* (AJ749800), a trpB gene for a putative transposase from *Photobacterium damselae* subsp. *piscicida* (AJ749797), and a partial atpA gene for the putative arginine ABC transported permease gene (AJ749789).

These findings indicate clearly that this bacterium faces the hostile environment in the host body by additional activation and secretion of factors, some of which are unknown, to enable them to establish the infection.

Figure 4.1. Surgical implantation of dialysis chambers containing *F. spartani* into adult lake trout (*Salvelinus namaycush*) for suppression subtractive hybridization studies. A, Dorsally recumbent and anesthetized lake trout (1.6 kg) in a foam supportive holding tray; 100 mg L^{-1} solution of MS-222 is being flushed over the gills using a recirculating pump; B, incision site of lake trout immediately after being sutured; C, incision site 6 days post-implantation; D, dialysis tube chamber containing *F. spartani* after being retrieved from the ceolomic cavity of lake trout and rinsed in sterile PBS.

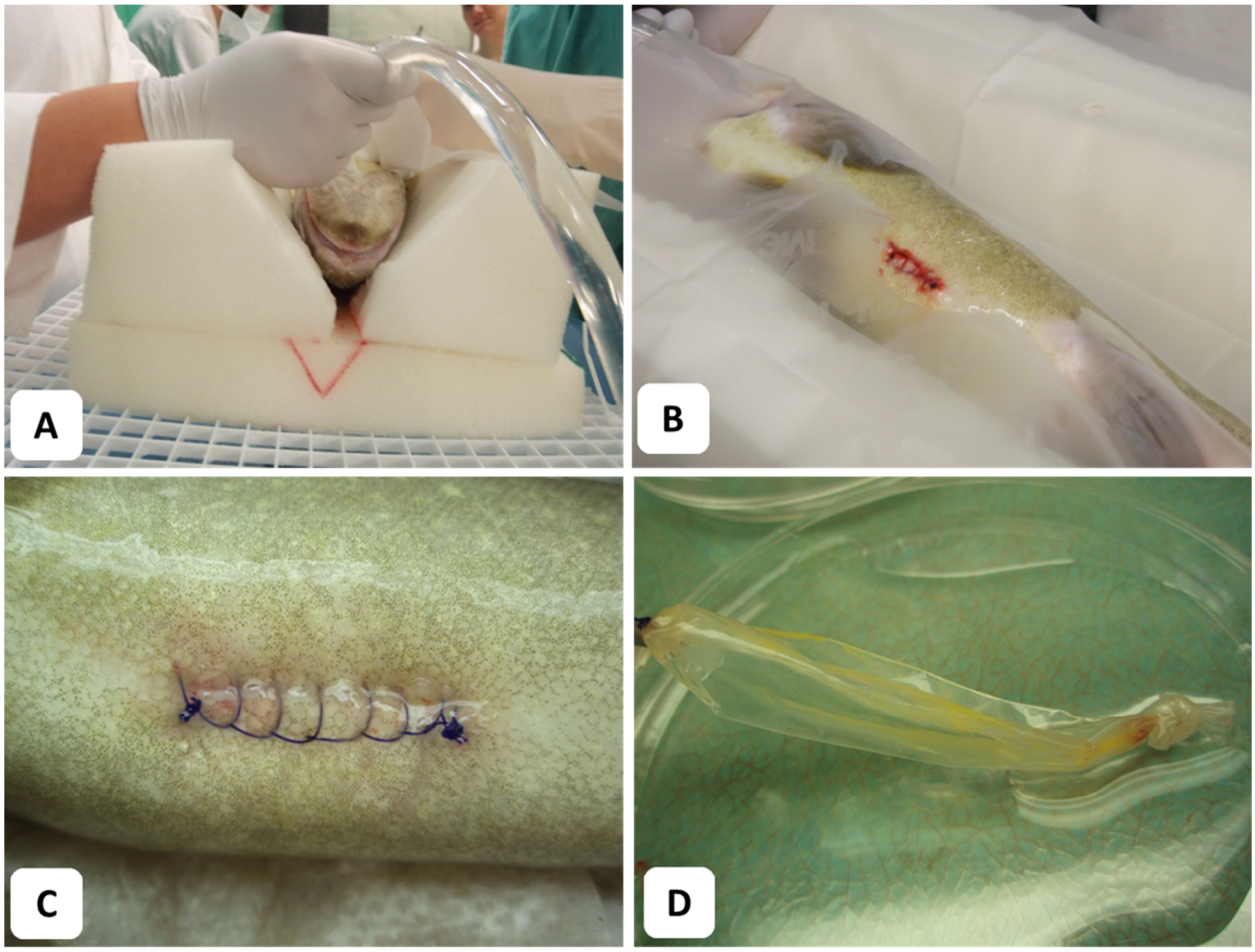


Figure 4.2. Colony counts for *in vitro* and *in vivo* dialysis chambers containing *F. spartani*.

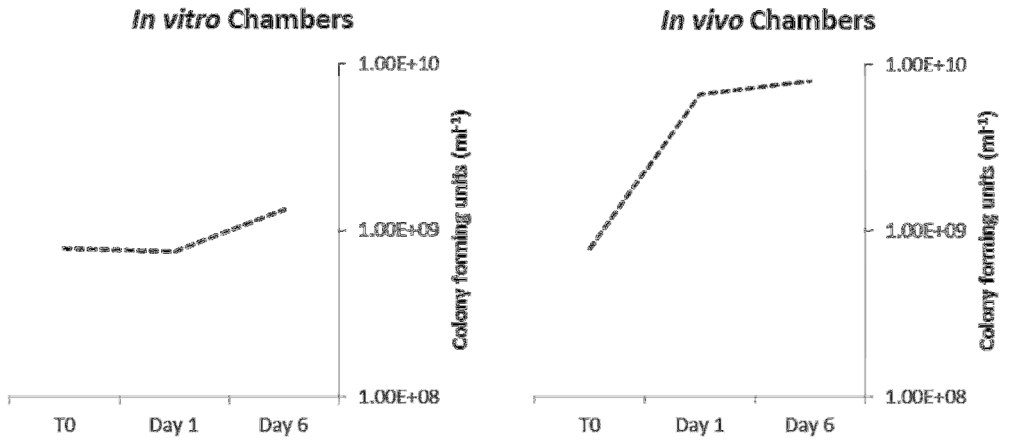
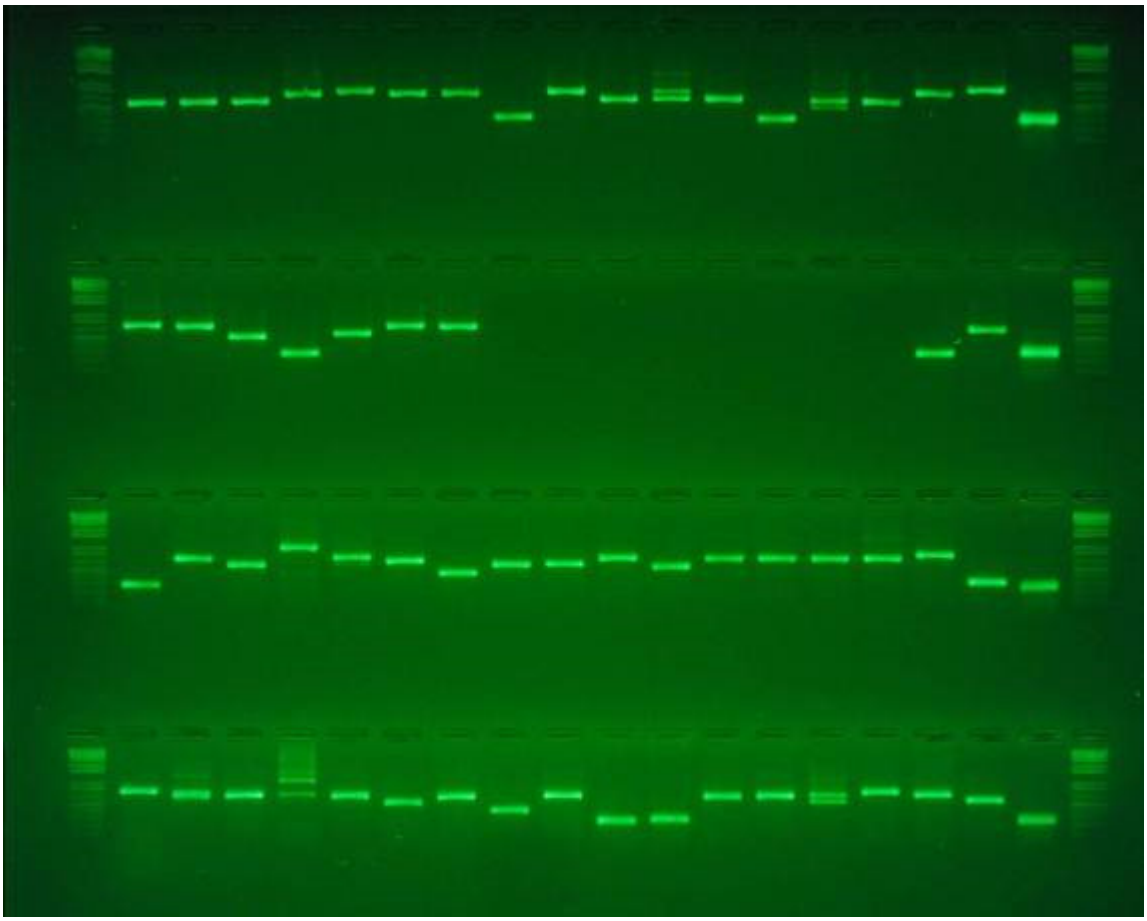


Figure 4.3. Gel electrophoresis results verifying uptake of a portion of the expressed sequence tags (ESTs) by DH5α competent cells as generated through the suppression subtractive hybridization assay and clone reactions.



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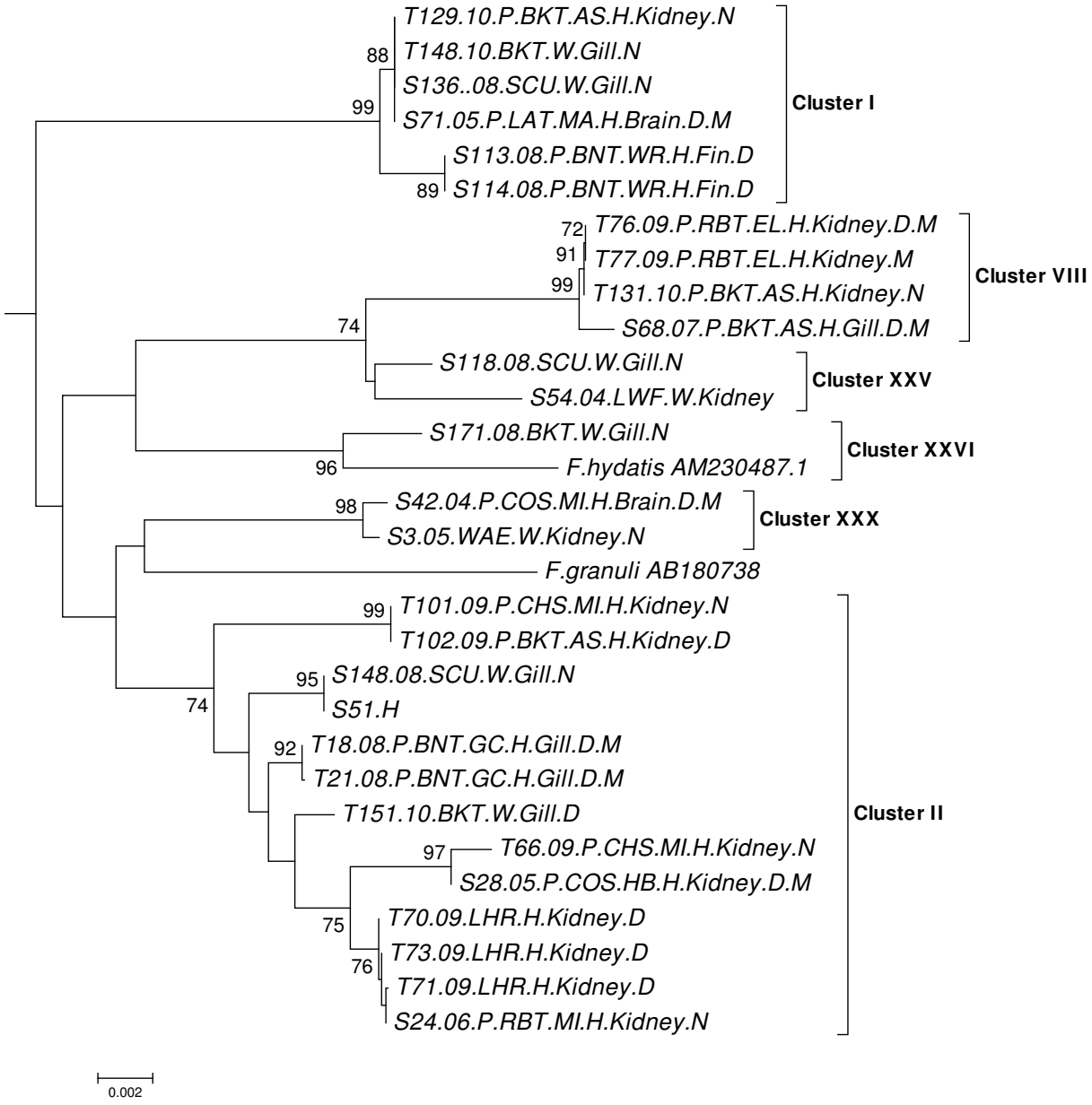
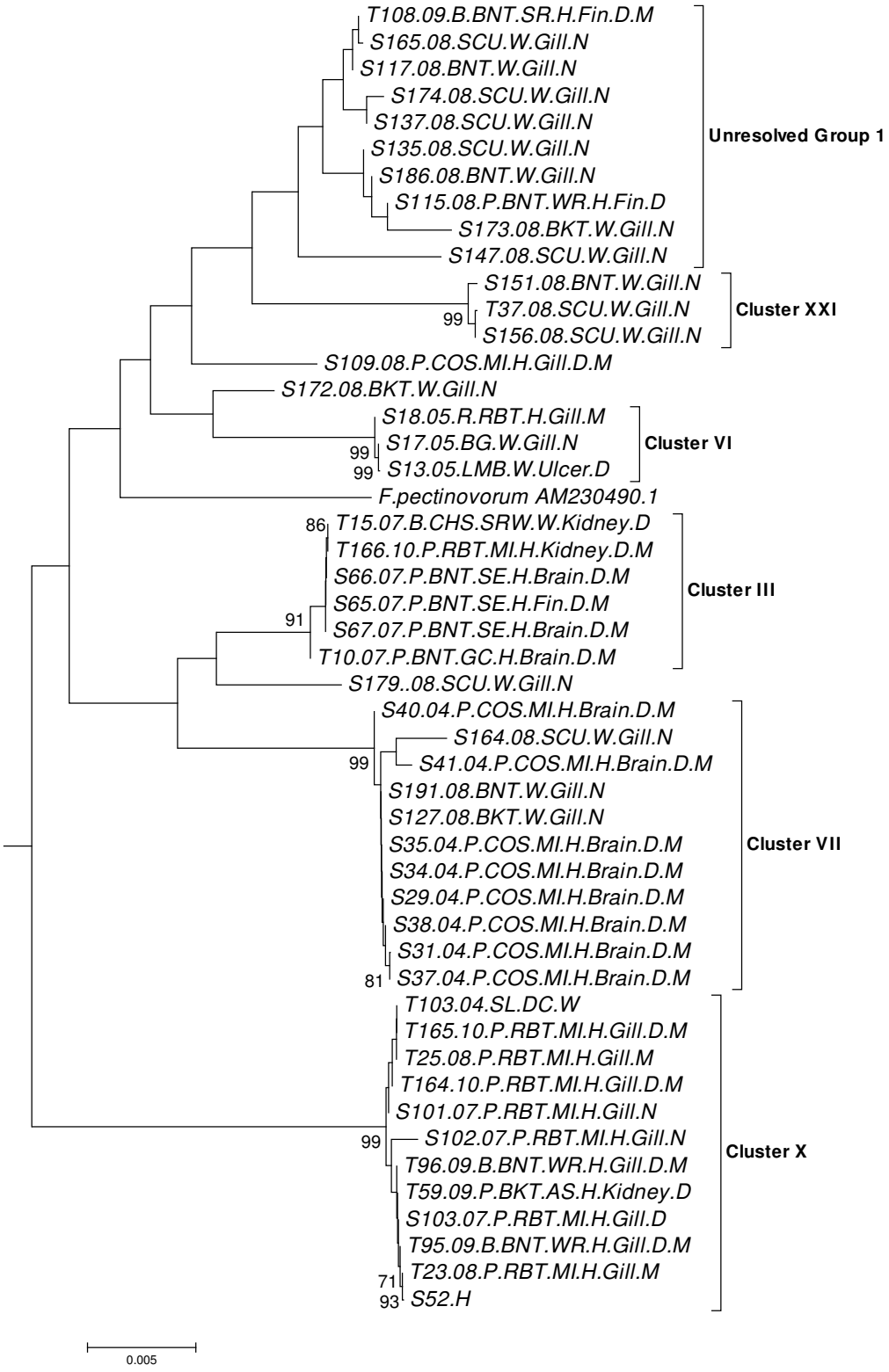
APPENDIX A

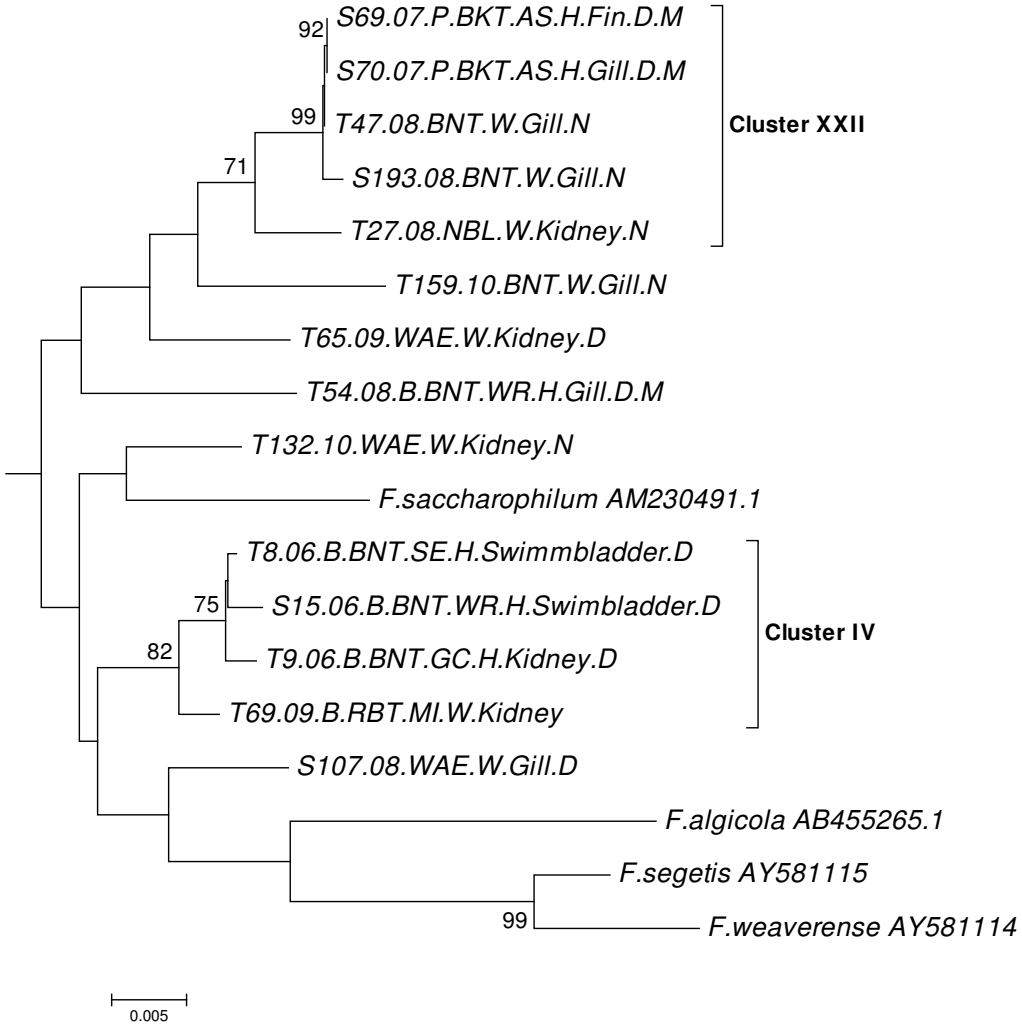
Supplementary Figure 1.2.1. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 6 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



Supplementary Figure 1.2.2. 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.

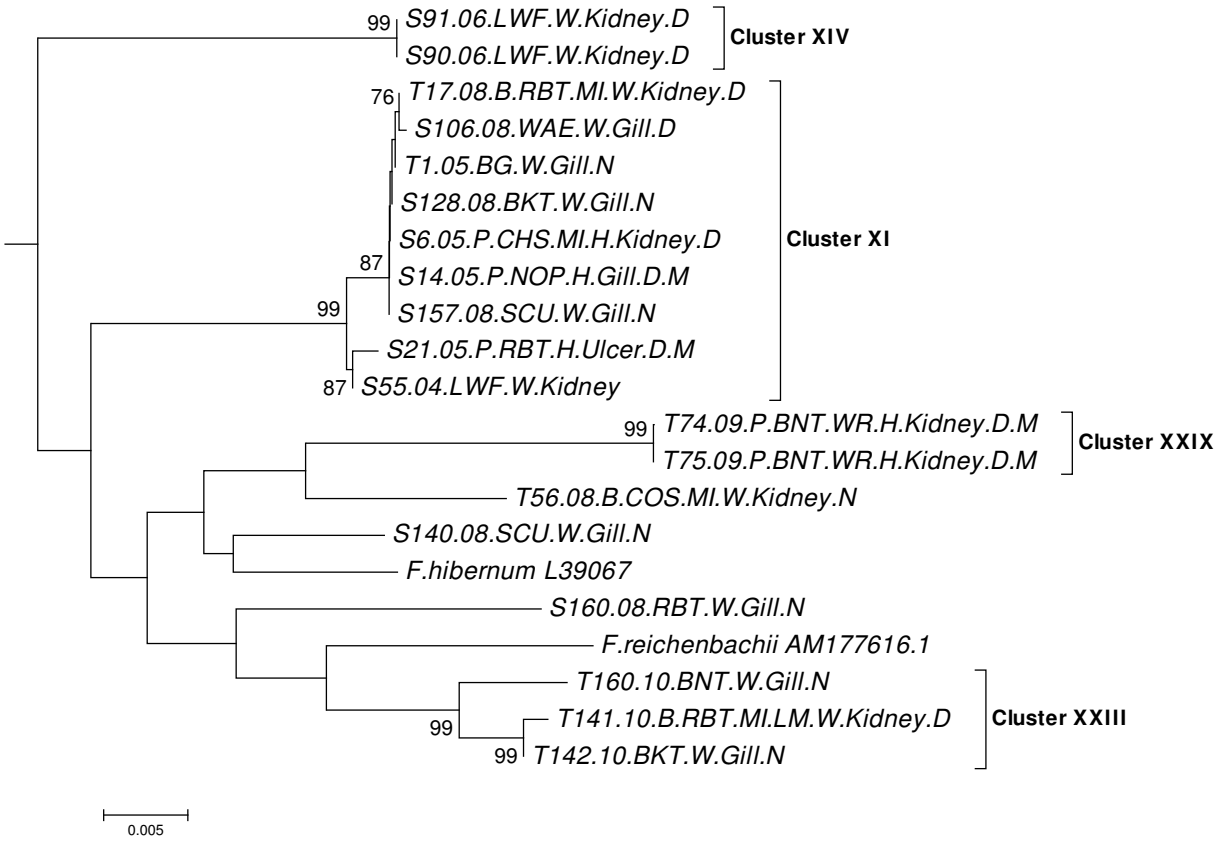


Supplementary Figure 1.2.3. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 2 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

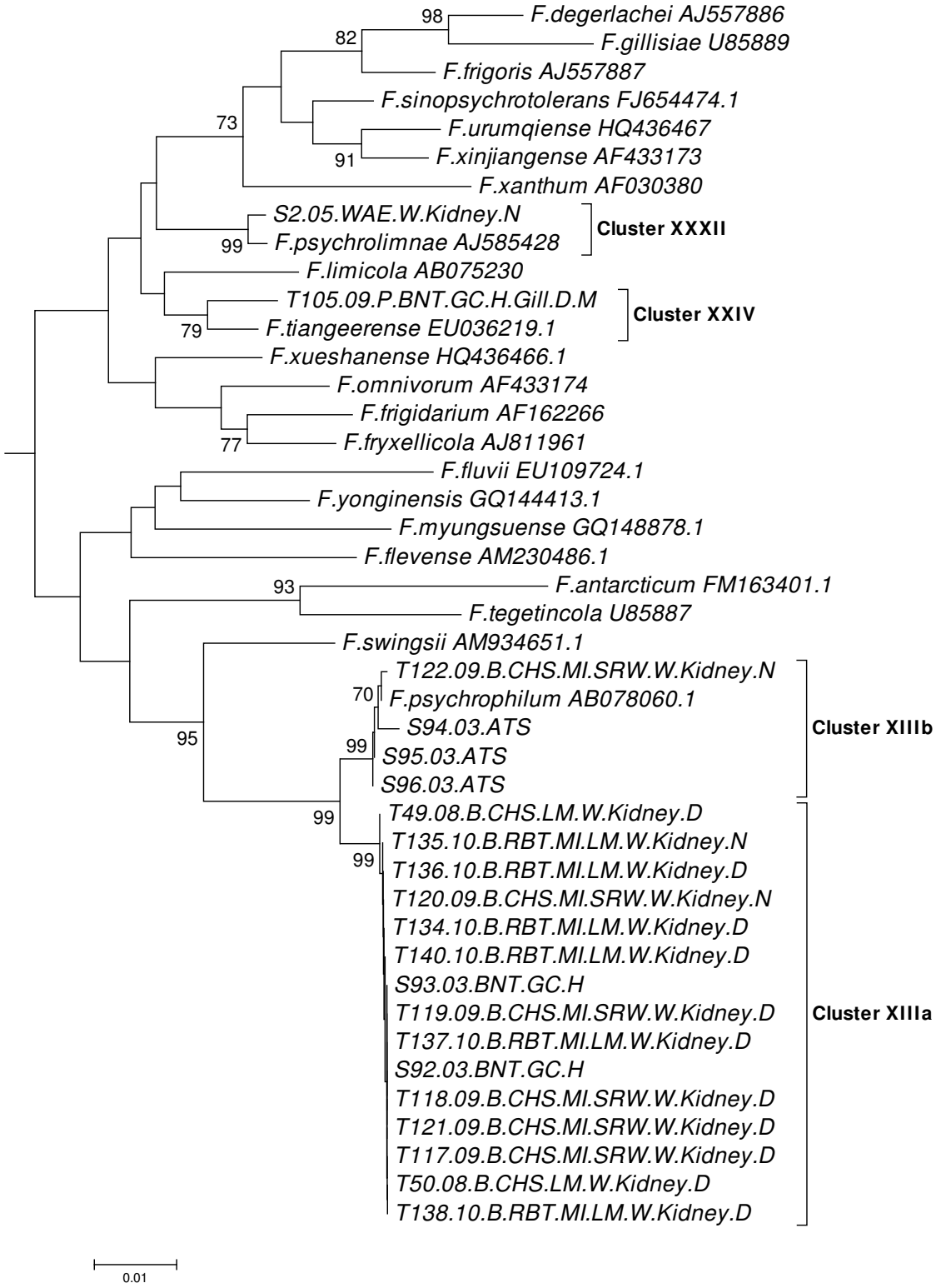


Supplementary Figure 1.2.4. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 6 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

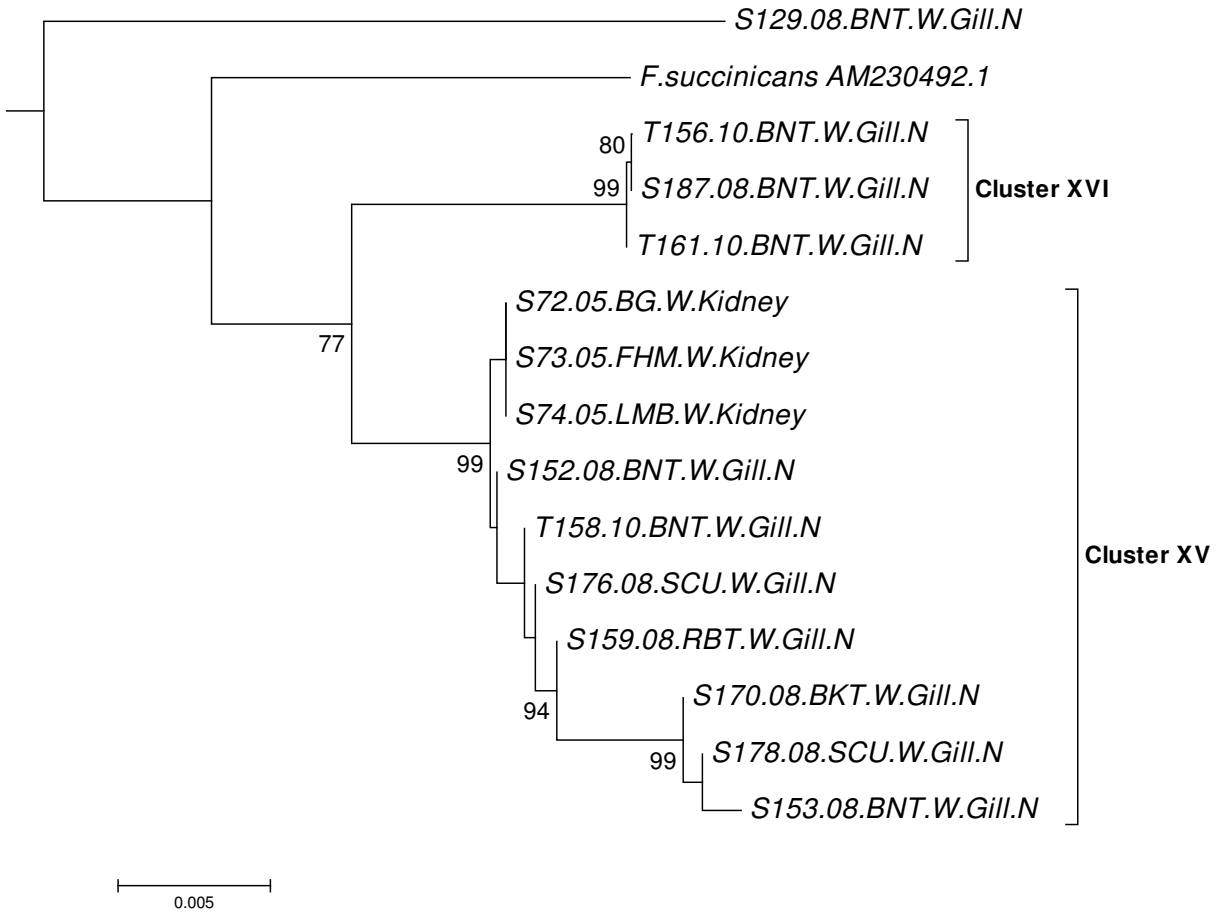
Supplementary Figure 1.2.5. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between 4 clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



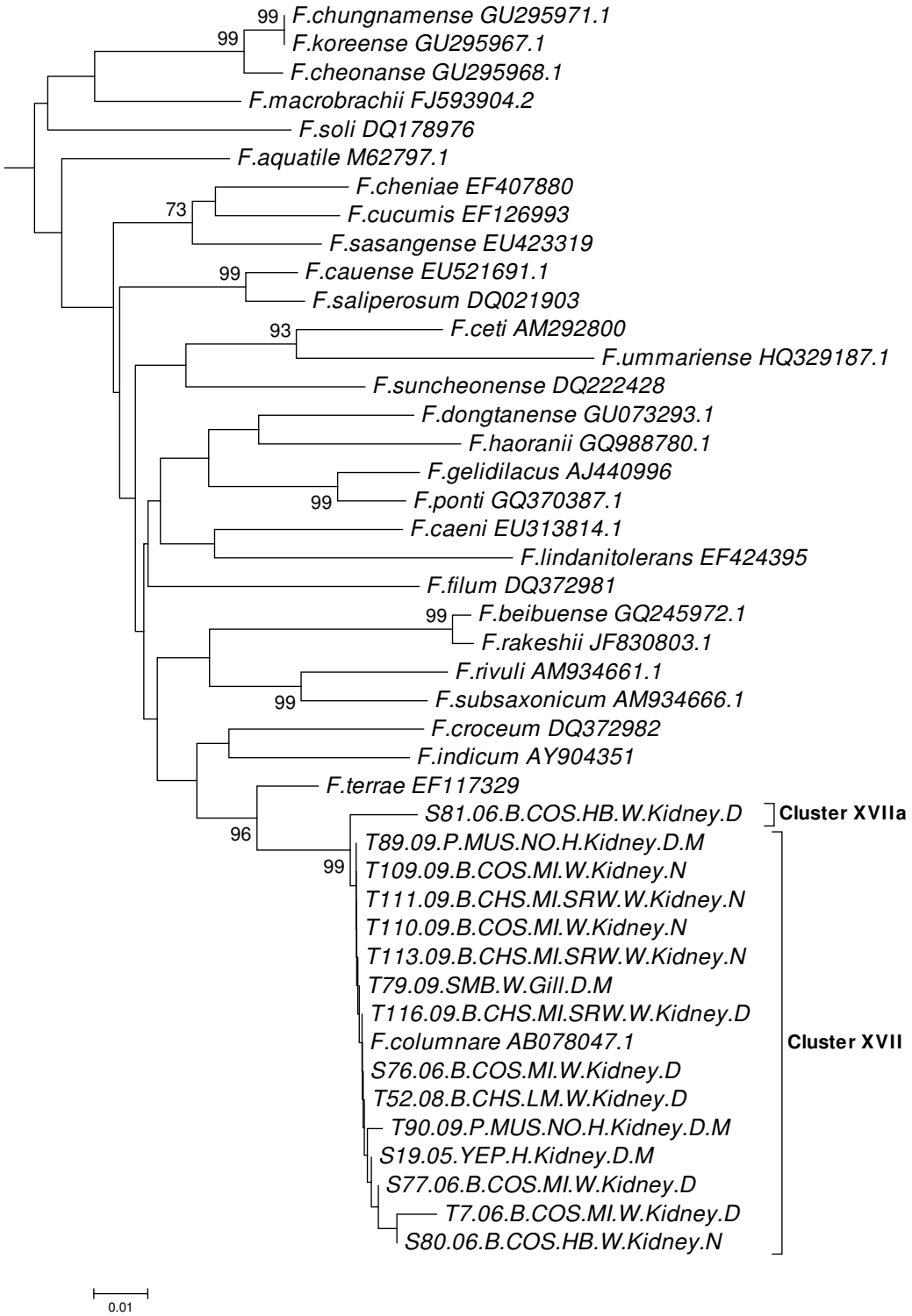
Supplementary Figure 1.2.6. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between four clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



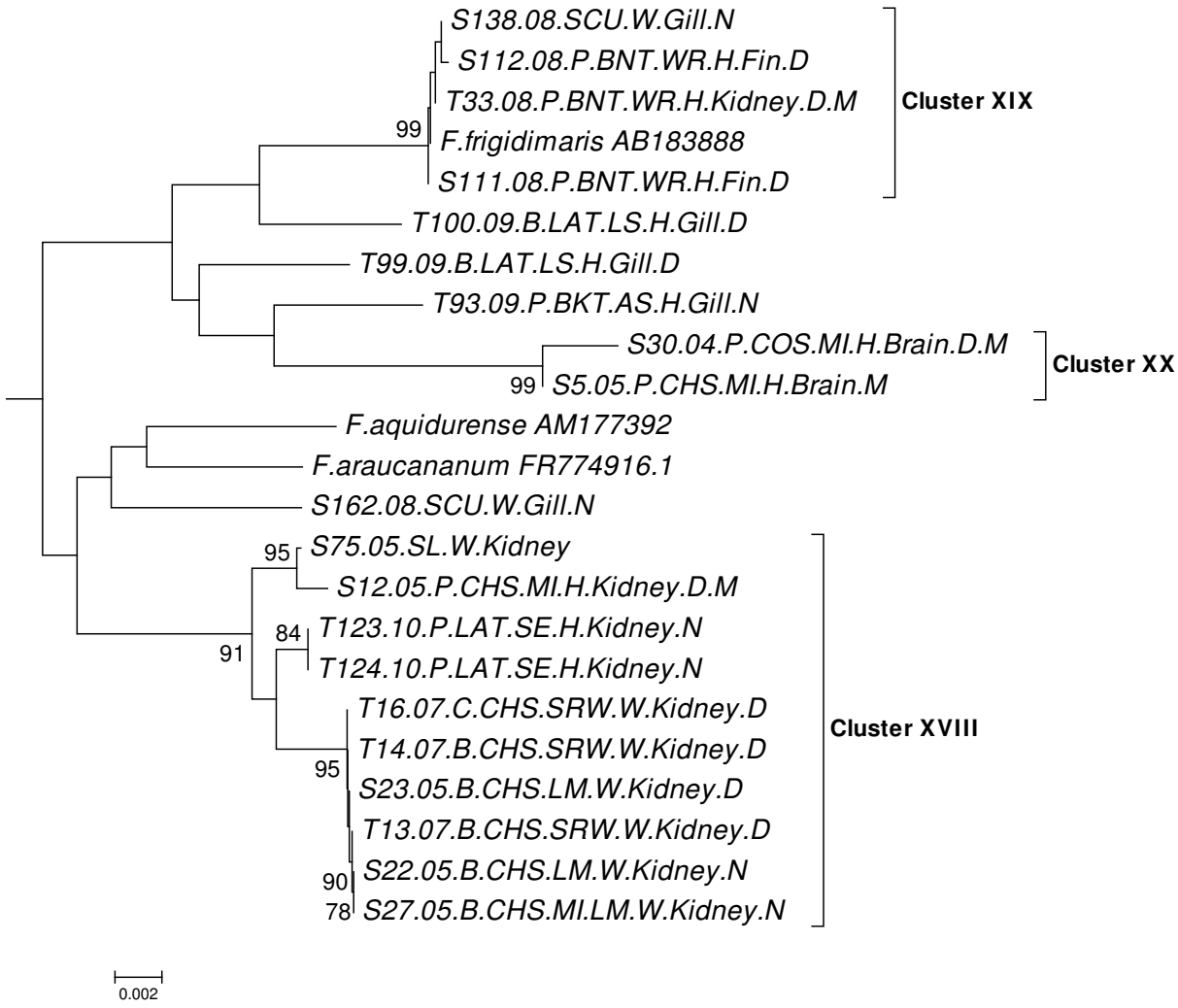
Supplementary Figure 1.2.7. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between two clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



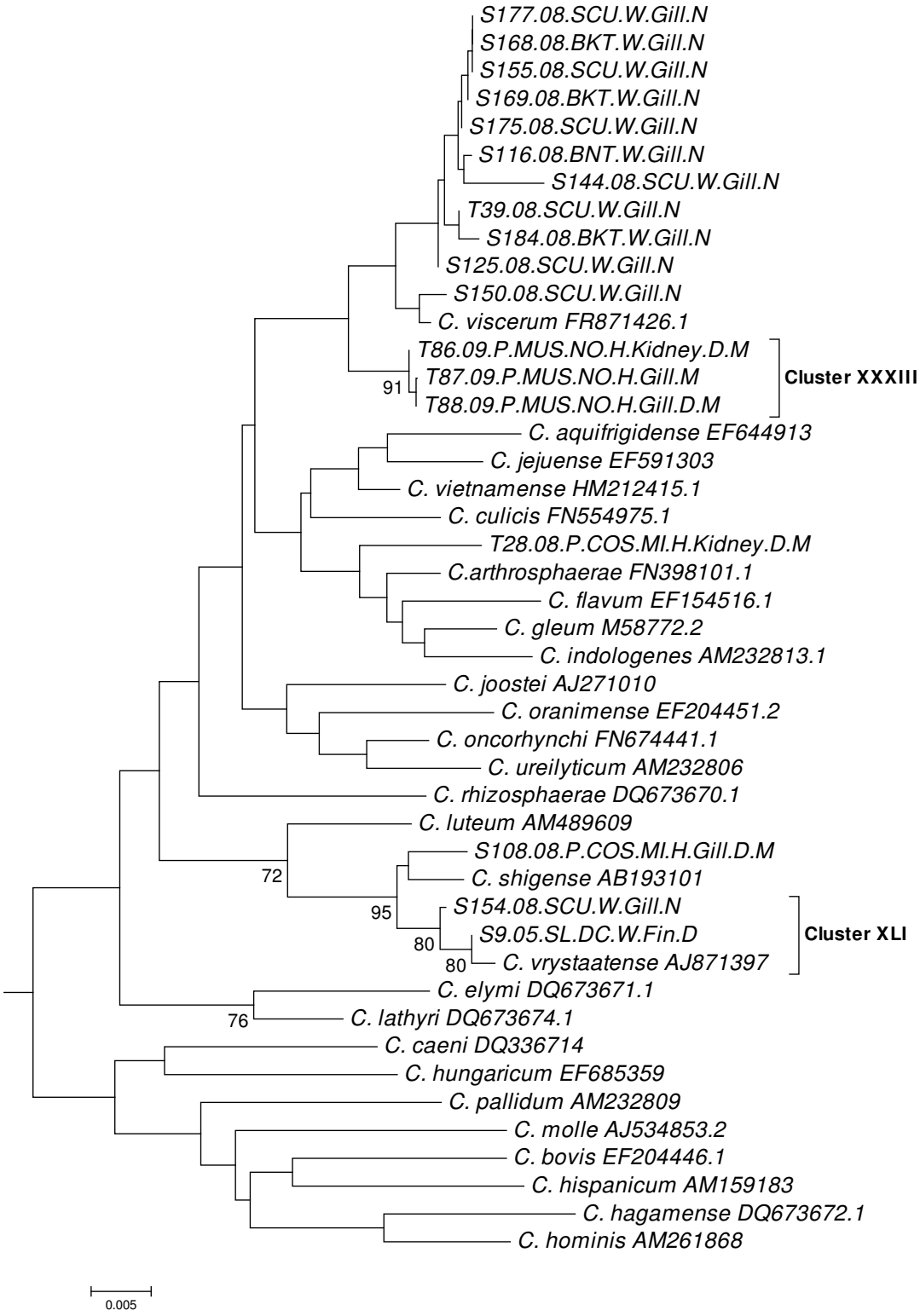
Supplementary Figure 1.2.8. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between Michigan fish-associated *F. columnare*. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



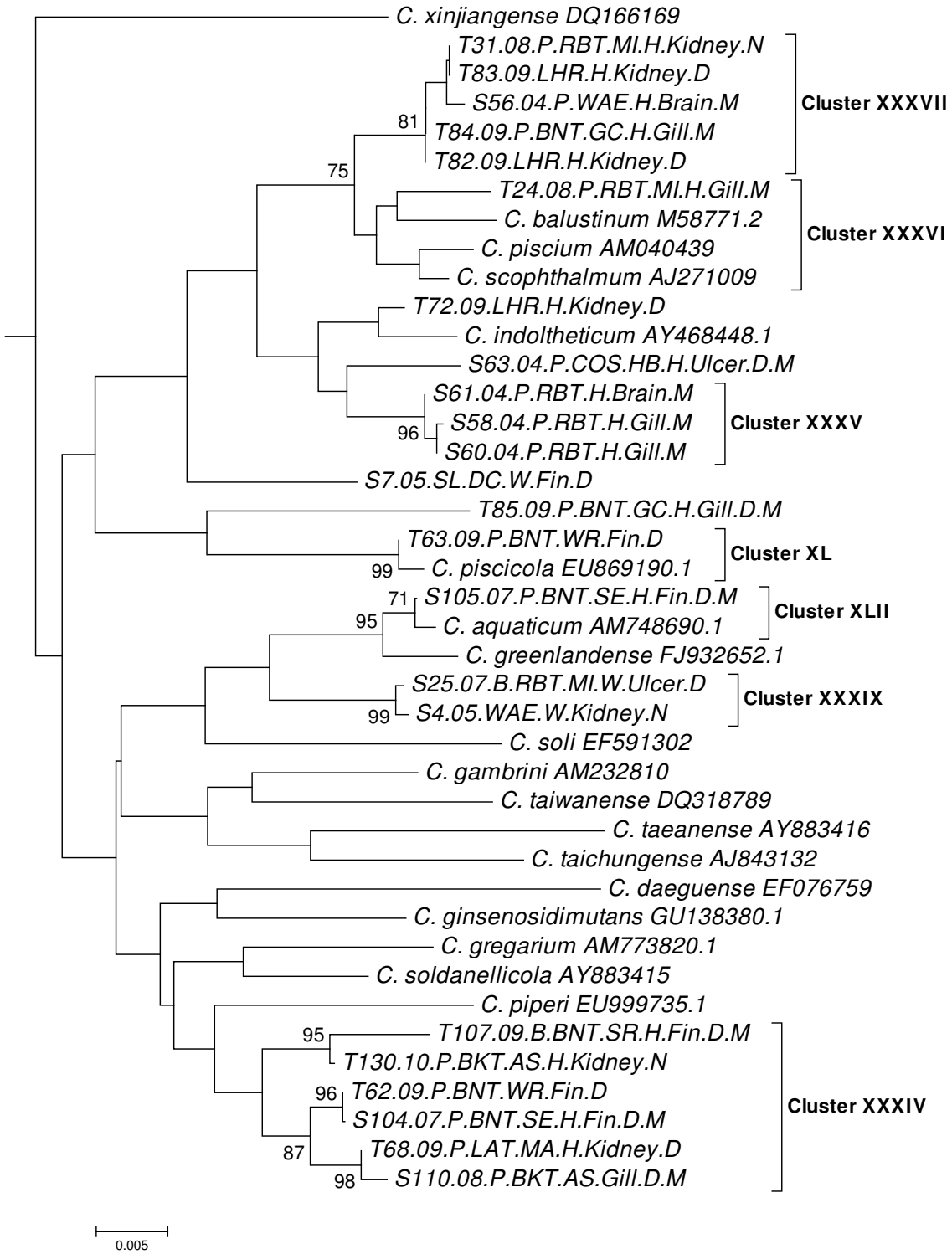
Supplementary Figure 1.2.9. Subtree of the dendrogram presented in Figure 1 depicting the phylogenetic relationship between three clusters of Michigan fish-associated *Flavobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



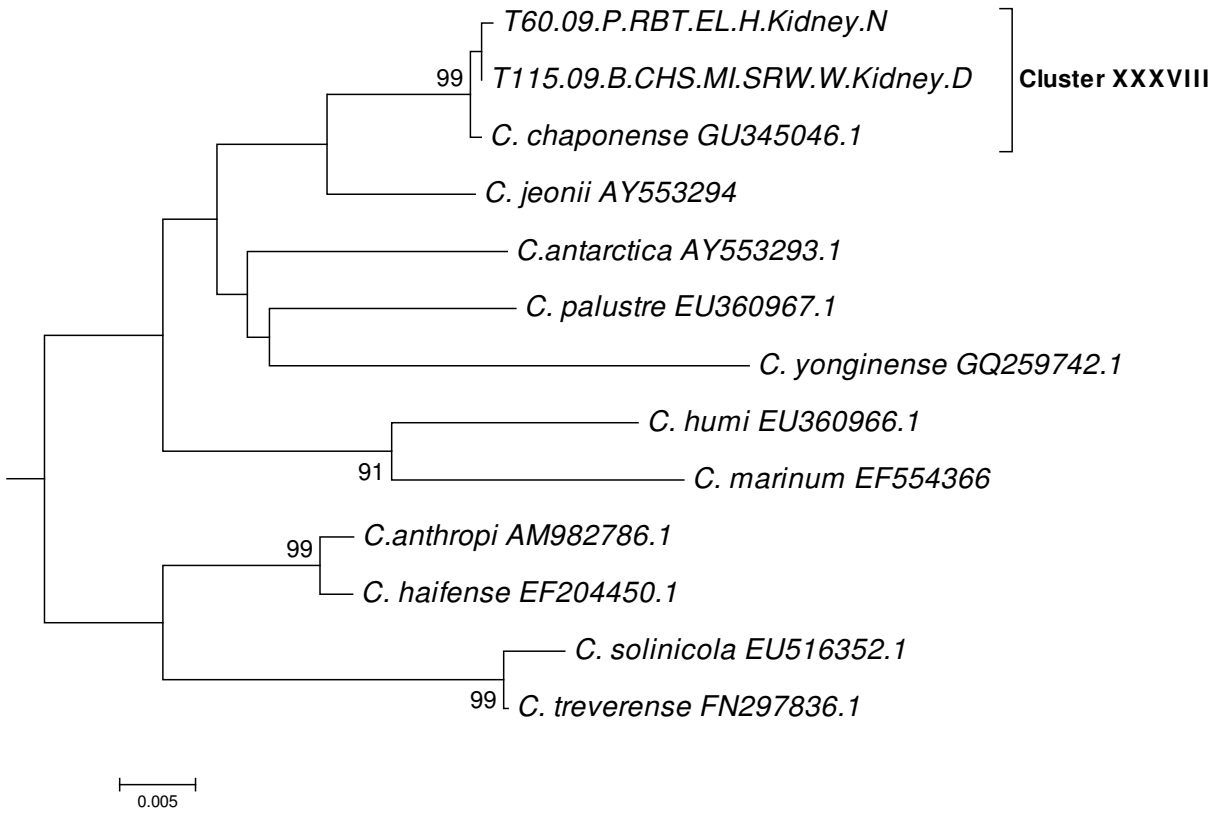
Supplementary Figure 1.2.10. Subtree of the dendrogram presented in Figure 2 depicting the phylogenetic relationship between two clusters of Michigan fish-associated *Chryseobacterium* spp. Bootstrap values >70% (expressed as percentages of 10,000 replicates) are presented at the branch nodes. The tree 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 1.2.11. 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 1.2.12. 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 Chinook salmon fingerlings	S	03/2009	Kidney	TSFH	JX287587
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 in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
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 brown trout fingerlings	M	05/2007	Brain	TSFH	JX287542
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-	S	11/2006	Kidney	OSFH	JX287541

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
			reared brown trout					
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 fingerlings	S	08/2005	Gills	OSFH	JX287707
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 coho salmon fry	M	02/2004	Brain	PRSFH	JX287712
Cluster VII	**S34**	*F. pectinovorum (97.7%)*	Hatchery-reared	M	02/2004	Brain	PRSFH	JX287687

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	**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
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 in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
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 fingerlings	S	07/2007	Gills	WLSFH	JX287637
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 in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
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 whitefish	S	Unknown	Kidney	Naubinway, Lake Michigan	JX287719
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 Chinook salmon	S	10/2009	Kidney	SRW	JX287601

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 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
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 in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
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 bass	S	05/2005	Kidney	Big Lake	JX287729
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 in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
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 Chinook salmon	S	10/2009	Kidney	SRW	JX287595
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

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
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 in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
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 yearlings	S	07/2009	Kidney	St. Mary's SFH, Ohio	JX287580
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	Tittabawasse e River	JX287708
Cluster XXXI	**S87**	*F. resistens (97.1%)*	Wild largemouth bass	S	08/2005	Kidney	Big Bass Lake	JX287735
Cluster	**S88**	*F. resistens (97.3%)*	Wild largemouth	S	08/2005	Kidney	Big Bass Lake	JX287736

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
XXXI			bass					
Cluster XXXII	**S2**	*F. psychrolimnae (99.6%)*	Wild spawning walleye	S	04/2005	Kidney	Tittabawasse e 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 fingerlings	M	07/2009	Gills	WLSFH	JX287758
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

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 brown trout fingerlings	M	07/2007	Fins	HSFH	JX287772
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.	**S147**	*F. pectinovorum (97.5%)*	Wild mottled	S	09/2008	Gills	Brundage	JX287671

Cluster in Tree	Bacterial Strain	Closest Relative (% 16S rDNA similarity)	Source of Isolate	Purpose of Sample	Month/ Year of Recovery	Tissue	Location	Accession Number
Group 1			sculpin				Creek	
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
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

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.	**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
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

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

APPENDIX B

Supplementary Table 3.4.1. Differential charateristics of *Flavobacterium* sp. T91 and related *Flavobacterium* spp. T91, results from this study; *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 ℃	+	-	-
Growth at 37 ℃	-	+	+
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 3.4.2. 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 3.4.3. 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^T, 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 ℃	+	-	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 3.4.4. 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^T, 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℃	+	-	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 3.4.5. 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 3.4.6. 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℃	+	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

APPENDIX D

Chryseobacterium aahli sp. nov., isolated from lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*) in Michigan

Running Title: *Chryseobacterium aahli* sp. nov.

Contents Category: New Taxa, Bacteroidetes

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Abstract

Two strains (T68^T and T62) of a Gram-negative, non-spore-forming, rod-shaped, yellow-pigmented bacterium containing a flexirubin-type pigment were recovered from the kidneys and necrotic fins of aqua-cultured lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*) during disease surveillance for the State of Michigan, USA, in 2009. In order to investigate the taxonomic status of these two strains, a polyphasic approach was undertaken. Both isolates possessed catalase and cytochrome oxidase activities, hydrolyzed esculin, and were proteolytic to multiple substrates (i.e.,

185 gelatin, casein, elastin, Tweens 20 & 80), but did not degrade agar. 16S rRNA gene
186 sequencing demonstrated that T68^T and T62 were nearly identical to one another
187 (≥99% 16S rRNA gene sequence similarity) and placed the organism within the genus
188 *Chryseobacterium*, where *C. ginsenosidimutans* (97.8%), *C. gregarium* (97.7%), *C.*
189 *soldanellicola* (97.6%), and *C. gambrini* (97.5%) were its closest relatives.
190 Subsequent phylogenetic analyses using neighbor-joining, maximum parsimony, and
191 Bayesian methodologies demonstrated that *Chryseobacterium* sp. strains T68^T and
192 T62 formed a well-supported clade (bootstrap values of 100 and 97; posterior
193 probability of 0.99, respectively) that was indeed distinct from other members of the
194 genus *Chryseobacterium*. The major fatty acid constituents according to fatty acid
195 methyl ester (FAME) analysis were *iso*-$C_{15:0}$, (30.9%), summed feature 3 ($C_{16:1}$ ω6*c*
196 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%).
197 Based upon the results of this study, strains T68^T and T62 represent a novel
198 *Chryseobacterium* sp., for which the name *Chryseobacterium aahli* is proposed. The
199 type strain is T68^T (GenBank accession number- JX287893).
200
201 Members of the family Flavobacteriaceae (Reichenbach, 1992; Bernardet *et al.*, 1996),
202 occupy an extremely wide range of ecological niches (reviewed in Jooste & Hugo 1999
203 and Bernardet & Nakagawa 2006) and can be associated with disease in an array of
204 organisms, including invertebrates (Li *et al.,* 2010), amphibians (Xie *et al.,* 2009),
205 reptiles (Hernandez-Divers *et al.,* 2009), birds (Segers *et al.,* 1993), and mammals
206 (Haburjak & Schubert 1997), including humans (Benedetti *et al.,* 2011). In fish, serious
207 diseases are caused by multiple species within the family Flavobacteriaceae, such as

208 *Flavobacterium* spp. (Shotts & Starliper 1999; Starliper 2011), *Tenacibaculum* spp.
209 (Suzuki *et al.,* 2001), and *Chryseobacterium* spp. (Muddarris & Austin 1989), the latter
210 of which have become an emerging problem on multiple continents (Bernardet *et al*.,
211 2005). Since its original description in 1994 by Vandamme and colleagues, the genus
212 *Chryseobacterium* has rapidly expanded from its original 6 species to over 60 species at
213 the time this manuscript was written. Concurrent with this rapid expansion are
214 descriptions of numerous novel fish-associated *Chryseobacterium* spp., such as *C.*
215 *piscium* (de Beer *et al.,* 2006), *C. piscicola* (Ilardi *et al.,* 2009), *C. arothri* (later
216 heterotypic synonym of *C. hominis*; Kämpfer *et al*., 2009) *C. chaponense* (Kämpfer *et*
217 *al*., 2011), and *C. viscerum* (Zamora *et al.,* 2012). Signs of disease in fish from which
218 *Chryseobacterium* spp. have been recovered include skin and muscle ulcerations
219 (Bernardet *et al*., 2005; Ilardi *et al.,* 2010; Kämpfer *et al*., 2011), gill hemorrhage and
220 hyperplasia (Muddarris & Austin 1989; Muddarris *et al.,* 1994), general signs of
221 septicemia (Muddarris & Austin 1989; Muddarris *et al.,* 1994; Bernardet *et al*., 2005;
222 Zamora *et al.,* 2012), or no overt signs of disease (de Beer *et al.,* 2006; Cambell *et al.,*
223 2008). Herein, we describe a novel fish-associated *Chryseobacterium* sp. recovered
224 from diseased salmonids in Michigan that is genotypically and phenotypically distinct
225 from all other described *Chryseobacterium* spp.
226
227 *Chryseobacterium* sp. strain T62 was recovered from the necrotic fins (Figure 1) of
228 hatchery-reared yearling brown trout (*Salmo trutta;* Harrietta State Fish Hatchery,
229 Wexford County, Michigan, USA) and *Chryseobacterium* sp. strain T68 from the kidneys
230 of 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 ℃ 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 in color , ranged in size 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 ℃ 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 were not motile via gliding according to the hanging-drop technique (as described in Bernardet & Nakagawa 2006) as viewed under a light microscope. Isolates were then cryopreserved at -80 ℃ in Hsu-Shotts broth supplemented with 20% glycerol.

In order to definitively determine the taxonomic position of the two bacterial strains, polyphasic characterization was performed. Bacterial colonies from pure 48 hr old 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 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. DNA amplification was carried out in a Mastercycler® Pro Thermalcycler (Eppendorf) with an initial denaturation step at 94℃ for 2 min, followed by 35 cycles of amplification, which included denaturation at 94℃ for 45 sec, annealing at 52℃ for 45 sec, and elongation at 72℃ for 90 sec. A final extension step was performed at 72℃ 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 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*

277 (outgroups) were downloaded from NCBI and the EzTaxon-e database (Kim *et al.,*
278 2012) and subsequently aligned with the sequences of strains T68 and T62. Neighbor-
279 joining (NJ) analysis was then performed (Saitou & Nei, 1987) using the Molecular
280 Evolutionary Genetics Analysis software (MEGA; Ver. 4.0), with evolutionary distances
281 being calculated using the Maximum Composite Likelihood method (Tamura *et al.*,
282 2004). Topology robustness was evaluated by bootstrap analysis based upon 1000
283 resamplings of the sequences. In order to confirm the phylogenetic validity of the initial
284 NJ analysis, *Chryseobacterium* sp. T68/T62 and the 13 most closely related
285 *Chryseobacterium* spp., along with 8 *Chryseobacterium* spp. recovered from fish/fish
286 products, the type species (*C. gleum*), Candidatus "*C. massiliae*", and members of the
287 genus *Elizabethkingia* and *Empedobacter* (outgroup) were aligned as described above
288 and further analyzed using Bayesian and Maximum Parsimony (MP) analyses.
289 Bayesian analysis was conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003)
290 using the General Time Reversible (GTR) model and gamma-shaped rate variation with
291 a proportion of invariable sites. Default settings were used for the
292 transition/transversion rate ratio (beta), topology (uniform), and prior probability
293 distribution on branch lengths (unconstrained). The Markov chain was run for up to ten
294 million generations, with a stopping rule in place once the analysis reached an average
295 standard deviation of split frequencies of <0.01%. Two independent analyses were
296 conducted, both with 1 cold and 3 heated chains using the default heating parameter
297 (temp=0.2). The initial 25% of Markov Chain Monte Carlo (MCMC) samples were
298 discarded as burnin and sampling occurred every 100 generations. Maximum
299 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 from 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, Figure 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 & 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℃, 15℃, 22℃, 37℃, and 42℃; 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 & 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℃ 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 trypticase soy broth and 15g of Bacto agar (Difco) per liter of distilled water for 24 h at 28 ℃. 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

368 *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-
369 OH (16.1%), $C_{16:0}$ (7.0%), $C_{16:0}$ 3-OH (5.1%), and smaller amounts of other fatty acids
370 (Table 2). The predominant fatty acids typical of the genus *Chryseobacterium* are *iso*-
371 $C_{15:0}$, *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
372 ω7*t*; Segers *et al*., 1993; Bernardet *et al.,* 2006). *Chryseobacterium* sp. T68 was quite
373 distinct from the other most closely related *Chryseobacterium* spp. in the high
374 percentage of $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c* (26.6%) compared to summed features
375 containing the same fatty acid for *C. ginsenosidimutans* (9.5%; Im *et al*., 2011), *C.*
376 *gregarium* (0%; Behrendt *et al.,* 2008), *C. soldanellicola* (9.7%; Park *et al.,* 2006), *C.*
377 *gambrini* (0%; Herzog *et al*., 2008), *C. defluvi* (9.4%; Kämpfer *et al*., 2003), and *C. piperi*
378 (12.6%; Strahan *et al.,* 2011). Interestingly, *Chryseobacterium* sp. T68 contained such
379 a high percentage of $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c* that it was even higher than what is
380 typical for members of the genus *Elizabethkingia* (17-19.6%; Kim *et al*., 2005).
381 *Chryseobacterium* sp. T68 was also unique in that it contained a much smaller
382 percentage of the hydroxy fatty acid *iso*-$C_{17:1}$ ω9*c* (1.4%) when compared to its closest
383 *Chryseobacterium* relatives, including *C. ginsenosidimutans* (9.3%; Im *et al*., 2011), *C.*
384 *gregarium* (16.9%%; Behrendt *et al.,* 2008), *C. soldanellicola* (14.6%; Park *et al.,* 2006),
385 *C. gambrini* (6.7%; Herzog *et al*., 2008), *C. defluvi* (4.8%; Kämpfer *et al*., 2003), and *C.*
386 *piperi* (22.0%; Strahan *et al.,* 2011). Other distinguishing characteristics of
387 *Chryseobacterium* sp. T68 included the relatively large percentage of $C_{16:0}$ (7.0%) and
388 $C_{16:0}$ 3OH (5.1%; Table 2). The biochemical characteristics of *Chryseobacterium* strains
389 T68/T62 are described in the species description, while those characters that
390 distinguish it from related *Chryseobacterium* spp. are listed in Table 3.

The results of the polyphasic characterization conducted in this study demonstrate 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 is likely a facultative fish-pathogen in multiple Great Lakes salmonid species (Loch and Faisal in preparation).

Description of *Chryseobacterium aahlii* sp. nov.

Chryseobacterium aahli (aah'li. N.L. gen. n. *aahlii* 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 ℃. 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 ℃, 15℃, and 22℃, but not at 37 or 42℃. 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 in acid production from sucrose. One of the two strains each 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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Figure 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 2. Dendrogram generated using the Neigbor-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.

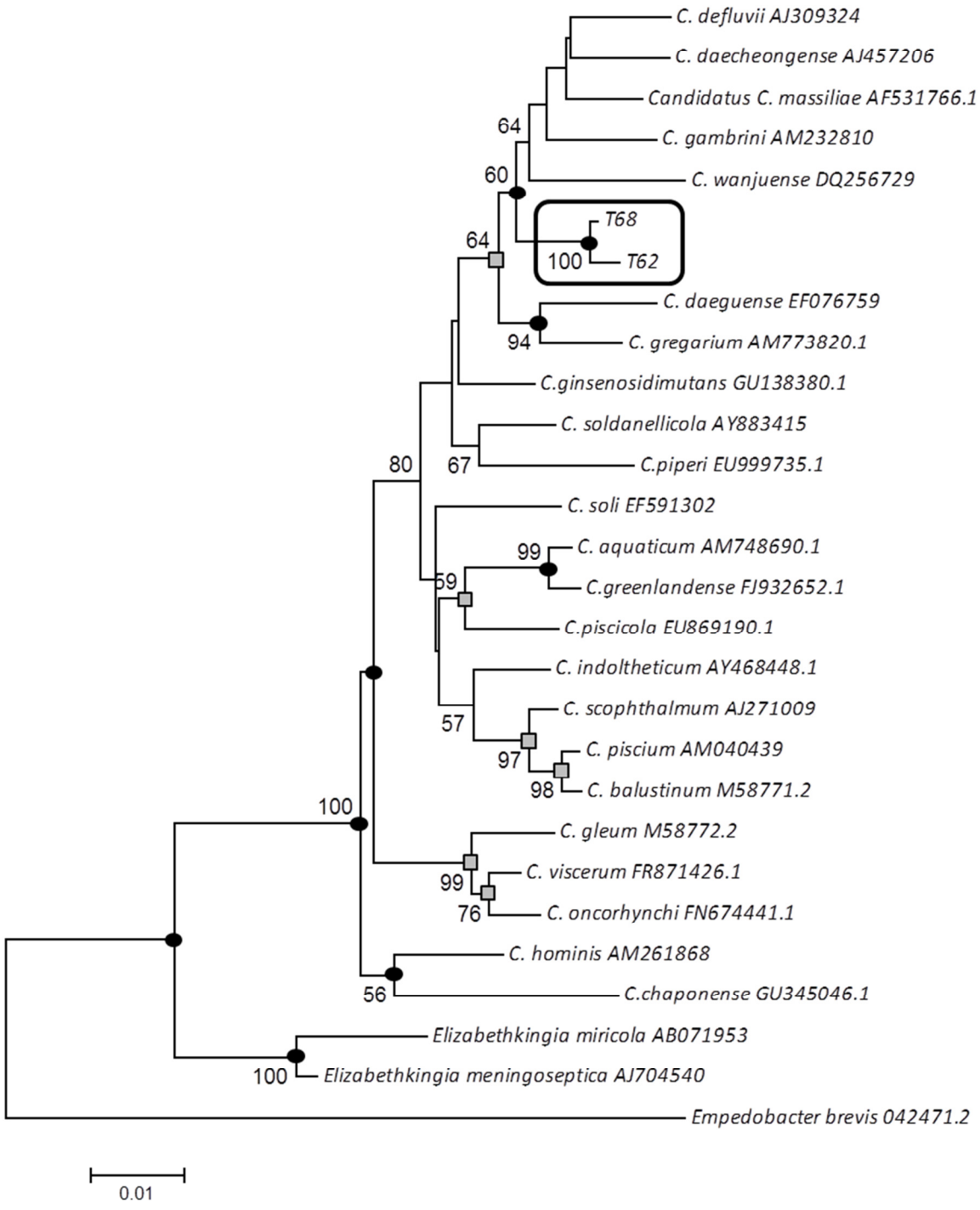

Table 1. Primers used for amplification and sequencing of the 16S rRNA gene of *Chryseobacterium* sp. strains T68 and T62.

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 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 ℃ in this study; 1, *C. ginsenosidimutans* grown on grown on nutrient agar for 48h at 27 ℃ and fatty acids comprising less than 1% were not published (Im *et al.,* 2011); 2, *C. gregarium* grown on TSA for 24h at 28 ℃ (Behrendt *et al.,* 2008); 3, *C. soldanellicola* grown on TSA for 48h at 30 ℃ (Park *et al.,* 2006); 4, *C. gambrini* grown on TSA for 24h at 30 ℃ (Herzog *et al.,* 2008); 5, *C. defluvi* ; 6, *C. piperi* grown on TSBA for 24h at 30 ℃ (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 ℃ (Kim *et al.,* 2005).

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 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.,* 1984; Bernardet *et al.* 2006); 9, *C. balustinum* (Kim *et al.,* 2005, Bernardet *et al.,* 2006), 10, *Elizabethkingia meningoseptica* (Lim *et al.,* 2003; 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
Growth On:													
MacConkey Agar	-	-	-	-	-	-	-	-	-	v	+	v	+
Cetrimide Agar	-	-	-	-	NR	+	NR	+	-	-	-	+	+
Able to grow at:													
pH 5.0	(+)	(+)	-	NR	+	NR	NR	-	+	NR	NR	NR	NR
4-5 ℃	+	+	-	(+)	+	-	-	-	+	-	(+)	-	-
37 ℃	-	-	+	-	+	+	+	+	[+]	+	-	+	+
42 ℃	-	-	-	NR	-	+	+	-	-	v	-	-	-
2% NaCl	(+)	(+)	-	NR	+	-	NR	+	+	NR	NR	NR	+
Production of:													
Indole	-	-	-	-	-	-	+	+	+	+	+	+	+
Hydrogen Sulfide	-	-	NR	-	-	+	-	-	-	-	v	-	+
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	-	-

+, 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.

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 ℃ 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℃ for 2- min, followed by 35 cycles of amplification, which included denaturation at 94℃ for 45- sec, annealing at 52℃ for 45- sec, and elongation at 72℃ for 90- sec. A final extension step was performed at 72℃ 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℃, 15℃, 22℃, 37℃, and 42℃; 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℃ 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 ℃. 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 ºC 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 -80ºC.

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 ℃. 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 ℃, 15℃, and 22℃, but not at 37 or 42℃. 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℃	+	+	-	+	+
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.

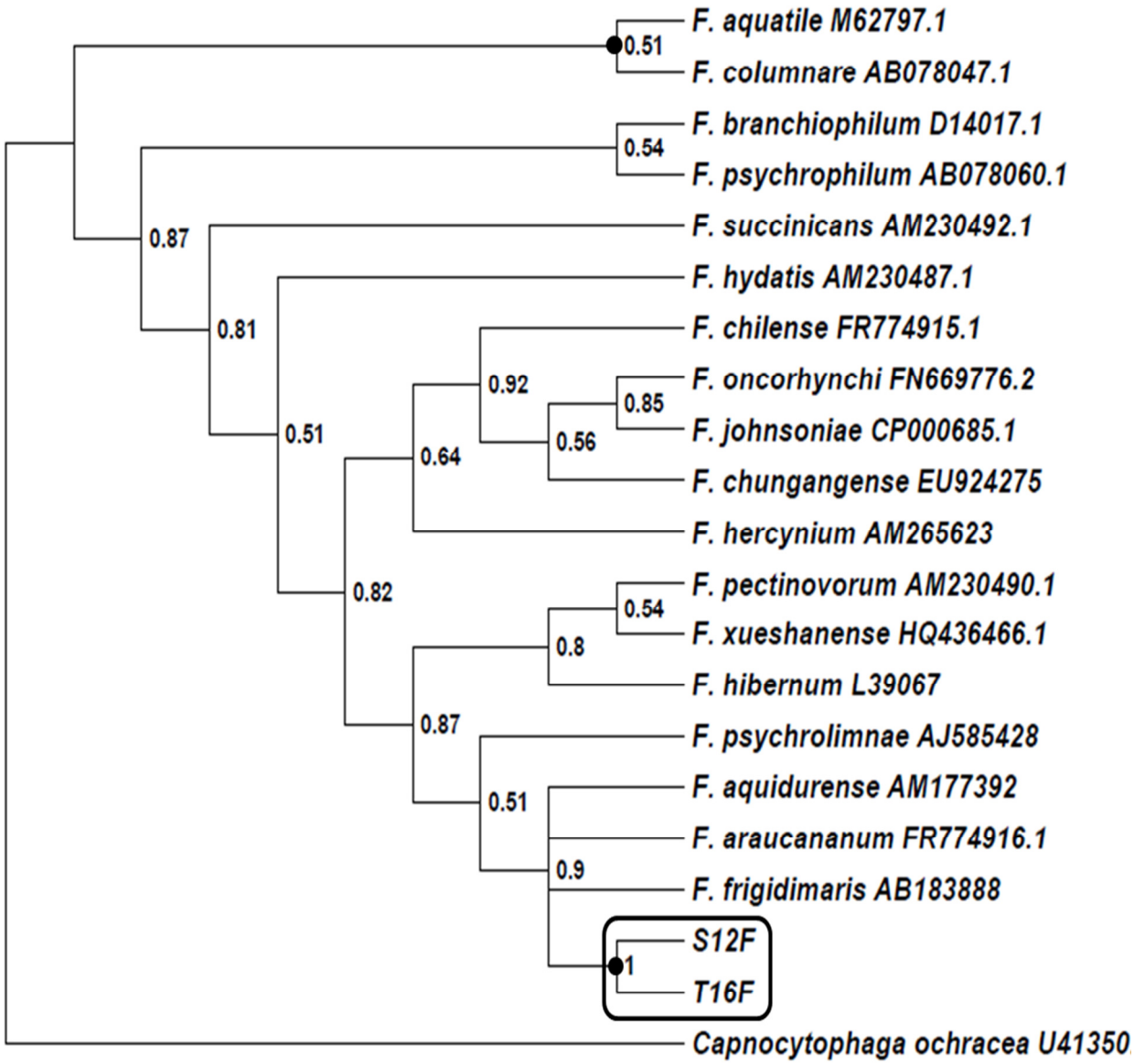


Figure 7.2. Growth kinetics for *Flavobacterium* sp. T16 and S12 as determined by 10 fold serial dilutions/plate counts and optical density (OD) readings taken at 600 nm (performed in duplicate). Isolates were inoculated into 40 ml of Hsu-Shotts broth supplemented with 5% (v/v) horse serum and 0.02% (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.

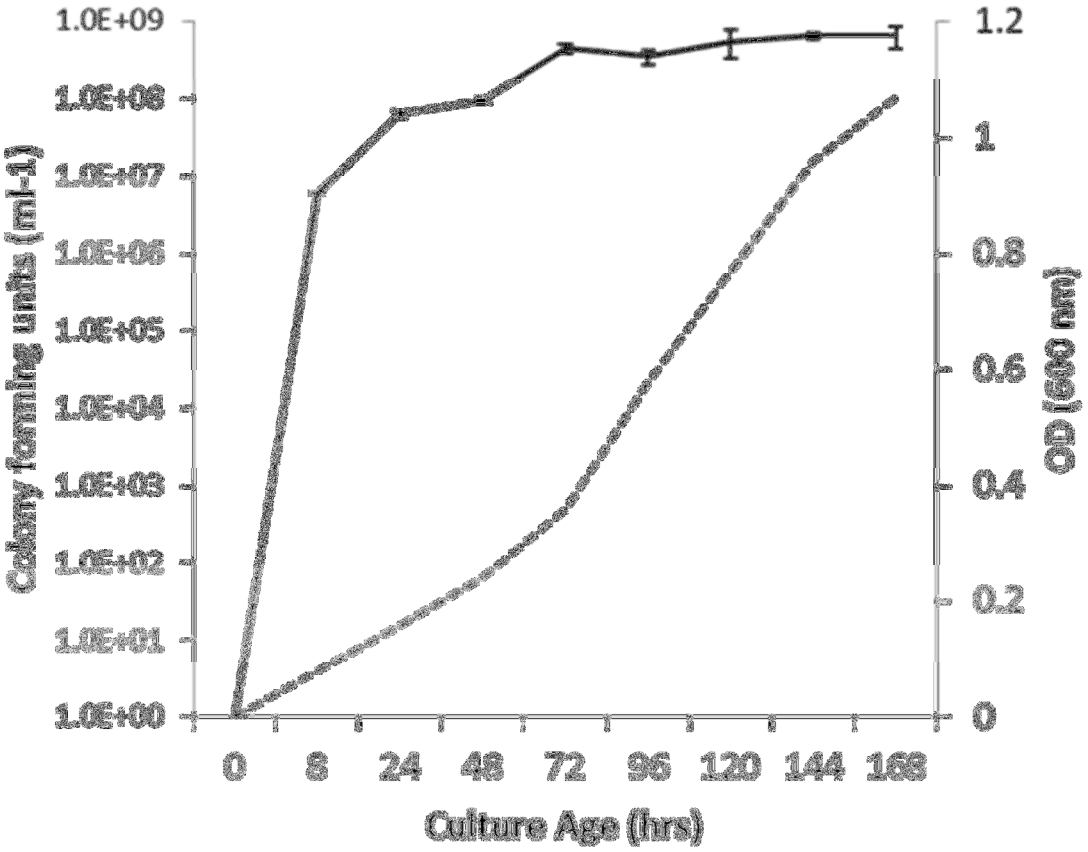


Figure 7.3. Gross lesions in fish intraperitoneally infected with *Flavobacterium* sp. T16 and S12. A) Petechial hemorrhage within the ventricle of the heart (arrow) of a Chinook salmon fingerling. Also note the hepatic pallor and red-tinged ascites within the pericardial and peritoneal cavities. B) 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.

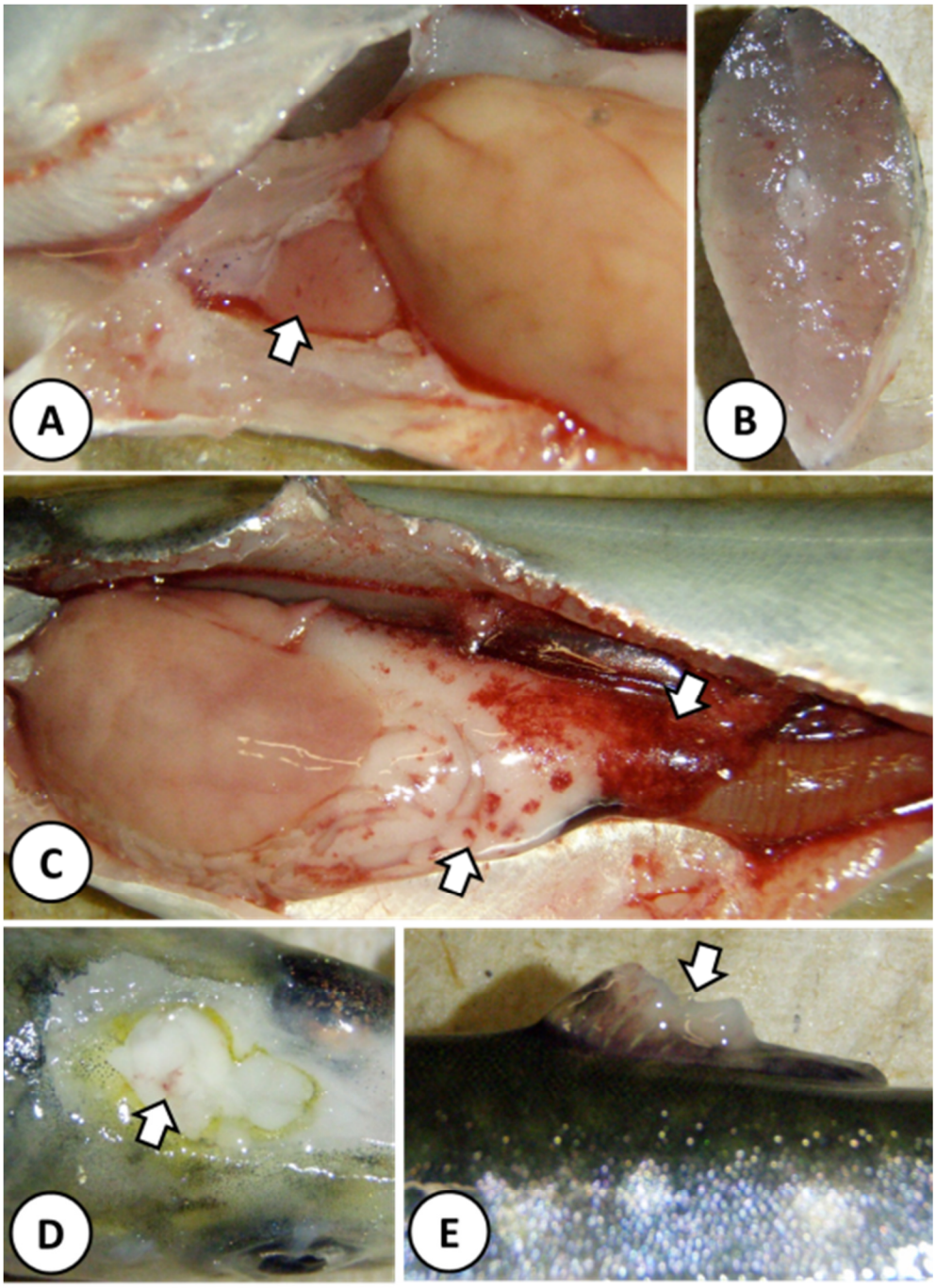


Figure 7.4. Hematoxylin and eosin (H & E) stained tissue sections from Chinook salmon intraperitoneally challenged with *Flavobacterium* sp. T16 and S12. A) Gills showing a proliferative branchitis consisting of epithelial hyperplasia of the secondary lamellae and interlamellar space resulting in secondary 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).

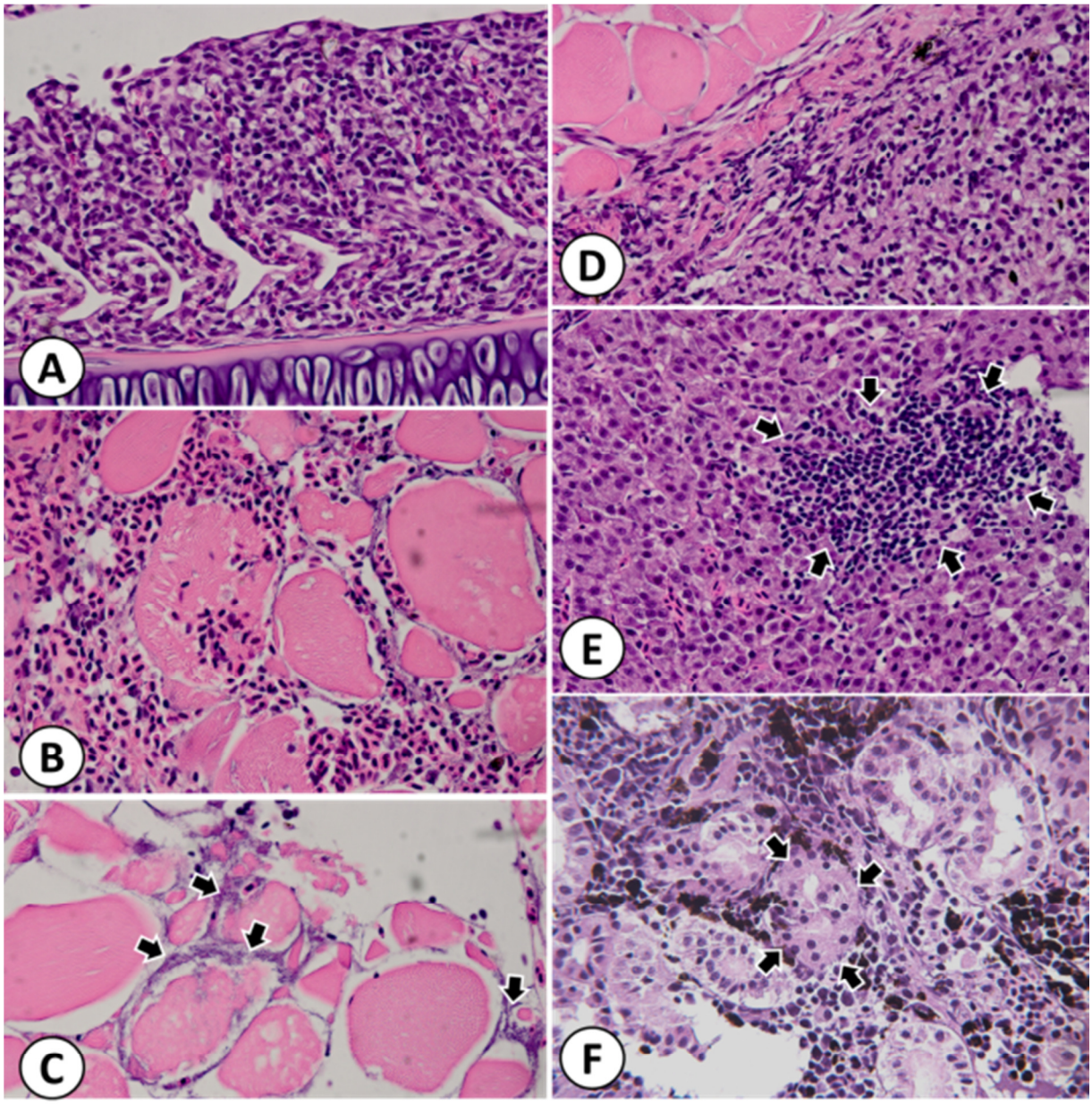
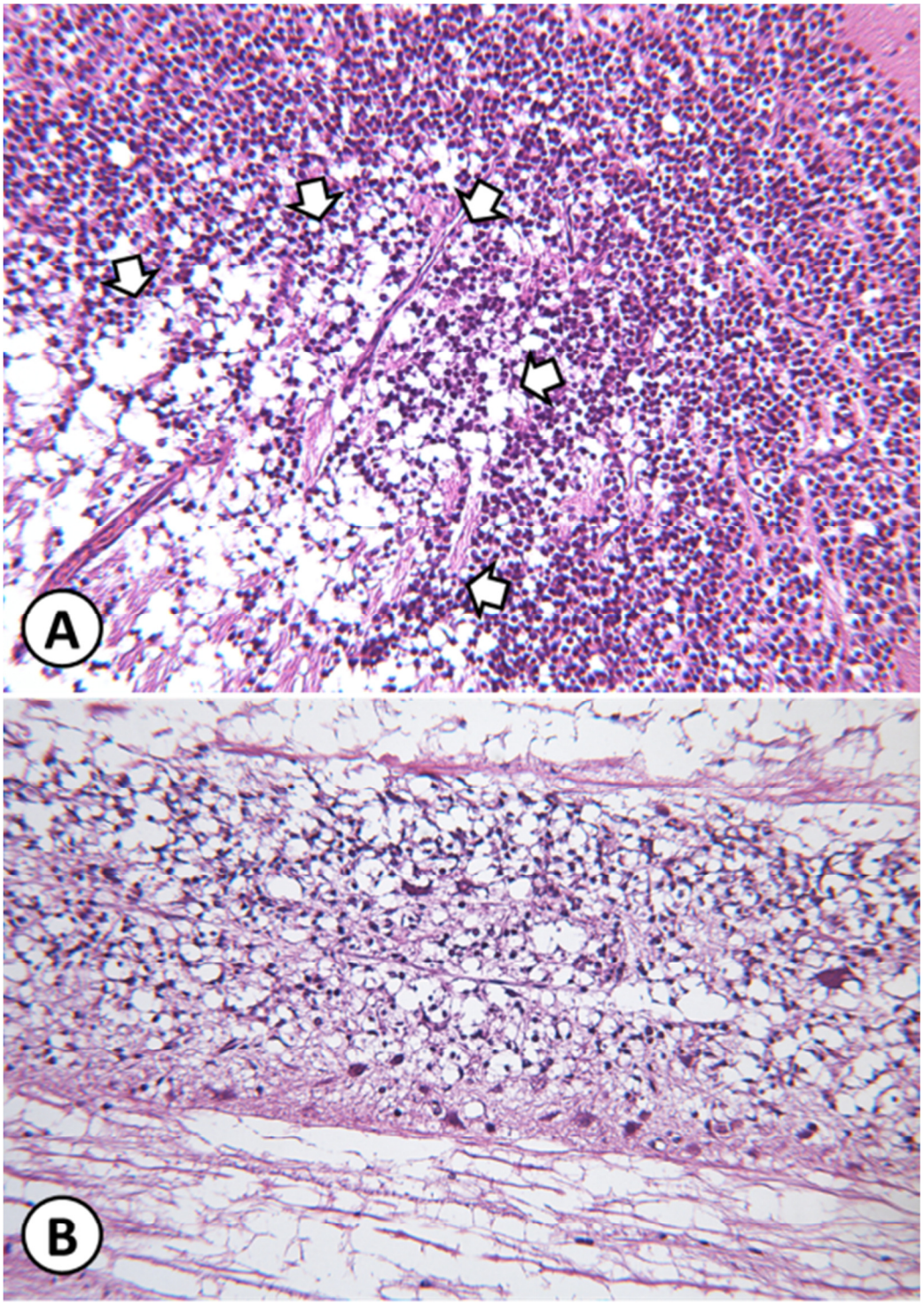


Figure 7.5. Hematoxylin and eosin (H & E) stained tissue sections from Chinook salmon intraperitoneally challenged with *Flavobacterium* sp. T16 and S12. A) Multifocal edema within the granular cell layer of the cerebellar cortex (arrows; 200x). B) Spongiosis and edema within the brain stem (200x).



APPENDIX 3-I

Appendix 3-I. Information on each of the 612 *Flavobacterium* spp. and *Chryseobacterium* spp. isolates that were recovered and characterized under Objective III.

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
600_TSD_5AH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
603_TSD_8AH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
604_TSD_10AH	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
605_TSD_11AH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
606_TSD_12BH	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
607_TSD_14EH	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
609_TSD_19AH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
610_TSD_20AH	F. hercynium_AM265623	97.8	T99.09.B.LAT.LS.H.Gill.D	98.1	Unres.	1	Eggs (TSFH)
611_TSD_21AH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
612_TSD_23AH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
613_TSD_27A?H	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Eggs (TSFH)
614_TSD_29A?H	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
617_TSD_42A?H	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
619_TSDR_25H	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
622_TSND_5AH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
626_TSND_10DH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
625_TSND_6CH	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Eggs (TSFH)
624_TSND_5FEH	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Eggs (TSFH)
635_TSND_24A?H	F. frigidimaris_AB183888	97.9	T99.09.B.LAT.LS.H.Gill.D	98.1	Unres.	1	Eggs (TSFH)
633_TSND_23A?H	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Eggs (TSFH)
664_TSD_28A?C	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
632_TSND_19FH	F. frigidimaris_AB183888	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
640_TSND_29AH	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
663_TSD_9AC	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
639_TSND_28AH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
662_TSD_8AC	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
630_TSND_18B?H	F. hercynium_AM265623	97.2	T99.09.B.LAT.LS.H.Gill.D	97.6	Unres.	1	Eggs (TSFH)
638_TSND_26AH	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Eggs (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
660_TSD_5AC	F. hercynium_AM265623	97.3	T99.09.B.LAT.LS.H.Gill.D	97.6	Unres.	1	Eggs (TSFH)
658_TSD_1AC	F. hercynium_AM265623	97.2	T99.09.B.LAT.LS.H.Gill.D	97.5	Unres.	1	Eggs (TSFH)
628_TSND_14A?H	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
621_TSND_2AH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
616_TSD_35AH	F. saccharophilum AB473208.1	97.4	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
669_TSND_2BC	F. saccharophilum AB473208.1	97.5	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
615_TSD_31BH	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
661_TSD_6BC	F. saccharophilum AB473208.1	97.3	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
677_TSND_12BC	F. saccharophilum AB473208.1	97.4	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
608_TSD_17A?H	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
659_TSD_3AC	F. saccharophilum AB473208.1	97.3	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
676_TSND_6BC	F. saccharophilum AB473208.1	97.5	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
674_TSND_5BC	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (TSFH)
671_TSND_3BC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
870_111130-1_1DLGH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
876_111130-1_27DSFJ?H	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
884_111130-1_20DCFJC	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
875_111130-1_24DSFJ?H	F. hercynium_AM265623	97.8	T99.09.B.LAT.LS.H.Gill.D	98.1	Unres.	1	Eggs (TSFH)
883_111130-1_11DCFJC	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
874_111130-1_15DFEH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
882_111130-1_1DCFJC	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
873_111130-1_8DumbH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (TSFH)
872_111130-1_7DSFJH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
871_111130-1_4DSFJH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (TSFH)
312_110118_P1UVADisC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
317_110118_P4UVADisC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
315_110118_P2UVBDisC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
314_110118_P2UVADisC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
313_110118_P1UVBDisC	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Fry (TSFH)
1018_120117-1_P18NDCFJH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
1015_120117-1_P14NDYLGH	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
987_120117-1_P9DFPC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
1017_120117-1_P18NDYLGH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Fry (TSFH)
136_110118_WS9undAC	F. hercynium_AM265623	97.8	T99.09.B.LAT.LS.H.Gill.D	98.1	Unres.	1	Water (TSFH)
299_110118_WS810-2BC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Water (TSFH)
278_110118_WS9undAH	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Water (TSFH)
276_110118_WS810-2D?H	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Water (TSFH)
303_110118_WS9undD?C	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Water (TSFH)
284_110118_WS10undD?H	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Water (TSFH)
300_110118_WS810-2CC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Water (TSFH)
298_110118_WS810-1BC	F. hercynium_AM265623	97.4	T99.09.B.LAT.LS.H.Gill.D	97.7	Unres.	1	Water (TSFH)
297_110118_WS810-1CC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Water (TSFH)
995_120117-1_WS810-1BC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Water (TSFH)
878_111130-2_6DERCH	F. hercynium_AM265623	97.5	T99.09.B.LAT.LS.H.Gill.D	97.8	Unres.	1	Eggs (WLSFH)
877_111130-2_3DGFJH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (WLSFH)
897_111130-2_25DFFJC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (WLSFH)
896_111130-2_12DFFJC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (WLSFH)
881_111130-2_22DGFJ?H	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	98.0	Unres.	1	Eggs (WLSFH)
895_111130-2_6DFFJC	F. hercynium_AM265623	97.6	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (WLSFH)
880_111130-2_18DGFJH	F. hercynium_AM265623	97.7	T99.09.B.LAT.LS.H.Gill.D	97.9	Unres.	1	Eggs (WLSFH)
879_111130-2_7DERCH	F. hercynium_AM265623	97.8	T99.09.B.LAT.LS.H.Gill.D	98.1	Unres.	1	Eggs (WLSFH)
150_110104_WS910-2BC	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.3	II	2	Water (PRSFH)
201_110104_WS710-3CH	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.4	II	2	Water (PRSFH)
210_110104_WS910-1CH	F. hercynium_AM265623	98.2	T151.10.BKT.W.Gill.D	99.1	II	2	Water (PRSFH)
183_110104_WS5undBH	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.3	II	2	Water (PRSFH)
160_110104_WS110-2DH	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.2	II	2	Water (PRSFH)
182_110104_WS5undAH	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.3	II	2	Water (PRSFH)
158_110104_WS110-2AH	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.4	II	2	Water (PRSFH)
256_110104_WS910-2AC	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.4	II	2	Water (PRSFH)
9_110512_WS1210-2EH	F. hercynium_AM265623	98.2	T151.10.BKT.W.Gill.D	99.3	II	2	Water (PRSFH)
12_110512_WS1310-2CH	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.2	II	2	Water (PRSFH)
17_110512_WS1410-3CH	F. hercynium_AM265623	98.2	T66.09.P.CHS.MI.H.Kidney	99.1	II	2	Water (PRSFH)
56_110512_WS110-2CH	F. hercynium_AM265623	98.2	T151.10.BKT.W.Gill.D	99.1	II	2	Water (PRSFH)
57_110512_WS110-2DH	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.4	II	2	Water (PRSFH)

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679_PLD_2AC	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.3	II	2	Eggs (PRSFH)
697_PLD_24CC	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.3	II	2	Eggs (PRSFH)
694_PLD_16CC	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
690_PLND_4AC	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
688_PLND_2AC	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
656_PLD_28A?H	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.5	II	2	Eggs (PRSFH)
687_PLD_15CC	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
652_PLD_6AH	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.5	II	2	Eggs (PRSFH)
646_PLND_5A?H	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
683_PLD_7CC	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
645_PLND_3AH	F. hercynium_AM265623	97.8	T70.09.LHR.H.Kidney.D	98.9	II	2	Eggs (PRSFH)
682_PLD_5CC	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (PRSFH)
247_110104_P18DH	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.4	II	2	Fry (PRSFH)
246_110104_P16DH	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Fry (PRSFH)
245_110104_P16C?H	F. hercynium_AM265623	98.3	T151.10.BKT.W.Gill.D	99.3	II	2	Fry (PRSFH)
239_110104_P6DH	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.2	II	2	Fry (PRSFH)
867_111130-2_14NDLCEH	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (WLSFH)
859_111130-2_6NDPFJH	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (WLSFH)
892_111130-2_11NDPFJC	F. hercynium_AM265623	98.4	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (WLSFH)
891_111130-2_4NDPFJC	F. hercynium_AM265623	98.5	T151.10.BKT.W.Gill.D	99.4	II	2	Eggs (WLSFH)
747_111005-1_5maleLYFJH	F. hercynium_AM265623	98.2	T71.09.LHR.H.Kidney.D	99.8	II	2	Brood (LMRW)
191_110104_WS610-1AH	F. glaciei_DQ515962	97.7	T148.10.BKT.W.Gill.N	99.7	I	3	Water (PRSFH)
216_110104_WS10undEH	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (PRSFH)
6_110512_WS1210-2BH	F.oncorhynchi_FR870076.1	97.5	T129.10.P.BKT.AS.H.Kidney	99.3	I	3	Water (PRSFH)
50_110512_WS910-1EH	F.oncorhynchi_FR870076.1	97.7	T129.10.P.BKT.AS.H.Kidney	99.5	I	3	Water (PRSFH)
39_110512_WS410-1BH	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (PRSFH)
279_110118_WS9undBH	F.oncorhynchi_FR870076.1	97.6	T129.10.P.BKT.AS.H.Kidney	99.4	I	3	Water (TSFH)
912_120106-1_WS8undCH	F.succinicans_AM230492.1	97.9	T129.10.P.BKT.AS.H.Kidney	99.7	I	3	Water (WLSFH)
959_120106-1_WS11undAC	F. glaciei_DQ515962	97.7	T148.10.BKT.W.Gill.N	99.7	I	3	Water (WLSFH)
958_120106-1_WS11undBC	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (WLSFH)
970_120106-1_WS5undAC	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.9	I	3	Water (WLSFH)
974_120106-1_WS6undBC	F. glaciei_DQ515962	97.7	T148.10.BKT.W.Gill.N	99.7	I	3	Water (WLSFH)

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1058_120416-1_WS910-1CH	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (WLSFH)
1049_120416-1_WS7undBH	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (WLSFH)
1047_120416-1_WS6undBH	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (WLSFH)
1078_120416-1_WS5undAC	F. glaciei_DQ515962	97.6	T148.10.BKT.W.Gill.N	99.6	I	3	Water (WLSFH)
1077_120416-1_WS3undBC	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (WLSFH)
1080_120416-1_WS6undBC	F. glaciei_DQ515962	97.7	T148.10.BKT.W.Gill.N	99.7	I	3	Water (WLSFH)
1086_120416-1_WS910-1AC	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Water (WLSFH)
967_120106-1_P15NDCFJ?C	F. glaciei_DQ515962	97.8	T148.10.BKT.W.Gill.N	99.8	I	3	Fry (WLSFH)
975_120106-1_WS8undAC	F.pectinovorum_AM230490.1	97.9	T76.09.P.RBT.EL.H.Kidney	99.7	VIII	4	Water (WLSFH)
909_120106-1_WS7undCH	F.pectinovorum_AM230490.1	98.2	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Water (WLSFH)
915_120106-1_WS9undAH	F.pectinovorum_AM230490.1	98.0	T76.09.P.RBT.EL.H.Kidney	99.7	VIII	4	Water (WLSFH)
902_120106-1_WS210-2AH	F.pectinovorum_AM230490.1	98.1	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Water (WLSFH)
935_120106-1_P6DYSH	F.pectinovorum_AM230490.1	98.0	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
950_120106-1_P10NDBH	F.pectinovorum_AM230490.1	98.1	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
948_120106-1_P20NDSFJH	F.pectinovorum_AM230490.1	98.0	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
933_120106-1_P4DCFJH	F.pectinovorum_AM230490.1	98.1	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
984_120106-1_P9DCFJC	F.pectinovorum_AM230490.1	97.8	T76.09.P.RBT.EL.H.Kidney	99.5	VIII	4	Fry (WLSFH)
966_120106-1_P4NDCFJC	F.pectinovorum_AM230490.1	97.9	T76.09.P.RBT.EL.H.Kidney	99.7	VIII	4	Fry (WLSFH)
986_120106-1_P19DCFJC	F.pectinovorum_AM230490.1	98.0	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
985_120106-1_P12DCFJC	F.pectinovorum_AM230490.1	98.1	T76.09.P.RBT.EL.H.Kidney	99.9	VIII	4	Fry (WLSFH)
937_120106-1_P10DFFJH	F.pectinovorum_AM230490.1	98.0	T76.09.P.RBT.EL.H.Kidney	99.7	VIII	4	Fry (WLSFH)
936_120106-1_P10PYE?H	F.pectinovorum_AM230490.1	98.1	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
931_120106-1_P2DCFJH	F.pectinovorum_AM230490.1	98.1	T76.09.P.RBT.EL.H.Kidney	99.8	VIII	4	Fry (WLSFH)
930_120106-1_P1DCFJH	F.pectinovorum_AM230490.1	97.9	T76.09.P.RBT.EL.H.Kidney	99.6	VIII	4	Fry (WLSFH)
951_120106-1_P1NDCFJC	F.pectinovorum_AM230490.1	98.2	T76.09.P.RBT.EL.H.Kidney	99.9	VIII	4	Fry (WLSFH)
745_111005-1_24femTFJH	F.pectinovorum_AM230490.1	97.9	T76.09.P.RBT.EL.H.Kidney	99.6	VIII	4	Brood (LMRW)
318_110118_P4UVDDisC	F. hercynium_AM265623	98.7	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Fry (TSFH)
121_110118_WS11undAC	F. hercynium_AM265623	98.5	T61.09.P.BNT.WR.Fin.D	99.7	V	5	Water (TSFH)
282_110118_WS10undBH	F. hercynium_AM265623	98.9	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (TSFH)
287_110118_WS11undAH	F. hercynium_AM265623	98.8	T61.09.P.BNT.WR.Fin.D	99.9	V	5	Water (TSFH)
301_110118_WS810-2DC	F. hercynium_AM265623	98.7	T61.09.P.BNT.WR.Fin.D	99.7	V	5	Water (TSFH)
101_110519_WS6undAH	F. hercynium_AM265623	98.6	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
1020_120117-1_WS1310-1CH	F. hercynium_AM265623	98.8	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (TSFH)
1004_120117-1_WS1610-1BC	F. hercynium_AM265623	98.7	T61.09.P.BNT.WR.Fin.D	99.7	V	5	Water (TSFH)
1011_120117-1_WS1310-1BC	F. hercynium_AM265623	98.6	T61.09.P.BNT.WR.Fin.D	99.6	V	5	Water (TSFH)
1098_120515-1_WS7undAH	F. hercynium_AM265623	98.8	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (TSFH)
1102_120515-1_WS11undCH	F. hercynium_AM265623	98.5	T61.09.P.BNT.WR.Fin.D	99.5	V	5	Water (TSFH)
1100_120515-1_WS8undAH	F. hercynium_AM265623	98.7	T61.09.P.BNT.WR.Fin.D	99.7	V	5	Water (TSFH)
1099_120515-1_WS7undBH	F. hercynium_AM265623	98.9	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (TSFH)
920_120106-1_WS1010-1BH	F. hercynium_AM265623	98.5	T61.09.P.BNT.WR.Fin.D	99.5	V	5	Water (WLSFH)
956_120106-1_WS1010-1EC	F. hercynium_AM265623	98.6	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (WLSFH)
928_120106-1_WS1310-1EH	F. hercynium_AM265623	98.7	T61.09.P.BNT.WR.Fin.D	99.8	V	5	Water (WLSFH)
204_110104_WS810-1CH	F. frigidimaris_AB183888	97.9	S21.05.P.RBT.H.Ulcer.D.M	99.7	XI	6	Water (PRSFH)
206_110104_WS810-1EH	F. frigidimaris_AB183888	97.9	S21.05.P.RBT.H.Ulcer.D.M	99.6	XI	6	Water (PRSFH)
4_110512_WS1110-2DH	F. aquidurense_AM177392	98.2	T1.05.BG.W.Gill.N	99.4	XI	6	Water (PRSFH)
8_110512_WS1210-2DH	F. frigidimaris_AB183888	97.9	T1.05.BG.W.Gill.N	99.7	XI	6	Water (PRSFH)
18_110512_WS1410-3DH	F. frigidimaris_AB183888	97.8	S21.05.P.RBT.H.Ulcer.D.M	99.4	XI	6	Water (PRSFH)
24_110512_WS510-1DH	F. aquidurense_AM177392	98.6	T1.05.BG.W.Gill.N	99.6	XI	6	Water (PRSFH)
25_110512_WS110-3EH	F. frigidimaris_AB183888	97.9	S21.05.P.RBT.H.Ulcer.D.M	99.7	XI	6	Water (PRSFH)
41_110512_WS410-1DH	F. frigidimaris_AB183888	97.9	T17.08.B-RBT-MI.W.Kidney	99.6	XI	6	Water (PRSFH)
44_110512_WS810-1DH	F. frigidimaris_AB183888	97.8	S21.05.P.RBT.H.Ulcer.D.M	99.6	XI	6	Water (PRSFH)
45_110512_WS810-1EH	F. frigidimaris_AB183888	97.9	S21.05.P.RBT.H.Ulcer.D.M	99.8	XI	6	Water (PRSFH)
48_110512_WS910-1CH	F. frigidimaris_AB183888	97.8	S21.05.P.RBT.H.Ulcer.D.M	99.6	XI	6	Water (PRSFH)
49_110512_WS910-1DH	F. aquidurense_AM177392	97.9	S21.05.P.RBT.H.Ulcer.D.M	99.6	XI	6	Water (PRSFH)
54_110512_WS1010-2DH	F. frigidimaris_AB183888	97.9	S21.05.P.RBT.H.Ulcer.D.M	99.5	XI	6	Water (PRSFH)
77_110512_P21-25CH	F. frigidimaris_AB183888	97.7	T17.08.B-RBT-MI.W.Kidney	99.6	XI	6	Fingerlings (PRSFH)
320_110512_WS1110-2EH	F. degerlachei_AJ557886	97.3	T75.09.P.BNT.WR.H.Kidney	99.6	XXIX	7	Water (PRSFH)
130_110118_WS310-1AC	F. degerlachei_AJ557886	97.4	T75.09.P.BNT.WR.H.Kidney	99.6	XXIX	7	Water (TSFH)
128_110118_WS1210-2A?C	F. degerlachei_AJ557886	97.3	T75.09.P.BNT.WR.H.Kidney	99.8	XXIX	7	Water (TSFH)
125_110118_WS12undBC	F. degerlachei_AJ557886	97.3	T74.09.P.BNT.WR.H.Kidney	99.6	XXIX	7	Water (TSFH)
133_110118_WS310-2DC	F. degerlachei_AJ557886	97.4	T75.09.P.BNT.WR.H.Kidney	99.8	XXIX	7	Water (TSFH)
310_110118_WS13undBC	F. degerlachei_AJ557886	97.5	T75.09.P.BNT.WR.H.Kidney	99.8	XXIX	7	Water (TSFH)
134_110118_WS310-2AC	F. degerlachei_AJ557886	97.3	T75.09.P.BNT.WR.H.Kidney	99.6	XXIX	7	Water (TSFH)

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114_110519_WS1510-1AH	F. degerlachei_AJ557886	97.4	T75.09.P.BNT.WR.H.Kidney	99.8	XXIX	7	Water (TSFH)
111_110519_WS1410-1AH	F. degerlachei_AJ557886	97.1	T75.09.P.BNT.WR.H.Kidney	99.4	XXIX	7	Water (TSFH)
109_110519_WS1310-1AH	F. degerlachei_AJ557886	97.4	T75.09.P.BNT.WR.H.Kidney	99.7	XXIX	7	Water (TSFH)
117_110519_WS1610-1AH	F. degerlachei_AJ557886	97.3	T75.09.P.BNT.WR.H.Kidney	99.5	XXIX	7	Water (TSFH)
1003_120117-1_WS1510-1BC	F. degerlachei_AJ557886	97.3	T75.09.P.BNT.WR.H.Kidney	99.8	XXIX	7	Water (TSFH)
174_110104_WS310-2B?H	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.8	XVI	8	Water (PRSFH)
172_110104_WS310-1EH	F.succinicans_AM230492.1	98.4	T161.10.BNT.W.Gill.N	99.8	XVI	8	Water (PRSFH)
188_110104_WS510-1H?H	F.succinicans_AM230492.1	98.4	T161.10.BNT.W.Gill.N	99.8	XVI	8	Water (PRSFH)
217_110104_WS10undFH	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.8	XVI	8	Water (PRSFH)
194_110104_WS610-1DH	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.6	XVI	8	Water (PRSFH)
223_110104_WS1110-1FH	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.7	XVI	8	Water (PRSFH)
205_110104_WS810-1DH	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.7	XVI	8	Water (PRSFH)
37_110512_WS810-1AH	F.succinicans_AM230492.1	98.2	T161.10.BNT.W.Gill.N	99.8	XVI	8	Water (PRSFH)
43_110512_WS810-1CH	F.succinicans_AM230492.1	98.2	T161.10.BNT.W.Gill.N	99.5	XVI	8	Water (PRSFH)
51_110512_WS1010-2AH	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.7	XVI	8	Water (PRSFH)
75_110512_P6-10AH	F.succinicans_AM230492.1	98.3	T161.10.BNT.W.Gill.N	99.6	XVI	8	Fingerlings (PRSFH)
847_111005-1_30OFFJH	F. frigidimaris_AB183888	99.1	T99.09.B.LAT.LS.H.Gill.D	99.3	Unres.	9	Brood (LMRW)
733_111005-1_2femFJLYH	F. frigidimaris_AB183888	99.0	T99.09.B.LAT.LS.H.Gill.D	99.1	Unres.	9	Brood (LMRW)
786_111005-1_6OFM	F. frigidimaris_AB183888	99.1	T99.09.B.LAT.LS.H.Gill.D	99.3	Unres.	9	Brood (LMRW)
728_111005-1_1femFJH	F. frigidimaris_AB183888	99.0	T1.05.BG.W.Gill.N	97.6	XI	9	Brood (LMRW)
785_111005-1_2OFFJH	F. frigidimaris_AB183888	99.2	T1.05.BG.W.Gill.N	97.9	XI	9	Brood (LMRW)
783_111005-1_30femSplH	F. frigidimaris_AB183888	99.3	T99.09.B.LAT.LS.H.Gill.D	99.4	Unres.	9	Brood (LMRW)
782_111005-1_2femSplH	F. frigidimaris_AB183888	99.2	T99.09.B.LAT.LS.H.Gill.D	99.4	Unres.	9	Brood (LMRW)
791_111005-1_23OFFJH	F. frigidimaris_AB183888	99.1	T99.09.B.LAT.LS.H.Gill.D	99.3	Unres.	9	Brood (LMRW)
787_111005-1_8OFFJH	F. frigidimaris_AB183888	99.2	T99.09.B.LAT.LS.H.Gill.D	99.4	Unres.	9	Brood (LMRW)
643_PLND_2AH	F.oncorhynchi_FR870076.1	99.4	T19.08.P.BNT.GC.H.Gill.D.M	99.8	IX	10	Eggs (PRSFH)
647_PLND_3A?H	F.oncorhynchi_FR870076.1	99.4	T19.08.P.BNT.GC.H.Gill.D.M	99.8	IX	10	Eggs (PRSFH)
911_120106-1_WS8undBH	F.oncorhynchi_FR870076.1	99.4	T19.08.P.BNT.GC.H.Gill.D.M	99.8	IX	10	Water (WLSFH)
976_120106-1_WS8undBC	F.oncorhynchi_FR870076.1	99.3	T20.08.P.BNT.GC.H.Gill.D.M	99.5	IX	10	Water (WLSFH)
1057_120416-1_WS910-1BH	F.oncorhynchi_FR870076.1	99.1	T20.08.P.BNT.GC.H.Gill.D.M	99.4	IX	10	Water (WLSFH)
1053_120416-1_WS8undCH	F.oncorhynchi_FR870076.1	99.3	T20.08.P.BNT.GC.H.Gill.D.M	99.5	IX	10	Water (WLSFH)

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1087_120416-1_WS910-1BC	F.oncorhynchi_FR870076.1	99.0	T20.08.P.BNT.GC.H.Gill.D.M	99.3	IX	10	Water (WLSFH)
824_111012-1_10femFJC	F.oncorhynchi_FR870076.1	99.7	T20.08.P.BNT.GC.H.Gill.D.M	99.9	IX	10	Brood (SRW)
261_110104_WS1210-2DC	F.pectinovorum_AM230490.1	98.5	T108.09.B.BNT.SR.H.Fin.D	99.8	Unres.	11	Water (PRSFH)
260_110104_WS1210-1CC	F.pectinovorum_AM230490.1	98.4	T108.09.B.BNT.SR.H.Fin.D	99.7	Unres.	11	Water (PRSFH)
910_120106-1_WS8undAH	F.pectinovorum_AM230490.1	98.6	T108.09.B.BNT.SR.H.Fin.D	99.8	Unres.	11	Water (WLSFH)
1059_120416-1_WS910-1DH	F.pectinovorum_AM230490.1	98.6	T108.09.B.BNT.SR.H.Fin.D	99.8	Unres.	11	Water (WLSFH)
1079_120416-1_WS6undAC	F.pectinovorum_AM230490.1	98.4	T108.09.B.BNT.SR.H.Fin.D	99.6	Unres.	11	Water (WLSFH)
1046_120416-1_WS6undAH	F.pectinovorum_AM230490.1	98.6	T108.09.B.BNT.SR.H.Fin.D	99.8	Unres.	11	Water (WLSFH)
1062_120416-1_WS1010-1BH	F.pectinovorum_AM230490.1	98.5	T108.09.B.BNT.SR.H.Fin.	99.7	Unres.	11	Water (WLSFH)
1052_120416-1_WS8undBH	F.pectinovorum_AM230490.1	98.5	T108.09.B.BNT.SR.H.Fin.D	99.7	Unres.	11	Water (WLSFH)
943_120106-1_P1NDPLH	T16F	98.4	T108.09.B.BNT.SR.H.Fin.D	99.8	Unres.	11	Fry (WLSFH)
829_111012-1_11NDC	F. cucumis_EF126993	95.7	T18.08.P.BNT.GC.H.Gill.D.M	92.9	II	12	Brood (SRW)
826_111012-1_6OFAC	F. cucumis_EF126993	96.0	T18.08.P.BNT.GC.H.Gill.D.M	93.1	II	12	Brood (SRW)
836_111012-1_17OFAC	F. cucumis_EF126993	95.9	T18.08.P.BNT.GC.H.Gill.D.M	93.1	II	12	Brood (SRW)
835_111012-1_7OFAC	F. cucumis_EF126993	95.9	T18.08.P.BNT.GC.H.Gill.D.M	93.2	II	12	Brood (SRW)
820_111012-1_21NDFP?C	F. cucumis_EF126993	95.9	T18.08.P.BNT.GC.H.Gill.D.M	93.1	II	12	Brood (SRW)
830_111012-1_1OF10-2AC	F. cucumis_EF126993	95.9	T18.08.P.BNT.GC.H.Gill.D.M	93.1	II	12	Brood (SRW)
794_111012-1_30OFH	F. cucumis_EF126993	95.9	T18.08.P.BNT.GC.H.Gill.D.M	93.1	II	12	Brood (SRW)
792_111012-1_17OFH	F. cucumis_EF126993	95.8	T18.08.P.BNT.GC.H.Gill.D.M	93.0	II	12	Brood (SRW)
1117_120515-1_P86-90BH	S12F	99.5	T124.10.P.LAT.SE.H.Kidney	99.7	XVIII	13	Water (TSFH)
800_111012-1_2femBrH	T16F	99.5	T124.10.P.LAT.SE.H.Kidney	99.6	XVIII	13	Brood (SRW)
852_111012-1_26NDH	S12F	99.5	T124.10.P.LAT.SE.H.Kidney	99.7	XVIII	13	Brood (SRW)
735_111005-1_5femSFJH	T16F	99.8	T14.07.B.CHS.SRW.W.Kid	99.8	XVIII	13	Brood (LMRW)
743_111005-1_23femFCH	S12F	99.5	S12.05.P.CHS.MI.H.Kid	99.5	XVIII	13	Brood (LMRW)
132_110118_WS310-2CC	F.tiangeerense_EU036219.1	97.9	T105.09.P.BNT.GC.H.Gill	98.3	XXIV	14	Water (TSFH)
124_110118_WS12undAC	F.tiangeerense_EU036219.1	98.2	T158.10.BNT.W.Gill.N	98.5	XV	14	Water (TSFH)
118_110519_WS1610-1BH	F.tiangeerense_EU036219.1	98.3	T105.09.P.BNT.GC.H.Gill	98.6	XXIV	14	Water (TSFH)
112_110519_WS1410-1CH	F.tiangeerense_EU036219.1	98.4	T158.10.BNT.W.Gill.N	98.7	XV	14	Water (TSFH)
1008_120117-1_WS1610-2FP?C	F.tiangeerense_EU036219.1	97.8	T105.09.P.BNT.GC.H.Gill	98.3	XXIV	14	Water (TSFH)
982_120106-1_WS1010-1AC	F.tiangeerense_EU036219.1	98.0	T105.09.P.BNT.GC.H.Gill	98.5	XXIV	14	Water (WLSFH)
240_110104_P7AH	F. saccharophilum AB473208.1	98.8	T69.09.B.RBT.MI.W.Kidney	98.7	IV	15	Fry (PRSFH)

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236_110104_P4BH	F. saccharophilum AB473208.1	99.2	T69.09.B.RBT.MI.W.Kidney	99.0	IV	15	Fry (PRSFH)
157_110104_P11CDisC	F. saccharophilum AB473208.1	98.9	T132.10.WAE.W.Kidney.N	98.8	Unres.	15	Fry (PRSFH)
233_110104_P2AH	F. saccharophilum AB473208.1	99.0	T69.09.B.RBT.MI.W.Kidney	98.9	IV	15	Fry (PRSFH)
629_TSND_16EH	F. saccharophilum AB473208.1	98.8	T69.09.B.RBT.MI.W.Kidney	98.6	IV	15	Eggs (TSFH)
71_110512_T7B1BH	F.oncorhynchi_FR870076.1	98.2	T59.09.P.BKT.AS.H.Kidney	99.7	X	16	Tools (PRSFH)
1110_120515-1_WS1610-1AH	F.oncorhynchi_FR870076.1	98.4	T59.09.P.BKT.AS.H.Kidne	99.9	X	16	Water (TSFH)
1084_120416-1_WS8undCC	F.oncorhynchi_FR870076.1	98.3	T59.09.P.BKT.AS.H.Kidney	99.8	X	16	Water (WLSFH)
1091_120416-1_WS1310-1CC	F.oncorhynchi_FR870076.1	98.3	T59.09.P.BKT.AS.H.Kidney	99.8	X	16	Water (WLSFH)
1073_120416-1_R19N1CH	F.oncorhynchi_FR870076.1	98.2	T59.09.P.BKT.AS.H.Kidney	99.8	X	16	Tools (WLSFH)
1092_120416-1_R19N1AC	F.oncorhynchi_FR870076.1	98.2	T59.09.P.BKT.AS.H.Kidney	99.7	X	16	Tools (WLSFH)
924_120106-1_WS1310-1AH	F. psychrolimnae_AJ585428	96.9	T8.06.B.BNT.SE.H.SB	96.9	IV	17	Water (WLSFH)
963_120106-1_WS1310-1AC	F. psychrolimnae_AJ585428	96.9	T8.06.B.BNT.SE.H.SB	96.9	IV	17	Water (WLSFH)
1064_120416-1_WS1310-2AH	F. psychrolimnae_AJ585428	97.1	T108.09.B.BNT.SR.H.Fin.D	97.0	Unres.	17	Water (WLSFH)
1089_120416-1_WS1310-1AC	F. psychrolimnae_AJ585428	96.6	T105.09.P.BNT.GC.H.Gill.D	96.6	XXIV	17	Water (WLSFH)
232_110104_WS1210-3B?H	F. frigidimaris_AB183888	98.6	T93.09.P.BKT.AS.H.Gill.N	98.8	Unres.	18	Water (PRSFH)
7_110512_WS1210-2CH	F. frigidimaris_AB183888	98.1	T93.09.P.BKT.AS.H.Gill.N	98.4	Unres.	18	Water (PRSFH)
14_110512_WS1310-2EH	F. aquidurense_AM177392	98.3	T17.08.B-RBT-MI.W.Kidney	98.5	XI	18	Water (PRSFH)
29_110512_WS210-2DH	F. frigidimaris_AB183888	98.3	T93.09.P.BKT.AS.H.Gill.N	98.7	Unres.	18	Water (PRSFH)
1095_120515-1_WS110-1BH	F. frigidimaris_AB183888	98.2	T17.08.B-RBT-MI.W.Kidney	98.6	XI	18	Water (TSFH)
326_110512_T2B1BH	F. saccharophilum AB473208.1	98.5	T93.09.P.BKT.AS.H.Gill.N	99.0	Unres.	19	Tools (PRSFH)
235_110104_P3DKCH	F. saccharophilum AB473208.1	98.2	T93.09.P.BKT.AS.H.Gill.N	98.6	Unres.	19	Fry (PRSFH)
234_110104_P3AH	F. saccharophilum AB473208.1	98.5	T93.09.P.BKT.AS.H.Gill.N	98.9	Unres.	19	Fry (PRSFH)
252_110104_P19FJDisH	F. saccharophilum AB473208.1	98.4	T93.09.P.BKT.AS.H.Gill.N	98.9	Unres.	19	Fry (PRSFH)
251_110104_P18FJDisH	F. saccharophilum AB473208.1	97.8	T93.09.P.BKT.AS.H.Gill.N	98.2	Unres.	19	Fry (PRSFH)
243_110104_P12A?H	F. saccharophilum AB473208.1	98.1	T93.09.P.BKT.AS.H.Gill.N	98.5	Unres.	19	Fry (PRSFH)
242_110104_P11A?H	F. saccharophilum AB473208.1	97.9	T93.09.P.BKT.AS.H.Gill.N	98.3	Unres.	19	Fry (PRSFH)
602_TSD_7CH	F. hercynium_AM265623	96.8	T108.09.B.BNT.SR.H.Fin.D	97.0	Unres.	20	Eggs (TSFH)
904_120106-1_WS210-2CH	F. hercynium_AM265623	96.3	T99.09.B.LAT.LS.H.Gill.D	96.6	Unres.	20	Water (WLSFH)
27_110512_WS210-2AH	F. limicola_AB075230	97.7	T141.10.B.RBT.MI.LM.W.Kid	98.6	XXIII	21	Water (PRSFH)
113_110519_WS1410-1DH	F. limicola_AB075230	97.7	T141.10.B.RBT.MI.LM.W.Kid	98.6	XXIII	21	Water (TSFH)
1010_120117-1_WS1310-1FP?C	F. limicola_AB075230	97.7	T141.10.B.RBT.MI.LM.W.Kid	98.6	XXIII	21	Water (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
1060_120416-1_WS910-1EH	F. limicola_AB075230	97.7	T141.10.B.RBT.MI.LM.W.Kid	98.6	XXIII	21	Water (WLSFH)
908_120106-1_WS7undBH	F.tiangeerense_EU036219.1	97.9	T141.10.B.RBT.MI.LM.W.Kid	98.3	XXIII	22	Water (WLSFH)
906_120106-1_WS4undAH	F.tiangeerense_EU036219.1	97.7	T141.10.B.RBT.MI.LM.W.Kid	98.3	XXIII	22	Water (WLSFH)
5_110512_WS1210-2AH	F. glaciei_DQ515962	97.6	T160.10.BNT.W.Gill.N	97.5	XXIII	23	Water (PRSFH)
129_110118_WS5undAC	F. glaciei_DQ515962	97.9	T160.10.BNT.W.Gill.N	97.7	XXIII	23	Water (TSFH)
971_120106-1_WS5undBC	F. glaciei_DQ515962	97.8	T160.10.BNT.W.Gill.N	97.8	XXIII	23	Water (WLSFH)
139_110104_WS110-3BC	F. glaciei_DQ515962	97.5	T160.10.BNT.W.Gill.N	97.8	XXIII	24	Water (PRSFH)
224_110104_WS1110-2HH	F. glaciei_DQ515962	97.5	T141.10.B.RBT.MI.LM.W.Kid	97.9	XXIII	24	Water (PRSFH)
35_110512_WS310-2FH	F.tiangeerense_EU036219.1	97.1	T141.10.B.RBT.MI.LM.W.Kid	97.6	XXIII	24	Water (PRSFH)
36_110512_WS310-2GH	F.tiangeerense_EU036219.1	97.2	T141.10.B.RBT.MI.LM.W.Kid	97.8	XXIII	24	Water (PRSFH)
268_110118_WS310-2CH	F. glaciei_DQ515962	97.2	T141.10.B.RBT.MI.LM.W.Kid	97.7	XXIII	24	Water (TSFH)
154_110104_WS1010-1CC	F. hibernum_L39067	98.3	T105.09.P.BNT.GC.H.Gill.D	98.0	XXIV	25	Water (PRSFH)
138_110104_WS110-2BC	F. hibernum_L39067	98.3	T105.09.P.BNT.GC.H.Gill.D	98.0	XXIV	25	Water (PRSFH)
143_110104_WS410-1AC	F. hibernum_L39067	98.3	T105.09.P.BNT.GC.H.Gill.D	98.0	XXIV	25	Water (PRSFH)
31_110512_WS310-2BH	F. hibernum_L39067	98.2	T105.09.P.BNT.GC.H.Gill.D	97.9	XXIV	25	Water (PRSFH)
691_PLND_4BC	F. hibernum_L39067	99.4	T1.05.BG.W.Gill.N	98.1	XI	26	Eggs (PRSFH)
957_120106-1_WS6undCC	F. saccharophilum AB473208.1	98.7	T6.06.LWF.W.Swimbladder	98.6	XII	27	Water (WLSFH)
1083_120416-1_WS7undBC	F. saccharophilum AB473208.1	98.6	T6.06.LWF.W.Swimbladder	98.4	XII	27	Water (WLSFH)
222_110104_WS1110-1DH	F. aquidurense_AM177392	99.2	T93.09.P.BKT.AS.H.Gill.N	99.0	Unres.	28	Water (PRSFH)
213_110104_WS10undBH	F. hercynium_AM265623	98.7	T6.06.LWF.W.Swimbladder	99.5	XII	29	Water (PRSFH)
19_110512_WS1410-3EH	F. hercynium_AM265623	98.7	T6.06.LWF.W.Swimbladder	99.5	XII	29	Water (PRSFH)
69_110512_T7N4BH	F. frigidimaris_AB183888	99.9	T33.08.P.BNT.WR.H.Kidney	99.8	XIX	30	Tools (PRSFH)
678_PLD_1AC	F. frigidimaris_AB183888	99.4	T33.08.P.BNT.WR.H.Kidney	99.3	XIX	30	Eggs (PRSFH)
684_PLD_8AC	F. frigidimaris_AB183888	99.1	T33.08.P.BNT.WR.H.Kidney	99.0	XIX	30	Eggs (PRSFH)
992_120117-1_WS310-2DC	F. aquidurense_AM177392	98.2	T157.10.BNT.W.Gill.N	99.5	Unres.	31	Water (TSFH)
1030_120117-1_WS310-2AH	F. aquidurense_AM177392	98.3	T157.10.BNT.W.Gill.N	99.6	Unres.	31	Water (TSFH)
990_120117-1_WS310-2AC	F. aquidurense_AM177392	98.4	T157.10.BNT.W.Gill.N	99.7	Unres.	31	Water (TSFH)
1088_120416-1_WS910-1CC	F. aquidurense_AM177392	98.3	T157.10.BNT.W.Gill.N	99.6	Unres.	31	Water (WLSFH)
323_110512_WS210-2CH	F. saccharophilum AB473208.1	98.2	T54.08.B.BNT.WR.H.Gill.D	98.5	Unres.	32	Water (PRSFH)
907_120106-1_WS7undAH	F. frigidimaris_AB183888	98.0	T10.07.P.BNT.GC.H.Brain.D	98.5	III	32	Water (WLSFH)
1007_120117-1_WS16unFPC	S12F	97.3	T57.08.B.COS.MI.W.SB	97.4	Unres.	33	Water (TSFH)
1025_120117-1_WS1510-2BH	S12F	97.6	T57.08.B.COS.MI.W.SB	97.7	Unres.	33	Water (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
989_120117-1_WS310-1FP?C	F.sinopsychrotolerans_FJ65447	99.6	T105.09.P.BNT.GC.H.Gill.D	97.3	XXIV	34	Water (TSFH)
137_110118_WS9undBC	F. limicola_AB075230	99.4	T105.09.P.BNT.GC.H.Gill.D	98.1	XXIV	35	Water (TSFH)
304_110118_WS910-1BC	F. limicola_AB075230	99.2	T105.09.P.BNT.GC.H.Gill.D	97.8	XXIV	35	Water (TSFH)
115_110519_WS1510-1BH	F.tiangeerense_EU036219.1	98.9	T105.09.P.BNT.GC.H.Gill.D	99.7	XXIV	36	Water (TSFH)
165_110104_WS210-1EH	F. psychrolimnae_AJ585428	99.1	T105.09.P.BNT.GC.H.Gill.D	97.9	XXIV	37	Water (PRSFH)
140_110104_WS210-1BC	F. psychrolimnae_AJ585428	99.4	T105.09.P.BNT.GC.H.Gill.D	98.1	XXIV	37	Water (PRSFH)
280_110118_WS9undCH	F. psychrolimnae_AJ585428	99.1	T105.09.P.BNT.GC.H.Gill.D	98.1	XXIV	37	Water (TSFH)
116_110519_WS1510-1CH	F. psychrolimnae_AJ585428	99.0	T105.09.P.BNT.GC.H.Gill.D	97.8	XXIV	37	Water (TSFH)
964_120106-1_WS1310-1BH	F. psychrolimnae_AJ585428	99.1	T3.05.LWF.W.Kidney.D	97.8	XXVIII	37	Water (WLSFH)
221_110104_WS1110-1CH	F. chungangense_EU924275	98.2	T69.09.B.RBT.MI.W.Kidney	98.2	IV	38	Water (PRSFH)
21_110512_WS110-2BH	F. chungangense_EU924275	98.2	T69.09.B.RBT.MI.W.Kidney	98.4	IV	38	Water (PRSFH)
322_110512_WS510-1BH	F. chungangense_EU924275	98.2	T69.09.B.RBT.MI.W.Kidney	98.3	IV	38	Water (PRSFH)
15_110512_WS1410-3AH	F. aquidurense_AM177392	98.6	T132.10.WAE.W.Kidney.N	99.1	Unres.	39	Water (PRSFH)
16_110512_WS1410-3BH	F. aquidurense_AM177392	98.6	T132.10.WAE.W.Kidney.N	99.1	Unres.	39	Water (PRSFH)
203_110104_WS810-1BH	F. saccharophilum AB473208.1	98.2	T159.10.BNT.W.Gill.N	99.4	Unres.	40	Water (PRSFH)
33_110512_WS310-2DH	F. saccharophilum AB473208.1	98.0	T159.10.BNT.W.Gill.N	99.3	Unres.	40	Water (PRSFH)
106_110519_WS12undAH	F. hercynium_AM265623	98.8	T157.10.BNT.W.Gill.N	98.6	Unres.	41	Water (TSFH)
1039_120117-1_WS11undCC	F. hercynium_AM265623	98.8	T157.10.BNT.W.Gill.N	98.7	Unres.	41	Water (TSFH)
1002_120117-1WS1510-1FPC	F. hercynium_AM265623	96.6	T157.10.BNT.W.Gill.N	96.4	Unres.	41	Water (TSFH)
22_110512_WS510-1AH	F.succinicans_AM230492.1	95.9	T158.10.BNT.W.Gill.N	96.9	XV	42	Water (PRSFH)
42_110512_WS810-1BH	F.reichenbachii_AM177616.1	95.4	T156.10.BNT.W.Gill.N	95.1	XVI	42	Water (PRSFH)
166_110104_WS210-2E?H	F.succinicans_AM230492.1	99.5	T158.10.BNT.W.Gill.N	98.7	XV	43	Water (PRSFH)
642_TSND_30AH	F.succinicans_AM230492.1	99.6	T158.10.BNT.W.Gill.N	98.4	XV	43	Eggs (TSFH)
1111_120515-1_WS1610-1BH	F.succinicans_AM230492.1	99.0	T158.10.BNT.W.Gill.N	98.8	XV	44	Water (TSFH)
1107_120515-1_WS1310-1CH	F.succinicans_AM230492.1	99.0	T158.10.BNT.W.Gill.N	98.8	XV	44	Water (TSFH)
1085_120416-1_WS8undDC	F.succinicans_AM230492.1	99.1	T158.10.BNT.W.Gill.N	99.0	XV	44	Water (WLSFH)
973_120106-1_WS6undAC	F.succinicans_AM230492.1	98.4	T158.10.BNT.W.Gill.N	99.3	XV	45	Water (WLSFH)
898_120106-1_WS6undAH	F.succinicans_AM230492.1	98.3	T158.10.BNT.W.Gill.N	99.2	XV	45	Water (WLSFH)
905_120106-1_WS3undAH	F.succinicans_AM230492.1	98.3	T158.10.BNT.W.Gill.N	99.2	XV	45	Water (WLSFH)
91_110519_T3N1BH	F.aquatile_M62797.1	97.3	T8.06.B.BNT.SE.H.SB	96.6	IV	46	Tools (TSFH)
274_110118_WS810-2B?H	F.aquatile_M62797.1	97.2	T148.10.BKT.W.Gill.N	96.4	I	46	Water (TSFH)
123_110118_WS11undDC	F.aquatile_M62797.1	98.2	T8.06.B.BNT.SE.H.SB	95.9	IV	47	Water (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
1000_120117-1WS1210-1FPC	F.aquatile_M62797.1	98.3	T8.06.B.BNT.SE.H.SB	96.0	IV	47	Water (TSFH)
998_120117-1_WS1110-1DC	F.aquatile_M62797.1	98.3	T8.06.B.BNT.SE.H.SB	96.0	IV	47	Water (TSFH)
996_120117-1_WS11undFPC	F.aquatile_M62797.1	98.2	T8.06.B.BNT.SE.H.SB	95.9	IV	47	Water (TSFH)
485_101012-1_23FEfemC	F. terrae_EF117329	97.4	T79.09.SMB.W.Gill.D.M	99.9	XVII	48	Brood (LMRW)
751_111005-1_30maleFCH	F. terrae_EF117329	97.4	T52.08.B.CHS.LM.W.Kidney	99.8	XVII	48	Brood (LMRW)
212_110104_WS10undAH	F. saccharophilum AB473208.1	98.8	T132.10.WAE.W.Kidney.N	99.1	Unres.	49	Water (PRSFH)
53_110512_WS1010-2CH	F. saccharophilum AB473208.1	98.8	T132.10.WAE.W.Kidney.N	99.1	Unres.	49	Water (PRSFH)
3_110512_WS1110-2CH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (PRSFH)
332_110512_P121-125EH	C. chaponense_GU345046.1	99.2	T115.09.B.CHS.MI.SRW	99.6	XXXVIII	50	Fingerlings (PRSFH)
93_110519_T3B1AH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Tools (TSFH)
131_110118_WS310-1B?C	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
269_110118_WS310-2DH	C. chaponense_GU345046.1	98.4	T115.09.B.CHS.MI.SRW	98.8	XXXVIII	50	Water (TSFH)
289_110118_WS1110-1BH	C. chaponense_GU345046.1	99.0	T115.09.B.CHS.MI.SRW	99.4	XXXVIII	50	Water (TSFH)
127_110118_WS1210-1DC	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
126_110118_WS12undDC	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
110_110519_WS1310-1BH	C. chaponense_GU345046.1	99.3	T115.09.B.CHS.MI.SRW	99.7	XXXVIII	50	Water (TSFH)
119_110519_WS1610-1CH	C. chaponense_GU345046.1	99.3	T115.09.B.CHS.MI.SRW	99.7	XXXVIII	50	Water (TSFH)
340_110519_WS1410-1BH	C. chaponense_GU345046.1	99.0	T115.09.B.CHS.MI.SRW	99.4	XXXVIII	50	Water (TSFH)
107_110519_WS12undBH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (TSFH)
991_120117-1_WS310-2CC	C. chaponense_GU345046.1	98.2	T115.09.B.CHS.MI.SRW	98.6	XXXVIII	50	Water (TSFH)
1006_120117-1_WS1610-2EC	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
997_120117-1_WS1110-1AC	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1027_120117-1_WS1610-1AH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (TSFH)
1037_120117-1_WS1210-1AH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (TSFH)
1035_120117-1_WS1110-1AH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1024_120117-1_WS1510-2AH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (TSFH)
1021_120117-1_WS1410-1AH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (TSFH)
1019_120117-1_WS1310-1AH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1031_120117-1_WS310-2BH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (TSFH)
1106_120515-1_WS1310-1BH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1097_120515-1_WS310-1BH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
1105_120515-1_WS1310-1AH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1103_120515-1_WS1210-1BH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1101_120515-1_WS11undBH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
1109_120515-1_WS1510-1BH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (TSFH)
983_120106-1_WS1010-1BC	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (WLSFH)
1051_120416-1_WS8undAH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (WLSFH)
1048_120416-1_WS7undAH	C. chaponense_GU345046.1	99.4	T115.09.B.CHS.MI.SRW	99.8	XXXVIII	50	Water (WLSFH)
1056_120416-1_WS910-1AH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (WLSFH)
1061_120416-1_WS1010-2AH	C. chaponense_GU345046.1	99.5	T115.09.B.CHS.MI.SRW	99.9	XXXVIII	50	Water (WLSFH)
426_101012-1_16BRfemH	C. chaponense_GU345046.1	97.3	T115.09.B.CHS.MI.SRW	97.7	XXXVIII	51	Brood (LMRW)
424_101012-1_16femH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.6	XXXVIII	51	Brood (LMRW)
422_101012-1_47AmaleH	C. chaponense_GU345046.1	97.1	T115.09.B.CHS.MI.SRW	97.5	XXXVIII	51	Brood (LMRW)
421_101012-1_36A?maleH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.5	XXXVIII	51	Brood (LMRW)
420_101012-1_32AmaleH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.5	XXXVIII	51	Brood (LMRW)
489_101012-1_15OF-1lowC	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.6	XXXVIII	51	Brood (LMRW)
583_101012-1_15CDEH	C. chaponense_GU345046.2	97.2	T115.09.B.CHS.MI.SRW	97.6	XXXVIII	51	Brood (LMRW)
419_101012-1_29A?maleH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.6	XXXVIII	51	Brood (LMRW)
582_101012-1_12CDEH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.6	XXXVIII	51	Brood (LMRW)
417_101012-1_14AmaleH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.5	XXXVIII	51	Brood (LMRW)
474_101012-1_10AmaleC	C. chaponense_GU345046.2	97.3	T115.09.B.CHS.MI.SRW	97.7	XXXVIII	51	Brood (LMRW)
816_111005-1_5maleMC	C. chaponense_GU345046.1	97.0	T115.09.B.CHS.MI.SRW	97.4	XXXVIII	51	Brood (LMRW)
812_111005-1_15femOFC	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.5	XXXVIII	51	Brood (LMRW)
845_111005-1_6OFH	C. chaponense_GU345046.1	97.5	T115.09.B.CHS.MI.SRW	97.9	XXXVIII	51	Brood (LMRW)
790_111005-1_20OFH	C. chaponense_GU345046.1	97.2	T115.09.B.CHS.MI.SRW	97.6	XXXVIII	51	Brood (LMRW)
258_110104_WS1210-1AC	C. piscicola_EU869190.1	99.7	T63.09.P.BNT.WR.Fin.D	99.7	XL	52	Water (PRSFH)
58_110512_T2N1AH	C. piscicola_EU869190.1	99.4	T63.09.P.BNT.WR.Fin.D	99.4	XL	52	Tools (PRSFH)
63_110512_T2B2AH	C. piscicola_EU869190.1	99.5	T63.09.P.BNT.WR.Fin.D	99.4	XL	52	Tools (PRSFH)
66_110512_T3B2BH	C. piscicola_EU869190.1	99.7	T63.09.P.BNT.WR.Fin.D	99.8	XL	52	Tools (PRSFH)
67_110512_T7N1AH	C. piscicola_EU869190.1	99.7	T63.09.P.BNT.WR.Fin.D	99.5	XL	52	Tools (PRSFH)
68_110512_T7N4AH	C. piscicola_EU869190.2	99.5	T63.09.P.BNT.WR.Fin.D	99.4	XL	52	Tools (PRSFH)
748_111005-1_6maleDRCH	C. piscicola_EU869190.1	99.6	T63.09.P.BNT.WR.Fin.D	99.5	XL	52	Brood (LMRW)
82_110519_P76-80GDH	C.antarctica AY553293.1	99.0	T115.09.B.CHS.MI.SRW	98.2	XXXVIII	53	Fingerlings (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
85_110519_P16-20GH	C.antarctica AY553293.1	99.0	T115.09.B.CHS.MI.SRW	98.3	XXXVIII	53	Fingerlings (TSFH)
86_110519_P76-80GAH	C.antarctica AY553293.2	98.9	T115.09.B.CHS.MI.SRW	98.2	XXXVIII	53	Fingerlings (TSFH)
87_110519_P76-80GBH	C. yonginense_GQ259742.1	98.4	T115.09.B.CHS.MI.SRW	98.3	XXXVIII	53	Fingerlings (TSFH)
89_110519_T1B2CH	C.antarctica AY553293.1	98.8	T115.09.B.CHS.MI.SRW	98.0	XXXVIII	53	Tools (TSFH)
96_110519_T3AP1BH	C.antarctica AY553293.1	99.0	T115.09.B.CHS.MI.SRW	98.3	XXXVIII	53	Tools (TSFH)
1001_120117-1_WS1210-1CC	C.antarctica AY553293.1	99.0	T115.09.B.CHS.MI.SRW	98.3	XXXVIII	53	Water (TSFH)
105_110519_WS1110-1AH	C. jeonii_AY553294	97.8	T115.09.B.CHS.MI.SRW	97.2	XXXVIII	54	Water (TSFH)
828_111012-1_15NDC	C. yonginense_GQ259742.1	97.6	T115.09.B.CHS.MI.SRW	97.9	XXXVIII	54	Brood (SRW)
586_101012-1_19CDEH	C. yonginense_GQ259742.1	98.5	T115.09.B.CHS.MI.SRW	97.8	XXXVIII	55	Brood (LMRW)
842_111005-1_18NDH	C. yonginense_GQ259742.1	98.5	T60.09.P.RBT.EL.H.Kidney	97.6	XXXVIII	55	Brood (LMRW)
793_111012-1_22OFH	C. yonginense_GQ259742.1	96.6	T115.09.B.CHS.MI.SRW.W	96.2	XXXVIII	56	Brood (SRW)
839_111005-1_14NDH	C. yonginense_GQ259742.1	96.7	T115.09.B.CHS.MI.SRW.W	96.3	XXXVIII	56	Brood (LMRW)
918_120106-1_WS9undDH	T28.08.P.COS.MI.H.Kidney.D.M	97.9	T28.08.P.COS.MI.H.Kidney	97.9	Unres.	57	Water (WLSFH)
1045_120416-1_WS5undAH	C. vrystaatense_AJ871397	97.3	T28.08.P.COS.MI.H.Kidney	97.1	Unres.	57	Water (WLSFH)
215_110104_WS10undDH	C. viscerum_FR871426.1	99.0	T39.08.SCU.W.Gill.N	99.2	Unres.	58	Water (PRSFH)
1009_120117-1_WS810-1CC	C. indoltheticum_AY468448.1	99.8	T72.09.LHR.H.Kidney.D	98.7	Unres.	59	Water (TSFH)
866_111130-2_3NDCEMH	C. indoltheticum_AY468448.1	99.9	T72.09.LHR.H.Kidney.D	98.8	Unres.	59	Eggs (WLSFH)
746_111005-1_4maleChryH	C. indoltheticum_AY468448.1	99.8	T72.09.LHR.H.Kidney.D	98.7	Unres.	59	Brood (LMRW)
814_111005-1_13femSplC	C. indoltheticum_AY468448.1	99.3	T72.09.LHR.H.Kidney.D	98.4	Unres.	60	Brood (LMRW)
841_111005-1_16NDH	C. indoltheticum_AY468448.1	99.4	T72.09.LHR.H.Kidney.D	98.3	Unres.	60	Brood (LMRW)
648_PLND_3BH	C. indoltheticum_AY468448.1	98.2	T72.09.LHR.H.Kidney.D	98.1	Unres.	61	Eggs (PRSFH)
851_111012-1_14NDH	C. indoltheticum_AY468448.1	98.8	T72.09.LHR.H.Kidney.D	99.2	Unres.	61	Brood (SRW)
738_111005-1_10femBYMH	C. indoltheticum_AY468448.1	98.6	T72.09.LHR.H.Kidney.D	98.4	Unres.	61	Brood (LMRW)
98_110519_R2B1AH	T68.09.P.LAT.MA.H.Kidney.D	97.2	T68.09.P.LAT.MA.H.Kidney	97.2	XXXIV	62	Tools (TSFH)
337_110519_T1B2BH	T68.09.P.LAT.MA.H.Kidney.D	97.1	T68.09.P.LAT.MA.H.Kidney	97.1	XXXIV	62	Tools (TSFH)
1071_120416-1_R19N1AH	T68.09.P.LAT.MA.H.Kidney.D	98.3	T68.09.P.LAT.MA.H.Kidney	98.3	XXXIV	63	Water (WLSFH)
1074_120416-1_R19B1AH	T68.09.P.LAT.MA.H.Kidney.D	98.6	T68.09.P.LAT.MA.H.Kidney	98.5	XXXIV	63	Tools (WLSFH)
342_110519_T1N1BH	C. xinjiangense_DQ166169	96.2	T63.09.P.BNT.WR.Fin.D	95.3	XL	64	Tools (TSFH)
1115_120515-1_R15N1AH	C. xinjiangense_DQ166169	96.4	T63.09.P.BNT.WR.Fin.D	95.7	XL	64	Tools (TSFH)
844_111005-1_27maleSplH	C. piscicola_EU869190.1	95.9	T63.09.P.BNT.WR.Fin.D	95.7	XL	64	Brood (LMRW)
945_120106-1_P6NDPLH	F. columnare M58781.2	99.0	T10.07.P.BNT.GC.H.Brain.D	99.5	III	F.col	Fry (WLSFH)
940_120106-1_P16DPLEH	F. columnare M58781.2	99.1	T10.07.P.BNT.GC.H.Brain.D	99.7	III	F.col	Fry (WLSFH)

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173_110104_WS310-1FH	F.succinicans_AM230492.1	98.4	T158.10.BNT.W.Gill.N	99.5	XV	U3A	Water (PRSFH)
218_110104_WS10undGH	F.succinicans_AM230492.1	98.3	T158.10.BNT.W.Gill.N	99.5	XV	U3A	Water (PRSFH)
142_110104_WS510-1AC	F.succinicans_AM230492.1	98.8	T158.10.BNT.W.Gill.N	100.0	XV	U3A	Water (PRSFH)
141_110104_WS310-2AC	F.succinicans_AM230492.1	98.7	T158.10.BNT.W.Gill.N	99.8	XV	U3A	Water (PRSFH)
180_110104_WS410-1FH	F.succinicans_AM230492.1	98.7	T158.10.BNT.W.Gill.N	99.8	XV	U3A	Water (PRSFH)
254_110104_110-2CC	F.succinicans_AM230492.1	98.4	T158.10.BNT.W.Gill.N	99.7	XV	U3A	Water (PRSFH)
20_110512_WS110-2AH	F.succinicans_AM230492.1	98.7	T158.10.BNT.W.Gill.N	99.9	XV	U3A	Water (PRSFH)
23_110512_WS510-1CH	F.succinicans_AM230492.1	98.7	T158.10.BNT.W.Gill.N	99.8	XV	U3A	Water (PRSFH)
30_110512_WS310-2AH	F.succinicans_AM230492.1	98.5	T158.10.BNT.W.Gill.N	99.6	XV	U3A	Water (PRSFH)
46_110512_WS910-1AH	F.succinicans_AM230492.1	98.6	T158.10.BNT.W.Gill.N	99.7	XV	U3A	Water (PRSFH)
321_110512_WS1310-2AH	F.succinicans_AM230492.1	98.6	T158.10.BNT.W.Gill.N	99.7	XV	U3A	Water (PRSFH)
926_120106-1_WS1310-1CH	F.succinicans_AM230492.1	98.1	T158.10.BNT.W.Gill.N	99.1	XV	U3A	Water (WLSFH)
1066_120416-1_WS1310-2CH	F.succinicans_AM230492.1	98.7	T158.10.BNT.W.Gill.N	99.5	XV	U3A	Water (WLSFH)
1065_120416-1_WS1310-2BH	F.succinicans_AM230492.1	98.7	T158.10.BNT.W.Gill.N	99.8	XV	U3A	Water (WLSFH)
1044_120416-1_WS3undAH	F.succinicans_AM230492.1	98.2	T158.10.BNT.W.Gill.N	99.3	XV	U3A	Water (WLSFH)
220_110104_WS1110-1BH	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.5	Unres.	U3B	Water (PRSFH)
199_110104_WS710-3AH	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.6	Unres.	U3B	Water (PRSFH)
208_110104_WS910-1AH	T16F	98.5	T108.09.B.BNT.SR.H.Fin.D	99.4	Unres.	U3B	Water (PRSFH)
214_110104_WS10undCH	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.6	Unres.	U3B	Water (PRSFH)
331_110512_T7APBH	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.7	Unres.	U3B	Tools (PRSFH)
654_PLD_20A?H	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.7	Unres.	U3B	Eggs (PRSFH)
653_PLD_14A?H	T16F	98.5	T108.09.B.BNT.SR.H.Fin.D	99.5	Unres.	U3B	Eggs (PRSFH)
649_PLD_1AH	T16F	98.3	T108.09.B.BNT.SR.H.Fin.D	99.3	Unres.	U3B	Eggs (PRSFH)
695_PLD_18AC	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.5	Unres.	U3B	Eggs (PRSFH)
700_PLD_38AC	T16F	98.5	T108.09.B.BNT.SR.H.Fin.D	99.5	Unres.	U3B	Eggs (PRSFH)
686_PLD_12A?C	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.5	Unres.	U3B	Eggs (PRSFH)
651_PLD_4AH	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.5	Unres.	U3B	Eggs (PRSFH)
698_PLD_31A?C	T16F	98.5	T108.09.B.BNT.SR.H.Fin.D	99.3	Unres.	U3B	Eggs (PRSFH)
681_PLD_4AC	T16F	98.4	T108.09.B.BNT.SR.H.Fin.D	99.4	Unres.	U3B	Eggs (PRSFH)
238_110104_P6CH	T16F	98.6	T108.09.B.BNT.SR.H.Fin.D	99.6	Unres.	U3B	Fry (PRSFH)
244_110104_P15C?H	F.pectinovorum_AM230490.1	98.6	T108.09.B.BNT.SR.H.Fin.D	99.2	Unres.	U3B	Fry (PRSFH)
655_TSD_41CC	F.pectinovorum_AM230490.1	97.8	T108.09.B.BNT.SR.H.Fin.D	98.4	Unres.	U3B	Eggs (TSFH)

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740_111005-1_13femMFJH	T16F	98.3	T108.09.B.BNT.SR.H.Fin.D	99.4	Unres.	U3B	Brood (LMRW)
750_111005-1_24maleFCH	F.pectinovorum_AM230490.1	98.6	T108.09.B.BNT.SR.H.Fin.D	99.2	Unres.	U3B	Brood (LMRW)
749_111005-1_14maleDFJH	T16F	98.7	T108.09.B.BNT.SR.H.Fin.D	99.7	Unres.	U3B	Brood (LMRW)
202_110104_WS810-1AH	F. aquidurense_AM177392	98.6	T108.09.B.BNT.SR.H.Fin.D	98.7	Unres.	U3C	Water (PRSFH)
200_110104_WS710-3BH	F. aquidurense_AM177392	98.5	T108.09.B.BNT.SR.H.Fin.D	98.8	Unres.	U3C	Water (PRSFH)
209_110104_WS910-1BH	F. aquidurense_AM177392	98.6	T108.09.B.BNT.SR.H.Fin.D	98.8	Unres.	U3C	Water (PRSFH)
262_110104_WS1210-2EC	F. aquidurense_AM177392	98.6	T108.09.B.BNT.SR.H.Fin.D	98.8	Unres.	U3C	Water (PRSFH)
259_110104_WS1210-1BC	F. aquidurense_AM177392	98.4	T108.09.B.BNT.SR.H.Fin.D	98.6	Unres.	U3C	Water (PRSFH)
680_PLD_2BC	F. aquidurense_AM177392	98.6	T108.09.B.BNT.SR.H.Fin.D	98.7	Unres.	U3C	Eggs (PRSFH)
693_PLND_5DC	F. aquidurense_AM177392	98.3	T108.09.B.BNT.SR.H.Fin.D	98.8	Unres.	U3C	Eggs (PRSFH)
699_PLD_33AC	F. columnare M58781.2	98.6	T166.10.P.RBT.MI.H.Kidney	99.1	III	U3C	Eggs (PRSFH)
696_PLD_20BC	F. columnare M58781.2	98.5	T166.10.P.RBT.MI.H.Kidney	99.1	III	U3C	Eggs (PRSFH)
685_PLD_10BC	F. columnare M58781.2	98.4	T10.07.P.BNT.GC.H.Brain.D	99.0	III	U3C	Eggs (PRSFH)
241_110104_P9CH	F. aquidurense_AM177392	98.5	T108.09.B.BNT.SR.H.Fin.D	98.8	Unres.	U3C	Fry (PRSFH)
237_110104_P5CH	F. aquidurense_AM177392	98.6	T108.09.B.BNT.SR.H.Fin.D	98.9	Unres.	U3C	Fry (PRSFH)
248_110104_P8DisH	F. aquidurense_AM177392	98.3	T108.09.B.BNT.SR.H.Fin.D	98.6	Unres.	U3C	Fry (PRSFH)
266_110104_P16CDisC	F. aquidurense_AM177392	98.3	T108.09.B.BNT.SR.H.Fin.D	98.7	Unres.	U3C	Fry (PRSFH)
265_110104_P14ADisC	F. aquidurense_AM177392	98.5	T108.09.B.BNT.SR.H.Fin.D	98.8	Unres.	U3C	Fry (PRSFH)
264_110104_P5BDisC	F. aquidurense_AM177392	98.5	T108.09.B.BNT.SR.H.Fin.D	98.9	Unres.	U3C	Fry (PRSFH)
955_120106-1_WS1010-1DC	F. columnare M58781.2	98.3	T10.07.P.BNT.GC.H.Brain.D	98.8	III	U3C	Water (WLSFH)
916_120106-1_WS9undBH	F.pectinovorum_AM230490.1	98.5	T10.07.P.BNT.GC.H.Brain.D	98.9	III	U3C	Water (WLSFH)
32_110512_WS310-2CH	T68.09.P.LAT.MA.H.Kidney.D	99.7	T68.09.P.LAT.MA.H.Kidney	99.6	XXXIV	U3D	Water (PRSFH)
61_110512_T2B1AH	T62.09.P.BNT.WR.Fin.D	99.7	T62.09.P.BNT.WR.Fin.D	99.7	XXXIV	U3D	Tools (PRSFH)
62_110512_T2B1CH	T68.09.P.LAT.MA.H.Kidney.D	99.8	T68.09.P.LAT.MA.H.Kidney	99.8	XXXIV	U3D	Tools (PRSFH)
620_TSND_1BH	T68.09.P.LAT.MA.H.Kidney.D	99.7	T68.09.P.LAT.MA.H.Kidney	99.7	XXXIV	U3D	Eggs (TSFH)
623_TSND_5BH	T62.09.P.BNT.WR.Fin.D	99.4	T130.10.P.BKT.AS.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
627_TSND_13BH	T68.09.P.LAT.MA.H.Kidney.D	99.5	T68.09.P.LAT.MA.H.Kidney	99.5	XXXIV	U3D	Eggs (TSFH)
668_TSND_2AC	T68.09.P.LAT.MA.H.Kidney.D	99.6	T68.09.P.LAT.MA.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
634_TSND_23B?H	T68.09.P.LAT.MA.H.Kidney.D	99.4	T68.09.P.LAT.MA.H.Kidney	99.4	XXXIV	U3D	Eggs (TSFH)
667_TSND_1AC	T68.09.P.LAT.MA.H.Kidney.D	99.7	T68.09.P.LAT.MA.H.Kidney	99.7	XXXIV	U3D	Eggs (TSFH)
641_TSND_29BH	T68.09.P.LAT.MA.H.Kidney.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.3	XXXIV	U3D	Eggs (TSFH)
631_TSND_19A?H	T68.09.P.LAT.MA.H.Kidney.D	99.4	T68.09.P.LAT.MA.H.Kidney	99.4	XXXIV	U3D	Eggs (TSFH)

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637_TSND_25BH	T68.09.P.LAT.MA.H.Kidney.D	99.5	T68.09.P.LAT.MA.H.Kidney	99.5	XXXIV	U3D	Eggs (TSFH)
636_TSND_24B?H	T68.09.P.LAT.MA.H.Kidney.D	99.5	T68.09.P.LAT.MA.H.Kidney	99.5	XXXIV	U3D	Eggs (TSFH)
670_TSND_3AC	T68.09.P.LAT.MA.H.Kidney.D	99.6	T68.09.P.LAT.MA.H.Kidney	99.5	XXXIV	U3D	Eggs (TSFH)
675_TSND_6AC	T68.09.P.LAT.MA.H.Kidney.D	99.7	T68.09.P.LAT.MA.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
673_TSND_5AC	T68.09.P.LAT.MA.H.Kidney.D	99.7	T68.09.P.LAT.MA.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
672_TSND_4AC	T68.09.P.LAT.MA.H.Kidney.D	99.7	T68.09.P.LAT.MA.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
887_111130-1_9NDPYFC	T62.09.P.BNT.WR.Fin.D	99.4	T130.10.P.BKT.AS.H.Kidney	99.7	XXXIV	U3D	Eggs (TSFH)
888_111130-1_29NDPYFC	T62.09.P.BNT.WR.Fin.D	99.4	T130.10.P.BKT.AS.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
886_111130-1_5NDPYFC	T62.09.P.BNT.WR.Fin.D	99.4	T130.10.P.BKT.AS.H.Kidney	99.8	XXXIV	U3D	Eggs (TSFH)
855_111130-1_1NDLCEH	T62.09.P.BNT.WR.Fin.D	99.3	T130.10.P.BKT.AS.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
854_111130-1_6NDLCEH	T62.09.P.BNT.WR.Fin.D	99.3	T130.10.P.BKT.AS.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
864_111130-1_29NDLCE?H	T62.09.P.BNT.WR.Fin.D	99.4	T130.10.P.BKT.AS.H.Kidney	99.7	XXXIV	U3D	Eggs (TSFH)
862_111130-1_15NDLCE?H	T62.09.P.BNT.WR.Fin.D	99.3	T130.10.P.BKT.AS.H.Kidney	99.6	XXXIV	U3D	Eggs (TSFH)
319_110118_P6UVEDisC	T62.09.P.BNT.WR.Fin.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.5	XXXIV	U3D	Fry (TSFH)
988_120117-1_P16DPYEC	T62.09.P.BNT.WR.Fin.D	99.3	T130.10.P.BKT.AS.H.Kidney	99.5	XXXIV	U3D	Fry (TSFH)
272_110118_WS810-1BH	T62.09.P.BNT.WR.Fin.D	99.1	T130.10.P.BKT.AS.H.Kidney	99.4	XXXIV	U3D	Water (TSFH)
296_110118_WS810-1AC	T62.09.P.BNT.WR.Fin.D	99.1	T130.10.P.BKT.AS.H.Kidney	99.4	XXXIV	U3D	Water (TSFH)
307_110118_WS1010-2BC	T62.09.P.BNT.WR.Fin.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.5	XXXIV	U3D	Water (TSFH)
306_110118_WS1010-1AC	T62.09.P.BNT.WR.Fin.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.5	XXXIV	U3D	Water (TSFH)
286_110118_WS1010-1E?H	T62.09.P.BNT.WR.Fin.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.5	XXXIV	U3D	Water (TSFH)
283_110118_WS10undCH	T62.09.P.BNT.WR.Fin.D	99.1	T130.10.P.BKT.AS.H.Kidne	99.4	XXXIV	U3D	Water (TSFH)
285_110118_1010-1CH	T62.09.P.BNT.WR.Fin.D	99.4	T130.10.P.BKT.AS.H.Kidney	99.7	XXXIV	U3D	Water (TSFH)
980_120106-1_WS9undCC	T68.09.P.LAT.MA.H.Kidney.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.8	XXXIV	U3D	Water (WLSFH)
919_120106-1_WS1010-1AH	T68.09.P.LAT.MA.H.Kidney.D	99.3	T130.10.P.BKT.AS.H.Kidney	99.8	XXXIV	U3D	Water (WLSFH)
1055_120416-1_WS8undEH	T68.09.P.LAT.MA.H.Kidney.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.8	XXXIV	U3D	Water (WLSFH)
934_120106-1_P5DMLCH	T62.09.P.BNT.WR.Fin.D	99.2	T130.10.P.BKT.AS.H.Kidney	99.8	XXXIV	U3D	Fry (WLSFH)
74_110512_37LH	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Fingerling (PRSFH)
857_111130-1_11NDPYH	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Eggs (TSFH)
856_111130-1_11NDCEH	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Eggs (TSFH)
863_111130-1_22NDPYH	C. piscium_AM040439	98.6	T31.08.P.RBT.MI.H.Kidney	99.6	XXXVII	U3E	Eggs (TSFH)
861_111130-1_14NDPYCH	C. piscium_AM040439	98.9	T31.08.P.RBT.MI.H.Kidney	99.9	XXXVII	U3E	Eggs (TSFH)

Isolate Number	Closest Described Relative	% Similarity	Closest Objective I Relative (% 16S rDNA Similarity)	% Similarity	AAHL Cluster	O-3 Cluster	Source
917_120106-1_WS9undCH	C. scophthalmum_AJ271009	98.7	T82.09.LHR.H.Kidney.D	99.7	XXXVII	U3E	Water (WLSFH)
913_120106-1_WS8undDH	C. piscium_AM040439	98.4	T31.08.P.RBT.MI.H.Kidney	99.4	XXXVII	U3E	Water (WLSFH)
1054_120416-1_WS8undDH	C. scophthalmum_AJ271009	97.9	T31.08.P.RBT.MI.H.Kidney	98.6	XXXVII	U3E	Water (WLSFH)
889_111130-2_1NDPYFC	C. piscium_AM040439	97.9	T82.09.LHR.H.Kidney.D	98.9	XXXVII	U3E	Eggs (WLSFH)
868_111130-2_17NDCEH	C. piscium_AM040439	98.7	T31.08.P.RBT.MI.H.Kidney	99.7	XXXVII	U3E	Eggs (WLSFH)
865_111130-2_1NDCEH	C. piscium_AM040439	98.7	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Eggs (WLSFH)
894_111130-2_19NDPYF?C	C. piscium_AM040439	98.7	T82.09.LHR.H.Kidney.D	99.9	XXXVII	U3E	Eggs (WLSFH)
893_111130-2_17NDPYFC	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Eggs (WLSFH)
949_120106-1_P2NDYCH	C. piscium_AM040439	98.7	T82.09.LHR.H.Kidney.D	99.8	XXXVII	U3E	Fry (WLSFH)
947_120106-1_P11NDPYC?H	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Fry (WLSFH)
946_120106-1_P7NDAH	C. piscium_AM040439	98.7	T82.09.LHR.H.Kidney.D	99.8	XXXVII	U3E	Fry (WLSFH)
932_120106-1_P3DMLCH	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Fry (WLSFH)
944_120106-1_P5NDPYC?H	C. piscium_AM040440	98.7	T31.08.P.RBT.MI.H.Kidney	99.7	XXXVII	U3E	Fry (WLSFH)
942_120106-1_P1NDPYCH	C. piscium_AM040439	98.8	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Fry (WLSFH)
953_120106-1_P11NDPPBC	C. piscium_AM040439	98.7	T31.08.P.RBT.MI.H.Kidney	99.8	XXXVII	U3E	Fry (WLSFH)
952_120106-1_P2NDPYCC	C. piscium_AM040439	98.8	T82.09.LHR.H.Kidney.D	100.0	XXXVII	U3E	Fry (WLSFH)
939_120106-1_P13DPYEH	C. piscium_AM040439	98.7	T82.09.LHR.H.Kidney.D	99.9	XXXVII	U3E	Fry (WLSFH)
192_110104_WS610-1BH	F. aquidurense_AM177392	99.1	T6.06.LWF.W.SB	98.9	XII	Unres.	Water (PRSFH)
219_110104_WS1110-1AH	F. frigidimaris_AB183888	98.9	T93.09.P.BKT.AS.H.Gill.N	99.2	Unres.	Unres.	Water (PRSFH)
228_110104_WS1210-2DH	F. glaciei_DQ515962	97.9	T1.05.BG.W.Gill.N	98.1	XI	Unres.	Water (PRSFH)
146_110104_WS410-2CC	S12F	98.4	T18.08.P.BNT.GC.H.Gill.D.M	98.5	II	Unres.	Water (PRSFH)
187_110104_WS510-1E?H	F. aquidurense_AM177392	98.6	T99.09.B.LAT.LS.H.Gill.D	98.6	Unres.	Unres.	Water (PRSFH)
227_110104_WS1210-2CH	F. aquidurense_AM177392	98.9	T8.06.B.BNT.SE.H.SB	98.8	IV	Unres.	Water (PRSFH)
225_110104_WS1210-2AH	F. saccharophilum AB473208.1	98.9	T69.09.B.RBT.MI.W.Kidney	99.2	IV	Unres.	Water (PRSFH)
181_110104_WS410-2F?H	F. glaciei_DQ515962	97.7	T141.10.B.RBT.MI.LM.W.Kid	98.2	XXIII	Unres.	Water (PRSFH)
193_110104_WS610-1CH	F. glaciei_DQ515962	97.4	T159.10.BNT.W.Gill.N	97.7	Unres.	Unres.	Water (PRSFH)
145_110104_WS310-3CC	F.dankookense_GU295970	97.7	T8.06.B.BNT.SE.H.SB	95.9	IV	Unres.	Water (PRSFH)
2_110512_WS1110-2BH	F. frigidimaris_AB183888	98.0	T108.09.B.BNT.SR.H.Fin.D	98.5	Unres.	Unres.	Water (PRSFH)
11_110512_WS1310-2BH	F. aquidurense_AM177392	99.4	T93.09.P.BKT.AS.H.Gill.N	98.7	Unres.	Unres.	Water (PRSFH)
26_110512_WS110-3FH	F. glaciei_DQ515962	97.6	T160.10.BNT.W.Gill.N	97.6	XXIII	Unres.	Water (PRSFH)
28_110512_WS210-2BH	C. yonginense_GQ259742.1	98.9	T115.09.B.CHS.MI.SRW.W	97.8	XXXVIII	Unres.	Water (PRSFH)
34_110512_WS310-2EH	F. saccharophilum AB473208.1	99.1	T132.10.WAE.W.Kidney.N	99.2	Unres.	Unres.	Water (PRSFH)

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38_110512_WS410-1AH	F. frigidimaris_AB183888	98.4	T99.09.B.LAT.LS.H.Gill.D	98.9	Unres.	Unres.	Water (PRSFH)
40_110512_WS410-1CH	F. chungangense_EU924275	98.9	T20.08.P.BNT.GC.H.Gill.D.M	98.8	IX	Unres.	Water (PRSFH)
47_110512_WS910-1BH	F. aquidurense_AM177392	97.8	T131.10.P.BKT.AS.H.Kidney	99.0	VIII	Unres.	Water (PRSFH)
52_110512_WS1010-2BH	F.pectinovorum_AM230490.1	98.2	T76.09.P.RBT.EL.H.Kidney	98.6	VIII	Unres.	Water (PRSFH)
55_110512_WS1010-2EH	F. frigidimaris_AB183888	98.2	T57.08.B.COS.MI.W.SB	98.5	Unres.	Unres.	Water (PRSFH)
59_110512_T2N1CH	F. chungangense_EU924275	98.4	T159.10.BNT.W.Gill.N	98.9	Unres.	Unres.	Tools (PRSFH)
60_110512_T2N1DH	S12F	97.9	T76.09.P.RBT.EL.H.Kidney	98.3	VIII	Unres.	Tools (PRSFH)
70_110512_T7B1AH	F. aquidurense_AM177392	97.8	T101.09.P.CHS.MI.H.Kidney	98.7	II	Unres.	Tools (PRSFH)
73_110512_T7APAH	C. indoltheticum_AY468448.1	98.6	T72.09.LHR.H.Kidney.D	99.7	Unres.	Unres.	Tools (PRSFH)
327_110512_T3B1AH	T68.09.P.LAT.MA.H.Kidney.D	97.9	T68.09.P.LAT.MA.H.Kidney	97.8	XXXIV	Unres.	Tools (PRSFH)
692_PLND_4CC	C. indoltheticum_AY468448.1	98.8	T72.09.LHR.H.Kidney.D	98.7	Unres.	Unres.	Eggs (PRSFH)
650_PLD_2AH	S12F	98.4	T108.09.B.BNT.SR.H.Fin.D	99.7	Unres.	Unres.	Eggs (PRSFH)
858_111130-1_21NDFJH	F. frigidimaris_AB183888	98.6	T99.09.B.LAT.LS.H.Gill.D	98.9	Unres.	Unres.	Eggs (TSFH)
90_110519_T3N1AH	C. chaponense_GU345046.1	97.4	T115.09.B.CHS.MI.SRW.W	97.8	XXXVIII	Unres.	Tools (TSFH)
95_110519_T3AP1AH	C. chaponense_GU345046.1	98.2	T115.09.B.CHS.MI.SRW.W	98.6	XXXVIII	Unres.	Tools (TSFH)
122_110118_WS11undCC	F.reichenbachii_AM177616.1	97.6	T141.10.B.RBT.MI.LM.W.Kid	98.4	XXIII	Unres.	Water (TSFH)
104_110519_WS11undBH	F. frigidimaris_AB183888	99.0	T93.09.P.BKT.AS.H.Gill.N	99.0	Unres.	Unres.	Water (TSFH)
102_110519_WS6undBH	F. glaciei_DQ515962	97.6	T57.08.B.COS.MI.W.SB	97.9	Unres.	Unres.	Water (TSFH)
339_110519_WS3undAH	F. aquidurense_AM177392	98.7	T10.07.P.BNT.GC.H.Brain.D	99.0	III	Unres.	Water (TSFH)
1005_120117-1_WS1610-2CC	F. segetis_AY581115	97.9	T69.09.B.RBT.MI.W.Kidney	98.2	IV	Unres.	Water (TSFH)
1036_120117-1_WS12undBH	T68.09.P.LAT.MA.H.Kidney.D	99.4	T68.09.P.LAT.MA.H.Kidney	99.4	XXXIV	Unres.	Water (TSFH)
1022_120117-1_WS1410-1BH	F. segetis_AY581115	98.0	T69.09.B.RBT.MI.W.Kidney	98.2	IV	Unres.	Water (TSFH)
1104_120515-1_WS12undCH	T68.09.P.LAT.MA.H.Kidney.D	97.6	T130.10.P.BKT.AS.H.Kidney	97.6	XXXIV	Unres.	Water (TSFH)
1112_120515-1_WS1610-2EH	F. cheniae_EF407880	98.3	T18.08.P.BNT.GC.H.Gill.D.M	93.1	II	Unres.	Water (TSFH)
1094_120515-1_WS1undAH	C. gambrini_AM232810	97.1	T31.08.P.RBT.MI.H.Kidney	96.6	XXXVII	Unres.	Water (TSFH)
981_120106-1_WS9undDC	F. aquidurense_AM177392	98.5	T10.07.P.BNT.GC.H.Brain.D	99.0	III	Unres.	Water (WLSFH)
965_120106-1_WS1310-2CC	F.succinicans_AM230492.1	98.1	T158.10.BNT.W.Gill.N	99.0	XV	Unres.	Water (WLSFH)
962_120106-1_WS1110-1CC	F.succinicans_AM230492.1	97.2	T18.08.P.BNT.GC.H.Gill.D.M	97.4	II	Unres.	Water (WLSFH)
925_120106-1_WS1310-1BH	F.succinicans_AM230492.1	98.9	T129.10.P.BKT.AS.H.Kidney	97.9	I	Unres.	Water (WLSFH)
954_120106-1_WS1010-1CC	S12F	98.7	T37.08.SCU.W.Gill.N	99.8	XXI	Unres.	Water (WLSFH)
927_120106-1_WS1310-1DH	F. frigidimaris_AB183888	98.0	T69.09.B.RBT.MI.W.Kidney	98.6	IV	Unres.	Water (WLSFH)
1050_120416-1_WS6undCH	C. aquaticum_AM748690.1	99.2	T31.08.P.RBT.MI.H.Kidney	97.1	XXXVII	Unres.	Water (WLSFH)

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1082_120416-1_WS7undAC	F.chilense_FR774915.1	97.0	T108.09.B.BNT.SR.H.Fin.D	97.5	Unres.	Unres.	Water (WLSFH)
1090_120416-1_WS1310-1BC	F. saccharophilum AB473208.1	98.7	T8.06.B.BNT.SE.H.SB	99.1	IV	Unres.	Water (WLSFH)
1081_120416-1_WS6undCC	F. aquidurense_AM177392	98.7	T10.07.P.BNT.GC.H.Brain.D	99.1	III	Unres.	Water (WLSFH)
890_111130-2_5NDDYFCC	F.pectinovorum_AM230490.1	97.9	T157.10.BNT.W.Gill.N	98.2	Unres.	Unres.	Eggs (WLSFH)
833_111012-1_21maleCSpChr	C. yonginense_GQ259742.1	97.5	T115.09.B.CHS.MI.SRW.W	97.1	XXXVIII	Unres.	Brood (SRW)
853_111012-1_30NDH	C. piscium_AM040439	99.1	T72.09.LHR.H.Kidney.D	99.9	Unres.	Unres.	Brood (SRW)
801_111012-1_11OFH	C. indoltheticum_AY468448.1	99.3	T72.09.LHR.H.Kidney.D	100.0	Unres.	Unres.	Brood (SRW)
798_111012-1_21femSplH	C. piscium_AM040439	98.6	T72.09.LHR.H.Kidney.D	99.3	Unres.	Unres.	Brood (SRW)
427_101012-1_5BRmaleH	C. chaponense_GU345046.1	98.2	T115.09.B.CHS.MI.SRW.W	98.6	XXXVIII	Unres.	Brood (LMRW)
739_111005-1_11femDYEH	C. vrystaatense_AJ871397	97.5	T28.08.P.COS.MI.H.Kidney	96.7	Unres.	Unres.	Brood (LMRW)
737_111005-1_9femFJH	F.oncorhynchi_FR870076.1	98.5	T19.08.P.BNT.GC.H.Gill.D.M	98.8	IX	Unres.	Brood (LMRW)
734_111005-1_3femPYEH	C. vrystaatense_AJ871397	97.8	T28.08.P.COS.MI.H.Kidney	97.6	Unres.	Unres.	Brood (LMRW)
817_111005-1_26femSplC	C. piscium_AM040439	99.1	T72.09.LHR.H.Kidney.D	99.9	Unres.	Unres.	Brood (LMRW)
784_111005-1_4maleMH	F. frigidimaris_AB183888	99.1	T100.09.B.LAT.LS.H.Gill.D	99.6	Unres.	Unres.	Brood (LMRW)