DETECTION OF NOVEL MULTILOCUS SEQUENCE TYPING GENOTYPES OF *FLAVOBACTERIUM PSYCHROPHILUM* IN NORTH AMERICA

By

Christopher Kay Knupp

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Fisheries and Wildlife—Master of Science

2018

ABSTRACT

DETECTION OF NOVEL MULTILOCUS SEQUENCE TYPING GENOTYPES OF *FLAVOBACTERIUM PSYCHROPHILUM* IN NORTH AMERICA

By

Christopher Kay Knupp

The fish pathogen, *Flavobacterium psychrophilum*, has caused substantial economic losses, particularly to farm/hatchery reared salmonids on at least five continents. *F. psychrophilum* genetic diversity has been explored using several techniques, including multilocus sequence typing (MLST), which has proven to be superior to other molecular typing assays. Although isolates of *F. psychrophilum* have been MLST genotyped in Europe, Asia, and South America, the genetic diversity of North American (NA) *F. psychrophilum* is largely unexplored. To address this knowledge gap, I genotyped 314 *F. psychrophilum* isolates that were recovered from 20 US states and 1 Canadian province from 10 fish species over nearly 4 decades, which revealed 66 sequence types (STs), 47 of which were novel, and 7 novel clonal complexes (CCs). CC-ST10 was the predominant CC in NA and its genotypes were recovered from rainbow trout/steelhead (*Oncorhynchus mykiss*) from nearly every sampled state. Several other CCs were also discovered, some of which appeared host specific, whereas others appeared to be "generalists". Recombination is hypothesized to have caused most variations among isolates of this study, although the mechanism by which this occurs remains unknown. To explore CC-ST10 pathogenicity in rainbow trout, I intramuscularly injected juvenile rainbow trout with several of its genotypes and compared them to the world's second largest CC, CC-ST191, which resulted in significant mortality. Overall, both CC-ST10 and CC-ST191 were highly pathogenic to rainbow trout, yet significant differences in mean times to death were observed among the genotypes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AICc	Corrected Akaike information criterion
AK	Alaska
ANCOVA	Analysis of covariance
AT	Allele type
atpA	Adenosine triphosphate synthetase, α subunit
BCWD	Bacterial cold water disease
BC	British Columbia
bp	Base pair
C	Celsius
CA	California or Cytophaga agar
CC	Clonal complex
CFU	Colony forming unit
CO	Colorado
CPM	Cumulative percent mortality
DLV	Double locus variant
DNA	Deoxyribonucleic acid
dnaK	Chaperone heat-shock protein 70
dN/dS	Ratio of non-synonymous to synonymous mutations
fumC	Fumarate hydratase class II
G	Gauge
g	Gram
g	Gravity

gyrB	Topoisomerase II, β subunit
H	Mean genetic diversity
HSU	Hsu-Shotts medium
I^S_A	Standardized index of association
ID	Idaho
IM	Intramuscular
IN	Indiana
IP	Intraperitoneal
L	Liter
MCMC	Markov chain Monte Carlo
MD	Maryland
mg	Milligram
MI	Michigan
min	Minute
mL	Milliliter
MLST	Multilocus sequence typing
MS-222	Tricaine methanesulfonate
MN	Minnesota
MT	Montana
mTYES	Tryptone yeast extract agar supplemented with 5% horse serum and 0.02% Lewin and Lounsbery mineral solution
NA	North America
NC	North Carolina
NaCl	Sodium Chloride

NM	New Mexico
OD	Optical density
OF	Ovarian fluid
OR	Oregon
PA	Pennsylvania
PF	Private facility
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PHI	Pairwise homoplasy index
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
R_{min}	Hudson and Kaplan lower bound on the minimum number of recombination events
rpm	Revolutions per minute
RTFS	Rainbow trout fry syndrome
rRNA	Ribosomal ribonucleic acid
S	Segregating sites
SD	South Dakota or standard deviation
SFH	State fish hatchery
SLV	Single locus variant
ST	Sequence type
trpB	Tryptophane synthetase, β subunit
tuf	Elongation factor tu
TYES	Tryptone yeast extract agar

UK	United Kingdom
Unk	Unknown
US	United States
USA	United States of America
UT	Utah
VA	Virginia
vol	Volume
VT	Vermont
WA	Washington
WE	Weir
WI	Wild or Wisconsin
WV	West Virginia
µl	Microliter
π	Nucleotide diversity

Introduction

Flavobacterium psychrophilum was first isolated during a mortality event among coho salmon (*Oncorhynchus kisutch*) in 1948 in the Pacific Norwest region of the United States (Borg 1948). Since then, *F. psychrophilum* has been recovered from numerous salmonids and in particular, rainbow trout (*O. mykiss*) and Atlantic salmon (*Salmo salar*; reviewed in Nematollahi et al. 2003). *F. psychrophilum* has been recovered from non-salmonid species as well, such as ayu (*Plecoglossus altivelis*) and common carp (*Cyprinus carpio*; reviewed in Nematollahi et al. 2003, Starliper 2011). Today, *F. psychrophilum* is known as the etiological agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) and is responsible for significant economic losses worldwide (reviewed in Loch & Faisal 2017).

Since its initial isolation, researchers noticed phenotypic diversity within this species, specifically in the form of smooth and rough colonies (Bernardet & Kerouault 1989). As serological and molecular biological techniques progressed, additional researchers began to investigate *F. psychrophilum* heterogeneity further. For example, Pacha (1968) examined 10 *F. psychrophilum* isolates recovered from Chinook salmon from Washington (USA) and discovered them to be highly related using a serological technique. Likewise, Wakabayashi et al. (1994) described two *F. psychrophilum* serotypes (i.e., O-1, O-2) and found that isolates belonging to serotype O-1 were recovered predominantly from coho salmon, whereas O-2 was recovered from ayu and rainbow trout. As of 2017, at least seven serotypes have been described (reviewed in Loch and Faisal 2017), although variations in techniques have hindered inter-laboratory comparisons. Molecular biology has taken advantage of numerous genetically-based techniques for detecting *F. psychrophilum* diversity, including pulsed field gel electrophoresis (PFGE), plasmid profiling, ribotyping, randomly amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP). Most recently, a multilocus sequence typing (MLST)

scheme was developed for use with *F. psychrophilum* by Nicolas et al. (2008), which has proven to be a highly discriminatory technique for describing *F. psychrophilum* heterogeneity. Since their pioneering study, numerous researchers worldwide have applied this technique to *F. psychrophilum* to better understand how genetic diversity may correlate with virulence, geographic distribution, and host specificity. To date, the majority of MLST typed *F. psychrophilum* isolates have originated from Europe; however, isolates from Asia, South America, Oceania, and North America have been examined as well. MLST has been used to reveal certain sequence types (STs) and clonal complexes (CCs) as host specific (Fujiwara-Nagata et al. 2013, Nilsen et al. 2014), geographically isolated or widespread (Nicolas et al. 2008, Fujiwara-Nagata et al. 2013, Avendaño-Herrera et al. 2014) and virulent (Fujiwara-Nagata et al. 2012, 2013, Nilsen et al. 2014). All previously published research articles have discovered STs belonging to a common CC; namely CC-ST10. CC-ST10 is the largest CC worldwide and contains hundreds of isolates from different regions of the world (Van Vliet et al. 2016). Of note, the majority of these isolates have been recovered from diseased/systemically infected rainbow trout and so it is hypothesized that this CC is particularly adapted and highly virulent to rainbow trout (Nilsen et al. 2014, Van Vliet et al. 2016).

In North America, few *F. psychrophilum* isolates have been genotyped using MLST. In fact, prior to 2016, only 10 *F. psychrophilum* isolates had been genotyped using this technique, which is surprising given the economic losses *F. psychrophilum* causes in North America. In 2016, Van Vliet et al. (2016) genotyped 95 new *F. psychrophilum* isolates although the majority of these came from Michigan (USA) and were recovered from three *Oncorhynchus spp.* (i.e., *O. mykiss*, *O. kisutch*, *O. tshawytscha*). Given the proportionally fewer *F. psychrophilum* isolates that have been MLST genotyped in North America in comparison to other regions of the world

that intensively rear salmonids (e.g., Europe), I opportunistically collected several hundred *F. psychrophilum* isolates from North America and evaluated their genetic diversity using MLST.

1. Study Objectives

In Chapter 1, I thoroughly reviewed the literature on *F. psychrophilum* and bacterial cold water disease (BCWD). I provided specific information in regards to *F. psychrophilum* diversity, including its phenotypic, serological, and molecular diversity. I also reviewed previous experimental challenge studies, which used numerous routes of exposure.

In Chapter 2, I reached out to fish health professionals across the US to gather 314 *F. psychrophilum* isolates, some of which were cryogenically preserved nearly four decades ago. These isolates originated from 10 fish host species, were recovered from 20 US states and 1 Canadian province, and I assessed their genetic diversity using MLST. I also evaluated the 7 MLST loci for recombination and mutation using multiple sequence and allelic profile based analyses in order to predict how *F. psychrophilum* achieved its genetic diversity.

In Chapter 3, I examined the *in vivo* pathogenicity of several CC-ST10 genotypes as well as those from the world's second largest MLST CC (i.e., CC-ST191) against their natural host, the rainbow trout. I hypothesized CC-ST10 genotypes were pathogenic to rainbow trout, isolates of the same genotype were similar in their pathogenicity, and the genotypes within and between the two largest CCs would differ in their pathogenicity.

Chapter 4 provided overall conclusions based upon the findings of these studies and offered suggestions for future research that could contribute to the control and management of this important fish pathogen.

REFERENCES

REFERENCES

Avendaño-Herrera, R., Houel, A., Irgang, R., Bernardet, J.F., Godoy, M., Nicolas, P., and E. Duchaud. 2014. Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms. Veterinary Microbiology 170:298-306.

Bernardet, J.F., and B. Kerouault. 1989. Phenotypic and genomic studies of Cytophaga psychrophila isolated from diseased rainbow trout Oncorhynchus mykiss in France. Applied Environmental Microbiology 55:1796-1800.

Borg, A.F. 1948. Ph.D. thesis. University of Washington, Seattle, Washington. Studies on myxobacteria associated with diseases in salmonid fishes.

Duchaud, E., Rochat, T., Habib, C., Barbier, P., Loux, V., Guérin, C., Dalsgaard, I., Madsen, L., Nilsen, H., Sundell, K., Wiklund, T., Strepparava, N., Wahli, T., Caburlotto, G., Manfrin, A., Wiens, G.D., Fujiwara-Nagata, E., Avendaño-Herrera, R., Bernardet, J-F., and P. Nicolas. 2018. Genomic Diversity and Evolution of the Fish Pathogen *Flavobacterium psychrophilum*. Frontiers in Microbiology 9:138.

Fujiwara-Nagata, E., Chantry-Darmon, C., Bernardet, J.F., M. Eguchi, M., Duchaud E., and P. Nicolas. 2013. Population structure of the fish pathogen *Flavobacterium psychrophilum* at a whole country and model river levels in Japan. Veterinary Research 44:34.

Fujiwara-Nagata, E., Ikeda, J., Sugahara, K., and M. Eguchi. 2012. A novel genotyping technique for distinguishing between *Flavobacterium psychrophilum* isolates virulent and avirulent to ayu, *Plecoglossus altivelis* (Temminck & Schlegel). Journal of Fish Diseases 35:471–480.

Loch T.P., M Faisal. 2017. *Flavobacterium* spp., p. 211–232. In Woo, PTK, Cipriano, RC (eds.), Fish Viruses and Bacteria: Pathobiology and Protection CABI.

Nematollahi, A., Decostere, A., Pasmans, F., and F. Haesebrouck. 2003. *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases 26:563-574.

Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J.F., and E. Duchaud. 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709.

Nilsen, H., Sundell, K., Duchaud, E., Nicolas, P., Dalsgaard, I., Madsen, L., Aspán, A., Jansson, E., Colquhoun, D.J., and T. Wiklund. 2014. Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. Applied and Environmental Microbiology 80:2728-2736.

Pacha, R.E. 1968. Characteristics of *Cytophaga psychrophila* (Borg) Isolated During Outbreaks of Bacterial Cold-Water Disease. Applied Microbiology 16:97-101.

Starliper, C.E. 2011. Bacterial coldwater disease of fishes caused *by Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108.

Van Vliet, D., Wiens, G.D., Loch, T.P., Nicolas, P., and M. Faisal. 2016. Genetic Diversity of *Flavobacterium psychrophilum* Isolates from Three Oncorhynchus spp. in the United States, as Revealed by Multilocus Sequence Typing. Applied and Environmental Microbiology 82:3246-3255.

Wakabayashi, H., Toyama, T., and T. Iida. 1994. A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathology 29:101-104.

Chapter 1

Literature Review

The family Flavobacteriaceae contains a multitude of fish pathogens, the most notorious of which is *Flavobacterium psychrophilum*, the causative agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS). In this chapter, I focused on bacterial cold water disease.

1. Bacterial Cold Water Disease

F. psychrophilum is the etiological agent of bacterial cold water disease and rainbow trout fry syndrome (Madsen & Dalsgaard 1999). Disease signs consistent with what is now known as BCWD were first reported by Davis (1946), at which time he coined the syndrome "Peduncle Disease". Affected rainbow trout had heavy loads of a bacterium associated with the affected tissues, yet the etiological agent could not be isolated at that time. However, in 1948, *F. psychrophilum* was isolated in the Pacific Northwest region of the United States after mass mortalities among coho salmon (*Oncorhynchus kisutch*; Borg 1948) occurred.

1.1. Host range

Flavobacterium psychrophilum predominately affects fish within the family Salmonidae, including coho salmon (*O. kisutch*) and rainbow/steelhead trout (*O. mykiss*), which are considered to be particularly susceptible (reviewed in Nematollahi et al. 2003, reviewed in Cipriano & Holt 2005). *F. psychrophilum* has been recovered from many other salmonids species, including Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), Chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), masou salmon (*O. masou*), chum salmon (*O. keta*), amago (*O. rhodurus*), cutthroat trout (*O. clarkii*), pink salmon (*O. gorbuscha*), brook trout (*Salvelinus fontinalis*), lake trout (*S. namaycush*), arctic char (*S. alpinus*), white spotted char (*S.*

leucomaenis), and grayling (*Thymallus thymallus*; Davis 1946, Rucker et al. 1953, Borg 1948, Schachte 1983, Holt 1987, Iida & Mizokami 1996, Ekman et al. 1999, and Madetoja et al. 2001, reviewed in Cipriano & Holt 2005, Fujiwara-Nagata et al. 2013). Additionally, *F. psychrophilum* can also infect non-salmonid fishes. For example, ayu (*Plecoglossus altivelis*), are particularly susceptible to infection with *F. psychrophilum* (Wakabayashi et al. 1994, Fujiwara-Nagata et al. 2013, Rochat et al. 2017), whereas isolation from other non-salmonid hosts, including European eel (*Anguilla Anguilla*), Japanese eel (*A. japonica*), forktongue goby (*Chaenogobius urotaenia*), common carp (*Cyprinnus carpio*), crucian carp (*Carassius carassius*), goldfish (*C. auratus*), Japanese crucian carp funbuna (*C. auratus langsdorfii*), Japanese dace (*Trybolodon hakonensis*), pale chub (*Zacco platypus*), lake goby (*Rhinogobius brunneus*), perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), tench (*Tinca tinca*), Japanese smelt (*Hypomesus nipponensis*), far eastern brook lamprey (*Lethenteron reissneri*), sea lamprey (*Petromyzon marinus* L.) takahaya (*Phoxinus jouyi*), European flounder (*Platichthys flesus*), numachichibu (*Tridentiger brevispinis*), white sturgeon (*Acipenser transmontanus*), Indian catfish (*Clarias batrachus*), and three-spined stickleback (*Gasteroosteus aculeatus*) is reported infrequently (Lehmann et al. 1991, Amita et al. 2000, Iida & Mizokami 1996, Madetoja et al. 2002, reviewed in Starliper 2011, Fujiwara-Nagata et al. 2013, reviewed in Loch & Faisal 2017). *F. psychrophilum* has also been recovered from other aquatic/semi-aquatic hosts, such as freshwater leeches (*Myzobdella lugubris;* Schulz and Faisal 2010), newts (Brown et al. 1997), and even aquatic plants (e.g., algae; Amita et al. 2000) and insects (e.g., caddisfly; Fujiwara-Nagata et al. 2013).

1.2. Geographic distribution

1.2.1. North America

Although *F. psychrophilum* was first described in the USA, its exact geographic distribution has not been determined. Nevertheless, *F. psychrophilum* is believed to be widespread across the USA (reviewed in Cipriano & Holt 2005, Starliper 2011, Loch & Faisal 2017) and has also been recovered from Canada (Allen et al. 2008, Hesami et al. 2008).

1.2.2. Europe

F. psychrophilum is widespread among European countries. For example, *F. psychrophilum* has been isolated in the countries of Belgium (reviewed in Nematollahi et al. 2003), Denmark (Lorenzen et al. 1991, Nilsen et al. 2014), Estonia (Madetoja et al. 2001), Finland (Dalsgaard & Madsen 2000, Madetoja et al. 2001), France (Siekoula-Nguedia et al. 2012), Germany (Nilz et al. 2009), Italy (Sarti et al. 1992), Ireland (Lorenzen et al. 1997), Norway (Nilsen et al. 2014), Scotland (reviewed in Starliper 2011), Spain (Toranzo & Barja 1993), Sweden (Ekman et al. 1999), Switzerland (Strepparava et al. 2013), and the United Kingdom (Austin & Stobie 1991).

1.2.3. Other continents

Compared to North America and Europe, reports of *F. psychrophilum* in Asia, South America, and Australia have been less frequent. The bacterium was first reported in Japan in 1987 (Wakabayashi et al. 1994) and has been regularly isolated since then (Fujiwara-Nagata et al. 2013). *F. psychrophilum* has also been reported in two other regions of Asia; namely Turkey (Kum et al. 2008) and the Korean Peninsula (Lee & Heo 1998). Within South America, most

reports of *F. psychrophilum* have come from Chile (Avendaño-Herrera et al. 2014), but *F. psychrophilum* has also been recovered in Peru (Leon et al. 2009). *F. psychrophilum* has been recovered in Australia (Schmidtke & Carson 1995).

1.3. Clinical signs

The most characteristic external sign of BCWD is the formation of deep ulcers on the caudal peduncle, where extensive erosion of the skin and musculature has occurred (reviewed in Cipriano and Holt 2005). However, the severity and distribution of BCWD clinical signs are influenced by many factors (reviewed in Nematollahi et al. 2003). For example, in sac-fry suffering from BCWD, erosion of the yolk sac has been reported (Holt et al. 1993), whereas in fingerlings a unilateral dark pigmentation has been accompanied by fin erosion (Pacha & Ordal 1970). Additional external disease signs include exophthalmia, frayed fins, abdominal distention, pale and/or hemorrhagic gills, and erosion of skin on the snout and posterior portion of the head (reviewed in Nematollahi et al. 2003, Starliper 2011). Internally, the kidneys and liver appear pale (reviewed in Starliper 2011), whereas the spleen is enlarged or hypertrophied (Bernardet et al. 1988). Petechial hemorrhages on visceral organs also occur (reviewed in Loch & Faisal 2017). Histologically, necrotic myositis, scleritis, and cephalic osteochondritis are common lesions in fish experiencing BCWD (Lumsden et al. 1996, reviewed in Nematollahi et al. 2003).

1.4. Losses and economic impact

F. psychrophilum causes millions of dollars in losses annually, much of which is due to mortality (Duchaud et al. 2018). For example, up to 70% mortality has been reported in *F.*

psychrophilum-infected fingerling rainbow trout (Avendaño-Herrera et al. 2014), whereas in older fish, losses < 20% are reported (Wood 1974). Fish that survive and recover from infection with *F. psychrophilum* often have spinal and ocular abnormalities (reviewed in Loch & Faisal 2017).

2. The Pathogen *Flavobacterium psychrophilum*

2.1. Taxonomy

F. psychrophilum has undergone many taxonomic re-classifications since its initial isolation nearly 70 years ago. *F. psychrophilum* was originally placed within the genus *Cytophaga* by Borg (1948); due to its affinity for low temperatures, it was given the name *Cytophaga psychrophila*. Since this bacterium did not produce fruiting bodies or degrade polysaccharides, *C. psychrophila* was re-classified as *Flexibacter psychrophilus* (Bernardet & Grimont 1989), but subsequent analysis of DNA G+C content indicated that *F. psychrophilus* was more similar to bacteria within the genus *Flavobacterium*. In 1996, Bernardet et al. (1996) emended the description of the genus *Flavobacterium* and renamed *Flexibacter psychrophilus* as *Flavobacterium psychrophilum*, as it is known today.

2.2. Phenotypic characteristics

F. psychrophilum is a weakly refractile (Pacha 1968) Gram-negative rod-shaped bacterium that is approximately 0.5 μm wide by 1-5 μm long (Bernardet & Kerouault 1989). This bacterium is strictly aerobic and produces yellow-pigmented (due to the presence of a flexirubin-type pigment) colonies with a raised center and smooth or thinly spreading edge (Bernardet & Kerouault 1989, Holt et al. 1993), although colony morphology can vary. *F.*

psychrophilum grows in the presence of up to 1.0% NaCl and at a pH range of 4.0 - 8.0 (Bernardet & Kerouault 1989). The bacterium hydrolyzes multiple substrates (e.g., gelatin, tributyrin, casein, tyrosine, lecithin, tween 20, and tween 80; Bernardet & Kerouault 1989) and also proteolyzes many host tissue constituents (e.g., collagen, fibrinogen, chondroitin sulphate, and fish muscle extract; Lorenzen et al. 1997). However, *F. psychrophilum* cannot hydrolyze xanthine, chitin, starch, agar, carboxymethylcellulose, or esculin or degrade simple or complex carbohydrates (Bernardet & Kerouault 1989, reviewed in Cipriano and Holt 2005). *F. psychrophilum* is weakly positive for both cytochrome oxidase and catalase, does not produce hydrogen sulfide, indole, arginine dihydrolase, lysine decarboxylase, or ornithine decarboxylase (Bernardet & Kerouault 1989, reviewed in Cipriano and Holt 2005). *F. psychrophilum* cannot absorb congo red nor reduce nitrate to nitrite (Bernardet & Kerouault 1989).

2.3. Bacterial culture conditions

F. psychrophilum is a somewhat fastidious bacterium and as its name implies, grows well at cooler temperatures, particularly between 10°C - 20°C although some strains will grow at temperatures of up to 25°C (Bernardet & Kerouault 1989). *F. psychrophilum* has a generation time of 2 hours at 15°C, which is considered to be its optimal growth temperature (Holt et al. 1993, Barnes & Brown 2011). *F. psychrophilum* grows poorly or not at all on nutrient-rich media such as trypticase soy agar (TSA; Wiklund et al. 1994, Ekman et al. 1999) and therefore is cultured on nutrient deplete media such as Cytophaga agar (Anacker & Ordal 1959), modified Cytophaga agar (Wakabayashi & Egusa 1974), Hsu-Shotts (Bullock et al. 1986), or tryptone yeast extract agar (TYES; Holt et al. 1993). The overgrowth of non-target bacteria when attempting to culture *F. psychrophilum* can occur, so supplementing primary isolation media

with antibiotics has been shown to aide in bacterial recovery (reviewed in Nematollahi et al. 2003). The supplementation of primary media with 10% fetal calf serum (Obach & Laurencin 1991) or 5-10% horse serum (Michel et al. 1999) has been shown to improve recovery of *F. psychrophilum*. Likewise, the addition of trace elements at a concentration below 0.05% was found to improve *F. psychrophilum* recovery (Lewin & Lounsberry 1996, Michel et al. 1999).

2.4. Diversity

2.4.1. Phenotypic diversity

Phenotypic variability within *F. psychrophilum* has been reported on numerous occasions. For example, variations in colony morphology (i.e., rough versus smooth; Bernardet & Kerouault 1989, Högfors-Rönnholm & Wiklund 2010) occur and are associated with differences in *in vitro* autoagglutination capacity and hydrophobicity. Högfors-Rönnholm & Wiklund (2010) provided evidence for phase variation (i.e., conversions between phenotypes) between the two phenotypes, whereby smooth phenotype colonies could convert to rough but not the other way around. Both phenotypes have proven equal in their abilities to cause mortality (Högfors-Rönnholm & Wiklund 2010). On the contrary, variability in elastase activity has been reported between *F. psychrophilum* strains (Madsen & Dalsgaard 1998), and this variability was associated with differential virulence (Europe). Likewise, variability in biofilm formation, enzymatic activity, and gliding motility have been reported (Madetoja et al. 2001, Hesami et al. 2008, Levipan & Avendaño-Herrera 2017).

2.4.2. Serological diversity

The serological diversity of *F. psychrophilum* has been examined for decades (Pacha 1968, reviewed in Cipriano & Holt 2005). Serotyping has relied upon multiple assays, which makes comparisons between laboratories difficult but some trends among several serotypes have been reported. For instance, Wakabayashi et al. (1994) described two *F. psychrophilum* serotypes; namely O-1 and O-2, where O-1 was recovered exclusively from coho salmon in the US or Japan and O-2 was non-host specific but localized to Japan. Similarly, Izumi and Wakabayashi (1999) reported the presence of three serotypes O-1, O-2, and O-3 with O-1 being recovered from multiple host species (i.e., coho salmon, rainbow trout, carp, and grayling), O-2 being recovered exclusively from rainbow trout, while O-3 was almost exclusively recovered from rainbow trout. In 2003, Izumi et al. (2003) added serotype O-4, which was recovered from multiple host species, and described mixed serotypes O-2/4 and O-3/4 as well. Lorenzen and Olesen (1997) identified and described three serotypes (i.e., Th, Fd, Fp^T) using slide agglutination and found the majority of isolates in their study belonged to Th (with subtypes Th-1 and Th-2), which were predominantly recovered from diseased rainbow trout. Likewise, many isolates identified as Fd were recovered from rainbow trout experiencing BCWD, whereas those identified as Fp^T were not recovered from BCWD outbreaks (Lorenzen & Olesen 1997). In agreement with this discovery, Madsen and Dalsgaard (2000) found that isolates belonging to serotype Fp^T did not appear to be highly virulent in comparison to other isolates. In 2002, Mata et al. (2002) described seven different serovars (i.e., 1, 2a, 2b, 3, 4, 5, 6, 7) using slide agglutination and an enzyme-linked immunosorbent assay (ELISA). These serovars appeared host specific as isolates recovered from rainbow trout belonged to serovars 2a, 2b, and 3, whereas coho salmon, European eel, common carp, tench, and ayu belonged to serovar 1, 4, 5, 6,

or 7, respectively. In summary, serotypes varied in their host specificities and virulence; however, many *F. psychrophilum* isolates were untypable with current protocols and serotyping techniques were not standardized across laboratories, which made comparisons difficult.

2.4.3. Molecular and genetic diversity

2.4.3.1. Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) has been used by multiple investigators to evaluate the genetic heterogeneity of *F. psychrophilum* isolates. For example, Arai et al. (2007) typed 80 *F. psychrophilum* isolates using PFGE, 64 of which were from ayu, and utilized the endonucleases *BlnI* and *XhoI* to identify 42 genotypes. Isolates recovered from ayu fell into three major clusters (i.e., XII, XVI, XVII), two of which (i.e., XII, XVII) contained isolates from multiple locations, whereas XVI contained isolates from a single location. Similarly, Chen et al. (2008) used PFGE to type 139 *F. psychrophilum* isolates recovered from salmonids to identify dozens of genotypes; however, the endonuclease used in this study was *SacI*. del Cerro et al. (2010) employed PFGE to genotype 25 *F. psychrophilum* isolates from Spain and revealed 17 PFGE patterns using the endonuclease *StuI*. Moreover, del Cerro et al. (2010) discovered that some *F. psychrophilum* isolates were specific to a particular aquaculture facility and the same genotypes were responsible for BCWD outbreaks over several months. Unfortunately, the lack of standardization in regard to the employed restriction enzyme impeded inter-laboratory comparisons.

2.4.3.2. Plasmid profiling

The plasmid repertoire of *F. psychrophilum* was reported to be homogenous by numerous investigators. For instance, Holt (1987) evaluated the plasmid profiles of 20 *F. psychrophilum* isolates and found many isolates contained an identical plasmid. However, plasmid sizes have varied between laboratories, making comparisons between laboratories difficult. The discrepancy in plasmid size has been attributed to differences in laboratory techniques and protocols (Izumi 2004). For example, the type strain of *F. psychrophilum*, NCIMB1947, has been estimated to have a plasmid size of 2.8kb, 2.7kb, or 2.4kb, depending on the investigator (Lorenzen et al. 1997, Chakroun et al. 1998, Izumi 2004, Madsen & Dalsgaard 2000). Recently, Duchaud et al. (2018) identified 15 plasmids within 41 *F. psychrophilum* isolates using whole genome sequencing and provided evidence that gel electrophoresis cannot adequately distinguish between all *F. psychrophilum* plasmids.

2.4.3.3. Randomly amplified polymorphic DNA analysis

Randomly amplified polymorphic DNA (RAPD) analysis has provided mixed results in regards to RAPD profile and fish host species (Chakroun et al. 1997, Valdebenito & Avendaño-Herrera 2009), whereby RAPD profiles appeared to differ among isolates recovered from ayu and tench but not among isolates recovered from Atlantic salmon and rainbow trout (Chakroun et al. 1997, Valdebenito & Avendaño-Herrera 2009). Chakroun et al. (1997) also noted that no clear demarcation between isolate and geographic origin could be detected among recovered RAPD profiles.

2.4.3.4. Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism (RFLP) analysis has been used to explore the genetic heterogeneity of *F. psychrophilum* isolates by utilizing the 16S rRNA and *gyrB* genes (Izumi et al. 2003). Restriction digest of *gyrB* using restriction enzyme *HinfI* yielded two distinct genotypes (i.e., genotypes A and B) and endonuclease *RsaI* generated another two genotypes (i.e., genotypes R and S) among 242 isolates of *F. psychrophilum* (Izumi et al. 2003). Isolates identified as genotype "A" were recovered exclusively from ayu, whereas genotype "B" isolates were non-host specific (Izumi et al. 2003). Furthermore, Izumi et al. (2003) found restriction digest of the 16S rRNA gene provided no distinct cleavage patterns among the examined isolates. Soule et al. (2005) identified two lineages of *F. psychrophilum* (i.e., lineages I and II), where lineage I was associated with an avirulent isolate of *F. psychrophilum* (i.e., ATCC 49418) and lineage II was associated with a virulent isolate (i.e., CSF 259-93). Furthermore, lineage II isolates had reduced susceptibility to tetracycline and were specific to trout, whereas lineage I was specific to salmon (Soule et al. 2005). Hesami et al. (2008) identified four RFLP variants; however, no host associations could be made.

2.4.3.5. Ribotyping

Cipriano et al. (1996) described ribotyping as a useful tool for discriminating between closely related strains of *F. psychrophilum*. Two years later, Charkroun et al. (1998) genotyped 85 *F. psychrophilum* isolates and described 31 ribotypes when using endonuclease *Hinc* II and an additional 35 ribotypes when using endonuclease *Pvu* II. However, clear correlations between ribotype and host species could not be drawn (Chakroun et al. 1998). Madsen and Dalsgaard (2000) ribotyped 299 *F. psychrophilum* isolates by discovered remarkable homogeneity in which

85% of isolates belonged to the same ribotype. Madsen and Dalsgaard (2000) utilized the restriction endonuclease *Eco*RI and concluded that this restriction enzyme was not useful for epidemiologic investigations, underscoring the importance of endonuclease selection.

2.4.3.6. Multilocus sequence typing

Multilocus sequence typing (MLST) was originally developed for the human pathogen *Neisseria meningitidis* (Maiden et al. 1998) and is a highly discriminatory genetic technique that is comparable among laboratories. This technique has since been successfully applied to numerous human, animal, plant, insect, and fish bacterial pathogens (Kotetishvili et al. 2005, Nemoy et al. 2005, Scally et al. 2005, Saunders & Holmes 2007, Clegg et al. 2016, Krongdang et al. 2017) and typically utilizes partial sequences of 6 - 8 highly conserved housekeeping genes to discriminate between isolates of the same species (Jolley & Maiden 2004). Each of the housekeeping genes are assigned an allele type (AT) based upon their nucleotide sequence. The specific combination of ATs (i.e., allelic profile), in turn, determines the sequence type (ST), which are then arranged into clonal complexes (CC) that are hypothesized to be closely related (Feil et al. 2004). In 2008, Nicolas et al. designed a MLST scheme for the genotyping of *F. psychrophilum* through the examination of 50 geographically diverse isolates from Asia, Europe, North America, and South America, which were recovered from 10 fish species. Out of the original 11 loci under investigation, 7 (i.e., *trpB, gyrB, dnaK, tuf, fumC, murG, atpA*) were selected to be used for future MLST investigations of *F. psychrophilum* (Nicolas et al. 2008). This study revealed the presence of 30 genotypes, some of which were more closely related to one another, or were correlated with a geographic location and/or host species. Following the initial study, Siekoula-Nguedia et al. (2012), typed 66 *F. psychrophilum* isolates collected from

farmed rainbow trout in France and identified 14 novel STs as well as previously identified ST2. Importantly, all ST2 isolates were recovered from rainbow trout experiencing episodes of BCWD. Moreover, they discovered an epidemic population structure in which a single clone (i.e., ST2) quickly gave rise to multiple new clones through recombination. Fujiwara-Nagata et al. (2013) analyzed 114 *F. psychrophilum* isolates recovered from 15 fish species in Japan. They discovered substantial *F. psychrophilum* genetic diversity within the country and revealed 32 STs unique to Japan. Additionally, the authors rediscovered 3 STs that had been previously reported in coho salmon and rainbow trout elsewhere in the world raising the possibility that the international trade of fish contributed to the spread this genotype. Strepparava et al. (2013) identified ST2 and its SLVs as the culprits of BCWD when 112 *F. psychrophilum* isolates were sampled from unhealthy rainbow trout, brown trout, and char in Swiss fish farms. Importantly, ST10, which is a SLV of ST2, was implicated in disease in these Swiss fish farms. ST10 had been previously recovered from diseased fish in other European countries, as well as from the state of Oregon in the United States. At this time, ST2 was designated the founding ST of a major clonal complex associated with an abundance of apparently virulent strains. However, this conclusion is now known to be inaccurate and was a product of insufficiently sampled *F. psychrophilum* isolates. In 2014, Nilsen et al. (2014) typed 560 *F. psychrophilum* isolates from 4 Nordic countries including, Denmark, Finland, Norway, and Sweden. This study identified numerous novel STs and CCs and it was at this time that ST10 was identified as the true founding ST of the major CC (i.e., CC-ST10) that has been repeatedly associated with BCWD outbreaks. Avendaño-Herrera et al. (2014) published a study on 91 *F. psychrophilum* isolates from Chile, which were recovered from Atlantic salmon, coho salmon, and rainbow trout – a majority of which presented with BCWD. Similar to previous studies, they identified STs

restricted to a particular geographic location as well as a few that were present elsewhere in the world (i.e., ST10, ST2, ST9, ST12, ST23). In 2016, Van Vliet et al. (2016) published the largest MLST study on 96 *F. psychrophilum* isolates from 3 *Oncorhynchus* spp. (e.g., rainbow/steelhead trout, chinook salmon, and coho salmon) and 9 states in the United States. However, the majority of these isolates (i.e., 50/96) were recovered from the state of Michigan, USA. Nevertheless, 34 *F. psychrophilum* STs, 28 of which were novel, were identified. Of note, novel ST78, was recovered from rainbow/steelhead trout experiencing acute mortality and belongs to CC-ST10. Furthermore, ST78 was recovered from 2 additional U.S. states, also experiencing BCWD mortalities. All ST10 and ST78 isolates were recovered exclusively from rainbow/steelhead trout, indicating this fish species may be the niche for these particular strains. This finding is in support of previous research, which has identified CC-ST10 isolates almost exclusively from rainbow trout. In comparison to the rest of the world, North America has had very few *F. psychrophilum* isolates evaluated using MLST, which is surprising considering the economic losses *F. psychrophilum* causes in US salmonid populations (reviewed in Cipriano & Holt 2005). Thus it remains unclear if the previous North American MLST studies on *F. psychrophilum* have adequately characterized the genetic heterogeneity in this area of the world and if the trends seen among Michigan *F. psychrophilum* isolates are also mirrored in other states, particularly those that intensively culture salmonids species. Furthermore, the contributions of recombination/mutation to the diversification of North American *F. psychrophilum* STs has not been evaluated previously.

2.4.3.7. Whole genome multilocus sequence typing

Recently, a whole genome analysis was completed on 41 *F. psychrophilum* isolates, 30 of which had not been whole genome sequenced previously (Duchaud et al. 2018). Within this study, 1,549 single copy genes were considered for whole genome MLST, where up to 26 allele types were present per loci. ST10 was still considered the founding ST of CC-ST10 and the authors concluded that whole genome analysis largely reflected the conclusions of the original MLST protocol based on 7 genes (Duchaud et al. 2018). In particular, *F. psychrophilum* was noted to have low levels of nucleotide divergence (i.e., 0.285% inside CDSs in pairwise sequence comparisons) and high amounts recombination among alleles. Furthermore, the genome of *F. psychrophilum* was described to be an "open" pan-genome (i.e., as additional *F. psychrophilum* isolates are sequenced, new genes are discovered) and of the genes identified within CC-ST10 isolates, some appeared to encode the machinery for a type II secretion system, which may contribute to the apparent virulence of CC-ST10 isolates. In the same context, the *tetX* gene was present within CC-ST10 isolates and is known to contribute to enzymatically inactivate tetracycline (McArthur et al. 2013, Duchaud et al. 2018).

2.5. *Flavobacterium psychrophilum* and experimental challenge models

Experimental challenge models are not only important for fulfilling Koch's postulates, but also for studying microbial pathogenesis, virulence mechanisms, and vaccine efficacy. For *F. psychrophilum*, many routes of infection have been utilized including subcutaneous, intraperitoneal, and intramuscular injection (Holt 1987, Obach & Laurencin 1991, Fredriksen et al. 2013a), as well as oral, anal, cohabitation and bath immersion (Lorenzen et al. 1991, Decostere et al. 2000). However, finding a robust and reproducible *F. psychrophilum* challenge

model has been challenging. For example, the reproducibility of *F. psychrophilum* challenge models are influenced by host factors (e.g., fish size, species, and origin), pathogen factors (e.g., bacterial strain, inoculum size and method of preparation), and environmental factors (e.g., the number of fish in each tank and their stress prior to infection; Madsen & Dalsgaard 1999, Decostere et al. 2000, Long et al. 2013, Long et al. 2014).

2.5.1. Bacterial inoculum preparations

Many different types of culture media have been utilized for growing *F. psychrophilum in vitro*, including TYES, iron-limited TYES, Shieh with or without iron, modified Cytophaga, Cytophaga supplemented with carbohydrates, and Cytophaga enriched with 5% horse serum (Madsen & Dalsgaard 1999, Decostere et al. 2000, Garcia et al. 2000, Madetoja et al. 2002, Aoki et al. 2005, Long et al. 2014). Likewise, different incubation temperatures (i.e., 15°C, 16°C, 17°C, 18°C), durations (i.e., 18, 24, 48, or 72 hours) with or without agitation, and the way in which inoculum size was estimated prior to infection (i.e., using incubation time to correlate with a particular growth phase or using optical density (OD) to correlate with viable cell counts) have been employed (Madsen & Dalsgaard 1999, Decostere et al. 2000, Garcia et al. 2000, Madetoja et al. 2002, Aoki et al. 2005, Long et al. 2014). Furthermore, Michel et al. (1999) provided evidence that the addition of 5% defibrinated horse serum, trace amounts of minerals (i.e., concentration < 0.05%), and stirring all improved the culturability of *F. psychrophilum*. The variability among laboratories in regards to inoculum preparation hinders reproducibility and comparability flavobacterial experimental challenge models. Furthermore, as Michel et al. (1999) provided evidence for, both the culture media and incubation environment can affect the accuracy of colony counts, which are used to determine the experimental inoculum size.

Therefore, a reliable and standardized approach to inoculum preparation must be adopted for inter-laboratory comparisons of results.

2.5.2. Immersion challenge studies

Immersion studies have proven useful for mimicking a natural route of infection (reviewed in Nematollahi et al. 2003); however, reproducibility has been an issue without scarification or pre-treatment with therapeutic compounds (i.e., formalin, H_2O_2; Madsen & Dalsgaard 1999, Garcia et al. 2000, Madetoja et al. 2000, Henriksen et al. 2013, Long et al. 2013). For example, Decostere et al. (2000) immersed rainbow trout in several strains of *F. psychrophilum* at 3 different concentrations (i.e., 10^6, 10^7, 10^8 cfu/mL^{-1}) for 45 minutes, which resulted in 0% mortality. Likewise, Decostere et al. (2000) was unable to produce mortalities in rainbow trout after skin abrasion with a 26 G needle or gill clipping followed by immersion in *F. psychrophilum*, again at varying concentrations (i.e., 10^6, 10^7, 10^8 cfu/mL^{-1}). Madsen & Dalsgaard (1999) were able to slightly elevate mortalities (i.e., 27 - 31% to 41 - 66%) among rainbow trout pre-treated with 0.005% formalin for 30 prior to immersion in 10^7 - 10^8 cfu/mL^{-1} of *F. psychrophilum* in comparison to the control group that received immersion in 10^7 cfu/mL^{-1} of *F. psychrophilum* only. However, the difference between these two groups was not statistically significant and a formalin-treated control group was not included. Henriksen et al. (2013) found a significant difference in mortality among rainbow trout pre-treated with a 200 mg/L^{-1} H_2O_2 for 60 minutes then immersed in *F. psychrophilum*; however, a standardized approach was not established.

2.5.3. Intraperitoneal injection studies

Intraperitoneal injection (IP) of *F. psychrophilum* has proven an effective means of eliciting BCWD among experimentally challenged fish. Decostere et al. (2000) produced similar mortality (i.e., 71% or 69%) among experimentally challenged rainbow trout challenged with either 4.5 x 10^5 cfu/fish^{-1} or 1.4 x 10^4 cfu/fish^{-1} and the observed difference was attributed to slightly different sizes of fish. Similarly, Decostere et al. (2001) IP-injected 1g (10 weeks old), 25g (20 weeks old), and 300g (15 months old) rainbow trout at a concentration of 10^6 cfu/fish^{-1} and was able to produce clinical signs and mortality among the 1g fish only. However, a drawback of this route of exposure is that it bypasses intrinsic mechanisms of host defense including physical barriers (e.g., skin, gills, mucus) and non-specific cells of the innate immune system (e.g., phagocytes, monocytes, cytotoxic cells; reviewed in Magnadóttir 2006).

2.5.4. Intramuscular injection studies

Intramuscular injection (IM) is the preferred route of exposure for larger fish (i.e., >5-10 grams; Fredriksen et al. 2013b) and has consistently produced significant mortality among experimentally challenged fish. For example, ayu (mass of ~1.0 gram per fish) challenged IM (in the dorsal musculature) with a concentration 1.5 x 10^5 cfu/fish^{-1} of *F. psychrophilum* produced 88-90% mortality and acute signs of BCWD disease such as bulging, discolored skin with uncoagulated blood collecting underneath the injection site; however, no open lesions developed (Miwa & Nakayasu 2005). Garcia et al. (2000) found IM injection to be the preferred route of infection when experimentally challenging rainbow trout. In their study, rainbow trout (mass of ~3-6 grams per fish) injected with 4.2 x 10^7 cfu/fish^{-1} experienced cumulative mortality of 100% by 18 days post infection and exhibited necrosis of the skin and underlying musculature near the

site of injection (Garcia et al. 2000). Similarly, Fredriksen et al. (2013b) achieved 100% mortality among juvenile rainbow trout (mass of ~36.6 grams per fish) following IM challenge of 1.0×10^6 cfu/fish^{-1} of *F. psychrophilum*. Of note, Fredriksen et al. (2013b) was unable to produce mortalities among their rainbow trout using an intraperitoneal (IP) route of exposure, even though at least one group was challenged with a dose (i.e., 5.0×10^7 cfu/fish^{-1}) higher than the one used for the IM challenge. Therefore, it was more advantageous to challenge their fish using IM injection since IP injection proved to be inadequate for reproducing BCWD. In the same context, Holt (1987) found that in order to produce comparable mortality in coho salmon, a much higher dose of *F. psychrophilum* was required for IP injection than IM injection. IM injection is an effective route of exposure for producing disease in fish of multiple sizes, whereas other routes of infection, such as immersion, are more appropriate for smaller fish (i.e., <5 grams; Fredriksen et al. 2013b). The reproducibility and consistently high mortality produced by IM injection makes this route of exposure useful not only for studying pathogenesis but also for testing vaccine efficacy.

F. psychrophilum causes disease in numerous fish species around the world, which results in substantial economic losses. For decades, researchers have been examining the genetic diversity of *F. psychrophilum* with the hope of better understanding important aspects of this pathogen, such as its epidemiology and pathogenicity. MLST has revealed substantial genetic diversity of *F. psychrophilum* in many regions of the world; however, the genetic diversity of *F. psychrophilum* is largely unknown in North America. Likewise, it is unclear if this diversity in North America correlates with pathogenicity, as this has not been examined in the previous studies.

REFERENCES

REFERENCES

Anacker, R.L., and E.J. Ordal. 1959. Study on the myxobacterium *Chondrococcus columnaris* I: serological typing. Journal of Bacteriology 78:25-32.

Arai, H., Morita, Y., Izumi, S., Katagiri, T., and H. Kimura. 2007. Molecular typing by pulsed-field gel electrophoresis of *Flavobacterium psychrophilum* isolates derived from Japanese fish. Journal of Fish Diseases 30:345-355.

Allen, K.J., Hesami, S., Lumsden, J.S., MacInnes, J.I., Metcalf, D., and V.E. Ostland. 2008. Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* isolates from Ontario salmonids with bacterial coldwater disease. Canadian Journal of Microbiology 54:619.

Amita, K., Hoshimo, M., Honma, T., and H. Wakabayashi. 2000. An investigation on distribution of *Flavobacterium psychrophilum* in the Umikava River. Fish Pathology 35:193-197.

Aoki, M., Kondo, M., Kawai, K., and S.I. Oshima. 2005. Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of rainbow trout *Oncorhynchus mykiss* fry syndrome. Disease of Aquatic Organisms 67:73-79.

Austin, B., and M. Stobie. 1991. Recovery of yellow pigmented bacteria from dead and moribund fish during outbreaks of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fry syndrome in England. Journal of Fish Diseases 14:677-682.

Avendaño-Herrera, R., Houel, A., Irgang, R., Bernardet, J.F., Godoy, M., Nicolas, P., and E. Duchaud. 2014. Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms. Veterinary Microbiology 170:298-306.

Barnes, M.E., and M.L. Brown. 2011. A Review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. Open Fish Science Journal 4:40-48.

Bernardet, J.F., Sergers, P., Vancanneyt, M., Berthe, F., Kersters, K., and P. Vandamme. 1996. Cutting a Gordian Knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga* a*quatilis* Strohl & Tait 1978). International Journal of Systematic Bacteriology 46:128-148.

Bernardet, J.F., and B. Kerouault. 1989. Phenotypic and genomic studies of Cytophaga psychrophila isolated from diseased rainbow trout Oncorhynchus mykiss in France. Applied Environmental Microbiology 55:1796-1800.

Bernardet, J.F., and P.A.D. Grimont. 1989. Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., and *Flexibacter maritimus* Wakabayashi, Hikida and Masumura 1986. International Journal of Systematic Bacteriology 39:346-354.

Bernardet, J.F., Baudin-Laurencin, F., and G. Tixerant. 1988. First identification of *Cytophaga psychrophila* in France. Bulletin of the European Association of Fish Pathologists 8:104-105

Borg, A.F. 1948. Ph.D. thesis. University of Washington, Seattle, Washington. Studies on myxobacteria associated with diseases in salmonid fishes.

Bruno, D.W. 1992. *Cytophaga psychrophila* (*Flexibacter psychrophilus*) (Borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the UK. Bulletin of European Association of Fish Pathologists 12:215-216.

Bullock, G.L., Hsu, T.C., and E.B. Shotts. 1986. Columnaris disease of salmonids. U.S. Fish and Wildlife Service Fish Disease Leaflet 72.

Castillo, D., Christiansen, R.H., Dalsgaard, I., Madsen, L., Espejo, R., and M. Middelboe. 2016. Comparative Genome Analysis Provides Insights into the Pathogenicity of *Flavobacterium psychrophilum*. PLoS ONE 11:e0152515.

Chakroun, C., Grimont, F., Urdaci, M.C., and J.F. Bernardet. 1998. Fingerprinting of *Flavobacterium psychrophilum* isolates by ribotyping and plasmid profiling. Diseases of Aquatic Organisms 33:167-177.

Chakroun, C., Urdaci, M.C., Faure, D., Grimont, F., and J.F. Bernardet. 1997. Random amplified polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen *Flavobacterium psychrophilum* and among *Flavobacterium* species. Diseases of Aquatic Organisms 31:187-196.

Chen, Y.C., Davis, M.A., LaPatra, S.E., Cain, K.D., Snekvik, K.R., and D.R. Call. 2008. Genetic diversity of *Flavobacterium psychrophilum* recovered from commercially raised rainbow trout, *Oncorhynchus mykiss* (Walbaum), and spawning Coho salmon, *O. kisutch* (Walbaum). Journal of Fish Diseases 31:765-773.

Cipriano, R.C., and R.A. Holt. 2005. *Flavobacterium psychrophilum*, cause of Bacterial Cold-Water Disease and Rainbow Trout Fry Syndrome. Fish Disease Leaflet No. 86, United States Department of the Interior. U.S. Geological Service, National Fish Health Research Laboratory, Kearneysville, West Virginia.

Cipriano, R.C., Schill, W.B., Teska, J.D., and L.A. Ford.W. 1996. Epizootiological Study of Bacterial Cold-Water Disease in Pacific Salmon and Further Characterization of the Etiologic Agent, *Flexibacter psychrophila*. Journal of Aquatic Animal Health 8:28-36.

Clegg, S.R., Carter, S.D., Birtles, R.J., Brown, J.M., Hart, C.A., and J.E. Evans. 2016. Multilocus Sequence Typing of Pathogenic *Treponemes* Isolated from Cloven-Hoofed Animals and Comparison to *Treponemes* Isolated from Humans. Applied and Environmental Microbiology 82:4523-4536.

Dalsgaard, I., and L. Madsen. 2000. Bacterial pathogens in rainbow trout *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. Journal of Fish Diseases 23:199-209.

Davis, H.S. 1946. Care and diseases of trout. United States Fish and Wildlife Service Research Report 12. Washington, DC.

Decostere, A., Lammens, M., and F. Haesebrouck. 2000. Difficulties in experimental infection with *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) using immersion, oral and anal challenges. Research in Veterinary Science 69:165-169.

del Cerro, A., Marquez, I., and J.M. Prieto. 2010. Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Spain. Journal of Fish Diseases 33:285-291.

Duchaud, E., Rochat, T., Habib, C., Barbier, P., Loux, V., Guérin, C., Dalsgaard, I., Madsen, L., Nilsen, H., Sundell, K., Wiklund, T., Strepparava, N., Wahli, T., Caburlotto, G., Manfrin, A., Wiens, G.D., Fujiwara-Nagata, E., Avendaño-Herrera, R., Bernardet, J-F., and P. Nicolas. 2018. Genomic Diversity and Evolution of the Fish Pathogen *Flavobacterium psychrophilum*. Frontiers in Microbiology 9:138.

Ekman, E., Borjeson, H., and N. Johansson. 1999. *Flavobacterium psychrophilum* in Baltic salmon Salmo salar brood fish and their offspring. Diseases of Aquatic Organisms 37: 159–163.

Faisal, M., Schulz, C., Loch, T., Kim, R., Hnath, J., Whelan, G., Taylor, W., Lynch, A., and N. Leonard. 2013. Current status of fish health and disease issues in the Laurentian Great Lakes: 2005–2010. Pages 259-302 in W.W. Taylor, A.J. Lynch, and N.J. Leonard, editors. Great Lakes fisheries policy 43 and management: A binational perspective. Michigan State University Press, East Lansing, Michigan.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., and B.G. Spratt. 2004. eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. Journal of Bacteriology 186:1518-1530.

Fredriksen, B.N., Olsen, R.H., Furevik, A., Souhoka, R. A., Gauthier, D., and B. Brudeseth, B. 2013a. Efficacy of a divalent and a multivalent water-in-oil formulated vaccine against a highly virulent strain of *Flavobacterium psychrophilum* after intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*). Vaccine 31:1994–1998.

Fredriksen, B.N., Furevik, A., Gauthier, D., Egenberg, M., Paulse, E.D., and B. Brudeseth. 2013b. Intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*) with two Norwegian field strains of *Flavobacterium psychrophilum*. Fish and Shellfish Immunology 35:595-598.

Fujiwara-Nagata, E., Chantry-Darmon, C., Bernardet, J.F., M. Eguchi, M., Duchaud E., and P. Nicolas. 2013. Population structure of the fish pathogen *Flavobacterium psychrophilum* at a whole country and model river levels in Japan. Veterinary Research 44:34.

Garcia, C., Pozet, F., and C. Michel. 2000. Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 42:191-197.

Henriksen, M.M.M., Madsen, L., and I. Dalsgaard. 2013. Effect of Hydrogen Peroxide on Immersion Challenge of Rainbow Trout Fry with Flavobacterium psychrophilum. PLoS ONE 8:e62590.

Hesami, S., Allen, K.J., Metcalf, D., Ostland, V.E., MacInnes, J.I., and J.S. Lumsden. 2008. Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* isolates from Ontario salmonids with bacterial coldwater disease. Canadian Journal of Microbiology 54:619-629.

Högfors-Rönnholm, E., and T. Wiklund. 2010. Phase variation in *Flavobacterium psychrophilum* characterization of two distinct colony phenotypes. Diseases of Aquatic Organisms 90:43-53.

Holt, R.A., Rohovec, J.S., and J.L. Fryer. 1993. Bacterial cold-water disease. Pages 3-23 in V. Inglis, R.J. Roberts, and N.R. Bromage, editors. Bacterial Diseases of Fish. Blackwell Scientific Publications, Oxford.

Holt, R.A. 1987. Ph.D. thesis. Oregon State University, Corvallis, Oregon. *Cytophaga psychrophila*, the causative agent of bacterial cold water disease in salmonid fish.

Iida, Y. and A. Mizokami. 1996. Outbreaks of coldwater disease in wild ayu and pale chub. Fish Pathology 31: 157–164.

Izumi, S., and F. Aranishi. 2004. Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. Applied and Environmental Microbiology 70:3968-3972.

Izumi, S., Liu, H., Aranishi, F., and H. Wakabayashi. 2003. A novel serotype of *Flavobacterium psychrophilum* detected using antiserum against an isolate from amago, *Oncorhynchus masou rhodurus* Jordan & Gilbert, in Japan. Journal of Fish Diseases 26:677-680.

Izumi, S., and H. Wakabayashi. 1999. Further study on serotyping of *Flavobacterium psychrophilum*. Fish Pathology 34:89-90.

Jolley, K.A., Chan, M.S., and M.C. Maiden. 2004. mlstdbNet – distributed multi-locus sequence typing (MLST) databases. BMC Bioinformatics 5:86.

Kotetishvili, M., Kreger, A., Wauters, G., Morris, J. G., Sulakvelidze, A., and O.C. Stine. 2005. Multilocus Sequence Typing for Studying Genetic Relationships among *Yersinia* Species. Journal of Clinical Microbiology 43:2674–2684.

Krongdang, S., Evans, J.D., Pettis, J. S., and P. Chantawannakul. 2017. Multilocus sequence typing, biochemical and antibiotic resistance characterizations reveal diversity of North American strains of the honey bee pathogen *Paenibacillus larvae*. PLoS ONE 12:e0176831.

Kum, C., Kirkan, S., Sekkin, S., Akar, F., and M. Boyacioglu. 2008. Comparison of in vitro antimicrobial susceptibility in *Flavobacterium psychrophilum* isolated from rainbow trout fry. Journal of Aquatic Animal Health 20:245-251.

Lee, K.B., and G.J. Heo. 1998. First isolation and identification of *Cytophaga psychrophila* from cultured ayu Korea. Fish Pathology 33:37-38.

Lehmann J., D. Mock, F.J., Sturenberg, and J.F. Bernardet. 1991. First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids. Diseases of Aquatic Organisms 10:217-220.

Leon, J., Avalos, R., and M. Ponce. 2009. *Flavobacterium psychrophilum* and its pathology of alevins from the El Ingenio fish farm, Huancayo. Reviews Peru Biology 15:117-124.

Levipan, H.A., and R. Avendaño-Herrera. 2017. Different Phenotypes of Mature Biofilm in *Flavobacterium psychrophilum* Share a Potential for Virulence That Differs from Planktonic State. Frontiers in Cellular and Infection Microbiology 7:76.

Lewin, R.A., and D.M. Lounsbery. 1969. Cultivation and Characterization of *Flexibacteria*. Microbiology, 58:145–170.

Loch T.P., M Faisal. 2017. *Flavobacterium* spp., p. 211–232. In Woo, PTK, Cipriano, RC (eds.), Fish Viruses and Bacteria: Pathobiology and Protection CABI.

Long, A., Fehringer, T.R., LaFrentz, B.R., Call, D.R., and K.D. Cain. 2014. Development of a waterborne challenge model *for Flavobacterium psychrophilum*. FEMS Microbiology Letters 359:154–160.

Long A., Fehringer T.R., Swain M.A., LaFrentz B.R., Call, D.R., and K.D. Cain. 2013. Enhanced efficacy of an attenuated *Flavobacterium psychrophilum* strain cultured under iron-limited conditions. Fish Shellfish Immunology 35:1477–1482.

Lorenzen, E., Dalsgaard, I., and J.F. Bernardet. 1997. Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome I: Phenotypic and genomic studies. Diseases of Aquatic Organisms 31:197-208.

Lorenzen, E., and N.J. Olesen. 1997. Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome II: serological studies. Diseases of Aquatic Organisms 31:209-220.

Lorenzen, E., Dalsgaard, I., From, J., Hansen, E.M., Horlyck, V., Korsholm, H., Mellergaard, S., and N.J. Olesen. 1991. Preliminary investigation of fry mortality syndrome in rainbow trout. Bulletin European Association of Fish Pathologists 11:77-79.

Lumsden, J.S., Ostland, V.E., and H.W. Ferguson. 1996. Necrotic myositis in cage cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), caused by *Flexibacter psychrophilus*. Journal of Fish Diseases 19:113–119.

Magnadóttir, B. 2006. Innate immunity of fish (overview). Fish and Shellfish Immunology 20:137-151

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Actman, M., and Spratt, B. G. 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences of the United States of America 95:3140-3145.

Madetoja, J., Dalsgaard, I., and T. Wiklund. 2002. Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. Diseases of Aquatic Organisms 52:109-18.

Madetoja, J., Hänninen, M-N., Hirvelä-Koski, V., Dalsgaard, I., and T. Wiklund. 2001. Phenotypic and genotypic characterization of *Flavobacterium psychrophilum* from Finnish fish farms. Journal of Fish Diseases 24: 469–479.

Madetoja, J., Nyman, P., and T. Wiklund. 2000. *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 43:27-38.

Madsen, L., and I. Dalsgaard. 2000. Comparative studies of Danish *Flavobacterium psychrophilum* isolates: ribotypes, plasmid profiles, serotypes and virulence. Journal of Fish Diseases 23:211-218.

Madsen, L., and I. Dalsgaard. 1999. Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 36:169-176.

Madsen, L., and I. Dalsgaard. 1998. Characterization of *Flavobacterium psychrophilum*; comparison of proteolytic activity and virulence of strains isolated from rainbow trout (*Oncorhynchus mykiss*). In: Methodology in Fish Disease Research (ed. by A.C. Barnes, G.A. Davidson, M.P. Hiney & D. McIntosh), pp. 45–52. Fisheries Research Services, Aberdeen.

Mata, M., Skarmeta, A., and Y. Santos. 2002. A proposed serotyping system for *Flavobacterium psychrophilum*. Letters in Applied Microbiology 35:166-170.

McArthur A.G, Waglechner N., Nizam F., Yan A., Azad M.A., Baylay A.J., Bhullar K., Canova M.J., De Pascale G., Ejim L., Kalan L., King A.M., Koteva K., Morar M., Mulvey M.R., O'Brien J.S., Pawlowski A.C., Piddock L.J.V., Spanogiannopoulos P., Sutherland A.D., Tang I., Taylor P.L., Thaker M., Wang W., Yan M., Yu T., and G.D. Wright. 2013. The Comprehensive Antibiotic Resistance Database. Antimicrobial Agents and Chemotherapy 57:3348-3357.

Michel, C., Antonio, D., and R.P. Hedrick.1999. Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. Research in Microbiology 150:351–358.

Miwa, S., and C. Nakayasu. 2005. Pathogenesis of experimentally induced bacterial cold water disease in ayu *Plecoglossus altivelis*. Diseases of Aquatic Organisms 67: 93–104.

Nemoy, L.L., Kotetishvili, M., Tigno, J., Keefer-Norris, A., Harris, A.D., Perencevich, E.N., Johnson, J.A., Torpey, D., Sulakvelidze, A., Morris, J.G., and Stine, O.C. 2005. Multilocus Sequence Typing versus Pulsed-Field Gel Electrophoresis for Characterization of Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolates. Journal of Clinical Microbiology 43:1776–1781.

Nematollahi, A., Decostere, A., Pasmans, F., and F. Haesebrouck. 2003. *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases 26:563-574.

Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J.F., and E. Duchaud. 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709.

Nilsen, H., Sundell, K., Duchaud, E., Nicolas, P., Dalsgaard, I., Madsen, L., Aspán, A., Jansson, E., Colquhoun, D.J., and T. Wiklund. 2014. Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. Applied and Environmental Microbiology 80:2728-2736.

Nilsen, H., Olsen, A.B., Vaagnes, O., Hellberg, H., Bottolfsen, K., Skjeslstad, H., and D.J. Colquhoun. 2011. Systemic *Flavobacterium psychrophilum* infection in rainbow trout *Oncorhynchus mykiss* (Walbaum) farmed in fresh and brackish water in Norway. Journal of Fish Diseases 34:403-408.

Nilz, J., Schaumburg, M., Eskens, U., Hamann, H.P., and M. Zschock. 2009. *Flavobacterium psychrophilum* in hessian fish farms. Tieraerztliche Umschau 64:191-199.

Obach, A., and F.B. Laurencin. 1991. Vaccination of rainbow trout *Oncorhynchus mykiss* against the visceral form of coldwater disease. Disease of Aquatic Organisms 12:13-15.

Pacha, R.E., and S. Porter. 1970. Myxobacterial disease of salmonids. In: Snieszko SF (ed) A symposium on disease of fishes and shellfishes, Special publication No. 5. Washington D.C, American Fisheries Society, pp 243-257.

Pacha, R.E. 1968. Characteristics of *Cytophaga psychrophila* (Borg) isolated during outbreaks of bacterial cold-water disease. Applied Microbiology 16:97-101.

Rochat, T., Fujiwara-Nagata, E., Calvez, S., Dalsgaard, I., Madsen, L., Calteau, A., Lunazzi, A., Nicolas, P., Wiklund, T., Bernardet, J.F., and E. Duchaud. 2017. Genomic Characterization of *Flavobacterium psychrophilum* Serotypes and Development of a Multiplex PCR-Based Serotyping Scheme. Frontiers in Microbiology 8:1752.

Rucker, R.R., Earp, B.J., and E.J. Ordal. 1953. Infectious diseases of Pacific salmon. Transactions of the American Fisheries Society 83:297-312.

Sarti, M., Giorgetti, G., and A. Manfrin. 1992. Method for rapid diagnosis of visceral myxobacteriosis in reared trout in Italy. Bulletin of the European Association of Fish Pathologists 12:209-210.

Scally, M., Schuenzel, E.L., Stouthamer, R., and L. Nunney. 2005. Multilocus Sequence Type System for the Plant Pathogen *Xylella fastidiosa* and Relative Contributions of Recombination and Point Mutation to Clonal Diversity. Applied and Environmental Microbiology 71:8491-8499.

Saunders, N. A., and A. Holmes. 2014. Multilocus Sequence Typing (MLST) of Staphylococcus aureus. In Y. Ji (Ed.), Methicillin-Resistant *Staphylococcus Aureus* (MRSA) Protocols (pp. 113–130). Totowa, NJ: Humana Press.

Schachte, J.H. 1983. Coldwater disease. Pages 193-197 in F.P. Meyer, J.W. Warren, and T.G. Carey, editors. A Guide to Integrated Fish Health Management in the Great Lakes Basin. Special Publication No. 83-2, Great Lakes Fishery Commission, MI, USA.

Schmidtke, L.M., and J. Carson. 1995. Characteristics of *Flexibacter psychrophilus* isolated from Atlantic salmon in Australia. Diseases of Aquatic Organisms 21:157-161.

Siekoula-Nguedia, C., Blanc, G., Duchaud, E., and S. Calvez. 2012. Genetic diversity of *Flavobacterium psychrophilum* isolated from rainbow trout in France: predominance of a clonal complex. Veterinary Microbiology 161:169-178.

Starliper, C.E. 2011. Bacterial coldwater disease of fishes caused *by Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108.

Strepparava, N., Nicolas, P., Wahl, T., Segner, H., and O. Petrini. 2013. Molecular epidemiology of *Flavobacterium psychrophilum* from Swiss fish farms. Diseases of Aquatic Organisms 105:203-210.

Soule, M., LaFrentz, S., Cain, K., LaPatra, S., and D.R. Call. 2005. Polymorphisms in 16S rRNA genes of *Flavobacterium psychrophilum* correlate with elastin hydrolysis and tetracycline resistance. Diseases of Aquatic Organisms 65:209-216.

Toranzo, A.E., and J.L. Barja. 1993. Fry mortality syndrome (FMS) in Spain. Isolation of the causative bacterium *Flexibacter psychrophilus*. Bulletin European Association of Fish Pathologists 13:30-32.

Valdebenito, S., and R. Avendaño-Herrera. 2009. Phenotypical, serological and genetic characterization of *Flavobacterium psychrophilum* strains isolated from salmonids in Chile. Journal of Fish Diseases 32:321-333.

Van Vliet, D., Loch, T.P., and M. Faisal. 2015. *Flavobacterium psychrophilum* Infections in Salmonid Broodstock and Hatchery-Propagated Stocks of the Great Lakes Basin. Journal of Aquatic Animal Health 27:192-202.

Wakabayashi, H., Toyama, T., and T. Iida. 1994. A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathology 29:101-104.

Wakabayashi H., and S. Egusa. 1974. Characteristics of myxobacteria associated with some freshwater fish diseases. Journal of the Japan Fisheries Society 40:751-757.

Chapter 2

Novel MLST *F. psychrophilum* Genotypes Infecting North American Salmonids

1. Abstract

Flavobacterium psychrophilum is the etiological agent of Bacterial Coldwater Disease (BCWD) and Rainbow Trout Fry Syndrome (RTFS), which cause significant economic losses in salmonid aquaculture, particularly to farm-raised rainbow trout (*Oncorhynchus mykiss*). The genetic heterogeneity of this fish pathogen has been explored recently by numerous investigators, whereby isolates from Europe, Asia, South America, Australia, and most recently, North America, have been genotyped using multilocus sequencing typing (MLST). These analyses revealed North American *F. psychrophilum* isolates to be genetically heterogeneous; nevertheless, much remains to be elucidated in regards to the population structure of this bacterium in North America. In this study, MLST was used to genotype 314 North American *F. psychrophilum* isolates that were opportunistically obtained from 10 fish host species from 20 US states and one Canadian province over a period of nearly four decades. My analyses placed these isolates into 66 sequence types (STs), 47 of which were novel. Furthermore, seven novel North American clonal complexes (CCs) were discovered, which brings the total number of North American CCs to 12. Many of the identified CCs have only been detected in North America, whereas others were found in North America and abroad. These CCs were diverse and varied in terms of host association, distribution, and association with disease. The largest *F. psychrophilum* CC identified in this study was CC-ST10, within which 10 genotypes were discovered, most of which came from *O. mykiss* experiencing BCWD. ST275 of CC-ST10 was recovered from wild/feral adult steelhead and the recovery of CC-ST10 from feral/wild fish in North America has not been reported previously. Furthermore, the progeny of infected broodstock were found to harbor the same ST, suggesting some STs are capable of circumventing current egg disinfection practices. The diversification of *F. psychrophilum* in

North America was found to be driven four times more frequently by recombination than point mutation and may be an indication of how new phenotypes emerge within this pathogen.

2. Introduction

Flavobacterium psychrophilum (Family Flavobacteriaceae; Phylum Bacteroidetes) is a Gram-negative, psychrophilic bacterium (reviewed in Nematollahi et al. 2003). It is the etiological agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS), both of which principally impact salmonids species (Family Salmonidae; reviewed in Cipriano & Holt 2005). These diseases cause significant mortality in farmed/hatchery-reared salmonids and can result in substantial economic losses (reviewed in Loch & Faisal 2017, Duchaud et al. 2018). Since its initial isolation in 1948 in Washington, USA (Borg 1948), this bacterium has been reported from at least five continents (e.g., Asia, Europe, North America, Australia, and South America), giving it a nearly worldwide distribution, particularly where salmonids are present (Nematollahi et al. 2003).

For decades, the degree of intraspecific genetic diversity within *F. psychrophilum* was unclear (Madsen & Dalsgaard 2000); however, advances in molecular typing techniques subsequently revealed considerable heterogeneity within this species. One such technique is multilocus sequence typing (MLST). Due to its discriminatory power, reproducibility, and repeatability (Cooper & Feil 2004), Nicolas et al. (2008) designed a MLST scheme for the genotyping of *F. psychrophilum* that is now being followed worldwide. In their original study, 50 *F. psychrophilum* isolates from Asia, Europe, North America, and South America, including 10 from North America (9 from the USA) were examined. Nicolas et al. (2008) revealed the presence of 30 genotypes, some of which were closely related or correlated to a geographic

location and/or host species. Since then, this genotyping technique has been applied to >1,000 *F. psychrophilum* isolates from different parts of the world, yielding nearly 200 distinct genotypes, which exhibited similar trends to those observed with the original 50 isolates tested (Siekoula-Nguedia et al. 2012, Fujiwara-Nagata et al. 2013, Strepparava et al. 2013, Avendaño-Herrera et al. 2014, Nilsen et al. 2014, Castillo et al. 2016, Van Vliet et al. 2016). To improve the comparison of all *F. psychrophilum* isolates, Jolley & Maiden (2010) developed a comprehensive and curated database (https://pubmlst.org/fpsychrophilum/) that is an invaluable resource for epidemiological studies of *F. psychrophilum*. In this context, as novel sequence types (STs; i.e., genotypes) were being discovered, some were found to be closely related, forming a clonal complex (CC), while others were genetically distinct from all other STs, described as a singleton. As the MLST database grew, a number of correlations became evident. For example, CC-ST10 encompasses hundreds of isolates and dozens of STs, yet almost all of them have been isolated from farm-raised rainbow trout (*Oncorhynchus mykiss*) on multiple continents. In the same context, members of CC-ST123 (12 isolates in 6 STs) have all been isolated from Atlantic salmon (*Salmo salar*) in Norway (Nilsen et al. 2014), whereas CC-ST52 and CC-ST48/CC-ST56 were recovered exclusively from non-salmonid species (Fujiwara-Nagata et al. 2013) and experimental challenge data provided evidence that these two CCs differ in their pathogenicity as well (Fujiwara-Nagata et al. 2012).

Despite the economic losses *F. psychrophilum* causes in North American salmonid populations (reviewed in Cipriano & Holt 2005), only 10 isolates had been MLST genotyped as of 2016 (Nicolas et al. 2008). Van Vliet et al. (2016) characterized an additional 95 *F. psychrophilum* isolates from 9 US states, finding 34 *F. psychrophilum* STs, 28 of which were previously undescribed. These STs varied spatially, by host species, and in association with

mortality events similar to those recovered outside of North America (Van Vliet et al. 2016). This study focused on isolates from three *Oncorhynchus* spp. (e.g., *O. mykiss*, *O. kisutch*, and *O. tshawytscha*) and over half were recovered from one US state (i.e., Michigan).

To better understand the genetic diversity of *F. psychrophilum* in North America, I undertook this study and MLST-genotyped 314 *F. psychrophilum* isolates recovered from 10 fish species, collected from 20 US states and one Canadian province. I took advantage of the presence of historical, cryopreserved *F. psychrophilum* isolates that were isolated over a period of four decades and included them in this study. This unique collection of isolates allowed for an evaluation of the genotypic relationships not only among North American isolates, but also among those in other parts of the world. An additional objective was to evaluate the role of point mutation and/or recombination in leading to the MLST diversity discovered in this study.

3. Materials and Methods

3.1. Fish collection and isolation of *F. psychrophilum*

A total of 314 *F. psychrophilum* isolates recovered from 20 U.S. states and one Canadian province over nearly four decades (e.g., 1981 - 2018) were examined in this study (Table 2.1a). The isolates originated from Idaho (n = 104), Utah (n = 43), Michigan (n = 38), Pennsylvania (n = 29), North Carolina (n = 24), Washington (n = 19), Oregon (n = 18), West Virginia (n = 7), Montana (n = 6), California (n = 5), South Dakota (n = 4), Virginia (n = 4), Minnesota (n = 3), New Mexico (n = 2), Colorado (n = 2), Alaska (n = 1), Indiana (n = 1), Maryland (n= 1), Wisconsin (n = 1), and Vermont (n = 1) (Figure 2.1). Additionally, a single isolate from British Columbia was included (Figure 2.1). The isolates were recovered from four fish genera and 10 species, including rainbow trout/steelhead (*O. mykiss*; n = 260), coho salmon (*O. kisutch*; n =

25), Chinook salmon (*O. tshawytscha*; *n* = 11), cutthroat trout (*O. clarkii*; *n* = 1), sockeye salmon (*O. nerka*; *n* = 1), Atlantic salmon (*S. salar*; *n* = 6), brown trout (*S. trutta*; *n* = 6), lake trout (*Salvelinus namaycush; n* = 1), splake (*S. fontinalis* x *S. namaycush; n* = 1), and white sturgeon (*Acipenser transmontanus*; *n* = 2; Table 2.1a). The isolates were recovered from external and internal tissues of fish either showing gross signs of BCWD or that were apparently healthy (Table 2.1a).

Recovery of the aforementioned *F. psychrophilum* isolates was completed by multiple investigators using flavobacterial-selective media, including cytophaga agar (CA; Anacker & Ordal 1959) and tryptone yeast extract agar (TYES; Holt 1987) with or without supplementation of 4 mg liter^{-1} of neomycin sulfate, or Hsu-Shotts medium (HSU; Bullock et al. 1986). *F. psychrophilum* isolates not collected in house were procured from project collaborators as actively growing cultures on solid media or as frozen cryo-stock and revived and subcultured on to TYES agar to verify isolate purity. A single colony was then inoculated into analagous broth media for generation of glycerol-supplemented cryo-stock (maintained at -80˚C) for further use.

3.2. Bacterial DNA extraction and *F. psychrophilum* identity confirmation

F. psychrophilum isolates were revived from cryostocks into TYES broth and incubated for 72-96 hours at 15˚C, at which time bacterial cells were subcultured on to fresh TYES medium with agar to verify purity. Bacterial genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, California) according to the manufacure's protocol for Gram-negative bacteria, quantified using a Qubit® Fluorometer (Life Technologies, Grand Island, New York), and then diluted to 20 ng/µl. Prior to MLST analyses, the identity of all

isolates was confirmed as *F. psychrophilum* using the conventional PCR assay of Toyama et al. (1994) as detailed previously (Van Vliet et al. 2015).

3.3. Multilocus sequence typing

Partial sequences of seven housekeeping genes (*trpB, gyrB, dnaK, fumC, murG, tuf, atpA*), previously selected by Nicolas et al. (2008), were PCR amplified as previously detailed (Van Vliet et al. 2016). Five µl of amplicon was electrophoresed in a 1.5% agarose gel prepared with 1 X SYBR®Safe DNA gel stain for 45 min at 100V. The gel was then viewed under ultraviolet transillumination to confirm the presence of an appropriately sized band. One µl of PCR product was then combined with 0.25 µl of ExoSAP-IT™ (Applied Biosystems) and 3.0 µl of 1 X PCR buffer with $MgCl_2$ (Sigma-Aldrich), incubating for 20 min at 37°C, followed by enzyme inactivation for 10 min at 95°C. The same primers employed for PCR amplification of each of the seven house-keeping genes were used to bidirectionally sequence purified PCR product at the Genomics Technology Support Facility (Michigan State University).

3.4. MLST data analysis

Chromatograms were manually verified for quality prior to assignation of allele types (ATs) and STs via an in-house script (P. Nicolas, INRA). eBURST v3 (eburst.mlst.net; Feil et al. 2004, Spratt et al. 2004) was used to dichotomize STs into CCs or as singletons based on locus variations in their allelic profiles. STs were considered single-locus variants (SLVs) when they differed at one of the seven ATs, whereas STs that varied at two of the seven ATs were considered double-locus variants (DLVs). CC were comprised of STs on the basis of their shared SLVs and were named after the predicted founding ST (i.e., the ST with the highest

number of SLVs). STs that did not form part of a CC were referred to as singletons. All isolates within the publicly accessible MLST database for *F. psychrophilum* (*n* = 1,097; https://pubmlst.org/fpsychrophilum/; Jolley & Maiden 2010), along with the 314 isolates of this study, were included in the eBURST v3 (Feil et al. 2004, Spratt et al. 2004) analysis. All previously typed isolates from North America (*n* = 107; Nicolas et al. 2008, Castillo et al. 2016, Van Vliet et al. 2016) were included in the eBURST v3 analyses.

3.5. Bayesian inference

Bayesian phylogenetic analysis was conducted using MrBayes v3.2.5 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) after concatenating the seven MLST loci sequences for each North American ST. Automated model selection was implemented in PAUP* v4.0a (Build 162; Swofford 2002) and the most appropriate model of DNA substitution for each MLST locus was selected using the corrected Akaike information criterion (AICc) score. Subsequently, a partitioned data file, with one partition per MLST locus, was used for Markov chain Monte Carlo (MCMC) analysis. The analysis consisted of two independent runs, both of which used four Markov chains (one heated, three cold) and began with a random starting tree. This analysis sampled every thousandth tree for 520,000 generations, at which point the average standard deviation of split frequency was <0.05. Prior to phylogenetic reconstruction, a 25% burn-in period (i.e., the first 25% of trees were discarded and not used for phylogenetic reconstruction) was utilized. FigTree v1.4.3 (Rambaut 2009) was used to visualize and annotate the Bayesian dendrogram.

3.6. MLST genetic analyses

Analysis of sequence variation among the seven housekeeping genes was implemented via multiple algorithms. DNAsp v6.10.01 (Rozas et al. 2017) was used to identify the number of alleles for each gene (i.e., allele types), the number of segregating sites (S), nucleotide diversity (π), and the Hudson and Kaplan lower bound on the minimum number of recombination events (R_{min}; Hudson & Kaplan 1985). Utilizing SLV STs from CCs and a method described by Feil et al. (2000), the contributions of recombination or point mutations to clonal diversification was estimated. Recombination was also evaluated among the concatenated sequences of representative STs using the pairwise homoplasy index (PHI) executed in SplitsTree v4.14.6 (Huson & Bryant 2006). START2 (Jolley et al. 2001) was utilized to calculate the ratios of non-synonymous to synonymous substitutions (dN/dS) for each locus. LIAN 3.7 (Haubold & Hudson 2000) identified the extent of linkage disequilibrium (I^S_A) between the ATs using a Monte Carlo simulation, whereby loci were resampled 1,000 times without replacement and observed mismatch variance was compared to the expected mismatch variance. LIAN 3.7 was also used to measure mean genetic diversity (H).

4. Results

4.1. Identification of novel *F. psychrophilum* MLST genotypes in North America

Out of the 314 North American *F. psychrophilum* isolates typed using MLST in the present study, 119 represented 47 novel MLST genotypes that have never been described anywhere in the world (Table 2.1b). Of these 47 genotypes, 11 formed four novel MLST clonal complexes (CC-ST288, CC-ST310, CC-ST281, CC-ST-287; Figure 2.2). CC-ST310 was recovered exclusively from juvenile rainbow trout (*O. mykiss*) suffering from BCWD at several

private facilities (Table 2.1a, Table 2.1b). Members of the CC-ST281were also recovered exclusively from steelhead trout (*O. mykiss*); however, 10/11 isolates belonging to this CC were from apparently healthy adult steelhead trout (*O. mykiss*), whereas the remaining isolate was recovered from recently hatched steelhead trout (*O. mykiss*) fry experiencing BCWD in a state fish hatchery (Table 2.1a, Table 2.1b). CC-ST288 was recovered exclusively from apparently healthy, adult Chinook salmon returning to spawn at a Michigan weir (i.e., MI-WE1). The fourth CC; CC-ST287, was recovered from both coho salmon and splake, both of which were being raised in two different state fish hatcheries (Table 2.1a, Table 2.1b).

Of the remaining 36 novel MLST genotypes, four clustered with several previously described North American MLST genotypes to form three additional novel MLST CCs (Figure 2.2). More specifically, former singleton ST29 clustered with novel genotype ST308 to form CC-ST29, which contains *F. psychrophilum* isolates recovered exclusively from Chinook salmon (Nicolas et al. 2008, Van Vliet et al. 2016; Table 2.1a, Table 2.1b). Likewise, former singleton ST257, which was previously recovered from a diseased juvenile steelhead trout (*O. mykiss*) from a state fish hatchery in Michigan (Van Vliet et al. 2016), was rediscovered in the present study in apparently healthy adult steelhead trout (*O. mykiss*) returning to spawn, and clustered with novel genotype ST276 to form CC-ST276 (Table 2.1a, Table 2.1b; Figure 2.2). Like ST257, ST276 had been recovered from both apparently healthy and diseased adult and juvenile steelhead trout (*O. mykiss*); however, all isolates identified as ST276 were from steelhead trout (*O. mykiss*) at state fish hatcheries in Pennsylvania (Table 2.1a, Table 2.1b). The remaining novel CC, CC-ST296, was comprised of ST265, ST296, and ST299 (Figure 2.2). All three of these STs were recovered from different host species, whereby previously described ST265 was recovered from Chinook salmon (Van Vliet et al. 2016), novel genotype ST296 was recovered

from both rainbow trout (*O. mykiss*) and sockeye salmon, and novel genotype ST299 was recovered from steelhead trout (*O. mykiss*) (Table 2.1a, Table 2.1b).

Of the remaining 32 novel MLST genotypes, 10 were placed into a single, preexisting North American CC (i.e., CC-ST10) that previously contained only six North American STs (Table 2.1a, Table 2.1b; Figure 2.2; https://pubmlst.org/fpsychrophilum/). Of these 10 novel genotypes, nine were recovered exclusively from *O. mykiss*, whereas the remaining genotype (i.e., ST306) was recovered from both rainbow trout (*O. mykiss*) and coho salmon (Table 2.1a, Table 2.1b). Additionally, 9/10 CC-ST10 genotypes were recovered from captive *O. mykiss*, whereas the remaining genotype (i.e., ST275) was recovered from both captive and wild/feral steelhead trout (*O. mykiss*; Table 2.1a, Table 2.1b). Furthermore, the wild/feral steelhead trout (*O. mykiss*) that novel genotype ST275 was recovered from was an apparently healthy adult, which is in contrast to all of the other nine genotypes as they were recovered from sub-adult *O. mykiss* that were displaying signs of BCWD disease (Table 2.1a, Table 2.1b). Nineteen of the remaining 22 novel genotypes were not placed into any previously or newly identified MLST CC (Figure 2.2) and thus were considered to be singleton genotypes, all of which were exclusive to a single fish host species (Table 2.1a, Table 2.1b).

When the genotypes of this study were combined with all previously reported genotypes from other regions of the world (https://pubmlst.org/fpsychrophilum/), the remaining three novel MLST genotypes recovered in this study belonged to two additional CCs whose members were recovered from Europe (i.e., Denmark, Finland, Norway, Switzerland). In particular, ST301 grouped within preexisting CC-ST191 as a single locus variant (SLV) of ST191, whereas ST277 and ST283 clustered with former singleton ST232 to form CC-ST232 (Figure 2.3).

4.2. *F. psychrophilum* MLST genotypes detected in this study that were reported previously from North America and/or abroad

The remaining 195 *F. psychrophilum* isolates of this study represented 19 MLST genotypes that have been previously reported in North America and/or abroad (Table 2.1a, Table 2.1b). Of these 19 genotypes, three (e.g., ST11, ST256, ST257) belonged to three CCs that are currently present only in North America. Of the remaining 16 previously described genotypes, seven belonged to one of three CCs that are present in both North America and/or Europe, Asia, and South America. For example, ST10, ST78, ST84, and ST86 were all recovered from diseased captive *O. mykiss* in the present study and belonged to CC-ST10, which is known to house numerous genotypes globally (Figure 2.3; https://pubmlst.org/fpsychrophilum/). Furthermore, ST10 was also recovered from coho salmon, Chinook salmon, and white sturgeon in this study and thus has the broadest host range of any North American genotype to date (Table 2.1a, Table 2.1b). Next, ST28 was recovered from cutthroat trout and belongs to CC-ST31, which is present not only in North America but Europe as well (Table 2.1a, Table 2.1b; Figure 2.2; https://pubmlst.org/fpsychrophilum/). Lastly, ST9 and ST13 were recovered herein from predominantly coho salmon; although one isolate belonging to ST9 was recovered from a rainbow trout (*O. mykiss*) as well. Nonetheless, these two genotypes form CC-ST9, which has been recovered predominantly from coho salmon in North America, Europe, Asia, and South America (Table 2.1a, Table 2.1b; Figure 2.2, Figure 2.4; http://pubmlst.org/fpsychrophilum).

When the genotypes of this study were combined with all previously reported genotypes from other regions of the world, two genotypes that were recovered in this study (i.e., ST267, ST70) belonged to two CCs whose members are from Europe or both Europe and South America. For example, ST267 belonged to CC-ST191 and was recovered from apparently

healthy, wild/feral adult steelhead trout (*O. mykiss*), whereas ST70 belonged to CC-ST124, which was recovered from an Atlantic salmon (Table 2.1a, Table 2.1b; Figure 2.3).

The remaining seven MLST genotypes were identified as singletons. Of these, five were recovered from a single fish host species, whereas the other two were recovered from multiple host species. In specific, ST27 and ST255 were recovered exclusively from steelhead trout (*O. mykiss*), both of which displayed signs of disease; ST30 and ST74 were recovered exclusively from coho salmon; and ST76 was recovered exclusively from Chinook salmon (Table 2.1a, Table 2.1b). Of the two singleton genotypes recovered from multiple host species, ST253 was recovered from an apparently healthy, captive adult rainbow trout (*O. mykiss*) and additionally from several brown trout, all of which displayed signs of BCWD disease. The final singleton genotype, ST258, was recovered predominantly from sub-adult, captive and diseased coho salmon but also from a single, apparently healthy adult steelhead trout (*O. mykiss*) returning to spawn (Table 2.1a, Table 2.1b).

4.3. Bayesian inference of North American *F. psychrophilum* genotypes

Phylogenetic analysis of the 86 currently recognized North American STs yielded robust support (i.e., posterior probability >70%) for all 12 North American CCs identified by eBURST (Figure 2.5). In addition, the three CCs (i.e., CC-ST191, CC-ST232, CC-ST124) that contained North American STs, which were formed when all STs from other regions of the world were combined with those in North America, were also robustly supported (Figure 2.5). Of these 15 CCs, CC-ST10 was the most basal, whereas CC-ST9 was the most derived (Figure 2.5). Of note, the 16 STs of CC-ST10 present in North America were separated into four clades (i.e., CC-ST10a-d; Figure 2.5), three of which (e.g., CC-ST10a-c) were robustly supported (Figure 2.5).

Of the three well-supported clades, CC-ST10a was most basal and contained ST303 and ST305, CC-ST10b contained eight STs, two of which (i.e., ST304, ST306) formed a robustly supported sub-clade (Figure 2.5). Lastly, CC-ST10c was comprised of five STs and was the most derived (Figure 2.5). In addition to the five STs of CC-ST10c, another ST, ST27, was found to share the same most recent common ancestor as all others in this clade (Figure 2.5). The remaining 14 CCs were reconstructed as independent clades, some of which shared a most recent common ancestor with a singleton ST (Figure 2.5). Furthermore, the Bayesian phylogeny provided support for the formation of several other clades made up solely of singleton STs (Figure 2.5). One clade in particular contained *F. psychrophilum* isolates belonging to three genotypes (i.e., ST251, ST254, ST266), which were recovered exclusively from feral Michigan Chinook salmon. Similarly, another *F. psychrophilum* clade housed genotypes that had been recovered from feral Michigan Chinook salmon; however, this clade housed not only singleton STs but CC-ST288 as well (Figure 2.5).

4.4. STs and geographic origin

Due to the inconsistent number of isolates recovered from each state, no statistical comparisons of STs in association with their geographical origin were conducted. Nevertheless, several observations were made. For example, the three most abundant STs (e.g., ST10, ST78, ST275), all of which belong to CC-ST10, also had the widest geographic distribution in our dataset (Table 2.1a, Table 2.1b; Figure 2.2). In specific, ST10, ST78, and ST275 were detected in 13, nine, and five states, respectively (Table 2.1a, Table 2.1b; Figure 2.2). The next most abundant STs (e.g., ST9, ST85, ST86, ST258, ST291, ST296, ST300, ST301, ST306) were recovered from two to four states, whereas the remaining 54 STs were recovered exclusively

from one state (Table 2.1a, Table 2.1b). In the state of Idaho, where 104/314 study isolates originated, 61/104 isolates were identified as ST10 and were detected in 9 distinct locations (Table 2.1a, Table 2.1b). The remaining 43 isolates fell into 16 additional STs, the bulk of which (12/16 STs) were recovered from a single location (Table 2.1a, Table 2.1b). Most isolates recovered from Idaho (n = 91/104 isolates) were affiliated with a ST that belonged to CC-ST10 (e.g., ST10, ST78, ST300, ST303, ST304, ST305, ST306, ST316). It appeared CC-ST10 had existed in Idaho for at least three decades since two of its genotypes (e.g., ST10, ST306) were recovered from this state in the early 1990s (Table 2.1a, Table 2.1b). In the state of Utah, most isolates were identified as ST10, ST78, or ST86, all of which belong to CC-ST10. Of these, ST10 was recovered most frequently (n = 37/43 isolates) followed by ST86 (n = 3 isolates), and then ST78 (n = 2 isolates). Of note, ST10 was recovered from 10 distinct locations between the years of 2004 and 2015 (Table 2.1a, Table 2.1b). The remaining isolate from Utah was identified as ST9 (Table 2.1a, Table 2.1b). In Michigan (38 isolates), 17 STs were identified among seven distinct locations between the years of 2010 and 2018 (Table 2.1a, Table 2.1b). Of these, most of the isolates were identified as ST253 (n = 6 isolates), ST256 (n = 5 isolates), or ST258 (n = 5 isolates), whereas the remaining 14 STs contained ≤ four isolates (Table 2.1a, Table 2.1b). In contrast to the isolates of Idaho and Utah, nearly half of the isolates recovered from Michigan (n = 16/38) were identified as singletons (e.g., ST253, ST258, ST278, ST282, ST286, ST290), whereas the remaining 22 isolates belonged to CC-ST10 (n = 5 isolates), CC-ST256 (n = 5 isolates), CC-ST288 (n = 4 isolates), CC-ST232 (n = 3 isolates), CC-ST191 (n = 2 isolates), CC-ST276 (n = 2 isolates), or CC-ST287 (n = 1 isolate; Table 2.1a, Table 2.1b). The geographical origins and genotypes for the remaining isolates are presented in Tables 2.1a and 2.1b.

4.5. *F. psychrophilum* genetic analyses

The mean genetic diversity (H) of all typed *F. psychrophilum* isolates recovered in North America (n = 421) to date was calculated to be 0.68 ± 0.04. H was also calculated for all other continents were *F. psychrophilum* isolates have been genotyped using MLST and ranged from 0.00 in Australia, 0.48 in South America, 0.59 in Europe, and 0.66 in Asia (https://pubmlst.org/fpsychrophilum/; Haubold & Hudson 2000). H was calculated to be 0.48 ± 0.06, 0.74 ± 0.05, and 0.90 ±0.01 for *F. psychrophilum* isolates recovered from North American *O. mykiss* (n = 318 isolates), *O. kisutch* (n = 44 isolates), and *O. tshawytscha* (n = 38 isolates), respectively. Mean genetic diversity among all other species (i.e., *S. salar*, n = 8; *S. trutta*, n = 6; *A. transmontanus*, n = 2; *O. clarkii*, n = 2; *O. nerka*, n = 1; *S. namaycush*, n = 1; *S. namaycush* x *S. fontinalis*, n = 1) was found to be 0.85 ± 0.02. The I^S_A value for all *F. psychrophilum* isolates recovered in North America was estimated to be 0.66 and differed significantly from 0 ($P < 0.001$), indicating linkage disequilibrium (i.e., nonrandom association between ATs).

As multiple isolates belong to the same ST, sampling bias may alter the overall mean genetic diversity among the North American *F. psychrophilum* isolates (Nilsen et al. 2014). As such, mean genetic diversity was calculated between the 86 currently identified *F. psychrophilum* STs in North America and found to be 0.92 ± 0.02. The I^S_A value was closer to 0 (I^S_A = 0.2648) among the 86 representative STs; however, it still differed significantly from 0 ($P < 0.001$), indicating linkage disequilibrium.

The 12 North American CCs presented in Figure 2.2 were combined with the three CCs in Figure 2.3 containing STs from North America (i.e., CC-ST124, CC-ST191, CC-ST232) and 30 SLVs were discovered among the 15 founding STs (Table 2.2). The CC with the highest

number of SLVs was CC-ST10 (n = 11), whereas the other 14 CCs had one to two SLVs. The loci *tuf* and *trpB* each accounted for the highest number of SLVs (n =7 SLVs per locus) followed by *gyrB* (n = 6 SLVs), *murG* (n = 4 SLVs), *atpA* (n =2 SLVs), *dnaK* (n = 2 SLVs), and *fumC* (n = 2 SLVs).

Among the 30 SLVs, 20 were the result of multiple nucleotide changes and thus were likely the result of recombination. Additionally, another four SLVs were likely the result of recombination because even though their variant AT differed from their ancestral AT by only one nucleotide, that variant AT was also present in another CC (Table 2.2). The remaining six SLVs differed from their ancestral AT by a single nucleotide and were not found elsewhere in the dataset, thereby suggesting those variant ATs likely arose from point mutation (Table 2.2). If 24/30 SLVs resulted from recombination and the remaining 6/30 SLVs resulted from point mutation, then North American *F. psychrophilum* loci are estimated to be four times more likely to diversify through recombination than by point mutation.

The potential for amino acid shifts resulting from these mutational events was also analyzed, revealing that 25 mutations were synonymous and five were non-synonymous. Of note is *murG*1 (i.e., locus *murG* designated with AT 1), which produced three distinct amino acid changes (e.g., proline to histidine, glutamic acid to lysine, and isoleucine to valine) resulting from seven nucleotide polymorphisms (Table 2.2). This contrasts with all other non-synonymous substitutions, which only produced a single amino acid change. In fact, of the five non-synonymous substitutions, four were due to variations at the *murG* locus and these four variations were the only ones found at this locus among the 15 CCs (Table 2.2).

When the seven loci used in the MLST scheme for *F. psychrophilum* were concatenated for each of the 86 representative STs in North America, a single stretch of DNA that was 5,799

base pairs (bp) long was produced. Among these STs, 139 SNPs were identified, meaning the nucleotide positions of the concatenated loci differed overall by 2.40% (Table 2.3). Pairwise comparisons among loci yielded a nucleotide diversity (π) ranging from 0.29% at *fumC* to 1.25% at *atpA* and the number of unique ATs varied from eight at *fumC* to 41 at *gyrB* (Table 2.3). The ratio of non-synonymous to synonymous mutations ranged from 0.0020 at *gyrB* to 0.2751 at *murG*, which indicates that non-synonymous mutations are rare and these loci are under purifying selection (dN/dS < 1). In addition, recombination was detected among all loci (R_{min} > 1) with *fumC* having the lowest R_{min} value (n = 2) and *atpA* having the highest (R_{min} = 10). The cumulative minimum number of recombination events among the seven loci was 38, whereas R_{min} for the concatenated sequences was 44, indicating additional recombination between the loci as well (Table 2.3). A PHI test conducted on the concatenated sequences revealed statistically significant evidence (P = < 0.001) of recombination between the 86 representative STs.

5. Discussion

Results presented herein reveal substantial genetic diversity within *F. psychrophilum* recovered from North America. In fact, combining data from this study with those of Nicolas et al. (2008) and Van Vliet et al. (2016) revealed that the mean genetic diversity of MLST-typed *F. psychrophilum* in North America is higher than all other studied continents (i.e., 0.68 in North America vs. 0.00 – 0.66 elsewhere). In this context, a recent study provided evidence that *F. psychrophilum* may have originated in North America, from where at least one successful genotype (e.g., ST10) was distributed to other regions of the world through the international trade of fish (Duchaud et al. 2018). If *F. psychrophilum* did indeed originate from North America, the bacterium would have had more time to diversify in this region. However, the

observed diversity in this study may be attributable, at least in part, to the life-history of some of the sampled fish host populations. For example, just 30 *F. psychrophilum* isolates were recovered from wild/feral fish but accounted for 15 STs (i.e., an average of two isolates for every ST), whereas 243 isolates were recovered from captive fish but belonged to 40 STs (i.e., >6 isolates for every ST). In this context, *F. psychrophilum* isolates from other regions of the world, such as Europe and South America, have been recovered primarily from captive host species (Siekoula-Nguedia et al. 2012, Strepparava et al. 2013, Avendaño-Herrera et al. 2014), which may have led to lower levels of diversity. For example, in Europe, Siekoula-Nguedia et al. (2012) discovered their 66 *F. psychrophilum* isolates from France belonged to 15 STs (i.e., >4 isolates for every ST) and in Switzerland, Strepparava et al. (2013) identified 27 STs among 112 *F. psychrophilum* isolates, equating to >4 isolates for every ST. Likewise, in South America, Avendaño-Herrera et al. (2014) utilized 94 *F. psychrophilum* isolates from Chile and discovered 15 STs (i.e., >6 isolates for every ST). On the other hand, in Asia, the majority of isolates originated from fish within a single river and of the 120 *F. psychrophilum* isolates typed from 15 different fish species, 35 STs were discovered (i.e., <3 isolates for every ST; Nicolas et al. 2008, Fujiwara-Nagata et al. 2013).

In addition to revealing substantial genetic diversity, MLST genotyping also revealed some important aspects of *F. psychrophilum* epidemiology in North America. Of the 31 currently described *F. psychrophilum* CCs (Figure 2.3), nearly a third (seven novel, two previously described) have, to date, been exclusively found in the USA, where they differ in their geographic distribution (Table 2.1a, Table 2.1b; Figure 2.2). Although six US-specific CCs were recovered from multiple states (i.e., CC-ST11, CC-ST29, CC-ST276, CC-ST287, CC-ST310), the exact distribution pattern and *F. psychrophilum* transmission dynamic in North America

requires additional typing of isolates originating from different regions and fish species. It is strongly believed that the common practice of transferring live fish and their gametes for aquaculture and stock enhancement purposes in North America contributes to pathogen dissemination (Parker 1989, Faisal 2007). In this context, it may not be a coincidence that Michigan Chinook salmon harbor members of CC-ST29, which in our study could not be found except in Oregon Chinook salmon, where the Chinook salmon that were transferred to Michigan originated (Hansen & Holey 2002). Likewise, it was evident that most North American CCs that originated from aquaculture facilities and hatcheries were found in multiple states, whereas those confined to one state were commonly associated with free roaming adult fish, underscoring the potential of pathogen transmission through trade.

This study revealed the diverse host ranges some of these US-specific CCs display, whereby some were collected from a single fish species (e.g., CC-ST29, CC-ST281, CC-ST288, CC-ST310) and others from multiple fish species (e.g., CC-ST11, CC-ST256, CC-ST276, CC-ST287, CC-ST296), which may be an indication that some North American CCs are adapted to a particular host species, whereas others are non-specific "generalists". The North American CC, CC-ST10, has been reported outside of North America as well, where its isolates were also predominantly recovered from farmed rainbow trout experiencing BCWD (Siekoula-Nguedia et al. 2012, Strepparava et al. 2013, Nilsen et al. 2014, Van Vliet et al. 2016). These previous reports correlate with our findings, whereby CC-ST10 was predominantly recovered from rainbow and steelhead trout (*O. mykiss*) associated with systemic infections/BCWD epizootics. However, two genotypes belonging to CC-ST10 (i.e., ST10, ST306) were recovered from fish species other than *O. mykiss* (i.e., Chinook salmon, coho salmon, white sturgeon) in this study and it remains to be determined if these genotypes have a broader host range or if the hosts these

isolates were recovered from were in proximity to *O. mykiss*. The relevance of CC-ST10 in North America and the world makes its genotypes targets for BCWD vaccine development. Within North America, CC-ST10 was the most widespread among currently recognized CCs where it has been detected in 16 out of the 20 states in the Northwest, West, Southwest, Midwest, Southeast, and Northeast regions of the USA that were examined in this study. Van Vliet et al. (2016) also found CC-ST10 to be the most widespread in the 9 states that were investigated therein. That study, along with Nilsen et al. (2014) and Duchaud et al. (2018), suggested that ST10, the predicted CC founder strain (Nilsen et al. 2014), along with other CC-ST10 STs, have been disseminated around the world as a result of the international salmonid egg trade, where they are also now widespread (Duchaud et al. 2018).

One novel genotype of CC-ST10 (i.e., ST275) was recovered from apparently healthy steelhead trout (*O. mykiss*) at a weir in Michigan and is to date the only ST of CC-ST10 reported to have been recovered from a wild/feral host in North America (Table 2.1a, Table 2.1b). Furthermore, the eggs from this steelhead trout (*O. mykiss*) were then reared at a Michigan state fish hatchery that later had an outbreak of BCWD, causing mass mortality among the resultant juvenile steelhead trout (*O. mykiss*). When the recovered *F. psychrophilum* isolates were genotyped, they were discovered to be ST275 (isolates US86 & US87; Table 2.1a, Table 2.1b), a ST not previously detected at this facility despite repeated MLST investigations. The original source of ST275 in the hatchery facility remains to be determined, but the possibility for vertical transmission and thus circumvention of modern egg disinfection strategies (i.e., iodophore disinfection) cannot be discounted. Indeed, *F. psychrophilum* has been shown to resist surface disinfection with povidone iodine in previous studies (Cipriano et al. 1995, Brown et al. 1997, Kumagai et al. 1998) and *F. psychrophilum* is believed to reside within the perivitellin space

(Kumagai et al. 2000), where it can resist salmonid egg lysozyme (reviewed in Cipriano & Holt 2005). However, other explanations for the occurrence of ST275 both at the weir and in the hatchery environment are possible, including improper disinfection of facility equipment at the weir that may have allowed for the transportation of ST275 to the hatchery facility, where it was introduced to the newly fertilized eggs. In any case, strict biosecurity protocols are needed to prevent the spread of *F. psychrophilum* and future research exploring additional egg and equipment disinfection protocols are required.

Although not nearly as widespread as CC-ST10, some STs within CC-ST191 (e.g., ST267, ST301) were also detected in multiple regions of the USA (e.g., Idaho, Michigan, Pennsylvania, West Virginia). CC-ST191 appeared to be confined to Europe (e.g., Denmark, Finland, France, Norway, Switzerland; Nicolas et al. 2008, Siekoula-Nguedia et al. 2012, Strepparava et al. 2013, Nilsen et al. 2014) until Van Vliet et al. (2016) detected it in Michigan, USA. The detection of this CC from the US on multiple occasions is of interest, because the founding ST and almost all of its SLVs were exclusively detected in Europe, suggesting this CC may have originated from Europe and subsequently was introduced to North America. Likewise, all STs within CC-ST124, which is the third largest currently described *F. psychrophilum* CC in the world and predominately recovered from *S. salar*, are found only within Europe except ST70, which was found in Chile by Avendaño et al. (2014) following the Atlantic salmon trade between Chile and Norway in the 1990's (Storebakken 2002). In addition, ST70 was discovered in the present study and by Nilsen et al. (2014), and in both instances from North American Atlantic salmon; however, any connection between the ST70 isolates from North America and those from Europe or Chile remains to be determined.

Thirty-one North America *F. psychrophilum* singletons were also detected in this study, bringing the total number of recognized North American singletons to 44 (Figure 2.2; Nicolas et al. 2008, Van Vliet et al. 2016). Based upon current data, most *F. psychrophilum* singletons appear to be geographically limited, where they were recovered from one specific aquaculture facility or site (Table 2.1a, Table 2.1b; Van Vliet et al. 2016). Although this may not be surprising, it was striking that some singletons were found repeatedly at the same facility over the years (e.g., ST253, 2010, 2013, 2017; Table 2.1a, Table 2.1b), emphasizing that some unique strains may find a niche and perpetuate themselves within aquaculture facilities.

By combining the 314 North America *F. psychrophilum* isolates presented herein and all previously typed North America *F. psychrophilum* isolates, it is evident that some North American STs have persisted longer than others. For example, some of the earliest recovered STs in North America (e.g., ST6, ST9, ST10, ST11, ST27, ST28, ST29, ST30, ST70, ST74, ST78, ST297, ST306, ST307, ST308, 1981-1996; Nicolas et al. 2008, Van Vliet et al. 2016) have not been rediscovered in the last 20 years (e.g., ST6, ST11, ST28, ST70, ST297, ST306, ST307, ST308), whereas others are still reappearing decades later (e.g., ST10, ST27, ST29, ST78), suggesting these variants have adapted mechanisms that promote their longevity even though great strides in biosecurity and disease control (i.e., antimicrobials) have been made. Interestingly, ST10 appears to be capable of producing equally successful and persistent clones. For example, ST10 was first recognized in North America (e.g., Oregon, USA) in 1990 (Table 2.1a, Table 2.1b), then its SLV, ST78, was recovered later in Colorado in 1996 (Van Vliet et al. 2016), and over the next 15 years, ST78 has been recovered from two additional US states (e.g., West Virginia, Michigan; Van Vliet et al. 2016) and one province of Canada (e.g., British Columbia; Castillo et al. 2016). Furthermore, ST78 has produced three of its own clones (e.g.,

ST84, ST316, ST317), which have been recovered from three additional US states (i.e., Idaho, North Carolina, Washington) where ST78 has yet to be recovered from, suggesting ST78 may be more geographically widespread than is currently recognized.

The phylogenetic relationships among the 86 North American STs were evaluated using eBURST v3, a program used in all previous MLST studies on *F. psychrophilum* (Nicolas et al. 2008, Siekoula-Nguedia 2012, Strepparava et al. 2013, Fujiwara-Nagata et al. 2013, Avendaño-Herrera et al. 2014, Nilsen et al. 2014, Van Vliet et al. 2016), and Bayesian inference. The Bayesian analysis, which was based upon the concatenated MLST loci, largely corroborated the results of the eBURST analysis, whereby all CCs established by eBURST were reconstructed with robust support (Figure 2.2, Figure 2.5); however, a few differences were noted. For example, ST27 and ST6 were placed within CC-ST10 via Bayesian analysis but not by eBURST (Figure 2.2, Figure 2.5); however, ST27 and ST6 share 5/7 MLST loci with several genotypes of CC-ST10 (i.e., DLV; data not shown) and would be allocated to CC-ST10 in a less stringent eBURST analysis. In this context, the hypothesized phylogenetic relationships depicted by eBURST are more conservative than those portrayed by Bayesian inference. The conservativeness of eBURST has been levied as a strength in that evolutionary relationships are not forced among genotypes (eburst.mlst.net; Feil et al. 2004, Spratt et al. 2004). Furthermore, Duchaud et al. (2018) recently suggested that recombination within *F. psychrophilum* may disrupt the relationships between *F. psychrophilum* genotypes. Therefore, inferences on the genealogical history of *F.* psychrophilum should be made with caution when using traditional phylogenetic analyses. Nonetheless, it is interesting that Bayesian inference robustly supported the eBURST CCs given the apparent frequency of recombination throughout the genome of *F. psychrophilum* (Duchaud et al. 2018). Furthermore, the Bayesian phylogeny provided support

for the formation of several other clades, which appeared to be rooted biologically (i.e., by host species) and geographically.

In this study, the contribution of recombination/mutation to the diversification among North America *F. psychrophilum* STs was best explained best by recombination, whereby 24/30 SLVs were hypothesized to have resulted from recombination and the remaining 6/30 SLVs resulted from mutation (i.e., ratio of 4:1), which is similar to what was reported initially by Nicolas et al. (2008; i.e., ratio of 4.5:1). This estimation, originally described by Feil et al. (2000), relies upon a visual inspection of gene sequences to estimate the mechanism of diversification. To validate this estimation, additional sequence-based computational tests (e.g., PHI, R_{min}) were utilized and both tests revealed evidence for recombination either within and/or between the MLST loci. Interestingly, all polymorphisms at the locus *murG*, which encodes for glycosyltransferase murein G, were non-synonymous. The exact function of this enzyme in *F. psychrophilum* has not been examined; however, in *Escherichia coli*, *murG* is required for biosynthesis of the peptidoglycan layer (Mohammadi et al. 2007) and therefore may confer an environmental advantage to epidemic clones of *F. psychrophilum*. The ratio of non-synonymous to synonymous mutations within *murG* however, is < 1 (i.e., dN/dS = 0.2751), and therefore accumulated non-synonymous mutations are selected against and are projected to be lost over time (Nilsen et al. 2014) but may create short-lived advantages during BCWD outbreaks. Although the natural mechanism(s) for recombination (e.g., transduction, transformation, conjugation) among *F. psychrophilum* isolates remain to be determined, the intermingling of isolates naturally and during the trade and introduction of fish and eggs to different facilities, bodies of water, or states provides a means for interaction of different *F. psychrophilum* genotypes. Indeed, multiple STs have been reported from a single infected host on multiple

occasions (Fujiwara-Nagata et al. 2013, Nilsen et al. 2014, Van Vliet et al. 2016). Even though recombination appears to be a driving force for diversity among *F. psychrophilum* isolates, the results of our analysis on linkage disequilibrium revealed statistically significant evidence for clonal populations (i.e., I^S_A value differs significantly from 0). Therefore, it would appear even in the presence of recombination, well adapted *F. psychrophilum* STs are capable of producing epidemic clones, some of which have persisted long enough to produce clones of their own (i.e., ST78, ST306). Nilsen et al. (2014) described an identical discovery among their 560 *F. psychrophilum* isolates, which were recovered from several Nordic countries.

In summation, MLST was used to reveal substantial diversity among 314 newly typed North American *F. psychrophilum* isolates, whereby 66 STs were identified, 47 of which were novel and recovered in this study for the first time. These isolates were recovered from 20 US states and one Canadian province over a period of four decades, which is the most comprehensive collection of *F. psychrophilum* isolates to be genetically analyzed in North America to date. These results expanded our knowledge on the population structure of *F. psychrophilum* in North America through the addition of seven novel CCs. These CCs have diverse host ranges and vary in their virulence. Additionally, for the first time, recombination was implicated as the driving force of *F. psychrophilum* diversification in North America, which may also be the mechanism for phenotypic changes, such as antimicrobial resistance, within this species, and warrants further investigation.

APPENDIX

Table 2.1a Complete information of 314 North American *F. psychrophilum* isolates presented in this study including year of isolation, location of isolation, host species, tissue of isolation, and host life stage. [a]AK = Alaska; BC = British Columbia; CA = California; CO = Colorado; ID = Idaho; IN = Indiana; MD = Maryland; MI = Michigan; MN = Minnesota; MT = Montana; NC = North Carolina; NM = New Mexico; OR = Oregon, PA = Pennsylvania; SD = South Dakota; UT = Utah; VA = Virginia; VT = Vermont; WA = Washington; WI = Wisconsin; WV = West Virginia; PF = private facility, SFH = state fish hatchery, WE = weir, WI = wild, Unk = Unknown. [c]Host was in captivity. [wf]Host was wild/feral. [D]Isolate was recovered from a fish experiencing BCWD. [H]Isolate was recovered from an apparently healthy fish. [U]Fish disease status was unknown. OF = Ovarian Fluid.

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US159	1989	OR-Unk	*O. kisutch*	Unk	Unk[U]
US160	1990	OR-Unk	*O. kisutch*	Unk	Unk[U]
US161	1990	OR-Unk	*O. kisutch*	Unk	Unk[U]
US165	1984	BC-Unk	*O. kisutch*	Unk	Unk[U]
US202	1990	OR-Unk	*O. kisutch*	Unk	Unk[U]
US248	2000	WA-SFH2	*O. kisutch*	OF	Unk[U]
US256	Unk	WA-PF2	*O. kisutch*	Unk	Unk[U]
US254	Unk	UT-SFH1	*O. mykiss*	Spleen	Unk[U]
US154	Unk	WA-Unk	*O. kisutch*	Unk	Unk[U]
US155	Unk	WA-Unk	*O. kisutch*	Unk	Unk[U]
US250	2000	WA-PF3	*O. tshawytscha*	OF	Unk[U]
US246	1998	WA-SFH1	*O. kisutch*	Kidney	Unk[U]
US284	2014	ID-PF7	*O. mykiss*[c]	Unk	Broodstock[D]
US121	2013	NC-PF3	*O. mykiss*[c]	Kidney	Fingerling[D]
US123	2011	UT-SFH2	*O. mykiss*[c]	Spleen	Fingerling[D]
US125	2011	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US126	2011	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US127	2011	UT-SFH1	*O. mykiss*[c]	Brain	Fingerling[D]
US128	2011	UT-SFH2	*O. mykiss*[c]	Lesion	Fingerling[D]
US129	2011	UT-SFH2	*O. mykiss*[c]	Spleen	Fingerling[D]
US130	2011	UT-SFH2	*O. mykiss*[c]	Spleen	Fingerling[D]
US135	2012	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US136	2012	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US137	2012	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US140	2013	NC-PF3	*O. mykiss*[c]	Brain	Fingerling[D]
US143	2012	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US188	2016	MD-SFH1	*O. mykiss*[c]	Unk	Fingerling[D]
US221	2011	UT-SFH2	*O. mykiss*[c]	Caudal Fin	Fingerling[D]
US226	2013	NC-PF3	*O. mykiss*[c]	Lesion	Fingerling[D]
US227	2012	UT-PF2	*O. mykiss*[c]	Spleen	Fingerling[D]
US229	2012	UT-PF2	*O. mykiss*[c]	Spleen	Fingerling[D]
US236	2011	UT-SFH2	*O. mykiss*[c]	Spleen	Fingerling[D]
US238	2011	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US240	2012	UT-PF2	*O. mykiss*[c]	Spleen	Fingerling[D]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US243	2013	NC-PF3	*O. mykiss*[c]	Kidney	Fingerling[D]
US293	2011	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US298	2005	UT-SFH3	*O. mykiss*[c]	Unk	Fingerling[D]
US299	2015	UT-SFH6	*O. mykiss*[c]	Spleen	Fingerling[D]
US311	2010	MT-SFH2	*O. mykiss*[c]	Kidney	Fingerling[D]
US312	2010	MT-SFH2	*O. mykiss*[c]	Kidney	Fingerling[D]
US315	2011	UT-SFH1	*O. mykiss*[c]	Spleen	Fingerling[D]
US316	2012	UT-PF2	*O. mykiss*[c]	Spleen	Fingerling[D]
US295	2009	UT-SFH2	*O. mykiss*[c]	Spleen	Fry[D]
US297	2004	UT-PF1	*O. mykiss*[c]	Unk	Fry[D]
US301	2013	UT-SFH7	*O. mykiss*[c]	Unk	Fry[D]
US302	2013	UT-SFH2	*O. mykiss*[c]	Spleen	Fry[D]
US303	2013	UT-SFH7	*O. mykiss*[c]	Spleen	Fry[D]
US304	2011	UT-SFH7	*O. mykiss*[c]	Spleen	Fry[D]
US305	2011	UT-SFH6	*O. mykiss*[c]	Spleen	Fry[D]
US306	2011	UT-SFH1	*O. mykiss*[c]	Spleen	Fry[D]
US366	2009	UT-SFH5	*O. mykiss*[c]	Spleen	Fry[D]
US369	2009	UT-SFH5	*O. mykiss*[c]	Spleen	Fry[D]
US058	2011	ID-PF4	*O. mykiss*[c]	Unk	Juvenile[D]
US114	2011	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US124	2011	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US146	2011	ID-PF4	*O. mykiss*[c]	Kidney	Juvenile[D]
US147	2011	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US169	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US182	2015	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US183	2015	ID-PF2	*O. mykiss*[c]	Kidney	Juvenile[D]
US184	2016	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US193	2011	ID-PF1	*O. mykiss*[c]	Unk	Juvenile[D]
US195	2013	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US206	2015	ID-PF6	*O. mykiss*[c]	Kidney	Juvenile[D]
US207	2015	ID-PF3	*O. mykiss*[c]	Spleen	Juvenile[D]
US209	2014	ID-PF3	*O. mykiss*[c]	Spleen	Juvenile[D]
US210	2016	ID-PF3	*O. mykiss*[c]	Spleen	Juvenile[D]
US211	2016	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US213	2014	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US222	2011	ID-PF-Unk	*O. mykiss*[c]	Unk	Juvenile[D]
US228	2013	ID-PF3	*O. mykiss*[c]	Spleen	Juvenile[D]
US230	2013	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US234	2011	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US235	2011	ID-PF4	*O. mykiss*[c]	Kidney	Juvenile[D]
US237	2011	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US262	2014	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US263	2014	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US264	2014	ID-PF5	*O. mykiss*[c]	Kidney	Juvenile[D]
US267	2014	ID-PF-Unk	*O. mykiss*[c]	Spleen	Juvenile[D]
US268	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US269	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US271	2015	ID-PF6	*O. mykiss*[c]	Spleen	Juvenile[D]
US272	2015	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US275	2015	ID-PF6	*O. mykiss*[c]	Kidney	Juvenile[D]
US279	2015	ID-PF3	*O. mykiss*[c]	Kidney	Juvenile[D]
US280	2015	ID-PF-Unk	*O. mykiss*[c]	Unk	Juvenile[D]
US281	2016	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US286	2014	ID-PF3	*O. mykiss*[c]	Kidney	Juvenile[D]
US288	2014	ID-PF2	*O. mykiss*[c]	Kidney	Juvenile[D]
US319	2012	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US320	2012	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US322	2012	ID-PF6	*O. mykiss*[c]	Spleen	Juvenile[D]
US326	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US327	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US330	2015	ID-PF3	*O. mykiss*[c]	Spleen	Juvenile[D]
US333	2014	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US334	2014	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US336	2014	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US342	2014	ID-PF-Unk	*O. mykiss*[c]	Kidney	Juvenile[D]
US345	2014	ID-PF5	*O. mykiss*[c]	Kidney	Juvenile[D]
US346	2015	ID-PF3	*O. mykiss*[c]	Kidney	Juvenile[D]
US347	2015	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US348	2015	ID-PF2	*O. mykiss*[c]	Kidney	Juvenile[D]
US350	2015	ID-PF6	*O. mykiss*[c]	Kidney	Juvenile[D]
US351	2015	ID-PF6	*O. mykiss*[c]	Spleen	Juvenile[D]
US353	2012	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US132	2012	SD-SFH1	*O. mykiss*[c]	Head swab	Unk[D]
US133	2012	SD-SFH2	*O. mykiss*[c]	Pool tissue	Unk[D]
US134	2012	SD-SFH1	*O. mykiss*[c]	Pool tissue	Unk[D]
US144	2012	SD-SFH1	*O. mykiss*[c]	Pool tissue	Unk[D]
US145	Unk	UT-SFH4	*O. mykiss*[c]	Unk	Unk[U]
US150	Unk	UT-SFH4	*O. mykiss*	Spleen	Unk[U]
US162	1990	OR-Unk	*O. mykiss*	Unk	Unk[U]
US173	2014	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US175	1994	ID-SFH1	*O. mykiss*[c]	Unk	Unk[D]
US179	2015	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US186	2014	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US253	Unk	CO-Unk	*O. mykiss*	Unk	Unk[U]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US291	2014	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US292	2014	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US294	2004	UT-SFH2	*O. mykiss*[c]	Spleen	Unk[D]
US296	2008	UT-SFH2	*O. mykiss*[c]	Spleen	Unk[U]
US307	2015	UT-Unk	*O. mykiss*	Unk	Unk[U]
US368	2002	NM-SFH1	*O. mykiss*	Kidney	Unk[U]
US059	2014	PA-SFH2	*O. mykiss*[c]	Kidney	Fingerling[D]
US075	2016	PA-SFH2	*O. mykiss*[c]	Kidney	Broodstock[H]
US079	2016	PA-SFH2	*O. mykiss*[c]	Kidney	Broodstock[H]
US180	2015	PA-SFH1	*O. mykiss*[c]	Unk	Unk[D]
US352	2010	MI-SFH4	*O. mykiss*[c]	Kidney	Fingerling[D]
US148	Unk	CA-SFH2	*A. transmontanus*	Kidney	Unk[U]
518	Unk	CA-Unk	*A. transmontanus*	Kidney	Unk[D]
US118	2013	NC-PF1	*O. mykiss*[c]	Unk	Fingerling[D]
US138	2013	NC-PF2	*O. mykiss*[c]	Spleen	Fingerling[D]
US365	2011	UT-SFH2	*O. mykiss*[c]	Kidney	Fingerling[D]
US367	2008	UT-SFH1	*O. mykiss*[c]	Spleen	Fry[D]
US120	2013	ID-PF6	*O. mykiss*[c]	Spleen	Juvenile[D]
US196	2012	ID-PF-Unk	*O. mykiss*[c]	Unk	Juvenile[D]
US204	2014	ID-PF6	*O. mykiss*[c]	Unk	Juvenile[D]
US208	2015	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US212	2014	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US241	2013	ID-PF6	*O. mykiss*[c]	Unk	Juvenile[D]
US266	2014	ID-PF3	*O. mykiss*[c]	Kidney	Juvenile[D]
US285	2014	ID-PF-Unk	*O. mykiss*[c]	Gill	Juvenile[D]
US287	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US289	2013	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US317	2012	ID-PF-Unk	*O. mykiss*[c]	Unk	Juvenile[D]
US318	2012	ID-PF6	*O. mykiss*[c]	Spleen	Juvenile[D]
US321	2012	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US335	2014	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US349	2015	ID-PF5	*O. mykiss*[c]	Kidney	Juvenile[D]
US251	2002	NM-SFH1	*O. mykiss*	Kidney	Unk[U]
US252	Unk	CO-Unk	*O. mykiss*	Kidney	Unk[U]
US172	2014	WA-PF1	*O. mykiss*[c]	Kidney	Yearling[D]
US177	2014	WA-PF1	*O. mykiss*[c]	Side lesion	Yearling[D]
US337	2014	WA-PF1	*O. mykiss*[c]	Skin	Yearling[D]
US338	2014	WA-PF1	*O. mykiss*[c]	Kidney	Yearling[D]
US167	2009	NC-PF3	*O. mykiss*[c]	Spleen	Fingerling[D]
US189	2016	CA-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US190	2016	CA-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US364	2014	WA-PF1	*O. mykiss*[c]	Heart	Yearling[D]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
551	2007	VT-Unk	*O. mykiss*	Unk	Unk[D]
US073	2008	UT-SFH4	*O. mykiss*[c]	Spleen	Fry[D]
US074	2010	UT-SFH6	*O. mykiss*[c]	Spleen	Fry[D]
US300	2010	UT-SFH4	*O. mykiss*[c]	Spleen	Fry[D]
US354	2015	WA-PF1	*O. mykiss*[c]	Skin	Yearling[D]
US104	2016	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US115	2011	NC-PF3	*O. mykiss*[c]	Brain	Fingerling[D]
US116	2013	NC-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US117	2013	WV-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US119	2013	WV-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US131	2011	NC-PF3	*O. mykiss*[c]	Kidney	Fingerling[D]
US170	2014	VA-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US171	2014	VA-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US185	2014	VA-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US192	2014	VA-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US194	2011	NC-PF3	*O. mykiss*[c]	Kidney	Fingerling[D]
US224	2013	NC-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US242	2013	NC-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US057	2014	PA-SFH5	*O. mykiss*[c]	Kidney	Fingerling[D]
US085	2016	MI-SFH4	*O. mykiss*[c]	Kidney	Fingerling[D]
US086	2016	MI-SFH4	*O. mykiss*[c]	Ascites	Fingerling[D]
US087	2016	MI-SFH4	*O. mykiss*[c]	Ext. lesion	Fingerling[D]
US151	Unk	WA-PF2	*O. mykiss*	Unk	Unk[U]
US313	2011	MT-SFH1	*O. mykiss*[c]	Kidney	Fingerling[D]
US314	2011	MT-SFH1	*O. mykiss*[c]	Kidney	Fingerling[D]
US223	2011	ID-PF4	*O. mykiss*[c]	Kidney	Juvenile[D]
US233	2013	ID-PF4	*O. mykiss*[c]	Spleen	Juvenile[D]
US239	2011	ID-PF4	*O. mykiss*[c]	Kidney	Juvenile[D]
US274	2015	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US276	2015	ID-PF6	*O. mykiss*[c]	Kidney	Juvenile[D]
US282	2016	ID-PF2	*O. mykiss*[c]	Kidney	Juvenile[D]
US290	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US332	2014	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US178	2015	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US197	2013	ID-PF-Unk	*O. mykiss*[c]	Spleen	Juvenile[D]
US323	2013	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US205	2015	ID-PF5	*O. mykiss*[c]	Kidney	Juvenile[D]
US245	2011	ID-PF4	*O. mykiss*[c]	Kidney	Juvenile[D]
US258	1990	OR-Unk	*O. kisutch*	Unk	Unk[U]
US259	1990	ID-Unk	*O. mykiss*	Unk	Unk[U]
US355	2015	WA-PF1	*O. mykiss*[c]	Unk	Yearling[D]
US356	2016	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US157	1991	OR-Unk	*S. salar*	Unk	Unk[U]
US163	1984	OR-Unk	*O. mykiss*	Unk	Unk[U]
US261	1981	OR-Unk	*O. tshawytscha*	Unk	Unk[U]
US164	1986	OR-Unk	*O. clarkii*	Unk	Unk[U]
US149	Unk	WA-Unk	*S. salar*	Unk	Unk[U]
US215	2017	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US216	2017	MI-WE1	*O. mykiss*[wf]	Spleen	Adult[H]
US339	2014	WV-PF2	*O. mykiss*[c]	Unk	Fingerling[D]
US340	2014	WV-PF2	*O. mykiss*[c]	Unk	Fingerling[D]
US341	2014	WV-PF2	*O. mykiss*[c]	Unk	Fingerling[D]
US343	2014	WV-PF2	*O. mykiss*[c]	Caudal Fin	Fingerling[D]
US344	2014	WV-PF2	*O. mykiss*[c]	Spleen	Fingerling[D]
US277	2015	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US181	2015	PA-SFH1	*O. mykiss*[c]	Unk	Unk[D]
US062	2012	MI-WE2	*S. salar*[wf]	Ext. lesion	Adult[D]
US064	2012	MI-WE2	*S. salar*[wf]	Kidney	Adult[H]
US084	2016	MI-WE2	*S. salar*[wf]	Kidney	Adult[H]
US097	2014	MI-WE1	*O. tshawytscha*[wf]	Kidney	Adult[H]
US101	2014	MI-WE1	*O. tshawytscha*[wf]	Kidney	Adult[H]
US217	2017	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US219	2017	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US220	2017	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US095	2016	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US105	2016	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US061	2014	PA-SFH3	*O. mykiss*[c]	Kidney	Fingerling[D]
US076	2016	PA-SFH2	*O. mykiss*[c]	Kidney	Broodstock[H]
US077	2016	PA-SFH2	*O. mykiss*[c]	Ulcer	Broodstock[D]
US078	2016	PA-SFH2	*O. mykiss*[c]	Kidney	Broodstock[H]
US080	2016	PA-SFH2	*O. mykiss*[c]	Eye	Broodstock[D]
US111	2017	PA-SFH2	*O. mykiss*[c]	Ext. lesion	Fry[D]
US112	2017	PA-SFH2	*O. mykiss*[c]	Kidney	Fry[D]
US113	2017	PA-SFH2	*O. mykiss*[c]	Kidney	Fry[D]
US199	2016	PA-SFH2	*O. mykiss*[c]	Kidney	Broodstock[D]
US200	2016	PA-SFH2	*O. mykiss*[c]	Ulcer	Broodstock[D]
US201	2016	PA-SFH2	*O. mykiss*[c]	Kidney	Broodstock[D]
US065	2016	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US068	2016	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US070	2016	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US071	2016	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US072	2016	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US067	2016	PA-SFH4	*O. mykiss*[c]	Kidney	Fry[D]
US069	2016	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US379	2017	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US380	2017	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US381	2017	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US382	2017	PA-WI1	*O. mykiss*[wf]	Kidney	Adult[H]
US066	2016	WI-SFH1	*S. namaycush* x *S. fontinalis*[c]	Unk	Unk[U]
US096	2013	MI-SFH3	*O. kisutch*[c]	Dorsal Fin	Yearling[D]
US106	2016	MI-WE1	*O. tshawytscha*[wf]	Kidney	Adult[H]
US107	2016	MI-WE1	*O. tshawytscha*[wf]	Kidney	Adult[H]
US108	2016	MI-WE1	*O. tshawytscha*[wf]	Kidney	Adult[H]
US109	2016	MI-WE3	*O. tshawytscha*[wf]	Kidney	Adult[H]
US174	2014	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US153	Unk	WA-PF2	*O. nerka*	Unk	Unk[U]
US176	2014	WA-PF1	*O. mykiss*[c]	Kidney	Yearling[D]
US122	2011	NC-PF3	*O. mykiss*[c]	Brain	Fingerling[D]
US198	2011	NC-PF3	*O. mykiss*[c]	Unk	Fingerling[D]
US231	2013	ID-PF1	*O. mykiss*[c]	Spleen	Juvenile[D]
US273	2015	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US331	2014	ID-PF1	*O. mykiss*[c]	Kidney	Juvenile[D]
US270	2015	ID-PF6	*O. mykiss*[c]	Kidney	Juvenile[D]
US329	2014	ID-PF4	*O. mykiss*[c]	Kidney	Juvenile[D]
US278	2015	ID-PF2	*O. mykiss*[c]	Kidney	Juvenile[D]
US328	2014	ID-PF2	*O. mykiss*[c]	Spleen	Juvenile[D]
US191	2016	CA-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US156	1981	OR-Unk	*O. kisutch*	Unk	Unk[U]
US203	1990	OR-Unk	*O. kisutch*	Unk	Unk[U]
US247	1998	OR-SFH1	*O. kisutch*	Kidney	Unk[U]
US257	1981	OR-Unk	*O. kisutch*	Unk	Unk[U]
US370	1981	OR-Unk	*O. kisutch*	Unk	Unk[U]
US255	1981	OR-SFH2	*O. kisutch*	Unk	Unk[U]
US249	2000	WA-PF3	*O. tshawytscha*	OF	Unk[U]
US094	2013	MI-SFH2	*S. trutta*[c]	Kidney	Fingerling[D]
US098	2013	MI-SFH2	*S. trutta*[c]	Spleen	Fingerling[D]
US102	2013	MI-SFH2	*S. trutta*[c]	Kidney	Fingerling[D]
US103	2013	MI-SFH2	*S. trutta*[c]	Kidney	Fingerling[D]
US060	2010	MI-SFH2	*S. trutta*[c]	Ascites	Adult[D]
US093	2017	MI-SFH2	*O. mykiss*[c]	Kidney	Broodstock[H]
US383	2018	MI-SFH4	*O. mykiss*[c]	Kidney	Fingerling[D]
US081	2016	MI-SFH3	*O. kisutch*[c]	Kidney	Fingerling[D]
US082	2016	MI-SFH3	*O. kisutch*[c]	Gill	Fingerling[D]
US099	2013	MI-SFH3	*O. kisutch*[c]	Gill	Yearling[D]
US100	2013	MI-SFH3	*O. kisutch*[c]	Ulcer	Yearling[D]
US214	2017	IN-SFH1	*O. kisutch*[c]	Kidney	Fry[D]

Table 2.1a (cont'd)

Isolate ID	Year	Location[a]	Species[c,wf]	Isolation tissue	Life stage[D,H,U]
US218	2017	MI-WE1	*O. mykiss*[wf]	Kidney	Adult[H]
US063	2012	MI-SFH1	*S. namaycush*[c]	Fin	Fingerling[D]
US083	2016	MI-WE2	*S. salar*[wf]	Eye	Adult[D]
US088	2016	PA-PF1	*S. trutta*[c]	Lesion	Adult[D]
US089	2016	MN-SFH1	*O. mykiss*[c]	Fin	Broodstock[D]
US090	2016	MN-SFH1	*O. mykiss*[c]	Kidney	Broodstock[H]
US091	2016	MN-SFH1	*O. mykiss*[c]	Kidney	Broodstock[H]
US092	2017	MI-SFH2	*O. mykiss*[c]	Kidney	Broodstock[H]
US110	2016	MI-WE3	*O. tshawytscha*[wf]	Kidney	Adult[H]
US139	2013	NC-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US141	2011	NC-PF3	*O. mykiss*[c]	Kidney	Fingerling[D]
US166	2011	NC-PF3	*O. mykiss*[c]	Spleen	Fingerling[D]
US225	2013	NC-PF1	*O. mykiss*[c]	Spleen	Fingerling[D]
US232	2011	NC-PF3	*O. mykiss*[c]	Kidney	Fingerling[D]
US244	2011	NC-PF3	*O. mykiss*[c]	Spleen	Fingerling[D]
US142	2011	NC-PF3	*O. mykiss*[c]	Brain	Fingerling[D]
US152	Unk	WA-PF2	*O. tshawytscha*	Unk	Unk[U]
US158	1989	OR-Unk	*O. kisutch*	Unk	Unk[U]
US168	2014	ID-PF5	*O. mykiss*[c]	Kidney	Juvenile[D]
US187	2014	ID-SFH1	*O. mykiss*[c]	Spleen	Unk[D]
US260	1985	OR-Unk	*O. mykiss*	Unk	Unk[U]
US265	2014	ID-PF5	*O. mykiss*[c]	Kidney	Juvenile[D]
US283	2014	ID-PF5	*O. mykiss*[c]	Spleen	Juvenile[D]
US309	2010	MT-SFH1	*O. mykiss*[c]	lesion	Fingerling[D]
US310	2010	MT-SFH1	*O. mykiss*[c]	Kidney	Fingerling[D]
US324	2014	NC-PF2	*O. mykiss*[c]	Spleen	Fry[D]
US325	2014	NC-PF2	*O. mykiss*[c]	Spleen	Fry[D]
US372	2011	AK-PF1	*O. kisutch*[c]	Kidney	Fingerling[D]

Table 2.1b Multilocus sequence typing allelic profiles, sequence types (STs), and clonal complexes (CCs) of the 314 North American *F. psychrophilum* isolates. STs in bold are found in North America and abroad, whereas STs in italics are novel.

	Allelic profile								
Isolate ID	***trpB***	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US159	4	7	6	5	6	5	4	**ST9**	CC-ST9
US160	4	7	6	5	6	5	4	**ST9**	CC-ST9
US161	4	7	6	5	6	5	4	**ST9**	CC-ST9
US165	4	7	6	5	6	5	4	**ST9**	CC-ST9
US202	4	7	6	5	6	5	4	**ST9**	CC-ST9
US248	4	7	6	5	6	5	4	**ST9**	CC-ST9
US256	4	7	6	5	6	5	4	**ST9**	CC-ST9
US254	4	7	6	5	6	5	4	**ST9**	CC-ST9
US154	4	7	6	5	6	8	4	**ST13**	CC-ST9
US155	4	7	6	5	6	8	4	**ST13**	CC-ST9
US250	2	8	2	2	2	2	2	**ST10**	CC-ST10
US246	2	8	2	2	2	2	2	**ST10**	CC-ST10
US284	2	8	2	2	2	2	2	**ST10**	CC-ST10
US121	2	8	2	2	2	2	2	**ST10**	CC-ST10
US123	2	8	2	2	2	2	2	**ST10**	CC-ST10
US125	2	8	2	2	2	2	2	**ST10**	CC-ST10
US126	2	8	2	2	2	2	2	**ST10**	CC-ST10
US127	2	8	2	2	2	2	2	**ST10**	CC-ST10
US128	2	8	2	2	2	2	2	**ST10**	CC-ST10
US129	2	8	2	2	2	2	2	**ST10**	CC-ST10
US130	2	8	2	2	2	2	2	**ST10**	CC-ST10
US135	2	8	2	2	2	2	2	**ST10**	CC-ST10
US136	2	8	2	2	2	2	2	**ST10**	CC-ST10
US137	2	8	2	2	2	2	2	**ST10**	CC-ST10
US140	2	8	2	2	2	2	2	**ST10**	CC-ST10
US143	2	8	2	2	2	2	2	**ST10**	CC-ST10
US188	2	8	2	2	2	2	2	**ST10**	CC-ST10
US221	2	8	2	2	2	2	2	**ST10**	CC-ST10
US226	2	8	2	2	2	2	2	**ST10**	CC-ST10
US227	2	8	2	2	2	2	2	**ST10**	CC-ST10
US229	2	8	2	2	2	2	2	**ST10**	CC-ST10
US236	2	8	2	2	2	2	2	**ST10**	CC-ST10
US238	2	8	2	2	2	2	2	**ST10**	CC-ST10
US240	2	8	2	2	2	2	2	**ST10**	CC-ST10
US243	2	8	2	2	2	2	2	**ST10**	CC-ST10
US293	2	8	2	2	2	2	2	**ST10**	CC-ST10
US298	2	8	2	2	2	2	2	**ST10**	CC-ST10
US299	2	8	2	2	2	2	2	**ST10**	CC-ST10
US311	2	8	2	2	2	2	2	**ST10**	CC-ST10
US312	2	8	2	2	2	2	2	**ST10**	CC-ST10

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US315	2	8	2	2	2	2	2	**ST10**	CC-ST10
US316	2	8	2	2	2	2	2	**ST10**	CC-ST10
US295	2	8	2	2	2	2	2	**ST10**	CC-ST10
US297	2	8	2	2	2	2	2	**ST10**	CC-ST10
US301	2	8	2	2	2	2	2	**ST10**	CC-ST10
US302	2	8	2	2	2	2	2	**ST10**	CC-ST10
US303	2	8	2	2	2	2	2	**ST10**	CC-ST10
US304	2	8	2	2	2	2	2	**ST10**	CC-ST10
US305	2	8	2	2	2	2	2	**ST10**	CC-ST10
US306	2	8	2	2	2	2	2	**ST10**	CC-ST10
US366	2	8	2	2	2	2	2	**ST10**	CC-ST10
US369	2	8	2	2	2	2	2	**ST10**	CC-ST10
US058	2	8	2	2	2	2	2	**ST10**	CC-ST10
US114	2	8	2	2	2	2	2	**ST10**	CC-ST10
US124	2	8	2	2	2	2	2	**ST10**	CC-ST10
US146	2	8	2	2	2	2	2	**ST10**	CC-ST10
US147	2	8	2	2	2	2	2	**ST10**	CC-ST10
US169	2	8	2	2	2	2	2	**ST10**	CC-ST10
US182	2	8	2	2	2	2	2	**ST10**	CC-ST10
US183	2	8	2	2	2	2	2	**ST10**	CC-ST10
US184	2	8	2	2	2	2	2	**ST10**	CC-ST10
US193	2	8	2	2	2	2	2	**ST10**	CC-ST10
US195	2	8	2	2	2	2	2	**ST10**	CC-ST10
US206	2	8	2	2	2	2	2	**ST10**	CC-ST10
US207	2	8	2	2	2	2	2	**ST10**	CC-ST10
US209	2	8	2	2	2	2	2	**ST10**	CC-ST10
US210	2	8	2	2	2	2	2	**ST10**	CC-ST10
US211	2	8	2	2	2	2	2	**ST10**	CC-ST10
US213	2	8	2	2	2	2	2	**ST10**	CC-ST10
US222	2	8	2	2	2	2	2	**ST10**	CC-ST10
US228	2	8	2	2	2	2	2	**ST10**	CC-ST10
US230	2	8	2	2	2	2	2	**ST10**	CC-ST10
US234	2	8	2	2	2	2	2	**ST10**	CC-ST10
US235	2	8	2	2	2	2	2	**ST10**	CC-ST10
US237	2	8	2	2	2	2	2	**ST10**	CC-ST10
US262	2	8	2	2	2	2	2	**ST10**	CC-ST10
US263	2	8	2	2	2	2	2	**ST10**	CC-ST10
US264	2	8	2	2	2	2	2	**ST10**	CC-ST10
US267	2	8	2	2	2	2	2	**ST10**	CC-ST10
US268	2	8	2	2	2	2	2	**ST10**	CC-ST10

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US269	2	8	2	2	2	2	2	**ST10**	CC-ST10
US271	2	8	2	2	2	2	2	**ST10**	CC-ST10
US272	2	8	2	2	2	2	2	**ST10**	CC-ST10
US275	2	8	2	2	2	2	2	**ST10**	CC-ST10
US279	2	8	2	2	2	2	2	**ST10**	CC-ST10
US280	2	8	2	2	2	2	2	**ST10**	CC-ST10
US281	2	8	2	2	2	2	2	**ST10**	CC-ST10
US286	2	8	2	2	2	2	2	**ST10**	CC-ST10
US288	2	8	2	2	2	2	2	**ST10**	CC-ST10
US319	2	8	2	2	2	2	2	**ST10**	CC-ST10
US320	2	8	2	2	2	2	2	**ST10**	CC-ST10
US322	2	8	2	2	2	2	2	**ST10**	CC-ST10
US326	2	8	2	2	2	2	2	**ST10**	CC-ST10
US327	2	8	2	2	2	2	2	**ST10**	CC-ST10
US330	2	8	2	2	2	2	2	**ST10**	CC-ST10
US333	2	8	2	2	2	2	2	**ST10**	CC-ST10
US334	2	8	2	2	2	2	2	**ST10**	CC-ST10
US336	2	8	2	2	2	2	2	**ST10**	CC-ST10
US342	2	8	2	2	2	2	2	**ST10**	CC-ST10
US345	2	8	2	2	2	2	2	**ST10**	CC-ST10
US346	2	8	2	2	2	2	2	**ST10**	CC-ST10
US347	2	8	2	2	2	2	2	**ST10**	CC-ST10
US348	2	8	2	2	2	2	2	**ST10**	CC-ST10
US350	2	8	2	2	2	2	2	**ST10**	CC-ST10
US351	2	8	2	2	2	2	2	**ST10**	CC-ST10
US353	2	8	2	2	2	2	2	**ST10**	CC-ST10
US132	2	8	2	2	2	2	2	**ST10**	CC-ST10
US133	2	8	2	2	2	2	2	**ST10**	CC-ST10
US134	2	8	2	2	2	2	2	**ST10**	CC-ST10
US144	2	8	2	2	2	2	2	**ST10**	CC-ST10
US145	2	8	2	2	2	2	2	**ST10**	CC-ST10
US150	2	8	2	2	2	2	2	**ST10**	CC-ST10
US162	2	8	2	2	2	2	2	**ST10**	CC-ST10
US173	2	8	2	2	2	2	2	**ST10**	CC-ST10
US175	2	8	2	2	2	2	2	**ST10**	CC-ST10
US179	2	8	2	2	2	2	2	**ST10**	CC-ST10
US186	2	8	2	2	2	2	2	**ST10**	CC-ST10
US253	2	8	2	2	2	2	2	**ST10**	CC-ST10
US291	2	8	2	2	2	2	2	**ST10**	CC-ST10
US292	2	8	2	2	2	2	2	**ST10**	CC-ST10

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US294	2	8	2	2	2	2	2	**ST10**	CC-ST10
US296	2	8	2	2	2	2	2	**ST10**	CC-ST10
US307	2	8	2	2	2	2	2	**ST10**	CC-ST10
US368	2	8	2	2	2	2	2	**ST10**	CC-ST10
US059	2	8	2	2	2	2	2	**ST10**	CC-ST10
US075	2	8	2	2	2	2	2	**ST10**	CC-ST10
US079	2	8	2	2	2	2	2	**ST10**	CC-ST10
US180	2	8	2	2	2	2	2	**ST10**	CC-ST10
US352	2	8	2	2	2	2	2	**ST10**	CC-ST10
US148	2	8	2	2	2	2	2	**ST10**	CC-ST10
518	2	8	2	2	2	2	2	**ST10**	CC-ST10
US118	2	8	2	2	2	41	2	ST78	CC-ST10
US138	2	8	2	2	2	41	2	ST78	CC-ST10
US365	2	8	2	2	2	41	2	ST78	CC-ST10
US367	2	8	2	2	2	41	2	ST78	CC-ST10
US120	2	8	2	2	2	41	2	ST78	CC-ST10
US196	2	8	2	2	2	41	2	ST78	CC-ST10
US204	2	8	2	2	2	41	2	ST78	CC-ST10
US208	2	8	2	2	2	41	2	ST78	CC-ST10
US212	2	8	2	2	2	41	2	ST78	CC-ST10
US241	2	8	2	2	2	41	2	ST78	CC-ST10
US266	2	8	2	2	2	41	2	ST78	CC-ST10
US285	2	8	2	2	2	41	2	ST78	CC-ST10
US287	2	8	2	2	2	41	2	ST78	CC-ST10
US289	2	8	2	2	2	41	2	ST78	CC-ST10
US317	2	8	2	2	2	41	2	ST78	CC-ST10
US318	2	8	2	2	2	41	2	ST78	CC-ST10
US321	2	8	2	2	2	41	2	ST78	CC-ST10
US335	2	8	2	2	2	41	2	ST78	CC-ST10
US349	2	8	2	2	2	41	2	ST78	CC-ST10
US251	2	8	2	2	2	41	2	ST78	CC-ST10
US252	2	8	2	2	2	41	2	ST78	CC-ST10
US172	2	8	2	2	2	41	2	ST78	CC-ST10
US177	2	8	2	2	2	41	2	ST78	CC-ST10
US337	2	8	2	2	2	41	2	ST78	CC-ST10
US338	2	8	2	2	2	41	2	ST78	CC-ST10
US167	2	41	2	2	2	41	2	ST84	CC-ST10
US189	2	8	2	2	2	46	2	*ST85*	CC-ST10
US190	2	8	2	2	2	46	2	*ST85*	CC-ST10
US364	2	8	2	2	2	46	2	*ST85*	CC-ST10

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
551	2	8	2	2	2	46	2	*ST85*	CC-ST10
US073	2	8	2	3	2	2	2	ST86	CC-ST10
US074	2	8	2	3	2	2	2	ST86	CC-ST10
US300	2	8	2	3	2	2	2	ST86	CC-ST10
US354	2	8	2	3	2	2	2	ST86	CC-ST10
US104	2	8	2	2	2	62	2	*ST275*	CC-ST10
US115	2	8	2	2	2	62	2	*ST275*	CC-ST10
US116	2	8	2	2	2	62	2	*ST275*	CC-ST10
US117	2	8	2	2	2	62	2	*ST275*	CC-ST10
US119	2	8	2	2	2	62	2	*ST275*	CC-ST10
US131	2	8	2	2	2	62	2	*ST275*	CC-ST10
US170	2	8	2	2	2	62	2	*ST275*	CC-ST10
US171	2	8	2	2	2	62	2	*ST275*	CC-ST10
US185	2	8	2	2	2	62	2	*ST275*	CC-ST10
US192	2	8	2	2	2	62	2	*ST275*	CC-ST10
US194	2	8	2	2	2	62	2	*ST275*	CC-ST10
US224	2	8	2	2	2	62	2	*ST275*	CC-ST10
US242	2	8	2	2	2	62	2	*ST275*	CC-ST10
US057	2	8	2	2	2	62	2	*ST275*	CC-ST10
US085	2	8	2	2	2	62	2	*ST275*	CC-ST10
US086	2	8	2	2	2	62	2	*ST275*	CC-ST10
US087	2	8	2	2	2	62	2	*ST275*	CC-ST10
US151	45	8	2	2	2	2	2	*ST294*	CC-ST10
US313	2	8	34	2	2	2	2	*ST300*	CC-ST10
US314	2	8	34	2	2	2	2	*ST300*	CC-ST10
US223	2	8	34	2	2	2	2	*ST300*	CC-ST10
US233	2	8	34	2	2	2	2	*ST300*	CC-ST10
US239	2	8	34	2	2	2	2	*ST300*	CC-ST10
US274	2	8	34	2	2	2	2	*ST300*	CC-ST10
US276	2	8	34	2	2	2	2	*ST300*	CC-ST10
US282	2	8	34	2	2	2	2	*ST300*	CC-ST10
US290	2	8	34	2	2	2	2	*ST300*	CC-ST10
US332	2	8	34	2	2	2	2	*ST300*	CC-ST10
US178	2	8	34	2	2	2	2	*ST300*	CC-ST10
US197	2	81	2	2	2	2	2	*ST303*	CC-ST10
US323	2	81	2	2	2	2	2	*ST303*	CC-ST10
US205	2	8	2	2	1	2	72	*ST304*	CC-ST10
US245	2	13	2	2	2	2	2	*ST305*	CC-ST10
US258	2	8	2	2	1	2	2	*ST306*	CC-ST10
US259	2	8	2	2	1	2	2	*ST306*	CC-ST10

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US355	2	8	2	3	2	41	2	*ST316*	CC-ST10
US356	2	8	2	1	2	41	2	*ST317*	CC-ST10
US157	6	9	7	3	7	5	7	ST11	CC-ST11
US163	6	9	7	3	7	5	7	ST11	CC-ST11
US261	15	17	14	9	13	17	16	*ST308*	CC-ST29
US164	3	16	13	9	12	16	15	ST28	CC-ST31
US149	1	13	15	14	1	7	1	ST70	CC-ST124
US215	4	73	22	3	3	3	3	ST267	CC-ST191
US216	4	73	22	3	3	3	3	ST267	CC-ST191
US339	46	3	22	3	3	3	3	*ST301*	CC-ST191
US340	46	3	22	3	3	3	3	*ST301*	CC-ST191
US341	46	3	22	3	3	3	3	*ST301*	CC-ST191
US343	46	3	22	3	3	3	3	*ST301*	CC-ST191
US344	46	3	22	3	3	3	3	*ST301*	CC-ST191
US277	46	3	22	3	3	3	3	*ST301*	CC-ST191
US181	46	3	22	3	3	3	3	*ST301*	CC-ST191
US062	28	75	15	2	25	1	54	*ST277*	CC-ST232
US064	28	75	15	2	25	1	54	*ST277*	CC-ST232
US084	8	62	15	2	25	1	54	*ST283*	CC-ST232
US097	1	28	4	2	25	25	59	ST256	CC-ST256
US101	1	28	4	2	25	25	59	ST256	CC-ST256
US217	1	28	4	2	25	25	59	ST256	CC-ST256
US219	1	28	4	2	25	25	59	ST256	CC-ST256
US220	1	28	4	2	25	25	59	ST256	CC-ST256
US095	1	2	2	3	46	12	2	ST257	CC-ST276
US105	1	2	2	3	46	12	2	ST257	CC-ST276
US061	1	2	2	3	25	12	2	*ST276*	CC-ST276
US076	1	2	2	3	25	12	2	*ST276*	CC-ST276
US077	1	2	2	3	25	12	2	*ST276*	CC-ST276
US078	1	2	2	3	25	12	2	*ST276*	CC-ST276
US080	1	2	2	3	25	12	2	*ST276*	CC-ST276
US111	1	2	2	3	25	12	2	*ST276*	CC-ST276
US112	1	2	2	3	25	12	2	*ST276*	CC-ST276
US113	1	2	2	3	25	12	2	*ST276*	CC-ST276
US199	1	2	2	3	25	12	2	*ST276*	CC-ST276
US200	1	2	2	3	25	12	2	*ST276*	CC-ST276
US201	1	2	2	3	25	12	2	*ST276*	CC-ST276
US065	6	9	7	2	49	5	3	*ST279*	CC-ST281
US068	6	9	7	2	49	5	3	*ST279*	CC-ST281
US070	6	9	7	2	49	5	3	*ST279*	CC-ST281

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US071	6	9	7	2	49	5	3	*ST279*	CC-ST281
US072	6	9	7	2	49	5	3	*ST279*	CC-ST281
US067	6	9	7	2	49	63	3	*ST281*	CC-ST281
US069	6	9	7	2	49	63	3	*ST281*	CC-ST281
US379	6	9	7	2	49	63	75	*ST331*	CC-ST281
US380	6	9	7	2	55	63	75	*ST332*	CC-ST281
US381	6	9	7	2	55	63	75	*ST332*	CC-ST281
US382	6	9	7	2	55	63	75	*ST332*	CC-ST281
US066	4	2	22	3	3	3	11	*ST280*	CC-ST287
US096	4	2	15	3	3	3	11	*ST287*	CC-ST287
US106	43	69	15	10	50	12	57	*ST288*	CC-ST288
US107	43	69	15	10	50	12	57	*ST288*	CC-ST288
US108	43	69	15	10	50	12	57	*ST288*	CC-ST288
US109	44	69	15	10	50	12	57	*ST289*	CC-ST288
US174	18	47	8	3	13	3	68	*ST296*	CC-ST296
US153	18	47	8	3	13	3	68	*ST296*	CC-ST296
US176	18	79	8	3	13	3	68	*ST299*	CC-ST296
US122	11	13	15	2	1	17	10	*ST291*	CC-ST310
US198	11	13	15	2	1	17	10	*ST291*	CC-ST310
US231	11	13	15	2	1	17	10	*ST291*	CC-ST310
US273	11	13	15	2	1	17	10	*ST291*	CC-ST310
US331	11	13	15	2	1	17	10	*ST291*	CC-ST310
US270	11	13	15	1	1	17	10	*ST310*	CC-ST310
US329	11	13	15	1	1	17	10	*ST310*	CC-ST310
US278	2	13	15	1	1	17	10	*ST311*	CC-ST310
US328	2	13	15	1	1	17	10	*ST311*	CC-ST310
US191	2	8	2	2	11	15	2	ST27	
US156	11	18	7	5	14	18	17	**ST30**	
US203	11	18	7	5	14	18	17	**ST30**	
US247	11	18	7	5	14	18	17	**ST30**	
US257	11	18	7	5	14	18	17	**ST30**	
US370	11	18	7	5	14	18	17	**ST30**	
US255	8	38	8	2	25	38	37	ST74	
US249	8	19	7	1	29	40	39	ST76	
US094	1	13	8	1	1	1	1	ST253	
US098	1	13	8	1	1	1	1	ST253	
US102	1	13	8	1	1	1	1	ST253	
US103	1	13	8	1	1	1	1	ST253	
US060	1	13	8	1	1	1	1	ST253	
US093	1	13	8	1	1	1	1	ST253	

Table 2.1b (cont'd)

Isolate ID	Allelic profile								
	trpB	***gyrB***	***dnaK***	***fumC***	***murG***	***tuf***	***atpA***	**ST**	**CC**
US383	41	68	10	9	12	18	14	ST255	
US081	42	18	26	5	47	8	14	ST258	
US082	42	18	26	5	47	8	14	ST258	
US099	42	18	26	5	47	8	14	ST258	
US100	42	18	26	5	47	8	14	ST258	
US214	42	18	26	5	47	8	14	ST258	
US218	42	18	26	5	47	8	14	ST258	
US063	2	28	8	14	10	4	14	*ST278*	
US083	18	2	8	3	13	3	11	*ST282*	
US088	8	18	8	10	8	12	32	*ST284*	
US089	2	14	16	10	51	64	14	*ST285*	
US090	2	14	16	10	51	64	14	*ST285*	
US091	2	14	16	10	51	64	14	*ST285*	
US092	1	76	9	5	1	65	64	*ST286*	
US110	13	69	15	5	52	12	26	*ST290*	
US139	2	8	2	3	36	66	65	*ST292*	
US141	2	8	2	3	36	66	65	*ST292*	
US166	2	8	2	3	36	66	65	*ST292*	
US225	2	8	2	3	36	66	65	*ST292*	
US232	2	8	2	3	36	66	65	*ST292*	
US244	2	8	2	3	36	66	65	*ST292*	
US142	11	77	8	10	23	39	66	*ST293*	
US152	8	78	8	7	36	67	67	*ST295*	
US158	8	28	33	3	17	61	69	*ST297*	
US168	29	12	8	10	33	68	70	*ST298*	
US187	26	80	4	5	20	69	71	*ST302*	
US260	18	28	35	3	25	39	73	*ST307*	
US265	2	82	36	9	10	6	21	*ST309*	
US283	30	29	36	5	2	6	21	*ST312*	
US309	18	8	7	3	49	5	7	*ST313*	
US310	18	8	7	3	49	5	7	*ST313*	
US324	42	3	11	5	14	7	1	*ST314*	
US325	43	83	2	7	53	36	12	*ST315*	
US372	47	85	4	2	43	71	74	*ST320*	

Table 2.2 Allelic variations among 15 North American clonal complexes (CCs). [a]Ancestral amino acid is listed first followed by SLV amino acid, Syn is a synonymous change

CC	ST of clonal ancestor	ST of SLV	Variant locus of SLV	Clonal ancestor AT	SLV AT	No. of nucleotide differences	Other CC(s) containing SLV	Other singletons containing SLV	Amino acid change[a]
CC-ST9	9	13	*tuf*	5	8	5	None	ST258	Syn
CC-ST10	10	78	*tuf*	2	41	5	None	None	Syn
CC-ST10	10	81	*tuf*	2	43	6	None	None	Syn
CC-ST10	10	82	*tuf*	2	44	1	None	None	P-S
CC-ST10	10	85	*tuf*	2	46	8	None	None	Syn
CC-ST10	10	86	*fumC*	2	3	2	CC-ST10; CC-ST11; CC-ST191; CC-ST287; CC-ST296; CC-ST276	ST254; ST261; ST264; ST266; ST282; ST292; ST297; ST307; ST313	Syn
CC-ST10	10	275	*tuf*	2	62	1	None	None	Syn
CC-ST10	10	294	*trpB*	2	45	1	None	None	Syn
CC-ST10	10	300	*dnaK*	2	34	1	None	None	Syn
CC-ST10	10	303	*gyrB*	8	81	4	None	None	Syn
CC-ST10	10	305	*gyrB*	8	13	3	CC-ST124; CC-ST310	ST253	Syn
CC-ST10	10	306	*murG*	2	1	7	CC-ST124; CC-ST310	ST253; ST286	P-H E-K I-V
CC-ST11	11	262	*murG*	7	49	1	CC-ST281	ST264; ST313	T-A
CC-ST29	29	308	*trpB*	8	15	1	None	None	Syn
CC-ST31	31	28	*gyrB*	19	16	9	None	None	Syn
CC-ST124	124	70	*trpB*	2	1	3	CC-ST10; CC-ST256; CC-ST276	ST6; ST83; ST253; ST286	Syn
CC-ST191	191	267	*gyrB*	3	73	10	None	None	Syn
CC-ST191	191	301	*trpB*	4	46	1	None	None	Syn
CC-ST232	232	277	*gyrB*	62	75	3	None	None	Syn

Table 2.2 (cont'd)

CC	ST of clonal ancestor	ST of SLV	Variant locus of SLV	Clonal ancestor AT	SLV AT	No. of nucleotide differences	Other CC(s) containing SLV	Other singletons containing SLV	Amino acid change[a]
CC-ST256	256	252	*murG*	25	16	2	None	ST250	H-P
CC-ST276	276	257	*murG*	25	46	6	None	None	K-E
CC-ST281	281	279	*tuf*	63	5	9	CC-ST9; CC-ST11	ST266; ST313	Syn
CC-ST281	281	331	*atpA*	3	75	4	None	None	Syn
CC-ST287	287	280	*dnaK*	15	22	2	CC-ST10; CC-ST191	None	Syn
CC-ST288	288	289	*trpB*	43	44	2	None	None	Syn
CC-ST296	296	265	*atpA*	68	62	1	None	None	Syn
CC-ST296	296	299	*gyrB*	47	79	4	None	None	Syn
CC-ST310	310	291	*fumC*	1	2	1	CC-ST10; CC-ST124; CC-ST232; CC-ST256; CC-ST281; CC-ST310	ST27; ST74; ST77; ST83; ST320	Syn
CC-ST310	310	311	*trpB*	11	2	1	CC-ST10; CC-ST124	ST27; ST278; ST285; ST292; ST309	Syn

Table 2.3 Summary of nucleotide sequence diversity among the 86 North American sequence types (STs). S, number of segregating sites. π, average pairwise nucleotide diversity per site. dN/dS, ratios of non-synonymous to synonymous mutations. R_{min}, Hudson and Kaplan's lower bound for the number of recombination.

Locus	Fragment size (bp)	No. of alleles	S	π	dN/dS	R_{min}
trpB	789	24	13	0.0042	0.0079	4
gyrB	1077	41	22	0.0068	0.0020	7
dnaK	873	20	29	0.0057	0.0586	3
fumC	750	8	5	0.0029	0.0422	2
murG	681	32	16	0.0066	0.2751	6
tuf	795	36	26	0.0074	0.0027	6
atpA	834	38	28	0.0125	0.0162	10
				Average	0.0578	
Sum			139			38
Concatenation	5,799		139	0.0055		44

Figure 2.1 Map of the United States and Canada. Distribution of 314 *F. psychrophilum* isolates recovered from the current North American study. These isolates were recovered from 20 U.S. states and one province of Canada. AK = Alaska; BC = British Columbia; CA = California; CO = Colorado; ID = Idaho; IN = Indiana; MD = Maryland; MI = Michigan; MN = Minnesota; MT = Montana; NC = North Carolina; NM = New Mexico; OR = Oregon, PA = Pennsylvania; SD = South Dakota; UT = Utah; VA = Virginia; VT = Vermont; WA = Washington; WI = Wisconsin; WV = West Virginia.

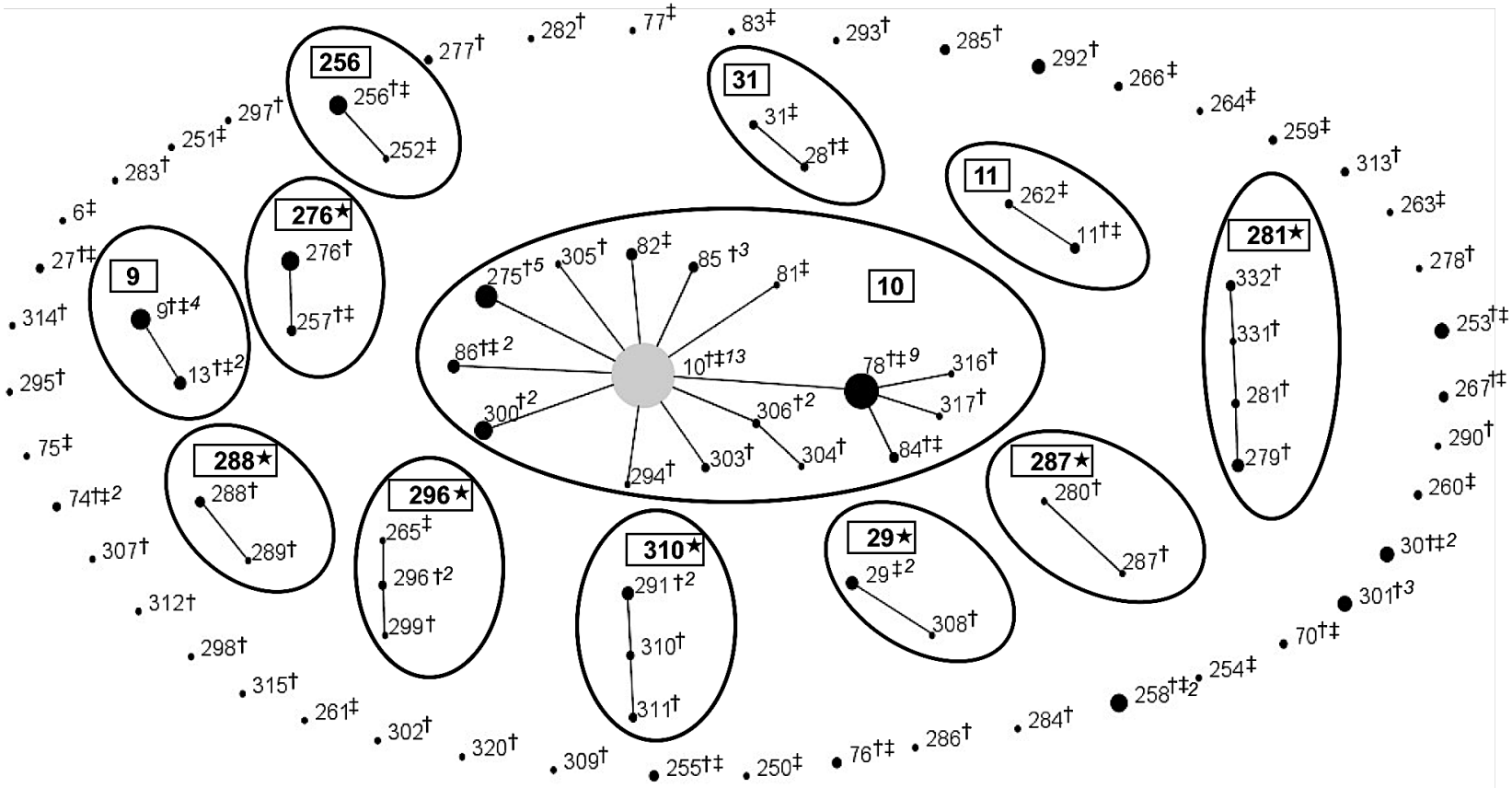


Figure 2.2 eBURST diagram of the 314 *F. psychrophilum* isolates typed in this study combined with all previously typed North American isolates (n = 107) in the *F. psychrophilum* MLST database. A dagger (†) denotes a sequence type (ST) that contains isolates typed in this study, whereas a double dagger (‡) denotes a ST containing previously typed isolates. Italicized numbers accompanying STs indicate the number of states that ST was found in (if no number, ST recovered from one state only). Clonal complexes (CC) are encircled and the founding sequence type (ST) for a CC is depicted as a number within a rectangle. Founding STs labeled with a star (★) indicate a newly described CC as a result of this study. The founding ST, or founder, is the ST with the highest number of single locus variants (SLVs). In instances where multiple STs have the same number of SLVs, the founder is named after the ST with the most isolates. If both STs have the same number of isolates, the CC is named for the earliest found ST.

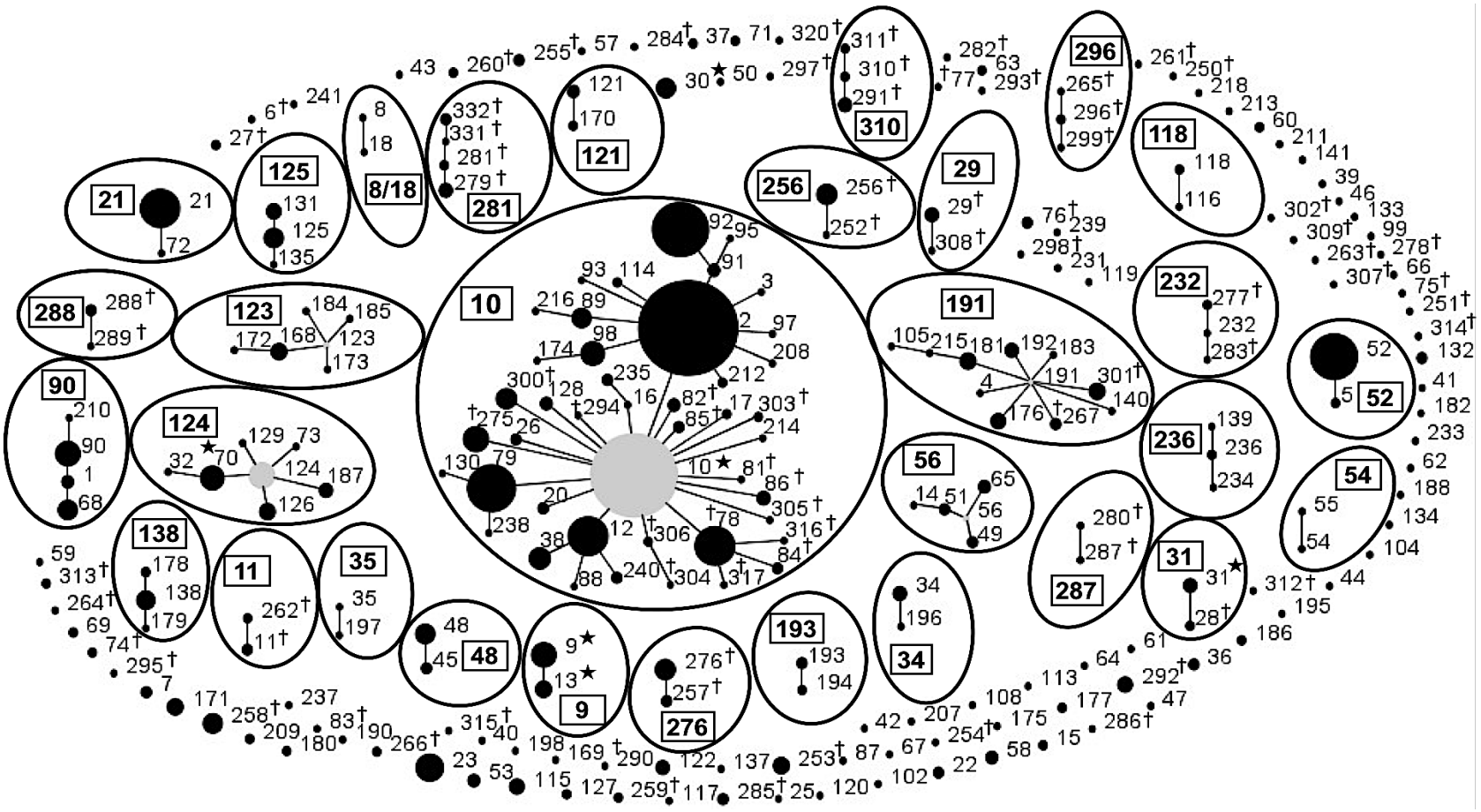


Figure 2.3 eBURST diagram of all 1,411 *F. psychrophilum* isolates. This includes the 1,097 isolates from the *F. psychrophilum* MLST database and the 314 newly typed isolates from this study. A dagger (†) indicates that ST is present in North America. A star (★) indicates that ST was found in North America and abroad. The founding sequence type (ST), or founder, is the ST with the highest number of single locus variants (SLVs; depicted as gray circles). In instances where multiple STs have the same number of SLVs, the founder is named after the ST with the most isolates. If both STs have the same number of isolates, the clonal complex (CC) is named for the earliest found ST. The founding genotype of a CC is enclosed within a box. The size of each circle indicates how many isolates belong to that ST.

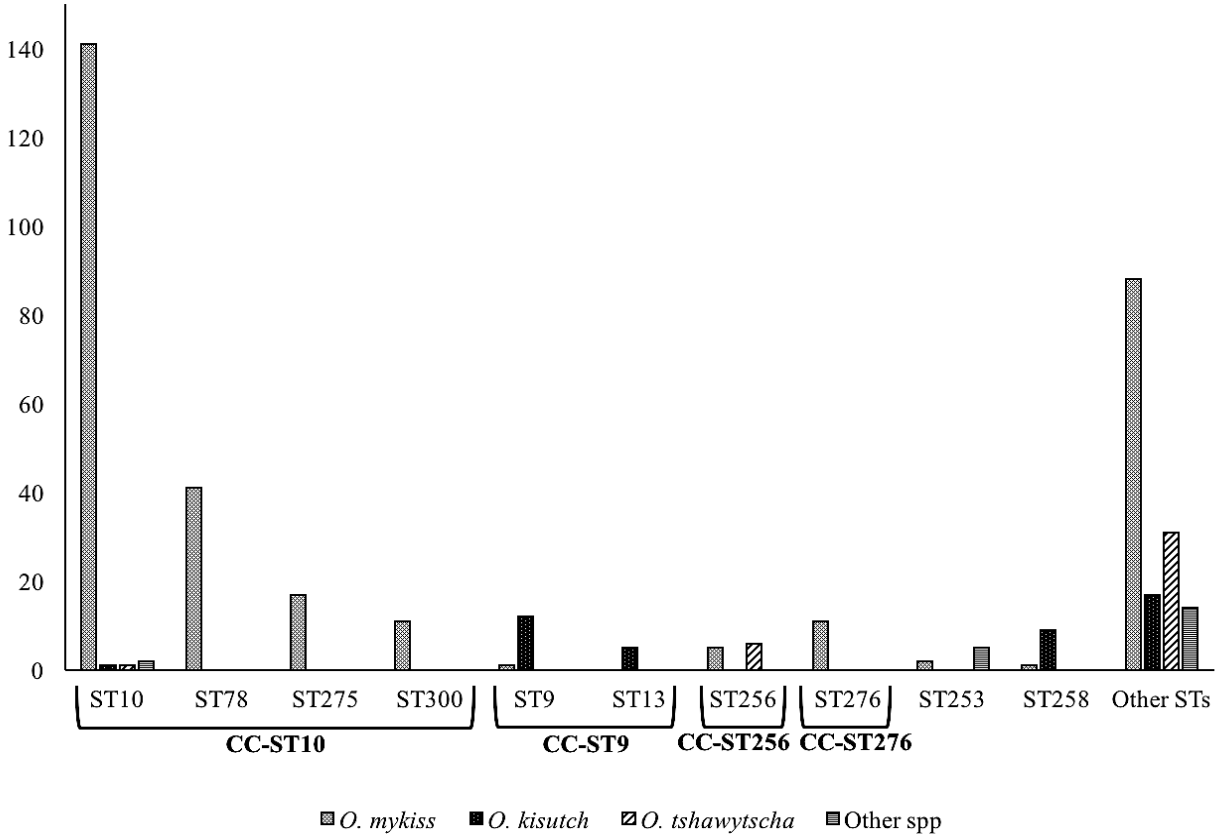


Figure 2.4 Frequency and host of origin among 10 North American *F. psychrophilum* sequence types (STs). STs are displayed along the X-axis with four clonal complexes (CCs; e.g., CC-ST10, CC-ST9, CC-ST256, CC-ST276) and two singletons (e.g., ST253, ST258) being shown. The Y-axis displays the number of recovered isolates. All 421 currently typed North American *F. psychrophilum* isolates are presented here.

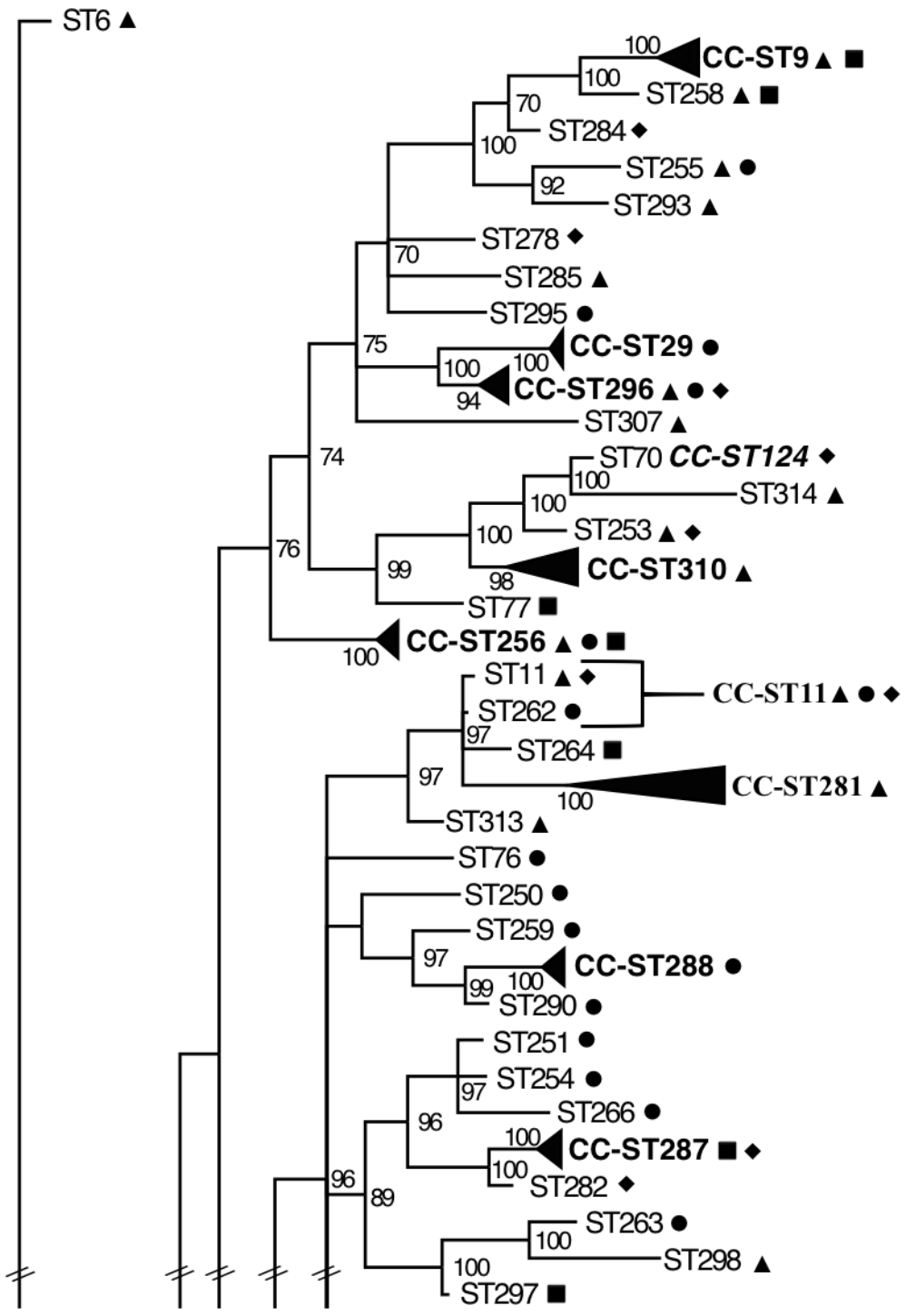
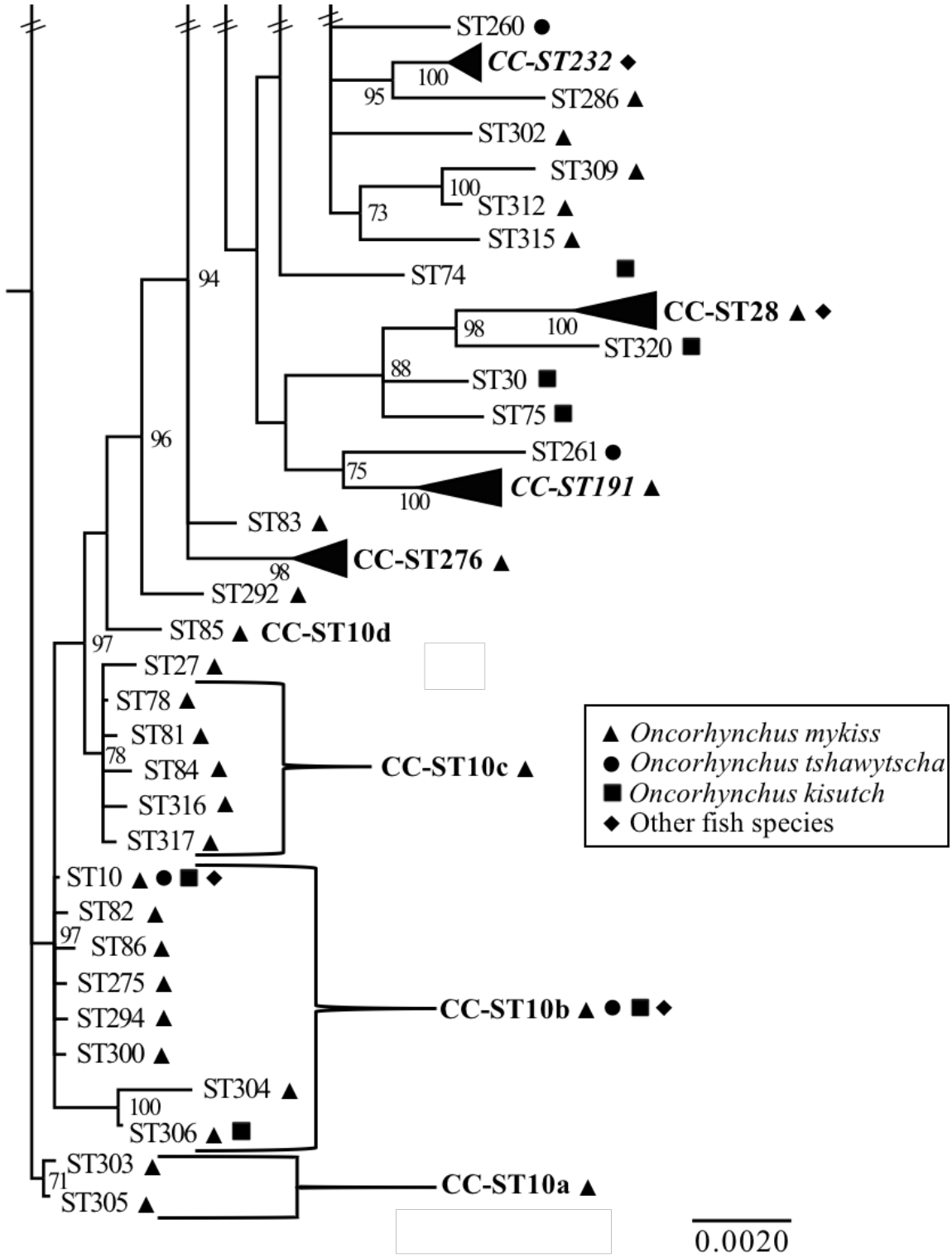


Figure 2.5 Phylogenetic reconstruction of the 86 *F. psychrophilum* sequence types (STs) currently described in North America using Bayesian inference. 15 clonal complexes (CCs) are in bold, 3 of which (in italics) are formed when the population structure of *F. psychrophilum* is evaluated in the context of the world. The fish host species each ST was recovered from is depicted as a symbol to the right of each ST and described in the key. Posterior probabilities ≥70% are displayed at branch nodes. The tree is drawn to scale and is unrooted.

Figure 2.5 (cont'd)



REFERENCES

REFERENCES

Anacker, R.L., and E.J. Ordal. 1959. Study on the myxobacterium *Chondrococcus columnaris* I: serological typing. Journal of Bacteriology 78:25-32.

Avendaño-Herrera, R., Houel, A., Irgang, R., Bernardet, J.F., Godoy, M., Nicolas, P., and E. Duchaud. 2014. Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms. Veterinary Microbiology 170:298-306.

Borg, A.F. 1948. Ph.D. thesis. University of Washington, Seattle, Washington. Studies on myxobacteria associated with diseases in salmonid fishes.

Brown, L.L., Cox, W.T., and R.P. Levine. 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Diseases of Aquatic Organisms 29:213-218.

Bullock, G.L., Hsu, T.C., and E.B. Shotts. 1986. Columnaris disease of salmonids. U.S. Fish and Wildlife Service Fish Disease Leaflet 72.

Castillo, D., Christiansen, R. H., Dalsgaard, I., Madsen, L., Espejo, R., and M. Middelboe. 2016. Comparative Genome Analysis Provides Insights into the Pathogenicity of Flavobacterium psychrophilum. PLoS ONE 11: e0152515.

Cipriano, R.C., and R.A. Holt. 2005. *Flavobacterium psychrophilum*, cause of Bacterial Cold-Water Disease and Rainbow Trout Fry Syndrome. Fish Disease Leaflet No. 86, United States Department of the Interior. U.S. Geological Service, National Fish Health Research Laboratory, Kearneysville, West Virginia.

Cipriano, R.C. 1995. Association of *Cytophaga psychrophila* with mortality among eyed eggs of Atlantic salmon (*Salmo salar*). Journal of Wildlife Diseases. 31:166-171.

Duchaud, E., Rochat, T., Habib, C., Barbier, P., Loux, V., Guérin, C., Dalsgaard, I., Madsen, L., Nilsen, H., Sundell, K., Wiklund, T., Strepparava, N., Wahli, T., Caburlotto, G., Manfrin, A., Wiens, G.D., Fujiwara-Nagata, E., Avendaño-Herrera, R., Bernardet, J-F., and P. Nicolas. 2018. Genomic Diversity and Evolution of the Fish Pathogen *Flavobacterium psychrophilum*. Frontiers in Microbiology 9:138.

Faisal, M. 2007. Health changes to aquatic animals in the globalization era. In Globalization: Effects on Fisheries Resources. (Taylor, W. W., Schechter, M. G., &Wolfson, L.G., ed.) Cambridge University Press, Cambridge.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., and B.G. Spratt. 2004. eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. Journal of Bacteriology 186:1518-1530.

Feil, E.J., Enright, M.C., and B.G. Spratt. 2000. Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*. Research in Microbiology 151:465–469.

Fujiwara-Nagata, E., Chantry-Darmon, C., Bernardet, J.F., M. Eguchi, M., Duchaud E., and P. Nicolas. 2013. Population structure of the fish pathogen Flavobacterium psychrophilum at a whole country and model river levels in Japan. Veterinary Research 44:34.

Fujiwara-Nagata, E., Ikeda, J., Sugahara, K., and M. Eguchi. 2012. A novel genotyping technique for distinguishing between *Flavobacterium psychrophilum* isolates virulent and avirulent to ayu, *Plecoglossus altivelis* (Temminck & Schlegel). Journal of Fish Diseases 35:471–480.

Hansen, M.J., and M.E. Holey. 2002. Ecological factors affecting the sustainability of Chinook and coho salmon populations in the Great Lakes, especially Lake Michigan. Sustaining North American salmon: perspectives across regions and disciplines. American Fisheries Society, Bethesda, Maryland, 155-179.

Haubold, B., and R.R Hudson. 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics 16:847–849.

Holt, R.A. 1987. Ph.D. thesis. Oregon State University, Corvallis, Oregon. *Cytophaga psychrophila*, the causative agent of bacterial cold water disease in salmonid fish.

Hudson, R.R., and N.L. Kaplan. 1985. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics 111:147–164.

Huelsenbeck, J.P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

Huson, D.H., and D. Bryant. 2006. Application of phylogenetic networks in evolutionary studies. Molecular Biology and Evolution 23:254–267.

Jolley, K.A., and M.C. Maiden. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11:595.

Jolley, K.A., Feil, E.J., Chan, M.S., and M.C Maiden. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics 17:1230–1231.

Kumagai, A., Yamaoka, S., Takahashi, K., Fukuda, H., and H. Wakabayashi. 2000. Waterborne Transmission of *Flavobacterium psychrophilum* in Coho Salmon Eggs. Fish Pathology 35:25-28.

Kumagai, A., Takahashi, S., Yamaoka, S., and H. Wakabayashi. 1998. Ineffectiveness of Iodophore Treatment in Disinfecting Salmonid Eggs Carrying *Cytophaga psychrophila*. Fish Pathology 33:123-128.

Loch T.P., M Faisal. 2017. *Flavobacterium* spp., p. 211–232. In Woo, PTK, Cipriano, RC (eds.), Fish Viruses and Bacteria: Pathobiology and Protection CABI.

Madsen, L., and I. Dalsgaard. 2000. Comparative studies of Danish *Flavobacterium psychrophilum isolates*: ribotypes, plasmid profiles, serotypes and virulence. Journal of Fish Diseases 23:211-218.

Mohammadi, T., Karczmarek, A., Crouvoisier, M., Bouhss, A., Mengin-Lecreulx, D., and T. den Blaauwen. 2007. The essential peptidoglycan glycosyltransferase MurG forms a complex with proteins involved in lateral envelope growth as well as with proteins involved in cell division in *Escherichia coli*. Molecular Microbiology 65:1106–1121.

Nematollahi, A., Decostere, A., Pasmans, F., and F. Haesebrouck. 2003. *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases 26:563-574.

Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J.F., and E. Duchaud. 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709.

Nilsen, H., Sundell, K., Duchaud, E., Nicolas, P., Dalsgaard, I., Madsen, L., Aspán, A., Jansson, E., Colquhoun, D.J., and T. Wiklund. 2014. Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. Applied and Environmental Microbiology 80:2728-2736.

Parker, N.C. 1989. History, status, and future of aquaculture in the United States. CRC Critical Reviews in Aquatic Sciences 1:97-109.

Rambaut, A. 2009. FigTree v1.3.1. http://tree.bio.ed.ac.uk/software/figtree/.

Ronquist, F., and J.P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Rozas J., Ferrer-Mata A., Sánchez-DelBarrio J-C., Guirao-Rico S., Librado P., Ramos-Onsins S.E., and A. Sánchez-Gracia. 2017. DnaSP v6: DNA Sequence Polymorphism Analysis of Large Datasets. Molecular Biology and Evolution 34:3299-3302.

Siekoula-Nguedia, C., Blanc, G., Duchaud, E., and S. Calvez. 2012. Genetic diversity of *Flavobacterium psychrophilum* isolated from rainbow trout in France: predominance of a clonal complex. Veterinary Microbiology 161:169-178.

Spratt, B.G., Hanage, W.P., Li, B., Aanensen, D.M., and E.J. Feil. 2004. Displaying the relatedness among isolates of bacterial species- the eBURST approach. FEMS Microbiology Letters 241:129-134.

Storebakken, T. 2002. Atlantic Salmon, *Salmo salar*. p. 79. In Nutrient Requirements and Feeding of Finfish for Aquaculture.

Strepparava, N., Nicolas, P., Wahl, T., Segner, H., and O. Petrini. 2013. Molecular epidemiology of *Flavobacterium psychrophilum* from Swiss fish farms. Diseases of Aquatic Organisms 105:203-210.

Swofford, D.L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Van Vliet, D., Wiens, G.D., Loch, T.P., Nicolas, P., and M. Faisal. 2016. Genetic Diversity of *Flavobacterium psychrophilum* Isolates from Three Oncorhynchus spp. in the United States, as Revealed by Multilocus Sequence Typing. Applied and Environmental Microbiology 82:3246-3255.

Van Vliet, D., Loch, T.P., and M. Faisal. 2015. *Flavobacterium psychrophilum* Infections in Salmonid Broodstock and Hatchery-Propagated Stocks of the Great Lakes Basin. Journal of Aquatic Animal Health 27:192-202.

Chapter 3

MLST genotypes of *F. psychrophilum* vary in their pathogenicity to naïve rainbow trout (*Oncorhynchus mykiss*)

1. Abstract

Flavobacterium psychrophilum is the etiological agent of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) in salmonids. Multilocus sequence typing (MLST) has demonstrated that *F. psychrophilum* isolates are genetically diverse and comprise multiple MLST clonal complexes (CC). Among the largest currently recognized CCs are CC-ST10 and CC-ST191, both of which primarily affect rainbow trout (*Oncorhynchus mykiss*); however, many studies have only highlighted the importance of CC-ST10. In the USA, 16 CC-ST10 genotypes have been identified and cause remarkable economic losses, particularly in farmed rainbow trout. Conversely, only two genotypes exist within CC-ST191 in the USA but also devastate farmed rainbow trout. In this study, differences in pathogenicity within and between the two main MLST CCs affecting rainbow trout were investigated. A series of intramuscular experimental infection studies were performed in triplicates using laboratory raised seven-month old rainbow trout. Each fish received ~10^7 - 10^8 cfu/fish^{-1} of each of 10 *F. psychrophilum* strains belonging to three STs of CC-ST10 (i.e., ST10, ST78, ST275) and two STs of CC-ST191 (i.e., ST267, ST301). All but one strain (i.e., US181) caused 100% mortality in their host; however, significant differences were found in mean days to death between isolates, including those of the same genotype. Overall, all strains appeared nearly equal in their ability to cause BCWD in rainbow trout and there were no significant differences between mean days to death for CC-ST10 and CC-ST191. Findings of this study underscore the ability of *F. psychrophilum* isolates of these two clonal complexes to cause significant mortalities in their host and suggest that multiple MLST CCs are highly virulent to rainbow trout.

2. Introduction

Flavobacterium psychrophilum is the etiological agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTSF), which are responsible for significant economic losses in farmed fish worldwide (reviewed in Loch & Faisal 2017). This bacterial pathogen primarily infects trout and salmon (Family Salmonidae), including rainbow trout/steelhead (*Oncorhynchus mykiss*), coho salmon (*O. kisutch)*, Chinook salmon (*O. tshawytscha*), and Atlantic salmon (*Salmo salar*; reviewed in Nematollahi et al. 2003, Loch & Faisal 2017).

Multilocus sequence typing (MLST) has revealed significant genetic heterogeneity within *F. psychrophilum* in the form of >240 sequence types (STs) that form >30 clonal complexes (CCs), some of which are present on multiple continents and appear highly virulent (Nicolas et al. 2008, Siekoula-Nguedia et al. 2012, Fujiwara-Nagata et al. 2013, Strepparava et al. 2013, Avendaño-Herrera et al. 2014, Nilsen et al. 2014, Van Vliet et al. 2016; Chapter 2). One such CC is CC-ST10, which has been reported on five continents (e.g., Asia, Europe, North America, Australia, South America) and is predominantly recovered from rainbow trout/steelhead suffering from BCWD. Likewise, previous research (i.e., Nicolas et al. 2008, Van Vliet et al. 2016), as well as Chapter 2 results, has shown that CC-ST10 isolates comprise a majority of those recovered from disease epizootics in the USA.

Although these isolates appeared highly pathogenic and some have highlighted CC-ST10 as virulent (Nilsen et al. 2014), particularly to rainbow trout/steelhead, the relationship of *F. psychrophilum* MLST genotype to virulence has not been adequately addressed. In fact, very few isolates from CC-ST10 have been used in experimental challenge studies but at least one has been used many times before; namely, CSF 259-93. CSF 259-93 was identified as ST10 in 2008

(Nicolas et al. 2008) and has proven to be highly virulent in both injection and immersion studies (LaFrentz et al. 2003, Long et al. 2014, Wagner & Oplinger 2014). It remains unclear; however, if this single ST10 isolate reflects the pathogenicity of other ST10 isolates, if this genotype's pathogenicity is mirrored by its single locus variants (SLVs), and if CC-ST10 is truly more virulent than other *F. psychrophilum* CCs.

The second largest currently recognized *F. psychrophilum* CC worldwide is CC-ST191. Similar to CC-ST10, the majority of CC-ST191 genotypes have been recovered from diseased rainbow trout/steelhead (Nicolas et al. 2008, Siekoula-Nguedia et al. 2012, Strepparava et al. 2013, Nilsen et al. 2014). Although most CC-ST191 genotypes have been reported in Europe, two (i.e., ST267, ST301) have now been reported in the US (Chapter 2). Furthermore, isolates belonging to these two genotypes were recovered from rainbow trout/steelhead, many of which were also diseased. However, there are no published reports of isolates recognized as CC-ST191 being evaluated for their virulence.

Although the comparative virulence of *F. psychrophilum* MLST genotypes has not been studied in salmonids, Fujiwara-Nagata et al. (2012, 2013), assessed the *in vivo* virulence of 40 *F. psychrophilum* isolates belonging to two different CCs (e.g., CC-ST52, CC-ST48/CC-ST56) in ayu (*Plecoglossus altivelis*) that were exclusively recovered from non-salmonids. Interestingly, the isolates belonging to CC-ST48/CC-ST56 caused BCWD-associated mortality, whereas CC-ST52 caused little to no mortality, thereby suggesting MLST CCs vary in their virulence.

To examine the correlation between *F. psychrophilum* pathogenicity and MLST genotype in rainbow trout, 10 *F. psychrophilum* isolates that were recently genotyped using MLST were selected for *in vivo* virulence experiments. The isolates included in my experiments belonged to ST10, ST78, and ST275 (CC-ST10) and ST267 and ST301 (CC-ST191). My first objective was

to confirm that multiple variants within CC-ST10 were indeed virulent to their natural host, the rainbow trout. My second objective was to determine if *in vivo* virulence varies between isolates and genotypes in the same CC, and my third objective was to compare the virulence of the two largest MLST CCs primarily affecting rainbow trout/steelhead in the USA.

3. Materials and Methods

3.1. *F. psychrophilum* isolates used in experimental challenge

Ten *F. psychrophilum* isolates that were genotyped using MLST and determined to belong to five STs within the two largest CCs were selected for experimental use (Table 3.1). These isolates were recovered from rainbow trout/steelhead that either displayed gross signs of BCWD or were apparently healthy and were recovered from three US states (e.g. Michigan, Pennsylvania, West Virginia) between the years of 2010 and 2017 (Table 3.1). Each of the two isolates within the five STs were either recovered from independent episodes of BCWD or during routine disease health surveillance (Table 3.1).

3.2. Fish and rearing conditions

Embryonated rainbow trout eggs (i.e., eyed eggs) were obtained from Trout Lodge (Bonney Lake, WA) and maintained in a vertical incubator supplied with dechlorinated pathogen-free water (12ºC ± 1ºC; ~19 L/min) at the Michigan State University (MSU) – University Research Containment Facility until hatching. After most of the yolk-sac was absorbed, sac-fry were then transferred into aerated flow-through tanks (~40 L; ~34 L/min; 12-h photoperiod) supplied with dechlorinated pathogen-free water (12ºC ± 1ºC) until exogenous feeding began, at which point fry were fed a commercial trout diet of appropriately sized food

via an automatic feeder. After several weeks, fish were fed by hand twice daily until satiation and the water volume was increased to ~400L (~19L/min; 12-h photoperiod). Upon commencement of feeding, all tanks were siphoned once to twice daily to remove detritus and uneaten food.

3.3. Experimental challenge via intramuscular injection

Intramuscular (IM) injection was selected as the route of infection because it has been established as a highly reproducible *F. psychrophilum* exposure route (Garcia et al. 2000). The 10 *F. psychrophilum* isolates (Table 3.1) were passed a single time through naïve rainbow trout via IM injection and re-isolated on tryptone yeast extract agar (TYES; Holt 1987) supplemented with 5% horse serum and 0.02% Lewin and Lounsbery mineral solution (referred to hereafter as mTYES; Lewin & Lounsbery 1996, Michel et al. 1999). After cultures were verified for purity, a single colony of each isolate was cryopreserved in mTYES supplemented with glycerol (20% [vol/vol]) for use in IM challenges.

For the experimental challenge, the 10 *F. psychrophilum* isolates were revived from cryo-stock on mTYES agar and incubated at 15ºC for 72 hours, after which, a single colony was sub-cultured on to fresh mTYES agar and incubated for 72 hours at 15ºC to verify cryo-stock purity. Next, an isolated colony of each isolate was inoculated into 400 mL of mTYES broth and incubated at 15ºC with constant shaking at a speed of 100 rpm for 72 hours. Bacterial pellets were harvested from mTYES broth via centrifugation (2,571 x *g*, 15 min; Thermo Scientific™ Sorvall™ Legend™ X1R centrifuge). The supernatant was decanted and the bacterial cell pellet was suspended in sterile 0.65% sodium chloride (NaCl) and adjusted to an optical density at 600-nm (OD_{600}) corresponding to a target of ~1 x 10^9 colony forming units (CFU) per mL using a

Biowave CO8000 Cell Density meter (WPA Inc., Cambridge, UK). To verify challenge dose, each challenge isolate suspension was serially diluted in ten-fold increments from 10^{-1} to 10^{-8} and 10 µl of each dilution was plated in duplicate on mTYES agar, incubated at 15ºC for 7 days, and final colony counts performed.

For the IM challenge, fish (n = 10 per isolate, in triplicate) were randomly selected, anesthetized in sodium bicarbonate-buffered tricaine methanesulfonate (MS-222) at a concentration of 100mg L^{-1}, and then IM-injected with 100 µl of the bacterial suspension (corresponding to 5.00 x 10^7 – 4.20 x 10^8 cfu/fish^{-1}; Table 3.2) proximal to the anterior portion of the dorsal fin on the left side using a 26 G needle. Control fish (n = 10, in triplicate) were anesthetized as described above and sham-inoculated with 100 µl of sterile 0.65% NaCl. After IM-injection, fish were immediately transferred into aerated flow-through glass tanks (37.85L; 12-h photoperiod; randomly selected for each isolate prior to challenge) supplied with dechlorinated pathogen-free water (12ºC ± 1). Fish were monitored daily for 28-days and cared for as described above; mortalities were necropsied, clinically examined, and multiple tissues (e.g., brain, gills, heart, kidney, liver, skin, and/or spleen) were bacteriologically analyzed. In addition, a representative of each challenge isolate recovered from a challenged fish was extracted and confirmed as *F. psychrophilum* using an endpoint PCR assay as detailed in Chapter 2. All challenge experiments were conducted in accordance with the MSU-Institutional Animal Care and Use Committee.

3.4. Statistical analyses

The cumulative percent mortality (CPM) and mean days to death were calculated for all challenge isolates. A one-way analysis of covariance (ANCOVA) was conducted to determine if

a statistically significant difference between *F. psychrophilum* isolates on the mean days to death while controlling for the continuous covariate, fish mass, was present. Testing was not conducted on CPM observations as all isolates caused ≥96.7% CPM. Fish mass was chosen to be a covariate in the testing of mean days to death because it influences fish mortality during experimental challenge with *F. psychrophilum* (Madsen & Dalsgaard 1999). Fish mass (i.e., the covariate) was rescaled to have a mean of zero and a standard deviation of one to standardize observations for analysis. For the experiment and ANCOVA analysis, fish tanks (n = 3 tanks per isolate) composed the experimental units, whereas individual fish were observational units (n = 10 fish per tank). Differences in least squares mean days to death between each *F. psychrophilum* isolate were tested with the Tukey-Kramer method used to adjust P-values for the pairwise tests. Differences between STs and CCs were tested using linear contrasts of means, where the elements of the contrast statement were based on which ST and CC each isolate belonged (see earlier description of isolates). Furthermore, to determine if the inoculum size influenced the mean days to death, a correlation analysis was conducted. P-values were considered statistically significant at an alpha level of 0.05. All statistical analyses were computed using SAS® software, Version 9.4 for Windows.

4. Results

4.1. Clinical signs

4.1.1. External signs

Within 24-hours post infection, all fish challenged with *F. psychrophilum* were lethargic and inappetent, except for a single fish inoculated with strain US181. All challenged fish showed a combination of external signs consistent with BCWD (Figure 3.1), including

superficial ulceration that deepened and extended into the underlying muscle as infection progressed (Figure 3.1c, e-g). Ulcerations were often accompanied by a prominent yellowish discoloration of the affected tissues (Figure 3.1c, e-g). In some instances, ulcerations became focally extensive and would spread either cranially or caudally (Figure 3.1f, g). Additional disease signs included hemorrhage within the eye, isthmus, and fins, and severe gill pallor (Figure 3.1a, b) accompanied by either erythema or diffuse petechial hemorrhage (Figure 3.1b). In most challenged fish, a focal area of hemorrhage and/or pallor occurred near the site of injection (Figure 3.1d). The percent of fish showing lesions in the gills, skin or fins are shown in Table 3.3.

4.1.2. Internal signs

Gross internal disease signs in rainbow trout infected with *F. psychrophilum* included renal, hepatic, and cardiac pallor; splenic swelling and/or enlargement; hemorrhage within the ovaries, serosal surface of the swim bladder, adipose tissue overlying the pyloric cecae, ventricle of the heart, and the liver (Figure 3.2a-d). The percent of fish showing lesions in the heart, kidney, liver, spleen, ovary, and/or swim bladder are shown in Table 3.3.

4.2. Cumulative percent mortality

Cumulative percent mortality (CPM) was 100% for all tested isolates, except for US181, in which a single fish survived for the duration of the study (96.7% CPM). Given the high CPM among all isolate groups, no statistical comparisons were made. All negative control fish survived for the duration of the study (Table 3.2).

4.3. Mean days to death

Overall, the mean days to death and the covariate, fish mass, were significantly different among all isolates (data not shown). Pairwise comparisons of the mean days to death for each combination of isolates revealed these differences further (Table 3.2). For example, although isolates CC10-US352 (i.e., isolate US352 belongs to CC-ST10), CC10-US26, and CC191-US215 were not significantly different from one another ($P > 0.99$), they were significantly different from all other isolates and overall had the highest mean days to death ($P \leq 0.0083$; Table 3.2). Isolates CC10-US75, CC10-US57, CC10-US87, CC191-US54, CC191-US181, and CC191-US343 did not differ significantly in their mean days to death either ($P > 0.17$; Table 3.2). Of these six isolates, five (i.e., CC10-US75, CC10-US57, CC10-US87, CC191-US54, CC191-US181) did not differ significantly from CC10-US53 ($P > 0.14$), whereas one (i.e., CC191-US343) did ($P < .05$; Table 3.2). On average, fish infected with isolate CC10-US53 died the quickest (i.e., 2.0 ± 1.4 days), whereas those infected with CC191-US215 survived the longest (i.e., 6.8 ± 3.8 days; Table 3.2).

The mean days to death among STs were found to differ significantly from one another too. In specific, CC10-ST10, CC10-ST78, and CC191-ST267 were not significantly different from one another in their mean days to death ($P > 0.13$), but were significantly different from CC10-ST275 and CC191-ST301 ($P \leq 0.0072$). On average, fish inoculated with CC10-ST10, CC10-ST78, or CC191-ST267 survived longer than those infected with CC10-ST275 or CC191-ST301 (Table 3.2). Likewise, CC10-ST275 and CC191-ST301 were not found to be significantly different from one another and had nearly identical mean days to death (i.e., 3.3 ± 2.0 versus 3.2 ± 1.7, respectively; $P = 0.8957$). On average, fish infected with a CC10-ST10 isolate survived the longest (i.e., 5.1 ± 3.2 days), whereas those infected with a CC191-ST301

isolate died the quickest (i.e., 3.2 ± 1.7). No significant difference in mean days to death was discovered between the two CCs (i.e., CC-ST10, CC-ST191; P = 0.3414).

The correlation analysis revealed no statistically significant relationship between isolate inoculum size (i.e., the dose) and the mean days to death (P = 0.5020). In fact, a positive correlation was seen between inoculum size and mean days to death (i.e., as inoculum size increases, fish survive longer; correlation coefficient = 0.2412). This positive relationship, however, was influenced by isolate US352 (ST10), which had the highest dose of *F. psychrophilum* (i.e., 4.20 x 10^8 cfu/fish^{-1}) as well as one of the longest mean days to death (i.e., 6.4 ± 3.9). When this isolate was removed from the analysis, a negative correlation between dose and mean days to death (correlation coefficient = -0.1486) that was not statistically significant (P = .7027) was discovered.

4.4. Reisolation of *F. psychrophilum*

A total of 242 *F. psychrophilum* isolates were recovered from the 115 fish that were sampled. Of these, 99 isolates were recovered from the kidney, followed by 68 from the skin near the injection site, 30 from the gills, 20 from the spleen, 13 from the liver, six from the brain, two from the dorsal fin, two from the heart, one from the adipose tissue overlying the pyloric cecae, and one from the musculature at the site of injection. All representative isolates were PCR confirmed as *F. psychrophilum*.

5. Discussion

This study examined the pathogenicity of 10 North American *F. psychrophilum* strains belonging to five STs within two distinct MLST CCs (i.e., CC-ST10, CC-ST191), both of which

are widespread globally. Indeed, CC-ST10 is regarded as a putatively virulent CC (Nilsen et al. 2014), but despite CC-ST191 being recovered repeatedly from BCWD epizootics in farmed rainbow trout on multiple continents (Nilsen et al. 2014, Van Vliet et al. 2016), no mention of its virulence has been made. Although similarities between these two CCs exist (i.e., both are predominantly recovered from diseased rainbow trout and found in overlapping geographic locations), their housekeeping genes are genetically distinct from one another (Chapter 2) and previous studies have shown that *F. psychrophilum* strains vary in their virulence (Madsen & Dalsgaard 1998, Madsen & Dalsgaard 2000, Fujiwara-Nagata et al. 2012, Fujiwara-Nagata et al. 2013). This encouraged me to explore *F. psychrophilum* MLST genetic diversity in the context of pathogenicity, particularly because heterogeneity in the core (i.e., conserved) genome may equate to differences in the accessory genome, which is more variable and associated with virulence (Segerman 2012). Despite their genetic distinctness, representative strains within CC-ST10 and CC-ST191 proved to be highly pathogenic, causing fulminant mortality (i.e., ≥96.7%) in a short amount of time (i.e., ~two to seven days; Table 3.2). Correlation analysis provided evidence that the observed mean days to death was influenced by the isolate and not the inoculum size, thereby indicating that all utilized CC-ST10 and CC-ST191 isolates were highly pathogenic irrespective of the challenge dose. Still, the rapid mortality caused by these strains was unexpected. Garcia et al. (2000) used a similar challenged dose (i.e., 4.2×10^7 cfu/fish^{-1}) when IM-injecting rainbow trout of a smaller size (i.e., average mass of ~three to six grams per fish), and yet 100% CPM was not reached until 18-days post infection compared to two to seven days, on average, in the current study. However, the MLST genotype of the *F. psychrophilum* isolate (i.e., JIP 02-97) used by Garcia et al. (2000) is unknown and may partially explain the observed differences in time to death.

The significant differences in mean days to death in rainbow trout challenged with *F. psychrophilum* isolates of the same MLST genotype suggests that MLST genotype may not entirely reflect an isolate's pathogenicity, which is determined in part by its virulence factors, of which *F. psychrophilum* has many (reviewed in Nematollahi et al. 2003, Starliper 2011, Castillo et al. 2016, Duchaud et al. 2018). Although CC-ST10 isolates that have been genomically characterized possess multiple factors that are believed to be important for virulence (e.g., putative hemolysin D transporter, type II secretion system, metalloproteases, Por secretion system; Castillo et al. 2016, Duchaud et al. 2018), the factors important for CC-ST191 virulence and whether these overlap or differ from those of CC-ST10 are unknown and warrant further investigation. With this in mind, previous research has shown that *F. psychrophilum* isolates belonging to different genotypes of the same CC share the majority of their proteins encoding genes, yet differences exist. For example, the protein encoding genes of two *F. psychrophilum* isolates belonging to CC-ST9 (i.e., OSU THC02-90 identified as ST9, NCMB 1947^{T} identified as ST13; Nicolas et al. 2008) differed by <100 genes according to whole genome sequencing (Wu et al. 2015, Rochat et al. 2017). In a similar context, recent research has shown that dozens of gene families within *F. psychrophilum* genotypes of the same CC are shared and are not present in other CCs or stand-alone genotypes (i.e., singletons). For example, within CC-ST10, multiple isolates belonging to several different genotypes (i.e., ST2, ST10, ST20, ST21, ST78) have been examined using whole genome sequencing (Duchaud et al. 2007, Wu et al. 2015, Castillo et al. 2016, Duchaud et al. 2018), and all isolates that were analyzed shared >80 gene families not present in other CCs (Duchaud et al. 2018). Interestingly, genes putatively linked to virulence, such as *tetX* and those encoding for a type II secretion system, were discovered among these gene families specific to CC-ST10. Furthermore, isolates of the same genotype

(i.e., ST2, ST12) have been found to differ in their gene families, which may have differential consequences for pathogenicity. For example, *F. psychrophilum* isolates 950106-1/1 and VQ50 share an identical MLST genotype (i.e., ST2); however, 950106-1/1 contains bacteriophage 6H, which is known to contain putative virulence factors that may aid in pathogenicity (Castillo et al. 2014), whereas VQ50 does not (Castillo et al. 2014, Duchaud et al. 2018). In this context, comparative studies linking the differential expression of genes linked to *F. psychrophilum* virulence in multiple MLST CCs and how these relate to *in vivo* virulence will greatly clarify the molecular pathogenesis of this fish-pathogenic bacterium and guide the development of future BCWD control strategies.

Previous research has shown that some *F. psychrophilum* genotypes, including those investigated in this study, are widely distributed across multiple continents (Avendaño-Herrera et al. 2014, Nilsen et al. 2014, Van Vliet et al. 2016) and some have implicated the trade of salmonids and their eggs as a factor in global flavobacterial dissemination (Nicolas et al. 2008, Avendaño-Herrera et al. 2014, Duchaud et al. 2018). Indeed, millions of eyed rainbow trout eggs are distributed every year both intra- and trans-continentally (Adeli & Baghaei 2013), which increases the likelihood of disseminating *F. psychrophilum*, a non-reportable pathogen that is transmitted vertically and can survive routinely employed iodophore egg-surface disinfection practices (Cipriano et al. 1995, Brown et al. 1997, Kumagai et al. 1998, Kumagai & Nawata 2010, reviewed in Loch & Faisal 2017). Furthermore, *F. psychrophilum* is transmitted horizontally through direct (i.e., fish to fish) and indirect contact (i.e., through the water column; Madetoja et al. 2000). Both transmission routes raise the possibility for multiple *F. psychrophilum* genotypes to intermingle in the fish farming environment and may help to

partially explain the relatively high recombination rates (Chapter 2, Nicolas et al. 2008, Duchaud et al. 2018) within the species.

In addition to potentially facilitating flavobacterial dissemination, it is possible that current aquaculture practices may also select for highly pathogenic *F. psychrophilum* strains, including those that were the focus of this study. Indeed, previous studies on *Flavobacterium columnare*, the causative agent of columnaris disease (reviewed in Loch & Faisal 2017), have suggested that intensive fish farming in combination with biological factors of the pathogen (i.e., *F. columnare* sheds at a rapid rate from dead fish and can survive for extended periods outside of its host; Kunttu et al. 2009) have contributed to its increased virulence over both long and short time scales (Pulkkinen et al. 2010, Sundberg et al. 2016). In this context, the numerous susceptible hosts that are reared in artificially high densities in aquaculture facilities likely facilitates the transmission of virulent *F. psychrophilum* isolates, whereby fish infected with and killed by *F. psychrophilum* shed the bacterium at substantially higher numbers and for longer periods of time compared to live infected fish (Madetoja et al. 2000). Moreover, the abundance of nutrients that are typically present in hatchery rearing units from sources such as uneaten food, may create an energetically inexpensive environment for *F. psychrophilum*, allowing strains to retain their virulence making subsequent infections during an outbreak easier, which has been documented for *F. columnare* (Penttinen et al. 2016). As such, the rapid rate at which CC-ST10 and CC-ST191 strains killed juvenile rainbow trout in this study could be advantageous for *F. psychrophilum* in aquaculture facilities, where successive transmission may occur before a treatment strategy is implemented. From a practical perspective, this highlights the need to remove dead fish during BCWD epizootics, as well as keep organic material (i.e., uneaten food, feces) to a minimum.

In the current study, I selected isolates from two predominating MLST CCs (i.e., CC-ST10, CC-ST191) that primarily affect rainbow trout/steelhead, which comprise the majority of trout species raised in US aquaculture (USDA, 2009). Although CC-ST10 and CC-ST191 are highly virulent in rainbow trout/steelhead, it remains to be determined if they are differentially virulent in other salmonid species. In this context, *F. psychrophilum* genotypes belonging to CC-ST9, which have been predominantly recovered from coho salmon (Nicolas et al. 2008, Avendaño-Herrera et al. 2014, Nilsen et al. 2014, Van Vliet et al. 2016, Chapter 2), induced minimal mortality (i.e., 12% CPM) in experimentally infected rainbow trout (Madsen & Dalsgaard, 2000), possibly indicating a lack of adaptation(s) (i.e., virulence factors) necessary to cause disease in rainbow trout.

In conclusion, results herein have highlighted the pathogenic capacity of two of the largest and most widespread *F. psychrophilum* CCs (e.g., CC-ST10, CC-ST191) in their natural host, the rainbow trout. Their highly pathogenic nature, host association, and global distribution highlights their importance in the development of future targeted prevention and control strategies, including the development of autogenous and/or multivalent vaccine preparations. However, much remains to be explored, not only within these CCs, but in other *F. psychrophilum* CCs that have different host specificities. Until an effective control strategy is discovered, strict biosecurity and fish husbandry practices at aquaculture and fish farming facilities around the world are needed to prevent the spread of this devastating fish pathogen.

APPENDIX

Table 3.1. Origin information for the 10 *F. psychrophilum* isolates that were selected for use in *in vivo* virulence experiments. All isolates were originally recovered from rainbow trout/steelhead. [A]Isolates denoted with [D] were recovered from a fish suffering from BCWD, whereas [H] indicates an isolate was recovered from an apparently healthy fish. *Sequence types denoted with [2016] were recovered and genotyped by Van Vliet et al. (2016).

Isolate ID[A]	State of isolation	Location of isolation	Year of isolation	Clonal complex	Sequence type[*]
US352[D]	MI	SFH4	2010	CC-ST10	ST10
US75[H]	PA	SFH2	2016	CC-ST10	ST10
US53[D]	MI	SFH4	2011	CC-ST10	ST78[2016]
US26[D]	MI	SFH4	2010	CC-ST10	ST78[2016]
US57[D]	PA	SFH4	2014	CC-ST10	ST275
US87[D]	MI	SFH4	2016	CC-ST10	ST275
US215[H]	MI	WE1	2017	CC-ST191	ST267
US54[D]	MI	SFH4	2013	CC-ST191	ST267[2016]
US181[D]	PA	SFH1	2015	CC-ST191	ST301
US343[D]	WV	PF2	2014	CC-ST191	ST301

Table 3.2 Statistical comparisons of mean days to death among 10 *F. psychrophilum* isolates, five sequence types (STs), and two clonal complexes (CCs). [#]Isolates with the same superscript letter (i.e., a, b, c), STs with the same superscript number (i.e., 1, 2) and CCs with the same symbol (i.e., *) are not significantly different. All significance testing was assessed at an alpha of 0.05. CFU/fish^{-1}, colony forming units injected per fish; CPM, cumulative percent mortality; OD, optical density at 600nm; SD, standard deviation.

CC	ST	Isolate ID	Mean body weight (g)	Total no. of fish	Challenge dose (CFU/fish^{-1})	CPM ± SD	Mean days to death ± SD[#]
		Sham-inoculated	16.6	30		0.00	
CC-ST10	ST10	US352	14.6	30	4.20×10^8	100 ± 0	6.4 ± 3.9^{a}
	ST10	US75	14.6	30	5.00×10^7	100 ± 0	$3.7 \pm 2.5^{b,c}$
	ST78	US53	15.7	30	2.55×10^8	100 ± 0	2.0 ± 1.4^{c}
	ST78	US26	15.7	30	1.90×10^8	100 ± 0	6.9 ± 2.1^{a}
	ST275	US57	12.7	30	2.10×10^8	100 ± 0	$3.3 \pm 1.7^{b,c}$
	ST275	US87	14.5	30	1.45×10^8	100 ± 0	$3.3 \pm 2.2^{b,c}$
CC-ST191	ST267	US215	14.9	30	5.50×10^7	100 ± 0	6.8 ± 3.8^{a}
	ST267	US54	13.0	30	5.50×10^7	100 ± 0	$2.8 \pm 1.3^{b,c}$
	ST301	US181	14.1	30	1.05×10^8	96.7 ± 4.7	$2.4 \pm 2.0^{b,c}$
	ST301	US343	15.3	30	1.45×10^8	100 ± 0	4.0 ± 1.4^{b}
CC-ST10	ST10		14.6	60	2.35×10^8	100 ± 0	5.1 ± 3.2^{1}
	ST78		15.7	60	2.23×10^8	100 ± 0	4.6 ± 1.8^{1}
	ST275		13.6	60	1.78×10^8	100 ± 0	3.3 ± 2.0^{2}
CC-ST191	ST267		14.0	60	5.50×10^7	100 ± 0	4.8 ± 2.6^{1}
	ST301		14.7	60	1.25×10^8	98.9 ± 3.7	3.2 ± 1.7^{2}
CC-ST10			14.6	180	2.87×10^8	100 ± 0	4.3 ± 2.3*
CC-ST191			14.4	120	9.00×10^7	99.5 ± 3.1	4.0 ± 2.2*

Table 3.3 Percent of *F. psychrophilum* isolates, sequence types (STs), and clonal complex (CCs) presenting with gross external (i.e., skin erosion/hemorrhage/swelling/pallor, gill pallor/hemorrhage/erythema) and internal (i.e., hemorrhage, swelling, pallor) pathology.

	External		Internal					
ID	Skin or Fin	Gill	Heart	Kidney	Liver	Spleen	Ovary	Swim Bladder
US352	66.7%	96.7%	96.7%	96.7%	100%	16.7%	3.3%	3.3%
US75	80.0%	100%	100%	96.7%	100%	6.7%	0.0%	0.0%
US53	73.3%	100%	100%	100%	100%	6.7%	0.0%	0.0%
US26	83.3%	83.3%	100%	100%	100%	16.7%	3.3%	6.7%
US57	73.3%	100%	100%	100%	100%	6.7%	0.0%	0.0%
US87	56.7%	100%	93.3%	93.3%	93.3%	20%	0.0%	3.3%
US215	70.0%	93.3%	100%	93.3%	96.7%	3.3%	0.0%	0.0%
US54	53.3%	100%	100%	100%	96.7%	6.7%	0.0%	3.3%
US181	53.3%	83.3%	83.3%	83.3%	83.3%	13.3%	0.0%	0.0%
US343	90.0%	100%	100%	100%	100%	3.3%	0.0%	0.0%
ST10	73.4%	98.4%	98.4%	96.7%	100%	11.7%	1.7%	1.7%
ST78	78.6%	91.7%	100%	100%	100%	11.7%	1.7%	3.4%
ST275	65.0%	100%	96.7%	96.7%	96.7%	13.4%	0.0%	1.7%
ST267	61.7%	96.7%	100%	96.7%	96.7%	5.0%	0.0%	1.7%
ST301	71.7%	91.7%	91.7%	91.7%	91.7%	8.3%	0.0%	0.0%
CC-ST10	72.3%	96.7%	98.4%	97.8%	98.9%	12.3%	1.1%	2.3%
CC-ST191	66.7%	94.2%	95.9%	94.2%	94.2%	6.7%	0.0%	0.9%

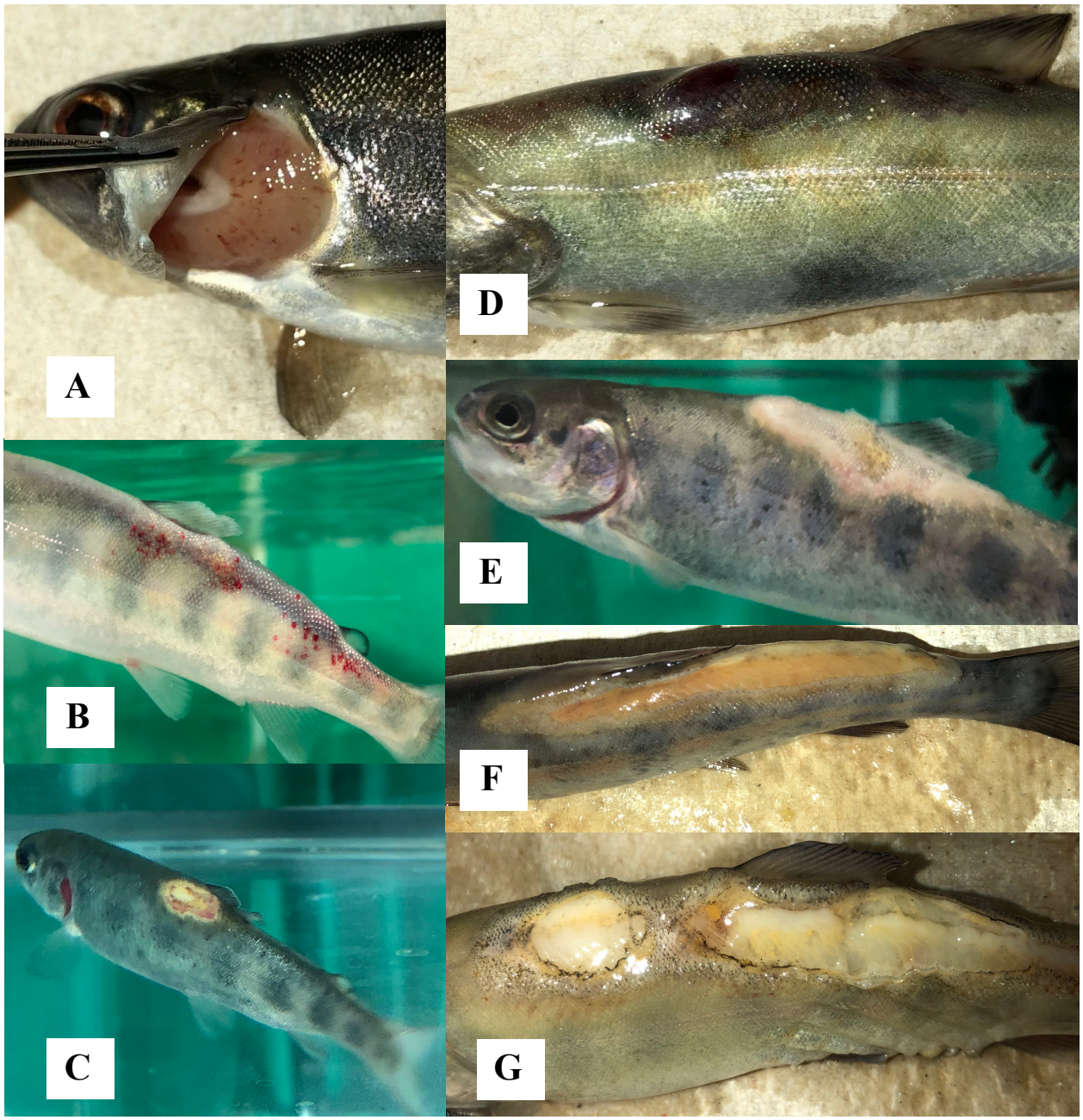


Figure 3.1 External signs of BCWD in rainbow trout (*Oncorhynchus mykiss*) fingerlings that were intramuscularly injected with *F. psychrophilum*. A. Severe gill pallor with concurrent diffuse petechial hemorrhage. B. Multifocal to diffuse petechial hemorrhage on the trunk and congestion at the base of the pelvic fin. C. Ulceration of the skin and underlying musculature on the dorsum. D. Severe swelling and congestion at the injection site. E-G. Progression of experimentally induced BCWD, beginning with superficial ulceration (E) that extended (F) and deepened into the underlying muscle (G) as the infection progressed. Note also the yellowish discoloration overlying the lesions in C and E-G.

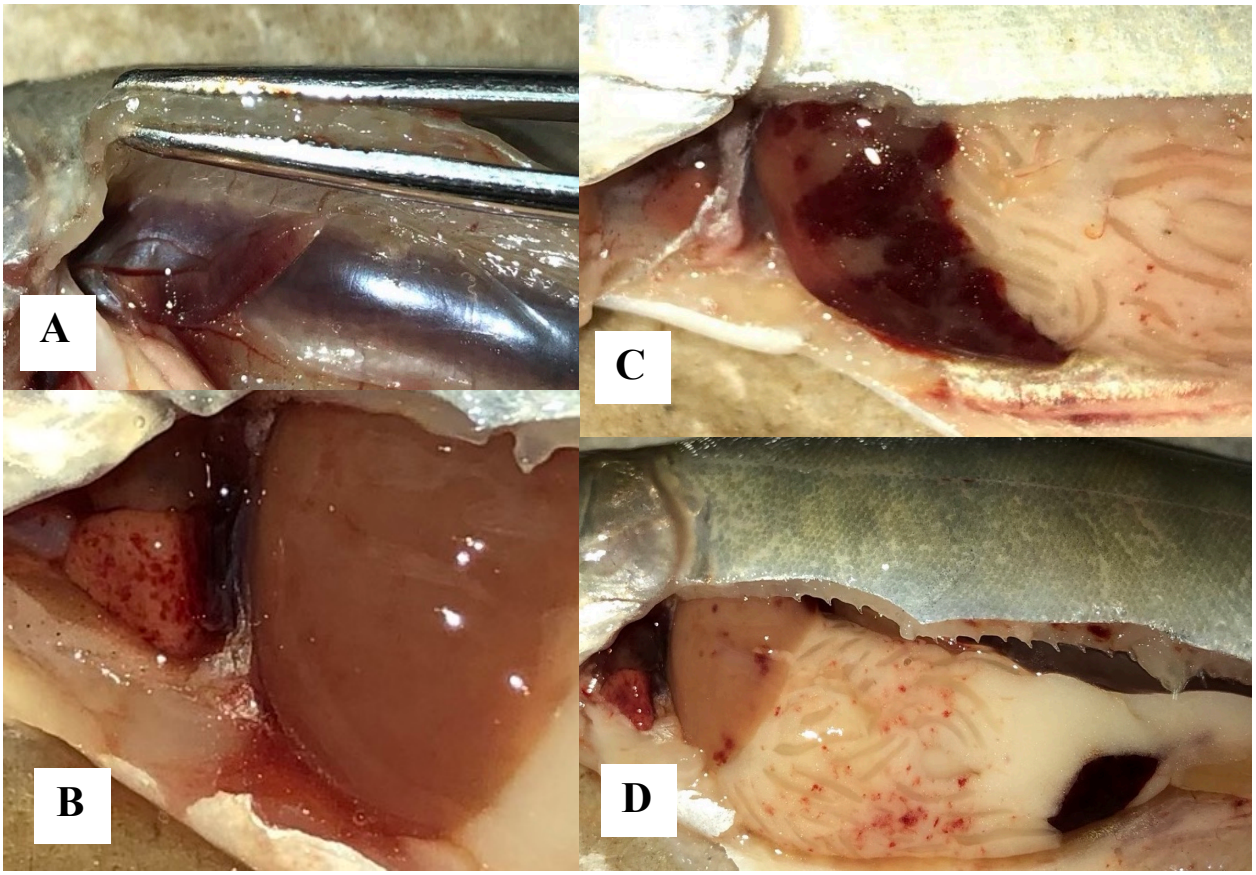


Figure 3.2 Internal signs of BCWD in rainbow trout (*Oncorhynchus mykiss*) fingerlings that were intramuscularly injected with *F. psychrophilum*. A. Hemorrhagic ovary. B. Diffuse petechial hemorrhage of the ventricle and bulbous arteriosus of the heart, as well as a moderately pale liver. C. Severely hemorrhagic liver accompanied by multifocal petechial hemorrhage of the adipose tissue overlying the pyloric cecae. D. Diffuse petechial hemorrhage of the ventricle of the heart accompanied by a severely pale liver with multifocal petechial hemorrhage and multifocal to diffuse petechial hemorrhage of the adipose tissue overlying the pyloric cecae.

REFERENCES

REFERENCES

Adeli, A., and F. Baghaei. 2013. Production and Supply of Rainbow Trout in Iran and the World. World Journal of Fish and Marine Sciences 5:335-341

Brown, L.L., Cox, W.T., and R.P. Levine. 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Diseases of Aquatic Organisms 29:213-218.

Avendaño-Herrera, R., Houel, A., Irgang, R., Bernardet, J.F., Godoy, M., Nicolas, P., and E. Duchaud. 2014. Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms. Veterinary Microbiology 170:298-306.

Castillo, D., Christiansen, R. H., Dalsgaard, I., Madsen, L., Espejo, R., and M. Middelboe. 2016. Comparative Genome Analysis Provides Insights into the Pathogenicity of *Flavobacterium psychrophilum*. PLoS ONE 11: e0152515.

Castillo, D., Espejo, R., and M. Middelboe. 2014. Genomic structure of bacteriophage 6H and its distribution as prophage in *Flavobacterium psychrophilum* strains. FEMS Microbiology Letters. 351:51-58.

Cipriano, R.C. 1995. Association of *Cytophaga psychrophila* with mortality among eyed eggs of Atlantic salmon (*Salmo salar*). Journal of Wildlife Diseases. 31:166-171.

Duchaud, E., Rochat, T., Habib, C., Barbier, P., Loux, V., Guérin, C., Dalsgaard, I., Madsen, L., Nilsen, H., Sundell, K., Wiklund, T., Strepparava, N., Wahli, T., Caburlotto, G., Manfrin, A., Wiens, G.D., Fujiwara-Nagata, E., Avendaño-Herrera, R., Bernardet, J-F., and P. Nicolas. 2018. Genomic Diversity and Evolution of the Fish Pathogen *Flavobacterium psychrophilum*. Frontiers in Microbiology 9:138.

Duchaud, E., Boussaha, M., Loux, V., Bernardet, J.F., Michel, C., Keroault, B., Mondot, S., Nicolas, P., Bossy, R., Caron, C., Bessières, P., Gibrat, JF., Claverol, S., Dumetz, F., Le Hénaff, M., and A. Benmansour. 2007. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. Nature Biotechnology. 25.763-769.

Fujiwara-Nagata, E., Chantry-Darmon, C., Bernardet, J.F., M. Eguchi, M., Duchaud E., and P. Nicolas. 2013. Population structure of the fish pathogen *Flavobacterium psychrophilum* at a whole country and model river levels in Japan. Veterinary Research 44:34.

Fujiwara-Nagata, E., Ikeda, J., Sugahara, K., and M. Eguchi. 2012. A novel genotyping technique for distinguishing between *Flavobacterium psychrophilum* isolates virulent and avirulent to ayu, *Plecoglossus altivelis* (Temminck & Schlegel). Journal of Fish Diseases 35:471–480.

Garcia, C., Pozet, F., and C. Michel. 2000. Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 42:191-197.

Holt, R.A. 1987. Ph.D. thesis. Oregon State University, Corvallis, Oregon. *Cytophaga psychrophila*, the causative agent of bacterial cold water disease in salmonid fish.

Kumagai, A., and A. Nawata. 2010. Mode of the Intra-ovum Infection of *Flavobacterium psychrophilum* in Salmonid Eggs. Fish Pathology 45:31-36.

Kumagai, A., Takahashi, S., Yamaoka, S., and H. Wakabayashi. 1998. Ineffectiveness of Iodophore Treatment in Disinfecting Salmonid Eggs Carrying *Cytophaga psychrophila*. Fish Pathology 33:123-128.

Kunttu, H.M.T., Valtonen, E.T., Jokinen, E.L., and LR Suomalainen. 2009. Saprophytism of a fish pathogen as a transmission strategy. Epidemics. 1:96-100.

LaFrentz, B.R., LaPatra, S.E., Jones, G.R., and K.D. Cain. 2003. Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. Journal of Fish Disease. 26:377-384.

Lewin, R.A., and D.M. Lounsbery. 1969. Cultivation and Characterization of *Flexibacteria*. Microbiology, 58:145–170.

Loch T.P., M Faisal. 2017. *Flavobacterium* spp., p. 211–232. In Woo, PTK, Cipriano, RC (eds.), Fish Viruses and Bacteria: Pathobiology and Protection CABI.

Long, A., Fehringer, T.R., LaFrentz, B.R., Call, D.R., and K.D. Cain. 2014. Development of a waterborne challenge model *for Flavobacterium psychrophilum*. FEMS Microbiology Letters 359:154–160.

Madetoja, J., Nyman, P., and T. Wiklund. 2000. *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 43:27-38.

Madsen, L., and I. Dalsgaard. 2000. Comparative studies of Danish *Flavobacterium psychrophilum* isolates: ribotypes, plasmid profiles, serotypes and virulence. Journal of Fish Diseases 23:211-218.

Madsen, L., and I. Dalsgaard. 1999. Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 36:169-176.

Madsen, L., and I. Dalsgaard. 1998. Characterization of *Flavobacterium psychrophilum*; comparison of proteolytic activity and virulence of strains isolated from rainbow trout (*Oncorhynchus mykiss*). In: Methodology in Fish Disease Research (ed. by A.C. Barnes, G.A. Davidson, M.P. Hiney & D. McIntosh), pp. 45–52. Fisheries Research Services, Aberdeen.

Michel, C., Antonio, D., and R.P. Hedrick.1999. Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. Research in Microbiology 150:351–358.

Nematollahi, A., Decostere, A., Pasmans, F., and F. Haesebrouck. 2003. *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases 26:563-574.

Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J.F., and E. Duchaud. 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709.

Nilsen, H., Sundell, K., Duchaud, E., Nicolas, P., Dalsgaard, I., Madsen, L., Aspán, A., Jansson, E., Colquhoun, D.J., and T. Wiklund. 2014. Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. Applied and Environmental Microbiology 80:2728-2736.

Penttinen, R., Kinnula, H., Lipponen, A., Bamford, J.K.H., and LR Sundberg. 2016. High Nutrient Concentration Can Induce Virulence Factor Expression and Cause Higher Virulence in an Environmentally Transmitted Pathogen. Microbial Ecology. 72:955-964.

Pulkkinen, K., Suomalainen, LR., Read, A.F., Ebert, D., Rintamäki, P., and E.T. Valtonen. 2010. Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proceedings of the Royal Society B. 277-599-600.

Sandkvist, M. 2001. Type II Secretion and Pathogenesis. Infection and Immunity 69:3523-3535.

Segerman, B. 2012. The genetic integrity of bacterial species: the core genome and the accessory genome, two different stories. Frontiers in Cell and Infection Microbiology. 2:1-8.

Siekoula-Nguedia, C., Blanc, G., Duchaud, E., and S. Calvez. 2012. Genetic diversity of *Flavobacterium psychrophilum* isolated from rainbow trout in France: predominance of a clonal complex. Veterinary Microbiology 161:169-178.

Starliper, C.E. 2011. Bacterial coldwater disease of fishes caused *by Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108.

Strepparava, N., Nicolas, P., Wahl, T., Segner, H., and O. Petrini. 2013. Molecular epidemiology of *Flavobacterium psychrophilum* from Swiss fish farms. Diseases of Aquatic Organisms 105:203-210.

Sundberg, LR., Ketola, T., Laanto E., Kinnula H., Bamford J.K.H., Penttinen R., and J. Mappes. 2016. Intensive aquaculture selects for increased virulence and interference competition in bacteria. Proceedings of the Royal Society B. 283:20153069.

Van Vliet, D., Wiens, G.D., Loch, T.P., Nicolas, P., and M. Faisal. 2016. Genetic Diversity of *Flavobacterium psychrophilum* Isolates from Three Oncorhynchus spp. in the United States, as Revealed by Multilocus Sequence Typing. Applied and Environmental Microbiology 82:3246-3255.

Wagner, E.J., and R.W. Oplinger. 2014. Comparison of the Susceptibility of Four Rainbow Trout Strains to Cold-Water Disease. Journal of Aquatic Animal Health, 26:160–167.

Wickham, E., Brown, N.F., Boyle, E.C., Coombes, B.K., and B.B. Finlay. 2007. Virulence is positively selected by transmission success between mammalian hosts. Current Biology 17:783-788.

Chapter 4

Conclusions and Future Research

1. Conclusions

Prior to these studies, very little was known about the genetic diversity of *Flavobacterium psychrophilum* in North America and how this diversity was related to fish host species, geographic distribution, and virulence. Furthermore, it was unclear how *F. psychrophilum* was acquiring this diversity and if this was a product of recombination or mutation. Likewise, the most prevalent *F. psychrophilum* MLST genotypes, all of which belonged to CC-ST10, had never had their pathogenicity confirmed or compared either within or between clonal complexes. The answers to these question may improve the control and spread of this serious pathogen of fish.

In Chapter 2, I examined the most comprehensive set of *F. psychrophilum* isolates in North America to date using MLST. This study revealed North American *F. psychrophilum* isolates to be more genetically diverse than anywhere else in the world ($H = 0.68 \pm 0.04$). This information supports a recent notion that *F. psychrophilum* originated in North America and subsequently spread to other regions of the world through the international trade of fish and eggs. In addition, I identified several genotypes within North America that have been reported in other locations around the world and interestingly, the majority of those isolates came from identical fish species. Although it currently remains to be proven, it would appear that the transportation of eggs and fish within North America has led to the spread of many genotypes as well. In this context, we recovered CC-ST29 from Chinook salmon in Oregon, USA, a state that distributed Chinook to Michigan, USA (a state where Chinook salmon are non-native) and it would appear *F. psychrophilum* isolates belonging to this CC were transported along with these fish as CC-ST29 has been detected among Chinook salmon in Michigan as well. In the same context, we discovered strong evidence that at least one genotype, ST275, was transported from an egg

collection facility to a state fish hatchery that later had a severe outbreak of BCWD in the resultant progeny due to ST275. This scenario, although unfortunate, is enlightening because even though strict biosecurity measures are implemented in order to limit the spread different pathogens, *F. psychrophilum* appears to be circumventing our current prevention strategies. Indeed, it is unknown whether this genotype was transported in or on the surface of the egg, and/or on equipment, nevertheless, this is cause for alarm. With this unique collection of *F. psychrophilum* isolates, we were able to utilize strains recovered as early as 1981. Interestingly, it appears that some genotypes (e.g., ST10, ST27, ST29, ST78) have existed in North America for decades and thus, are likely established within the salmonid population, which may be due to evolutionary adaptations to their hosts; however, this remains to be determined. Furthermore, multiple sequence analyses indicated recombination is responsible for the diversification of *F. psychrophilum* in North America, even though clonal populations (i.e., clonal complexes) persist. Although it remains to be determined, clonal populations of *F. psychrophilum* established in a particular state or facility may contain genes related to virulence or antimicrobial resistance. In a situation where biosecurity fails, this may lead to a virulent complex coming into contact with a less or non-virulent complex with recombination permitting the less or non-virulent population to become virulent.

In Chapter 3, I examined the pathogenicity of the most widespread genotypes in North America, which belonged to CC-ST10, a CC known for its apparent virulence, and compared them to one another, as well as to two other genotypes, which belong to the second largest CC (i.e., CC-ST191) but does not receive notoriety for its apparent virulence. Not surprisingly, the genotypes of CC-ST10 were very pathogenic towards rainbow trout; however, not all genotypes or strains within the same genotype were equal in their pathogenicity. Interestingly, ST10 did not appear

to be the most virulent genotype, ST10 actually took the longest to cause death in its host. However, this may be an adaptive mechanism that allows for a longer-lasting infection and thereby allows ST10 more time to colonize its next host. Furthermore, CC-ST191 was nearly identical to CC-ST10 in terms of pathogenicity towards rainbow trout and raises the question of how pathogenic other CCs may be toward their respective hosts. Overall, findings of this study pave the way for multiple research directions including the development of effective vaccines that encompass most genotypes, host-pathogens interactions, and evolution of genetic diversity among *F. psychrophilum* strains.

2. Future Research

My studies have provided a framework upon which future studies can be developed. I was able to genotype over 300 isolates of *F. psychrophilum*, none of which have ever had their genetic diversity explored previously. Despite the uniqueness of these isolates, in that they were recovered from multiple fish species and geographic areas; some facilities, fish species, and states were represented more than others, a matter that did not allow for the application of rigorous statistical analyses. As such, there is a need for more isolates that have been recovered from salmonids and from all locations in which they exist. The isolates used in this study included all but one isolate from the USA and therefore, there is a need to obtain more isolates (fresh and archived) from Canada and Mexico so that we can better understand the genetic diversity and pathogen transmission dynamics of this serious pathogen over a larger spatial and temporal landscape in North America. Integrated into that is the need to better understand the degree of host specificity and temporal persistence of *F. psychrophilum* genotypes. This is of paramount importance because previous attempts to use a single strain for vaccine production

have been unsuccessful. Therefore, there is a need for a polyvalent vaccine against *F. psychrophilum*, one for that is effective for each salmonid species, in particular those that are intensively reared. Future studies should also examine how *F. psychrophilum* MLST genotypes relate to flavobacterial mechanisms of virulence (i.e., pathogen-host interactions), as well as mechanisms for environmental persistence, especially given my findings that some strains seem to persist in aquaculture facilities over extended periods of time/rearing cycles. The results of Chapter 3 suggest that those five genotypes are highly pathogenic to rainbow trout. Future experiments should explore their pathogenicity in other salmonids species in order to evaluate whether they are truly adapted to a rainbow trout or are equally pathogenic to multiple salmonid species. Furthermore, it remains unclear how well these genotypes can reproduce BCWD through a more natural route of exposure (i.e., immersion). However, immersion studies remain to be standardized, a matter that should must be addressed first and foremost.