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Genetic Diversity of *Flavobacterium psychrophilum* Isolates from Three *Oncorhynchus* spp. in the United States, as Revealed by Multilocus Sequence Typing

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ABSTRACT

The use of a multilocus sequence typing (MLST) technique has identified the intraspecific genetic diversity of U.S. *Flavobacterium psychrophilum*, an important pathogen of salmonids worldwide. Prior to this analysis, little U.S. *F. psychrophilum* genetic information was known; this is of importance when considering targeted control strategies, including vaccine development. Herein, MLST was used to investigate the genetic diversity of 96 *F. psychrophilum* isolates recovered from rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), and Chinook salmon (*Oncorhynchus tshawytscha*) that originated from nine U.S. states. The isolates fell into 34 distinct sequence types (STs) that clustered in 5 clonal complexes (CCs) ($n = 63$) or were singletons ($n = 33$). The distribution of STs varied spatially, by host species, and in association with mortality events. Several STs (i.e., ST9, ST10, ST30, and ST78) were found in multiple states, whereas the remaining STs were localized to single states. With the exception of ST256, which was recovered from rainbow trout and Chinook salmon, all STs were found to infect a single host species. Isolates that were collected during bacterial cold water disease outbreaks most frequently belonged to CC-ST10 (e.g., ST10 and ST78). Collectively, the results of this study clearly demonstrate the genetic diversity of *F. psychrophilum* within the United States and identify STs of clinical significance. Although the majority of STs described herein were novel, some (e.g., ST9, ST10, ST13, ST30, and ST31) were previously recovered on other continents, which demonstrates the transcontinental distribution of *F. psychrophilum* genotypes.

IMPORTANCE

***Flavobacterium psychrophilum* is the causative agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) and is an important bacterial pathogen of wild and farmed salmonids worldwide. These infections are responsible for large economic losses globally, yet the genetic diversity of this pathogen remains to be fully investigated. Previous studies have identified the genetic diversity of this pathogen in other main aquaculture regions; however, little effort has been focused on the United States. In this context, this study aims to examine the genetic diversity of *F. psychrophilum* from the United States, as this region remains important in salmonid aquaculture.**

The causative agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS), *Flavobacterium psychrophilum*, is an important bacterial pathogen of wild and farmed salmonids worldwide (1). In addition to horizontal transmission, *F. psychrophilum* is suspected of being vertically transmitted (2–5) and appears to resist standard povidone-iodine treatment during egg disinfection (4, 6, 7), which make efforts to control this bacterium particularly problematic. Since the initial isolation of *F. psychrophilum* in North America (8), *F. psychrophilum* infections have been reported in Europe, South America, Asia, and Australia (9–11) and from all of the major areas of intensive salmonid aquaculture that have been studied (12).

Despite the fact that the trade of live fish and their eggs has been hypothesized as a major factor that drove the transcontinental spread of *F. psychrophilum* (13, 14), the epidemiological details to support this have not been fully elucidated. A number of molecular biology-based assays have been employed to study the genetic diversity of *F. psychrophilum* in an attempt to define its host specificity, geographical associations, and virulence (15–18). However, despite these concerted efforts, the lack of standardized, reproducible, and comparable assays (18–20) left the intraspecific heterogeneity of *F. psychrophilum* as it relates to distribution and transmission routes incompletely understood.

Multilocus sequence typing (MLST) is a robust, reproducible, and established technique to identify and characterize the strain diversity of human and animal bacterial pathogens (21, 22), including those affecting fish (23–27). MLST is based on the sequencing of, typically, 7 housekeeping gene loci, whereby an isolate is characterized by the allele types (ATs) found at the loci.

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FIG 1 The number of *F. psychrophilum* isolates from each location where samples were collected in the United States. Isolates were recovered from three *Oncorhynchus* spp.: *O. mykiss*, $n = 54$; *O. tshawytscha*, $n = 26$; and *O. kisutch*, $n = 16$.

Each specific combination of ATs is referred to as a sequence type (ST), which can be further grouped into clonal complexes (CCs) based upon their suspected evolutionary relatedness. An MLST scheme for *F. psychrophilum* was developed (19) and optimized (28, 29), and its use has since linked some *F. psychrophilum* STs/CCs recovered from infected salmonids in Europe (28, 30–32), Chile (30, 33), and Japan (29) with enhanced virulence (28, 31–33) and host species predilections (19, 29, 32, 33).

However, the genetic diversity of *F. psychrophilum* in the United States has not yet been adequately addressed; this is a matter of importance considering the potential of this knowledge to contribute to targeted control strategies, including vaccine development. Particular attention should be focused on deciphering the host specificity and virulence levels of certain *F. psychrophilum* genotypes found in the United States, where *F. psychrophilum*-susceptible salmonids have been artificially propagated since the end of the 19th century (34). Similarly, captive and feral salmonid populations that were intentionally introduced into the Great Lakes basin (GLB) over the last century (35) continue to suffer from *F. psychrophilum* infections (36), yet little is known about the pathogen population structure there. Here we investigated the population structure of *F. psychrophilum* within the United States by utilizing a comprehensive MLST approach, with the goal of characterizing the distribution of clonal complexes and their association with local BCWD outbreaks in feral and farmed *Oncorhynchus* species stocks.

MATERIALS AND METHODS

Fish collection and isolation of *F. psychrophilum*. This study analyzed 96 *F. psychrophilum* isolates originating from 9 states within the United States (Fig. 1). Among these, 50 isolates originated from Michigan and accounted for two watersheds (i.e., Lake Michigan and Lake Huron) of the GLB. The remainder originated from Idaho ($n = 18$), Washington ($n = 8$), Utah ($n = 6$), North Carolina ($n = 5$), West Virginia ($n = 3$), Colorado ($n = 2$), New Mexico ($n = 2$), and Oregon ($n = 2$). Isolates were recovered from rainbow trout (*Oncorhynchus mykiss*; $n = 54$), Chinook salmon (*Oncorhynchus tshawytscha*; $n = 26$), and coho salmon (*Oncorhynchus kisutch*; $n = 16$) sampled between 1981 and 2013 (between 2009 and 2013 for the GLB isolates) (Table 1). Samples originated from various types of host tissues (i.e., kidney, spleen, brain, ovarian fluid, and external lesions) and life stages (i.e., eggs, fry, juveniles, and sexually mature adults) (Table 1). The majority of the *F. psychrophilum* isolates were recovered from fish exhibiting gross disease signs commonly associated with BCWD (e.g., muscle ulceration, fin erosion, exophthalmia, and swollen internal organs), whereas others were occasionally recovered from apparently healthy fish.

Bacterial isolation was performed by a number of investigators. For this process, tissues were inoculated onto flavobacterium-selective media, such as cytophaga agar (CA) (37) supplemented with neomycin sulfate at 4 mg · liter^{-1} and tryptone-yeast extract-salts (TYES) medium (38). In several rainbow trout outbreaks, multiple isolates were saved from single fish to examine the presence of coinfection. Otherwise, 1 CFU from each fish was subcultured for further analyses. The isolates were then cryopreserved in CA or TYES broth supplemented with glycerol (20% [vol/vol]) and then immediately frozen at −80°C for future analyses. Strain CSF259-

TABLE 1 MLST allele and STs and association with CCs of 96 U.S. *F. psychrophilum* isolates[a]

Isolate identification no.[b]	Isolate designation	Yr of isolation	U.S. state	Salmonid species	Isolation tissue[c]	Feral/captive	Life stage	Allelic profile[d]							ST[d]	CC
								trpB	*gyrB*	*dnaK*	*fumC*	*murG*	*tuf*	*atpA*		
526	CSF201-91	1991	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
527	CSF408-92	1992	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
50	CSF259-93	1993	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
529	CSF060-99	1999	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
530	CSF016-00	2000	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
531	CSF054-01	2001	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
533	CSF088-03	2003	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
534	CSF352-04	2004	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
535	CSF226-05	2005	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
536	CSF009-06	2006	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
537_A	ARS-01S-08	2008	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
539_B	ARS-01L-08	2008	ID	*O. mykiss*	Lesion	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
542	ARS-03S-08	2008	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
545V_B	ARS-05K1-09	2009	NC	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
548_B	ARS-05S2-09	2009	NC	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
519	F12 K1 17	2002	NM	*O. mykiss*	Kidney	Captive	Fry[e]	2	8	2	2	2	2	2	ST10	CC-ST10
520	F12 6 17	2002	NM	*O. mykiss*	Kidney	Captive	Fry[e]	2	8	2	2	2	2	2	ST10	CC-ST10
523	03-009	2003	UT	*O. mykiss*	Unknown	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
556	08-114	2008	UT	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
553	09-041	2009	UT	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
554	09-080	2009	UT	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
557	09-083	2009	UT	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
552	ARS-002-07	2007	WV	*O. mykiss*	Kidney/brain	Captive	Juvenile[e]	2	8	2	2	2	2	2	ST10	CC-ST10
515_C	463-96 Fsp RBT5	1996	CO	*O. mykiss*	Kidney/lesion	Captive	Unknown[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
521_C	464-96 Fsp RBT1B	1996	CO	*O. mykiss*	Kidney/lesion	Captive	Unknown[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US17	WLSFH	2010	MI	*O. mykiss*	Brain	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US26	WLSFH	2010	MI	*O. mykiss*	Brain	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US32	WLSFH	2010	MI	*O. mykiss*	Brain	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US45	WLSFH	2010	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US38	WLSFH	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US40	TSFH	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US42_D	WLSFH	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US53_D	WLSFH	2011	MI	*O. mykiss*	Ext. lesion	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US44	TSFH	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US49	TSFH	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
US51	TSFH	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	41	2	**ST78**	CC-ST10
549	ARS-001-06	2006	WV	*O. mykiss*	Fin	Captive	Adult	2	8	2	2	2	41	2	**ST78**	CC-ST10
550	ARS-002-06	2006	WV	*O. mykiss*	Fin	Captive	Adult	2	8	2	2	2	41	2	**ST78**	CC-ST10
538_A	ARS-01K-08	2008	ID	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	8	2	2	2	**44**	2	**ST82**	CC-ST10
540_E	ARS-02S-08	2008	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	**44**	2	**ST82**	CC-ST10
541_E	ARS-02L-08	2008	ID	*O. mykiss*	Lesion	Captive	Juvenile[e]	2	8	2	2	2	**44**	2	**ST82**	CC-ST10
543	ARS-07S-08	2008	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	**44**	2	**ST82**	CC-ST10
546_B	ARS-05K2-09	2009	NC	*O. mykiss*	Kidney	Captive	Juvenile[e]	2	**41**	2	2	2	41	2	**ST84**	CC-ST10
547_B	ARS-05S1-09	2009	NC	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	**41**	2	2	2	41	2	**ST84**	CC-ST10
532	CSF067-02	2002	ID	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	2	2	**43**	2	**ST81**	CC-ST10
555	09-032	2009	UT	*O. mykiss*	Spleen	Captive	Juvenile[e]	2	8	2	3	2	2	2	**ST86**	CC-ST10
US18	LMRW	2013	MI	*O. mykiss*	Kidney	Feral	Adult	1	28	4	2	25	25	**59**	**ST256**	CC-ST256
US39	LMRW	2013	MI	*O. mykiss*	Kidney	Feral	Adult	1	28	4	2	25	25	**59**	**ST256**	CC-ST256
US46	LMRW	2011	MI	*O. tshawytscha*	Kidney	Feral	Adult	1	28	4	2	25	25	**59**	**ST256**	CC-ST256

US47	SRW	2011	MI	*O. tshawytscha*	Egg	Feral	Egg	1	28	4	2	25	25	**59**	**ST256**	CC-ST256
US14	LMRW	2013	MI	*O. tshawytscha*	Kidney	Feral	Adult	1	28	4	2	25	25	**59**	**ST256**	CC-ST256
US30	LMRW	2013	MI	*O. tshawytscha*	Kidney	Feral	Adult	1	28	4	2	25	25	**59**	**ST256**	CC-ST256
US08	PRW	2011	MI	*O. kisutch*	Kidney	Feral	Adult	1	28	4	2	16	25	**59**	**ST252**	CC-ST256
503	SH3-81	1981	OR	*O. kisutch*	Kidney	Captive	Juvenile[e]	4	7	6	5	6	5	4	ST9	CC-ST9
502	Quilcene C7	2000	WA	*O. kisutch*	OF	Feral	Adult	4	7	6	5	6	5	4	ST9	CC-ST9
504	Quilcene C5	2000	WA	*O. kisutch*	OF	Feral	Adult	4	7	6	5	6	5	4	ST9	CC-ST9
US07	PRW	2010	MI	*O. kisutch*	Kidney	Feral	Adult	4	7	6	5	6	8	4	ST13	CC-ST9
US19	PRW	2010	MI	*O. kisutch*	Kidney	Feral	Adult	4	7	6	5	6	8	4	ST13	CC-ST9
US28	LMRW	2010	MI	*O. mykiss*	Kidney	Feral	Adult	3	19	13	9	12	16	15	ST31	CC-ST31
US29	LMRW	2010	MI	*O. mykiss*	Kidney	Feral	Adult	3	19	13	9	12	16	15	ST31	CC-ST31
US33	LMRW	2010	MI	*O. tshawytscha*	Kidney	Feral	Adult	6	9	7	3	**49**	5	7	**ST262**	CC-ST262
US50	LMRW	2010	MI	*O. tshawytscha*	Kidney	Feral	Adult	6	9	7	3	**49**	5	7	**ST262**	CC-ST262
US54	WLSFH	2013	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	4	**73**	22	3	3	3	3	**ST267**	CC-ST191
US04	SRW	2011	MI	*O. tshawytscha*	Egg	Feral	Egg	8	17	14	9	13	17	16	ST29	
US25	SRW	2011	MI	*O. tshawytscha*	Kidney	Feral	Adult	8	17	14	9	13	17	16	ST29	
US41	LMRW	2011	MI	*O. tshawytscha*	Kidney	Feral	Adult	8	17	14	9	13	17	16	ST29	
US55	SRW	2011	MI	*O. tshawytscha*	Kidney	Feral	Adult	8	17	14	9	13	17	16	ST29	
US48	PRW	2012	MI	*O. kisutch*	Kidney	Feral	Adult	**42**	18	26	5	**47**	8	14	**ST258**	
US52	PRW	2012	MI	*O. kisutch*	Kidney	Feral	Adult	**42**	18	26	5	**47**	8	14	**ST258**	
US20	PRW	2013	MI	*O. kisutch*	Kidney	Feral	Adult	**42**	18	26	5	**47**	8	14	**ST258**	
US27	PRW	2013	MI	*O. kisutch*	Kidney	Feral	Adult	**42**	18	26	5	**47**	8	14	**ST258**	
505	W98-317-16K	1998	OR	*O. kisutch*	Kidney	Captive	Unknown	11	18	7	5	14	18	17	ST30	
506	EC98-305-5402K	1998	WA	*O. kisutch*	Kidney	Captive	Unknown	11	18	7	5	14	18	17	ST30	
511	AFTC P-3	2000	WA	*O. tshawytscha*	OF	Feral	Adult	8	19	7	1	**29**	**40**	**39**	**ST76**	
512	AFTC C2	2000	WA	*O. tshawytscha*	OF	Feral	Adult	8	19	7	1	**29**	**40**	**39**	**ST76**	
US13	LMRW	2012	MI	*O. tshawytscha*	Kidney	Feral	Adult	**41**	**68**	10	9	12	18	14	**ST255**	
US31	LMRW	2012	MI	*O. tshawytscha*	Kidney	Feral	Adult	**41**	**68**	10	9	12	18	14	**ST255**	
US21	SRW	2009	MI	*O. tshawytscha*	Kidney	Feral	Adult	27	**69**	15	5	13	36	26	**ST259**	
US23	SRW	2013	MI	*O. tshawytscha*	Kidney	Feral	Adult	27	**69**	15	5	13	36	26	**ST259**	
US22	SRW	2010	MI	*O. tshawytscha*	Kidney	Feral	Adult	11	23	28	1	**48**	1	8	**ST260**	
US56	SRW	2010	MI	*O. tshawytscha*	Kidney	Feral	Adult	11	23	28	1	**48**	1	8	**ST260**	
US36	LMRW	2008	MI	*O. tshawytscha*	Kidney	Feral	Adult	18	**72**	9	3	13	5	11	**ST266**	
US43	LMRW	2008	MI	*O. tshawytscha*	Kidney	Feral	Adult	18	**72**	9	3	13	5	11	**ST266**	
508	03-398-1	2003	WA	*O. kisutch*	Kidney	Captive	Adult	8	**38**	8	2	25	**38**	**37**	**ST74**	
510	03-449-5	2003	WA	*O. kisutch*	Kidney	Captive	Adult	27	22	15	7	12	39	**38**	**ST75**	
513	03-169	2003	WA	*O. kisutch*	Kidney	Captive	Juvenile[e]	28	**39**	4	2	30	12	4	**ST77**	
544	ARS-03B-09	2009	NC	*O. mykiss*	Brain	Captive	Juvenile[e]	1	**40**	16	2	**31**	**45**	2	**ST83**	
US05	LMRW	2011	MI	*O. tshawytscha*	Egg	Feral	Egg	**40**	**66**	4	5	16	47	**57**	**ST250**	
US06	LMRW	2011	MI	*O. tshawytscha*	Egg	Feral	Egg	4	**67**	15	10	13	12	**58**	**ST251**	
US09	OSFH	2013	MI	*O. mykiss*	Ext. lesion	Captive	Adult	1	13	8	1	1	1	1	**ST253**	
US12	SRW	2013	MI	*O. tshawytscha*	Kidney	Feral	Adult	4	47	15	3	13	3	11	**ST254**	
US16	WLSFH	2013	MI	*O. mykiss*	Kidney	Captive	Juvenile[e]	1	2	2	3	**46**	12	2	**ST257**	
US24	SRW	2009	MI	*O. tshawytscha*	Kidney	Feral	Adult	21	14	**32**	3	34	13	**60**	**ST261**	
US34	SRW	2012	MI	*O. tshawytscha*	Kidney	Feral	Adult	11	**70**	**33**	10	**50**	13	**61**	**ST263**	
US35	PRW	2011	MI	*O. kisutch*	Kidney	Feral	Adult	6	**71**	7	3	**49**	**60**	7	**ST264**	
US37	SRW	2012	MI	*O. tshawytscha*	Kidney	Feral	Adult	18	47	8	3	13	3	**62**	**ST265**	

[a] Isolates designated WLSFH, TSFH, LMRW, PRW, and OSFH were recovered from the Lake Michigan watershed. Isolates designated SRW were recovered from the Lake Huron watershed. ST, sequence type; CC, clonal complex.
[b] Isolates originating from the same fish are denoted by the same letter (A, B, C, D, or E).
[c] Ext. lesion, external lesion; OF, ovarian fluid.
[d] Novel allele types and STs are shown in bold.
[e] Isolates recovered during high levels of morbidity and mortality.

TABLE 2 STs identified in this study that have been found in other locations in the world

	Current analysis		Prior analyses	
ST[a]	Host	Location(s)	Host	Location(s) [reference(s) and/or source]
9	*O. kisutch*	OR, WA	*O. kisutch*	OR (19), British Columbia (19), Chile (19, 33), Japan (29)
10	*O. mykiss*	ID, NC, NM, UT, WV	*O. mykiss*, *Salvelinus* sp., tank water	ID (19), OR (19), Chile (33), Denmark (Inger Dalsgaard, http://pubmlst.org/fpsychrophilum/ [32]), Finland (Tom Wiklund, http://pubmlst.org/fpsychrophilum/), Scotland (19), Spain (19), Switzerland (31), Sweden (32), Japan (29)
13	*O. kisutch*	MI	*O. kisutch*, *Salmo trutta*	WA (19), Finland (Tom Wiklund, http://pubmlst.org/fpsychrophilum/), Japan (19)
29	*O. tshawytscha*	MI	*O. tshawytscha*	OR (19)
30	*O. kisutch*	OR, WA	*O. kisutch*	Japan (19, 29)
31	*O. mykiss*	MI	*O. mykiss*	Denmark (Inger Dalsgaard, http://pubmlst.org/fpsychrophilum/), Switzerland (19, 33)

[a] ST, sequence type.

93, whose genotype was already available (19), was also included in this analysis.

DNA was extracted from each suspected *F. psychrophilum* isolate using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's protocol for Gram-negative bacteria. For a portion of the isolates, the *F. psychrophilum*-specific primers of Toyama et al. (39) were used to PCR amplify a partial stretch of the 16S rRNA gene as previously described (36). The remaining isolates were confirmed to be *F. psychrophilum* using PCR amplification with degenerate universal primers (40) and Sanger sequencing as previously reported (41).

MLST. PCR amplification of partial sequences of 7 housekeeping genes (*atpA*, *dnaK*, *fumC*, *gyrB*, *murG*, *trpB*, and *tuf*) as originally described by Nicolas et al. (19) and modified by Siekoula-Nguedia et al. (28) was performed using primer sequences previously described by Fujiwara-Nagata et al. (29) in a 50-μl reaction volume. Each reaction volume included 25 μl of 2× GoTaq green master mix (Promega), 20 ng of DNA template, and 0.25 μM each primer, with nuclease-free water comprising the remainder. Sterile nuclease-free water served as a negative control in all assays, and the type strain ATCC 49418 of *F. psychrophilum* was used as a positive control. All genes were amplified using the same touchdown protocol: 94°C for 5 min; 24 cycles at 94°C for 0.5 min, 55°C for 0.5 min (−0.4°C/cycle), and 72°C for 1 min (+2 s/cycle); 12 cycles at 94°C for 0.5 min, 45°C for 0.5 min, and 72°C for 2 min (+3 s/cycle); and a final extension step at 72°C for 10 min, as detailed by Fujiwara-Nagata et al. (29). The reactions were run on a 1.5% agarose gel at 100 V for 40 min, where the presence of an amplicon of the appropriate size under UV exposure confirmed amplification (29). The amplified PCR product was purified using the QIAquick purification kit (Qiagen), according to the manufacturer's protocol. Bidirectional sequencing was performed using the corresponding forward and reverse primers (29) or the *F. psychrophilum*-specific MLST sequencing primers (M13a, 5′-CAGGAAACAGCTATGACC-3′; M13b, 5′-TGTAAAACGACGGCCAGT-3′) (29).

MLST data analysis. All chromatograms were manually verified before ATs and STs were assigned using an in-house script (P. Nicolas, INRA). AT profiles were analyzed to delineate CCs and other relationships using eBURST v3 (eburst.mlst.net) (42, 43), on the basis of single-locus variants (SLVs). STs not belonging to any CC were referred to as singletons. The entire publically available *F. psychrophilum* MLST database at the time of examination (n = 995) (http://pubmlst.org/fpsychrophilum/) was used in the eBURST analysis. The average pairwise diversities at the gene (i.e., locus) and nucleotide levels were computed as the mean number of differences between pairs of STs. The genotype data collected in this study are available through the *F. psychrophilum* MLST database (http://pubmlst.org/fpsychrophilum/) (44). The statistical associations between the STs and the variables of interest (host species, geographical origin) were investigated using Fisher's exact test. Fisher's exact test was also used to investigate the associations for a specific ST by analyzing condensed contingency tables. All statistical analyses were conducted using SAS statistical software (version 9.1; SAS Institute, Inc.).

Nucleotide sequence accession numbers. All sequences have been deposited in GenBank (accession numbers KT809511 to KT810182).

RESULTS

Genetic diversity of *F. psychrophilum* in the United States. MLST analysis of the 96 U.S. *F. psychrophilum* isolates revealed 34 distinct STs (Table 1). Among these, 28 STs (n = 60 isolates) were novel, whereas the remaining 6 (n = 36 isolates) have previously been reported from other locations worldwide (Table 2). The most abundant ST was ST10, which accounted for 23/96 (23.9%) of our isolates. The second most abundant ST (n = 15/96, 15.6%) was the novel ST78. The remaining 34 STs were each identified fewer than 6 times in our collection of isolates (Table 1).

The mean gene diversity of the U.S. *F. psychrophilum* isolates was 0.75 ± 0.03, and the mean nucleotide diversity was 4.4 kbp^{-1}. The diversity indices between the 34 STs were also computed; the mean gene diversity was 0.925 ± 0.02, and the mean nucleotide diversity was 5.4 kbp^{-1}. The gene diversity indices varied between isolates retrieved from different fish host species. The highest gene diversity was from Chinook salmon isolates (0.89 ± 0.02), followed by that from coho salmon isolates (0.79 ± 0.05). The lowest gene diversity of 0.32 ± 0.07 was computed from isolates collected from rainbow trout.

Identification of clonal complexes. The eBURST analysis identified a few clonal complexes with the SLV link criterion (Fig. 2). The largest CC in this analysis was CC-ST10, which contains 6 STs (Fig. 2), including the two most abundant ones (ST10 and ST78). Besides CC-ST10, which accounted for nearly half of the isolates in the U.S. data set (n = 46), two other smaller complexes, CC-ST256 (n = 7 isolates, n = 2 STs) and CC-ST9 (n = 5 isolates, n = 2 STs), were detected (Fig. 2).

An eBURST analysis was also conducted to depict the connection between the 96 isolates and the 995 isolates currently in the *F. psychrophilum* MLST database as of June 2015 (Fig. 3). This revealed additional SLV links between the STs of this study and those identified in other studies. Namely, ST31, ST262, and ST267 were identified as part of small clonal complexes (designated CC-ST31, CC-ST262, and CC-ST191, respectively). In particular, ST267 was discovered in Michigan clusters with CC-ST191 and is the first ST from North America in this CC. Nevertheless, most of the STs identified in this study (21/34) were singletons (Fig. 2).

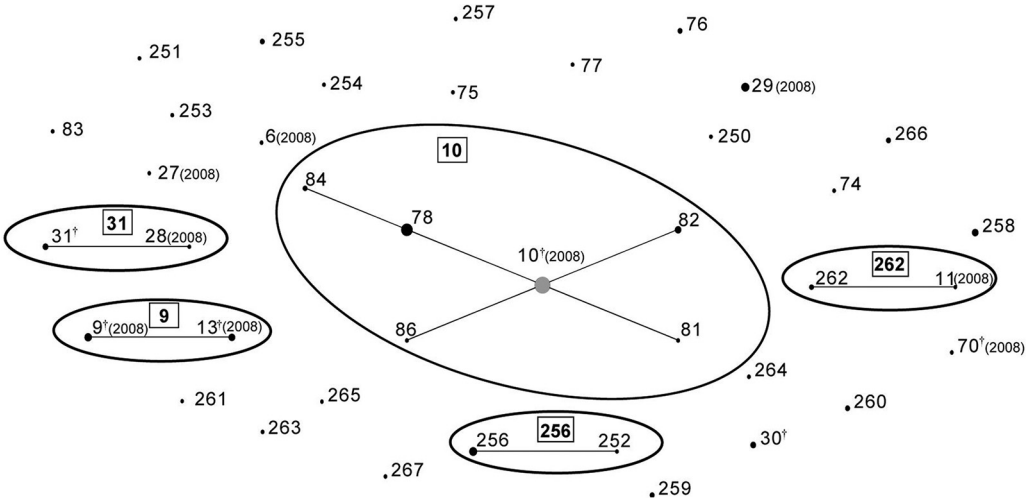

FIG 2 eBURST diagram depicting the relationships of the 96 U.S. *F. psychrophilum* isolates of this study and the 10 previously typed U.S. isolates (19). Sequence types (STs) followed by "(2008)" denote isolates previously typed by Nicolas et al. (19). STs 9, 10, 13, and 29 were detected in the U.S. in both the current U.S. study and that of Nicolas et al. (19). A dagger (†) identifies STs found in both the U.S. and abroad; all other isolates are unique to the U.S. Light gray denotes the predicted founder ST. Clonal complexes (CC; numbers within rectangles) are named after the predicted founding ST. In CCs composed of two STs, the CC is named after the most abundant ST; if both STs are equally represented, then the CC is named for the earliest found ST.

Out of the 21 singleton STs, most ($n = 13$) were represented by a unique isolate. When several isolates were identified as the same novel singleton ST, all isolates were recovered from the same geographic location (i.e., U.S. state) during a short time period (i.e., 1 to 3 years).

Association between STs and fish host species. When the association between the ST and fish host species was investigated, Fisher's exact test revealed an overall association between these two variables ($P < 0.001$). Indeed, all but one of the 34 STs of this study were retrieved from a single host fish species (Table 3).

For rainbow trout, which was the best represented fish host ($n = 54$ isolates) (Table 1), 23 isolates belonged to ST10 and 15 to ST78 (Table 3). Both ST10 and ST78 were statistically significantly associated with rainbow trout ($P < 0.001$). The association between genotype and host fish species extended beyond the ST level, since all CC-ST10 isolates were retrieved exclusively from rainbow trout. Overall, the 6 STs in CC-ST10 accounted for 85% ($n = 46$) (Table 1) of the isolates from this fish host. A larger sample size may show statistically significant associations between rainbow trout and the other STs in CC-ST10. Of note, all isolates that belonged to CC-ST10 were retrieved from captive fish and the vast majority ($n = 44/46$ isolates) were recovered during high mortality and/or morbidity events (Table 1). Furthermore, on five

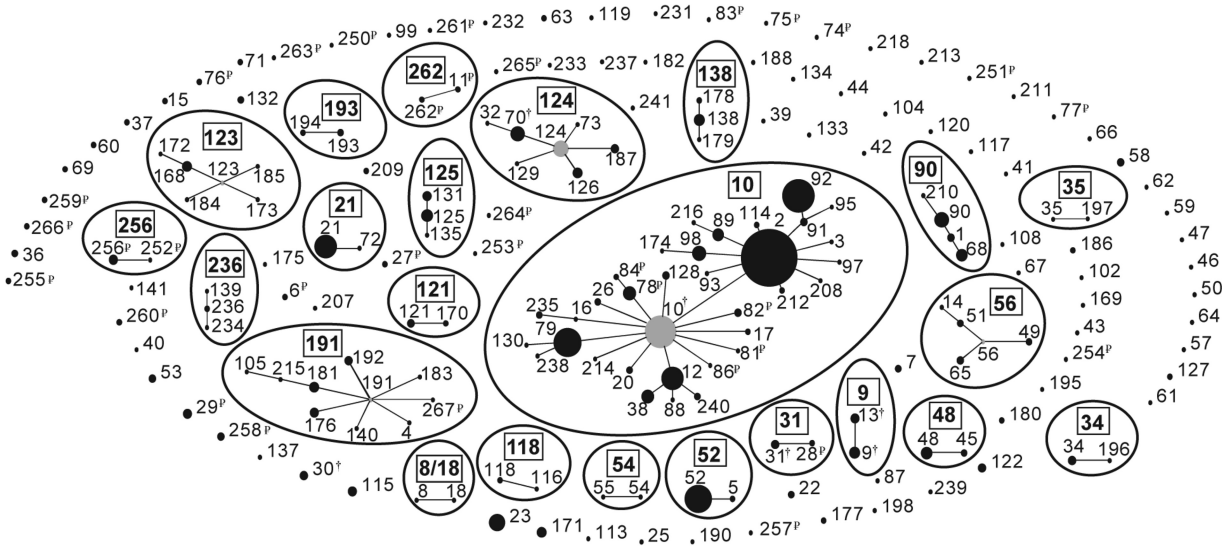

FIG 3 eBURST diagram depicting the relationships among global *F. psychrophilum* isolates, including the isolates of this North American study ($n = 1{,}091$). Daggers indicate sequence types (STs) found in both North America and abroad; ℘ denotes STs currently present only in North America. Light gray denotes the predicted founder ST. Clonal complexes (CCs) (numbers within rectangles) are named after the predicted founding genotype. In CCs composed of two STs, the CC is named after the most abundant ST; if both STs are equally represented, then the CC is named for the earliest found ST.

TABLE 3 MLST STs in this study summarized by fish host species[a]

Salmonid host species	STs unique to fish host species	ST found in >1 fish host species
O. mykiss	ST10 (23), ST78 (15), ST82 (4), ST31 (2), ST84 (2), ST81 (1), ST83 (1), ST86 (1), ST253 (1), ST257 (1), ST267 (1)	ST256 (2)
O. tshawytscha	ST29 (4), ST76 (2), ST255 (2), ST259 (2), ST260 (2), ST262 (2), ST266 (2), ST250 (1), ST251 (1), ST254 (1), ST261 (1), ST263 (1), ST265 (1)	ST256 (4)
O. kisutch	ST258 (4), ST9 (3), ST13 (2), ST30 (2), ST252 (1), ST74 (1), ST75 (1), ST77 (1), ST264 (1)	

[a] Numbers in parentheses indicate the numbers of isolates identified as that sequence type (ST) in this study.

occasions, multiple isolates from the same rainbow trout host were retrieved and analyzed. In three of those cases, analysis of both external lesions and internal tissues (kidney or spleen) led to the identification of the same ST (i.e., ST78 or ST82) (Table 1). However, in two instances, multiple STs were found to be coinfecting the same individual fish (i.e., ST10 with either ST82 or ST84) (Table 1).

The 26 Chinook salmon isolates fell into 14 STs (Table 3), among which the two most abundant STs were ST29 and ST256 (represented by 4 isolates each). ST29 was isolated exclusively from Chinook salmon, while ST256 was isolated from rainbow trout as well (Table 3). The 16 isolates recovered from coho salmon belonged to 9 different STs, among which the most abundant ST was ST258 ($n = 4$) (Table 3). It was noticeable that all isolates in CC-ST9 (i.e., ST9 and ST13) originated from coho salmon (Table 1).

The only detected exception to the strict association between STs and host fish was for ST256, which was isolated from both rainbow trout ($n = 2$) and Chinook salmon ($n = 4$) (Table 3). Interestingly, another ST belonging to the same CC-ST256 was also identified in coho salmon ($n = 1$) (Table 1). All of these hosts were feral fish from the GLB (Table 1).

Geographical origin of the STs. Our sampling scheme combined with the strong association between the ST and the host fish did not allow a global analysis of the statistical association between the ST and the geographical origin. Nevertheless, in this study, a particularly striking association between the ST and the geographical origin of the two most abundant STs (i.e., ST10 [$n = 23$] and ST78 [$n = 15$]) was observed. ST10 and ST78 isolates were recovered exclusively from captive rainbow trout and differed clearly by their geographical distributions (Table 1). ST10 isolates originated from Idaho ($n = 13$), Utah ($n = 5$), North Carolina ($n = 2$), New Mexico ($n = 2$), and West Virginia ($n = 1$). ST78 was identified primarily in Michigan ($n = 11$) and secondarily in Colorado ($n = 2$) and West Virginia ($n = 2$).

The state of Michigan accounted for 50 of our isolates, which were divided in two subgroups, reflecting two of the watersheds within the GLB (i.e., Lake Michigan and Lake Huron). This provided the opportunity to further examine a potential link between the ST and the geographical origin within a particular geographical area. Feral fish in the Lake Michigan watershed accounted for the highest number of isolates ($n = 24$) and number of distinct STs ($n = 12$), 9 of which are newly described herein (Table 1). The most abundant ST in the Lake Michigan watershed was ST256 ($n = 5$ isolates). From the Lake Huron watershed, 12 isolates recovered from feral fish resulted in the identification of 8 STs, 7 of which are also newly described herein (Table 1). Captive hosts in the GLB were represented by 14 isolates from 3 Michigan state fish hatcheries and resulted in 4 novel STs (i.e., ST78, ST253, ST257, and ST267) (Table 1). The most abundant ST observed in Michigan state fish hatcheries was ST78 ($n = 11$ isolates; 22%; CC-ST10). Fisher's exact test (based on STs) revealed a significant association between all 6 locations within the state of Michigan and the ST ($P < 0.001$). Most STs were unique to single locations within the GLB (e.g., ST259 and ST260 were recovered only from the Lake Michigan watershed), but others were widespread throughout the state (e.g., ST256 was recovered from both the Lake Michigan and Lake Huron watersheds) (Table 1).

DISCUSSION

Previous MLST studies have demonstrated the genetic heterogeneity of *F. psychrophilum* populations in other parts of the world. Despite the fact that the 96 isolates of this study may not fully represent the entire diversity of the U.S. *F. psychrophilum* population, the observed 34 distinct STs, of which 28 were novel, clearly depict a similar genetic heterogeneity within the United States. Upon comparison to the global MLST database, it also became clear that some U.S. *F. psychrophilum* STs and/or CCs have transcontinental distributions, whereas others seem to be more geographically limited. The results of this study shed light on the population structure of *F. psychrophilum* in the United States and highlight the similarities and differences between this population and *F. psychrophilum* populations elsewhere.

Average pairwise diversity measurements allow direct comparison of data sets from distinct regions of the world. The average gene diversity of the 96 U.S. isolates (i.e., 0.75 ± 0.03) was higher than any previously reported values from other regions of the world, where it varied from 0.43 in France (28) to 0.48 in Chile (33), 0.61 in Nordic countries (32), and 0.68 in Japan (29). However, the observed diversity may depend on the sampling scheme and the epidemiological characteristics of each *F. psychrophilum* population under investigation. In particular, the number of fish species in these previous studies varied from 1 to 15 (28, 29, 32, 33), whereas this study focused on 3 *Oncorhynchus* spp. Interestingly, the average pairwise nucleotide diversity of the 34 STs in the current study (i.e., 5.4 kbp^{-1}) is strikingly similar to that reported in Japan (i.e., 5.4 kbp^{-1}, based on 35 STs) (29), which may suggest that the genetic diversity of *F. psychrophilum* may be roughly comparable between the temperate regions of the Northern Hemisphere.

Studies on other fish-pathogenic flavobacteria have linked genetic differences to virulence and host specificity. For example, Olivares-Fuster et al. (45) found that genomovar I of fish-pathogenic *Flavobacterium columnare* is predominantly associated with the threadfin shad (*Dorosoma petenense*), while Shoemaker et al. (46) linked genomovar II of the same bacterium to channel catfish (*Ictalurus punctatus*). Host-specific trends for different *F. psychrophilum* genotypes are also becoming evident

(19, 29, 32), and the findings from this study suggest that the same may be true for some genotypes found in the United States (Table 2). For example, isolates belonging to CC-ST10 were recovered exclusively from rainbow trout, thus supporting the hypothesis that CC-ST10 strains are particularly adapted to this species, which is highly susceptible to BCWD, or its rearing conditions (32). A statistical association between ST10 and ST78 (both CC-ST10) and rainbow trout hosts has been demonstrated; however, statistically significant associations between rainbow trout and the other STs in CC-ST10 may become evident as more isolates are analyzed. Likewise, Avendaño-Herrera et al. (33) indicated a probable association between CC-ST9 strains and coho salmon, which coincides with the findings of this study, as all CC-ST9 isolates recovered in the United States were isolated from coho salmon. Further investigation into the mechanisms responsible for the apparent association between STs and host species is greatly needed.

However, other factors may also contribute to these observed associations. The STs belonging to CC-ST10 have been found to circulate in more than one host species in different regions of the world: rainbow trout and Atlantic salmon in Chile (33) and rainbow trout and brown trout in Switzerland (31). These patterns might be explained by the interconnection between the rearing systems of the different fish species. In this study, the absence of CC-ST10 in feral rainbow trout raises the suspicion that the observed association between CC-ST10 and this host species may result from a combination of some degree of host specificity and intensive culture conditions. It is also interesting that for the feral fish in the GLB and in particular for Chinook salmon (which harbor the highest *F. psychrophilum* infection prevalence among the 3 *Oncorhynchus* spp. that were recently examined) (36), data from this study do not seem to indicate an epidemic population structure, as demonstrated by the lack of dominant STs or CCs. It is also only among these fish that we observed infections by the same ST (e.g., ST256) or CC (e.g., CC-ST256) in different fish host species. Collectively, these observations give additional support to the idea that fish life conditions are also an important determinant of the *F. psychrophilum* genetic population structure.

Similar to the virulence trends identified in *F. columnare* (46), previous *F. psychrophilum* MLST studies suggested that some STs may be associated with higher virulence. For example, the founding and subfounding genotypes of CC-ST10 (i.e., ST2 and ST10) caused severe disease outbreaks in Europe and Chile and were proposed to have given rise to multiple highly pathogenic sublineages (28, 31–33). The findings of this study support this hypothesis, as all but 2 CC-ST10 isolates recovered in the United States were isolated from rainbow trout undergoing clinical BCWD. Within this complex, ST10 and its SLV ST78 seem to be dominant in North America. While ST10 has worldwide distribution (31, 32), ST78 has emerged as an ST of clinical significance, at least in Michigan. Indeed, only 2 isolates recovered during clinical disease epizootics in Michigan state fish hatcheries did not belong to this ST. Similarly, CC-ST191 contains isolates from disease outbreaks in farmed rainbow trout (32), including ST267, which was isolated during a disease outbreak at a Michigan state fish hatchery. The reason(s) for the apparent increase in virulence that certain STs display remains to be determined. However, a recent study demonstrated that members of CC-ST10 exhibit enhanced adherence to fish mucus, thereby facilitating their colonization, and are also resistant to antibiotics commonly used in aquaculture (7). Both the adherence and antimicrobial resistance properties, in conjunction with the high susceptibility of rainbow trout to *F. psychrophilum* in general, may be contributing to the dominance of these STs in aquaculture facilities.

This study brings new data on the history of CC-ST10, which is the largest established and most widespread CC based on all available *F. psychrophilum* MLST data. CC-ST10 is composed of a total of 34 STs recovered in North America, Europe, Asia, and South America (19, 28, 29, 31–33). Likewise, this study demonstrated that CC-ST10 is widespread in U.S. rainbow trout farms. This finding, along with the history of dissemination of rainbow trout/eggs from the United States to other countries, suggests that CC-ST10 originated from North America. Inclusion of U.S. isolates in the global *F. psychrophilum* MLST database and eBURST analyses suggests that ST10 is the likely founder of CC-ST10, rather than ST2 (19, 28, 29, 33), as the novel U.S. STs are SLVs of ST10 rather than ST2. Furthermore, it is notable that neither ST2 nor its SLVs (other than ST10) have yet to be observed in the United States, suggesting that evolution and diversification of the ST2 lineage within CC-ST10 may have occurred outside this country.

In addition to the globally distributed STs in the United States, this study revealed the presence of *F. psychrophilum* populations with lower prevalences and probably more limited geographical distributions. This was exemplified by the relatively high number of novel singletons in the salmonid populations of the GLB, which, aside from two occasions (i.e., ST257 and ST267), were not associated with morbidity or mortality. Furthermore, the majority (e.g., 6/8) of the singletons from outside the GLB were also not associated with morbidity or mortality, which coincides with the suggestion that many of these singletons may correspond to less virulent STs (7, 28, 32). These may be representatives of endemic *F. psychrophilum* populations whose characteristics with respect to pathogenicity, fish host, and geographical distribution remain to be clarified.

In conclusion, the MLST investigation of *F. psychrophilum* isolates recovered from feral and hatchery-reared salmonids in the United States revealed marked genetic diversity. Several of the U.S. *F. psychrophilum* STs are found worldwide, whereas others seem specific to the continental United States. These results shed light on the historical links between the different *F. psychrophilum* populations worldwide. Furthermore, we demonstrated the association between particular STs and host species, as well as high virulence, in the United States. This information can be used to more appropriately investigate preventative control measures to reduce the spread and severity of BCWD.

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