Distribution of *Flavobacterium psychrophilum* and its *gyrA* genotypes in a river

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Abstract

Flavobacterium psychrophilum can be divided into three genotypes, G-C type (ayu type), A-C type (multi-fish type), and A-T type (salmon-trout type), by two single-nucleotide polymorphisms in the DNA gyrase subunit A (*gyrA*) gene. We isolated *F. psychrophilum* from various samples collected from the lower basin of a river flowing into Lake Biwa, as a model for bacterial survey, in Shiga Prefecture in June, September, and December from 2010 to 2013 and investigated their *gyrA* genotypes. All three types of *F. psychrophilum* were isolated in June when ayu went up the river from the lake. In September, ayu gathered in the lower river basin for spawning and became high in density, almost all of the isolates were of the G-C genotype and the A-T genotype was never isolated. In December, only the A-T type was isolated from the river samples, when Biwa trout *Oncorhynchus masou rhodurus* came to the river to spawn and were present in the sampling area. In accordance with the seasonal change of the host fish species in the river, the genotype of *F. psychrophilum* isolated from the environment seems to have changed as well.

Keywords *Flavobacterium psychrophilum* · Bacterial cold-water disease · Ayu · *Plecoglossus altivelis* · Lake Biwa basin · *gyrA* genotyping · Distribution

Introduction

Bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS) are a problem mainly with salmonid fish such as coho salmon *Oncorhynchus kisutch*, rainbow trout *Oncorhynchus mykiss*, and ayu *Plecoglossus altivelis*. The causative agent *Flavobacterium psychrophilum* is a Gram-negative, aerobic, long, rod-shaped bacterium

(Bernardet et al. 1996). The first report of *F. psychrophilum* infection was from North America in the 1930s. Since then, many countries have been affected, with economic losses reaching millions of dollars (Antaya 2008). The disease caused by *F. psychrophilum* has been reported as the peduncle disease of rainbow trout (Davis 1946), and the bacterium was isolated from juvenile coho salmon (Borg 1960). In Japan, the ayu affected by *F. psychrophilum* was

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found in 1987 (Wakabayashi et al. 1994). Since then, almost all parts of Japan have been affected by *F. psychrophilum*, and it has spread not only in aquaculture facilities but also in natural rivers of Japan. *F. psychrophilum* has been isolated from various wild fish including akaza *Liobagrus reinii*, oikawa *Zacco platypus*, ookuchibass *Micropterus salmoides*, kadayashi *Gambusia affinis*, kawamutsu *Nipponocypris temminckii*, numa-chichibu *Tridentiger brevispinis*, and honmoroko *Gnathopogon caeruleus* (Arai et al. 2006; Izumi et al. 2007; Fujii et al. 2009), and from environmental samples such as river water and algae (Amita et al. 2000).

Treatments designed to cure BCWD include chemotherapy using sulfisoxazole sodium (Ninomiya and Yamamoto 2001) and florfenicol, which are the officially approved drugs to cure BCWD in ayu, and warmed water (28°C) treatment (Yamamoto et al. 2001; Sugahara et al. 2010a). However, chemotherapy is restricted and undesirable because of the occurrence of drug-resistant bacteria (Rangdale et al. 1997; Bruun et al. 2000, 2003). In addition, warmed water treatment may cause heat stress, oxygen deficiency, water quality degradation, and other diseases. Furthermore, although both methods are effective for closed systems in onshore aquaculture sites, it is impossible to use them in open natural rivers. Thus, prevention of BCWD is an essential and realistic approach to controlling its spread. Many countries and companies have been working toward the development of vaccines against BCWD such as injection, oral treatment, immersion, and live vaccines; however, they have not yet reached the practical stage.

In order to prevent the occurrence of BCWD, it is important to elucidate the infection route of this bacterium. Attempts have been made to investigate the distribution of the bacterium, but satisfactory results have yet to be obtained, in part because of its host specificity (Izumi et al. 2003; Yoshiura et al. 2006; Fujiwara-Nagata et al. 2012). We have reported that *F. psychrophilum* can be divided into four genotypes using single nucleotide polymorphisms of the DNA gyrase subunit A (*gyrA*) gene (Fujiwara-Nagata et al. 2012). The first is a G-C type which was isolated from ayu, and the second is the A-T type which was isolated from salmonid fish. The third, G-T type, and the fourth, A-C type, are multi-fish types which were isolated from various fish species including ayu, salmonids, and cyprinids. The G-C type shows virulence in ayu, but the A-T and G-T types show no virulence in the fish. The A-C type shows both high and low virulence in ayu, that is, the A-C type isolated from ayu shows high virulence and the A-C type isolated from other than ayu shows low virulence in ayu. The G-T type was isolated from rainbow trout, yamame *Oncorhynchus masou masou*, amago *Oncorhynchus masou ishikawae*, and ayu from 2002 to 2005, and the isolation areas were also limited. The use of *gyrA* genotyping makes it possible to identify the host fish species of *F. psychrophilum*. As a next step to prevent the occurrence of BCWD in rivers, it is necessary

to accurately understand the distribution of *F. psychrophilum* and the potential impact on fisheries owing to its occurrence in important fish species.

To contribute to the establishment of effective prevention measures for BCWD occurring in rivers, the purpose of this study was to elucidate the distribution status of each genotype of *F. psychrophilum* in the natural environment. We chose a river which flows into Lake Biwa as a model for bacterial survey, because it was clear and supported various fish species including ayu, salmonids, and cyprinids. The landlocked lake-produced ayu living in Lake Biwa, the largest freshwater lake in Japan, primarily spawns and dies in the downstream area of the inflowing rivers from September to October. The larvae grow in the lake from October to February of the following year, and from the beginning of March they return to the rivers around the lake. In the present study, samples of ayu, other fish species, river water, aquatic insects and their nests, algae adhering to the surface of stones, sand, and floating mud were collected, separated, and subjected to bacterial isolation. For the isolates identified as *F. psychrophilum*, *gyrA* genotyping was performed.

Materials and methods

Field sampling

Our field surveys were conducted in the lower basin of the river, which flowed into the northwestern part of Lake Biwa, in Shiga Prefecture in June, September, and December of 2010–2013 (sampling was missed in December 2010 and June 2012).

Fish including ayu, environmental samples such as river water, aquatic insects and their nests, algae adhering to the surface of rocks, and mud from the bottom of the river were sampled. With authorization from Shiga Prefecture, fish caught by electrofishing were kept at low temperatures in tanks with ice cubes and quickly transported to our laboratory. We caught only fish bigger than 5 cm in length.

Water samples were collected using sterilized plastic bottles and transferred to the laboratory at a low temperature with ice cubes. Water temperature was measured with a water temperature gauge. Furthermore, from June 2012 to December 2013, the river water temperature was continuously recorded by a data logger (UA-002-64, Onset Computer Corporation, MA, USA).

Sample processing

Fish (except lamprey)

Fish were euthanized by severing the spinal cord. Gill samples were cut into pieces of ~25 mm² and suspended in 1 ml of phosphate-buffered saline (PBS). When gill samples were

collected, up to five fish of the same fish species were pooled in one microtube to make one specimen. Samples from internal organs and skin lesions were collected using a sterile loop and streaked directly onto FLP agar plates (Cepada et al. 2004).

Lamprey

Using sterilized dissection scissors and tweezers, the head of the lamprey was cut out, minced, and homogenized in 1 ml of PBS. As much as possible, the meat pieces adhering to the homogenizer pestles were put in PBS, and the samples were weighed.

Caddisfly, shrimp, leech, and melania snail

The insects were separated from their nests using sterilized tweezers and then homogenized with pestles. As much as possible, the meat pieces adhering to the pestles were put in PBS, and the samples were weighed. Shrimp, leech, and snail samples were treated in the same way as aquatic insects.

Algae, sediments, and caddisfly cases

The algae attached to stones, sediments, and caddisfly cases collected from the river were weighed (0.5 g) and placed in tubes. The samples were briefly centrifuged, and their supernatants were then removed. One ml of PBS was added to the tubes.

Isolation of bacteria

All specimens were mixed by vortexing at maximum speed (3000 rpm) for 30 s, after which the samples were briefly (approximately 5 s) precipitated at $2840\times g$ with a small tabletop centrifuge. The supernatants were further diluted to 10^{-4} in PBS to obtain well-separated colonies, and 0.1 ml of each diluted sample was spread onto FLP agar plates. All plates were incubated at 4 °C for 14 days.

DNA extraction

Yellow colonies up to around 30 colonies in each plate were selected for identification as *F. psychrophilum* based on the PCR amplification according to the guidelines of the Council for the Control of BCWD of ayu (Japanese Ministry of Agriculture, Forestry, and Fisheries: https://www.maff.go.jp/j/syoutan/suisan/suisan_yobo/ayu_reisui/pdf/sisin_2008.pdf Accessed 01 June 2019). Briefly, DNA was extracted directly from yellow-pigmented colonies using 1 µl of proteinase K in 200 µl of PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.0). The extraction and

inactivation were performed at 55 °C for 30 min and at 95 °C for 10 min, respectively.

Polymerase chain reaction (PCR)

PCR was performed targeting the 16S rRNA gene with nested PCR (Toyama et al. 1994) and DNA topoisomerase IV subunit B (*parE*) gene (Izumi and Wakabayashi 2000). When the PCR results were positive for both genes, the isolate was considered to be *F. psychrophilum*.

For the first round of 16S rDNA nested PCR, a universal primer pair, 20F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1500R (5'-GGTTACCTTGTTACGACTT-3'), was used (Weisburg et al. 1991). For the second round of the nested PCR, we used the specific primer pair PSY1 (5'-GTTGGC ATCAACACACT-3') and PSY2 (5'-CGATCCTACTTG CGTAG-3') (Toyama et al. 1994). The amplification for the first 16S rRNA PCR and the *parE* PCR were performed in a total volume of 12 µl with the following ingredients: 1× reaction buffer (Bioline, London, UK), 0.25 mM of d-NTP mix, 1.2 mM of MgCl₂, 0.4 µM of forward and reverse primers, 0.3 U of BIOTAQ™ (Bioline), and 2 µl of DNA template. As a template for the second 16S rDNA PCR, 2 µl of the PCR product of the first round diluted to 1/20 was used. The PCR conditions were as follows: pre-denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 20 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and a final cycle at 74 °C for 5 min. The PCR products (10 µl) and 3 µl of tracking dye were mixed and electrophoresed on 0.8% agarose gels with ethidium bromide to confirm the presence of a 1089-bp band for the 16S rDNA nested PCR and a 1017-bp band for the *parE* PCR.

gyrA on/off switch assay

A DNA sample positive for *F. psychrophilum* by the above PCR procedures was subjected to an on/off switch assay. Amplification primers with 3'-terminal phosphorothioate modification were as follows: *gyrA*-125-G forward primer (5'-TATCACCCACACACGGAGATG-3'), *gyrA*-125-A forward primer (5'-TATCACCCACACACGGAGAT-3'), *gyrA*-202-C reverse primer (5'-CTCCATCTACAGATC CAAAG-3'), and *gyrA*-202-T reverse primer (5'-CTCCAT CTACAGATCCAAAG-3') (Fujiwara-Nagata et al. 2012). The amplification was performed in a total volume of 14 µl with the following ingredients: 1× PS buffer (Takara, Shiga, Japan), 0.25 mM of dNTPs, 0.4 µM of each primer, 0.45 U of Prime STAR (Takara), and 1 µl of template DNA. The PCR amplification conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 98 °C for 30 s, annealing at 61.5 °C for 5 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Subsequently, 5 µl of the PCR product was electrophoresed on

3% agarose gel with ethidium bromide to confirm the presence of a 115-bp band characteristic of *F. psychrophilum*.

Results

Prevalence of *F. psychrophilum* in each sample for each season

Although there were variations in the number of isolates, *F. psychrophilum* was found to be present in the model river throughout the year from 2010 to 2013 (Table 1). The gills of ayu caught in the river carried *F. psychrophilum* at a high percentage, 89% and 100% in June and September, respectively. *F. psychrophilum* was isolated from the kidney of 39 and 47% of individuals in June and September, respectively. December was not subject to survey, as there were no ayu in the river. The proportion of *F. psychrophilum* isolated from the gills of fish species other than ayu was 60%, 52%, and 14% in June, September, and December, respectively. However, isolation of *F. psychrophilum* from their kidneys was low, 0%, 4%, and 0%, respectively. The proportion of *F. psychrophilum* isolated from the other samples including river water, caddisflies, their nests, algae adhering to the surface of stones, and sediment was 14%, 17%, and 4% in June, September, and December, respectively. During the field surveys, the symptoms of BCWD were seen only in ayu.

F. psychrophilum isolated from ayu

F. psychrophilum isolated from ayu was of the G-C and A-C types, and the A-T type was not detected (Table 2). The G-C type was isolated from 74% (42/57 individuals) of the kidneys from *F. psychrophilum*-positive ayu collected in June and from 99% (109/110 individuals) of the kidneys from *F. psychrophilum*-positive ayu in September. The A-C type was isolated from the kidneys of ayu in June and September, but the rate of isolation was lower than that of the G-C type. Interestingly, there was no case where the A-C type alone was isolated from the gills of ayu. Both the G-C and A-C types were isolated from the gills of ayu at the same time in June (24%, 6/25 samples) and September (2%, 1/43 samples). The G-C and A-C types were also simultaneously isolated from the kidney of nine ayu individuals collected in June. It was confirmed that the G-C and A-C types of *F. psychrophilum* co-infected the same fish.

F. psychrophilum isolated from fishes other than ayu

F. psychrophilum was isolated from aburahaya *Phoxinus steindachneri*, ugui *Tribolodon hakonensis*, kamatsuka *Pseudogobio esocinus*, nigo *Hemibarbus barbus*, hasu *Opsariichthys uncirostris*, biwa-higai *Sarcocheilichthys variegatus microoculus*, ukigori *Gymnogobius urotaenia*, utsusemi-kajika *Cottus reinii*, numa-chichibu, touyoshinobori *Rhinogobius* sp., ookuchibass, and sunayatsume *Lethenteron reissneri* (Table 2). From the *F. psychrophilum*-positive

Table 1 Number of positive and negative samples of *Flavobacterium psychrophilum* and detection rate

Origin of samples	Number of <i>F. psychrophilum</i> -positive samples	Number of <i>F. psychrophilum</i> -negative samples	Detection rate
June, 2010, 2011, and 2013			
Gill (ayu)	25	3	89%
Kidney (ayu)	57	89	39%
Gill (other fish)	12	8	60%
Kidney (other fish)	0	14	0%
Other samples	5	30	14%
September, 2010–2013			
Gill (ayu)	43	0	100%
Kidney (ayu)	110	125	47%
Gill (other fish)	13	12	52%
Kidney (other fish)	1	27	4%
Other samples	8	38	17%
December, 2011–2013			
Gill (ayu)	ND	ND	ND
Kidney (ayu)	ND	ND	ND
Gill (other fish)	4	25	14%
Kidney (other fish)	0	14	0%
Other samples	2	54	4%

ND no data

Table 2 Number of fish samples from which *Flavobacterium psychrophilum* was isolated from 2010 to 2013 and the number of samples isolated for each genotype

Year	Month	Order	Family	Fish species	gyrA genotyping (no. of the genotype/no. positive)		Tissue	Num- ber of samples ^a	gyrA genotyping (no. of the genotype/no. positive)				
					Scientific name	Common name			G-C	A-C	A-T	G-C/A-C ^b	G-C/A-T ^c
2010, 2011, 2013	June	Salmoniformes	Plecoglossidae	<i>Plecoglossus altivelis altivelis</i>	Ayu	Gill	28	19/25			6/25		
						Kidney	146	42/57	6/57		9/57		
		Cypriniformes	Cyprinidae	<i>Phoxinus steindachneri</i>	Aburahaya	Gill	2	1/1					
				<i>Tribolodon hakonensis</i>	Ugui	Kidney	3						
						Gill	1	1/1					
				<i>Pseudogobio esocinus</i>	Kamatsuka	Kidney	3						
						Gill	1	1/1					
				<i>Hemibarbus barbus</i>	Nigoi	Kidney	1					1/2	
				<i>Opsariichthys uncirostris</i>	Hasu	Gill	2	1/2			1/1		
						Gill	2						
						Kidney	2						
		Perciformes	Gobiidae	<i>Gymnogobius urotaenia</i>	Ukigori	Gill	2						
				<i>Tridentiger brevispinis</i>	Numa-chichibu	Gill	2						
				<i>Rhinogobius</i> sp. type OR	Touyoshinobori	Gill	1						
		Scorpaeniformes	Cottidae	<i>Cottus reinii</i>	Utsusemi-kajika	Gill	4	1/3	1/3		1/3		
						Kidney	5						
		Petromyzontiformes	Petromyzontidae	<i>Lethenteron reissneri</i>	Sunayatsume	Gill	3	1/3	1/3		1/3		
						Subtotal	Gill	20	6/11	2/11	0	2/11	2/11
							Kidney	14	0	0	0	0	0

Table 2 (continued)

Year	Month	Order	Family	Fish species	Common name		Tissue	Num- ber of samples ^a	gyrA genotyping (no. of the genotype/no. positive)			
					Scientific name				G-C	A-C	A-T	G-C/A-C ^b
2010, 2011, 2012, 2013	September	Salmoniformes	Plecoglossidae	<i>Plecoglossus altivelis altivelis</i>	Ayu	Gill	43	42/43			1/43	
						Kidney	235	109/110	1/110			
		Cypriniformes	Cyprinidae	<i>Phoxinus steindachneri</i>	Aburahaya	Gill	1					
				<i>Pseudogobio esocinus</i>	Kamatsuka	Gill	1	1/1				
				<i>Opsariichthys uncirostris</i>	Hasu	Gill	3	1/1				
						Kidney	3					
		Perciformes	Gobiidae	<i>Gymnogobius urotaenia</i>	Ukigori	Gill	1	1/1				
						Kidney	5					
				<i>Tridentiger brevispinis</i>	Numa-chichibu	Gill	4	2/2				
						Kidney	8					
				<i>Rhinogobius</i> sp. type OR	Touyoshinobori	Gill	2	1/1				
						Kidney	1					
Scorpaeniformes	Odontobutidae	<i>Odontobutis obscura</i>	Donko	Gill	1							
				Kidney	1							
		<i>Cottus reinii</i>	Utsusemi-kajika	Gill	4	3/3						
Petromyzontiformes	Cottidae			Kidney	10	1/1						
		<i>Lethenteron reissneri</i>	Sunayatsume	Gill	8	3/3		1				
			Subtotal	Gill	25	12/13	0	0	1/13	0		
				Kidney	28	1/1	0	0	0	0		

Table 2 (continued)

Year	Month	Order	Family	Fish species	Common name	Tissue	Num- ber of samples ^a	gyrA genotyping (no. of the genotype/no. positive)			
								G-C	A-C	A-T	G-C/A-C ^b G-C/A-T ^c
2011, 2012, 2013	December	Cypriniformes	Cyprinidae	<i>Phoxinus steindachneri</i>	Aburahaya	Gill	2				
						Kidney	1				
				<i>Pseudogobio esocinus</i>	Kamatsuka	Gill	1				
				<i>Sarcocheilichthys variegatus microoculus</i>	Biwa-higai	Gill	1			1/1	
						Kidney	1				
			Cobitidae	<i>Misgurnus anguillicaudatus</i>	Dojou	Gill	1				
				<i>Tridentiger brevispinis</i>	Numa-chichibu	Gill	2				
		Perciformes	Gobiidae			Kidney	1				
				<i>Rhinogobius</i> sp. type OR	Touyoshinobori	Gill	2				
		Centrarchidae		<i>Micropterus salmoides</i>	Ookuchibass	Gill	1			1/1	
						Kidney	1				
		Scorpaeniformes	Cottidae	<i>Cottus reinii</i>	Utsusemi-kajika	Gill	6				
						Kidney	8				
		Petromyzontiformes	Petromyzontidae	<i>Lethenteron reissneri</i>	Sunayatsume	Gill	13			2/2	
					Subtotal	Gill	4	0	0	4/4	0
						Kidney	14	0	0	0	0

^a Kidney samples were taken from each fish; gill samples were pooled from up to five individuals of a fish species and used as one sample^b G-C type and A-C type were detected from one specimen^c G-C type and A-T type were detected from one specimen

fishes other than ayu, the G-C type, A-C type, A-T type, G-C and A-C types, and G-C and A-T types were isolated at 64% (18/28 samples), 7% (2/28 samples), 14% (4/28 samples), 11% (3/28 samples), and 7% (2/28 samples), respectively.

Aburahaya, kamatsuka, utsusemikajika, numa-chichibu, touyoshinobori, and sunayatsume were caught every surveyed month. Among these, only sunayatsume was confirmed as carrying *F. psychrophilum* each month. Co-infection of the G-C and A-C types was confirmed in sunayatsume in June and September. In the case of sunayatsume, it was confirmed that the G-C and A-C types were single or co-infected in June and September, respectively. In December, only the A-T type was isolated from sunayatsume. No symptoms of BCWD appeared in the sunayatsume.

When the kidneys of fishes other than ayu were investigated, of the 14, 28, and 14 specimens collected in June, September, and December, respectively, only one specimen of utsusemi-kajika, collected in September 2013, had the G-C type. No symptoms of BCWD appeared in the utsusemi-kajika.

F. psychrophilum isolated from environmental samples

F. psychrophilum was isolated from aquatic insect nests, river water, algae, bottom sediments, shrimp, leech, and deposits such as leaves and roots of plants adhered to concrete blocks submerged in the river (Table 3). The bacterium was not isolated from aquatic insects or freshwater snails. In June, the G-C and A-T types from the river water and G-C and A-C types from the bottom sediment were isolated. In September, only the G-C type was isolated from aquatic insect nests, river water, algae, shrimp, and leech samples. In December, only the A-T type was isolated from deposits of aquatic insect nests and concrete blocks.

Water temperature in the surveyed river

Figure 1 shows the daily water temperature of the model river from June 2012 to December 2013. Temperatures ranged from 14.5 °C to 23.7 °C (average 18.8 °C) in June, 13.2 °C to 25.9 °C (average 19.5 °C) in September, and 1.5 °C to 13.3 °C (average 8.4 °C) in December.

Table 3 The number of samples with *Flavobacterium psychrophilum* isolated from 2010 to 2013 and each genotype of *F. psychrophilum*

Year	Month	Sample type	Number of samples used	Number of positive samples	gyrA genotyping			
					G-C	A-C	A-T	G-C/A-T ^a
2010, 2011, 2013	June	Aquatic insect	17	0				
		Nest of aquatic insect	6	0				
		River water	3	2	1			1
		Algae	5	0				
		Bottom sediment	4	3	2	1		
2010, 2011, 2012, 2013	September	Aquatic insect	18	0				
		Nest of aquatic insect	9	2	2			
		River water	4	3	3			
		Algae	5	1	1			
		Sediment to a concrete brick	1	0				
		Bottom sediment	5	0				
		Shrimp	2	1	1			
		Leech	1	1	1			
		Freshwater snail	1	0				
2011, 2012, 2013	December	Aquatic insect	24	0				
		Nest of aquatic insect	8	1			1	
		River water	3	0				
		Algae	5	0				
		Sediment to a concrete brick	1	1			1	
		Bottom sediment	5	0				
		Shrimp	7	0				
		Leech	1	0				
		Freshwater snail	2	0				

^aG-C type and A-T type were detected from one specimen

Discussion

Conventionally, two possibilities have been considered regarding the infection route of *F. psychrophilum* in ayu in the next season (Amita et al. 2000). One is egg-associated transmission and the other is horizontal transmission from resident algae in the river as the infection source. Besides these two possibilities, it has been reported that the overwintering ayu present in a river carries the bacterial agent (Miyazaki 2008). However, no overwintering ayu exists in the surveyed river investigated in this research. In this study, to clarify the mode of transmission of *F. psychrophilum* in ayu in a river, a target survey of *F. psychrophilum* in a river flowing into lake Biwa, as a model for bacterial survey, was conducted over 4 years by bacterial isolation from various samples including ayu, other fish species, and river environmental specimens.

The results of this study clearly show that the isolation rate of *F. psychrophilum* and the genotype present in the river varies depending on the season. In June and September, the G-C and A-C types of *F. psychrophilum* were isolated from various samples of the river but were not isolated at all in December. This was consistent with the existence of ayu, a host fish species of the G-C and A-C types, in the river. Ayu living in Lake Biwa spawns in a downstream area of an influx of a river into the lake from mid-September to late October and then dies. The hatched larvae grow in the lake from November to February of the following year, and from the beginning of March, they return to the rivers around the lake. Thus, ayu did not exist in the surveyed river in December, when the G-C and A-C types were not isolated.

Fig. 1 Daily water temperature of the model river. Black diamond: June–December 2012, white circle: January–December 2013 (missing in November)

This suggests that the presence of ayu affects the genotype appearance of *F. psychrophilum* present in rivers.

In December, the A-T type that shows host specificity against salmonid fish was isolated from the river, but the G-C type was not. At the sampling area in the river, *Oncorhynchus masou rhodurus*, a local trout called Biwa trout, swims upstream of the river to spawn in the season. In accordance with the seasonal change of the host fish species in the river, the genotype of the bacterium isolated from the environment seems to have also changed.

Various genotypes of the bacterium were isolated in the June survey; however, almost all of the isolates were of the G-C type in the September survey. One possibility is that this might be caused by the rise of river water temperature. Kumagai et al. (2010) reported that prevalence of *F. psychrophilum* in ayu was less than 10% in August, but it increased to above 90% in October. This difference in the prevalence of bacterium in ayu between August and October might be caused by a drop in water temperature to 15–20 °C, which is optimum for the bacteria (Uddin and Wakabayashi 1997), and immune-suppression due to the maturation of ayu (Kumagai et al. 2010). The surveyed river in this study recorded the highest water temperature of 22 °C at night and 26 °C during daytime in August (2012 and 2013). Sugahara et al. (2010a) reported that the growth temperature of the bacteria might differ among the strains, nevertheless, the upper permissive temperature for growth was suggested to be 25–27 °C. In the future, it is necessary to investigate whether there is a difference in the temperature resistance ability among *F. psychrophilum* isolates and to investigate the possibility that the water temperature affects the seasonal genotype variation.

Amita et al. (2000) described the possibility that fishes other than ayu were carriers of *F. psychrophilum*. As a result of this study, it became clear that the G-C type of the bacterium was widely distributed in the river in June and September. Genotypes of the bacterium detected from the samples collected from 2010 to 2013 were almost all G-C type detected from ayu, fishes other than ayu, and environmental samples. In December, the G-C type was no longer detected from any samples. Considering that fishes other than ayu must be persistently infected with the bacterium to become carriers, it is unlikely that fishes other than ayu would be carriers.

Furthermore, there was only one case where the G-C type was detected from the kidney samples of fishes other than ayu, which was in utsusemikajika, in September 2013. In the river, from spring to early summer, as the ayu moves in from Lake Biwa, the density of ayu rises sharply. This high density of ayu continues until ayu spawns in autumn and then dies. The reason the G-C was detected in fishes other than ayu was that as the amount of infected ayu increased, the amount of discharged bacteria from ayu accumulated,

and the concentration of the bacteria in the rivers became high; therefore, the gills which have a contact surface with the river water were temporarily colonized with *F. psychrophilum*.

In fish samples other than ayu, only sunayatsume, or lamprey fish, had the G-C and A-C types of *F. psychrophilum* in June and September and the A-T type in December. Three genotypes of *F. psychrophilum* have been isolated from sunayatsume through 1 year. Juveniles of sunayatsume spend several years eating organic matter and diatoms in the middle and downstream of rivers. In late summer and autumn, juveniles transform into adults and their digestive organs disappear. Then, they spend the winter and spawn in next spring and die. Owing to this lifestyle, it is thought that it is easy for sunayatsume to possess the bacteria, and the bacterial types colonized in sunayatsume reflect the type of *F. psychrophilum* present in the river at the time. *F. psychrophilum* was isolated from sea lamprey in Lake Ontario (Elsayed et al. 2006). Sunayatsume and umiyatsume belong to the order Petromyzontiformes and are grouped into Cyclostomata along with nutaunagi (hagfish). Cyclostomes present many primitive features, so they are regarded as important in evolutionary research. It would be interesting to know why sunayatsume are colonized by various types of BCWD bacteria, so this will be a topic for further research.

There is concern of the possibility that the environmental samples are a source of infection of BCWD. One report suggests that algae samples may be a source of *F. psychrophilum* among environmental samples (Amita et al. 2000). Ayu eats aquatic insects at the early stages of moving up to the river and then it eats algae adhering to stones. Therefore, it is considered that the ayu that goes up a river has a greater chance of contact with environmental samples such as algae and aquatic insects. *F. psychrophilum* capable of showing pathogenicity in ayu has high physiological activity that retains colony-forming ability (Sugahara et al. 2010b). Thus, we focused on *F. psychrophilum* as having colony-forming ability in this study. Our method for cultivating *F. psychrophilum* worked very well even from the gills of fish other than ayu and environmental samples. As a result of the distribution survey, the ratio of isolation of *F. psychrophilum* from the environmental samples was low even in June and September, and in December, the G-C and A-C types were not isolated from the environmental samples. A DNA survey distinguishing the *F. psychrophilum* genotype will be required in future study.

The results of this study reveal that the possibility of horizontal transmission of *F. psychrophilum* to ayu via a fish other than ayu or environmental samples is low. Although there are reports showing the possibility of vertical transmission of the bacterium in salmonid fish (Kumagai and Nawata 2010; Kohara et al. 2012), the possibility of the transmission in ayu is low (Kumagai et al. 2004). In 2007, we collected

fertilized eggs from a riverbed, fry, and juveniles, and tried to detect viable cells and DNA of *F. psychrophilum*. As a result, DNA of *F. psychrophilum* was detected from fertilized eggs and hatched larvae, but *F. psychrophilum* was isolated only from hatched larvae (data not shown). Neither DNA nor viable cells were detected from the juveniles. *F. psychrophilum* isolated from hatched larvae were of the G-C and A-C types. It was confirmed that these types of *F. psychrophilum* were in the surveyed river at this time, so it was understandable that hatched larvae could carry this bacterium. Further research should be conducted to investigate whether the juveniles are colonized by the bacterium. Yamamoto et al. (2015) reported that there were fewer juveniles of ayu infected with *F. psychrophilum* (less than 3%) according to a survey dealing with a large number of juveniles collected off the coast of Lake Biwa from November to February. In the case of ayu larvae descending to the sea, *F. psychrophilum* cannot survive in seawater, so it can be expected to be removed naturally; however, it is considered difficult to remove the bacterium from Lake Biwa, as it is a freshwater lake.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest with regard to the content of this article.

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References

- Amita K, Hoshino M, Honma T, Wakabayashi H (2000) An investigation on the distribution of *Flavobacterium psychrophilum* in the Umikawa river. Fish Pathol 35:193–197 (In Japanese with English abstract)
- Antaya C (2008) Current eco-economical impacts of *Flavobacterium psychrophilum*. MMG 445 basic biotechnology. eJournal 4:16–21
- Arai H, Fujita M, Suzuki K, Katagiri T, Kuge T (2006) Pathogenicity of *Flavobacterium psychrophilum* isolated from pale chub *Zacco platypus* and reddish bullhead *Liobagrus reini* to ayu *Plecoglossus altivelis altivelis*. Aquac Sci 54:575–576 (In Japanese with English abstract)
- Bernardet JF, Sergers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P (1996) Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description

- of the family Flavobacteriaceae, and proposal of *Flavobacterium hydatidis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int J Syst Bacteriol 46:128–148
- Borg AF (1960) Studies on myxobacteria associated with diseases in salmonid fishes. Wildl Dis 8:1–85
- Bruun MS, Schmidt AS, Madsen L, Dalsgaard I (2000) Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 187:201–212
- Bruun MS, Madsen L, Dalsgaard I (2003) Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro antibiotic susceptibilities. Aquaculture 215:11–20
- Cepeda C, García-Márquez S, Santos Y (2004) Improved growth of *Flavobacterium psychrophilum* using a new culture medium. Aquaculture 238:75–82
- Davis HS (1946) Care and Diseases of Trout. US Department of Interior Research Report, No. 12. US Government Printing Office, Washington, DC, USA.
- Elsayed EE, Eissa AE, Faisal M (2006) Isolation of *Flavobacterium psychrophilum* from sea lamprey, *Petromyzon marinus* L., with skin lesions in Lake Ontario. J Fish Dis 29:629–632
- Fujii H, Harada S, Kotouge T (2009) *Flavobacterium psychrophilum* carrying situation in native fish species caught from the Arida river and the pathogenicity of *F. psychrophilum* isolated from dark chub *Zacco temminckii* on ayu *Plecoglossus altivelis altivelis*. Aquacult Sci 57:621–622 (In Japanese with English abstract)
- Fujiwara-Nagata E, Ikeda J, Sugahara K, Eguchi M (2012) A novel genotyping technique for distinguishing between *Flavobacterium psychrophilum* isolates virulent and avirulent to ayu, *Plecoglossus altivelis altivelis* (Temminck and Schlegel). J Fish Dis 35:471–480
- Izumi S, Wakabayashi H (2000) Sequencing of *gyrB* and their application in the identification of *Flavobacterium psychrophilum* by PCR. Fish Pathol 35:93–94
- Izumi S, Aranishi F, Wakabayashi H (2003) Genotyping of *Flavobacterium psychrophilum* using PCR-RFLP analysis. Dis Aquat Organ 56:207–214
- Izumi S, Ouchi S, Kuge T, Arai H, Mito T, Fujii H, Aranishi F, Shimizu A (2007) PCR-RFLP genotypes associated with quinolone resistance in isolates of *Flavobacterium psychrophilum*. J Fish Dis 30:141–147
- Kohara M, Kasai H, Yoshimizu M (2012) Intra-ovum infection in salmonid eggs artificially contaminated with fish pathogenic bacteria: *Flavobacterium psychrophilum*, *Renibacterium salmoninarum* and *Aeromonas salmonicida*. Fish Pathol 47:49–55
- Kumagai A, Nawata A (2010) Mode of the intra-ovum infection of *Flavobacterium psychrophilum* in salmonid eggs. Fish Pathol 45:31–36
- Kumagai A, Nakayasu C, Oseko N (2004) No evidence for the presence of *Flavobacterium psychrophilum* within ayu eggs. Fish Pathol 39:183–187
- Kumagai A, Nawata A, Taniyai Y (2010) Monitoring of outbreaks of bacterial cold-water disease among ayu in a river where asymptomatic carriers of *Flavobacterium psychrophilum* were released. Fish Pathol 45:115–120
- Miyazaki T (2008) *Flavobacterium psychrophilum* isolated from overwintering ayu *Plecoglossus altivelis*. Fish Pathol 43:167–169 (In Japanese with English abstract)
- Ninomiya K, Yamamoto M (2001) Therapeutic attempt to control of coldwater disease in ayu, *Plecoglossus altivelis* by using Sulphisol-Na. Bull Shiga Pref Fish Exp Sta 48:17–20 (In Japanese with English abstract)
- Rangdale RE, Richards RH, Alderman DJ (1997) Minimum inhibitory concentrations of selected antimicrobial compounds against *Flavobacterium psychrophilum* the causal agent of rainbow trout fry syndrome (RTFS). Aquaculture 158:193–201
- Sugahara K, Fujiwara-Nagata E, Eguchi M (2010a) Dynamics of bacterial cold-water disease pathogen, *Flavobacterium psychrophilum*, in infected fish organs and rearing water after warmed water treatment. Fish Pathol 45:58–65
- Sugahara K, Fujiwara-Nagata E, Fukuda A, Eguchi M (2010b) Viable but non-culturable state of bacterial cold-water disease pathogen *Flavobacterium psychrophilum* at various temperatures. Fish Pathol 45:158–163
- Toyama T, Kita-Tsukamoto K, Wakabayashi H (1994) Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. Fish Pathol 29:271–275
- Uddin M, Wakabayashi H (1997) Effect of temperature on growth and protease production of *Cytophaga psychrophila*. Fish Pathol 32:225–226
- Wakabayashi H, Toyama T, Iida T (1994) A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathol 29:101–104
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703
- Yamamoto MK, Takahashi S, Ninomiya (2001) Efficacy of the combined elevated water temperature treatment and chemotherapy for the bacterial coldwater disease in ayu. Bull Shiga Pref Fish Exp Sta 48:11–16 (In Japanese with English abstract)
- Yamamoto M, Sugahara K, Endo M, Ishimaru K, Kato K (2015) Epidemiological study of *Flavobacterium psychrophilum* in ayu *Plecoglossus altivelis* caught in Lake Biwa and the inflowing rivers from 1988 to 2011. Fish Pathol 50:97–104
- Yoshiura Y, Kamaishi T, Nakayasu C, Ototake M (2006) Detection and genotyping of *Flavobacterium psychrophilum* by PCR targeted to peptidyl-prolyl cis-trans isomerase C gene. Fish Pathol 41:67–71 (In Japanese with English abstract)

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