Spatiotemporal distribution of Flavobacterium psychrophilum and ayu Plecoglossus altivelis in rivers revealed by environmental DNA analysis

Tenma, Hinako ; Tsunekawa, Koki ; Fujiyoshi, Riho ; Takai, Hajime ; Hirose, Masae ; Masai, Nanami ; Sumi, Kosuke ; Takihana, Yuta ;…

(Citation)
Fisheries Science,87:321-330

(Issue Date)
2021-04-01

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90009283

Fisheries Science (2021) 87:321–330
https://doi.org/10.1007/s12562-021-01510-z

ORIGINAL ARTICLE

Biology

Spatiotemporal distribution of *Flavobacterium psychrophilum* and ayu *Plecoglossus altivelis* in rivers revealed by environmental DNA analysis

Hinako Tenma[1] · Koki Tsunekawa[1] · Riho Fujiyoshi[1] · Hajime Takai[1] · Masae Hirose[1] · Nanami Masai[1] · Kosuke Sumi[1] · Yuta Takihana[1] · Sogen Yanagisawa[1] · Kota Tsuchida[1] · Kenichi Ohara[2] · Toshiaki Jo[3,4] · Masaki Takagi[1,5] · Akiko Ota[1] · Hiroyoshi Iwata[1] · Yuichi Yaoi[1,6] · Toshifumi Minamoto[3]

Received: 25 October 2020 / Accepted: 19 March 2021 / Published online: 1 April 2021

Abstract

Outbreaks of bacterial cold-water disease (BCWD), caused by *Flavobacterium psychrophilum*, are widespread in Japan, especially among ayu *Plecoglossus altivelis*. There are few investigations of *F. psychrophilum* in river water, and its seasonal distribution has not been clarified. We aimed to identify the spatiotemporal dynamics of *F. psychrophilum* and ayu to provide information that is useful for establishing a countermeasure for BCWD. Quantitative analysis of environmental DNA (eDNA) was used to clarify the year-round dynamics of ayu and *F. psychrophilum*. We sampled river water from the Nagara and Ibi rivers in Japan, and conducted monthly water sampling and eDNA quantification. Changes in the eDNA concentration of ayu were consistent with the known life histories of the fish. There was a strong negative correlation between the eDNA concentration of *F. psychrophilum* and water temperature, suggesting a strong dependence of *F. psychrophilum* dynamics in the river on water temperature. Furthermore, relatively high eDNA concentrations were recorded for both organisms in early summer and fall, suggesting that ayu is infected with *F. psychrophilum* during these seasons when experiencing up- and downmigration, respectively.

Keywords Ayu *Plecoglossus altivelis* · Bacterial cold-water disease · Environmental DNA (eDNA) · *Flavobacterium psychrophilum* · Seasonal dynamics

Introduction

Outbreaks of bacterial cold-water disease (BCWD) in rivers cause serious damage to inland fisheries globally. BCWD is caused by a Gram-negative aerobic bacillus, *Flavobacterium psychrophilum* (Bernardet et al. 1996). The first BCWD case was reported in North America, and the pathogen was detected in the kidney of juvenile coho salmon *Oncorhynchus kisutch* in Washington State, USA (Borg 1960). Many cases of infection in rainbow trout *Oncorhynchus mykiss* have been reported from at least five continents, that is, Asia (Lee and Heo 1998), Europe (Austin and Stobie 1991), North America, Australia (Schmidtke and Carson 1995), and South America (Bustos et al. 1995), especially where Salmonidae species are present throughout the year.

In Japan, the first outbreak of BCWD was reported from an aquaculture farm in Tokushima Prefecture in 1987 (Wakabayashi et al. 1994), and the first outbreak in a natural river was confirmed in 1993 (Iida and Mizokami 1996).

✉ Toshifumi Minamoto
minamoto@people.kobe-u.ac.jp

1 Nature and Science Club Bioscience Team, Gifu Senior High School, Gifu City, Gifu, Japan

2 Gifu Prefectural Research Institute for Fisheries and Aquatic Environments, Kakamigahara City, Gifu, Japan

3 Graduate School of Human Development and Environment, Kobe University, 3-11, Tsurukabuto, Nada-Ku, Kobe, Hyogo 651-8501, Japan

4 Research Fellow of Japan Society for the Promotion of Science, Chiyoda-ku, Tokyo, Japan

5 Ogaki-Kita Senior High School, Ogaki City, Gifu, Japan

6 Yaizu-Chuo Senior High School, Yaizu City, Shizuoka, Japan

Subsequently, BCWD has spread throughout the country, causing serious damage to inland water industries nationwide (Inoue 2000). The dynamics of BCWD in rivers have been investigated in several previous studies. The occurrence of BCWD is significantly influenced by water temperature, and low water temperatures (15–20 °C) are considered to be the optimum temperature for the pathogen (Uddin and Wakabayashi 1997). In addition, the stress-induced decrease in immunity of ayu fish due to rapid changes in water temperature (Valle and Taguchi 1995), high population density (Iguchi et al. 2002, 2003), and high concentration of suspended matter in river water (Awata et al. 2011) is also considered to be a factor in the development of BCWD. There has been a debate regarding the carriers of BCWD in rivers. Farmed ayu (Kumagai et al. 2010), juvenile ayu from the sea (Kumagai et al. 2011), and fish species other than ayu (Takeuchi et al. 2016) have been identified as carriers, and the pathogen has been isolated from attached algae, sediments, and aquatic insects in river beds (Amita et al. 2000; Fujiwara-Nagata et al. 2019). It has also been suggested that the pathogens may be present in the river even in the seasons when ayu is not present in the river (Hara et al. 2007). Thus, the dynamics of BCWD in rivers have not been fully elucidated, and it is important to accumulate knowledge on the dynamics of the pathogen in rivers to promote disease control in rivers.

To understand the dynamics of ayu and *F. psychrophilum* efficiently, we utilized environmental DNA (eDNA) analysis. eDNA is defined as the total pool of DNA isolated from environmental samples (Taberlet et al. 2018). Using eDNA detection, the distribution of target organisms can be clarified (Ficetola et al. 2008; Yamanaka and Minamoto 2016). In addition, it is known that the concentration of eDNA is proportional to the biomass of organisms (Takahara et al. 2012). Conventional survey methods, such as visual inspection and direct catch to investigate the ecology of ayu that migrates between rivers and the sea, require a lot of time and labor. In contrast, eDNA analysis, which requires low labor and is not harmful to the environment, is useful for monitoring such organisms (Thomsen and Willerslev 2015). This technology can also detect *F. psychrophilum* at low concentrations in river water. Because both ayu and *F. psychrophilum* can be detected simultaneously in river water samples, the dynamics of pathogens and their host species can be monitored at the same time and place, under the same conditions.

Ayu is an amphidromous fish: its larvae hatch in the middle to lower reaches of the river in autumn and downmigrate to and stay along the ocean coast in winter; grown-up juveniles run up the river in spring, mature fish inhabit upper and middle reaches in the summer, and return to spawning grounds in autumn (Wakabayashi 2009: https://colloque.inra.fr/flavobacterium2009/content/download/3303/33796/file/23Wakabayashi.pdf). It is also known that some populations do not migrate and do not spawn eggs in autumn, and these are called overwintering ayu (Miyazaki 2008). In addition to these natural ayu, many ayu are released as resources for fisheries. For example, approximately 40,000 kg of ayu were released in the Nagara River and about 9000 kg in the Ibi River both in 2018 and 2019 (Communication with the Gifu Prefecture Fisheries Association). As a result, about 70% of ayu in the Nagara River are of natural origin (Aino et al. 2015). In addition to its importance in the fisheries industry, ayu has deep cultural relationships with people in Japan. In Gifu Prefecture, the relationship between ayu and people in the Nagara River basin has been evaluated, and ayu of the Nagara River system has been designated as a Globally Important Agricultural Heritage System since 2015 (Council to Promote the Ayu of the Clear Nagara River, a World Agricultural Heritage Site: https://giahs-ayu.jp/en/, accessed 7 October 2020). The spread of BCWD not only causes a great loss in commercial value but also affects the cultural relationship between ayu and people.

In this study, we aimed to clarify the seasonal distribution of both the pathogens and hosts. Therefore, we compared the spatiotemporal eDNA dynamics of ayu and *F. psychrophilum* from the same eDNA sample through year-round water sampling in the Nagara and Ibi rivers. Both rivers are famous for the fishing and farming of ayu, have similar lengths, and share the river mouth and estuary. However, the Nagara River has an estuary weir, which divides the river from the sea beside the installed fish ladder, whereas the Ibi River has no estuary weir. Therefore, we investigated the seasonal dynamics of ayu and *F. psychrophilum* in these two rivers with different topographical conditions. In addition to the eDNA survey, to validate the time of infection by *F. psychrophilum*, we tested the infection status of ayu.

Materials and methods

Water sampling and DNA extraction for eDNA estimation

eDNA surveys were conducted throughout the year to clarify the distribution and relative biomass of ayu in the Nagara River (length, about 110 km) and Ibi River (about 88 km). From 27 July 2017 to 23 June 2018, we sampled 1 L of river water almost every month at 16 sites (9 and 6 in the Nagara and Ibi rivers, respectively, and 1 at the river mouth estuary, which is shared by both rivers) (Fig. 1). Sampling was conducted from 6 a.m. to 4 p.m. on each sampling day. Water samples were collected using plastic bottles sterilized with chlorine bleaching. One milliliter of 10% benzalkonium chloride solution was added to prevent the degradation of DNA (Yamanaka et al. 2017). After water collection, the

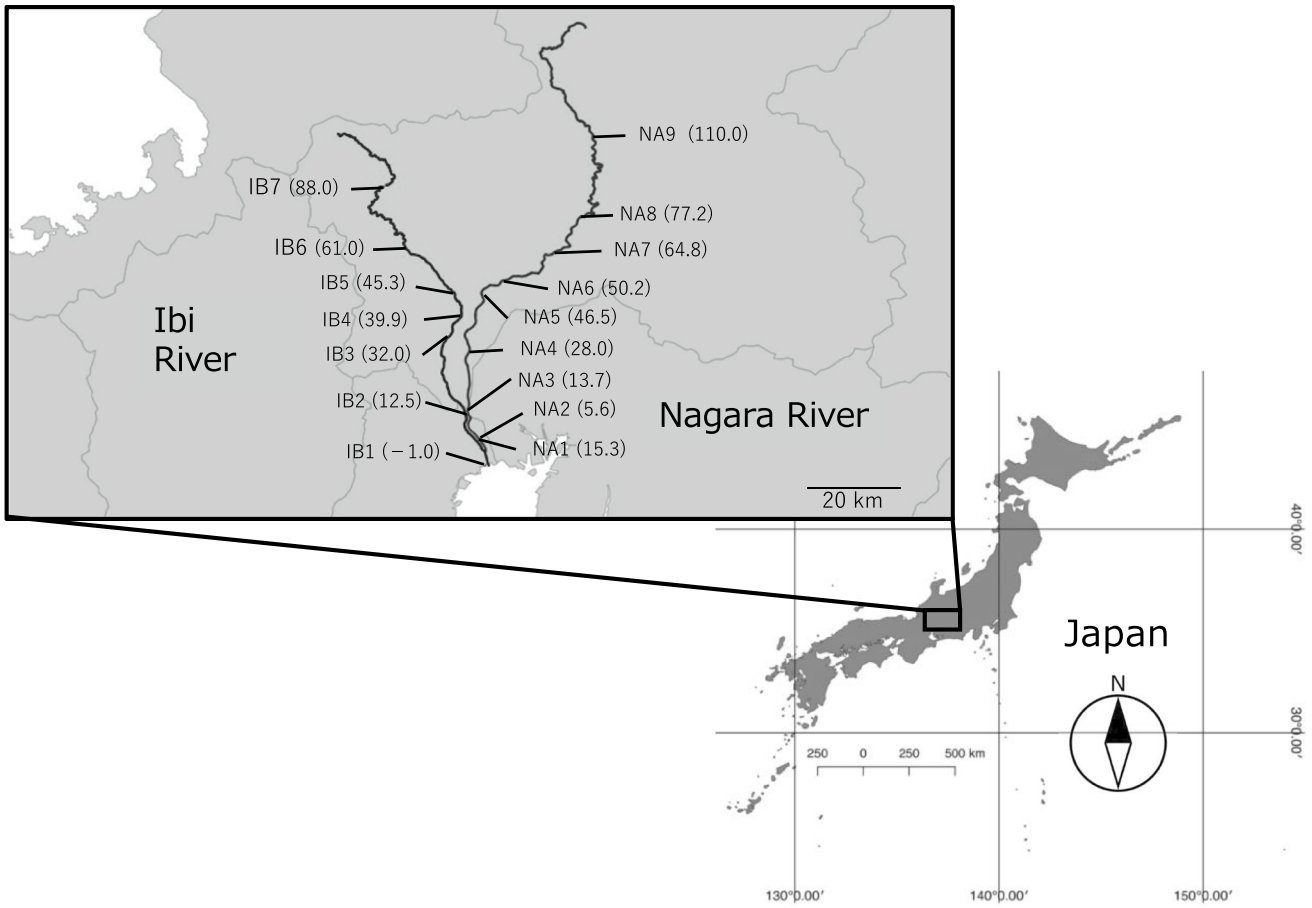


Fig. 1 Sampling locations along the Nagara and Ibi rivers (bold lines) in Japan. We selected nine sites along the Nagara River (NA1 to NA9), six sites along the Ibi River (IB2 to IB7), and one site at the mouth of both rivers (IB1). Numerals in parentheses indicate the distance of each sampling site from the river mouth (km), and gray lines represent the prefectural borders

bottles were kept in a cooler box filled with ice. The temperature of the river surface water was measured on-site.

One liter of sampled river water was filtered with a glass fiber filter (GE Healthcare, GF/F 0.7 µm). Through this filtration, we could collect eDNA from macro- and microorganisms without distinguishing between intracellular and extracellular origins. DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN) as described by Miya et al. (2015). Briefly, 220 µL solutions comprising 20 µL proteinase K and 200 µL Buffer AL were placed on the filter and incubated at 56 °C for 30 min. After incubation, the samples were processed according to the manufacturer's protocol to obtain a final elution volume of 100 µL (The eDNA Society 2019). The DNA solution was stored at −20 °C. Disposable gloves were worn throughout the experimental procedure to avoid contamination.

Table 1 Primers and probes used for real-time PCR for ayu and *Flavobacterium psychrophilum*

Target species	Name	Sequence (5′–3′)	Reference
Ayu	Paa-Cytb (forward)	CCTAGTCTCCCTGGCTTTATTCTCT	Yamanaka and Minamoto (2016)
	Paa-Cytb (reverse)	GTAGAATGGCGTAGGCGAAAA	Yamanaka and Minamoto (2016)
	Paa-Cytb (probe)	FAM-ACTTCACGGCAGCCAACCCCC-TAMRA	Yamanaka and Minamoto (2016)
Flavobacterium psychrophilum	PPIC-Q (forward)	CCTTCGATGTAGTTTCTGTGCCATA	Ohara et al. (2010)
	PPIC-Q (reverse)	TTCTAATTCACGAGATTCGTCTGCT	Ohara et al. (2010)
	PPIC-Q (probe)	FAM-AAGTAGAACCTACAGATGCGGAAC-TAMRA	This study

Real-time PCR for ayu eDNA

Real-time polymerase chain reaction (PCR) was used for quantitative analysis of ayu eDNA, using primers and probes described by Yamanaka and Minamoto (2016) targeting a part of the mitochondrial Cytb gene (131 bp) (Table 1). Real-time PCR reactions were performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) with the following conditions: initial steps of 50 °C for 2 min and 95 °C for 10 min, and 55 cycles of 95 °C for 15 s and 60 °C for 60 s. Real-time PCR was performed in triplicate for all samples, quantification standards, and negative controls. The artificially synthesized gene of partial ayu Cytb DNA (131 bp) was diluted to contain 3.0×10^1, 3.0×10^2, 3.0×10^3, and 3.0×10^4 copies per reaction and used as quantification standards. Pure water was used as a negative control. The average value of the triplicates was calculated. When a nonamplified replicate was observed, the DNA concentration of the replicate was set to 0 and included in the calculation of triplicates. The DNA concentration per 5 µL eDNA sample was multiplied by 20 to convert the value to DNA concentration per 1 L of water.

Real-time PCR for *F. psychrophilum* eDNA

Real-time PCR was performed for the eDNA of *F. psychrophilum* using the samples from seven representative sites that were selected to overview the dynamics of *F. psychrophilum* in the river. Samples collected from NA3, NA6, NA9, IB1, IB2, IB5, and IB6 from 30 September 2017 to 2 September 2018 were analyzed. We used the primers described by Ohara et al. (2009) targeting the peptidyl-prolyl cis–trans isomerase C (PPIC) gene region (131 bp) (AB254195) (Table 1). A TaqMan probe was designed for this study. The amplification and calculation of eDNA concentrations were conducted as described above.

A total of six samples of NA3 and NA9 collected on 4 November 2017, and on 6 January and 28 April 2018, were used for direct sequencing. The PCR products were purified using the Exo SAP-IT Kit (GE Healthcare). The purified DNA products were directly sequenced with the same primers as those used for amplification using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 in an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific).

Statistical analysis

Statistical analysis was performed using R version 3.6.1 (R Core Team 2019). We performed a generalized linear mixed model (GLMM) analysis with a binomial distribution to assess the relationship between water temperature and survival of *F. psychrophilum*. We treated the detection or nondetection of *F. psychrophilum* eDNA in a given sample (i.e., 1 or 0) as the dependent variable, water temperature as the explanatory variable, and the sampling site as the random effect. Target eDNA was considered as detected in the sample if any of the PCR replicates showed a positive PCR amplification. Moreover, based on the logistic regression, we calculated the water temperature at which the detection rate of *F. psychrophilum* eDNA was 50%, assuming that the value represented the temperature threshold at which *F. psychrophilum* could survive in water.

Infection status of ayu during the upmigration period

To understand the infection status of *F. psychrophilum* before the prevalence of an epidemic, specimens were collected from the Nagara River estuary weir from April to May 2019, the upstream migration period of ayu. The survey was conducted at the Nagara Estuary Barrage Operating and Maintenance Office, with a special permit for capture from Mie Prefecture. In addition, on 22 June 2019, ayu was collected from the middle Nagara River basin by fishing. The collected ayu was fixed with ethanol and stored at −20 °C. Gills and kidneys were removed from each individual, and 0.01 g of each was used to extract DNA with the DNeasy Blood and Tissue Kit to obtain a final elution amount of 150 µL. The obtained DNA solution was stored at −20 °C. *F. psychrophilum* was detected using real-time PCR in the same manner as described above.

Results

Seasonal dynamics of ayu eDNA

We investigated the seasonal distribution of eDNA of ayu to determine its dynamics. The results of eDNA analysis and fluctuation of water temperature for each point are shown in Fig. 2. No amplification was confirmed from any negative control.

In the Nagara River, a high concentration of ayu eDNA was detected from June to December, and the concentration was relatively low from January to April. In spring, the concentration was high in the midstream region (NA5–NA6); in summer, in the upstream region (NA6–NA9); and it sharply increased in autumn. The maximum amount of 849,425.6 copies/L was recorded from NA6 on 30 September 2017.

In the Ibi River, a high concentration of ayu eDNA was detected from May to November, and it decreased from January to April. It was detected in high concentrations in the midstream region (IB3) from spring to summer, and from midstream to downstream region (IB2–IB5) in autumn. The maximum amount of 92,798.0 copies/L was recorded from IB4 on 30 July 2017.

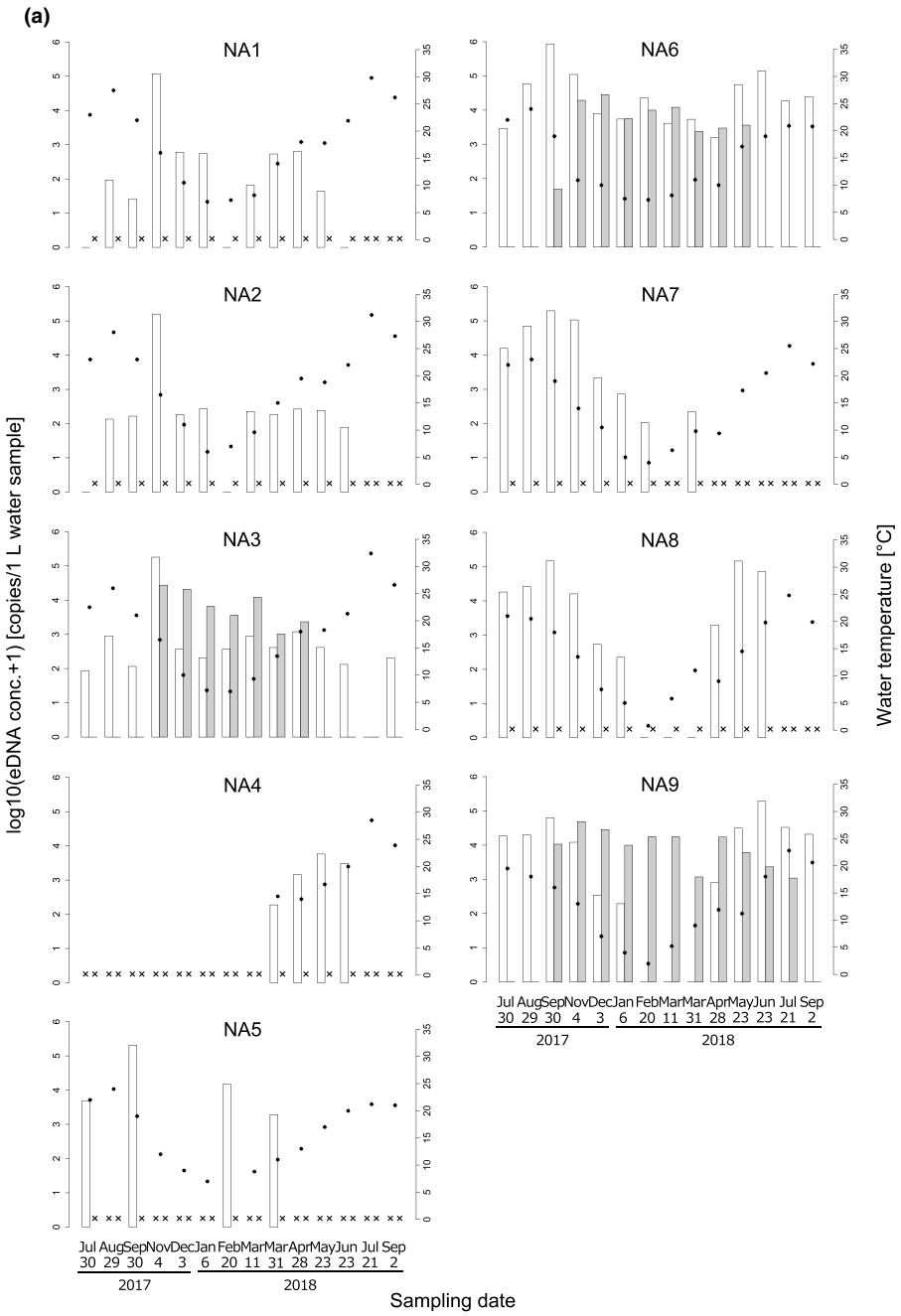


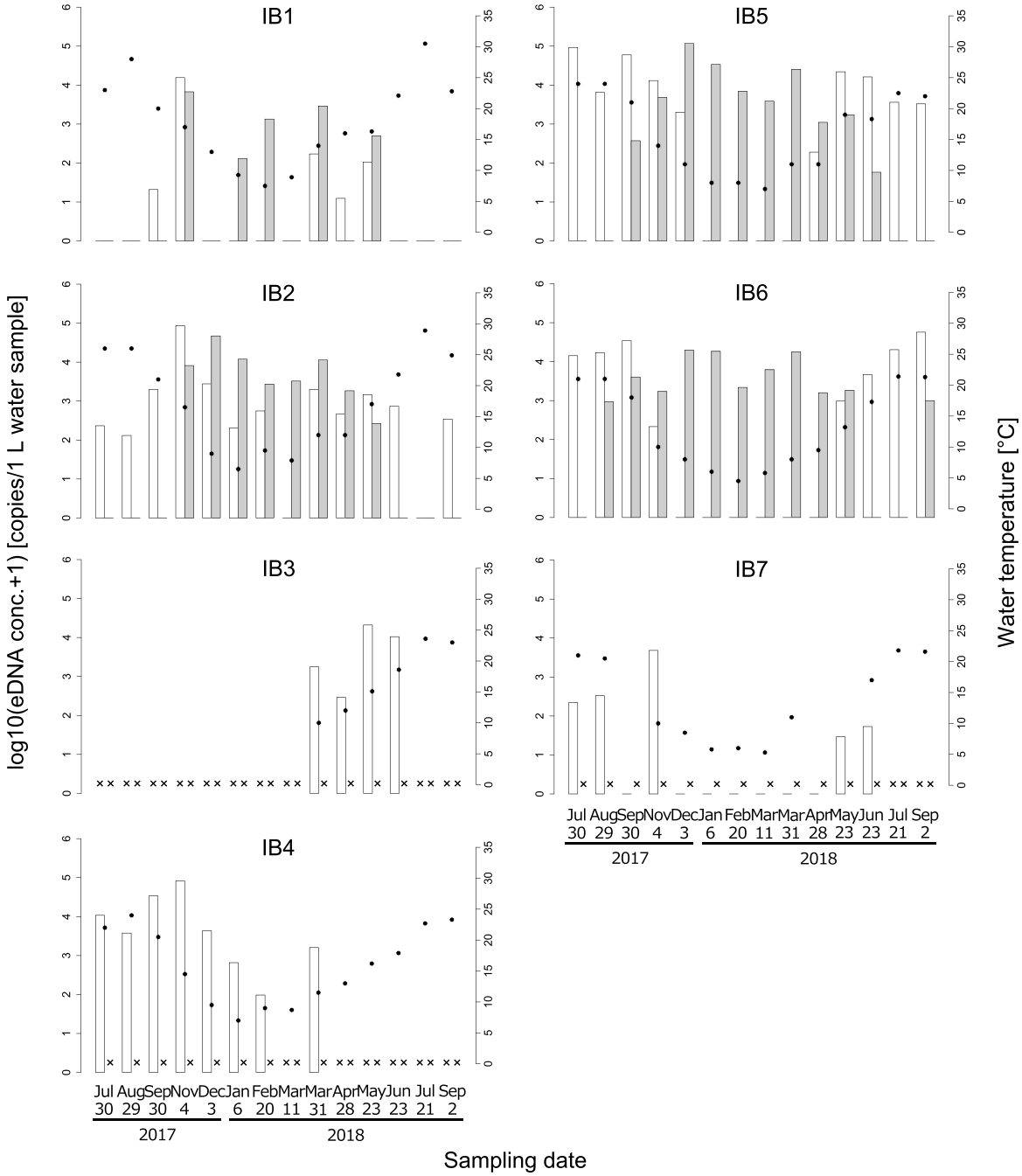
Fig. 2 Seasonal fluctuations in environmental DNA (eDNA) concentrations from ayu (white bar) and *Flavobacterium psychrophilum* (gray bar) and water temperature (black dot) in the Nagara (**a**) and Ibi (**b**) rivers. Left and right axis represent the eDNA concentrations (log-transformed, copies/1 L water sample) and water temperature (°C), respectively. Symbol "×" represents that riverine water samples for detection of ayu or *F. psychrophilum* eDNA were not collected on a given date

Seasonal dynamics of *F. psychrophilum* eDNA

We surveyed the seasonal distribution of *F. psychrophilum* eDNA to clarify the dynamics of bacteria in the rivers and to compare them with the dynamics of ayu. eDNA of *F. psychrophilum* was not detected in any of the samples on 30 July 2017. On 3 November 2017, 48,533.8 copies/L were detected in NA9, and on 6 January 2018, 11,913.4 copies/L were detected in IB2. On 28 April 2018, 17,222.1 copies/L were detected in NA9. No PCR amplification was confirmed from any of the negative controls. The sequences of amplicons derived from river water eDNA matched with those of *F. psychrophilum* genotype A (Izumi et al. 2003; Yoshiura et al. 2006).

Results of the GLMM analysis showed that water temperature significantly explained the detection/nondetection of *F. psychrophilum* eDNA (Table 2; $P < 0.001$); the detectability of *F. psychrophilum* eDNA decreased as

Fig. 2 (continued) **(b)**



water temperature increased. The detection rate of *F. psychrophilum* was 50% at a water temperature of 19.8 °C (Fig. 3).

Infection status of ayu during the upmigration period

From the real-time PCR test, DNA of *F. psychrophilum* was detected in the gills and kidneys of ayu during the upmigration period (Table 3). Sixty-seven percent of ayu

Table 2 Result of the generalized linear mixed model assessing the relationship between water temperature and detectability of *Flavobacterium psychrophilum* environmental DNA

	Estimate	SE	*P* value
Intercept	12.0729	3.6661	***
Water temperature	−0.6095	0.1833	***

***$P < 0.001$

carried *F. psychrophilum* in the gills on 2 April 2019, before the apparent occurrence of the disease, and the carriage rate detected from the gills increased to 90% on 22 April, and was 100% on 13 May and 22 June. The carriage rates in the kidney were 80% and 60% on 22 April and 13 June, respectively.

Discussion

We conducted a quantitative analysis of the eDNA of ayu and *F. psychrophilum* in the rivers in Gifu Prefecture, Japan. In Gifu Prefecture, BCWD caused by *F. psychrophilum* was first identified in in 1996 (Hara et al. 2007) and has been a threat to fishery industries as well as to river ecosystems, and understanding its dynamics in rivers is expected to lead to the control of disease outbreaks. The eDNA distribution of both ayu and *F. psychrophilum* matched the known dynamics of both organisms: up- and downmigration of ayu in spring and autumn, respectively, and that of *F. psychrophilum* increased from November to May and decreased from June to September. Ayu and *F. psychrophilum* coexisted for a limited period in the river, during the up- and downmigration season of ayu, and therefore, the infection could be occurring during these seasons.

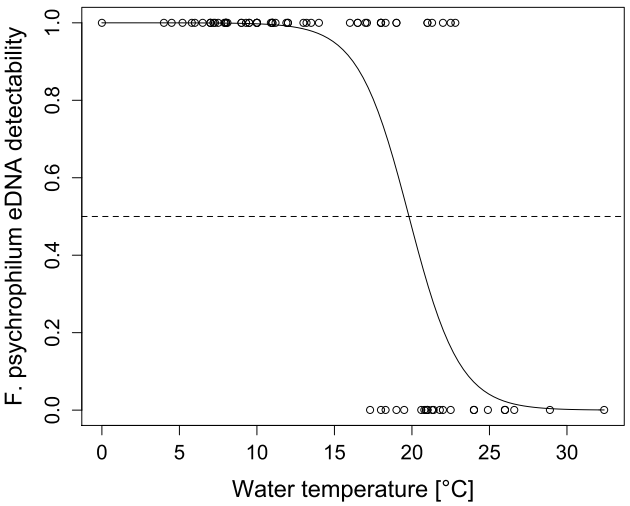


Fig. 3 Relationship between water temperature and the detectability of *Flavobacterium psychrophilum* environmental DNA (eDNA) in the Ibi and Nagara rivers. Each plot indicates the detection or non-detection of *F. psychrophilum* eDNA in water samples. A logistic regression curve was described using the parameters obtained using a generalized linear mixed model, both of which were statistically significant (Table 2). The intersection between the regression curve and the dashed line indicates the water temperature (19.8 °C) at which the detectability of *F. psychrophilum* eDNA was 50%

Distribution of ayu eDNA in the Nagara and Ibi rivers

The overall results of the eDNA survey for ayu were consistent with the known life history of ayu. In particular, the decrease in DNA concentration in the upstream region (NA9, IB6, and IB7) and the increase in DNA concentration in the middle reaches of the river (NA6 and IB5) on 30 September 2017, accurately reflected the spawning migration of ayu in these rivers (Komada 2016). In addition, NA5 and NA6 are known spawning grounds where many ayu spawn every year and the result matched the local ecology. This suggests that the eDNA survey is efficient for surveying the seasonal distribution of ayu in rivers over 100 km in length in addition to the shorter rivers surveyed previously (Saba River, Doi et al. 2017; Yodo River, Yamanaka and Minamoto 2016). The maximum eDNA concentration of ayu obtained in this study (8.3×10^6 copies/L) was larger than that recorded in other rivers (2.2×10^4 copies/L in the Gonokawa River [Inui et al. 2017] and 1.5×10^7 copies/L in the Tama River [Naito et al. 2018]; both rivers known to be rich ayu habitats), suggesting the presence of large ayu resources in the Nagara and Ibi rivers. However, since DNA concentrations increase during the spawning season, we need to be careful when comparing fish abundance based on eDNA levels. As a new finding, the eDNA of ayu was detected in some spots in January and February, after the spawning migration of most individuals, suggesting the

Table 3 Details of ayu collected from the Nagara River

Date	Location	No. of individuals	Mean ± SD of body weight (g)	Mean ± SD of body length (mm)	% (number of analyzed samples) of individuals positive for *Flavobacterium psychrophilum* using qPCR	
					Gill	Kidney
02 April 2019	NA2	9	–	–	66.7(9)	–
22 April 2019	NA2	10	–	–	90 (10)	80 (5)
13 May 2019	NA2	10	1.7 ± 0.6	69.9 ± 7.1	100 (10)	60 (5)
22 June 2019	NA6	12	7.7 ± 5.4	101.1 ± 19.3	100 (12)	–

presence of overwintering ayu that stay in a wide area of the river through winter. Overwintering ayu (those which live in the river in winter when other populations of ayu go down the river and live along the coast) have been reported in many rivers in Japan (Suzuki 1939; Sakae et al. 1996; Miyazaki 2008; Suzuki 2016). Although there are no official records of overwintering ayu in the Nagara and Ibi rivers, there is a possibility they may be present. In a previous study (Miyazaki 2008), *F. psychrophilum* was detected in the overwintering ayu in the Sho River, and they were suspected to be hosts of *F. psychrophilum* in winter and cause continuous infection by *F. psychrophilum* in the river.

Seasonal distribution of *F. psychrophilum* in the Nagara and Ibi rivers

Detection rate of *F. psychrophilum* in the rivers was very low from May to August. The eDNA concentration of *F. psychrophilum* in the downstream regions of the river increased in November, and maintained a higher concentration throughout the river during winter and early spring. The eDNA concentration of *F. psychrophilum* increased and decreased in the rivers without necessarily depending on the distribution of ayu, which suggested that the period in which ayu and *F. psychrophilum* coexisted in the river was limited. Generally, BCWD of ayu prevails before and after the rainy season, from May to July, which led to the hypothesis that *F. psychrophilum* would be detected mainly from May to July. However, our results suggest that ayu might be infected with *F. psychrophilum* earlier, during the up- and downmigration stages, before the apparent epidemic of BCWD.

The results of our surveys showed that *F. psychrophilum* was detected during the period of low water temperature and not during the high-temperature period. The GLMM results showed a negative effect of water temperature on the detection of *F. psychrophilum* eDNA, and the temperature threshold for the presence or absence of *F. psychrophilum* was 19.8 °C. In a previous laboratory-based heating experiment on *F. psychrophilum*, the bacterial population decreased at 23 °C and was inactivated at 28 °C. The decrease in detection rate of *F. psychrophilum* at higher temperatures is in line with previous findings, and shows the usefulness of eDNA analysis for the survey of *F. psychrophilum*.

eDNA of *F. psychrophilum* was absent from river surface water during summer, and their whereabouts remain unknown. It is possible that *F. psychrophilum* lives inside the body of ayu, in other fish that do not develop the disease, in attached algae on the riverbed, or in the deeper layers of the river, and requires further investigation. Furthermore, detailed investigation is needed to determine the localization of *F. psychrophilum* in winter. One possibility is that *F. psychrophilum* is held by the overwintering ayu. As mentioned above, the wintering ayu behaves differently from those with the well-known life cycle, and a case has been reported (Miyazaki 2008), in which *F. psychrophilum* was isolated from a wintering ayu when it was caught in April. There is a possibility that the overwintering ayu transmits the pathogen to the next generation of ayu.

Infection status of ayu during the upmigration period

From the above results, we hypothesized that ayu is likely to be infected with *F. psychrophilum* during the up- and downmigration periods, and the infectious status was checked during the upmigration period. We found that ayu harbors *F. psychrophilum* in early April when it migrates from the ocean coast to the river. This result is consistent with previous reports that showed that ayu harbors *F. psychrophilum* just after freshwater acclimation in April and May before the epidemic (Amita et al. 2000; Fujii 2009).

Considering the overall results, we suggest a mechanism of *F. psychrophilum* infection: during the up- and downmigration periods, when the ayu concentrates in a particular area of the river, ayu is infected with *F. psychrophilum*, which causes its death in June or October. Although the mechanism of transgenerational transmission of BCWD is not clearly understood, it has been shown that intra-egg infection is caused by the invasion of *F. psychrophilum* when it attaches to the egg surface in salmonid fish (Kumagai and Nawata 2010). Because eDNA of *F. psychrophilum* was also detected at a high concentration in areas below the spawning area of ayu, it is considered that *F. psychrophilum* is concentrated in the river water near the spawning ground. Additionally, because it is possible that a high concentration of eDNA of *F. psychrophilum* is released from the carcasses of ayu that have finished spawning (Ohara et al. 2010), there is a possibility of horizontal infection of eggs at the spawning ground.

In our study, because more than 90% of individuals were infected with *F. psychrophilum* after June, we suggest that many ayu have the possibility of developing the disease. The factors involved in the development of BCWD are suggested to be: (1) the increase in the growth efficiency of *F. psychrophilum* in ayu owing to the decrease in water temperature during the rainy season, and (2) the decrease in the immunity of ayu due to various stresses, such as rapid changes in water temperature, territorial conflict, and spawning behavior (Iguchi and Matsubara 2002; Iguchi 2003). In the spawning and downmigration seasons, it is possible that ayu larvae are infected with *F. psychrophilum*.

Perspectives for future countermeasures against BCWD

Based on the results of this study, the optimization of the timing of the release of ayu is considered to be an effective measure against damage from BCWD. In Japan, ayu has been released when the water temperature is higher than 13 °C according to the guidelines of the Ayu BCWD Countermeasures Council, the Ministry of Agriculture (Sato 2018). While this approach is believed to be empirically correct, it is not based on the mechanism of infection. This study revealed the period when *F. psychrophilum* spreads in rivers. In the future, by examining the release time using the amount of *F. psychrophilum* in rivers as an index, the damage caused by BCWD can be further suppressed. In Gifu Prefecture, the improvement in ayu seedlings resistant to BCWD is underway (Kuwada et al. 2010). Constructing an effective release plan for ayu possessing this resistance and conventionally farmed ayu in the future and conducting eDNA surveys in rivers will help to promote epidemic prevention in rivers by using the habitat survey of *F. psychrophilum* as an index.

As described above, *F. psychrophilum* invades and settles in the river ecosystem and becomes a threat to ayu owing to past release projects. In addition to ayu, various biological release projects are currently being conducted globally, which can cause the spread of alien organisms and the spread of pathogenic bacteria such as *Edwardsiella ictaluri* (Nagai and Iida 2008). By utilizing the eDNA survey used in this research as a method for host and pathogen monitoring, early countermeasures against various problems caused by alien pathogens can be taken and damage to wild and planted organisms can be reduced or prevented. Through such surveys, it is possible to control the disease by identifying the infection time and onset time of the disease. Through such a control measure of BCWD, we can protect ayu not only as commercial resources but also as cultural heritage.

Acknowledgements We thank Prof. Takahiko Mukai (Gifu University) for helping with genetic sequencing; Prof. Hideki Innan (The Graduate University for Advanced Studies) for helping with the statistical analysis; and Masataka Tagami (World Freshwater Aquarium Aquatotto Gifu), Prof. Morihiro Harada (Gifu University), Yasuo Niimura (freelance photo-ecologist), Tsuyoshi Ookuma (Nagaragawa Fishery Cooperative), and Isao Takahashi (Takahashi Research Office of Freshwater Biology) for helping with field surveys. We appreciate the help of the River Management Division of Gifu Prefecture for providing the map of the rivers; Chubu Regional Development Bureau of the Japanese Ministry of Land, Infrastructure, Transport and Tourism for supporting field surveys; Nagaragawa Estuary Barrage Operating and Maintenance Office of Incorporated Administrative Agency Japan Water Agency for helping with the field survey and sampling ayu; and Nagaragawa-Chuo Fishery Cooperative and Nagaragawa Fishery Cooperative for permitting ayu collection. This study was partly supported by the Ito Scholarship Foundation and by the Tohnetsu-Foundation.

References

Aino S, Yodo T, Yoshioka M (2015) Changes in the composition of stock origin and standard length of ayu *Plecoglossusaltivelisaltivelis* during the Tomozuri angling season in the Nagara River, central Japan. Fish Sci 81:37–42. https://doi.org/10.1007/s12562-014-0822-y

Amita K, Hoshino M, Honma T (2000) An investigation of the distribution of *Flavobacterium psychrophilum* in the Umikawa river. Fish Pathol 35:193–197. https://doi.org/10.3147/jsfp.35.193 (**in Japanese with English abstract**)

Austin B, Stobie M (1991) Recovery of yellow pigmented bacteria from dead and moribund fish during outbreaks of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fry syndrome in England. J Fish Dis 14:677–682. https://doi.org/10.1111/j.1365-2761.1991.tb00626.x

Awata S, Tsuruta T, yada T, Iguchi K (2011) Effects of suspended sediment on cortisol levels in wild and cultured strains of ayu *Plecoglossus altivelis*. Aquaculture 314:115–121. https://doi.org/10.1016/j.aquaculture.2011.01.024

Bernardet JF, Sergers P, Vancannteyt 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 hydatids* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int J Syst Bacteriol 46:128–148. https://doi.org/10.1099/00207713-46-1-128

Borg AF (1960) Studies on myxobacteria associated with diseases in salmonid fishes. J Wildl Dis 8:1–85

Bustos PA, Calbuyahue J, Montana J, Opazo B, Entrala P, Solervicens R (1995) First isolation of *Flexibacter psychrophilus*, as causative agent of rainbow trout fry syndrome (RTFS), producing rainbow trout mortality in Chile. Bull Eur Assoc Fish Pathol 15:162–164

Doi H, Inui R, Akamatsu Y, Kanno K, Yamanaka H, Takahara T, Minamoto T (2017) Environmental DNA analysis for estimating the abundance and biomass of stream fish. Freshw Biol 62:30–39. https://doi.org/10.1111/fwb.12846

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biol Lett 4:423–425. https://doi.org/10.1098/rsbl.2008.0118

Fujii H (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*. Aquac Sci 54:621–622 (**in Japanese with English abstract**)

Fujiwara-Nagata E, Shindoh Y, Yamamoto M, Okamura T, Takegami K, Eguchi M (2019) Distribution of *Flavobacterium psychrophilum* and its gyrA genotypes in a river. Fish Sci 85:913–923. https://doi.org/10.1007/s12562-019-01355-7

Hara T, Kuwada T, Saitou K (2007) The movement of cold water disease in river instance of stocking of ayu not infected with

cold-water disease. Bull Gifu Pref Fish Exp Sta 52:1–4 (**in Japanese**)

Iguchi K (2003) The influence of rearing density on stress response and disease susceptibility of ayu. Aquaculture 220:515–523. https://doi.org/10.1016/S0044-8486(02)00626-9

Iguchi K, Matsubara N (2002) Reduction of transport stress of ayu by obligated schooling. Fish Sci 68:849–853. https://doi.org/10.1046/j.1444-2906.2002.00502.x

Iida Y, Mizokami A (1996) Outbreaks of coldwater disease in wild ayu and pale chub. Fish Pathol 31(3):157–164. https://doi.org/10.3147/jsfp.31.157

Iguchi K, Ito F, Ogawa K, Matsubara N, Yodo T, Yamasaki T (2002) Reduction of transport stress of ayu by obligated schooling. Fish Sci 68:849–853. https://doi.org/10.1046/j.1444-2906.2002.00502.x

Iguchi K, Ogawa K, Nagae M, Ito F (2003) The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*). Aquaculture 220:515–523. https://doi.org/10.1016/S0044-8486(02)00626-9

Inoue K (2000) Cold water disease in ayu. Kaiyo to Seibutsu 22:35–38 (**in Japanese**)

Inui R, Goto M, Kono T, Kakenami Y, Hitotsumatsu A, Kakenami Y, Hitotsumasu A (2017) Environmental DNA analysis for estimating the resources of *Plecoglossus altivelis* in Gonokawa river. J Japan Soc Civil Eng Ser B1 73:1105–1100. https://doi.org/10.2208/jscejhe.73.I_1105 (**in Japanese with English abstract**)

Izumi S, Aranishi F, Wakabayashi H (2003) Genotyping of *Flavobacterium psychrophilum* using PCR-RFLP analysis. Dis Aquat Org 56:207–214. https://doi.org/10.3354/dao056207

Komada Y (2016) Ayu in Nagara River: from 40 years of field research. Gifu Shimbun, Gifu, Japan (**in Japanese**)

Kumagai A, Nawata A (2010) Mode of the intra-ovum infection of *Flavobacterium psychrophilum* in salmonid eggs. Fish Pathol 45(1):31–36

Kumagai A, Nawata A, Taniai Y (2010) Monitoring of out breaks of bacterial cold water disease among ayu in a river where asymptomatic carriers of *Flavobacterium psychrophilum* were released. Fish Pathol 45(3):115–120. https://doi.org/10.3147/jsfp.45.115

Kumagai A, Nawata A, Ototake M (2011) Prevalence of *Flavobacterium psychrophilum* among wild ayu in rivers that do not have a history of ayu stocking. Fish Pathol 46(3):91–94. https://doi.org/10.3147/jsfp.46.91

Kuwada T, Kageyama T, Ohara K, Hara T, Saito K (2010) Increased resistance to an infectious cold-water disease and the improved catchability on "TOMO-ZURI" angling, using selective breeding in ayu (*Plecoglossus altivelis altivelis*)—difference in the resistance to cold-water disease, catchability on "TOMO-ZURI" angling and genetic profile among the three strains. Report of Gifu Prefectural Research Institute for Freshwater Fish and Aquatic Environments 55:5–15 (**in Japanese**)

Lee KB, Heo GJ (1998) First isolation and identification of *Cytophaga psychrophila* from cultured ayu in Korea. Fish Pathol 33:37–38. https://doi.org/10.3147/jsfp.33.37

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R Soc Open Sci 2:150088. https://doi.org/10.1098/rsos.150088

Miyazaki T (2008) *Flavobacterium psychrophilum* isolated from overwintering ayu *Plecoglossus altivelis*. Fish Pathol 43:167–169. https://doi.org/10.3147/jsfp.43.167 (**in Japanese with English abstract**)

Nagai T, Iida Y (2008) Characterization of *Edwardsiella ictaluri* isolated from wild ayu *Plecoglossus altivelis* in Japan. Fish Pathol 43:158–163. https://doi.org/10.3147/jsfp.43.158

Naito D, Akamatsu Y, Inui R, Goto M, Kobayashi T, Imamura F (2018) Habitats *Plecoglossus altivelis* in the Tama river basin by environmental DNA. J Japan Soc Civil Eng Ser B1 74:517–522. https://doi.org/10.2208/jscejhe.74.I_517 (**in Japanese with English abstract**)

Ohara K, Kageyama T, Kuwada T, Umino T, Furusawa S, Yoshiura Y (2009) Quantitative estimation of *Flavobacterium psychrophilum* infected ayu *Plecoglossus altivelis altivelis* by real-time PCR. Nippon Suisan Gakkaishi 75:258–260. https://doi.org/10.2331/suisan.75.258 (**in Japanese**)

Ohara K, Kageyama T, Kuwada T, Umino T, Furusawa S (2010) Quantitative estimation of *Flavobacterium psychrophilum* discharged from infected ayu *Plecoglossus altivelis altivelis*by real-time PCR. Nippon Suisan Gakkaishi 76:705–707. https://doi.org/10.2331/suisan.76.705 (**in Japanese with English abstract**)

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Sakae K, Umino T, Takahara Y, Arai K, Nakagawa H (1996) Biological and biochemical characteristics of yearling ayu in the Ohta River of Hiroshima Prefecture. Nippon Suisan Gakkaishi 62:46–50. https://doi.org/10.2331/suisan.62.46 (**in Japanese with English abstract**)

Sato M (2018) Stocking effectiveness of ayu Plecoglossus altivelis during the early season in a tributary of the Yoneshiro River basin. Aquac Sci 66:227–233 (**in Japanese with English abstract**)

Schmidtke LM, Carson J (1995) Characteristics of *Flexibacter psychrophilus* isolated from Atlantic salmon in Australia. Dis Aquat Organ 21:157–161. https://doi.org/10.3354/dao021157

Suzuki K (1939) Regeneration of gonads in *Plecoglossus altivelis* after Spawning Season. Cytologia 10:113–126. https://doi.org/10.1508/cytologia.10.113

Suzuki K (2016) Age and migration history of yearling ayu that appeared in Kawazu-ratsu River. Bull Shizuoka Pref Res Inst Fish 49:21–26 (**in Japanese with English abstract**)

Taberlet P, Bonin A, Zinger L, Coissac E (2018) Environmental DNA: for biodiversity research and monitoring https://doi.org/10.1093/oso/9780198767220.001.0001

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of fish biomass using environmental DNA. PLoS ONE 7:e35868. https://doi.org/10.1371/journal.pone.0035868

Takeuchi H, Hiratsuka M, Oniuma H, Umino Y, Nakano D, Iwadare M, Tomono R, Hori K, Imai T, Ishikawa T, Takai N, Mano N (2016) Infection status of ayu and other wild fish with *Flavobacterium psychrophilum* and *Edwardsiella ictaluri* in the Tama river. Japan Fish Pathol 51(4):184–193. https://doi.org/10.3147/JSFP.51.184

The eDNA Society (2019) Environmental DNA sampling and experiment manual version 2.1. Otsu, Japan: http://ednasociety.org/eDNA_manual_Eng_v2_1_3b.pdf

Thomsen PF, Willerslev E (2015) Environmental DNA—an emerging tool in conservation for monitoring past and present biodiversity. Biol Conserv 183:4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Uddin N, Wakabayashi H (1997) Effects of temperature on growth and protease production of *Cytophaga psychrophile*. Fish Pathol 32(4):225–226. https://doi.org/10.3147/jsfp.32.225

Valle GD, Taguchi N (1995) Genetic variation of some physiological traits of clonal ayu (*Plecoglossus altivelis*) under stressed and non-stressed conditions. Aquaculture 137:193–202. https://doi.org/10.1016/0044-8486(95)01112-9

Wakabayashi H, Toyama T, Iida T (1994) A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathol 29:101–104. https://doi.org/10.3147/jsfp.29.101

Yamanaka H, Minamoto T (2016) The use of environmental DNA of fishes as an efficient method of determining habitat connectivity.

Ecol Indic 62:147–153. https://doi.org/10.1016/j.ecolind.2015.11.022

Yamanaka H, Minamoto T, Matsuura J, Sakurai S, Tsui S, Motozawa H, Hongo M, Sogo Y, Kakimi N, Teramura I, Sugita M, Baba M, Kondo A (2017) A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. Limnology 17:233–241. https://doi.org/10.1007/s10201-016-0508-5

Yoshiura Y, Kamaishi T, Nakayashu C, Ototake M (2006) Detection and genotyping of Flavobacterium psychrophilum by PCR targeted to peptidyl-prolyl cis-trans isomerase C gene. Fish Pathol 41(2):67–71. https://doi.org/10.3147/jsfp.41.67