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Real-time PCR assays for environmental DNA detection of three salmonid fish in Hokkaido, Japan: application to winter surveys

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Abstract

Dolly Varden (*Salvelinus malma*) and Whitespotted Char (*S. leucomaenis*) are representative native fish of the family Salmonidae that inhabit the upper reaches of rivers on Hokkaido Island, Japan. They are threatened by the invasive Rainbow Trout (*Oncorhynchus mykiss*). In this study, environmental DNA (eDNA) real-time PCR assays to detect these three salmonids were developed and used to clarify the distribution pattern of these fish. A specificity test for each assay was conducted using DNA extracted from both target and closely related fish, and the specificity of each assay was confirmed. Then, we carried out eDNA surveys in two mountainous rivers around Mt. Daisetsu in winter, when snow depth was maximized. In the winter surveys, eDNA of all three species were successfully detected from river water samples, including under-ice water samples. The results of eDNA detection corresponded with the results of an earlier distribution survey performed with Japanese-style fly-fishing and lure-fishing. These results suggested that the eDNA assays developed in this study are applicable for inter-seasonal surveys for these species.

Keywords: Dolly Varden (*Salvelinus malma*), environmental DNA (eDNA), Rainbow Trout (*Oncorhynchus mykiss*), winter, Whitespotted Char (*S. leucomaenis*)

Introduction

Two *Salvelinus* species, Dolly Varden (*S. malma*) and Whitespotted Char (*S. leucomaenis*) are representative native species of the family Salmonidae in the upper streams of Hokkaido Island, the northernmost area of Japan. Both species show partial migration: some individuals migrate to the sea, while the others remain in rivers (Yamamoto et al. 1999; Koizumi et al. 2006). Populations of an exotic salmonid, Rainbow Trout (*Oncorhynchus mykiss*), on Hokkaido Island have rapidly increased in recent years, and this exotic trout threatens the endemic salmonids through competition for resources and breeding sites (Taniguchi et al. 2000; Baxter et al. 2007). Because some salmonids aggregate on specific sites during winter, effective removal of non-native species is possible during this season (Koizumi et al. 2017). Thus, species distribution in winter should be important information for managing these species. Previous studies reported the seasonal habitat use and winter distribution of salmonid species in Hokkaido (Morita et al. 2011; Sahashi and Morita 2014; Koizumi et al. 2017). However, conventional survey methods, such as electrofishing and netting, are labor-intensive, especially in the mountain areas of the island during winter due to heavy snow and low temperature. In addition, the ice covering the river prevents conventional surveys. Therefore, a simple method is required for distribution surveys of salmonids in Hokkaido Island during winter.

Environmental DNA (eDNA) analysis has been developed and applied to many aquatic species in the last decade (Ficetola et al. 2008; Jerde et al. 2011; Minamoto et al. 2012). To date, there have been many eDNA studies on freshwater fish species (Laramie et al. 2015; Sigsgaard et al. 2015; Yamanaka & Minamoto 2016). Such eDNA analysis has been intensively applied for surveying salmonids, especially *Salvelinus* species. For

example, eDNA detection of Brook Trout (*S. fontinalis*), Bull Trout (*S. confluentus*), and Lake Trout (*S. namaycush*) in North America (Wilcox et al. 2015; Lacoursière-Roussel et al. 2016; McKelvey et al. 2016; Wilcox et al. 2016) has been reported. Recently, eDNA assays have also been reported for Rainbow Trout (Rusch et al. 2018). On Hokkaido Island, Japan, eDNA detection of Sakhalin Taimen (*Hucho perryi*), a member of the Salmonidae, was successful (Mizumoto et al. 2017).

An eDNA survey only requires water collection at the site, and therefore the survey can be conducted even in winter, when traditional survey methods such as fishing and netting are ineffective. On the other hand, eDNA release from individuals may decrease in winter because poikilothermic animals generally have lowered metabolisms at low temperatures. In general, eDNA concentration or detection frequency fluctuates among seasons and peaks in the breeding season (Bylemans et al. 2017; Takahashi et al. 2018). For an amphibian species, the detection probability of eDNA was lowest in winter (Buxton et al. 2018). For salmonid species, most studies reporting the efficacy of eDNA analysis were conducted from spring to autumn (e.g. Lacoursière-Roussel et al. 2016; McKelvey et al. 2016; Wilcox et al. 2016; Rusch et al. 2018), and there is no report on winter eDNA surveys.

In the present study, we developed species-specific eDNA assays for three salmonid species: Dolly Varden, Whitespotted Char, and Rainbow Trout. We used these assays for species-specific detection in two mountainous rivers on Hokkaido Island in the winter. Our study demonstrated the feasibility of a seasonal-distribution survey using eDNA in an area with heavy snowfall.

Materials & Methods

Designing real-time PCR assays

Sequences of the mitochondrial cytochrome b gene of the target species Dolly Varden, Whitespotted Char, and Rainbow Trout as well as that of Masu Salmon (*Oncorhynchus masou*), a closely related and sympatric salmonid species in Hokkaido, and that of Brook Trout, a potential sympatric species, were downloaded from the database of the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov). We used sequences including almost the entire length of the cytochrome *b* region of each species. Because we found intra-species variation between Japanese Dolly Varden individuals and those from other regions, we used sequences of Japanese individuals only. As we found no full-length cytochrome b sequences for Whitespotted Char, partial cytochrome *b* sequences were used. The sequences used for designing primers are shown in Table 1. The sequences were aligned using eBioX 1.5.1 (Barrio et al. 2009), and we identified species-specific nucleotides for each species by eye. Primers with at least one species-specific site in the five nucleotides from the 3' ends with Tm values of around 60˚C were selected. TaqMan MGB probes (Thermo Fisher Scientific, Waltham, MA, USA) for Dolly Varden and Whitespotted Char and a TaqMan probe for Rainbow Trout were designed within the inner sequences of designed primer sets using Primer Express 3.0 (Thermo Fisher Scientific) with default settings. The designed primers and probes are shown in Table 2. The lengths of the amplicons were 139, 126, and 153 bp for Dolly Varden, Whitespotted Char, and Rainbow Trout, respectively.

Specificity test of designed assays

The specificities of the designed primer sets were first checked *in silico* by Primer-Blast

(https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with default settings, and then in *in vitro* tests using DNA of related species. Total DNA was extracted from two Dolly Varden individuals, and one each individual of Whitespotted Char, Rainbow Trout, and Masu Salmon using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Brook Trout was not tested because it is exotic to Japan, rare in Hokkaido, and considered to be absent from the study rivers.

DNA concentration was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). The specificity of each assay was tested using 1 pg of target and non-target species DNA as template. Real-time PCRs were carried out using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Three assays were performed for each sample. Reaction conditions of all three assays were the same, and only primers and probes were different among the assays. Each 20 μL TaqMan reaction contained 1 pg of DNA template, 900 nM of each primer, 125 nM of TaqMan probe, 0.1 μL of AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific), and 10 μL of 2 × Environmental Master Mix 2.0 (Thermo Fisher Scientific). The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 15 s at 95°C and 1 min at 60°C. All real-time PCRs for eDNA samples included PCR negative controls, and were performed in triplicate. The amplified fragments were directly sequenced by a commercial sequencing service (Fasmac, Atsugi, Japan). To reduce the risk of carry-over contamination, the pre- and post-PCR experiments were performed in independent rooms.

Winter eDNA survey in a mountainous river

To check the applicability of eDNA surveys in the heavy snow season on Hokkaido

129 Island, water was sampled from two stony-bed mountainous rivers surrounding Mt.
130 Daisetsu. Surveys were conducted in the Antaroma River (Kamikawa, Hokkaido, Japan)
131 and Piukenai River (Higashikawa, Hokkaido, Japan) on March 9 and 10, 2017,
132 respectively. The width and depth of the Antaroma River are 1.5–10 m and 0.3–1 m,
133 respectively, and those of Piukenai River are 5–15 m and 0.5–1 m, respectively. The
134 discharge of Piukenai River at Site 6 is 5–10 m^3/s (Ministry of Land, Infrastructure,
135 Transport and Tourism, Japan; http://www1.river.go.jp/ [in Japanese]) but no data is
136 available for the discharge of Antaroma River. Six sampling sites, three in each river,
137 were set (Fig. 1). In these sites, the presence of target species had been preliminarily
138 surveyed by Japanese-style fly-fishing and lure-fishing by one of the authors (AI).
139 Eighty-three times of 30-min fishing surveys were conducted from June to October in
140 2013–2016 for Piukenai River, and 14 times of 30-min surveys were conducted from
141 July to September in 2014–2016 for Antaroma River. Water samples from Sites 2 and 3
142 were collected from under-ice, and from running water at the other sites. Water
143 temperature was measured at each site. At each site, a 900 mL water sample was
144 collected using a plastic bottle, and benzalkonium chloride was added to a final
145 concentration of 0.1% to suppress degradation of DNA (Yamanaka et al. 2017). The
146 water samples were transported to the laboratory and filtrated within eight hours of
147 sampling. The sample water was filtrated using glass-fiber filters with nominal pore size
148 of 0.7 μm (GF/F, GE Healthcare, Chicago, IL). A 900 mL sample of pure water was
149 filtrated to monitor contamination at the filtration and subsequent extraction steps.
150 Filters were preserved at -20°C until DNA extraction. All equipment used in water
151 collection and water filtration steps, including plastic bottles, filter funnels, and
152 tweezers, were bleached with diluted commercial bleach solution (> 0.1% sodium

chloride solution) before use to prevent contamination.

eDNA extraction and real-time PCR

Extraction of eDNA from filters was performed according to a previous study (Uchii et al. 2016) with slight modification. In brief, each filter was placed in a Salivette tube (Sarstedt, Nümbrecht, Germany), and 440 µL lysis solution, composed of 400 µL of Buffer AL (Qiagen) and 40 µL of Proteinase K (Qiagen), was added to the filter. Then, the tube was incubated at 56°C for 30 min, and the lysed DNA solution was collected by centrifugation. After adding 220 µL of TE buffer to the filter, the Salivette tube was re-centrifuged to collect the remaining DNA from the filter. After 400 µL of ethanol was added to the collected DNA solution, the mixture was transported to the DNeasy mini spin column (Qiagen). Subsequently, we followed the manufacturer's instructions for the DNeasy Blood & Tissue Kit and total eDNA was eluted in 100 µL of AE buffer.

Real-time PCRs were carried out under the conditions described above, except for the volume of template eDNA and controls (5 µL in this case). All real-time PCRs for eDNA samples included filtration negative controls, PCR positive controls (DNA extracted from tissue samples of target species), and PCR negative controls, and were performed in triplicate. If any of the three replicates showed a positive signal (an amplification signal within 55 cycles), the sample was treated as positive. The amplified fragments were directly sequenced by a commercial Sanger sequencing service (Fasmac).

Results and Discussion

We succeeded in designing specific eDNA assays for three species of Salmonidae:

177 Dolly Varden, Whitespotted Char, and Rainbow Trout. The Primer-Blast procedure for
178 the primers targeting Dolly Varden hit two potential sympatric species (Whitespotted
179 Char and Big-scaled Redfin, *Tribolodon hakonensis*). However, both species had one
180 substitution in the second nucleotide from the 3' end of both primers, suggesting that
181 these primers could not amplify sequences from these species. In fact, the *in vitro*
182 specificity test using DNA of Whitespotted Char revealed no amplification signal. The
183 Primer-Blast procedure run for the primers targeting Whitespotted Char and Rainbow
184 Trout hit no potential sympatric species, and the results of *in vitro* specificity tests
185 corroborated the specificity of the designed eDNA primers. All six real-time PCRs
186 using DNA from the two Dolly Varden individuals showed amplification signals with Ct
187 values between 33.9 and 36.0, and no amplification signal was observed for the PCRs
188 using non-target species DNA as template. Similarly, in the eDNA assays performed for
189 Whitespotted Char and Rainbow Trout, only the target species DNA was amplified with
190 Ct values between 34.3 and 35.0, and between 24.7 and 29.1, respectively. The DNA
191 fragments amplified in the specificity tests were confirmed as the target sequences by
192 Sanger sequencing. In addition, amplified fragments from eDNA surveys were also
193 confirmed as target sequences. Thus, the three assays developed in this study were
194 specific to each target fish species in Hokkaido.

195 Table 3 shows the results of real-time PCR and fishing surveys. By fishing
196 surveys, Dolly Varden and Whitespotted Char were found at the two downstream sites
197 (Sites 2 and 3), but only Dolly Varden was found at the uppermost site (Site 1) in the
198 Antaroma River. In Piukenai River, Dolly Varden and Rainbow Trout were found at all
199 three sites (Sites 4 to 6). Our winter eDNA survey resulted in positive detection of Dolly
200 Varden at all six sites. In addition, Whitespotted Char was detected at Site 2, and

Rainbow Trout was detected at Sites 4 and 5 (Fig. 1; Table 3). Most eDNA samples showed Ct values between 34 and 40, and three out of 17 positive reactions of Dolly Varden showed Ct values above 40 (between 40 and 52). There was no site where the eDNA assay result was positive and that of fishing was negative. No negative controls, including filtration negative controls and PCR negative controls, showed positive signals. Therefore, positive signals were from organisms near the sampling sites, not from contamination.

The overall result of eDNA detection corresponded well with the results of the previous distribution survey performed using Japanese-style fly-fishing and lure-fishing, although Whitespotted Char was not detected at Site 3 and Rainbow Trout was not detected at Site 6, despite the presence of the species (Table 3). This kind of "false-negative" result is common for eDNA studies (Ficetola et al. 2015; Guillera-Arroita et al. 2017). Some previous studies reported the detection probabilities (rate of positive amplifications among PCR replicates) of eDNA at sites where the target species were known to be present; the probabilities varied among these studies. For example, Erickson et al. (2017) reported detection probabilities of 30.7% (375/1222 replicates) and 27.2% (332/1222) for Silver Carp (*Hypophthalmichthys molitrix*) and Bighead Carp (*H. nobilis*), respectively, based on more than 1,000 eDNA samples. Baldigo et al. (2017) reported a probability of 74.5% (76/102) for Brook Trout. These studies were conducted in warm seasons, and the detection probabilities in our study conducted in winter were 94.4% (17/18), 33.3% (2/6) and 66.7% (6/9) for Dolly Varden, Whitespotted Char, and Rainbow Trout, respectively. Although we did not directly compare the detection probabilities among seasons, the probabilities in our study were comparable with those of the previous studies. These results suggested that eDNA

surveys in winter are as effective as that in other seasons. The average water temperature across the six sites was 2.27 ± 1.75°C (mean ± SD). Thus, it is possible to apply eDNA analysis in seasonal distribution surveys, including in mid-winter, when traditional survey methods are difficult.

In the Piukenai River (Sites 4 to 6), the distances between sampling sites were relatively short (~1,000 and 1,500 m between sites 4 and 5, and between 5 and 6, respectively). The distance eDNA can flow downstream is controversial. Previous studies reported flowing distances from less than 50 m (Pilliod et al. 2014) to more than 10 km (Deiner and Altermatt, 2014). The results obtained here suggest a short flow distance because the eDNA of Rainbow Trout was detected at Site 5 but not at Site 6. However, this should be investigated in more detail in future studies.

We did not quantify eDNA concentration in this study. However, because our assays are based on TaqMan chemistry, which is generally used in quantification of target genes, we can quantify eDNA using these assays combined with quantification standards (known copies of target DNA). In future studies, seasonal changes of eDNA concentrations in the streams can be investigated, and biomass or abundance may be estimated by eDNA quantification (Doi *et al.* 2017; Lacoursière-Roussel *et al.* 2016; Takahara *et al.* 2012).

This study revealed that the eDNA analysis for three species of Salmonidae is possible in mountainous rivers even in winter, when snow depth is the greatest. We succeeded in developing specific eDNA assays for Dolly Varden, Whitespotted Char, and Rainbow Trout; two of these are native to the study area and the other is invasive and threatening the native species. The assays described here can be used in surveys to clarify the current distributions of these species, as well as changes in these distributions.

These assays are also applicable for determining how competitive exclusion of native species by Rainbow Trout might proceed in rivers in Hokkaido, and for describing the effects of river-crossing structures on the migration of salmonid fish species.

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258

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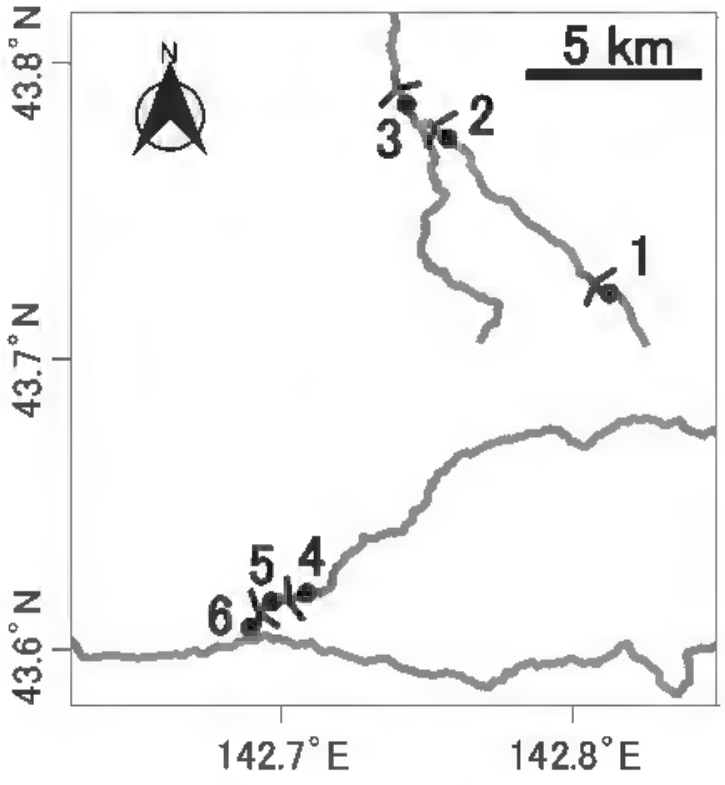
372
373

374 Figure legend

375

376 Figure 1. Location of the survey sites in Mt. Daisetsu area. Sites 1 to 3 and 4 to 6 are
377 located in Antaroma River and Piukenai River basins, respectively. Sites (black dots)
378 are separated by dams (black lines) and fish migration between sites is not possible.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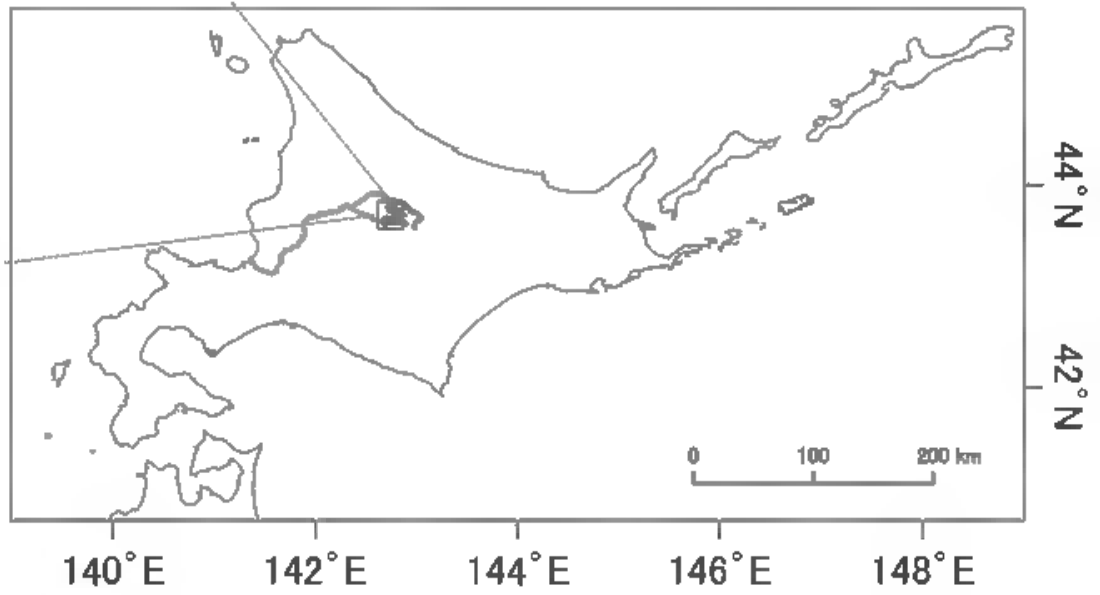
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Table 1. Accession numbers of the nucleotide sequences used for designing environmental DNA assays.

	Species	Accession Numbers
Target Species	Dolly Varden	AB612339, AB612338, AB612337
	Whitespotted Char	AB863624, AB612333, AB612335, AB612332
	Ranbow Trout	JX960815, AF125209, FJ435601, AF125208, FJ435602, HQ167694, L29771, LC050735, FJ435588, FJ435586, DQ288270, DQ288268, D58401, KP013084, FJ435598, FJ435597, KU872710, AY032631, JX960813, KP085590, NC_001717, MF621750
Related Species	Brook Trout	AF154850, D58399, JX960852, KU872718, MF621737
	Masu Salmon	AF125210, KC733791, KC733793, KC733792, LC098721, NC_008747

Table 2. Sequences of primers and probes used in this study

Target species	Name of primer / probe	Sequence (5' --> 3')	Amplicon length
Dolly Varden	S_malma_J_CytB_F	CTTATTTGCCTACGCAATTCTCC	139 bp
	S_malma_J_CytB_R	GTGAGGATGAGTATGTCTGCTACCA	
	S_malma_CytB_P	FAM-TTGTCCCGATCCTCCACA-MGB-NFQ	
Whitespotted Char	S_leucomaenis_CytB_F	CCCAGCAGGGATCAACTCAG	126 bp
	S_leucomaenis_CytB_R	GGGTTGGCTGGCGTGA	
	S_leucomaenis_CytB_P	FAM-CCTAACAGCCCTAGCTC-MGB-NFQ	
Rainbow Trout	O_mykiss_CytB_F	CCCTAGTGACCCCACCTCATA	153 bp
	O_mykiss_CytB_R	CAAAATAAGAATTGGGTGAGCG	
	O_mykiss_CytB_P	FAM-ACGATCCATCCCCAACAAGCTGGG-TAMRA	

Table 3. Results of the environmental DNA (eDNA) survey and previous distribution survey using fishing. The “+” and “-” in the eDNA column indicate the positive and negative signals obtained in the three replicates, respectively. The “+” and “-” in the Fishing column indicate the detection and non-detection of the corresponding fish by traditional fishing survey.

River	Site	eDNA			Fishing		
		DV*	WC**	RT***	DV*	WC**	RT***
Antaroma	1	+++	---	---	+	-	-
	2	+++	++-	---	+	+	-
	3	+++	---	---	+	+	-
Piukenai	4	+++	---	+++	+	-	+
	5	+++	---	+++	+	-	+
	6	++-	---	---	+	-	+

* Dolly Varden

** Whitespotted Char

*** Rainbow Trout