Research article

Quantitative Analysis of Diplomonad Flagellate *Spironucleus salmonis* Infection in Intestines of Hatchery and Wild Salmonid Fishes in Hokkaido

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ABSTRACT—Diplomonad flagellate *Spironucleus salmonis* infection in hatchery-reared juvenile chum salmon *Oncorhynchus keta* and masu salmon *O. masou* has been previously reported in Hokkaido, northern Japan. We established a quantitative real-time PCR (qPCR) assay for the *S. salmonis* ribosomal RNA gene (rDNA) using a fish intestine nucleic acid template, which we then use to investigate the infection intensity of *S. salmonis* in hatchery-reared, farmed, and wild salmonids throughout Hokkaido. Our new qPCR assay enabled the measurement of rDNA from $1.0\text{--}1.0 \times 10^8$ copies/ μL . Our study reported a significantly positive correlation between parasite trophozoite cell number and its rDNA copy number, and presented this as a simple regression (parasite rDNA copy number = $-768 + 338 \times$ parasite cell number). By reporting *S. salmonis* infection in juvenile and/or adult chum and masu salmon collected from three hatcheries, one fish farm, and one river in Hokkaido, we demonstrate the utility of our qPCR assay for the surveillance of *S. salmonis* infection in hatchery-reared, farmed and wild fish.

Key words: *Spironucleus salmonis*, *Oncorhynchus keta*, *Oncorhynchus masou*, hatchery, fish farm, wild fish, Hokkaido, salmon

Infection of diplomonad flagellate *Spironucleus salmonis* was found from the intestinal lumen of juvenile chum salmon *Oncorhynchus keta*, pink salmon *O. gorbuscha*, and masu salmon *O. masou* reared for stock enhancement purposes in hatcheries in Hokkaido, northern Japan (Awakura and Matsumoto, 1970; Urawa and Awakura, 1994; Urawa, 2011; Urawa *et al.*, 2018^{*1}; Mizuno *et al.*, 2020). Experimental infection of juvenile masu salmon with *S. salmonis* for 10 weeks resulted in cumulative mortality of 17.9% of salmon, accompanied by a low condition factor (Urawa, 2011; Urawa *et al.*, 2018^{*1}), and 16% mortality of juvenile chum salmon (Urawa and Mizuno, 2019^{*2}). Systemic infection with *Spironucleus* parasites have also caused epithelial

necrosis of the intestinal lumen, internal hemorrhaging, splenomegaly, and granulomatous lesions in the liver and spleen of farmed juvenile salmon, commonly resulting in heavy host mortality (Mo *et al.*, 1990; Kent *et al.*, 1992; Poppe *et al.*, 1992; Poppe and Mo, 1993; Sterud *et al.*, 1998). While mortality caused by *S. salmonis* infection has not been reported for hatcheries in Hokkaido, given the severity of afflictions attributed to this parasite, this would seem likely.

Given their body size (7–9 μm), flagellar swimming trophozoites of *S. salmonis* are typically observed microscopically in fresh smear of chopped intestine obtained from heavily infected fish to diagnose its presence. However, using this method it is difficult to detect instances of slight infection, for which an alternative approach is required. A partial nucleotide sequence of

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^{*1} Urawa, S., S. Mizuno and M. Ban (2018): Impact of *Spironucleus salmonis* on the growth and mortality of juvenile masu salmon *Oncorhynchus masou*. Abstracts of 8th International Symposium on Aquatic Animal Health, September 2–6, 2018, Charlottetown, PEI, Canada. Available at: <https://isaah2018.com/>

^{*2} Urawa, S. and S. Mizuno (2019): Effects of water exchange rate on the diplomonad flagellate *Spironucleus salmonis* infection in juvenile salmonid fish. Abstract Book of 19th International Conference on Diseases of Fish and Shellfish, pp 315, September 9–12, 2019, Porto, Portugal. Available at: <https://www.eafp2019.com/docsBookOfAbstracts.pdf>

the small subunit ribosomal RNA gene (SSUrDNA) for *S. salmonis* was identified, and two oligonucleotide primers (Salmonis-1f and Salmonis-4r) that amplify a 745-base pair (bp) partial *S. salmonis* SSUrDNA sequence using polymerase chain reaction (PCR) were reported for its detection (Fard *et al.*, 2007). Thereafter, partial SSUrDNA nucleotide sequences of three *Spironucleus* species, *S. torosa* from Atlantic cod *Gadus morhua* (Jørgensen and Sterud, 2007), *S. meleagridis* from turkey *Meleagris gallopavo* (Levy *et al.*, 2015), and *S. muris* from house mouse *Mus musculus* (Kolisko *et al.*, 2008), have been newly reported. Oligonucleotide primers for specific detection of *S. salmonis* SSUrDNA should be reexamined by multiple sequence alignment analysis of all *Spironucleus* SSUrDNAs registered in GenBank (National Center for Biotechnology Information, Rockville, MD, USA).

While no system for quantitative real-time PCR (qPCR) of *S. salmonis* SSUrDNA has been established, a qPCR for *S. muris* has (Jackson *et al.*, 2013). Either fluorescent labeling probe methods, or intercalated methods could be used to develop a qPCR system for *S. salmonis* SSUrDNA. Because fluorescent labeling probe methods would be prohibitively expensive for many hatcheries and fish farms to implement on a continuous monitoring basis, intercalated methods are less expensive. To prevent amplification of nonspecific PCR products, recommended nucleotide numbers of the PCR product amplified with qPCR using intercalated methods generally range of 60–300 bp (Thermo Fisher Scientific Inc., 2014^{*3}). As the Salmonis-1f and Salmonis-4r primers amplify a 745-bp partial *S. salmonis* SSUrDNA sequence, they are inappropriate for development of a qPCR system because primers products exceed 300 bp. Accordingly, new primers must be developed.

We first establish a qPCR assay for *S. salmonis* SSUrDNA using an intercalated SYBR Green method. We then examine the relationship between parasite number and parasite SSUrDNA copy number using our new qPCR assay. Finally, using our qPCR assay, we survey infection by *S. salmonis* in hatchery-reared, farmed, and wild salmonids in Hokkaido.

Materials and Methods

Sampling

Fish samples were collected from 11 hatcheries, two fish farms and one river in Hokkaido in 2017 and 2018 (Table 1). Hatchery 1 is the Chitose Field Station

of the Hokkaido National Fisheries Research Institute, located on Chitose River, a tributary of Ishikari River, central Hokkaido, Japan, where *S. salmonis* infection of juvenile chum and masu salmon has been previously reported (Mizuno *et al.*, 2020). Hatchery-reared juveniles and farmed fish were collected using landing nets from a different pond for every fish species.

For hatchery-reared juvenile chum and masu salmon, diplomonad flagellate infection was assessed by microscopic observation (Microscopy GmbH 37081; Carl Zeiss, Göttingen, Germany) of pooled intestinal smears from five fish selected randomly from each pond just before collecting fish samples for qPCR. Adult chum and masu salmon in the process of migrating upstream for spawning were captured in a salmon wheel about 7 km downstream of hatchery 1, and other wild fishes composed of juvenile chum and masu salmon, brown trout *Salmo trutta*, stone loach *Barbatula barbatula*, floating goby *Gymnogobius urotaenia*, chestnut goby *G. castaneus*, lamprey *Lethenteron* sp., and sculpin *Cottus* sp. were caught upstream of hatchery 1 by casting net or electrofishing (LR-24 Backpack Electrofisher; Smith-Root, Vancouver, WA, USA). The origin of adult salmon, whether from wild or hatchery fish, was unknown. Rearing water for juvenile chum and masu salmon at hatchery 1 was sourced from Chitose River.

Sex ratios of fish samples were unbiased. All fish were weighed, and dissected using sterilized forceps and scissors. The entire intestine excluding the pyloric caeca and vent was sampled and used for extraction of nucleic acid. In addition, the intestines of chum salmon juveniles ($n = 5$) reared in hatchery 1 were pooled and used for *in vitro* culture of diplomonad flagellates.

In vitro culture of diplomonad flagellates

In vitro culture of diplomonad flagellates was performed following Buchmann and Uldal (1996). In brief, pooled intestines of infected juvenile chum salmon ($n = 5$) were placed in a 50 mL culture flask containing 25 mL of culture medium, and then incubated at 7.8°C for 15 days as a primary culture. The culture medium (pH 7.5) was composed of Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum inactivated at 56°C for 30 min, and 4% Keister's modified bile-supplemented TYI-S-33 medium (Keister, 1983), including the antibiotics penicillin (2,000 U/mL), gentamicin (50 µg/mL), and nystatin (100 U/mL) for inhibition of bacterial and fungal growth. The 5-mL primary cell culture suspension and 15 mL new culture medium were introduced into a new 50 mL culture flask, and incubated at 10°C for 3 days as a secondary culture. Nitrogen gas was not added at all stages of the culture.

A sample of 100 µL of secondary culture medium (including only trophozoites of diplomonad flagellates) was pipetted into a single well on a 24-well microplate, and the total number of trophozoites in the well was

^{*3} Thermo Fisher Scientific Inc. (2014): Real-time PCR handbook. Thermo Fisher Scientific Inc., MA, Waltham, 68 p. Available at: <https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/real-time-pcr-handbook.pdf#search=%27applied+biosystems+realtime+PCR+handbook%27>

Table 1. Sampling details, infection intensity and prevalence of *Spironucleus salmonis* fish used for the present study

Origin	Sampling place	Sampling time	Fish	Age	Average body weight (g)	Sample numbers	Infection intensity of <i>S. salmonis</i> (copies)*	Infection prevalence of <i>S. salmonis</i> (%) [†]
Hatchery	Hatchery-1	April 20th, 2018	Chum salmon (<i>Oncorhynchus keta</i>)	0+	0.547	10	$1.41 \times 10^5 \pm 2.17 \times 10^5$	100
			Masu salmon (<i>O. masou</i>)	1+	16.7	10	$8.97 \times 10^6 \pm 1.54 \times 10^7$	100
	Hatchery-2	March 1st, 2018	Chum salmon	0+	1.22	10	Not detected	0
	Hatchery-3	March 20th, 2018	Chum salmon	0+	0.963	10	Not detected	0
	Hatchery-4	March 20th, 2018	Chum salmon	0+	0.891	10	$4.02 \times 10^5 \pm 5.41 \times 10^5$	70
	Hatchery-5	April 4th, 2018	Chum salmon	0+	0.742	10	Not detected	0
	Hatchery-6	April 5th, 2018	Chum salmon	0+	0.868	10	Not detected	0
	Hatchery-7	April 5th, 2018	Chum salmon	0+	1.10	10	$1.34 \times 10^3 \pm 3.17 \times 10^3$	30
	Hatchery-8	April 5th, 2018	Chum salmon	0+	1.00	10	Not detected	0
	Hatchery-9	April 19th, 2018	Chum salmon	0+	1.06	10	Not detected	0
	Hatchery-10	April 19th, 2018	Chum salmon	0+	0.929	10	Not detected	0
Fish farm	Hatchery-11	May 2nd, 2018	Chum salmon	0+	1.04	10	Not detected	0
	Farm-1	April 20th, 2018	Masu salmon	0+	0.794	10	$5.49 \times 10^5 \pm 1.22 \times 10^6$	60
			Rainbow trout (<i>O. mykiss</i>)	0+	0.631	10	Not detected	0
	Farm-2	July 5th, 2018	Rainbow trout	0+	2.86	10	Not detected	0
			Dolly Varden (<i>Salvelinus malma</i>)	0+	1.54	10	Not detected	0
Wild or hatchery	Chitose River	September 20th, 2017	Chum salmon	4+	2.90×10^3	27	$6.05 \times 10^8 \pm 3.14 \times 10^9$	14.8
		October 18th, 2017	Masu salmon	2+	1.20×10^3	20	$1.79 \times 10^2 \pm 3.37 \times 10^2$	20
Wild	Chitose River	April 20th, 2018	Chum salmon	0+	0.543	33	$6.86 \times 10^2 \pm 1.95 \times 10^3$	18.1
			Masu salmon	0+	0.549	31	$1.22 \times 10^2 \pm 3.01 \times 10^2$	16.1
				1+	9.48	22	36	4.54
			Brown trout (<i>Salmo trutta</i>)	Unknown	14.0	21	Not detected	0
			Stone loach (<i>Barbatula barbatula</i>)	Unknown	3.43	2	Not detected	0
			Floating goby (<i>Gymnogobius urotaenia</i>)	Unknown	1.87	2	Not detected	0
			Chestnut goby (<i>Gymnogobius castaneus</i>)	Unknown	1.15	2	Not detected	0
			Lamprey (<i>Lethenteron</i> sp.)	Unknown	5.47	4	Not detected	0
			Sculpin (<i>Cottus</i> sp.)	Unknown	1.77	7	Not detected	0

* Infection intensity of *S. salmonis* showed total copy number of the parasite small subunit ribosomal RNA gene detected in intestine nucleic acid template of fish, and was expressed as means $\pm$ standard deviations.

[†] Infection prevalence of *S. salmonis* showed percentage of the parasite-positive sample number/ total sample number.

	FQ1 primer		RQ2 primer
<i>S. salmonis</i> -1 (qPCR standard)	1 CGACTCGAGGAAGCTCTCGACGCCAACGTAGCGAGCGCG---GTGAGCTGCAGCGAGTTACGGCAACAACACTAT-CG---CTAT 79	80	AGGACAGGGGAGGGCTCTCTATTATAGGGGGACAGTGAAATAGGATGATGCCTATAGGAGGAACAGTGGCGAGGCACTGAG 164
<i>S. salmonis</i> -2	1 79	80	 164
<i>S. vortens</i>	1 A.....CTA.....TT.....TCT.....T--A.G...TA.....G.T...C...GTGT.T.G.GT.GC....G 83	84	AG.CA---C.C.AC...C.GC.A.....C...T.C.A...T.GGC...T.A.TG.G.G. 166
<i>S. meleagridis</i>	1 AC.T...A.A.GGAGT.TT.CACC...GGGC...CGCT.CAC.C...A.GC...GTGGCGCT.CGCG.ACCG.C.C 86	87	T.G.G.C.CGG.A.CGGCGG...CCGG.....G.C.C.CGG...CCC.GGCA...G...GGCGC. 171
<i>S. muris</i>	1 CCTC.....A.GGGAG.TT.TGC---GA.T.....ACAG.C---GAG.G...GGCG---CG.CGGG.C 73	74	T.G.G.CACGG...CGT.CGG...GACT.....TG.....A.....C.AGTC...C.G.GGCA...G...GAGC. 158
<i>S. salmonicida</i>	1 ACTATA.T.A.CAAGT.TTT.TAAC--TACTTAACT--TTTT.AT.TAT.AA.TTAT.TGCG-----G.C. 69	70	TT.G.GT---A.T.TGGGG.C.CG.TA.....CTA.CG.A.CC...GGTA...G...T.CC.A 149
<i>S. barkhanus</i>	1 ACTATA.C.A.G.CGA.T.T.C.AA---TTTTAGCA---T.TT.AT.TAT.AA.TTAT.G.GCG-----GC. 69	70	T.A.GT---A.T.TGGGG.C.CG.TA.....CTA.CG.A.CC...GGTA...G...T.CC.A 149
<i>S. torosa</i>	1 ACTGT...A.A.AAAG.TT.T.C--TT.TTA.CA--ATCATCA...AAT.G--C.T...GCG-----G.C. 67	68	T...GT---A.T.AAAGG.C.TGCT.....C.AGCA.A.CC...GGTA...G...GT.T.A 147

Fig. 1. Alignment of partial small-subunit ribosomal RNA gene sequences from seven *Spironucleus* species obtained from the GenBank database: *S. salmonis* isolated from chum salmon from hatchery-1 (*S. salmonis*-1, accession no. LC496562; present study), *S. salmonis* isolated from rainbow trout (*S. salmonis*-2, DQ394703), *S. salmonicida* (DQ181595), *S. barkhanus* (DQ186581), *S. vortens* (U93085), *S. torosa* (EF050055), *S. meleagridis* (EF050054), and *S. muris* (EU043230). Underlined sequences show forward (FQ1) and reverse primers (RQ2) for qPCR. Differences from *S. salmonis*-1 are given with the respective base. (•): sequence similarity; (–): gaps.

counted by observation at $\times 200$ using an inverted microscope (Primo Vert; Carl Zeiss). The culture medium was diluted to adjust trophozoite concentration to 2,000 cells/mL using new culture medium, and used for preparation of the qPCR standard, and for analysis of relationships between parasite number and parasite SSUrDNA copy number.

Preparation of nucleic acid template and qPCR standard

Nucleic acid comprising total DNA and partial RNA was extracted from the intestine or cultured trophozoites using a nucleic acid extraction kit (SepaGene; Sekisui Medical, Tokyo, Japan). Intestines of fish excepting adult chum and masu salmon were homogenized in 100 μ L of Tris-EDTA buffer (10 mM of Tris-HCl and 1 mM of EDTA; pH 8.0). Nucleic acids were extracted from the homogenized sample using a phenol-chloroform method and suspended in 25 μ L of Tris-EDTA buffer. Nucleic acid from the intestines of adult chum and masu salmon was extracted using a method at 10-fold volume scale and suspended in 250 μ L of Tris-EDTA buffer. qPCR primers were designed to specifically amplify the partial 164-base *S. salmonis* SSUrDNA by multiple sequence alignment analysis (ClustalW 2.1 software available from DNA Data Bank of Japan, <https://www.ddbj.nig.ac.jp/index.html>) using partial SSUrDNA nucleotide sequences of seven *Spironucleus* species obtained from GenBank: *S. salmonis* isolated from chum salmon in hatchery 1 (accession no. LC496562), *S. salmonis* isolated from rainbow trout (DQ394703), *S. salmonicida* (DQ181595), *S. barkhanus* (DQ186581), *S. vortens* (U93085), *S. torosa* (EF050055), *S. meleagridis* (EF050054), and *S. muris* (EU043230). Primer nucleotide sequences were 5'-CGACTCGAGGAAGTCTCG-3'

(forward, FQ1), and 5'-CTCAGTGCCTGCGCACTT-3' (reverse, RQ2) (Fig. 1). The continuous stretch of 3 nucleotides forming each 3' end of FQ1 and RQ2 for *S. salmonis* differed from that of the six other *Spironucleus* species (Fig. 1). Nucleic acid extracted from 100 μ L cultured trophozoites ($n = 1$) was used to prepare the qPCR standard. In addition, nucleic acids extracted from the intestines of age-0+ chum salmon from hatcheries 1 and 4–6 ($n = 3$ for each hatchery) and age-0+ masu salmon from hatchery 1 ($n = 3$), and Tris-EDTA buffer (as a negative control) were used to examine whether FQ1 and RQ2 primers could specifically amplify the partial 164-bp *S. salmonis* SSU rDNA using the following PCR. A 50- μ L aliquot of the PCR mixture comprised 1 μ L of nucleic acid template, 25 μ L of 2 $\times$ PCR mixture (AmpliAq Gold 360 Master Mix; Applied Biosystems, Foster City, CA, USA), 0.5 μ L of 50 μ M FQ1 primer, 0.5 μ L of 50 μ M RQ2 primer, and 23 μ L of distilled water. PCR thermal cycles comprised one cycle of enzyme activation for 10 min at 95°C, 40 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 60°C, extension for 30 s at 72°C, and one cycle of complete extension for 7 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis and stained in 0.5 μ g/mL ethidium bromide. Their electrophoretic patterns were photographed using a gel imaging system (Wise Uv WUV-M20; ATTO, Tokyo, Japan). Deduced 164-bp PCR products were ligated into the pCR2.1 plasmid vectors, and isolated using a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). At least 20 PCR products were sequenced for each nucleic acid template in both directions using an automated ABI PRISM 377 DNA Sequencer (Applied Biosystems), dye terminators, and M13 forward and T7 reverse primers. The PCR product originating from cultured *S. salmonis*

was digested from the pCR2.1 plasmid vector using the *EcoRI* restriction enzyme (Nippon Gene, Toyama, Japan), then refined as the qPCR standard using a gel extraction kit (QIAquick Gel Extraction Kit; Qiagen, Hilden, Germany) after electrophoresis on a 2% agarose gel including 0.5 µg/mL ethidium bromide. The standard was dissolved in 20 µL Tris-EDTA buffer, and the absorbance value at a wavelength of 260 nm (A260) was measured using a spectrophotometer (2030 Multi Label Reader ARVO X4; PerkinElmer, MA). The standard concentration (copies/µL) was calculated by $A_{260} \times 50 \times 10^{-9} \text{ (g/µL)} / [164 \text{ (bp)} \times 2 \text{ (nucleotides/bp)} \times 330 \text{ (g/m)}] \times [6.02 \times 10^{23} \text{ (copies/m)}]$ (Applied Biosystems, 2006^{*4}), and the original standard was prepared by dilution to adjust it to 1.0×10^8 (copies/µL) using the Tris-EDTA buffer.

qPCR analysis

Each reaction mixture (total volume 50 µL) was loaded into a single well of a 96-well microplate; it comprised 25 µL of 2× PCR mixture (Power SYBR Green PCR Master Mix, Thermo Fisher Scientific), 0.5 µL of each 50 µM FQ1 primer and RQ2 primer, 23 µL distilled nuclease-free water, and 1.0 µL of either the standard or nucleic acid template. Thermal cycles were conducted using a qPCR system (QuantStudio 6 Flex Real-Time PCR System; Thermo Fisher Scientific) with 40 cycles after an initial activation of 95°C for 10 min. Each cycle consisted of denaturation step at 95°C for 30 s, and an annealing and elongation step at 60°C for 30 s. Amplification of qPCR products was monitored by luminescence reading at the end of the elongation step of each cycle. The standard curve was constructed in duplicate for each assay, and shown as a regression line of scatter plots between the threshold cycle of the assay and the logarithmic concentration of 10-fold dilution series of the original standard (using the mean threshold cycle of the duplicate reactions in each assay). The absolute copy numbers of *S. salmonis* SSUrDNA present in the nucleic acid template were estimated from the standard curve and threshold cycle detected using the template. Detectable samples were defined as *S. salmonis*-positive, while undetectable samples were regarded as *S. salmonis*-negative.

Accuracy of qPCR

After preparation of the qPCR standard, remaining

chum salmon intestinal nucleic acid templates from hatcheries 1 and 5 ($n = 1$ from each hatchery), and of masu salmon from hatchery 1 ($n = 1$), were used to test qPCR accuracy. A 10-fold dilution series for each of the original standard, the three intestine nucleic acid templates, and the Tris-EDTA buffer (as a negative control) were analyzed three times in duplicate (six times in total) by qPCR, to verify the accuracy of qPCR standard curves, similarity between the standard and calibration curves of *S. salmonis*-positive samples, and undetectability of *S. salmonis*-negative samples. Calibration curves were constructed as for the standard curves, although the x-axis to construct the standard and calibration curves expressed the logarithmic dilution factor of the original standard and the original *S. salmonis*-positive nucleic acid template (rather than logarithmic concentration, as for the former qPCR analysis). The slopes, amplification efficiencies [E (%)], and coefficients of determination (R^2) of the regression lines were calculated using qPCR software (QuantStudio Real-Time PCR Software version 1.1; Thermo Fisher Scientific). In the only standard curve, y-intercept at -8 of logarithmic dilution factor (1 copy of *S. salmonis* rDNA) on the x-axis was examined. qPCR analyses of chum and masu salmon intestinal nucleic acid templates from hatchery 1 were performed four times, in four separate microwells (16 times in total) to calculate the intra-assay coefficient of variation (CV) (across the quadruplicate samples), and inter-assay CV (among the four-replicate assays, for each of four-microwell reactions). The CV was expressed as percentage of standard deviation/mean.

Application of qPCR system

To examine the relationship between parasite cell number and copy number of *S. salmonis* SSUrDNA, 78 trophozoite samples (100 µL) including from 1 to 200 cells were prepared by dilution of cultured trophozoites (2,000 cells/mL) using the new culture medium described above. The total numbers of parasite SSUrDNA copies included in the nucleic acid template extracted from each sample were estimated by qPCR. Scatter plots of parasite number against parasite SSUrDNA copy number were constructed. The total numbers of absolute parasite SSUrDNA copies included in the nucleic acid template extracted from intestines of hatchery-reared, farmed, and wild fish were then analyzed to determine infection intensity and prevalence of *S. salmonis* by qPCR. Infection intensity of *S. salmonis* was expressed as total copy numbers of parasite SSUrDNA contained in the intestinal nucleic acid template (copies), with data summarized as means ± standard deviations. The prevalence of *S. salmonis* is presented as a percentage (%) of *S. salmonis*-positive samples over the total number of samples.

*4 Applied Biosystems (2006): Foundation and application for analysis of gene expression using real-time PCR. Thermo Fisher Scientific Inc., MA, Waltham, 77 p. Available at: <https://web.sapmed.ac.jp/bme/service/realtimepcr.pdf#search=%27%E3%83%AA%E3%82%A2%E3%83%AB%E3%82%BF%E3%82%A4%E3%83%A0PCR%E3%81%AB%E3%82%88%E3%82%8B%E9%81%BA%E4%BC%9D%E5%AD%90%E7%99%BA%E7%8F%BE%E8%A7%A3%E6%9E%90%27>

Statistical analysis

A comparison of slope, E (%), and R^2 between the standard and calibration curves for *S. salmonis*-positive samples was performed by nonparametric Kruskal-Wallis test. A relationship between parasite number and parasite SSUrDNA copy number was determined by Pearson's correlation coefficient after a data normality test; the regression function was examined by a simple regression analysis. Statistical significance was accepted when $P < 0.05$. All statistical analyses were performed using Statcel4 (OMS Publishing, Tokyo, Japan).

Results

Microscopic observation

Trophozoites of diplomonad flagellates were detected in intestinal smears of juvenile chum salmon reared in hatcheries 1, 4 and 7, and juvenile masu salmon from hatchery 1. Parasites were not found in juvenile chum salmon from hatcheries 2, 3, 5, 6, and 8–11.

Characteristics of qPCR assay

Amplification of PCR products was detected as a clear single band at the 164-bp position in all lanes of hatchery 1 and 4 reared chum salmon, hatchery 1 reared masu salmon, and the qPCR standard originating from cultured trophozoites (Fig. 2). In contrast, no amplification of PCR products occurred in any lane for

hatcheries 5 and 6 for reared chum salmon, or the negative control. Sequencing of PCR products revealed that 200 isolated PCR products (20 each from lanes 1–9 and 17, Fig. 2) had a 100% homology to the partial *S. salmonis* rDNA of the 164 base nucleotide sequence (Fig. 1). In three replicate assays, standard curves showed a simple linear regression between 1.0×10^0 (dilution factor 10^{-8}) and 1.0×10^8 (dilution factor 1) copies/ μ L for parasite SSUrDNA copy numbers (Fig. 3), a slope ranging -3.408 to -3.390 , E (%) ranging 96.53 to 97.25, R^2 ranging 0.9990 to 0.9997, and y-intercept ranging 36.59 to 37.36 (Table 2). In three replicate assays, calibration curves presented a simple linear regression for the logarithmic dilution factor of intestinal nucleic acid template between -5 and 0 in hatchery 1 reared chum salmon, and between -6 and 0 in hatchery 1 reared masu salmon (Fig. 3). No *S. salmonis* rDNA was detected in a 10-fold dilution series for intestinal nucleic acid templates of hatchery 5 reared chum salmon, or for Tris-EDTA buffer. There were no significant differences in the slope ($P = 0.147$), E (%) ($P = 0.147$), or R^2 ($P = 0.393$) among the standard and calibration curves for hatchery 1 reared chum and masu salmon for three replicate assays (Table 2). In *S. salmonis*-positive samples, the intra-assay CV ranged 2.02%–7.34%, and the inter-assay CV ranged 8.95%–12.1% (Table 3).

Quantitative analysis of *S. salmonis*

Data for the 78 samples revealed a normal distribution for both parasite number ($P = 8.77 \times 10^{-10}$) and

Fig. 2. Electrophoretic pattern of polymerase chain reaction (PCR) products derived from a partial ribosomal RNA gene sequence of *Spironucleus salmonis* from intestinal nucleic acid templates of hatchery-reared chum and masu salmon using the primer set for quantitative real-time PCR (qPCR) assay. Lanes: 1–3, hatchery 1 reared chum salmon; 4–6, hatchery 1 reared masu salmon; 7–9, hatchery 4 reared chum salmon; 10–12, hatchery 5 reared chum salmon; 13–15, hatchery 6 reared chum salmon; 16, DNA molecular weight marker (M); 17, qPCR standard as positive control (PC); 18: Tris-EDTA as negative control (NC). Before electrophoretic analysis, infection of diplomonad flagellates was determined microscopically in reared chum salmon from hatcheries 1 and 4, and reared masu salmon from hatchery 1, but not from reared chum salmon from hatcheries 5 and 6. The arrowhead shows the 164-base pair (bp) position.

Fig. 3. Standard and calibration curves with 10-fold dilution series of nucleic acid templates extracted from intestines of *Spironucleus salmonis*-positive fish in quantitative real-time PCR. While regression lines are not shown, each curve is indicated by a scatter of points between the logarithm of the sample dilution factor (x-axis) and threshold cycle of the assay (y-axis). Circles, triangles, and squares depict standards, and samples of hatchery 1 reared masu salmon and hatchery 1 reared chum salmon, respectively. Large-striped, medium-open, small-closed marks express first, second and third replicate assays, respectively. The original standard (1.0×10^8 copies/ μ L) was set as dilution factor 1.

Table 2. The slope, amplification efficiency [E(%)], coefficients of determination (R^2) and y-intercept of standard curves and of calibration curves from *Spironucleus salmonis*-positive nucleic acid template of intestines

Assay time	Factors of quantitative PCR assay	Nucleic acid template		
		Standard	Intestine of chum salmon (hatchery-1)	Intestine of masu salmon (hatchery-1)
First	Slope	-3.408	-3.421	-3.370
	E (%)	96.53	96.04	98.02
	R^2	0.9997	0.9990	0.9993
	y-intercept	37.29	No data	No data
Second	Slope	-3.406	-3.429	-3.395
	E (%)	96.60	95.71	97.03
	R^2	0.9996	0.9990	0.9996
	y-intercept	37.36	No data	No data
Third	Slope	-3.390	-3.392	-3.377
	E (%)	97.25	97.15	97.73
	R^2	0.9990	0.9992	0.9990
	y-intercept	36.59	No data	No data

Table 3. The intra-assay coefficient of variation (CV) and the inter-assay CV of *Spironucleus salmonis* small-subunit ribosomal RNA gene (SSUrDNA) copy number of *S. salmonis*-positive samples estimated by the quantitative PCR assay

Sample	Assay time	<i>S. salmonis</i> rDNA copy numbers (copies/ μ L)				Intra-assay CV* (%)	Inter-assay CV [†] (%)			
		Microwell number					Microwell number			
		No. 1	No. 2	No. 3	No. 4		No. 1	No. 2	No. 3	No. 4
Chum salmon (hatchery-1)	First	8.05×10^4	7.74×10^4	7.79×10^4	7.70×10^4	2.02	9.06	11.4	8.95	9.83
	Second	8.03×10^4	8.51×10^4	8.28×10^4	9.02×10^4	4.98				
	Third	7.74×10^4	7.45×10^4	7.91×10^4	7.38×10^4	3.26				
	Fourth	9.42×10^4	9.58×10^4	9.43×10^4	8.81×10^4	3.67				
Masu salmon (hatchery-1)	First	3.34×10^5	3.17×10^5	3.29×10^5	3.36×10^5	2.59	10.1	9.37	9.35	12.1
	Second	3.29×10^5	3.64×10^5	3.61×10^5	3.94×10^5	7.34				
	Third	4.01×10^5	3.86×10^5	3.79×10^5	4.02×10^5	2.89				
	Fourth	3.28×10^5	3.25×10^5	3.07×10^5	3.12×10^5	3.18				

* Intra-assay CV (across the quadruplicate samples) calculated according to the formula: $100 \times \text{Standard deviation}/\text{Mean}$

† Inter-assay CV (among the four-replicate assays, for each of four separate microwells reactions) calculated according to the formula: $100 \times \text{Standard deviation}/\text{Mean}$

Fig. 4. Correlation between numbers of parasite trophozoites and parasite trophozoite small-subunit ribosomal RNA gene (SSUrDNA) copies in *Spironucleus salmonis*. Trophozoite samples ($n = 78$) expressed as scatter plots between numbers of trophozoites (x-axis) and SSUrDNA copies (y-axis).

parasite SSUrDNA copy number ($P = 1.57 \times 10^{-11}$). There was a significant positive correlation between parasite number and parasite SSUrDNA copy numbers ($P = 2.65 \times 10^{-83}$, $R = -0.996$; Fig. 4). This correlation was shown as a simple regression formula: (parasite SSUrDNA copy number) = $-768 + 338 \times$ (parasite number) [P (slope) = 2.66×10^{-83} , P (y-intercept) = 5.93×10^{-4}]. Infection of *S. salmonis* was detected at three of the 11 hatcheries (1, 4, 7), one of two fish farms (1), and one river (Table 1). Parasite infection was detected in juvenile and adult chum and masu salmon, but not rainbow trout, Dolly Varden, brown trout, stone loach, floating goby, chestnut goby, lamprey, or sculpin. The average infection intensity on *S. salmonis*-positive samples ranged 1.34×10^3 – 8.97×10^6 copies in hatchery-reared fish, 5.49×10^5 copies in farmed fish, and 36 – 6.05×10^8 copies in adult salmon and wild fish (Table 1). Prevalence of *S. salmonis* ranged 30%–100% in hatchery-reared fish, 60% in farmed fish, and 4.54%–20% in adult salmon and wild fish (Table 1).

Discussion

Based on light-microscopic analysis, intestinal smears from hatchery-reared juveniles, and fish from hatcheries 1 and 4, were regarded as *S. salmonis*-positive controls, and those from hatcheries 5 and 6 were *S. salmonis*-negative controls. Amplification and sequencing of PCR products revealed FQ1 and RQ2 primers (a set of primers for qPCR) successfully amplified partial *S. salmonis* rDNA, suggesting correct design of the qPCR primer set. Simple PCR using FQ1 and RQ2 primers enabled detection of infection of *S. salmonis* from an intestinal nucleic acid template.

Standard curves for qPCR are generally deemed highly accurate when the slope is between -3.58 and

-3.10 , E (%) is between 90% and 100%, and R^2 is ≥ 0.999 (Thermo Fisher Scientific Inc., 2014^{*3}). Our standard curves constructed in three replicate assays satisfied all these conditions, indicating the standard was highly accurate. The only other qPCR assay system reported a *Spironucleus* species, for *S. muris* (Jackson *et al.*, 2013), enabled measurement in the range of 1.0 – 1.0×10^5 copies. Our standard covers a broader range (to 1.0×10^8 copies), with the same lower limit.

The calibration curves for two positive control samples satisfy all aforementioned conditions for accurate qPCR analysis. No differences in the slopes, E (%), or R^2 among them and our standard curve indicated the three curves were parallel, suggesting that our method of intestine sample preparation was correct. In addition, no *S. salmonis* rDNA was detected from the intestines of hatchery 5 reared chum salmon, or from Tris-EDTA, revealing definite undetectability from negative control samples in our qPCR system. The validation test of sample measurement is generally acceptable for qPCR assay when the inter-assay CV is $< 15\%$ and the intra-assay CV is $< 10\%$ (Pfaffl, 2004). Our validation test of measurement using two positive control samples (hatchery 1 reared chum and masu salmon) satisfied all conditions for an acceptable assay, indicating our qPCR assay was highly accurate and reliable regardless of the species of fish used as a test sample. *Spironucleus* species generally have both trophozoite stage and dormant cyst stages in their life cycle (Williams *et al.*, 2011), but we have yet to determine if our qPCR assay can detect rDNA of *S. salmonis* cysts.

In statistical analysis, a strong correlation between two factors is acceptable when $|R|$ is > 0.7 (Mari and Kotz, 2001). The present correlation between parasite number and parasite SSUrDNA copy number satisfies this condition. Our simple regression formula allows

any user to estimate the total cell number of *S. salmonis* infecting the intestine of fish based on the rDNA copy number. An estimate is arithmetically possible when the intestine is infected with more than two *S. salmonis* cells. Our regression formula also estimates there to be 338 rDNA copies in a single *S. salmonis* cell. Previous studies on protists infecting aquatic organisms have estimated one dinoflagellate (*Hematodinium* sp.) cell to contain 8.0×10^2 – 1.0×10^5 copies (Nagle *et al.*, 2009), and the ectoparasitic flagellate *Ichthyobodo salmonis* to contain 1.0×10^4 – 2.5×10^4 copies per cell (Isaksen *et al.*, 2012; Mizuno *et al.*, 2016). Therefore, rDNA copy number included in a single protist cell may vary widely by species.

Quantitative analysis of *S. salmonis* infecting hatchery-reared and farmed salmonid fishes using our qPCR assay reveals the prevalence of this parasite to differ throughout Hokkaido. qPCR analysis of wild freshwater fishes suggests *S. salmonis* is naturally host-specific to chum and masu salmon. Our record of this parasite on adult and wild juvenile chum and masu salmon might be a first, and both species may be infected with *S. salmonis* throughout their life cycle. This raises the possibility that wild individuals of these two salmon species in Chitose River might be sources of infection for juvenile salmon reared in hatchery 1. Infection has been previously reported for hatchery-reared juveniles of brook trout *Salvelinus fontinalis*, lake trout *Salvelinus namaycush*, brown and rainbow trout, Chinook *O. tshawytscha*, and chum, pink and masu salmon (Moore, 1922; Davis, 1926; Awakura and Matsumoto, 1970; Sano and Ushiyama, 1970; Ferguson, 1979; Kent *et al.*, 1992; Urawa and Awakura, 1994; Buchmann and Uldal, 1996; Tojo and Santamaria, 1998; Poynton *et al.*, 2004; Fard *et al.*, 2007; Urawa, 2011; Guz and Puk, 2015; Mizuno *et al.*, 2020). Unlike previous studies, we did not detect infection in farmed rainbow trout or wild brown trout. This discrepancy is possibly due to not only regional or habitat differences but differences in susceptibility to *S. salmonis* among fish species in low prevalence of the parasite. To prevent possible mortality of hatchery-reared juvenile fish, it is necessary to determine if infection by *S. salmonis* is via wild fish.

We estimate the total number of *S. salmonis* cells infecting parasite-positive fish to range 2.37 – 1.78×10^6 based on their infection intensity (36 – 6.05×10^8 copies) using our regression formula. The total estimated number of parasite cells infecting juvenile chum salmon reared at hatchery 7 [which had the lowest infection intensity (1.34×10^3 copies) among all hatchery-reared juveniles infected with the parasite] was 6.23 cells. We could not observe infection in adult masu and wild juvenile chum and masu salmon using microscopy, because infection intensities (36 – 6.86×10^2 copies) were less than the minimum detection limit (1.34×10^3 copies).

In conclusion, we establish an accurate and reliable

qPCR for *S. salmonis* SSUrDNA, and demonstrate the usefulness of this assay for surveying infection of this species in hatchery and wild fishes.

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References

- Awakura, T. and H. Matsumoto (1970): Studies on the *Octomitus* disease of yamabe and amago. *Annual Report of the Hokkaido Fish Hatchery for Fiscal 1969*, pp. 125–126. (In Japanese)
- Buchmann, K. and A. Uldal (1996): Temperature, pH and bile dependent *in vitro* cultivation of *Hexamita salmonis* from rainbow trout *Oncorhynchus mykiss* intestine. *Dis. Aquat. Org.*, **24**, 169–172.
- Davis, H. S. (1926): *Octomitus salmonis*, a parasitic flagellate of trout. *Bull. Bur. Fish. Wash.*, **42**, 9–26.
- Fard, M. R. S., A. Jorgensen, E. Sterud, W. Bleiss and S. L. Poynton (2007): Ultrastructure and molecular diagnosis of *Spironucleus salmonis* (Diplomonadida) from rainbow trout *Oncorhynchus mykiss* in Germany. *Dis. Aquat. Org.*, **75**, 37–50.
- Ferguson, H. W. (1979): Scanning and transmission electron microscopical observation on *Hexamita salmonis* (Moore, 1922) related to mortalities in rainbow trout fry *Salmo gairdneri* Richardson. *J. Fish Dis.*, **2**, 57–67.
- Guz, L. and K. Puk (2015): First molecular identification of *Spironucleus salmonis* (Diplomonadida) from diseased rainbow trout *Oncorhynchus mykiss* in Poland. *Med. Weter.*, **71**, 497–499.
- Isaksen, T. E., E. Karlsbakk, O. Repstad and A. Nylund (2012): Molecular tools for the detection of *Ichthyobodo* spp. (Kinetoplastida), important fish parasites. *Parasitol. Int.*, **61**, 675–683.
- Jackson, G. A., R. S. Livingston, L. K. Riley, B. A. Livingston and C. L. Franklin (2013): Development of a PCR assay for the detection of *Spironucleus muris*. *J. Am. Ass. Lab. Anim. Sci.*, **52**, 165–170.
- Jørgensen, A. and E. Sterud (2007): Phylogeny of *Spironucleus* (eopharyngia: diplomonadida: hexamitinae). *Protist*, **158**, 247–254.
- Keister, D. B. (1983): Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Trans. R. Soc. Trop. Med. Hyg.*, **77**, 487–488.
- Kent, M. L., J. Ellis, J. W. Fournie, S. C. Dawe, J. W. Bagshawe and D. J. Whitaker (1992): Systematic hexamitid infection in seawater pen-reared chinook salmon. *Dis. Aquat. Org.*, **14**, 81–89.
- Kolisko, M., I. Cepicka, V. Hampl, J. Leigh, A. J. Roger, J. Kulda, A. G. B. Simpson and J. Flegr (2008): Molecular

- phylogeny of diplomonads and enteromonads based on SSU rDNA, alpha-tubulin and HSP90 genes: implications for the evolutionary history of the double karyomastigont of diplomonads. *BMC Evol. Biol.*, **8**, 205.
- Levy, M. G., L. V. Powers, K. C. Gore and H. S. Marr (2015): *Spironucleus meleagridis*, an enteric diplomonad protozoan of cockatiels (*Nymphicus hollandicus*): preliminary molecular characterization and association with clinical disease. *Vet. Parasitol.*, **208**, 169–173.
- Mari, D. D. and S. Kotz (2001): Correlation and dependence. Imperial College Press, London, 236 p.
- Mizuno, S., S. Urawa, M. Miyamoto, M. Hatakeyama, N. Koide and H. Ueda (2016): Quantitative analysis of *Ichthyobodo salmonis* an ectoparasitic flagellate infecting juvenile chum salmon *Oncorhynchus keta* in hatcheries. *Fish Sci.*, **83**, 283–290.
- Mizuno, S., S. Urawa, Y. Katsumata, T. Morishita and M. Ban (2020): Ultrastructural and molecular phylogenetic identification of the diplomonad flagellate *Spironucleus salmonis* infecting hatchery-reared salmonid fishes in Hokkaido. *Fish Pathol.*, **55**, 8–17.
- Mo, T. A., T. Poppe and L. Iversen (1990): Systematic hexamitosis in salt-water reared Atlantic salmon. *Bull. Eur. Assoc. Fish Pathol.*, **10**, 69–70.
- Moore, E. (1922): *Octomitus salmonis*, a new species of intestinal parasite in trout. *Trans. Am. Fish. Soc.*, **52**, 74–97.
- Nagle, L., A. R. Place, E. J. Schott, R. Jagus, J. S. Pitula and G. Messick (2009): Real-time PCR assay-based assay for quantitative detection of *Hematodinium* sp. in the blue club *Callinectes sapidus*. *Dis. Aquat. Org.*, **84**, 79–87.
- Pfaffl, M. W. (2004): Quantification strategies in real-time PCR. In "A–Z of quantitative PCR" (ed. by S. A. Bustin). International University Line, La Jolla, pp. 87–112.
- Poppe, T. and T. A. Mo (1993): Systematic, glanulomatous hexamitosis of farmed Atlantic salmon: interactions with wild fish. *Fish. Res.*, **17**, 147–152.
- Poppe, T., T. A. Mo and L. Iversen (1992): Disseminated hexamitosis in sea-caged Atlantic salmon. *Dis. Aquat. Org.*, **14**, 91–97.
- Poynton, S. L., M. R. S. Fard, J. Jenkins and H. W. Ferguson (2004): Ultrastructure of *Spironucleus salmonis* n. comb. (formerly *Octomitus salmonis* sensu Moore 1922, Davis 1926, and *Hexamita salmonis* sensu Ferguson 1979), with a guide to *Spironucleus* species. *Dis. Aquat. Org.*, **60**, 49–64.
- Sano, T. and M. Ushiyama (1970): Control measures and some biological observations of hexamitiasis in trout. *Fish Pathol.*, **4**, 125–129. (In Japanese)
- Sterud, E., T. A. Mo and T. Poppe (1998): Systematic spironucleosis in sea-farmed Atlantic salmon *Salmo salar*, caused by *Spironucleus barkhanus* transmitted from feral Arctic char *Salvelinus alpinus*? *Dis. Aquat. Org.*, **33**, 63–66.
- Tojo, J. L. and M. T. Santamaria (1998): Oral pharmacological treatments for parasitic diseases of rainbow trout *Oncorhynchus mykiss*. I: *Hexamita salmonis*. *Dis. Aquat. Org.*, **33**, 51–56.
- Urawa, S. (2011): Spironucleosis. In "New atlas of fish diseases", 2nd edition (ed. by K. Hatai and K. Ogawa). Midori Shobo Co., Ltd, Tokyo. p. 35. (In Japanese)
- Urawa, S. and T. Awakura (1994): Protozoan diseases of freshwater fishes in Hokkaido. *Sci. Rep. Hokkaido Fish Hatchery*, **48**, 47–58.
- Williams, C. F., D. Lloyd, S. L. Poynton, A. Jorgensen, C. O. M. Millet and J. Cable (2011): *Spironucleus* species: Economically-important fish pathogens and enigmatic single-celled eukaryotes. *J. Aquac. Res. Development*, **1**, 1–13.