Hybridization between native white-spotted charr and nonnative brook trout in the upper Sorachi River, Hokkaido, Japan

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Abstract Invasion status and impacts of nonnative brook trout (*Salvelinus fontinalis*) in a Hokkaido stream were investigated with field surveys and genetic analyses. Nonnative brook trout was detected in nine (41 %) of 22 sampled reaches in three tributaries of the Sorachi River, Hokkaido, Japan. Based on the external pigmentation, twelve putative hybrids between brook trout and native white-spotted charr (*Salvelinus leucomaenis*) were collected in two reaches. Microsatellite and mitochondrial DNA data established that 58% of these hybrids were first generation (F_1) progenies between male brook trout and female white-spotted charr. Our results suggest potential negative impacts of nonnative brook trout on native charr populations in Hokkaido through interspecific interactions.

Keywords Brook trout · White-spotted charr · Invasion · Directional hybridization

Introduction

Invasion by nonnative trout is a serious threat to the conservation of freshwater ecosystems (Fuller et al. 1999; Rahel 2002). Rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and brook trout (*Salvelinus fontinalis*) are among the most widely introduced fluvial salmonid species worldwide in cool-temperate regions (Elliott 1994; Fausch et al. 2001). Evidence suggests these species have negative impacts on native biota through competition, predation, indirect cascade effects, and hybridization (Fausch 1988, 2007; Leary et al. 1993; Townsend 1996; Baxter et al. 2004).

In Hokkaido, northern Japan, rainbow trout originated from western North America and brown trout from Europe have rapidly expanded their distributions during the last four decades by both human-mediated introductions and natural dispersal (Takami and Aoyama 1999; Arai et al. 2002). Concerns have consequently emerged over the impacts of rainbow and brown trout on native salmonids, including masu salmon (*Oncorhynchus masou*), white-spotted charr (*Salvelinus leucomaenis*), Dolly Varden (*Salvelinus malma*), and Sakhalin taimen *(Parahucho perri*) (Kitano 2004; Nomoto et al. 2010; Hasegawa et al. 2012a, 2012b). Previous studies have demonstrated competition or niche segregation between masu salmon and rainbow trout (Taniguchi et al. 2000, 2002; Inoue et al. 2009; Hasegawa et al. 2010) and replacement of white-spotted charr by brown trout (Takami et al. 2002; Morita et al. 2004; Hasegawa and Maekawa 2009).

Stocking of brook trout in Japan is not as popular and expansion is less evident when compared to rainbow or brown trout (Kitano 2004); yet brook trout originated from eastern North America could be one of concerned invasive species in headwater drainages because of their short life cycle, wider habitat preference, and tendency to overpopulate small streams (Scott and Crossman 1973). In western North America, introduced brook trout have displaced native cutthroat trout (*Oncorhynchus clarki*)

through interspecific competition after the widespread establishment of reproducing populations in headwater streams and lakes (Griffith 1988; Dunham et al. 2002; Benjamin et al. 2007). Moreover, hybridization between native bull trout (*Salvelinus confluentus*) and introduced brook trout occurs over a wide geographic area in the western North America (Kanda et al. 2002). For example, Leary et al (1993) described a rapid and almost complete displacement of native bull trout by introduced brook trout in Montana streams, in which initial phases were characterized by frequent hybridization. Although this is not introgressive hybridization forming hybrid swarms mainly due to low fertility of F_1 hybrids, wasted reproductive potential can promote displacement of bull trout by brook trout since hybridization tends to occur predominantly between female bull trout and male brook trout (Leary et al. 1993, 1995; Kanda et al. 2002). Also in Japanese headwater streams, introduced brook trout and white-spotted charr hybrids have been documented based on appearance in streams in Honshu, central Japan (Suzuki and Kato 1966; EAGJ 1982). Little is known about mechanism of hybridization between these two species due to lacking of genetic analysis, but we should pay attention to interspecific hybridization when brook trout invaded into native charr habitats in Japan.

In the present study, we focus on the invasion of brook trout and their potential impacts on native white-spotted charr in upper reaches of the Sorachi River, Hokkaido where brook trout have already been documented (Kondo et al. 2000). We predicted that interspecific hybrids between brook trout and white-spotted charr should be present within this river and utilized genetic analyses to verify putative hybrids identified by external appearance.

Materials and methods

Study area. The study was conducted during 24–27 June, 2003 at three tributaries of upper Sorachi River in the Sorachi district in central Hokkaido, Japan (Fig. 1). Twenty-two study sites (reach length: ca. 50–100 m) were established over the three streams to investigate fish distribution and abundance. Streams at the study sites were generally small (first to third order), with low to moderate channel gradient (Table 1). The upper Sorachi River is inhabited by white-spotted charr, Dolly Varden, brook trout, rainbow trout, Sakhalin taimen, crucian carp (*Carassius auratus langsdorfii*), Siberian stone loach (*Noemacheilus barbatula toni*), sculpin (*Cottus nozawae*) and brook lamprey (*Lethenteron reissneri*).

Field surveys. We made one pass electrofishing to estimate the relative abundance of fish using an electrofishing unit (Model 12 Backpack Electrofisher, Smith-Root Inc.). The captured fish were identified to species based on appearance (Nakabo 2000) and standard lengths were measured. Individuals with ambiguous wavy lines on the dorsal fin were marked as putative hybrids between brook trout and white-spotted charr (Suzuki and Fukuda, 1973; Fig. 2). For *Salvelinus* spp. which were well known for interspecific hybridization (e.g., Suzuki and Fukuda 1974; Leary et al. 1993), fin clips (less than several square millimeters) were preserved in 99 % ethanol for subsequent DNA analyses.

We recorded physical environmental variables (water temperature, reach length, channel width, a maximum depth, and dominant substrate) by measuring at the center of each reach. The channel gradient, expressed as percent change in relative height to reach length, was estimated on 1:25,000 topography maps (Published by Geographical survey institute, government of Japan) by measuring stream length at 10m-incremental changes in elevation.

Genetic analyses. Total genomic DNA of fish were isolated from fin tissue by Proteinase K/SDS digestion at 55 ^{o}C, and followed by phenol-chloroform extractions. Samples were precipitated in 2.5 volumes of EtOH and 0.1 volume of 2 M NaCl.

We used three microsatellite loci (SFO-12, SSA-197, MST-85), which are expected to be diagnostic between brook trout and white-spotted charr, to determine nuclear DNA ancestry of putative hybrids (Angers et al. 1995; O'Relly et al. 1996; Presa and Guyomard 1996; Angers and Bernatchez 1998). Microsatellite amplification was performed on a thermal cycler (Perkin-Elmer Inc.) in a 10 µl reaction containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTP, 0.5µM of each primer and 0.25 units of *Taq* DNA polymerase. Microsatellites were analyzed on an ABI 310 (Applied Biosystems) automated sequencer. Scoring of allele sizes was performed using Genescan version 2.1 and Genotyper version 2.0 (Applies Biosystems), with reference to the internal standard. We included a reference white-spotted charr sample with known allelic sizes on all runs.

PCR-RFLP of NADH dehydrogenase 1 region (ND1: ca. 2,000 bp) of mitochondrial DNA were used for determining maternal ancestry of putative hybrids (Cronin et al. 1993). The ND1 region was chosen because it is expected to have a less intraspecific variation when compared to other mtDNA regions (see Kanda and Allendorf 2001). Amplifications were in 20-µL reaction mixtures containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTP, 0.5 units of *Taq* DNA polymerase. The PCR profile consisted of 95 ^{o}C, 9 min followed by 30 cycles of denaturation (94 ^{o}C, 0.5 min), annealing (55 ^{o}C, 0.5 min) and extension (72 ^{o}C, 2 min). Amplified segments were initially screened for variation with 8 different enzymes: *Hinf*I, *Hpa*II, *Hae*III, *Xba*I, *Taq*I, *Alu*I, *Afa*I (*Rsa*I), and *Dde*I. Digests were performed in 12-µL of PCR product and 2–3 units of restriction enzymes. Digested fragments were separated with 6 % polyacrylamide gels and ethidium bromide staining then visualized by ultraviolet transillumination. Based on the results of an initial screening of baseline samples of *Salvelinus leucomaenis* and *S. fontinalis*, *Taq*I was selected as the restriction enzyme with highest interspecific resolution. *Taq*I digested ND1 into six segments (657, 525, 510, 208, 90, and 21 bp) for brook trout (DDBJ: AF154850) and six segments (ca.

925, 510, 420, 110, 80, and 20 bp) for white-spotted charr.

The software NewHybrids (Anderson and Thompson 2002) was used to estimate the posterior probabilities that each individual belongs to one of six genotypic classes: two parental (P_0, P_1), first generation hybrids (F_1), second generation hybrids (F_2), backcrosses of F_1 with the first parental (B_0), backcrosses of F_1 with the second parental (B_1). Software parameters were set as follows: without individual or allele frequency prior information and independent of "Jeffreys-like" or "Uniform" priors for both mixing proportions and allele frequencies (posterior probabilities were not affected by these priors). Posterior distributions were evaluated after discarding an initial "burnin" of 25,000 sweeps and10^5 iterations of the Monte Carlo Markov Chain. Individuals were assigned to the class with the highest posterior probability. Individuals with probability under 0.9 were not assigned.

Results

Fish distribution and abundance. Eight fish species and 12 putative interspecific hybrid individuals were caught in this study (Table 1). Salmonid fish were major taxa occurring in 19 study sites, and native white-spotted charr was the predominant salmonid fish occurring in 13 (59 %) of 22 sites. Dolly Varden was caught in only one headwater site. Nonnative trout were common, brook trout occurred in nine (41 %) sites and rainbow trout in six (27 %) sites. Five (23 %) study sites were comprised only of nonnative salmonids, and in eight (36 %) sites nonnative salmonids were sympatric with native charr. The sites with highest occurrence of brook trout were steeper in gradient ($F_{1,11} = 7.81$, $P = 0.02$ by ANOVA for arcsin-square-root-transformed gradient) than those of rainbow trout. The density of white-spotted charr was not significantly correlated with that of brook trout (Pearson's $r = -0.31$, $P > 0.05$) nor with that of

177 rainbow trout (r = -0.029, P > 0.05) among 17 sites (F1–3, F5, N1–4, N6–11, T1, T3–4)
178 where either of these salmonid fish (SL > 6 cm) was caught. Putative hybrids between
179 white-spotted charr and brook trout were detected in two sites where these two species
180 co-occurred or inhabited closely. Abundant hybrids were found on the site F5 with the
181 highest salmonid density. The benthic fish abundance differed greatly among streams,
182 rather than among the study sites. For example, stream loaches were abundant in the
183 Furebetsu and Nishitappu streams, but uncommon in the Nunobe stream. Similarly,
184 sculpin commonly occurred in the Nunobe and Nishitappu streams but not in the
185 Furebetsu. Negative effects of invasive nonnative trout on the benthic fish were not
186 clear.
187 Body length distribution of white-spotted charr and brook trout was bimodal
188 with fry (age-0: 4–6 cm in standard length, SL) and older fish (age > 0: SL > 8 cm),
189 indicating self-reproducing populations (Fig. 3). Body lengths of rainbow trout and
190 putative hybrids also varied among individuals, although age-0 cohorts were not clearly
191 identified for them.
192 **DNA analyses of charr.** Genetic variation at microsatellite loci was assessed in
193 63 individuals with >10 cm SL identified by appearance as white-spotted charr, brook
194 trout, and putative hybrids. All three microsatellite loci were polymorphic and variable
195 in both white-spotted charr and brook trout with certain differences in allele size
196 distributions [Table 2, Electronic Supplemental Material (EMS) Table S1].
197 Of these individuals, most were assigned to pure white-spotted charr (n = 23),
198 pure brook trout (n = 29) or F_1 hybrid category (n = 7) with a posterior probability
199 higher than 0.9 (Table 2). The remaining 4 individuals (#119, #120, #121 and #127)
200 could not be assigned to a particular class and may present backcrosses or later
201 generation hybrids (Table 3). Among these, only individual #120 (14.7 cm SL) obtained
202 relatively high support P = 0.72 for parental brook trout. One small putative hybrid
203 #117 (12.7 cm SL) was assigned to pure white-spotted charr. These may partly due to

phenotypic variability along developmental stage.

Of the seven individuals (#1, #118, #122–126) assigned to the F_1 category with high probability ($P \geq 0.9$) by NewHybrids, white-spotted charr was identified as the maternal parent in all cases, i.e., the first generation hybrids were from mating between male brook trout and female white-spotted charr. Variable genotypes of microsatellite markers also indicate that they were not derived from single clutch, though these hybrids were from almost single study site.

Discussion

We found a broad zone of brook trout and rainbow trout invasion in the upper Sorachi River in central Hokkaido. Moreover, we report the first instance of interspecific hybridization between introduced brook trout and native white-spotted charr in Hokkaido. Nonnative trout invasions and following interspecific interactions potentially have negatively impacts on native salmonid species.

Nonnative brook and rainbow trout were most likely introduced into the Sorachi River area during the 1950's to 1990's for aquaculture. Based on a questionnaire to a local angler's shop, aquaculture escapees of brook trout had successfully established self-reproducing populations in tributaries of Nishitappu Stream as early as the 1980's (M. Yamamoto, personal communication). Rainbow trout might be occasionally stocked by private anglers since we found some had deformed fins, a common occurrence on aquaculture fish. Water temperature, especially maximum summer temperature, is probably the chief factor determining the success in establishment of nonnative trout (e.g., Dunham et al. 2002; Benjamin et al. 2007). The temperatures recorded in this study (10–15 °C) are consequently conducive to survival of nonnative brook and rainbow trout.

Although negative relationship was not clearly observed between nonnative trout and native white-spotted charr abundance, the absence of native salmonids in some study sites might result from strong ecological interactions between natives and nonnatives. The potential impacts of nonnative salmonines on native species are widely reported (e.g., Allendorf and Leary 1988; Dunham et al. 2002). Because nonnative salmonids are ecologically very similar to native salmonids, there is a strong potential for common resource requirements (i.e., niche overlap) and for interspecific competition. Interspecific competition is the most widely recognized mechanism of displacement of native cutthroat trout by nonnative brook trout in western North America (Griffith 1988; Dunham et al. 2002). Furthermore, interspecific hybrids between native white-spotted charr and nonnative brook trout were detected, as we had predicted. However, such hybrids were not caught on all co-occur sites. Therefore, ecological factors such as high density, relatively narrow spawning space or time, may play a role in determining the occurrence of interspecific hybridization. Further studies are necessary to examine the relative importance of each effect of competition, predation, pathogen transmission, and hybridization, which may vary in time and space (Taylor et al. 1984).

The genetic analyses of three microsatellite loci and ND1 region RFLPs of mtDNA clearly showed interspecific hybridization between white-spotted charr and brook trout, although possibilities of misidentifying individual fish to each criterion were still remained to some extent based on a limited number of genetic marker (e.g., Allendorf et al. 2001). The hybrids were comprised of abundant F_1, with the near absence of F_2, when we confined to data with reliable identification. This composition may imply that the hybridization is not introgression. Similar interspecific hybridization has been documented between bull trout and introduced brook trout in North America which produce nearly sterile progeny (Leary et al. 1993, 1995; Kanda et al. 2002). This idea would be supported by the experimental data that survival rates of hybrid progenies between brook trout and white-spotted charr decreased with increased generation of

backcrosses (Suzuki and Fukuda 1974). More diagnostic nuclear loci may help with resolution for this hybridization.

Directional hybridization has been implicated in the population decline of endangered species (Leary et al. 1993). Because eggs are generally a crucially limited resource for population growth of a species than sperms, it is possible that the population of white-spotted charr suffers more detrimental effects from hybridization due to reduced egg availability. According to the maternally inherited mtDNA analysis, most F_1 hybrids had white-spotted charr mtDNA. This indicates that the detected hybridization between native white-spotted charr and introduced brook trout is unidirectional, with brook trout males mating with female white-spotted charr in the study streams. Directional hybridization can be caused by various pre- and post-mating factors (Taylor 2004). For post-mating factor of salmonid species, it is often observed that progeny of one direction of hybridization displays higher survival than the reciprocal cross (Suzuki and Fukuda 1974). However, since the rates of survival and growth of F_1 hybrids differ little irrespective of parent combination between brook trout and white-spotted charr (Suzuki and Fukuda 1971), post-mating factors would be less important for hybridization between white-spotted charr and brook trout. Pre-mating factors, such as differences in mating tactics, competitive ability, and/or reproductive timing between parental species, may be the strongest determinants of directional hybridization (Wirtz 1999; Taylor 2004). In cases where there are differences in size at maturity between species, sneaking tactics employed by smaller species have been proposed as an explanation for directional hybridization (Baxter et al. 1997; Taylor 2004). However, size range of adult fish was quite similar for white-spotted charr and brook trout in the study area, which also indicates that they have similar competitive potential for mate acquisition. The observed directional hybridization may be due to the asynchronous spawning patterns of these species. In a stream in Honshu, white-spotted charr spawn from late October to early November, whereas brook trout spawn from

November to December (Uehara and Yoshida 1984). The reproductively active period of males generally begins earlier than that of females in stream charr (e.g., Kitano 1996). The spawning period of brook trout males is more likely to overlap with white-spotted female charr than *vice versa*. Such hybridization processes have also been suggested in other species of salmonids (e.g., Rosenfield et al. 2000; Kitano et al. 2009).

Our results indicate the occurrence of hybridization between native white-spotted charr and nonnative brook trout, which may play a role in the displacement of native charr by nonnative trout in Hokkaido streams. Further ecological studies should attempt to reveal mechanisms and impacts of nonnative trout invasion so that managers can develop effective conservation strategies of native endangered species.

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446

Figure Captions

Fig. 1 Study area location in the Sorachi River with relative percent composition of salmonid species (pie chart). Site and species codes correspond to Table 1

Fig. 2 Appearances of *Salvelinus fontinalis* (top: 18.4 cm SL), a putative hybrid (middle: 25.3 cm SL), and *Salvelinus leucomaenis* (bottom: 15.3 cm SL)

Fig. 3 Size-frequency distribution of salmonid fishes. *OM Oncorhynchus mykiss, HB* putative hybrids between *Salvelinus leucomaenis* and *Salvelinus fontinalis, SF Salvelinus fontinalis, SM Salvelinus malma, SL Salvelinus leucomaenis*

Table 1 Habitat variables and fish data of each sampling site in Furebetsu stream (F1–5), Nunobe Shimonosawa stream (N1–5), Nunobe main stream (N6-12), and Nishitappu stream (T1–5)

Sites	Elevation	Gradient	Width	Depth	Substrate	WT	Salmonids density[a]	Fish species								
	(m)	(%)	(m)	(cm)		(°C)	(N·100m^{-2})	*SM*	*SL*	*SF*	*OM*	*HB*	*CA*	*BT*	*CN*	*LR*
F1	185	0.8	5.0	80	Boulder/Sand	11.9	0.5		2		2		1	48		1
F2	260	0.8	4.0	50	Pebble/Sand	11.3	1.3		14		1			8		
F3	270	1.5	3.0	60	Boulder/Pebble	11.7	4.5		8					12		
F4	290	4.0	3.0	40	Pebble/Sand	—	0.0		4					1		
F5	320	1.5	2.5	50	Boulder/Pebble	11.5	29.6		22	4		11		19		
N1	260	0.7	3.5	80	Boulder/Sand	11.5	6.9		2		10			15		
N2	290	1.3	3.0	50	Pebble	9.9	2.0			6	2			1	28	1
N3	300	1.0	1.5	40	Pebble/Sand	10.1	3.3			7	1				16	
N4	310	2.0	2.0	40	Pebble/Sand	10.1	6.9			12					12	
N5	300	2.9	1.0	40	Pebble/Sand	11.5	0.0								23	
N6	310	2.0	4.0	40	Boulder/Pebble	10.3	0.5		1						8	
N7	310	1.7	5.0	80	Bedrock/Pebble	12.2	1.3		4			1				
N8	310	1.5	2.0	50	Bedrock/Boulder	18.3	5.0		1	1			11	1	2	
N9	315	1.5	2.0	60	Pebble/Sand	—	2.7		1	1					1	
N10	350	4.0	2.0	20	Pebble	8.5	2.5			5					1	
N11	320	1.5	5.0	80	Boulder/Pebble	15.5	0.4			2				29		
N12	670	6.7	8.0	150	Bedrock/Boulder	11.0	0.1	1								
T1	330	2.2	4.0	40	Bedrock/Pebble	14.3	0.8		2	1				12	3	1
T2	310	7.5	1.0	30	Boulder/Pebble	11.1	0.0							14	2	
T3	330	1.3	2.0	30	Pebble/Sand	10.8	4.2		5					10	6	1
T4	340	1.0	2.5	50	Pebble/Sand	11.1	12.0		9		9			6	13	4
T5	360	1.5	7.0	120	Boulder	9.3	0.0								7	

SM Salvelinus malma, *SL Salvelinus leucomaenis*, *SF Salvelinus fontinalis*, *OM Oncorhyncus mykiss*, *HB* putative hybrids between *SL* and *SF*, *CA Carassius auratus langsdorfii*, *BT Noemacheilus barbatulus toni*, *CN Cottus nozawae*, *LR Lethenteron reissneri*

a An underestimate, because based on number of salmonids (SL > 6 cm) caught by one pass electrofishing

Table 2 Results of hybrid analyses of two salmonid fish (*SL Salvelinus leucomaenis*, *SF Salvelinus fontinalis*) implemented by NewHybrids (Anderson and Thompson 2002), with associated species identification based on appearance

Appearance	Range of microsatellite loci (bp)			No of individuals assigned by NewHybrids			No of individuals with each mtDNA	
	SFO-12	SSA-197	MST-85	Pure *SL*	Pure *SF*	F_1 hybrid	*SL*	*SF*
SL	199–235	114–120	138–150	22	0	0	22	0
SF	269–273	146–160	174–190	0	29	0	0	29
Putative hybrids*	199–273	114–158	138–176	1	0	7	8	0

* Four individuals with low posterior probabilities ($P < 0.90$) were not included in this Table

Table 3 Individual posterior probabilities output by NewHybrids which was not able to be assigned to a particular class with $P \geq 0.90$ and mtDNA type

Individual	Output by NewHybrids	mtDNA
#119	$B_{SF} = 0.53$; $F_1 = 0.25$; $F_2 = 0.20$	*SL*
#120	$P_{SF} = 0.72$; $B_{SF} = 0.22$	*SF*
#121	$B_{SF} = 0.46$; $P_{SF} = 0.22$; $F_1 = 0.18$; $F_2 = 0.14$	*SF*
#127	$F_1 = 0.66$; $B_{SF} = 0.22$	*SF*

P_{SF} parental *S. fontinalis*, B_{SF} backcross F_1 × *S. fontinalis*, F_1 first generation hybrid, F_2 second generation hybrid

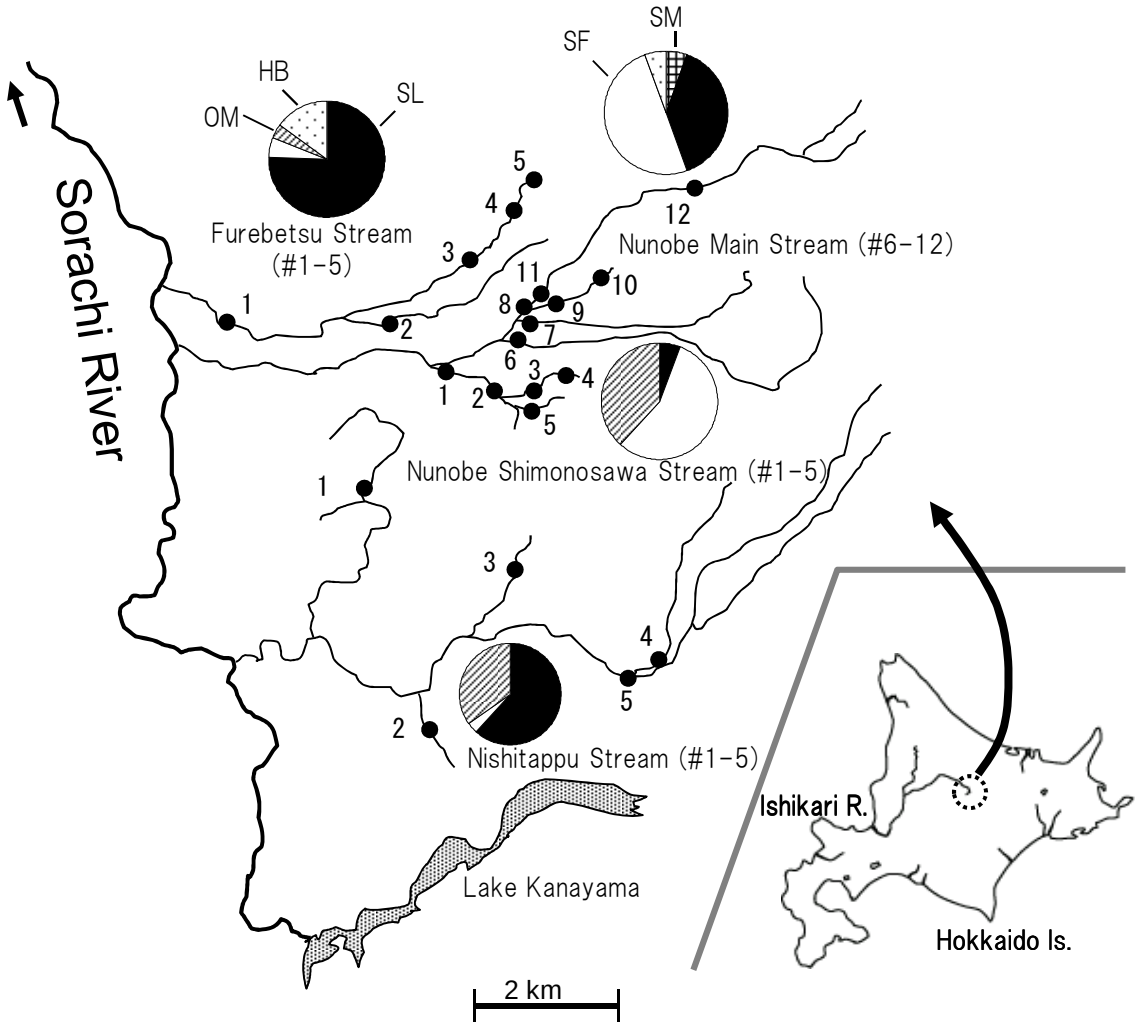

Fig 1 Kitano et al

Fig 2 Kitano et al

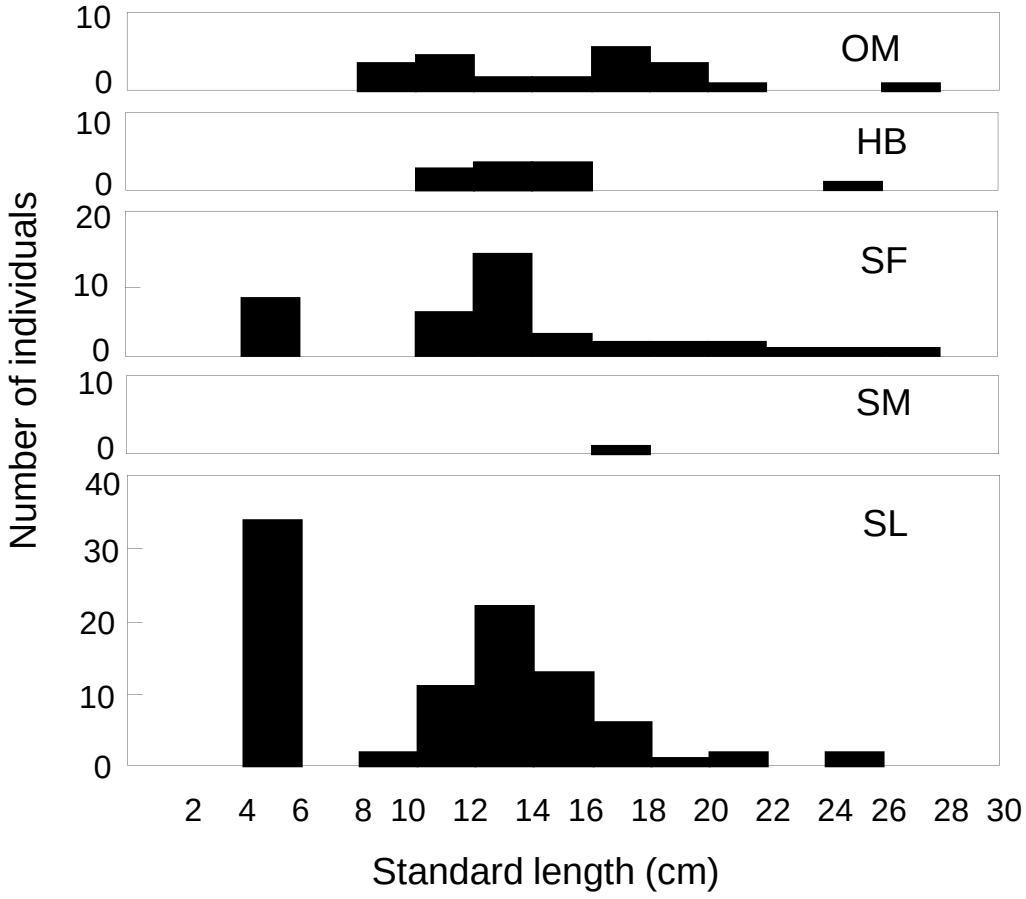

Fig 3 Kitano et al