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1 **Temporal change in the distribution and composition of native, introduced, and**  
2 **hybrid charrs in northern Japan**

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10 Short title: Hybridization between native and non-native charr

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23 **Abstract**

24       Introductions of non-native species have caused various negative impacts on native  
25 species and their ecosystems. Hybridization is particularly prevalent among closely  
26 related species, and can result in displacement, hybrid swarms, or the disruption of a  
27 locally adapted gene complex. Although hybridization between native and non-native  
28 species is widespread, long-term monitoring is generally lacking. In this study, we  
29 compared the distribution and composition of native white-spotted charr (*Salvelinus*  
30 *leucomaenis*), introduced brook trout (*Salvelinus fontinalis*), and their hybrids in the  
31 upper Sorachi River, Hokkaido, Japan in 2003 and 2013, especially focusing on (1) if  
32 genetic introgression or hybrid swarm has occurred and (2) if white-spotted charr have  
33 declined, since a previous study indicated a potentially harmful asymmetric hybridization  
34 with the mothers of hybrids being all white-spotted charr. We found no evidence of  
35 decline in native white-spotted charr; rather, the distribution and abundance of introduced  
36 brook trout had decreased. Of 142 charr (i.e., genus *Salvelinus*) collected, 18 individuals  
37 (13%) were hybrids but no unidirectional hybridization was observed. However, most of  
38 the hybrids were post-F1 individuals with biased mating with white-spotted charr. The  
39 effects of long-term introgression on native white-spotted charr should be further  
40 examined.

41

42 **Keywords:** hybrid swarm; introgression; invasive species; rainbow trout; microsatellite;  
43 mtDNA

## 44 **Introduction**

45        Introductions of non-native species are a major threat to biodiversity and can have  
46 serious economic impacts (Vitousek et al., 1997; Allendorf & Lundquist, 2003; Gozlan et  
47 al., 2010). Introduced species interact with native species in various ways, such as  
48 through competition, predation, hybridization, or spread of diseases and parasites (Mack  
49 et al., 2000; Allendorf et al., 2001; Peeler et al., 2011). These effects have contributed to  
50 the decline or extinction of many populations of plants and animals  
51 (Allan & Flecker, 1993; Rhymer & Simberloff, 1996).

52        Hybridization is more common among fish species than among other vertebrate taxa  
53 because most fishes have external fertilization (Hubbs, 1955; Leary et al., 1995; Scribner  
54 et al., 2001). In addition, many fish species have been introduced around the world for  
55 both recreational and commercial purposes. This can exacerbate non-native and native  
56 species interactions through invasive hybridization which may become more serious with  
57 increases in land use and global climate change (Allendorf et al., 2001; Muhlfeld et al.,  
58 2014). Hybridization and introgression of native with non-native species can cause the  
59 breakdown of inherent gene complexes and ecological adaptations in native populations,  
60 which can threaten the persistence of rare or endangered taxa  
61 (Rhymer & Simberloff, 1996).

62        Hybridization can result in three major consequences. First, if hybrids are fertile and  
63 have relatively high fitness, hybrid swarms can form in which the majority or all of the  
64 individuals in a population are of hybrid origin resulting in genomic extinction of parental  
65 species (Epifanio & Philipp, 2000; Allendorf et al., 2001). A famous example of a hybrid  
66 swarm is the one between native cutthroat *Oncorhynchus clarki* and introduced rainbow  
67 trout *O. mykiss* (Allendorf & Leary, 1988). Alternatively, if hybrids are sterile or have

68 very low fitness, there will be two outcomes depending on the direction of hybridization.  
69 If the mating is unidirectional, the maternal species may decline or even be displaced by  
70 the paternal species because the production of eggs has higher energetic costs than sperm  
71 and is often limited as a resource for population growth. For example, this unidirectional  
72 hybridization might have significantly reduced native bull trout *Salvelinus confluentus*  
73 populations in cases where brook trout *S. fontinalis* have been introduced (Leary et al.,  
74 1993). Finally, if hybrids have low fitness and the direction of mating is more or less  
75 random, two interbreeding species might be able to coexist, as in the many cases of  
76 natural hybrid zones (Taylor, 2004).

77       Although hybridization between native and introduced species is globally widespread  
78 (reviewed in Rhymer & Simberloff, 1996), long-term monitoring is generally lacking (but  
79 see Muhlfeld et al., 2014). Displacement of native by non-native species can be rapid  
80 (e.g., < 10 years) especially when unidirectional hybridization occurs and the fitness of  
81 hybrids is very low (Leary et al., 1993; Konishi & Takata, 2004). To assess the impacts of  
82 introduced species appropriately and develop management schemes, long-term  
83 monitoring is necessary.

84       In Japan, hybridization between native white-spotted charr (*Salvelinus leucomaenis*)  
85 and non-native brook trout (brook charr, *S. fontinalis*) has been documented in the upper  
86 Sorachi River of Hokkaido (Kitano et al., 2014). In addition, mitochondrial DNA  
87 (mtDNA) of all F1 hybrids ( $N = 7$ ) were identical to that of white-spotted charr,  
88 indicating unidirectional hybridization (Kitano et al., 2014). Suzuki (1974) indicated in a  
89 breeding experiment that the fertility of F1 hybrids was lower than that of parental  
90 species, although the fitness in the wild is unknown. Therefore, we can predict either  
91 ongoing genetic introgression, which might result in hybrid swarm, or decline of native

92 white-spotted charr through the seemingly unidirectional hybridization in the upper  
93 Sorachi River. However, populations of native white-spotted charr have not been  
94 monitored since the initial survey in 2003 (Kitano et al., 2014). In addition, because the  
95 previous study only used three microsatellite loci with a relatively small number of  
96 individuals for genetic analysis ( $N = 63$ ), a more detailed survey is required.

97 In the present study, the current status of the native and introduced charr was  
98 evaluated to determine (1) whether genetic introgression or hybrid swarm has occurred  
99 and (2) whether decline of native white-spotted char has occurred via unidirectional  
100 hybridization. A follow-up survey in the same 22 study sites as the previous study (Kitano  
101 et al., 2014) was conducted, and using a greater number of microsatellite markers and  
102 hybrid individuals, we compared the distributions of parental species and their hybrids  
103 between the two sampling times separated by a 10-year interval.

104

105

## 106 **Materials and methods**

107

### 108 *Study area and field surveys*

109 The study area was located in three major tributaries of the upper Sorachi River,  
110 central Hokkaido, Japan (Fig. 1). The same 22 sites were sampled as in the previous  
111 survey (Kitano et al., 2014) in order to directly compare the distribution and abundance  
112 of individuals between 2003 and 2013. Reach lengths of each site were set at least 100 m,  
113 the same length, or longer than the previous survey (Kitano et al., 2014) for more  
114 accurate sampling. Brook trout, white-spotted charr, their hybrids, rainbow trout, Dolly  
115 Varden (*Salvelinus malma*), freshwater sculpin (*Cottus nozawae*), Siberian stone loach

116 (*Nemacheilus barbatulus toni*), Japanese dace (*Tribolodon hakonensis*), and brook  
117 lamprey (*Lethenteron reissneri*) inhabit this study area.

118 Brook trout are coldwater-adapted species originally distributed in northeastern  
119 North America. In Japan, brook trout have been recorded in 14 prefectures of Honshu and  
120 Hokkaido but self-reproducing populations have been reported in only four freshwater  
121 systems of spring-fed streams or cold mountain lakes (Kitano, 2004). Although the  
122 current distribution is limited, introduced populations could affect native salmonids via  
123 hybridization, redd superimposition, and possibly competition (Kitano, 2004). Brook  
124 trout were most likely introduced into the study area during the 1950s to 1990s for  
125 aquaculture purposes (Kitano et al., 2014).

126 A one-pass backpack electrofishing survey (200–300 V) was conducted during July,  
127 2013 at the 22 study sites. The fish collected were anesthetized with clove oil, identified  
128 to species using phenotypic characteristics, and measured to the nearest 1 mm (fork  
129 length for salmonids and total body length for other species) (Nakabo, 2000). Hybrid  
130 individuals often showed intermediate physical characteristics and coloration between  
131 white-spotted charr and brook trout, especially with regard to the dorsal fins. Individuals  
132 with ambiguous wavy lines on the dorsal fin were marked as putative hybrids according  
133 to Kitano et al. (2014). Adipose fin tissues were collected from all charr and preserved in  
134 99% ethanol for subsequent DNA analysis to verify species identification.

135

#### 136 *DNA analysis*

137 Total genomic DNA was extracted from fin tissues with a PureGene DNA isolation  
138 kit (Applied Biosystems) following manufacturer's instructions. Eight microsatellite loci  
139 were used (Sfo12, Ssa197, Sco200, Ots101, Sle6, u-85, Sco211, Sle5), of which Sfo12,

140 Ssa197, and MST-85 were expected to be diagnostic markers to identify brook trout,  
141 white-spotted charr, and their hybrids (Kitano et al., 2014). Allele sizes of other loci were  
142 partially overlapped but significantly differentiated between white-spotted charr and  
143 brook trout ( $F_{ST} > 0.4$  for all loci combined), resulting in high resolution for species  
144 identification (e.g., Vähä & Primmer, 2006). PCR reactions were performed in 10 ul  
145 volumes using a thermal cycler (Takara; Thermal cycler TP650). The reaction mixture  
146 contained 0.5 U Master Mix (GoTaq, Promega), 0.2  $\mu$ M of each primer, 0.2 mM dNTP,  
147 50 mM KCL, 15 mM Tris-HCl (pH 8.0), 1.5 mM MgCl<sub>2</sub>, and approximately 50–100  
148 ng/ $\mu$ l of genomic DNA as a template. PCR was carried out for 2 min at 95°C followed by  
149 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at  
150 72°C for 30 s. The amplified products were analyzed on the genetic analyzer ABI 3130  
151 (Applied BioSystems) and allele sizes were scored by GeneMapper (GeneMapper v.4.0;  
152 Applied BioSystems).

153 To evaluate the direction of hybridization between brook trout and white-spotted  
154 charr, maternally inherited mtDNA was amplified using the primers HN20 (5'-GTG TTA  
155 TGC TTT AGT TAA GC3') and Tpro2 (5'-ACC CTT AAC TCC CAA AGC3'), which  
156 are located in the proline and phenylalanine tRNAs, respectively (Brunner et al., 2001).  
157 The PCR condition was 2 min at 94°C followed by 40 cycles of denaturation at 93°C for  
158 60 s, annealing at 47°C for 60 s, and extension at 72°C for 60 s. The PCR products were  
159 purified by PEG precipitation. Purified products were cycle sequenced using Big dye  
160 terminator v.3.1 (Applied BioSystems) and run on the ABI 3130 (Applied BioSystems).  
161 The base sequences were analyzed with the software MEGA ver.6.0 (Tamura et al., 2013).  
162 The mother species was determined by comparing the mtDNA sequences with known  
163 sequences of brook trout (AF154850) and white-spotted charr (KF513161) from DDBJ

164 (DNA Data Bank of Japan) database.

165

166 *Data analysis*

167 To evaluate the changes of salmonid compositions, proportions of captured  
168 salmonids were compared between 2003 and 2013 with a  $\chi^2$  test. Following Kitano et al.  
169 (2014), data were summarized to four regions due to small sample sizes in some of the 22  
170 sites (Fig. 1). We also compared the proportion of salmonids in site FB5 where most  
171 hybrids were collected in 2003. Parental species and hybrids were genetically determined  
172 by the software NewHybrids (Anderson & Thompson, 2002). We assigned each  
173 individual to one of six genotypic classes based on the posterior probabilities: two  
174 parental (P0, P1), first-generation hybrids (F1), second-generation hybrids (F2),  
175 backcrosses of F1 with the first parental (B0), and backcrosses of F1 with the second  
176 parental (B1). Software parameters were set as follows: without individual or allele  
177 frequency prior information and the “Jeffreys-like” priors for both mixing proportions  
178 and allele frequencies. Posterior distributions were evaluated after discarding an initial  
179 “burnin” of 25,000 sweeps and  $10^5$  iterations of the Monte Carlo Markov Chain.  
180 Individuals were assigned to the class with the highest posterior probability. Since  
181 NewHybrids detects only F1 and second hybrid generations, we also performed a  
182 Bayesian clustering method to infer potential later generation hybrids by the software  
183 STRUCTURE (Pritchard et al., 2000). Although STRUCTURE cannot determine the  
184 generations of hybrid classes, we assumed the presence of introgressive hybridization  
185 when different levels of genetic admixture were observed among hybrid individuals. We  
186 ran the program for a user-defined number of clusters  $k$  (1–5) under the following  
187 conditions:  $10^6$  replicates after a burn-in of  $10^5$ , admixture model, correlated allele

188 frequency, and no prior population information. When all charr samples (i.e.,  
189 white-spotted charr, brook trout, and their hybrid) were analyzed, the most likely number  
190 of genetic clusters was  $k = 2$ , representing white-spotted charr and brook trout (see  
191 Results). We determined F1 hybrids when the probability of assignments of either one of  
192 the parental species was larger than 40% and smaller than 60% (theoretically 50%),  
193 whereas we assumed post-F1 hybrids when it was 5–40% or 60–95%.

194

195

## 196 **Results**

197

198       Seven fish species and putative hybrids were caught in the distribution survey, of  
199 which a total of 188 individuals were salmonids (Supplementary Table 1). The results  
200 from NewHybrids and STRUCTURE were largely consistent: the former indicated 18  
201 hybrids and the latter indicated 18 hybrids (Fig. 2; Table 1). Both analyses suggested that  
202 only 1–2 hybrids were F1 and the rests were post-F1, with backcrosses between F1 and  
203 white-spotted charr being dominant. Hereafter, we will only report the result from  
204 STRUCTURE due to the consistency with NewHybrids. Of the 22 sites surveyed,  
205 occurrence sites of white-spotted charr, brook trout, and their hybrids were 16 (73%), 4  
206 (18%), and 5 sites (23%), respectively.

207       Contrary to the prediction, white-spotted charr have not declined: rather, opposite  
208 trends were observed. The occurrence site of white-spotted charr increased from 13 sites  
209 in 2003 to 16 sites in 2013, whereas that of brook trout decreased from 9 to 4 sites. The  
210 occurrence of hybrids increased from 2 sites in 2003 to 5 sites in 2013. In site FB5, where  
211 unidirectional hybridization had been observed in 2003, pure brook trout were not

212 detected, however pure white-spotted charr and hybrids were observed in the current  
213 survey (Fig. 3). Additional sampling in and around the site was conducted several times,  
214 but pure brook trout were not captured. The composition of salmonid species at the  
215 Shimonosawa stream was significantly different between 2003 and 2013 ( $\chi^2 = 22.469$ ,  $P <$   
216  $0.01$ ) with the proportion of brook trout having decreased (Fig. 3). In sites NS2–NS4,  
217 which are above an erosion control dam, few salmonids were collected, even during  
218 additional intensive sampling in and around the site. Compositions of salmonids were  
219 relatively stable in the other three regions (Furebetsu  $\chi^2 = 6.488$ ,  $P = 0.09$ ; Nunobe main  
220  $\chi^2 = 1.915$ ,  $P = 0.59$ ; Nishitappu  $\chi^2 = 1.561$ ,  $P = 0.46$ ).

221 In total, 98 (69%) white-spotted charr, 25 (18%) brook trout, and 18 (13%) hybrids  
222 were collected from the present survey. Contrary to the previous study, no evidence for  
223 sex-specific unidirectional hybridization was observed (Table 1). In site FB5 half of the  
224 hybrids had white-spotted charr and brook trout mtDNA, respectively. In site NM10, all  
225 the six hybrids had brook trout mtDNA. However, direction of mating was biased after  
226 post-F1: most were either backcrosses between F1 and white-spotted charr or later  
227 generations mating with white-spotted charr. The varying degrees of genetic admixture  
228 shown in STRUCTURE suggest ongoing introgression.

229

230

## 231 **Discussion**

232

233 We found no evidence of a decrease in native white-spotted charr. Rather, the  
234 distribution and abundance of introduced brook trout has decreased in the past 10 years.  
235 This is especially the case for site FB5 where in 2003 pure brook trout and hybrids had

236 been observed, yet presently pure brook trout individuals have disappeared. Moreover, in  
237 the Shimonosawa stream, brook trout are now rare when in 2003 they dominated. Post-F1  
238 hybrids were detected in several sites, indicating ongoing introgressive hybridization,  
239 although no hybrid swarm was observed in any site. All the hybrids found 10 years ago  
240 had white-spotted charr mtDNA, but hybrids found in this study were produced from  
241 both mothers of the native and non-native charr. Taken together, these results suggest that  
242 hybridization levels between native and introduced charr fluctuate substantially even  
243 within a relatively short time span of 10 years, which highlights the need for long-term  
244 monitoring of introduced populations.

245       Most hybrids were post-F1 individuals with varying degrees of genetic admixtures  
246 from the two parental species. This indicates that hybrids of white-spotted charr and  
247 brook trout are fertile even after F1 generations (e.g., Suzuki, 1974) but their fitness is not  
248 so high as to cause a hybrid swarm. Alternatively, 20–60 years (i.e., since the introduction  
249 of brook trout, Kitano et al., 2014) may not be enough time for a hybrid swarm to form.  
250 Few studies have examined direct fitness of hybrids between native and non-native  
251 salmonids so far, but Muhlfeld et al. (2009) suggest that even lowered reproductive  
252 success of hybrids can cause long-term introgression. We should continue to monitor the  
253 introgression and also investigate the ecological consequences of the introgression, such  
254 as losses in locally adapted gene complexes (Rhymer & Simberloff, 1996).

255       Our data also indicate that patterns of hybridization change both spatially and  
256 temporally. For example, all the hybrids in the upper Furebetsu stream (FB3–FB5) in  
257 2003 were produced by female white-spotted charr and male brook trout, but the hybrids  
258 in 2013 were produced from both mothers of the native and non-native charr. In addition,  
259 all the hybrids found in MN10 had brook trout mtDNA. Interestingly, most hybrids

260 collected in this study were post-F1 hybrids with higher proportions of genetic admixture  
261 from white-spotted charr. This is probably because the native white-spotted charr have  
262 been dominating the upper Sorachi River, leading to the higher probability of mating  
263 between hybrids and white-spotted charr. It is known that population sizes should affect  
264 the frequency and direction of inter-specific hybridization (Wirtz, 1999). Hybridization is  
265 often unidirectional in salmonids (Redenbach & Taylor, 2003; Baumsteiger et al., 2005;  
266 Kozfkay et al., 2007), but spatial and temporal variations in the patterns of hybridization  
267 have also been reported (Kanda et al., 2002; Rubidge & Taylor, 2004; Gunnell et al.,  
268 2008; DeHaan et al., 2010). It seems that many different factors affect the frequency and  
269 directionality of hybridization, such as population size (Kanda et al., 2002; Rubidge &  
270 Taylor, 2004; DeHaan et al., 2010), sneaking mating behavior (Kitano et al., 1994), and  
271 breeding periods. In our system, relative abundance may be one of the important factors,  
272 but a more detailed survey is certainly required.

273 Populations of non-native brook trout have been declining in the last 10 year period,  
274 which may be partly due to hybridization. In the upper Furebetsu stream (FB3–FB5)  
275 where a relatively large number of hybrids were observed in 2003, no pure brook trout  
276 were collected in this survey, whereas post-F1 hybrids were still observed. White-spotted  
277 charr have dominated in this stream and pure brook trout might have had lower chances  
278 to mate with conspecifics, resulting in the near disappearance. However, many other  
279 ecological factors could affect the decline of brook trout other than hybridization. For  
280 example, the compositions of non-native rainbow trout increased in some tributaries,  
281 which may be replacing brook trout because rainbow trout are much larger (e.g., 15-25  
282 cm in brook trout compared to 20–50 cm in rainbow trout) and fecund (e.g., Clark &  
283 Rose, 1997). Also, rainbow trout could be a potential threat to native white-spotted charr

284 (Morita et al., 2004). In the upper Shimonosawa stream (NS2–NS4) a local population of  
285 brook trout have almost collapsed in the past 10 years. Interestingly, rainbow trout have  
286 also significantly declined in this stream (I. Koizumi, personal observation), whereas  
287 freshwater sculpin have increased. Some portions of the Shimonosawa stream flow in  
288 pristine natural forests and conditions should be favorable for salmonids. Therefore, it is  
289 difficult to imagine why certain salmonids declined dramatically. One possible factor for  
290 near local extinction could be loss of genetic diversity (Saccheri et al., 1998). Local  
291 population sizes of brook trout and rainbow trout would have been small because the  
292 populations had been isolated by an erosion control dam (ca. 3 m in height, no fish  
293 passage) in the middle of the stream. Strong genetic drift, as well as founder effects,  
294 would have lowered the genetic diversity of the introduced species.

295       Climate change might also have mediated the distributions and species compositions,  
296 including hybrids. In this region, mean annual air temperature has been increased by one  
297 degree Celsius over the past 30 years (Pearson's correlation,  $r = 0.529$ ,  $P = 0.001$ , data  
298 source: the Japan Meteorological Agency, the Rokugo station, Fig. 1). A similar trend was  
299 observed during 2001–2013 (Pearson's correlation,  $r = 0.739$ ,  $P = 0.003$ ) and the mean  
300 summer temperature (June–August) differed by 2.17 °C between the years 2001–2003  
301 and 2011–2013 (considering the years affecting the dominant year classes, i.e., age-0+,  
302 1+, and 2+, during the study periods). Increase in water temperature alone, or in  
303 conjunction with temperature-dependent competition (Taniguchi & Nakano, 2000) might  
304 have influenced populations of white-spotted charr, brook trout, rainbow trout, as well as  
305 hybrids in the last decade. More detailed surveying, as well as long-term monitoring, will  
306 be required.

307

308

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314

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427 **Figure Captions**

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429 **Fig. 1** Study location in central Hokkaido, Japan showing the location of 22 study sites in  
430 the upper Sorachi River where distributions of brook trout, white-spotted charr and  
431 hybrids were compared with that of 10 year ago. Site numbers correspond to  
432 Supplementary Table 1. The location of the Rokugo meteorological station from the  
433 Japan Meteorological Agency is indicated by a star.

434

435 **Fig. 2** Distruct plots for STRUCTURE runs of white-spotted charr, brook trout, and  
436 hybrids collected in the upper Sorachi River. Each fish is represented by a vertical bar  
437 that denotes membership fractions ( $K = 2$ ). Red bars represent pure white-spotted charr,  
438 and green bars represent pure brook trout. Bars which have both red and green indicate  
439 hybrid individuals

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441 **Fig. 3** Relative compositions of salmonid species in 2003 and 2013. Abbreviations of  
442 species names are as follows *WSC* white-spotted charr, *BT* brook trout, *HYB* hybrid  
443 between white-spotted charr and brook trout, *RT* rainbow trout. One Dolly Varden charr  
444 caught in NM12 in 2003 was not included.

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452 **Table 1** Number of hybrids collected and their mtDNA haplotypes. The number in the

453 Extra represents hybrids caught in extra surveys.

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Sites	No. of hybrids				mtDNA haplotype	
	F1	F1 × WSC	F1 × BT	F2	WSC	BT
FB3	-	1	-	-	-	1
FB4	-	-	-	1	1	-
FB5	-	6	-	-	3	3
NS5	-	1	-	-	1	-
NM10	-	6	-	-	-	6
Extra	1	2	-	-	2	1

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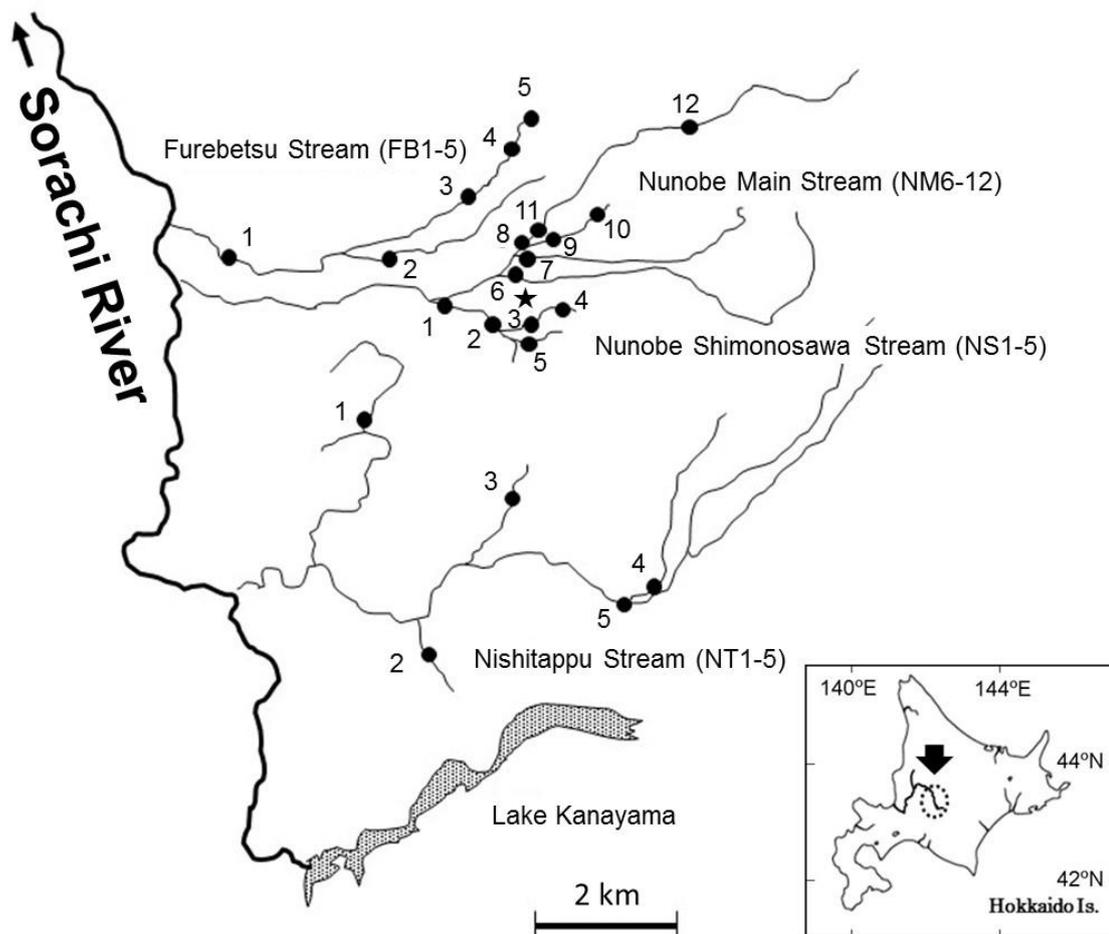
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459 **Fig. 1**

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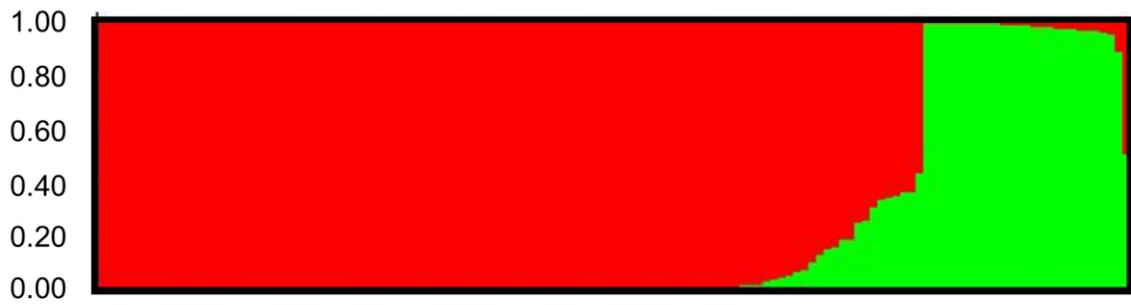
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471 **Fig. 2**

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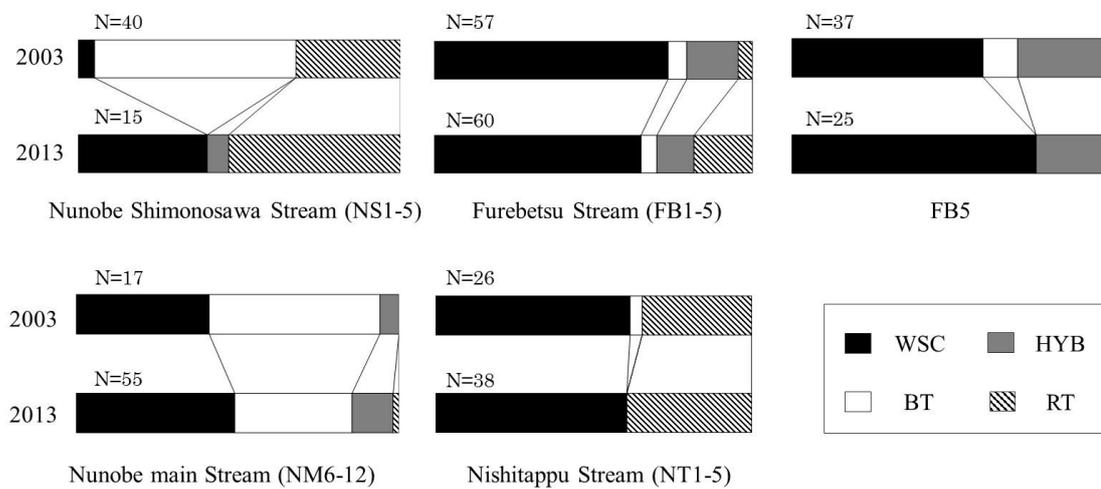
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491 **Fig. 3**

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