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

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Short title: Hybridization between native and non-native charr

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

Materials and methods

Study area and field surveys

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

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

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

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

DNA analysis

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

Ssa197, and MST-85 were expected to be diagnostic markers to identify brook trout, white-spotted charr, and their hybrids (Kitano et al., 2014). Allele sizes of other loci were partially overlapped but significantly differentiated between white-spotted charr and brook trout (F_{ST} > 0.4 for all loci combined), resulting in high resolution for species identification (e.g., Vähä & Primmer, 2006). PCR reactions were performed in 10 ul volumes using a thermal cycler (Takara; Thermal cycler TP650). The reaction mixture contained 0.5 U Master Mix (GoTaq, Promega), 0.2 µM of each primer, 0.2 mM dNTP, 50 mM KCL, 15 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, and approximately 50–100 ng/µl of genomic DNA as a template. PCR was carried out for 2 min at 95ºC followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72ºC for 30 s. The amplified products were analyzed on the genetic analyzer ABI 3130 (Applied BioSystems) and allele sizes were scored by GeneMapper (GeneMapper v.4.0; Applied BioSystems).

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

(DNA Data Bank of Japan) database.

Data analysis

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

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

Results

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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315 **References**

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Figure Captions

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

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

Fig. 3 Relative compositions of salmonid species in 2003 and 2013. Abbreviations of species names are as follows *WSC* white-spotted charr, *BT* brook trout, *HYB* hybrid between white-spotted charr and brook trout, *RT* rainbow trout. One Dolly Varden charr 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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458

Fig. 1

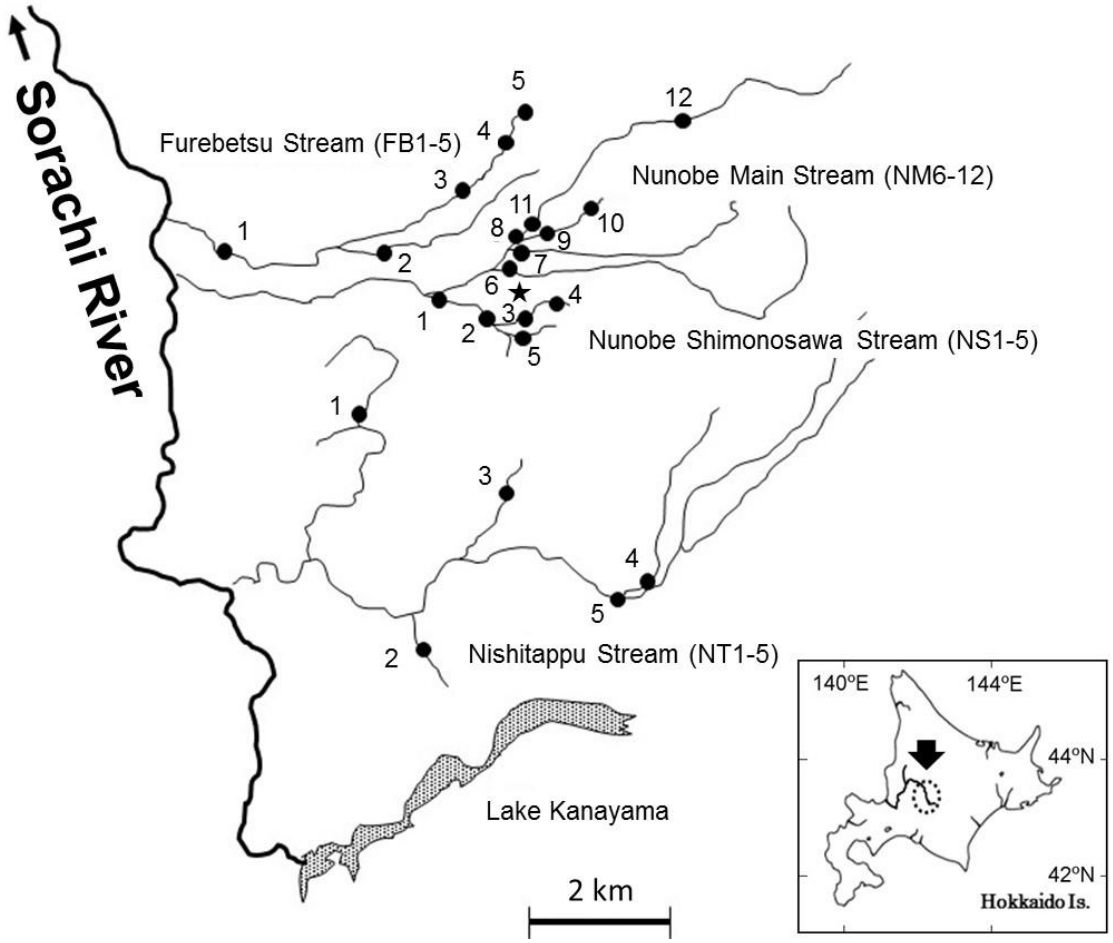

Sorachi River
Furebetsu Stream (FB1-5)
Nunobe Main Stream (NM6-12)
Nunobe Shimonosawa Stream (NS1-5)
Nishitappu Stream (NT1-5)
Lake Kanayama
2 km
140°E
144°E
44°N
42°N
Hokkaido Is.

Fig. 2

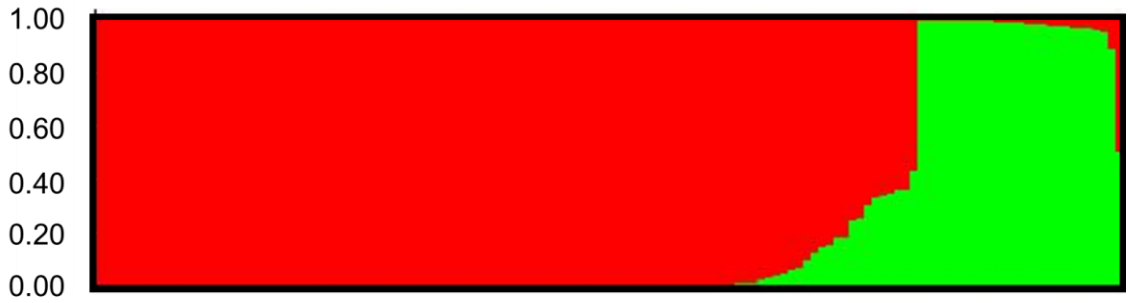
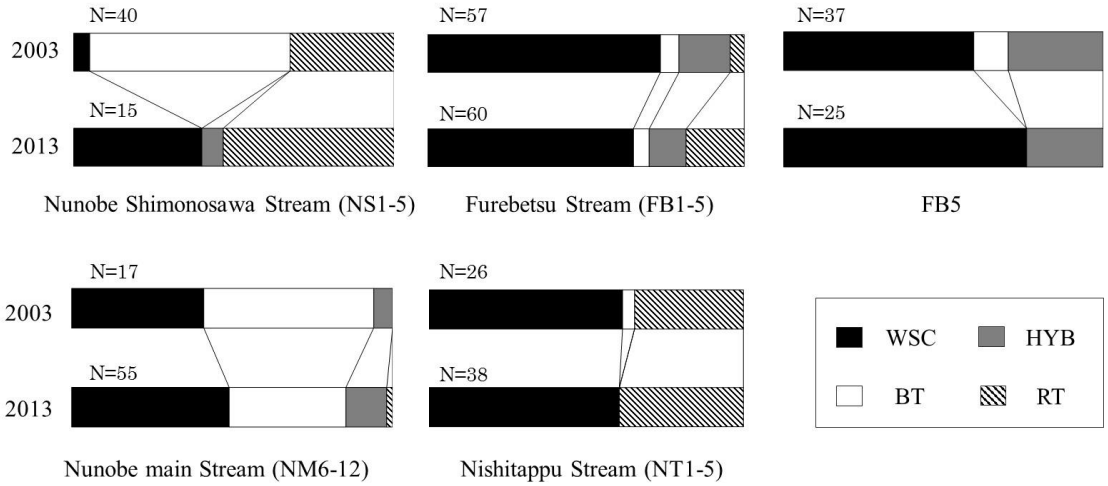


Fig. 3


N=40
2003
N=15
2013
Nunobe Shimonosawa Stream (NS1-5)
N=57
N=60
Furebetsu Stream (FB1-5)
N=37
N=25
FB5
N=17
2003
N=55
2013
Nunobe main Stream (NM6-12)
N=26
N=38
Nishitappu Stream (NT1-5)
WSC
HYB
BT
RT