Spatial distribution of juvenile masu salmon (*Oncorhynchus masou*) with incongruent genotypic and phenotypic sex in Hokkaido, Japan

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Abstract To clarify the spatial distribution of masu salmon *Oncorhynchus masou* with incongruent genotypic and phenotypic sex, we compared the phenotypic sex determined by gonad analysis with the genotypic sex identified by a male-specific genetic marker, Growth hormone pseudogene (GHp), in 584 individuals inhabiting 23 rivers in Hokkaido, Japan. We found that the genotypic sex is different from the phenotypic sex for 39 individuals in 10 rivers. Among them, 35 individuals (89.7 %) were GHp+ females phenotypically. The opposite feature was noted in the remaining four individuals (10.3 %). This study also revealed that GHp+ phenotypic females are distributed mainly in the Sea of Japan, whereas GHp- phenotypic male tend to appear

in the Sea of Okhotsk. In addition, there was a significant negative correlation between the distance from the Bishabetsu River, where frequency of GHp+ phenotypic females was the highest, and the frequency of those individuals in each river. These results suggest that the distribution of masu salmon with incongruent genotypic and phenotypic sex would be related to the movement of individuals among the populations and regions.

Keywords Growth hormone pseudogene · Male specific marker · Masu salmon · Region · Spatial distribution

Introduction

Sex is determined genetically by a combination of sex chromosomes in all eukaryotes. In salmonid fish, genetic sex determination is thought to follow a XX-XY system or a male heterogamety (Thorgaard 1977). Sex-determining genes exist in sex chromosomes. Although mammals have a common sex-determining gene (SRY) (Gubbay et al. 1990), the sex-determining genes of other organisms are not known. As for fish, only the sex-determining genes of medaka *Oryzias latipes* have been identified (Matsuda et al. 2002) and those of salmonid fish remain unknown.

A few genetic markers linked to the Y chromosome in salmonid fish have been developed, enabling identification of sex by genetic analysis (Oty1, Devlin et al. 1991; Oty2, Brunelli and Thorgaard 2004; GHp,

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Du et al. 1993). One of the major genetic markers for the sex identification of salmonid fish is the growth hormone pseudogene (GHp). It has been reported that this marker can identify the sex of many salmonid fish species (Chinook salmon *Oncorhynchus tshawytscha*, Du et al. 1993; chum salmon *Oncorhynchus keta*, Devlin et al. 2001; and masu salmon *Oncorhynchus masou*, Nakayama et al. 1999, Zhang et al. 2001). Phillips et al. (2005) clarified that GHp is located at the short arm of Y chromosome in Chinook salmon and coho salmon by means of fluorescence in situ hybridization (FISH) assay, indicating that this marker is effective for identifying sex.

Following the identification of GHp as a sex-specific genetic marker in salmonids, several studies aimed at locating GHp in a chromosome (Phillips et al. 2005; Williamson et al. 2008) and clarifying how consistent phenotypic sex is with genotypic sex (e.g., Nagler et al. 2001; Brykov et al. 2010) have been conducted. In order to discuss the effectiveness of sex-specific genetic markers from a broad perspective, Devlin et al. (2005) examined the phenotypic and genotypic sexes of a total of 2,458 individuals in 55 populations of Chinook salmon in Canada and the U.S. and found that phenotypic sex was consistent with genotypic sex in 96.7 % of the individuals. From this result, they concluded that the marker is effective for most populations. However, for some individuals, phenotypic sex differed from genotypic sex and such individuals were more abundant in the U.S. than in Canada.

Incongruent genotypic and phenotypic sex has also been documented in masu salmon populations. For example, Zhang et al. (2001) investigated 131 masu salmon individuals in Japan and found that the genotypic sex is not consistent with the phenotypic sex in nine individuals (6.9 %). In addition, Brykov et al. (2010) examined masu salmon populations in Russia and reported individuals with incongruent genotypic and phenotypic sex. However, there has been no large-scale research of the spatial distribution of individuals with incongruent genotypic and phenotypic sex. The finding that the frequency of those individuals differs with region or geography would contribute to elucidating the appearance mechanism of individuals with incongruent genotypic and phenotypic sex. This study aimed to compare phenotypic sex determined by gonad analysis and genotypic sex confirmed with the sex-specific genetic marker GHp for masu salmon

populations in Hokkaido, in order to clarify the spatial distribution pattern of individuals with incongruent genotypic and phenotypic sex on a large scale.

Materials and methods

Study site and sample collection

In this study, a total of 584 juveniles (0+ year old) were captured with an electrofisher (Smith-Root Inc. Model-12) and by angling in the six rivers flowing into the Sea of Okhotsk, the six rivers flowing into the Pacific Ocean, and the 11 rivers flowing into the Sea of Japan in Hokkaido between 2005 and 2007 (Fig. 1, Table 1). The Toikanbetsu (no. 1) and Sanru (no. 2) Rivers are the primary and secondary tributaries of the Teshio River (length: 256 km), and are located 40 km and 150 km upstream of the estuary, respectively. In most of the rivers investigated, there is no record of hatchery fish release and there are no artificial obstacles between the rivers and the sea. Accordingly, all of the investigated rivers are considered to be nearly intact. The captured individuals were anesthetized with 2-phenoxy ethanol (0.2 mL/L) to measure body length to the nearest 0.1 cm, and transported to a laboratory at Hokkaido University. At the laboratory, all individuals were dissected to determine their sexes

Fig. 1 Sampling locations of masu salmon in Hokkaido, the northernmost island of Japan. Numbers correspond to populations listed in Table 1

Table 1 Site location, sampling date, and sampling number of each river in this study

Region	No.	River	Location		Sampling date	n
Sea of Japan	1	Sanru	44°18'N	142°37'E	2005 Aug	24
	2	Toikanbetsu	44°54'N	141°59'E	2005 Aug	26
	3	Utsutsu	44°45'N	141°46'E	2006 Aug	29
	4	Poro	43°38'N	141°21'E	2006 Sep	29
	5	Kunbetsu	43°37'N	141°21'E	2007 July	27
	6	Bishabetsu	43°33'N	141°22'E	2007 July	25
	7	Atsuta	43°23'N	141°25'E	2007 July	35
	8	Shakotan	43°20'N	140°27'E	2005 Aug	26
	9	Horonaifu	43°19'N	140°24'E	2005 Aug	28
	10	Ruubetsunai	42°51'N	140°20'E	2006 Sep	27
Sea of Okhotsk	12	Chiraibetsu	45°22'N	142°05'E	2007 Aug	21
	13	Onishibetsu	45°20'N	142°09'E	2006 Aug	24
	14	Sarukotsu	45°18'N	142°12'E	2007 Aug	20
	15	Sarufutsu	45°16'N	142°13'E	2007 Aug	23
	16	Rurochi	44°27'N	143°09'E	2006 Aug	24
	17	Kotanuka	43°45'N	145°03'E	2006 Aug	27
	18	Bewase	43°03'N	145°05'E	2006 Oct & Nov	31
Pacific Ocean	19	Touberi	42°30'N	143°27'E	2006 Sep	31
	20	Betsubetsu	42°34'N	141°25'E	2006 Sep	23
	21	Shirarika	42°21'N	140°17'E	2006 Sep & Oct	32
	22	Nodaoi	42°13'N	140°23'E	2005 Aug	19
	23	Haraki	41°43'N	141°02'E	2005 Aug	16

by visual inspection of the gonad (Nakamura et al. 1974). In addition, in order to confirm their sex genetically, the adipose fins were collected and stored in 99 % ethanol. For all individuals, age was estimated from their scales.

Genetic analysis

From the stored adipose fins, a 3 mm piece was removed and ethanol was evaporated from it. Then, DNA extraction was carried out with Chelex 100. For all of the sampled individuals, sex was analyzed by the polymerase chain reaction (PCR) using a pair of primers (Forward, 5'-CAATGACTCTCAGCATCTGCC-3'; Reverse, 5'-GCCTCCAGCGACTTCCTGCAC-3') reported by Zhang et al. (2001). These primers amplified two distinct fragments in masu salmon males, about 400 bp fragment from GH-2 and about 280 bp fragment from GHp, but only a GH-2 fragment in females (Fig. 2). In the PCR, 10 μ L of the reaction solution was mixed with 1 μ L of 10x PCR buffer, 1.5 mM

MgCl₂, 0.2 mM dNTP, 0.5 μ M of each primer, 0.5 unit of EX Taq polymerase (Takara), and 20 ng of template DNA. Amplification profile included pre-cycling denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at

Fig. 2 Agarose gel showing growth hormone 2 (GH-2) and GHp genes amplified by polymerase chain reactions using GH-specific primers: duplex PCR amplification of about 400 bp GH-2 fragment (*males and females*) and 280 bp GHp fragment (*male only*). GHp+female indicates that phenotypic sex is female and genotypic sex is male

72°C for 30 s, with post-cycling extension at 72°C for 7 min. The fragment size of the PCR product was measured by 2 % agarose gel electrophoresis.

Statistical analysis

For the comparison of the frequency of individuals with incongruent genotypic and phenotypic sex among regions, arcsine transformation and ANOVA were used. To examine the correlation between the frequency of GHp+phenotypic female and inter-population distance, the Spearman correlation test was conducted. Inter-population distance was defined as the distance between the estuary of the Bishabetsu River, where the frequency of those individuals was highest, and the estuary of each river. As for the Sanru and Toikanbetsu Rivers, which are the tributaries of the Teshio River, inter-population distance means the distance between the estuary of the Bishabetsu River and the confluence part of the Teshio River. Inter-population distance was measured by using Mapion software (<http://www.mapion.co.jp/>). Unfortunately, GHp- phenotypic males were excluded because of their small number.

In the comparison of body size between GHp+ females and normal females, both of which were 0+ year old, the factor was square-root transformed to standardize variances and improve normality, and then the two-way ANOVA was conducted based on two variables of the populations and female types (normal females vs. GHp+ females), using the data of only the rivers where individuals with incongruent genotypic and phenotypic sex were sampled ($n=10$).

Results

Analysis of a total of 584 individuals collected in the 23 rivers of Hokkaido revealed that the genotypic sex is consistent with the phenotypic sex for 546 individuals (93 %). Meanwhile, for 39 individuals captured in 10 rivers, the genotypic sex was inconsistent with the phenotypic sex (Table 2). Among 39 fish with incongruent genotypic and phenotypic sex, 35 were GHp+ phenotypic females and the remaining four were GHp-phenotypic males. Then, the body size of 0+ year old fish was compared between GHp+ females and normal females in each river. Some difference was noted among populations, but the difference was not

significant (Table 3). The difference in body size among populations was considered to be due to the difference in the timing of sampling.

In addition, the appearance rate of individuals with incongruent genotypic and phenotypic sex was compared among regions (Sea of Okhotsk, Sea of Japan, and Pacific Ocean), and it was found that the appearance rate varies significantly ($F_{2,20}=4.787$, $P<0.05$) and most of the genotypic males having a female phenotype inhabit the Sea of Japan side. Although their number is small, incongruent individuals with genotypic females and phenotypic males tend to appear in the Sea of Okhotsk side. In addition, a significant correlation was noted between the inter-population distance from the Bishabetsu River (No. 6) and the frequency of those individuals in each river ($\rho=-0.630$, $P<0.001$, Fig. 3).

Discussion

In this study, sex was identified with GHp, a genetic marker for sex identification, and it was found that the phenotypic sex determined by gonad analysis is not consistent with the genotypic sex in 39 individuals (6.7 %). Most of the incongruent individuals are GHp+phenotypic females. Three major factors contribute to the appearance of individuals with incongruent genotypic and phenotypic sex. The first factor is that GHp may have moved to the X chromosome or autosomal chromosome through rearrangement between chromosomes. Most reports of individuals with incongruent genotypic and phenotypic sex have indicated that there are many females that have male-specific genetic markers and in rare cases, males lose their genetic markers (Williamson and May 2002; Chown and Nagler 2004). Similarly in this study, it was found that phenotypic females with male genetic markers were approximately 90 % of individuals with incongruent genotypic and phenotypic sex, suggesting that GHp on the Y chromosome may be rearranged to some other location. Williamson et al. (2008) analyzed the chromosomes of Chinook salmon individuals having incongruent genotypic and phenotypic sex with the FISH method, and the results indicate that some Y chromosomes, including GHp and Oty1, may have undergone recombination with X chromosomes and autosomes. The second factor is that the regions associated with the sex-determining genes on the Y

Table 2 Numbers and fork lengths (Average $\pm$ S.D.) of 0+ year old masu salmon whose phenotypic sex was determined by gonad analysis and genotypic sex was determined with GHp

Region	No.	Population	Phenotype SEX	Genotype GHp+	0+ individuals F.L. $\pm$ S.D.	Genotype GHp-	0+ individuals F.L. $\pm$ S.D.	GHp+Female (%)
Sea of Japan	1	Sanru	F	0	-	11	9.38 $\pm$ 2.05	0
			M	13	10.5 $\pm$ 0.78	0	-	
	2	Toikanbetsu	F	0	-	10	10.60 $\pm$ 0.42	0
			M	15	9.82 $\pm$ 1.29	1	7.0	
	3	Utsutsu	F	3	10.43 $\pm$ 1.37	17	10.70 $\pm$ 1.93	15.0
			M	9	10.93 $\pm$ 1.31	0	-	
	4	Poro	F	8	10.88 $\pm$ 1.36	9	10.09 $\pm$ 0.69	47.1
			M	12	10.28 $\pm$ 0.73	0	-	
	5	Kunbetsu	F	3	7.73 $\pm$ 1.16	9	8.61 $\pm$ 1.76	25.0
			M	15	7.93 $\pm$ 1.21	0	-	
	6	Bishabetsu	F	5	7.84 $\pm$ 1.39	4	7.48 $\pm$ 1.39	55.6
			M	16	7.51 $\pm$ 1.09	0	-	
	7	Atsuta	F	5	6.96 $\pm$ 0.69	20	6.21 $\pm$ 0.77	20.0
			M	10	6.29 $\pm$ 0.41	0	-	
	8	Shakotan	F	1	7.0	9	7.86 $\pm$ 3.22	10.0
			M	16	7.62 $\pm$ 2.23	0	-	
	9	Hironaifu	F	5	8.86 $\pm$ 2.18	13	9.24 $\pm$ 2.54	27.8
			M	10	11.7 $\pm$ 1.55	0	-	
	10	Rubetsunai	F	0	-	10	11.83 $\pm$ 0.70	0
			M	17	12.26 $\pm$ 0.12	0	-	
	11	Ooanzai	F	1	11.4	7	11.55 $\pm$ 0.31	12.5
			M	11	6.49 $\pm$ 0.49	0	-	
Sea of Okhotsk	12	Chiraihetsu	F	0	-	10	6.67 $\pm$ 0.76	0
			M	11	6.49 $\pm$ 0.49	0	-	
	13	Onishibetsu	F	0	-	17	8.50 $\pm$ 0.79	0
			M	7	8.70 $\pm$ 0.78	0	-	
	14	Sarukotsu	F	0	-	10	7.13 $\pm$ 0.94	0
			M	10	7.38 $\pm$ 0.77	0	-	
	15	Sarufutsu	F	0	-	11	6.56 $\pm$ 0.98	0
			M	12	6.43 $\pm$ 0.54	0	-	
	16	Rurochi	F	2	8.05 $\pm$ 0.50	7	8.94 $\pm$ 0.88	22.2
			M	15	9.24 $\pm$ 0.82	2	8.20 $\pm$ 0.14	
Pacific Ocean	17	Kotanuka	F	0	-	6	8.73 $\pm$ 0.47	0
			M	20	9.30 $\pm$ 0.82	1	7.8	
	18	Bewase	F	0	-	20	9.25 $\pm$ 0.69	0
			M	11	9.77 $\pm$ 1.00	0	-	
	19	Touberi	F	0	-	20	10.26 $\pm$ 0.95	0
			M	11	10.46 $\pm$ 1.12	0	-	
	20	Betsubetsu	F	0	-	14	10.86 $\pm$ 1.09	0
			M	9	10.79 $\pm$ 1.89	0	-	
	21	Shirarika	F	2	10.10 $\pm$ 0.42	13	10.03 $\pm$ 1.06	13.3
			M	17	10.10 $\pm$ 1.72	0	-	
	22	Nodaoi	F	0	-	10	8.71 $\pm$ 1.31	0

Table 2 (continued)

Region	No.	Population	Phenotype SEX	Genotype GHp+	0+ individuals F.L.±S.D.	Genotype GHp-	0+ individuals F.L.±S.D.	GHp+Female (%)
Total	23	Haraki	M	9	12.00±1.4	0	-	0
			F	0	-	6	9.32±2.01	
			M	10	12.50±1.22	0		
			F	35	8.97±1.94	263	9.08±2.04	11.7
			M	284	9.33±2.12	4	7.80±0.57	

chromosome may have mutated and as a result, the phenotypic sex became female although the genetic type is XY. This phenomenon was observed in medaka fish *O. latipes*. It has been reported that the sex-determining gene of medaka is DMY and that when this part mutates, the phenotypic sex becomes female even when the sex chromosome is XY (Matsuda et al. 2002). The third factor is the effect of temperature and sex steroid hormones at the early life stage. It has been reported that sex can be controlled by temperature or sex steroid hormones for most fish species, including salmonid fish (Okada 1985; Pifferer 2001; Pifferer and Guiguen 2008). However, as the results of this study indicate that individuals with incongruent genotypic and phenotypic sex appear in mainly the sea of Japan side, it is difficult to consider that there exists a common factor in sex reversal. In this study, a few male individuals that do not have GHp were discovered, and so GHp could have moved to the X chromosome or an autosome through chromosome rearrangement in the sex-determining gene of masu salmon.

One intriguing finding of this study is that GHp+ phenotypic females were found mainly in the Sea of Japan side and hardly in the sides of the Sea of Okhotsk and the Pacific Ocean. On the other hand, GHp- phenotypic males were distributed mainly in the Sea of Okhotsk. There are some reports on the migration timing

and place of masu salmon in the sea (Kato 1991; Mayama et al. 2005). According to those studies, in the case of masu salmon inhabiting Hokkaido, most populations in the rivers of the Sea of Japan side undergo the smolt phase in early spring, enter the ocean, migrate northward in the Sea of Japan, and spend the summer in the Sea of Okhotsk. Then, as water temperature decreases, they migrate southward to reach the area around the Tsugaru Peninsula and return to the north in early spring. Meanwhile, the populations in the Pacific Ocean side migrate to the Sea of Okhotsk via the Nemuro Strait where they spend the summer, and then go southward to reach the area around the Tsugaru Peninsula for overwintering. Therefore, the populations of the Sea of Japan side and the Pacific Ocean side have different migration routes. Furthermore, Okazaki (1986) investigated the genetic structure of 18 masu salmon populations, including 10 Hokkaido populations, by allozyme electrophoresis and found that those populations were divided into two groups, the Sea of Japan and

Table 3 Result of two-way ANOVA using the fork lengths of 0+ years old juvenile of each population. Female types indicate GHp+ females and normal females

Variable	Sum of Squares	df	Means Sum of Square	F-value	P-value
Female type	0.0007	1	0.0007	0.022	0.881
Population	2.980	9	0.331	10.141	<0.001
Interaction	0.120	9	0.013	0.409	0.928
Error	4.016	123	0.033

Fig. 3 Relationship between the percentage of GHp+ females in each river and the distance from Bishabetsu River (No. 6), where GHp+ females is the highest ($\rho = -0.630$ $P < 0.001$)

Sea of Okhotsk group and the Pacific and eastern Hokkaido group. This result indicates that gene flow, such as straying into non-natal rivers, occurs more frequently in the populations in the Sea of Japan and the Sea of Okhotsk than in the populations in the Pacific Ocean side. It was concluded that the differences in migration routes and gene flow levels among populations in different regions influence the distributions of individuals with incongruent genotypic and phenotypic sex, and individuals with incongruent genotypic and phenotypic sex exist mainly in the Sea of Japan side and the Sea of Okhotsk.

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