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SHORT COMMUNICATION

A comparison of sex-specific markers for two wild masu salmon populations in Hokkaido, Japan

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Abstract

We evaluated the utility of three male-specific molecular markers, *sdY*, *sdY 227U* and *OtY2m*, in two wild populations of masu salmon (*Oncorhynchus masou*) in southwestern Hokkaido, Japan. Male-specific fragments amplified in all phenotypic males across the markers. Phenotypic females were genetically identified as males using *sdY* (57%) and *sdY 227U* (4%), but no phenotypic females were identified as males using *OtY2m*. We conclude that *OtY2m* was the most reliable sex marker, followed closely by *sdY 227U*, among those tested in our study populations. Additional research is warranted to test the applicability of these markers in other populations and *Oncorhynchus* species.

KEYWORDS
genotype, *Oncorhynchus masou*, Pacific salmon, PCR, phenotype, sex identification

1 | MAIN BODY

Efficient and reliable identification of sex in salmonids is an invaluable tool in understanding their ecology, management and aquaculture. However, identifying sex in immature salmonids is often challenging because they lack secondary characteristics externally, and fish need to be sacrificed for sex determination (Kinami & Matsuyama, 2022; Morita & Nagasawa, 2010). Genetic sex identification offers a cost-effective and non-lethal solution to this challenge (Brunelli & Thorgaard, 2004; Yano et al., 2013). Salmonids are characterized by a male heterogametic sex determination system (Thorgaard, 1977), and male-specific molecular markers have proliferated in Pacific salmon (genus *Oncorhynchus*) (Brunelli & Thorgaard, 2004; Hsu & Gwo, 2010; Yano et al., 2012). However, phenotypic females have often been identified as genetic males using these markers (Hsu & Gwo, 2010; Podlesnykh et al., 2017; Ueda et al., 2022), indicating the complexity and variation in their sex determination process. Thus, the development and applications of male-specific sex markers remain an important inquiry in the management and ecology of Pacific salmon.

Masu salmon (*Oncorhynchus masou*) are commercially and recreationally important in their native range in East Asia (Morita et al., 2018). In Hokkaido, Japan, their populations are typically partially anadromous, meaning that some individuals migrate to the ocean and return to their natal rivers for spawning, but others remain and

mature in the freshwater environment (Kato, 1991). Male-specific molecular markers correctly identified males and females of masu salmon using *sdY (sexually dimorphic on the Y-chromosome)* (Yano et al., 2013) and *OtY2m* primers (Hsu & Gwo, 2010). However, Ueda et al. (2022) reported that these male-specific markers were also amplified in female red-spotted masu salmon (*O. masou ishikawae*). Kinami and Matsuyama (2022) have recently developed a new marker, *sdY 227U*, and used it to identify males and females of farmed masu salmon; however, this marker has not previously been tested in wild populations of masu salmon. Farmed salmonids, including masu salmon, typically originate from multiple but imperfectly documented broodstocks (Kinami & Matsuyama, 2022), and a successful application of sex-specific markers to farmed fish does not necessarily guarantee their transferability to all wild populations. Accordingly, our current knowledge is limited on the utility of these male-specific markers in determining sex of wild masu salmon.

Here, we applied the three male-specific markers (*sdY*, *OtY2m*, and *sdY 227U*) for genetic sex identification of wild masu salmon collected in Horonai River (42°41′21″N, 141°34′28″E) and Yufutsu River (42°43′05″N, 141°32′01″E), southwestern Hokkaido, Japan. Both rivers harbour wild masu salmon populations, and stock enhancement of masu salmon has not been conducted in either river at least in the past 6 years (R. Futamura., *personal communication*). The masu salmon population in Horonai River is partially anadromous; males are a mix of sea-run and resident individuals, but nearly all females are sea-run (Futamura et al., 2022). Sea-run individuals attain much larger body sizes at maturity than resident individuals. A long-term intensive mark-recapture study of masu salmon has been conducted in Horonai River since 2018 (Futamura et al., 2022). The masu salmon population in Yufutsu River is located above an artificial barrier to movement, and all individuals mature as residents. Masu salmon populations composed exclusively of residents are uncommon in Hokkaido. To increase sample size, sampling occurred at a second site, located in Yuburi River at its confluence with Yufutsu River; the two sites were hydrologically connected in terms of fish movement, and the samples were pooled (hereafter referred to as Yufutsu River). We collected masu salmon by using a backpack electrofishing unit (Model 12B, Smith-Root Inc.) and dipnets during their spawning season in autumn (Table S1), and adipose fin was partially clipped (~0.5 cm^2) and preserved in 99.5% ethanol for genetic analysis. To prevent the carryover of tissues to the other specimens, iris scissors for fin clipping were sanitized with 99.5% ethanol between samples. Phenotypic sex was determined by secondary characteristics and the presence of eggs and milt in the field, and fork length was measured for each individual.

In the laboratory, adipose fin (1 mm^2, ≧0.5 mg) was suspended in a 15-μL solution of a 3% suspension of the Chelex 100 resin (Bio-Rad) in ddH_2O including 1 μL of Proteinase K (≧0.6 mAnson U) (Takara Bio Inc.). To prevent cross contamination, tweezers and iris scissors for trimming the fin were initialized for each specimen by wiping with 99.5% ethanol and sterilized with a gas torch. Genomic DNA was extracted by incubating at 56°C for 20 min, a modified incubation period and temperature setting from Casquet et al. (2012), followed by 70°C for 10 min (Stec et al., 2020). Fragments were amplified by the three multiplex combinations of male-specific markers with positive control markers (Table S2). A 10-μL solution for multiplex PCR contained 5 μL of 2×MightyAmp Buffer Ver. 3, 0.2 μL of MightyAmp DNA Polymerase Ver. 3 (Takara Bio Inc.), 1 μL of template DNA, and the primers with quantities described in Table S2. Thermal cycling profiles for the multiplex combinations were as follows: for *sdY 227U/18S 514U* (Kinami & Matsuyama, 2022), 2 min 98°C and 30 cycles for 98°C for 10 s and 68°C for 40 s; for *sdY/18S* (Yano et al., 2013), 2 min 98°C, followed by 98°C for 10 s, 60°C for 15 s and 68°C for 40 s for 30 cycles, and 72°C for 3 min; for *OtY2m/16S* (Hsu & Gwo, 2010), 2 min 98°C, followed by 98°C for 10 s, 56°C for 15 s and 68°C for 40 s for 30 cycles and 72°C for 3 min. A 10-μL volume of the PCR products was electrophoresed through 2% agarose gels in TAE buffer. Amplifications of the fragments were evaluated by visual inspection of the presence of bands on the gel under the BLook blue-LED transilluminator system (Genedirex) by staining with Midori Green Xtra (Nippon Genetics), regardless of band intensity. Specimens amplified with a male-specific fragment and a positive control fragment were assigned to be genotypic males, whereas specimens amplified with only a positive control fragment were assigned to be genotypic females. The sizes of expected fragment are as follows: 225 bp for *sdY 227U* and 532 bp for *18S 514U* (Kinami & Matsuyama, 2022), 680 bp for *sdY* (Ueda et al., 2022) and 370 bp *18S* (Yano et al., 2013) and 300 bp for *Oty2* and 200 bp for *16S* (Hsu & Gwo, 2010). When no positive control fragments appeared, the PCR of that specimen was classified to have failed.

We found that the three male-specific markers differed in their accuracy of sex identification (Table 1; Figure 1). Except for individuals with failed PCR amplifications, male-specific fragments of the three markers were amplified in all phenotypic males. Male-specific fragments were observed in 7% of females (2/27) in Horonai River for *sdY 227U* (Kinami & Matsuyama, 2022), in 46% of females (11/24) in Horonai River, and 70% of females (14/20) from Yufutsu River for *sdY* (Yano et al., 2013), but were not observed for *OtY2m* (Hsu & Gwo, 2010). When both rivers were combined, 4% of phenotypic females (2/47) were identified as males using *sdY 227U* and 57% (25/44) using *sdY*. Overall, when fragments were amplified, 100% of the individuals (91 out of 91) were sexed correctly using *OtY2m*, and 98% of the individuals (93/95) were sexed correctly using *sdY 227U*. The overall assignment accuracy was 73% (67/92) in *sdY*. These amplification patterns indicated that *OtY2m* had the most practical utility for genetic sex identification of masu salmon in these populations followed closely by *sdY 227U*. It should be noted that non-specific fragments were also amplified using *OtY2m* primers. The expected fragments for *OtY2m* and 16S are approximately 300 and 200 bp, respectively (Hsu & Gwo, 2010), whereas unexpected fragments of approximately 100 and 500 bp, which do not overlap with *OtY2m*, also appeared (Figure 1c). These non-specific bands have not been detected in previous studies on the subspecies of masu salmon (Hsu & Gwo, 2010; Ueda et al., 2022) and may therefore be attributable to population-specific genetic polymorphisms used in this study and/or minor modification of PCR protocols from previous studies, such as DNA extraction methods and enzymes. Although male-specific *Oty2m* and positive control fragments are visually distinguishable, the establishment of a

TABLE 1 A summary of PCR results with alignment between phenotypic and genotypic sexes and mean and range of fork length (FL) for each population and life history group.

						# Individuals with male-specific fragment (# failed amplification)		
Population	**Life history**	**Phenotypic sex**	***N***	**ID of electrophoresis**	**Mean (range) of FL (mm)**	***sdY 227U***	***sdY***	***OtY2m***
Horonai	Migrant	Male	4	1–4	459 (390–520)	4 (0)	4 (0)	4 (0)
Horonai	Resident	Male	28	5–32	110 (82–116)	28 (0)	28 (0)	27 (1)
Yufutsu	Resident	Male	16	33–48	207 (180–250)	16 (0)	16 (0)	16 (0)
Horonai	Migrant	Female	27	49–75	496 (395–560)	2 (0)	11 (3)	0 (3)
Yufutsu	Resident	Female	20	76–95	194 (160–230)	0 (0)	14 (0)	0 (0)

Note: Yufutsu River and Yuburi River are pooled as Yufutsu River samples due to their geographical proximity and connectivity. Sex markers are *sdY 227U* (Kinami & Matsuyama, 2022), *sdY* (Yano et al., 2013) and *OtY2m* (Hsu & Gwo, 2010).

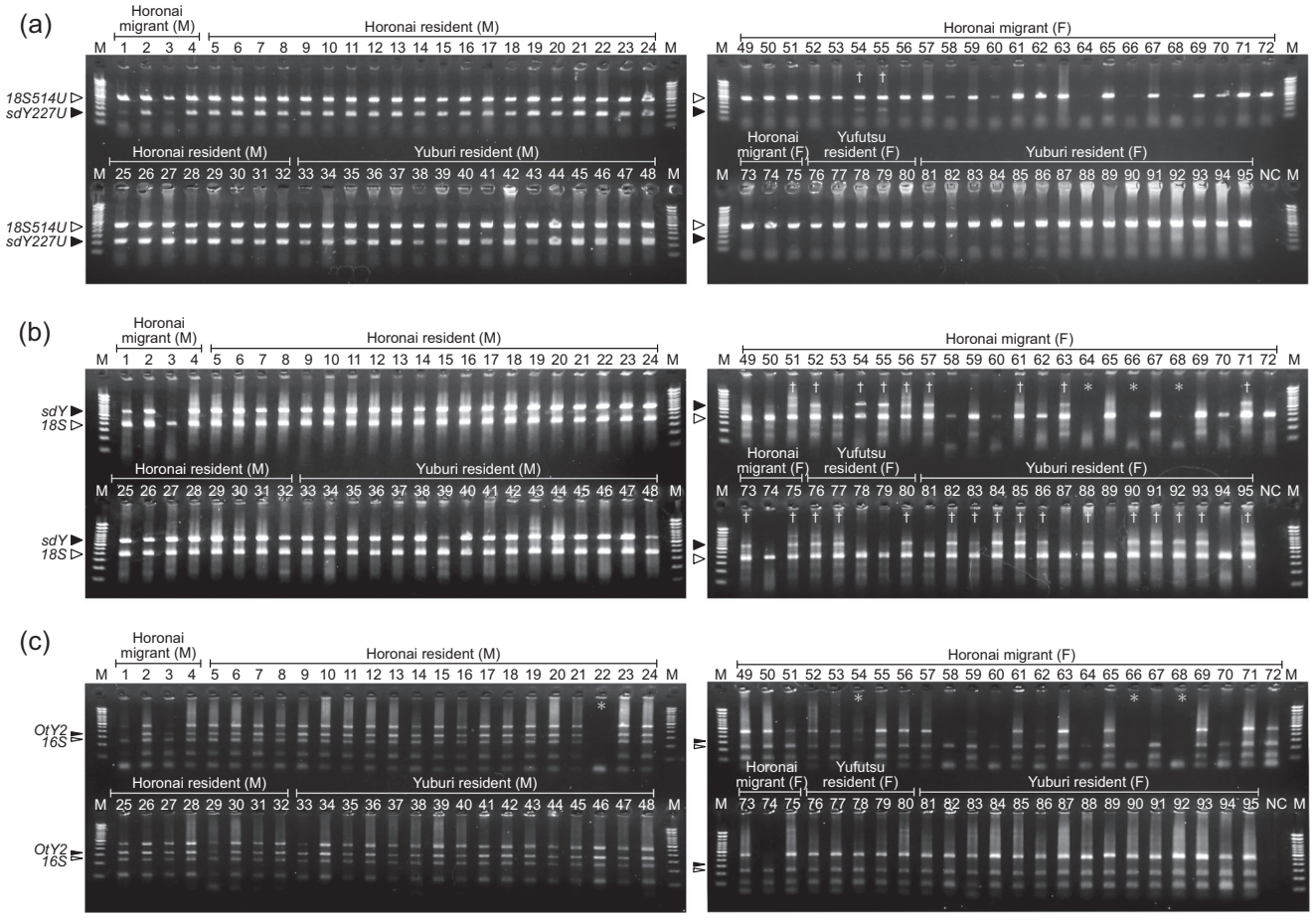

FIGURE 1 Electrophoresis of PCR products in 2% agarose gel: (a) *sdY 227U* (Kinami & Matsuyama, 2022), (b) *sdY* (Yano et al., 2013) and (c) *OtY2m* (Hsu & Gwo, 2010). Males appear on the left side and females on the right side of the panels. NC in the right panels denotes negative controls with the loading of dye and reagent solution, and M indicates the TrackIt 100 bp DNA Ladder (Thermo Fisher Scientific). Asterisks (*) and daggers (†) indicate that the specimens were classified as failed amplification and females with male-specific bands, respectively.

multiplex protocol without amplification of non-specific fragments or a new marker set that can be used in multiple populations would alleviate this aesthetic issue and facilitate its more widespread use as a sex marker.

In summary, we tested genetic sex identification using three male-specific markers with a PCR-based method on two wild masu salmon populations with different life history patterns in southwestern Hokkaido, Japan. We gained two key insights in this study. First,

the accuracy of sex determination differs among existing male-specific markers, despite previous successful applications of *sdY* (Yano et al., 2013) in wild masu salmon populations. The finding that no phenotypic females were misidentified as genetic males using *OtY2m* (Hsu & Gwo, 2010) was noteworthy. Second, this study provided novel evidence that a recently developed marker for farmed masu salmon, *sdY 227U*, was highly accurate in sex determination of wild populations. For our study populations, *sdY 227U* and *OtY2m* proved to be promising molecular tools for genetic sex identification. In our case, a simultaneous use of both markers may increase our ability to genetically sex masu salmon confidently and it would be logistically feasible given the low cost and rapid assessment afforded by these markers. The efficacy of sex-specific markers differs among populations of masu salmon (Yamamoto et al., 2012) and other Pacific salmon (Cavileer et al., 2015; Komrakova et al., 2018), and additional investigations are warranted to test for their applicability beyond the study populations.

AUTHOR CONTRIBUTIONS

Takuya K. Hosoki: Conceptualization; funding acquisition; investigation; methodology; resources; validation; visualization; writing—original draft. **Noël M. Clark**: Data curation; investigation; methodology; validation; writing—original draft. **Ryo Futamura**: Data curation; funding acquisition; writing—review and editing. **Senri Moriyama**: Data curation; methodology; writing—review and editing. **Osamu Kishida**: Funding acquisition; project administration; resources; supervision; writing—review and editing. **Yoichiro Kanno**: Investigation; project administration; supervision; writing—original draft.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

No new data were created or analysed in this study.

ETHICS STATEMENT

The fieldwork was conducted in accordance with a protocol approved by the Hokkaido University Field Science Center for Northern Biosphere Institutional Animal Care and Use Committee (Approval Number 2-6).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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