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Supplement: Development of Y-Chromosome-Specific Assays and Rules for Differentiating XY and YY Genotypes

For the presence–absence assay (SexY_Brook1), we included a 5′-6-FAM fluorescently labeled, unpublished forward primer (SexYBrookF) and two reverse primers. The first reverse primer (BSexY1), in combination with the SexYBrookF, amplifies a sequence 161 bp in length and is presumed to be a Y-specific (male) product (Figure S.1). The second reverse primer (BSexY2), in combination with SexYBrookF, amplifies a sequence 131 bp in length and is presumed to be interrogating an autosomal region (Figure S.1). These three primers were included with primers for six microsatellite loci in a multiplex PCR amplification. Primer sequences and PCR protocols for this assay are summarized in Table S.1. Thermal cycling conditions were 95°C for 15 min followed by 25 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 60 s, then a final extension of 60°C for 30 min. Amplification products were electrophoresed on a 3100 genetic fragment analyzer. The sex typing accuracy for the presence–absence assay was evaluated by genotyping 45 known phenotypic male broodstock and 45 known phenotypic female broodstock from the Iron River National Fish Hatchery. Samples that exhibited peaks of 131 bp and 161 bp and that amplified at the other loci in the panel were scored as “males”. Samples that exhibited peaks of 131 bp and that amplified at the other loci in the panel were scored as “females”. The genotypic sex call for all individuals matched the phenotypic sex call (90 out of 90 = 100% accuracy).

For the TaqMan-based allelic discrimination assay (SexY_Brook2), we used the same AFLP-produced sequence data to design a 5′ exonuclease assay that amplifies the suspected Y-specific product along with the suspected autosomal product to act as an internal control. These

products are interrogated using fluorogenic probes (TaqMan chemistry; Applied Biosystems, Foster City, California). Primer and probe sequences and PCR protocols for the TaqMan-based assay are summarized in Table S.2. Thermal cycling conditions were 95°C for 10 min followed by 60 cycles of 95°C for 15 s and 60°C for 1 min. Sex identification was accomplished through the analysis of allelic discrimination plots of endpoint fluorescence using an Applied Biosystems 7500 Real-Time PCR instrument (Figure S.2). The 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) fluorophore (*x*-axis) was associated with the probe for the Y-specific product (males), while the carboxyfluorescein (FAM) fluorophore (*y*-axis) labeled the autosomal product. Samples that exhibit fluorescence from both FAM and VIC were scored as XY (male). Samples that exhibit FAM fluorescence but not VIC fluorescence were scored as XX (female). Samples that exhibit low or no fluorescence for both FAM and VIC were scored as "no call." A no-template control was included in every PCR in which no DNA was added to a well to verify that there was no contamination across samples and reagents. The sex typing accuracy for the presence–absence assay was also evaluated by genotyping 45 known phenotypic male broodstock and 45 known phenotypic female broodstock from the Iron River National Fish Hatchery. The genotypic sex call for all individuals matched the phenotypic sex call (90 out of 90 = 100% accuracy).

The Y-chromosome-specific assays described above allow clear differentiation of XX and XY individuals, but because an X-chromosome-specific marker was not available and there were no known YY individuals, the differentiation of XY and YY genotypes was more complicated. Fortunately, when running these assays on sample sets that contained known XY individuals and suspected YY individuals, we noticed two significant diagnostic patterns. For the presence–absence assay (SexY_Brook1), the peak height of the *SFO-135* allele at *Sco102* was

always higher than the peak height of the sex marker in known XY males (Table S.3). When this assay is run on a presumed mixture of XY and YY individuals, a proportion of samples exhibit a higher peak at SexY_Brook1. This result is consistent with the expectation that individuals with two copies of the Y-specific product (YY) would exhibit higher peak heights than individuals with only one copy (XY).

For the TaqMan-based allelic discrimination assay (SexY_Brook2), we observed a third cluster of individuals, oriented closer to the *x*-axis, when sample sets with known XX and XY individuals and presumed YY individuals were screened (Figure S.3). This pattern is consistent with the expectation that individuals that contain two copies of the Y-specific product (YY) would produce twice the amount of VIC fluorescence than single copy XY individuals. We used results from both assays when identifying YY individuals for spawning. In addition, we completed confirmatory crosses between identified YY individuals and known XY individuals (see Methods and Results).

Supplementary Table S.1. Quantity and concentration of primer mix used in the presence–absence multiplex PCR configuration of the Y-chromosome-specific assay (SexY_Brook1). Primer sequences are also shown. Once the primer mix had been made, the PCR reaction was run in a 5-µL volume on a PCR thermal cycler with 0.50 µL of primer mix, 2.50 µL of Qiagen Master Mix (cat. 206143), 1.00 µL dH20, and 1.00 µL template DNA (unknown concentration).

Quantity (µL)	Concentration (µM)	Reagent	Primer sequence
3.00	100	Sco200F	5′-PET GTG CCT TGG TGG AGA TTA C-3′
3.00	100	Sco200R	5′-CCT TTA TGT GTC CCT GTA TGA-3′
2.00	100	Sco215F	5′-PET GAG AGA GAG AGA TGG GTG ACA-3′
2.00	100	Sco215R	5′-ATC CAC AAA ACA AGA TTC CTA-3′
2.00	100	Sco102F	5′-HEX CCA TCT CTT CTT ACC CTC CTC-3′
2.00	100	Sco102R	5′-CCA AAA AGC AGT TGA TAG ACC-3′
2.00	100	Sfo18F	5′-NED TGG TGT ATC CTG CTC CTG-3′
2.00	100	Sfo18R	5′-TGG AAT GTG TGT CTG TTT TCT-3′
2.00	100	Sco220F	5′-NED AAC GAG TTC TAA TGA CTC CAA C-3′
2.00	100	Sco220R	5′-ATC ATG CTC ATC ATC ACT CTC-3′
2.00	100	Sco110F	5′-FAM CCT TGT GAG AGC TAA GGT AGT G-3′
2.00	100	Sco110R	5′-GGA GGA CAT ATT CCA ACT TTG-3′
1.00	100	SexYBrookF	5′-5HEXGA CAG AGA CGT AGC CAG ACA AG-3′
0.50	100	BSexY1R	5′-ACC AAA TGT CAC CCT ATT AGC TAC C-3′
0.50	100	BSexY2R	5′-CCC ACC ACA CCA CTC CTA AG-3′

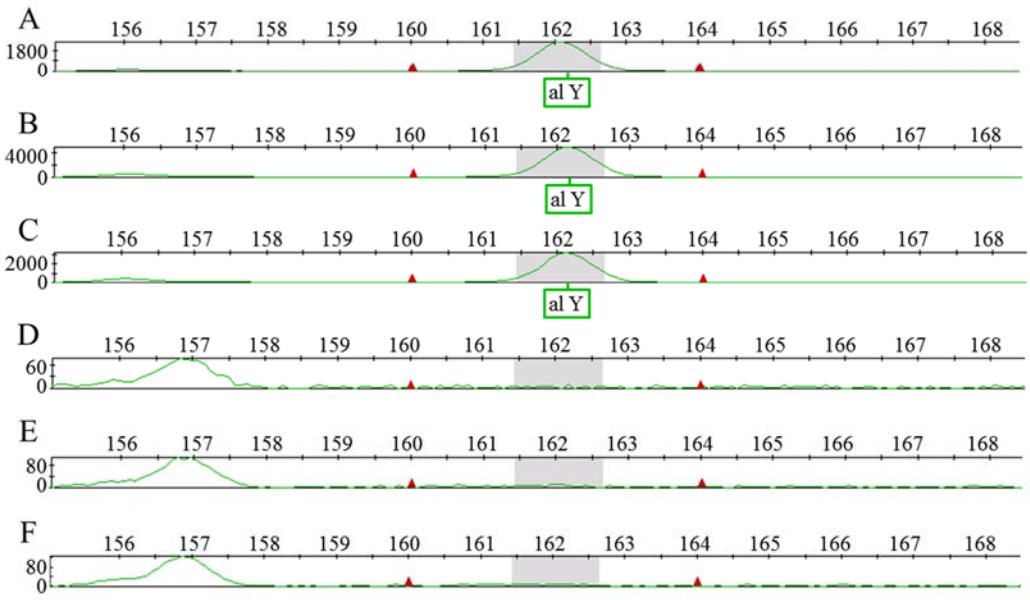
Supplementary Table S.2. Quantity and concentration of PCR reagents used in the TaqMan-based allelic discrimination configuration of the Y-chromosome-specific assay. Primer and probe sequences are also shown. The PCR reaction was run in a 10-µL volume on a thermal cycler with 0.06 µL of each primer and probe, 5.00 µL of TaqMan Master Mix, 3.94 µL dH20, and 1.00 µL template DNA (unknown concentration).

Quantity (µL)	Concentration	Reagent	Primer and Probe sequence
5.00	10×	TaqMan Master Mix	
0.015	100 µM	SexYBrook2F	5′-CCC TGA CAC AGA GAC CCA CT-3′
0.015	100 µM	SexYBrook2R	5′-GTGCACTACCTTTGACCAGG-3′
0.015	100 µM	SexYBP1	VICCGT CCC AAA TGG CAT CCT TCC CTAMGBNFQ
0.015	100 µM	SexYBP2	6FAM TGT CCC AAA TGG CAT CCC TT MGBNFQ
3.94	100 µM	dH20	
1.00	Unknown	Template DNA	

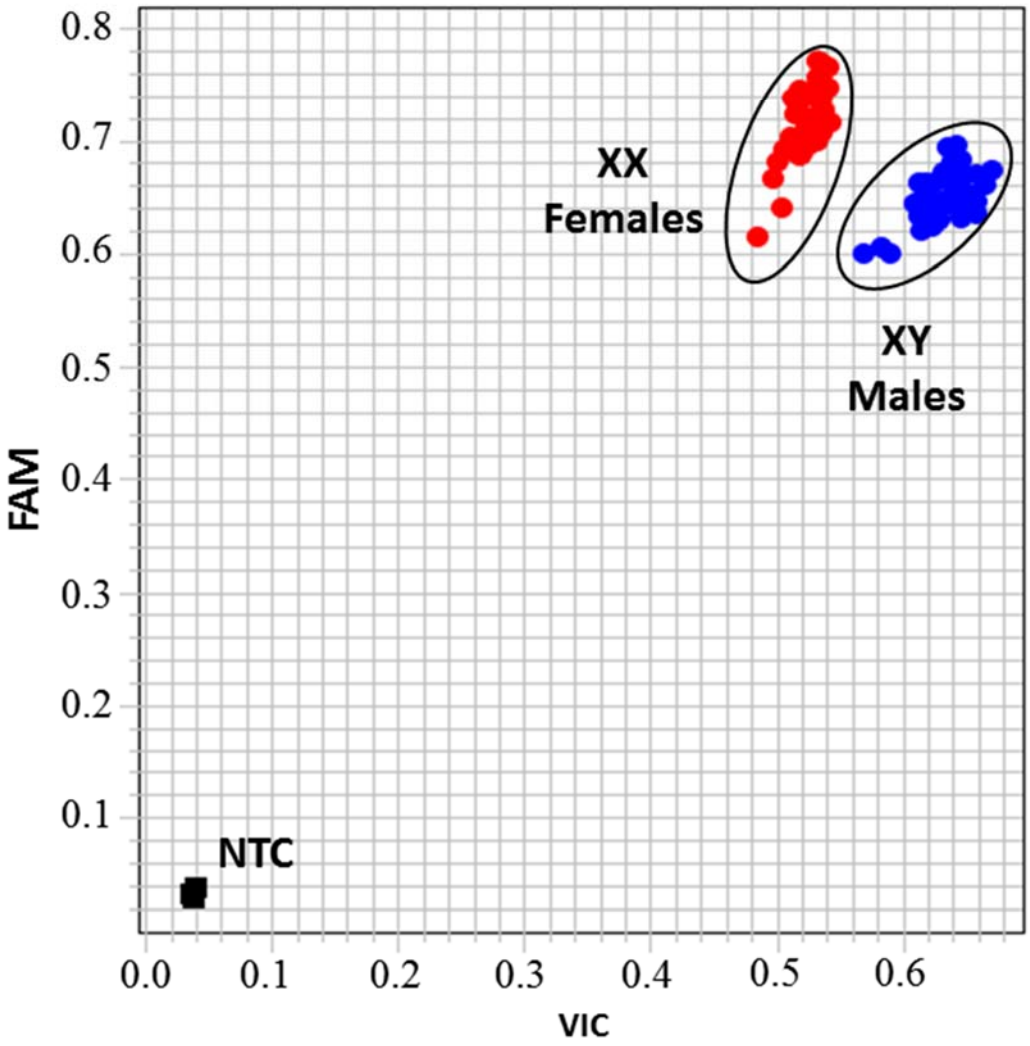
Supplementary Table S.3. Peak height of an allele *SFO-135* at the locus *Sco102*. The peak height of the *SFO-135* was higher than the peak height of the sex marker in all known XY males.

Sample number	Allele 1	Height 1	Allele 2	Height 1	Difference
SfoIRFH10C_0001	Male	7,055	*SFO-135*	7,075	–20
SfoIRFH10C_0002	Male	6,744	*SFO-135*	8,066	–1,322
SfoIRFH10C_0003	Male	9,044	*SFO-135*	9,760	–716
SfoIRFH10C_0004	Male	8,989	*SFO-135*	9,775	–786
SfoIRFH10C_0005	Male	9,106	*SFO-135*	10,155	–1,049
SfoIRFH10C_0006	Male	8,941	*SFO-135*	9,981	–1,040
SfoIRFH10C_0007	Male	7,864	*SFO-135*	9,035	–1,171
SfoIRFH10C_0008	Male	8,361	*SFO-135*	9,680	–1,319
SfoIRFH10C_0009	Male	8,663	*SFO-135*	9,681	–1,018
SfoIRFH10C_0010	Male	7,688	*SFO-135*	8,612	–924
SfoIRFH10C_0011	Male	8,766	*SFO-135*	9,798	–1,032
SfoIRFH10C_0012	Male	7,513	*SFO-135*	8,828	–1,315
SfoIRFH10C_0013	Male	6,925	*SFO-135*	8,523	–1,598
SfoIRFH10C_0014	Male	5,623	*SFO-135*	7,828	–2,205
SfoIRFH10C_0015	Male	7,979	*SFO-135*	9,446	–1,467
SfoIRFH10C_0016	Male	8,614	*SFO-135*	10,022	–1,408
SfoIRFH10C_0017	Male	5,320	*SFO-135*	6,563	–1,243
SfoIRFH10C_0018	Male	8,695	*SFO-135*	9,687	–992
SfoIRFH10C_0019	Male	8,287	*SFO-135*	9,299	–1,012
SfoIRFH10C_0020	Male	8,111	*SFO-135*	9,401	–1,290
SfoIRFH10C_0021	Male	9,093	*SFO-135*	10,067	–974
SfoIRFH10C_0022	Male	8,988	*SFO-135*	10,205	–1,217
SfoIRFH10C_0023	Male	5,937	*SFO-135*	7,767	–1,830
SfoIRFH10C_0024	Male	8,191	*SFO-135*	9,700	–1,509
SfoIRFH10C_0025	Male	8,657	*SFO-135*	9,884	–1,227
SfoIRFH10C_0051	Male	8,367	*SFO-135*	10,874	–2,507
SfoIRFH10C_0052	Male	8,910	*SFO-135*	9,664	–754
SfoIRFH10C_0053	Male	9,108	*SFO-135*	10,111	–1,003
SfoIRFH10C_0054	Male	9,081	*SFO-135*	10,328	–1,247
SfoIRFH10C_0055	Male	8,804	*SFO-135*	9,712	–908
SfoIRFH10C_0056	Male	8,871	*SFO-135*	9,994	–1,123
SfoIRFH10C_0057	Male	8,892	*SFO-131*	20,482	–11,590
SfoIRFH10C_0058	Male	8,041	*SFO-135*	11,138	–3,097

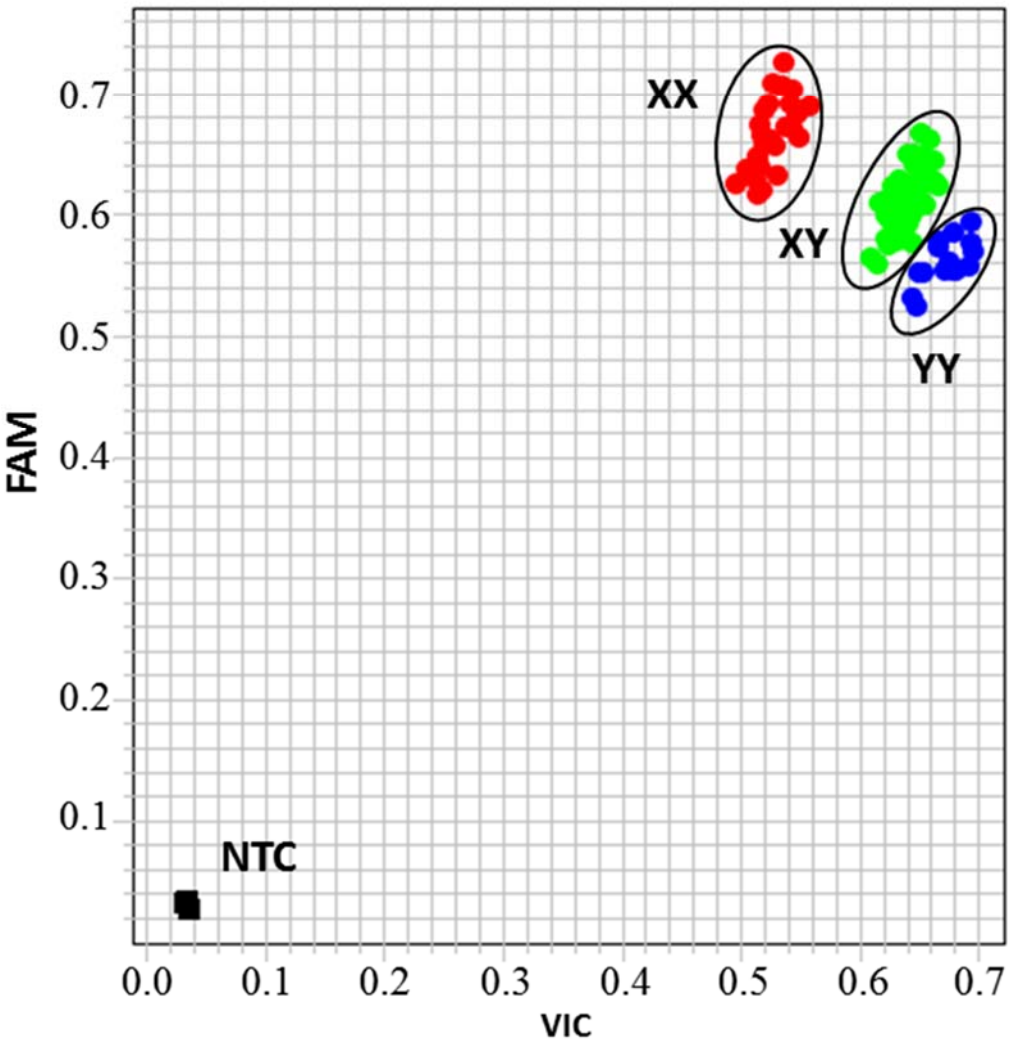
Sample number	Allele 1	Height 1	Allele 2	Height 1	Difference
SfoIRFH10C_0059	Male	8,881	*SFO-135*	14,617	–5,736
SfoIRFH10C_0060	Male	9,043	*SFO-135*	18,253	–9,210
SfoIRFH10C_0061	Male	8,776	*SFO-135*	9,584	–808
SfoIRFH10C_0062	Male	9,054	*SFO-135*	9,959	–905
SfoIRFH10C_0063	Male	8,778	*SFO-135*	13,746	–4,968
SfoIRFH10C_0064	Male	8,841	*SFO-135*	17,196	–8,355
SfoIRFH10C_0065	Male	8,676	*SFO-135*	12,237	–3,561
SfoIRFH10C_0066	Male	8,394	*SFO-135*	9,572	–1,178
SfoIRFH10C_0067	Male	7,124	*SFO-135*	8,415	–1,291
SfoIRFH10C_0068	Male	8,776	*SFO-135*	9,515	–739
SfoIRFH10C_0069	Male	9,038	*SFO-135*	17,641	–8,603
SfoIRFH10C_0070	Male	8,974	*SFO-135*	9,925	–951
SfoIRFH10C_0071	Male	9,040	*SFO-135*	9,855	–815
SfoIRFH10C_0072	Male	8,633	*SFO-135*	14,488	–5,855
SfoIRFH10C_0073	Male	8,886	*SFO-135*	10,143	–1,257
SfoIRFH10C_0074	Male	7,893	*SFO-135*	9,280	–1,387
SfoIRFH10C_0075	Male	9,066	*SFO-135*	10,077	–1,011



Supplementary Figure S.1. Examples of electropherograms showing the presence (first three samples: A–C) and absence (last three samples: D–F) of the Y-chromosome-specific product (~161 bp) amplified in a multiplex PCR. The *y*-axis shows relative fluorescence units (RFU) and the *x*-axis shows the estimated length of the Y-chromosome-specific product (male "peak").



Supplementary Figure S.2. An allelic discrimination plot showing diagnostic clustering of XX (females) and XY (males) using a modified Y-specific assay (SexY_Brook2). The VIC fluorophore (*x*-axis) is associated with the probe for the Y-specific product (males), while the FAM fluorophore (*y*-axis) labels the autosomal product. The black squares on the bottom left of the plot are no-template controls (NTCs), which omit DNA from the PCR reaction to verify that there is no contamination across samples and reagents.



SupplementaryFigure S.3. An allelic discrimination plot showing diagnostic clustering of XX (females), XY (males), and presumed YY individuals using a modified Y-specific assay (SexY_Brook2). The VIC fluorophore (*x*-axis) is associated with the probe for the Y-specific product (males), while the FAM fluorophore (*y*-axis) labels the autosomal product. The black squares on the bottom left of the plot are no-template controls (NTCs).