GENETIC ANALYSIS OF THE Y CHROMOSOME IN CHINOOK SALMON (*Oncorhynchus tshawytscha*)

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TO THE UNIVERSITY HONORS COLLEGE:

As thesis advisor for KELSEY WERTZLER,

I have read this paper and find it satisfactory.

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Date

PRÉCIS

Sex-determining mechanisms are intriguing systems that have yet to be elucidated in many organisms. In humans, sex-determination studies have potential applications mainly in the realm of healthcare and pharmacology as well as evolutionary studies. In organisms other than humans, sex-determining mechanisms can act as models to improve understanding of unknown systems. Occasionally it is advantageous in some organisms to have the capability to manipulate their genetic or expressed sex. In aquaculture of salmonid fishes it is favorable to raise all-female stocks due to the later onset of maturity expressed by females as opposed to males. Later maturity is desired in salmonid farming because maturing salmon show increased stress, lower quality flesh, and are more susceptible to disease (Benfey 2001). Thus, there is a need to understand sex-determination mechanisms in salmonids so that sex can be manipulated for commercial benefit.

In order to accomplish this, it is necessary to determine the sex of the fish without sacrificing it (e.g. DNA analysis). This creates an additional need for a DNA test that can easily and efficiently determine the genetic sex of an individual fish through DNA sex markers associated with the Y chromosome. While many studies have been conducted on salmonids and Chinook salmon *(Oncorhynchus tshawytscha)* in particular, there is still much to be discovered concerning their sexual differentiation processes and Y chromosomal features. This project studies the genetic features of the Y chromosome in Chinook salmon and examines their potential use in developing a DNA test for sex.

To address this research question, two approaches are used. First, a polymerase chain reaction (PCR)-based method is used to identify Chinook DNA sequences that are

specific to the Y chromosome. PCR functions to exponentially amplify specific fragments of DNA, and can be used to screen the DNA of male and female individuals in order to locate male-specific fragments. Secondly, a more complicated technique, amplified fragment length polymorphism (AFLP), is employed to screen a large number of individuals for male-specific sequences. AFLP has the capability to screen entire genomes by specifically fragmenting DNA and identifying single nucleotide polymorphisms (SNPs) between individuals. When the AFLP patterns of males and females are compared, male-specific sequences can be visualized and recovered.

Male-specific sequences are essentially markers in the DNA that correspond to sex, and are termed sex markers. This study identifies one particular sex marker (OtY3) that is used to develop an innovative and informative test for identifying genetic sex in Chinook salmon. OtY3 is a marker that PCR amplifies two regions, one that is Y chromosome linked and another that is autosomally linked. The developed test is unique in that the marker contains an inherent control, making it more easily utilized over other methods previously identified. The marker also is observed to detect Y chromosome polymorphisms—a feature that has not been observed in any fish until now.

In addition to locating the OtY3 sex marker, 12.5 Kilobases (Kb) of sequence data was accumulated and deposited into GenBank, a sequence database provided the National Center for Biotechnology Information. Together, the 12.5 Kb of characterized sequence and the new sex marker, OtY3, provide a significant source of information concerning the Chinook Y chromosome and a convenient, non-sacrificing method of sexing individuals.

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INTRODUCTION

Sex determination studies in fish, and salmonids in particular, are of interest for several reasons. Aquaculture of salmonids is a large and financially profitable industry in which it is more commercially valuable to grow all-female stocks. This is due to the higher cost-benefit ratio provided by females as they undergo a later onset of maturity than males. Thus, there is a need to understand sex-determining mechanisms in fish in order to identify sex during early stages of development and also to understand sex-reversal events so that they can be induced to increase female stock. Fish demonstrate multiple mechanisms of sex determination ranging from an entirely genetic-based regulation to an environmentally dependent system (e.g. temperature or population dynamics), as well as a partial genetic modulation in which environmental factors have an influence (Baroiller and Guirguen 2001). This diversity makes them a useful model for investigating evolution of sex-determining mechanisms as well as for understanding various contributors that may affect sex determination in other species. An additional benefit of studying the salmonid sex-determining mechanisms is that these mechanisms are thought to be at a primitive stage of evolution (Phillips et al. 2001). As a result, expanded knowledge of the salmonid systems can be applied to create a greater understanding of sex-determining mechanisms in general, as well as those conserved in vertebrates.

While it is generally accepted that salmonids express an X/Y sex chromosome system (male heterogamety) as the method for genetic sex determination (Allendorf and Thorgaard 1984; Donaldson and Hunter 1982), the master gene for sex-determination in nearly all teleosts remains unknown. Sex-determining loci have been localized to a

specific chromosome or chromosome region using sex-specific sequences and fluorescence in situ hybridization (FISH) in Chinook salmon (*Oncorhynchus tshawytscha*), Coho salmon (*O. kisutch*), rainbow trout (*O. mykiss*), and recently Atlantic salmon (*Salmo Salar*) (Stein et al. 2001; Iturra et al. 2001; Artieri et al. 2006). However, the medaka (*Oryzias latipes*) is the only fish species in which the gene for sex has been elucidated, indicating *DMY* as the master gene residing in the DM-domain of the Y chromosome (Matsuda 2003). *DMY* shows similarity to a mammalian male sex-determining gene *DMRT1* which is located near *SRY*, the principal sex-determining gene found in most mammals (Nanda 2002).

Although a major sex-determining gene has not been isolated among salmonids, sex-specific loci have been detected in several species from this group. The phenotypic sex-determining locus (denoted *SEX*) has been assigned to linkage groups in lake trout (*Salvelinus namaycush*), brook trout (*S. Fontinalis*), Arctic charr (*S. alpinus*), rainbow trout, and Atlantic salmon, however this locus is not uniformly localized on the genetic linkage map of all species in which it is present (Woram et al. 2003). Other sex-specific loci have been identified in Pacific salmon which allow for the determination of genotypic sex. Currently, in Chinook salmon, there are four DNA sequences that have been discovered to be sex-specific. Devlin and colleagues (1991, 1994) identified OtY1, a male-specific locus that is part of an 8 kilobase (Kb) sequence containing 300 repeats in diploid male Chinook which can be used to differentiate genotypic sex through polymerase chain reaction (PCR). Du and others (1993) located a male-linked growth hormone pseudogene appearing in all Pacific salmon species except sockeye salmon (*O. nerka*) and rainbow trout which was later used by Nagler et al. (2004) to develop a high-

throughput real time PCR protocol that indicates genetic sex. OT-24, found by Clifton and Rodriguez (1997) identified a 950 bp sequence that was more prevalent in males than females. This locus can be quantitatively amplified to show sex specificity, although it is infrequently used. Most recently, Brunelli and Thorgaard (2003) isolated OtY2, a male specific region unassociated with OtY1 or the growth hormone pseudogene that can be PCR amplified to determine genotypic sex in multiple Pacific salmon species. While these techniques are reliable, they are not necessarily easily utilized. Thus, the need remains for a simple and straightforward approach to genetically distinguish sex in Pacific salmon. This study examines the Chinook Y chromosome with the aim of fulfilling this remaining need.

There are numerous methods to identify sex markers and male-specific sequences. PCR screening of male and female genomic DNA is a convenient and cost-effective way to accomplish this. PCR essentially functions to exponentially synthesize specific portions of DNA molecules. To do this, at least a small portion of the DNA to be synthesized must be known so that oligonucleotide primers can be designed on the 5' and 3' ends of the template DNA. When the template DNA strand is heated to 94° C, it is denatured to form two single stranded molecules. The temperature can then be lowered sufficiently to allow annealing of the oligonucleotide primer to the single stranded DNA template. At about 72° C, the annealed primers then allow a thermostable DNA polymerase to synthesize the remainder of the complementary strand on the template DNA molecule. This three-step temperature variation is successively cycled around 30 times to create and exponentially increasing number of DNA molecules.

PCR is a widely used research tool with an infinite amount of applications. To identify sex markers, primers can be used that anneal to various regions on the Y chromosome. Although the Y chromosome is only found in males, much of its sequence is homologous to the X and other chromosomes. The comparison of male and female DNA can be PCR screened for male-specific amplification to isolate sex-specific markers.

Amplified fragment length polymorphism (AFLP) is also a versatile molecular tool. This technique has been shown to be an effective method to isolate sex-specific markers with increased power over other commonly used methods (Griffiths and Orr 1999). AFLP essentially combines the reliability and accuracy of restriction enzymes with the power of PCR to detect single nucleotide polymorphisms (SNPs). To perform AFLP, genomic DNA is cut by restriction enzymes (ERs), fragmenting the DNA molecule into many smaller pieces of varying sizes (depending on the frequency of restriction sites in the sequence). Oligonucleotide adapters, with overhanging sequences complementary to the RE cut sites are then ligated to the fragmented DNA. Adapted DNA fragments are amplified with primers designed to anneal to the adapter sequences plus one (+1) nucleotide beyond the RE cut site into the genomic DNA. These PCR products are then amplified again with primers that extend three (+3) nucleotides beyond the RE site into the genomic DNA. Varying combinations of +1 and +3 primers are used to screen through the genome of the organism being analyzed.

The main feature of AFLP that makes it effective in screening genomes containing such a large amount of DNA (for reference, the human genome is about 3 x 10^9 base pairs) is that it is extremely discriminatory. The amount of analyzed fragments

is narrowed down in three successive steps. First, REs with varying frequency of cut sites will cut only where there is a cut site in the DNA specific to that enzyme. If there is even one nucleotide difference, the enzyme will not cut. Second, upon +1 amplification only those fragments with sequences containing the specific target nucleotide will be amplified. Finally, the +3 amplification discriminates even further by selecting for an additional two nucleotides further into the fragment. Each of theses steps functions to select for the specific DNA fragments visualized in order to meaningfully analyze SNPs in an entire genome of DNA.

For identification of sex specific markers, AFLP can be used to compare male and female DNA. While males and females share a large percentage of their genomes, differences seen in the AFLP banding patterns can be attributed to sex. Fragments seen in males that are absent in females can be recovered for use as male-specific markers.

This study uses both PCR and AFLP based studies to located sex-specific markers in Chinook salmon. A particularly useful marker, labeled OtY3, was discovered using the PCR method.

CENTRAL QUESTION

The objective of this study is to isolate additional DNA features that uniquely characterize the Chinook salmon Y chromosome and also to utilize these features to

provide insight into the genetic structure of the Y chromosome. To accomplish these goals, two main approaches are employed: first a PCR-based method is used to sequence and locate male specific regions of the Chinook genome, and secondly an AFLP-based approach is used to broadly search the genomes of multiple Chinook individuals for Y chromosome-specific sequences.

METHODS

Chinook genomic DNA Samples.

Each population consisted of Chinook salmon that were phenotypically sexed at the time of sample collection and genotypic sex was verified using the OtY2 marker as described by Brunelli and Thorgaard (2004). Six populations were studied and labeled according to the region where the samples were obtained. These six populations were: 15 females and 15 males from the Chilliwack River, Canada; 45 females and 51 males from the Copper River/Gulkana River, AK; 13 females and 13 males from the Dworshak Hatchery, ID; 50 females and 50 males from the Priest Rapids Hatchery, WA; 22 females and 28 males from the Tuluksak River, AK; and 14 females and 16 males from the Willamette River, OR.

Isolation and sequencing of clones from a genomic library.

Cloned genomic DNA fragments of the OtY2 region were obtained from a Stratagene λZAPII genomic library constructed from a single Chinook male individual from the Willamette river population. Library subpools representing each resuspended primary phage plate from the library construct were screened for clones containing the OtY2 fragment using the corresponding primers (Table 1). OtY2 positive subpools were plated at a subconfluent titer on NZCYM media as per Sambrook (1989). These plates were further screened using the same OtY2 PCR primer set by using an 8-well multichannel pipette to touch the top agar surface of the phage plate and then placing the tips into a 96-well microtiter plate containing prepared PCR reactions. In this way, an 8 x 12 grid was formed on the phage plate that corresponded to the grid of PCR reactions in the microtiter plate. Reactions that were found to be positive indicated the close proximity of an OtY2-containing plaque and were located on the screened plate for isolation by plaque coring and additional PCR with the same primers. The region surrounding the original scar was screened by touching each proximal plaque and PCR testing to identify the strongly positive plaque which indicated that its insert contained the OtY2 region. The specific plaque containing the genomic region of interest was cored and re-plated at a lower titer for further clone isolation through PCR screening. This original OtY2 clone was sequenced and flanking primers were designed on the border sequence of the locus and used to rescreen the phage library for chromosome walking.

The resulting sequence data yielded 12.5 Kb flanking the OtY2 locus. This sequence was deposited into Genbank, accession no. 788926. All sequencing was

performed by the Washington State University Laboratory of Biotechnology and Bioanalysis.

Sex marker identification through PCR.

The OtY2 and flanking regions were evaluated for male specificity by using PCR amplification of numerous loci throughout the 12.5 Kb of new sequence data (see Table 1 for notable primer combinations). Male specific amplification, indicating Y-chromosome sequence, was determined using genomic DNA of male and female individuals from the Willamette river population. Male distinguishing amplification products were purified and sequenced for determining copy number and Y chromosome polymorphisms between male individuals. One particular locus, denoted OtY3, was found to distinguish male Chinook DNA and was focused upon for further characterization (see Table 1 for primers). The corresponding autosomal fragment of the OtY3 locus was sequenced and deposited into GenBank, accession no. 789758.

Polymerase chain reaction amplification was conducted on 50 ng of genomic DNA in 20 µL reactions with the following ingredients: 2 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates, 10X Taq Polymerase buffer (Invitrogen), 10 pM of each primer (Life Technologies), and 1 unit of *taq* polymerase (New England Biolabs). Thermocycling was as follows: initial denaturation of 94° C for 3 min, then 30 cycles of 94° C for 50 s, 60° C for 50 s, and 72° C for 1 min, concluding with a final elongation at 72° C for 2 min. Electrophoresis was performed with a 2% agarose gel in TAE buffer.

AFLP.

DNA used for AFLP was phenol-chloroform extracted from fin clips of phenotypically and genotypically sexed Chinook samples originating from the Copper river/Gulkana river population. DNA was consolidated into sex specific pools in order to neutralize possible autosomal allelic variances among individuals. Eight pools were generated, 4 male and 4 female, each containing 11 individuals contributing 2 mg of DNA to the pool. The pools were then used to construct a BamHI-Taqα1 AFLP template which was screened using Cy5-labeled BamHI (+3) primers against all combinations of unlabeled TaqαI A, C, G, or T (+3) primers (Vos et al. 1995; Brunelli and Thorgaard 2004).

Identification of a male specific fragment through AFLP.

Once a male-specific fragment was identified, it was excised from the gel and reamplified with the corresponding +1 primer set. The amplified fragment was then cloned by insertion into a pGemTEasy plasmid (Promega), transformed into DH5α cells (Invitrogen Life Technologies) and sequenced yielding a 374 bp AFLP fragment (GenBank accession # 776961). Various primers were then designed, based on the recovered sequence, with the goal of obtaining male-specific PCR amplification.

TABLE 1: Notable primers used

This table describes the primers used to conduct PCR and AFLP reactions as discussed throughout the paper. The 5' start site indicated relates to the GenBank sequence deposition of the recovered male Chinook library sequence (accession no. 788926).

Primer	Sequence 5'- 3'	5' start
OtY2-F2	CTGGTTCGAGCCTAAGTAG	2592
OtY2-R2	CATCTGCTCCTACTGCATC	2860
OtY3F	CAGAT ACTGATGGTGCAAC	7037

OtY3R	CATGCTCAGCACTGACACTG	7742
2083F	CACGTCAGTCACTGAGAATG	2083
3404R	CAGCAATGTGCACATGGTAG	3404
BamHI-gcg	GACTGCCTACTGATCCGCG	n/a
TaqαI-acg	GATGAGTCCTGACCGAACG	n/a

RESULTS

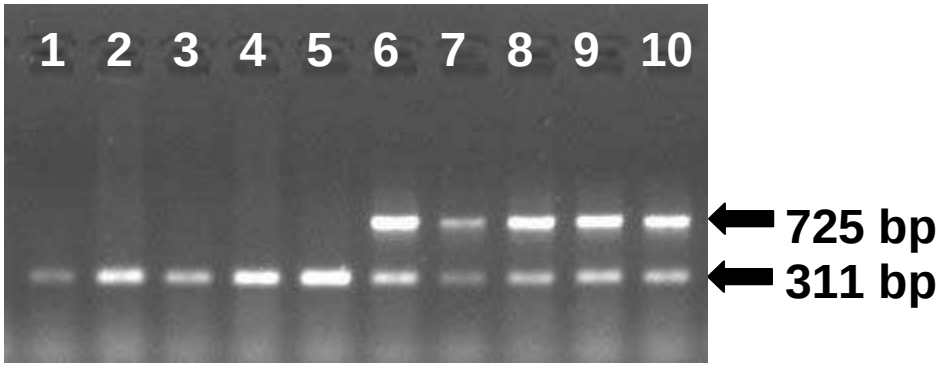
PCR Based Approach.

Using the flanking region of OtY2 as a starting point, clones from a male Chinook genomic library were sequenced to add 12.5 Kb of sequence data to the original locus. This region was analyzed for sequence characteristics and was found to contain no open reading frames, due to the widespread presence of stop codons, and was also extremely repetitive. Additionally, BLASTn searches of this area using the National Center for Biotechnology Information (NCBI) database revealed little homology to previously characterized sequences with the exception of one region that appeared to show homology to a gene for reverse transcriptase (nucleotides 885-2889, GenBank accession no. 788926). The sequence was also analyzed for male-specific fragments by PCR amplifying various regions and comparing male and female products. Products that were seen in males, but not females, were determined to be localized on the Y chromosome and male-specific.

PCR screening was carried out on male and female individuals from the Willamette river population with multiple primer combinations developed from the 12.5 Kb of recovered sequence. One specific primer combination denoted OtY3 (Table 1)

simultaneously exhibited both male-specific amplification and autosomal amplification. When separated in a 2% agarose gel, this primer set amplified an autosomal 311 base pair (bp) band seen in both males and females along with an additional higher molecular weight band of 725 bp seen only in male individuals (Fig. 1). With a simple PCR amplification, this locus shows the ability to identify Chinook as male (two bands) or female (one band). This result is particularly auspicious because the autosomal fragment acts as an inherent control for the reaction, removing the need for multiplexing to verify negative results. Also, the two bands have an easily discernable size difference which facilitates the ease of band resolution and identification of genotypic sex.

FIGURE 1: Isolation of sex-specific locus OtY3
Willamette individual females (lanes 1-5) and males (lanes 6-10) after OtY3 DNA PCR amplification.



Recovery of the male-specific and autosomal bands followed by subsequent cloning and sequencing revealed the male band to contain a minisatellite insertion of multiple 33 bp repeats, absent in females (Fig. 2). The minisatellite in the male-specific band was found to contain approximately 22 repeats, while the autosomal band seen in both sexes exhibited the 33 bp sequence unrepeated.

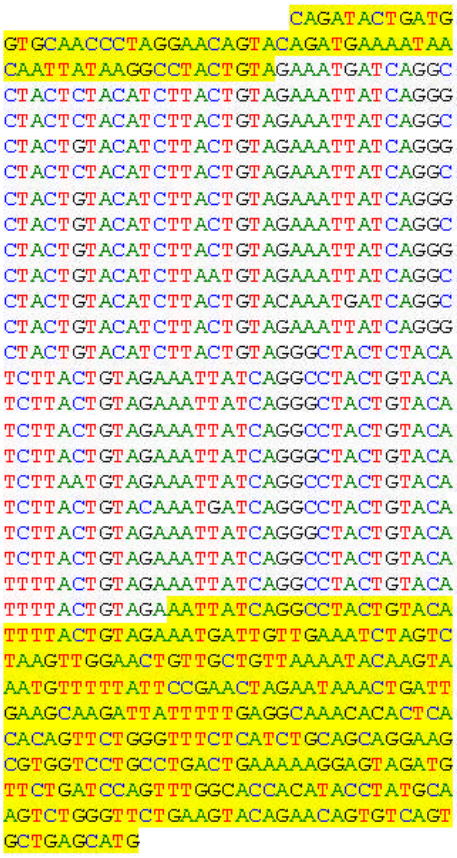


FIGURE 2: Sequence differences among autosomal and male-specific products indicate an inserted minisatellite. This Chinook sequence obtained from a male individual from the Willamette population shows the OtY3 locus as amplified by OtY3F and OtY3R primers. The apparent 33 bp minisatellite is easily seen repeating throughout the sequence. The highlighted segments represent sequence recovered from the autosomal fragment (GenBank accession no.789758), while the unhighlighted region denotes the male-specific repetitive insert (entire fragment, GenBank accession no. 788926).

Amplification of several regions throughout the OtY2 and OtY3 loci sequence, revealed the presence of multiple copies of amplified regions upon sequencing. Clones derived from the male-specific band of a single individual showed numerous SNPs indicating that multiple distinct copies of the male-specific fragment exist in a single individual (Fig. 3). Regions examined for multiple copy number included primer

combinations surrounding the OtY3 locus as well as a primer set designed to amplify a region spanning OtY2 (primers 2083F and 3404R). Two populations were focused upon for this study, Willamette and Tuluksak. Upon isolation of the male-specific band, cloning the fragment, and sequencing of several clones, it was determined that at least 2 copies of the OtY2 spanning region existed in the Willamette population and at least 3 copies of this locus were present in the Tuluksak population.

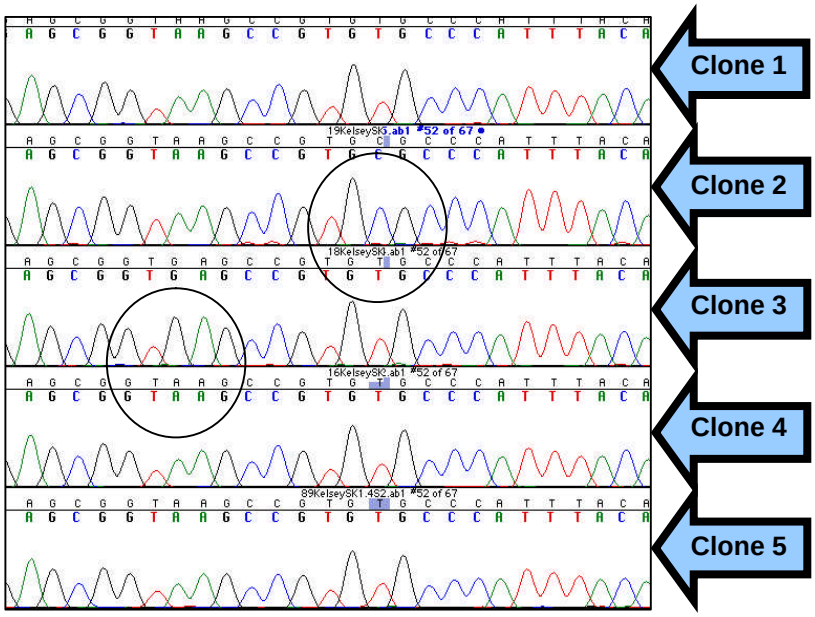


FIGURE 3: Sequence evidence of multiple copies within an individual. Chromoatogram results of sequence taken from cloned DNA of a single Chinook male individual from the Tuluksak population illustrate the SNPs present in the region. This indicates the existence of multiple copies of this locus within the male genome. Analysis of Chum, Coho and Sockeye also revealed polymorphic sequences, suggesting multiple copies.

When other species of Pacific salmon were examined for the OtY3 locus, this multi-copy phenomenon was also observed. At least 2 to 3 copies of the locus were detected in single male Chum and Coho individuals, and a male Sockeye individual was found to contain 6 different copies of the fragment in six different clones (data not shown).

Screening for the OtY3 PCR product in additional populations also revealed polymorphisms in the male-specific band. While all of the males in the Willamette population exhibited same-sized male-specific products, other populations were found to amplify two distinct sizes of the male-specific band, suggesting the presence of two different alleles for this locus (Fig.4). The males from the Willamette, Chilliwack, and Priest Rapids populations contained only allele A, whereas individuals in the Gulkana and Tuluksak populations possessed either allele A (725 bp) or allele B (approximately 500 bp). The Dworshak population was the only group that showed male individuals simultaneously possessing both alleles, seen as three bands comprised of two male-specific bands consistent in size with alleles A and B along with the smaller autosomal band (Fig. 4; Table 2). Interestingly, the Dworshak population either exhibited both alleles or allele A only.

FIGURE 4: Y chromosome polymorphisms exist within and among populations.
Male Polymorphisms within the Gulkana population upon PCR amplification of OtY3. Lane 1, female autosomal fragment; lane 2, male with allele B, lane 3, female autosomal fragment; lane 4, male with allele A.

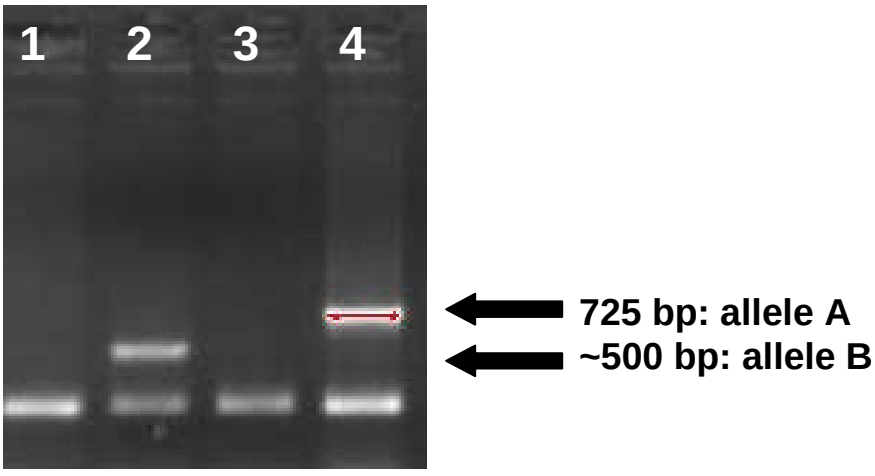


TABLE 2: Genotypic distribution within populations.
Y chromosome polymorphisms were seen in the OtY3 locus as the presence of two different male-specific alleles upon PCR amplification with the corresponding primers. Allele A indicates the 725 bp product, and allele B indicates the approximately 500 bp product. Some individuals from the Dworshak population showed both alleles.

Population	Allele A	Allele B	Alleles A & B
Chilliwack River, Canada	100%	0%	0%
Dworshak Hatchery, ID	80%	0%	20%
Copper River/Gulkana River, AK	30%	70%	0%
Priest Rapids Hatchery, WA	100%	0%	0%
Tuluksak River, AK	91%	9%	0%
Willamette River, OR	100%	0%	0%
Total	77.2%	21.2%	1.6%

Based on phenotypic identification of sex, determined at the time of sample collection, this locus has proved to be extremely accurate for genotyping sex in Chinook salmon. All successfully tested individuals were correctly sexed through amplification of the OtY3 locus (Table 3). When other species of Pacific salmon were tested for sex typing with this locus, Coho salmon tentatively showed functional typing; however Chum, Sockeye, and Pink salmons did not demonstrate distinct sex-related banding patterns (data not shown).

TABLE 3: OtY3 accurately sexes Chinook individuals.
The OtY3 locus was used to screen six populations of male and female Chinook DNA. DNAs were phenotypically sexed at the time of collection and genotypically verified using the OtY2 marker (Brunelli and Thorgaard, 2004). The accuracy of the OtY3 marker to identify sex is shown. These figures include all reactions that produced a visible product. Failed reactions were not counted as accurate or inaccurate.

Population	Number of Individuals in Sample	Number of Successfully Typed individuals	Genotypic Accuracy
Chilliwack River, Canada	30	25	100%
Dworshak Hatchery, ID	26	20	100%

Copper River/Gulkana River, AK	96	76	100%
Priest Rapids Hatchery, WA	100	77	100%
Tuluksak River, AK	50	49	100%
Willamette River, OR	30	30	100%

AFLP based results.

Throughout the AFLP screening, 80 AFLP polyacrylamide gels were run, encompassing some 480 different primer combinations that revealed one potential fragment for a chromosomal sex marker. This fragment (Fig. 5), showing an intense association with males, was recovered and sequenced.

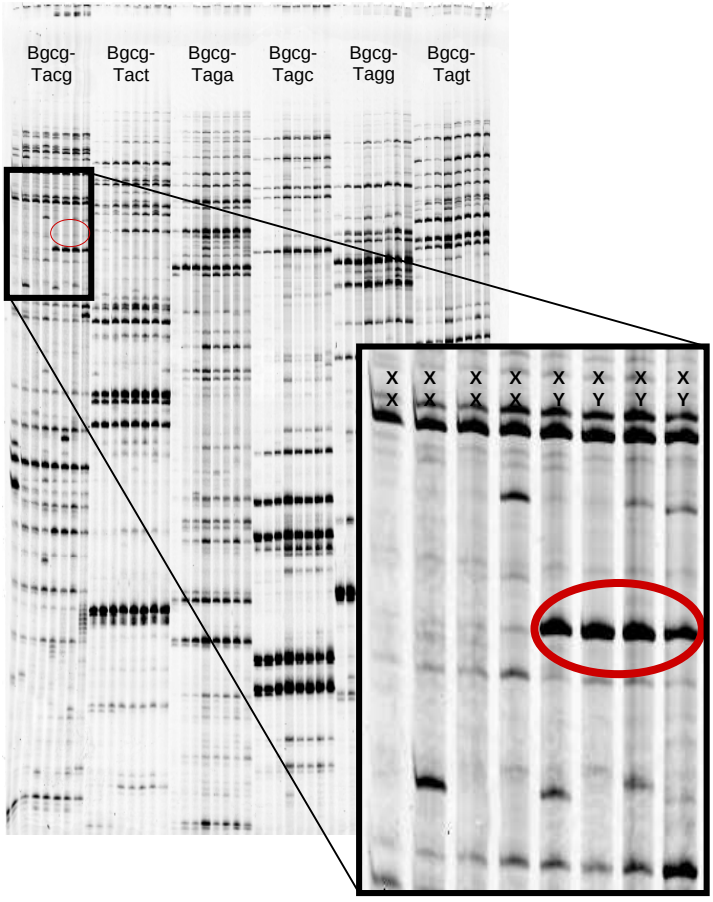


FIGURE 5: Isolation of a male-specific locus through AFLP.
This AFLP gel illustrates the different +3 primer combinations examined in a typical gel. The first primer set (BamHI-gcg to TaqαI-acg) shows the first four pools (female) have a very faint product, while the second four pools (male) have a very strong product. This band was recovered from the gel for sequencing.

The resulting 274 bp sequence (Fig. 6) was searched for homology to known sequences using the NCBI BLASTn search engine.

FIGURE 6: Sequence of the recovered AFLP male-specific fragment.
The male-associated fragment from Fig. 5 was recovered and sequenced as shown here, GenBank Accession # 996961.

```
  1 ggatccgcgt cgcgaaaaac atattcttgg tcatactgat ggtgagttga
 51 cgctgatctt atattcagta gttcttgtcg gctgtatgta aagaaaccta
101 agatgacctg gggtactagt gtaagaaata acacgtaaaa aaacaaaaaa
151 ctgcatagtt tcctaggaac gcgaagcgag gcggccatct ctgtcggcgc
201 cggagtgtga gtctctcttg ctctctagtg aaccatggga agtttactaa
251 gctggaggac tcgtatgcgt tcga
```

A short, 50 bp region of the fragment was revealed to show homology to the first intron region of the gene for the piscine hormone, somatolactin—a pituitary hormone from the prolactin/growth hormone family (Fig. 7). The exact function of somatolactin remains unclear, although there is some speculation that it may be involved in such physiological processes as maturation, calcium regulation, stress response, and acid base regulation (Kaneko, 1996).

FIGURE 7: AFLP male-specific product shows homology to somatolactin gene.
An NCBI BLAST search showed alignment of the male-associated sequence (Fig. 6) with the first intron region of the gene for somatolactin.

```
Score = 83.8 bits (42), Expect = 2e-13
Identities = 51/54 (94%), Gaps = 0/54 (0%)
Strand=Plus/Plus

Query  145
CTGCATAGTTTCCTAGGAACGCGAAGCGAGGCGGCCATCTCTGTCGGCGCCGGA  198
            ||||||||||||||||||||| ||| ||||
||||||||||||||||||||
Sbjct  745
CTGCATAGTTTCCTAGGAACGCAAAGAGAGGTGGCCATCTCTGTCGGCGCCGGA  798
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Multiple PCR primers were designed to anneal to varying locations within the recovered AFLP fragment with the goal of producing male-specific PCR amplification. At this time, PCR with these primers has shown sex-specific amplification such that males amplify a product and females do not, however not all male individuals reliably and consistently show products.

DISCUSSION

Sequence characterization.

As shown in the results section, the 12.5 Kb of new sequence data flanking the OtY2 locus appeared to contain repetitive regions with multiple copies. When various regions throughout the OtY2 and OtY3 loci were examined for evidence of multiple copies, all results indicated the presence of at least 2 copies. Sequence analysis of the OtY2 spanning region in the Willamette population, using 2083F and 3404R primers (Table 1), showed a convincing SNP, providing evidence that another version of this locus is present within the male genome (Fig. 3). Similar efforts with the Tuluksak population yielded SNPs in 2 positions suggesting the presence of 3 copies of the locus within this population's genome. Multiple loci were tested in this fashion, all indicating that at least 2 versions of the examined locus were present in Chinook.

OtY3 marker.

The OtY3 locus amplifies two DNA regions in Chinook salmon: an autosomal region shared by both sexes, and a Y chromosome region only in males. Visualization of the amplification of this locus yields a single band in females, and at least 2 bands in males—one identical to the band seen in females, and another of higher molecular weight. Upon sequencing of the male-specific and autosomal fragments from Willamette population individuals, it was found that the larger male-linked product differed only in that it contained a 33 bp segment that was repeated numerous times. While this 33 bp fragment was seen in the autosomal product, it was not repeated. The Y chromosome product seemed to have an additional 22 repeats of the fragment incorporated into the region.

This repeated region, or minisatellite, was found to be particularly interesting when it was discovered to contain Y chromosome polymorphisms within and across populations. Slight size variations of the male-linked product were seen, representing a deviation in the number of repeats inserted into the minisatellite. This resulted in a male-specific product of 725 bp (denoted allele A) and another of approximately 500 bp (denoted allele B). The majority of males screened showed the presence of only allele A (77.2%). Allele 2 was seen in 21.2% of males screened (mainly attributed to the Tuluksak population), and it was very rare (1.6%) to identify males that contained both allele A and allele B (Table 2). This indicates that there is likely a gene conversion event occurring to normalize the number of repeats on the Y chromosome, yielding a relatively uniform genotype across individuals. It is possible that individuals with both the A and B alleles represent the failure of such gene conversion.

The OtY3 marker is the first Y chromosome polymorphism to be identified in fish. Polymorphisms on the Y chromosome can be valuable tools for studying evolutionary processes. In humans, polymorphisms on the Y chromosome have been used to track evolution geographically throughout the globe (Hammer et al. 1997; Jorde et al. 2000). The OtY3 marker has the potential to track evolutionary changes in Chinook and to provide additional insight into their phylogeny.

The OtY3 locus provides a convenient method to genotypically distinguish males from females. While other methods have been previously developed that essentially perform the same function, the method described here is more easily utilized for several reasons. First, PCR amplification of this locus yields an autosomal product which acts as a built-in control, and negates the need for verifying negative reactions through multiplexing. Secondly, the size difference between the autosomal and male-specific fragments is sufficient to easily and relatively quickly resolve the sex-differentiating banding pattern. The only potential pitfall found when using this locus to identify sex in individuals was that rarely a female sample would show a very faint second product corresponding to the male-specific band. This faint product was only seen before the PCR reaction conditions were optimized, thus it should only cause inaccuracy if PCR is being performed at non-optimal conditions. It is also possible that this effect may be seen in populations not studied as there could be slight variations in optimal PCR conditions for such uncharacterized groups.

AFLP analysis.

Using AFLP to screen a large number of male and female individuals yielded the detection of one male associated fragment. This fragment was very strongly apparent in all four male DNA pools, while extremely faint in the female pools. When excised and sequenced, the fragment was seen to contain a run of adenine nucleotides appearing to correspond to a poly-A tail of a terminating gene sequence. Although the region preceding the poly-A sequence did not show homology to any known sequences, a NCBI BLASTn search revealed that the region immediately following the run of adenine nucleotides aligned with the first intron region of the piscine gene for somatolactin. The combination of potential coding sequence (somatolactin) interrupted by another gene (poly-A region) implies the presence of a pseudogene.

Somatolactin has been characterized as a pitutitary hormone originating from the prolactin/growth hormone family. This hormone is found only in fish and resides in the pars intermedia. Although the exact function and purpose of the hormone has yet to be elucidated, it is thought to have some association with a variety of physiological processes including maturation, calcium regulation, stress response, osmoregulation and ionoregulation (Kaneko 1996). Of these potential roles for somatolactin, the possible involvement in maturation is an interesting feature, providing that a partial sequence of the gene was found to be male-linked in the Chinook AFLP screening. In Coho salmon, somatolactin has been found to be highly correlated to plasma estradiol levels in females and 11-ketotestosterone levels in males (Rand-Weaver et al. 1992). Changes in plasma somatolactin levels were also found to occur during smoltification and spawning in Coho salmon (Rand-Weaver and Swanson 1993). It is apparent that this hormone could play a significant role in physiological events in which sex-specific and maturation inducing

genes are being transcribed and expressed. It is interesting then, that an intron sequence from this hormone appears in the Y chromosome and has been apparently disrupted by the insertion of another gene sequence, as indicated by the presence of the poly-A tail. Further sequencing of this AFLP recovered region would ascertain if additional somatolactin-related sequences are present and would also elucidate the proposed insertion of a separate gene which disrupts the somatolactin sequence.

In order to exploit the male-associated AFLP fragment, several PCR primers were designed from its sequence to develop a PCR test that would distinguish male and female DNA. Despite the fact that primers were designed to scan multiple regions of the fragment, a reliable test had not been discovered at the time of this submission. Although the developed PCR primers did not show amplification in females, not all male individuals showed successfully amplified products. The potential remains to design more informative primer combinations and to further optimize the PCR reaction to provide a reliable method for determining genetic sex through PCR amplification of this AFLP fragment.

An interesting result of the AFLP screen was that the use of pooled DNA from outbred individuals produced a very low ratio of potential sex markers to the number of primers screened. When a similar technique was utilized by Brunelli and Thorgaard (2004), using pooled DNA from androgenetic individuals, a much higher ratio of potential markers to number of primers screened was seen. Despite the recovery and characterization of approximately 50 potential markers located through the androgenetic individuals, only one was actually verified to be a male-specific marker, while all others were found to be artifacts. The comparison of these results indicate that while use of

DNA from outbred individuals yields fewer markers, the located markers are more likely to be authentic and tightly male-associated.

The overall goal of this project was to isolate genetic sex markers in Chinook salmon using a PCR-based technique and an AFLP-based technique. The PCR method yielded OtY3, a new and useful system for detecting genetic sex in Chinook. OtY3 also has great potential for further use in evolutionary studies, as it is the first Y chromosome polymorphism observed in fish. The AFLP-based study also produced a Y chromosome sex marker, although it has not been fully developed. While the marker itself has been isolated, further work must be done to optimize its use in PCR for easily sexing Chinook DNA.

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