**Task 4: Sex Marker**

***Background***

Sex determination in fish is a highly variable trait62 and understanding its mechanisms is crucial not only for understanding the biology of the individual species of fish but also for gaining insight into the evolution of sex chromosomes and genetic mechanisms underlying sex determination.63 Fish represent the most diverse group of vertebrates on earth with over 30,000 described species.64 With this diversity and constant exposure to variable environments comes a vast array of morphological, physiological, behavioral, developmental and sexual mechanisms.65–68 In teleost fishes, sex determination can be genetic or environmental and varies even between closely related species.62,69–71 Delta smelt are a unisexual species that do not appear to have environmental regulation of sex determination which suggests sex may be determined genetically. In teleost fishes, endogenous genetic sex determination mechanisms can occur at the chromosomal level, where heterogametic males (XY) or females (ZW) exist, or mechanisms can be at the genic level where single or multiple genes influence sex determination.70 While clarifying the mechanism of sex determination in delta smelt will increase our biological knowledge, it will also allow us to identify and develop diagnostic markers for the practical management of the species.

The ability to non-invasively identify sex in delta smelt will assist in management of the captive colony and develop knowledge of the biology of wild delta smelt. Currently, wild fish can only be sexed by the expression of gametes from ripe adult fish or through dissection, both sacrifice the life of the fish or gametes. When sex is identified by expression, pressure is put on the abdomen of fish until eggs are excreted (in females) or running milt (in males).72 Because sexual identification of wild fish primarily relies on the physiological status of an individual fish, only about two-thirds of wild adult delta smelt sampled can be sexed (Hammock pers. comm.). Knowledge of the genetic underpinnings of sex determination in fishes is a vital asset to the better management of captive populations, basic knowledge of life history characteristics of the species, ecological surveys and management regarding population metrics, species modeling, demographic inference, and sex-based survival.73

In order to non-invasively identify the sex of wild and captive delta smelt, we sought to identify potential candidate allele(s) which could be used as genetic diagnostics for classifications of sex.

***DNA sampling & sequencing***

*Methods*

To identify a sex specific marker or markers for delta smelt, we sampled adipose fin clips from 24 female and 24 male captive-bred individuals taken from the FCCL and sexually identified through either dissection or gametic expression. DNA was extracted using the Qiagen DNEasy 96 Blood & Tissue Kit with a modification of elution in100uL of H2O rather than the proprietary AE Buffer included with the kit.

Prior analyses that attempted to determine sex markers in delta smelt used the *Sbf1* restriction enzyme, which cuts DNA approximately every 65,000 base pairs, but no sex markers were identified. For this library preparation we sought to maximize the number of restriction enzyme cut sites and acquire reads from more locations throughout the genome. To do this we digested extracted DNA using the *Pst1* restriction enzyme, which shears DNA sixteen times as often as the *Sbf1* restriction enzyme, or roughly once every 4,100 base pairs. RAD sequencing libraries were prepared at the GVL according to Ali et al (2016)<CITE>, and sequenced at the UC Davis Sequencing Center with 150 bp paired-end reads on an Illumina HiSeq.

***Genome wide association study***

*Methods*

We performed two rounds of a genome wide association study (GWAS) using the new male and female reference genomes. For each GWAS, we first aligned raw RAD sequencing data from 24 male and 24 female sexed fish to the reference genome. Next, we looked for the association of an allele at any location in the genome with sex classification by carrying out a dominant model case-control GWAS using males as controls (0) and female as cases (1) in the program ANGSD12. If we found alleles that associate with a particular sex that locus could be used as a diagnostic for genetically identifying sex. The association of a particular allele with sex category was reported as likelihood ratio test (LRT) statistic and is chi square distributed with one degree of freedom. We applied a conservative significance cutoff with a Bonferroni corrected p-value of 0.05 using the formula where is the number of loci analyzed, is the desired p-value or significance threshold (0.05), and is the adjusted p-value given the number of loci used in the analysis.

*Results*

We analyzed 848,444 and 922,975 loci spread across the male and female genome assemblies, respectively. These loci correspond to a Bonferroni corrected p-value cutoff of 5.893141e-08 in loci found within the male reference genome and 5.417265e-08 in loci analyzed within the female reference genome. No significant association in sex was found using the female reference genome. Two loci located in the male reference genome on Chromosome 5 were significantly associated with sex in delta smelt (Figure 7). The two SNPs most associated with sex in delta smelt had an LRT scores of 37.854854 and 35.802804 which correspond to p-values of 7.621e-10 and 2.183e-9, respectively.

***Depth analysis***

*Methods*

If sex determination in delta smelt were caused by chromosomal differences, it would be expected that the heterogametic sex (i.e. XY) would have roughly half the sequencing depth of the homogametic sex (i.e. XX) at the large region that determines sex in the fish. Additionally, the heterogametic sex would be expected to have novel sequence content. To look for signs of sex specific sequencing depth differences, we looked for male and female differences in the presence and depth of RAD markers spread throughout the genome. To do this we performed two experiments, one using the male genome as a reference and another using the female genome as a reference. Each experiment used the 24 male and 24 female alignment files from the previous GWAS. First, we acquired the depth of aligned reads at each location in the reference genome using samtools depth. Next, using custom bash and perl scripts we threw out all locations where no male or female RAD sequencing data aligned, and we totaled the number of male alignments and gathered the total coverage for each sex (<https://github.com/shannonekj/DS_sex-marker/blob/master/analysis/>). Finally, we sorted the coverage difference files and looked for locations in the genome where one sex had high and consistent coverage and the other sex had shallow or no coverage.

*Results*

We found no large areas that corresponded to an inflation of any one particular sex having higher depth of coverage compared to the sex and could not identify any sex specific loci in this analysis.

***K-mer analysis***

*Methods*

In our k-mer analysis we sought to identify unique differences of sequence content in males versus females. To do this, we used 10X Genomics linked-read data from one male and one female. First, we created a signature of all k-mers belonging to each sex with sourmash compute -scaled 1000 to sample one k-mer from ever 1000 base pairs. The signature compute step was followed by sourmash signature merge to incorporate all data from the R1 and R2 files for each sex. We purged the signature files of low abundance k-mers (abundance < 5) to eliminate k-mers that are more likely sequencing errors and threw out k-mers that were shared between male and females to only leave sex-specific k-mers. The resulting high abundance, single sex k-mers were used in our analysis.

First, we plotted and compared k-mer abundance for each sex to determine if there were observable differences between sexes. A difference in k-mer abundance could correspond to a sex determining regions within a sex (i.e. sex chromosome). Next, we extracted contigs which contain five or more k-mers within one contig which corresponds to a contig length of roughly 5,000bp. We then compared the abundance of male and female k-mers found within those contigs. After, we took the median abundance of k-mers within a contig to find the contigs abundance in each sex. We compared the male contig abundance to the female contig abundance and isolated male-only contigs deemed “putative Y” sequences for further validation.

To validate our results, we mapped RAD sequencing data to the putative Y sequences and ran a depth analysis. In order to ensure the putative Y reads were indeed mapping to one location in the genome, we performed a stringent end-to-end alignment of the putative Y sequencing data using bowtie2. Only reads that entirely aligned to regions in the genome were used in the subsequent analysis. To find depth locations, we aligned the RAD sequencing data to the male reference genome using samtools <CITE>. We then pulled RAD alignment depth information from all of the locations where the putative Y sequencing data had also aligned using the software bedtools<CITE> and custom bash and perl scripts. After we obtained depth information across all of the putative Y regions, we ran the same depth analysis as above.

*Results*

After abundance filtration, there were approximately 118,191,000 male-only k-mers and 494,251,000 female-only k-mers. There was a clear distinction between the distribution of male and female abundances, where males had more high abundance k-mers compared to females (Figure 8). Upon filtering k-mers for those found on long contigs (contigs containing 5 k-mers or more) there was a clear increase of male-specific k-mers at half the abundance of the female and male peak on the right (Figure 9). This is consistent with the male sequencing data potentially having heterogametic regions in its genome. We found 44 contigs with k-mer mean abundance in the male sequencing data that had zero abundance in the female sequencing data (Figure 10).

We mapped the putative Y data back to the male reference genome and found the reads mapped to multiple regions within the genome (Table 6). We did not find a significant difference in male versus female read depth at locations across the putative Y regions.