**Sequencing the delta smelt genome: improved annual monitoring of Ne and further understanding of the wild and hatchery delta smelt populations**

June 30, 2021 Final Report

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This document will serve as the final report for A19-1844 contract between State Water Contractors and the Regents of the University of California.

**Background**

For this project we are assembling a reference genome for delta smelt in order to 1) calculate a more precise estimation of contemporary effective population sizes (NE) for the 2017 to 2019 wild cohorts, 2) examine domestication selection at discrete locations in the genome, and 3) identify sex-specific markers and/or chromosomes in delta smelt. The creation of a reference genome for delta smelt will enable scientists to more fully take advantage of the decreasing samples available and use archival samples more effectively. All deliverable statuses are listed in Table 1.

The use of next generation sequencing (NGS) technologies in conjunction with a highly contiguous and accurate reference genome, increases the power and precision of inferences made in population genetic studies (such as structure, diversity, understanding local adaptation).1 DNA sequences unaligned to a reference genome fail to account for how each piece of sequenced DNA interacts with all other sequences (i.e. linkage patterns). However, by aligning to a reference genome we have knowledge of where each piece of sequenced DNA lies within the genome and relative to other sequenced DNA. Additionally, by knowing each markers distance from one another, and how it is or is not inherited with other markers we can survey a greater number of informative markers spread throughout the genome. This creates the ability to better answer questions involving demography, adaptation, fitness, and disease susceptibility.2,3 For example, previous work in Chinook salmon using the closely related rainbow trout reference genome found small amount of markers that were associated with runtime.4 In 2014, these markers were located next to a gap in the rainbow trout reference assembly. Reanalysis using a highly contiguous 2018 Chinook salmon reference genome found diagnostic markers located within the gap in the 2014 reference genome (Prince *et al.* unpublished).5 This example underscores how reference genomes can propel conservation research forward.

Recently, genome sequencing technologies have become both more cost effective and efficient. Hybrid assemblies, that is assemblies that use multiple NGS technologies, are the most reliable way to achieve a chromosome-scale hi-quality genome assembly. The Vertebrate Genomes Project, a consortium aimed towards developing an assembly pipeline and quality standards for genome assemblies of all vertebrates, established quality goal metrics for the continuity, completeness and accuracy of reference genomes6. Therefore, a main goal of this project is to develop a highly accurate chromosome-scale reference genome, hereafter called “reference genome”, using linked-read, long-read hi-c chromatin confirmation capture and a genetic linkage map (Figure 1).

**Task 1: Genome assembly**

***Sample collection (Figure 1A)***

*Methods*

The first step in a genome assembly is to collect tissue for the extraction of high molecular weight (HMW) genomic DNA, which is extracted DNA that is longer in length (>50 kilobases) than standard DNA extractions. Because all of the sequencing technologies utilize sequence fragments on the order of tens of thousands of base pairs long or rely on long range interactions throughout the genome, extracted DNA for generating a contiguous genome assembly must be sufficiently long. Due to the difficulties in getting sufficient quantity of HMW DNA, we conducted four trips to sample different tissue types from fish 600 days post hatch at the FCCL (Table 2). At the start of this project long-read sequencing was costly and we sought to only incorporate the sequencing technology if absolutely necessary; because of this our first and second sampling trips sought to acquire enough tissue to provide sufficient quantity of HMW DNA for linked-read and hi-c sequencing. However, at the end of 2019 the price of long-read sequencing dropped dramatically and based on our results from trips 1 and 2, for trip 3 we sought to acquire enough tissue to sequence a single male fish with all three of the technologies and a female fish with the last long-read technology. However, prior to acquiring the last sequencing technology for the male fish, our samples were thrown out by the sequencing center and it was necessary to sample one additional male specimen on a fourth trip. On each occasion fish were euthanized according to the approved animal care protocols/standard operating procedures. Back muscle, internal organ and/or scale tissues were sampled onsite at the FCCL and cooled for transportation directly to the UC Davis Sequencing Center.

*Results*

We took a total of four trips to sample tissue. On the first trip we sampled back muscle from three males and three females (Table 2). These samples were flash frozen using liquid nitrogen and transported on dry ice. While we obtained sufficient HMW DNA from a female specimen, none of the sampled male fish from this trip produced enough HMW DNA. Therefore, we returned to the FCCL on a second trip and selected larger males. Extraction lengths from these male fish were also insufficient. A decision to take a third trip was made due to the availability of a new sampling method that uses additional tissue types (not just back muscle tissue) and new tissue preservation storage solution of samples in cooled propylene glycol rather than only flash freezing in liquid nitrogen. On the third trip we sampled back muscle tissue, internal organs, and scales from a total of two males and two females. Additionally, because DNA can be fragmented in the freeze-thaw process, we hedged our bets for sampling on third trip by preserving half of all sampled tissues in propylene glycol at 4°C, and half with the protocol used on trips 1 and 2 (flash freezing with liquid nitrogen and transporting on dry ice) which was successful for a prior female fish. Due to a sampling being thrown out we had to carry out one additional sampling in order to acquire tissue for hi-c sequencing. On the fourth trip we sampled back muscle, scales and internal organs from one male fish.

***Isolation of high molecular weight genomic DNA (Figure 1B)***

*Methods*

For all three sequencing types (linked-read, long-read and hi-c), HMW DNA is required. For the linked-read and long-read sequencing, HMW DNA extractions from the fish tissues occurred at the UC Davis Sequencing Center using the protocol described in Wasko et al. (2003)7. We acquired samples to extract DNA from both sexes to examine sex determining regions in delta smelt, which is currently unknown. The size range of extracted DNA fragments were determined using a pulse field gel run at a low frequency, to not shear the DNA, for 24 hours. Physical sampling and extractions were repeated until the mean distribution of extraction lengths was 50kb or greater and there was sufficient quantity of HMW DNA to carry out the required sequencing technologies for each sex. For extractions and subsequent hi-c sequencing, a male and female sample was sent to Phase Genomics and the Vertebrate Genome Project, respectively.

*Results*

Trip 1: DNA from one male (T1M03) and one female (T1F02) DNA was extracted from the first sampling (See Table 2 for fish identifications corresponding to trips). HMW DNA from the T1F02 had DNA fragments of sufficient length for sequencing (Figure 2A).

Trip 2: We performed three separate rounds of extractions on tissue samples from two different males (T2M02 and T2M03). Yet despite multiple attempts at extracting HMW DNA, we did not obtain fragments of sufficient length for sequencing (Figure 2B-D).

Trip 3: We performed two separate rounds of extractions on multiple different tissue types (Table 2). From these extractions we acquired DNA of sufficient length from flash frozen back muscle tissue from one male (T03M02\_BM\_FF, Figure 2E) and flash frozen scales from one female (T3F02\_SC, Figure 2F). We did not observe that storing tissue in propylene glycol had an effect on increasing the fragment length of extracted DNA (Figure 2E & F).

Trip 4: The tissue samples from the male specimen sampled on trip 4 were sent directly to the Vertebrate Genome Project for subsequent extraction and sequencing.

***Long-read library prep & sequencing (Figure 1C)***

*Methods*

The inclusion of long-read data dramatically assists in creating a more contiguous assembly by spanning repetitive elements and resolving chimeric sequences throughout the genome. Long-reads provide greater continuity of scaffolded contigs and resolving repetitive sequences within genome assemblies, because the reads (sequence fragments produced from the sequencer) are thousands rather than hundreds of base pairs long. Previously, long-read sequencing was both costly and had an error rate up to 10%. Within the past year, new PacBio HiFi chemistry has come out to dramatically reduce the number of errors from almost 10% to under 1%. Additionally, the price of PacBio long-read sequencing has dropped dramatically, making sequencing a male and female fish affordable for this project.

PacBio HiFi SMRTbell® Libraries (<https://www.pacb.com/>) were prepped following the SMRTbell Express Template Prep Kit 2.0 procedure. The UC Davis Sequencing Center used a Megaruptor to shear DNA to an average length of ~15kb. The input for the library prep was 5.6ug of DNA and the library was size selected to 11kb. Prepped DNA was run on a Sequel II machine and sequencing data was collected as recorded “movies” of nucleotides. Each movie collection lasted for 30 hours per run. Additional movies were collected until the amount of sequencing data for each sex was ~25-30x coverage.

*Results*

A total of five movie collections or 150 hours of sequencing data were collected. Two male movie collections generated roughly 25x coverage (data combined into Supplemental Data 1) and three female movie collections generated roughly 28x coverage (Run 1 & 2 combined into Supplemental Data 2, Run 3 data in Supplemental Data 3).

***Long-read post-sequencing quality control (Figure 1C)***

*Methods*

Sequencing data stored in binary bam files were downloaded from Bioshare, the UC Davis Sequencing Center’s host service. We used CCS software’s (<https://github.com/PacificBiosciences/ccs>) statistical model on raw reads to generate highly accurate consensus sequences with known base quality values and converted binary data to fastq format for downstream analysis. A base quality value assigns a score to each letter of sequencing data to denote the level of confidence in each base called by the sequencer. Reads with quality scores over Q20, denoting an error probability of 0.01, were used for subsequent assembly.

*Results*

A total of 3,095,133 male and 2,741,504 female reads passed quality control.

***Linked-read library prep & sequencing (Figure 1C)***

*Methods*

Once sufficient extracted HMW genomic DNA fragments were acquired, DNA was adjusted to a concentration of 0.91 ng/µl. We selected the 10X Genomics platform (<https://www.10xgenomics.com/technology/>) for our linked-read sequence data. 10X Genomics library preparation takes extracted HMW gDNA, shears the DNA into 50kb long segments, inserts each fragment into an oil coated gel emulsion bead (GEM), further shears DNA within each bead, and attaches unique barcodes to all DNA fragments within each GEM for identification post-sequencing. Whole genome sequencing libraries were prepared using Chromium Genome Library & Gel Bead Kit v.2 (10X Genomics, cat. 120258), Chromium Genome Chip Kit v.2 (10X Genomics, cat. 120257), Chromium i7 Multiplex Kit (10X Genomics, cat. 120262), and Chromium controller according to manufacturer’s instructions. After library preparation 1.14 ng of template gDNA was loaded on a Chromium Genome Chip and sequenced to roughly 80x coverage on an Illumina NovaSeq6000 150bp PE lane (Illumina, San Diego, CA).

*Results*

Previous RAD-based estimates of genome size predicted the genome to be approximately 0.6Gb. Since we acquired sufficient HMW DNA from the female individual first, we used the previous RAD estimate of genome size to generate a projected 80x coverage of data, or roughly 45 gigabytes of raw sequencing data. However, when we received the linked-read sequencing data we used a k-mer based estimation technique through Genomescope <CITE> to estimate a haploid genome size of 0.49Gb. As such, we sequenced the female assembly to roughly 120x coverage. We amended the amount of data generated for the male linked-read sequencing run to produce roughly 30 gigabytes of sequencing data for roughly 80x coverage (Table 3).

***Linked-read post-sequencing quality control (Figure 1C)***

*Methods*

After sequencing, bioinformatics quality control (QC) steps are necessary at multiple stages in the workflow of assembling of a reference genome. Immediate sources of error in sequencing data can result from sequenced DNA being contaminated with off-target DNA in the wet lab during preparation for sequencing, or biases in base calls from the sequencing machine. To QC for these two types of error, we conducted three computational quality control steps (kat hist, kat gcp and kat comp) using the software program KAT8. Each step splits sequencing data into sub-sequences of a given length, or k-mers, (where k is equal to a specified sequence length), and plots out frequencies, or comparisons, to visually inspect the data for quality issues. All bioinformatics work was conducted on the UC Davis farm compute cluster (the farm).

*Results*

Step 1 (contamination):

We looked for signs of contamination using various functions within the software KAT<CITE>. First, we used the kat hist function to check for signs of contamination by plotting a histogram of the observed number of distinct k-mers at different frequencies for lengths k=21, 31, and 41 for female (Figure 3A-C) and male sequencing data (Figure 3D-F). Uncontaminated samples are expected to have a single peak with a surplus of k-mers at a very low frequency due to sequencer errors. We saw clear peaks for each sample, and at each value of k indicating no signal of contamination.

Next we used the kat gcp function to plot the proportion of the k-mer comprised of the G and C nucleotides against the frequency of the that k-mer in the sequencing data and the number of distinct k-mers for a given GC count vs. frequency. Contaminated samples are expected to have a non-normal distribution. For example, samples contaminated with bacteria will have more k-mers with GC counts above 50%. We plotted GC counts against the frequency of k-mers of length k=21, 31, and 41 for the female (Figure 4A-C) and male (Figure 4D-F) sequencing data. All GC count frequency plots show a normal distribution of distinct k-mers, indicating no observable signs of bacterial or organelle contamination.

Lastly, we evaluated the data for sequencing bias between the forward (R1) and reverse (R2) files. We used the kat comp function to plot the frequency of a given k-mer in each of the paired-end sequence data files. Sequencing bias in either of the two files would result in an irregular pattern in the number of distinct k-mers. We plotted the number of distinct k-mers at different frequencies for the R1 and R2 files against one another for k-mers of length k=21, 31, and 41 for the female (Figure 5A-C) and male (Figure 5D-F) sequencing data. The number of distinct k-mers does not appear to be heavily skewed in the male or female sequencing data indicating the raw data shows no signs of major sources of sequencing bias (Figure 5A-F).

***Hi-C chromatin conformation capture sequencing & post-seq QC (Figure 1C)***

*Methods*

Genomic DNA in eukaryotes has high levels of repetition, leading to unresolved gaps surrounding large repetitive elements such as in the middle and at the ends of each chromosome. In order to resolve these gaps, we are using hi-c chromatin conformation capture (hi-c). We outsourced hi-c library prep and sequencing to Phase Genomics in Seattle, WA. Tissue from the same female that was sequenced with linked-read sequencing was sent to Phase Genomics for hi-c library prep and subsequent sequencing. Raw sequencing data and an initial scaffolding report were received for the female sample.

*Results*

Sequencing data reports from Phase Genomics indicate a successful library prep and sequencing (Supplemental Data 4). A total of 87,444,477 read pairs were analyzed of which 56.38% where high quality with a minimum mapping quality of greater than or equal to 20, a maximum edit distance of less than or equal to 5, and no duplications. The data contained an average of 2,966.33 read pairs per contig greater than 5kb. These data appear normal and indicate they will be useful in creating a more contiguous assembly.

***Genome assembly (Figure 1D-H)***

*Methods*

We initially generated two phased assemblies, one for each sex, by inputting the long-read sequencing data into the Improved Phased Assembly tool (IPA)<CITE>. The assembly product was polished of errors, purged of duplicate haplotigs, and phased into primary and alternative assembly files. The primary assembly file contains a contiguous haploid assembly, while the alternate assembly file contains the alternate haplotype of the diploid delta smelt. After creating the initial draft assembly, we incorporated linked-read information to first break the assembly at locations that were incorrectly joined, then to scaffold the assembly into larger, more contiguous chunks using the software scaff10x <CITE>. In order to use long distance information, we indexed the assembly produced in the scaff10x step and mapped hi-c reads to the draft assembly using bwa<CITE> and samtools<CITE>. We used the arima mapping pipeline perl scripts<CITE> to pair reads, and quality filter the 5’ end and for mapping quality. After we added read group information, marked duplicated reads, and sorted the mapped read files with picard<CITE>. These data were then converted into mapped bed files using bedtools<CITE>. We then used the mapped bed files, scaffolded assembly and the initial alternative assembly as input to further scaffold the assembly using the SALSA2 pipeline<CITE> with non-default parameters: -i 5 -x GATC -m yes. SALSA2 uses the frequency of how often reads pair together to determine how close locations in the genome are to one another. It links sequences in a draft assembly to one another in order to close gaps and produce a more contiguous assembly. Finally, we anchored our assembly into chromosomes by using a genetic linkage map produced in Lew et al (2015)<CITE> with the output from the hi-c assembly step and the software chromonomer<CITE>.

We used a combination of software and metrics to evaluate each draft assembly after every step of the assembly process (Table 4). The assembly length should be as close to the estimated genome size as possible. The N50 metric is the length of the scaffold where half of the assembly is held in scaffolds of that size or larger. The L50 metric tells the number of scaffolds that contain half of the assembly. BUSCO scores were used to evaluate the completeness of each assembly as expected from a core set of highly conserved single copy genes in the Actinopterygii lineage.

*Results*

In each step the assembly length and N50 sizably increased and the L50 dropped precipitously. The final metrics for the male genome assembly were an N50 of 12,200,365 bp, an L50 =15, a total assembly length of 472,157,411 bp, with a total of 549 scaffolds. The final metrics for the female genome assembly were an N50 of 14,850,352 bp, an L50 =13, a total assembly length of 437,273,953 bp, with a total of 376 scaffolds. The final assemblies had BUSCO scores of 88.4% and 89.3% complete genes in the final male assembly female assembly, respectively (Table 4).

***Cytogenic (Karyotype) Chromosome Validation***

*Methods*

We collaborated with Dr Mary Delaney’s lab to determine the diploid number of chromosomes in delta smelt, which has not been previously reported. This cytogenetic work does not use next generation sequencing-based methods to produce results and allows for an independent validation of the number of chromosomes expected in our final delta smelt genome assemblies.

Fish were anesthetized with Colchicine (10 microliters of 1 mg/ml stock) and returned to water. Individual fish lengths were measured. In both cases organs were rinsed, stored in PBS at ambient temperature of the CABA environment. We established two spleen pools, one of male (n = 15), and one of female (n = 13) specimens and one additional male gonad was harvested. Organ pools were gently aspirated into single cell suspensions by pipetting in hypotonic solution (0.56% KCl) for 15-20 min. Cells were centrifuged at ~1000 rpm for 10 min, supernatant hypotonic solution was removed and a 3:1 fixative (methanol:glacial acetic acid) was added. Cell pellets were resuspended and stored at 4ºC. Two to three more fixative washes (centrifugation, resuspension in new fixative) were conducted, and cells were applied to slides one week later. Slides were stained using the DNA staining fluorescent dye (DAPI) and cells were examined using an Olympus BX-40 Microscope. Images of cells were captured and stored using the Cytovision Software and the number of chromosomes in the species were determined from those images.

*Results*

Seventy-five cell images were collected from the three pooled sample sets (mixed sex, males-only, females-only). However, only 18 cells were of the quality suitable for counting/analysis with intact cells, clearly defined chromosomes, and limited to no overlap of chromosomes. From these 18 cells, we determined the 2n (diploid) chromosome count for the delta smelt to be 56, with 15 cells exhibiting 2n=56 and 3 cells with hypomodal counts (1 cell with 2n=54, 2 cells with 2n=55, Table 5). Figure 6 shows a representative mitotic metaphase cell from a male spleen cell exhibiting 56 chromosomes.