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

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# Prologue and Project Objectives

Delta smelt (*Hypomesus transpacificus*) abundances have been in decline for decades1,2. Genetic tools have been useful for monitoring overall diversity and effective population size () in the wild population, genetic management in the captive population, developing assays to detect hybrids between delta smelt, wakasagi, and longfin smelt, and develop eDNA assays3,4. Attempts at quantifying in the wild population have been hampered by several interacting factors. 1) a lack of power using microsatellite markers, and 2) a very large historical in the delta smelt population5,6. In order to improve estimates of , we assembled the delta smelt genome. We then leveraged the genome to estimate , examine domestication selection, and search for a sex marker. While is useful for monitoring purposes, we strongly advice against the use of in isolation for making management decisions.

For this project our tasks were to 1) assemble a high-quality reference genome for delta smelt that is publicly available for all researchers; 2) estimate contemporary effective population sizes (NE) for the wild 2017 to 2019 birth year cohorts of delta smelt, 3) examine domestication selection across the genome, and 4) search for sex-specific markers and/or chromosomes in delta smelt. The status of each deliverable is listed in Table 1. We have divided this final report into 4 sections, each with its own background, methods, results and discussion.

# Task 1: Genome assembly

* Background
* Sample collection
* DNA isolation
* Long-read library prep & sequencing
* Linked-read library prep & sequencing
* Hi-C chromatic confirmation capture prep & sequencing
* Genome assembly
* Cytogenetic (karyotype) analysis
* Discussion

## Background

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 including analyses of population structure, genetic diversity and local adaptation7. DNA sequences which are captured by the sequencer but not aligned 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 gain the knowledge of where each segment of sequenced DNA lies within the genome and relative to other sequenced DNA. Additionally, an assembled genome vastly increases the power to answer questions concerning demography, adaptation, fitness, and disease susceptibility.8,9 For example, previous work in Chinook salmon, using the closely related rainbow trout reference genome, found a small number of markers that were associated with runtime. In 2014, these markers were located on five different scaffolds and next to gaps in the highly fractured rainbow trout assembly. Reanalysis using long-reads to span gaps, and reorient and link fragmented scaffolds found diagnostic markers located within the gap in the 2014 reference genome10. Since the 2017 study, Chinook salmon now have their own reference genome that has enabled more detailed mapping of life-history variation and phenotypic traits in the same location11. These examples underscore how reference genomes can propel conservation research forward.

Recently, genome sequencing technologies have become both more cost effective and efficient. “Hybrid assemblies” (assemblies that use multiple NGS technologies) are a reliable way to achieve a chromosome-scale high-quality genome assembly12,13. 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 genomes13. Therefore, a main goal of this project is to develop a highly accurate chromosome-scale reference genome, hereafter called “reference genome”, using linked-read (Box 1), long-read (Box 2), hi-c chromatin confirmation capture (Box 3), and a genetic linkage map14 (Figure 1).

**BOX 1: Linked-read Sequencing**, such as 10X Genomics used in this study, takes large segments of extracted DNA (around 50kbp) found in an organism’s genome and puts them into individual oil beads. Within each oil bead, the segment of DNA is sheared to a length that an Illumina sequencer can read (~300-500bp) and barcodes corresponding to the oil bead the fragments are found in are attached on either side. All of the oil beads are then pooled together and sequenced to get highly accurate base calls with pseudo-long reads.



Image taken from: https://genome.med.harvard.edu/services/singleCell/ViewOverview.action

**BOX 2:** Long-read Sequencing, such as PacBio HiFi used in this study, takes long segments of DNA (over 50 kbp), shears them into a few segments (10-20kbp) and circularizes each. The circular molecules are then run through a sequencer to get an accurate circular consensus sequence over 20kbp in length.

Image taken from: https://www.pacb.com/smrt-science/smrt-sequencing/hifi-reads-for-highly-accurate-long-read-sequencing/

**BOX 3:** Hi-C Chromatin Confirmation Capture takes DNA in the nucleus and links all of the locations that are touching together. It then shears the linked DNA into lengths appropriate for short-read sequencing (300-500bp). The frequency two segments of DNA are found linked together is inversely proportional to their distance in the genome. That is, if two segments of DNA are close to one another, they will be sequenced together many times.



Image taken from: https://data.4dnucleome.org/experiment-types/dilution-hi-c/

## Sample collection (Figure 1A)

*Methods*

The first step in our genome assembly was to collect tissue for the extraction of high molecular weight (HMW) genomic DNA from both male and female fish. HMW DNA is extracted using a special protocol that produces longer DNA fragment lengths (>50 kilobases) compared with standard DNA extractions (<10 kilobases). HMW DNA is required for each of the three sequencing technologies (linked-read, long-read and hi-c) that we used to create our genome assembly. In order to examine potential sex-determining regions we sequenced both male and female samples to create two sex-specific genome assemblies.

Though we had intended to take a single trip to the FCCL for this project, we found recovering sufficient HMW DNA difficult, and ended up conducted four separate trips to sample different tissue types from 600 days post hatch (dph) male and female delta smelt (Table 2) with the final goal of producing enough HMW DNA to sequence each sex with each of the three technologies. Additionally, at the start of this project long-read sequencing was costly, and we sought to only incorporate the sequencing technology if absolutely necessary. Therefore, 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 only. However, at the end of 2019 the price of long-read sequencing dropped dramatically. Based on our mixed results from Trips 1 and 2, we made a third trip to acquire enough tissue to sequence a single male fish with all three of our chosen technologies, and enough tissue from a female fish to sequence with long-reads (as already we had enough HMW DNA for a female for hi C and linked reads). An error committed by the sequencing center required us to make one additional trip, Trip 4, to sample one additional male specimen. 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 DNA Technologies & Expression Analysis Core Laboratory (UC Davis Sequencing Center).

*Results*

We took a total of four trips to sample tissue. On the Trip 1 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 male fish produced enough HMW DNA. Therefore, we returned to the FCCL and selected larger males on Trip 2. The extracted DNA lengths from these male fish were also insufficient. A decision to take Trip 3 was made due to the discovery of a tissue sampling method that uses additional tissue types (not just back muscle tissue) and new tissue preservation storage solution of samples in cooled propylene glycol15 rather than solely flash freezing in liquid nitrogen. On Trip 3, 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 Trip 3 and preserved half of all sampled tissues in propylene glycol at 4°C, and half flash frozen and transported both on dry ice. On Trip 4, we sampled back muscle, scales and internal organs from one male fish. All sampled tissue was flash frozen and stored on dry ice for transportation.

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

*Methods*

For 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)16. 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 HMW DNA extractions and subsequent hi-c sequencing, a female tissue sample was sent to Phase Genomics and a male tissue sample was sent to the Vertebrate Genome Project for isolation of HMW DNA. Samples were sent to different locations due to COVID-19 delays at Phase Genomics during the time of sampling the male fish.

*Results*

Trip 1: DNA from one male (T1M03) and one female (T1F02) DNA was successfully 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 from both males and females (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 where it was successfully extracted and sequenced.

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

*Methods*

The inclusion of long-read data dramatically assists in creating a more contiguous genome assembly because it spans repetitive elements and resolves chimeric sequences throughout the genome. Long-reads provide greater continuity of scaffolded **contigs** (a stretch of DNA sequence created from a consensus of reads) and resolving repetitive sequences. This is because the reads (sequence fragments produced by the sequencer) are thousands rather than hundreds of base pairs long. Previously, long-read sequencing was both costly and had a high error rate of up to 10%. During 2020, a new PacBio HiFi chemistry was developed which dramatically reduces the number of errors to under 1%.

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 fragment length of ~15kb. The input for the library prep was 5.6ug of DNA and the library was size selected down to be 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 (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). This amount of long-read sequencing data was sufficient to continue on to subsequent steps.

## 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/>) to generate 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 on an Illumina NovaSeq6000 150bp PE lane (Illumina, San Diego, CA). We used a previous RAD-based estimate of a haploid genome size (0.6Gb) to sequence the first sample to an estimated 80x coverage.

Since we successfully extracted HMW DNA from a female individual first, we used the female linked-read data to improve our estimate of delta smelt genome size with a more accurate k-mer (where k is equal to a specified sequence length) based approach with the software Genomescope217. We then used the updated genome size estimate to adjust the amount of linked-read sequencing data collected for the male sample.

*Results*

The k-mer based haploid genome size from the female was estimated to be 0.49Gb. We generated approximately 45 gigabytes of female linked-read sequencing data and 30 gigabytes of male linked-read sequencing data for a total of roughly 120x and 80x coverage, respectively (Table 3). This coverage was sufficient to continue on to subsequent assembly steps.

## Hi-C chromatin conformation capture prep & sequencing (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 chromosome18. In order to resolve these gaps, we used hi-c chromatin conformation capture (hi-c). We outsourced the female hi-c library prep and sequencing to Phase Genomics in Seattle, WA in 2019. Phase used their in-house proprietary library preparation and sequencing protocols. Raw sequencing data and an initial scaffolding report were received for the female sample. Unfortunately, when we had male tissue available for hi-c sequencing the global pandemic in response to COVID-19 was underway. Due to the lack of availability of sequencing centers accepting new material, we had to outsource the male hi-c sequencing to the Vertebrate Genome Project at Rockefeller. The data from these sequencing runs have yet to be returned to us at the GVL and we have not been able to acquire a return date. However, because hi-c links long-range interactions we used the female sequencing data for both male and female scaffolding.

*Results*

We received sequence files of 87,444,477 read pairs in total which is sufficient for resolving gaps and further scaffolding the linked & long-read combined assembly (Supplemental Data 4).

**Quality Control**: 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. Other sources of error may come from having too great of a proportion of poor-quality reads.

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

*Methods*

Sequencing data 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 convert binary data to fastq format for downstream analysis. A base quality value assigns a score to each letter (nucleotide) 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 reads and 2,741,504 female reads representing 35,841,976,770 and 28,549,585,055 base pairs, respectively, passed quality control and was sufficient to be used for subsequent assembly.

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

*Methods*

To quality control for contamination and sequencing bias errors, we conducted three computational quality control steps (kat hist, kat gcp and kat comp) using the software program KAT19. Each step splits sequencing data into sub-sequences of a given length, or k-mers, 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).

We looked for signs of contamination using the kat hist and kat gcp functions within the software KAT. 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 and male sequencing data. Uncontaminated samples are expected to have a single peak with a surplus of k-mers at a very low frequency due to sequencer errors. Second, 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 and male sequencing data.

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 and male sequencing data.

*Results*

We saw clear peaks in the histograms for each sample and at each value of k (Figure 3). All GC count frequency plots show a normal distribution of distinct k-mers (Figure 4). Additionally, the number of distinct k-mers does not appear to be heavily skewed in the male or female sequencing (Figure 5). These data together indicate no observable signs of bacterial or organelle contamination or major sources of sequencing bias in our sequencing data.

## Hi-C chromatin conformation capture post-sequencing quality control (Figure 1C)

*Methods*

In order to assess if our sequencing data would be useful in linking scaffolds. We looked at a percentage of high-quality reads (minimum mapping quality of greater than or equal to 20, a maximum edit distance of less than or equal to 5, and no duplications) that mapped to our draft assembly created from the next step. We also observed the number of reads which aligned to each contig (>600 desired) and the number of reads that are sufficiently far apart (1-15% expected).

*Results*

Sequencing data reports from Phase Genomics indicate a successful library prep and sequencing (Supplemental Data 4). A total of 56.38% of reads were considered high quality. The data contained an average of 2,966.33 read pairs per contig greater than 5kb and 18.78% of the read pairs mapped to greater than 10 kilobases apart. These data appear normal and indicate they will be useful in creating a more contiguous assembly.

## Genome assembly (Figure 1D-H)

*Methods*

We have broken this section into various steps for clarity and flow when describing this iterative process.

Step 1: Use long-read sequencing data to create Draft Assembly A

To begin, we generated two phased assemblies, one for each sex, by inputting the long-read sequencing data into the Improved Phased Assembly tool (IPA, <https://github.com/PacificBiosciences/pbipa>). 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.

Step 2: Incorporate linked-reads into Draft Assembly A to produce Draft Assembly B

After creating the initial draft assembly, we incorporated the linked-read data 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 (<https://github.com/wtsi-hpag/Scaff10X>).

Step 3: Incorporate hi-c data into Draft Assembly B to produce Draft Assembly C

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 bwa20 and samtools21. We used the Arima Mapping pipeline perl scripts (<https://github.com/ArimaGenomics/mapping_pipeline>) to pair reads, and quality filter the 5’ end and for mapping quality. Next, we added read group information, marked duplicated reads, and sorted the mapped read files with picard(<http://broadinstitute.github.io/picard/>). These data were then converted into mapped bed files using bedtools22. We then used the mapped bed files, scaffolded assembly and the initial alternative assembly as input to close gaps and further scaffold the assembly using the SALSA2 pipeline23 with non-default parameters: -i 5 -x GATC -m yes. SALSA2 uses the frequency of how often reads pair (or are sequenced) together to determine how close any two locations in the genome are to one another, it finds the locations of the paired reads in the draft genome and links the two locations to close gaps and produce a more contiguous assembly.

Step 4: Use linkage map with Draft Assembly C to produce Final Assembly

Finally, we anchored our assembly into chromosomes by using a genetic linkage map produced in Lew et al (2015)14 with the output from the hi-c assembly step and the software chromonomer24.

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 (Box 4). BUSCO25 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).

**BOX 4**: Assembly statistics

The N50 of an assembly is the length of contigs/scaffolds that contain 50% or more of the assembly.

The L50 of an assembly is the number of contigs/scaffolds that contain 50% or more of the assembly.



**N50 = 60**

**L50 = 3**

Figure taken from: https://www.molecularecologist.com/2017/03/29/whats-n50/

## Cytogenic (Karyotype) Chromosome Validation

*Methods*

We collaborated with Dr Mary Delaney 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 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 after sampling. 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). Quality control steps discarded cells that were not intact, did not contain clearly defined chromosomes, or had significantly overlapping chromosomes. After quality control filtration, 18 cells were retained for counting/analysis. 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.

## Genome Assembly Discussion

The diploid chromosome number of 56 for delta smelt aligns with that reported for other smelt species, 2n=54, 56 or 58 for European smelt26,27 and 2n=56 for the Japanese pond smelt28. As others have noted, Robertsonian fusions/fissions of chromosomes (acrocentrics fusing to form metacentrics or vice versa) may be the source of the karyotype variation, which is also the basis for karyotype variation observed within and among salmonid species27,29. No sex chromosomes have been reported to date for those smelts studied cytogenetically and here we found no evidence for sex-specific chromosomes although a more detailed study is necessary to secure such a determination given the small sample size and low resolution of images. Similar to other reports, we note a preponderance of subtelocentric/acrocentric chromosome pairs over metacentric chromosome pairs.

The primary objective of this study was to create a highly contiguous genome assembly for use within and beyond the scope of this project. We have achieved the gold-standard for constructing reference genome and have assembled a chromosome-level reference genome for delta smelt and have validated our number of chromosomes with an independent cytogenetic study. To assemble the delta smelt genome, we combined PacBio long reads, 10X Chromium linked-reads, Phase hi-c chromatin conformation capture and a linkage map to create two sex-specific reference assemblies for male and female fish.

The final total lengths for the male and female assemblies were 0.47Gb and 0.44Gb, respectively which is similar to the pond smelt genome (*Hypomesus nipponensis*) which has a total length of 0.50Gb30. Our final male and female assemblies had 376 and 549 scaffolds with N50’s of 0.12Gb and 0.15Gb, respectively. The first 28 contigs, representing number of haploid chromosomes confirmed by cytogenetic karyotyping contain 73.3% and 81.6% of the sequencing data of total assemblies in male and females, respectively. For comparison the 3.2Gb human genome––considered one of the leaders in genome assemblies and has been actively worked on for over a decade––has an N50 of 0.67Gb, has been assembled into 23 chromosomes and still contains 92 unplaced scaffolds for a total of 115 scaffolds. Thus, our reference assembly in a species that has a smaller genome with more chromosomes is quite a successful first pass. Our final genome assemblies are roughly 25-30 times more contiguous that the newly published pond smelt assembly and our final assemblies contained 88.4% (in male reference) and 89.3% (in female reference) of core genes expected in the *Actinopterygii* BUSCO database.

In summation, our final reference genomes are highly contiguous and contain a high number of ultra-conserved conserved genes. This indicates that this project was successful in assembling a useful resource for current and future studies involving delta smelt and closely related species.

# Task 2: NE Estimation

* Background
* Sample acquisition and estimation
* Read processing and alignment
* Principal component analysis for hybrid detection
* Contemporary temporal calculation
* Historical genetic diversity calculation
* Discussion

## Background

The of a given population is defined as the size of a Wright-Fisher population that would have the same rate of change of a genetic parameter as the given population study31. A Wright-Fisher population is one that is closed (no migration) and randomly mating. Since wild populations inevitably violate these assumptions, is typically much lower than the true census size.

A number of methods exist to estimate and examine , which can be broadly separated into two categories: contemporary and historical. Contemporary describes the effective population size of recent generations as little as one generation back. We used a temporal method of estimating contemporary through genetic drift that requires two time points to yield the harmonic mean of the years between the two time points. Contemporary temporal methods are widely used and a reliable way of measuring in multi-generational datasets.32–37. Temporal methods are commonly used in conservation genetic studies and management as they can detect population declines up to as rapidly as one generation post-population decline38 and reliably detect population declines ten generations post decline39. These estimates can give us a broad understanding of the genetic diversity of delta smelt over a longer timeframe––from 1995 to 2019––and using more markers than previous studies in delta smelt which only used microsatellites5,6.

Historical detects the effective population size of the population that led to the amount of genetic diversity currently observed in the modern-day population and can be derived from the equation where (theta) is a measure of genetic diversity and is the organism’s mutation rate. Since the mutation rate in delta smelt is unknown, but it is a constant conversion to , we simply observed genetic diversity over time.

In combination with information on various population parameters, is a useful tool for monitoring endangered populations as it can inform the likelihood of a given allele to be lost or fixed within a population., which tells us how quickly a population is losing genetic diversity40,41. Several recommendations have evolved over the years regarding how large needs to be in a population for it to be “healthy” (citations and mini review), but there is no way to predict how affects a population, and there are many examples of populations that have persisted and recovered after experiencing severe bottlenecks (e.g. northern elephant seal). Therefore, in a wild population, point estimates are not useful as an absolute value taken alone and without further modeling. Here we use the genome assemble to observe trends in the delta smelt population through time.

***Sample acquisition & sequencing***

*Methods*

We collected and sequenced archived samples obtained in state and federal trawls from 1993-2024. Contemporary samples were collected and transferred into the custody of the Genomic Variation Laboratory as fin clips by Interagency Ecological Program surveys from 2015-2020. Genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. In order to produce a large number of loci in a cost-effective manner, restriction site associated DNA (RAD) sequencing was carried out for all individuals. RAD libraries were prepared using the *Sbf1* restriction enzyme according to the ‘new RAD protocol’ described in Ali et al.42, and sequenced 100 bp paired-end reads on an Illumina HiSeq 4000.

*Results*

A total of 2,976 samples from 24 years spanning 27 generations were sequenced creating a rich dataset for further analysis.

## Read processing and alignment

*Methods*

In order to make demographic inferences, alleles spread across the genome of different individuals and contained within and across generations need to be compared. To do this, we split sequencing data into files corresponding to single individuals, using custom perl scripts to separate raw sequencing data into plates, then individuals. We then aligned the separated (or split) individual sequencing files to the assembled genome using bwa, which resulted in sequence alignment map (SAM) files. We then further processed the SAM files by sorting according to read name (samtools sort), filling in mate coordinates (samtools fixmate -m), removing duplicate reads (samtools markdup -r), and indexing the resulting files to create binary alignment map (BAM) files (samtools index) for downstream analyses.

## Principal component analysis for hybrid detection

*Methods*

When conducting population genetic analyses on a species, it is important to make sure that erroneous individuals are excluded. Delta smelt have been observed to hybridize with wakasagi smelt (*Hypomesus nipponensis*) in the San Francisco estuary4. Due to the possibility of visual misidentification or technical error, we ran a principal component analysis to identify and exclude hybrid individuals or individuals with outlying genotypes.

To do this, we used the program ANGSD43 to randomly sample a single read at all sites contained in at least half of the samples for each individual (angsd -doMajorMinor 1 -minMapQ 20 -minQ 20 -SNP\_pval 1e-12 -GL 1 -doMaf 1 -doCov 1 -doIBS 1 -doCounts 1). This creates a 0 to 1 matrix for each individual’s sampled allele at all locations in the form of a covMat file. We then calculated obtained eigenvalues using the program R44, calculated the observed variance for PC1 and PC2, and visualized the first and second principal components (PC) and removed outlier individuals.

*Results*

The first principal component (PC1) showed 30.7% variance across the x-axis while the principal component 2 (PC2) had 24.5% variance across the y-axis. These large proportions of variance indicate the presence of hybrids or technical artifacts from sequencings. We identified and removed a total of 19 individuals with a PC1 > 0.01 and/or PC2 < -0.01 from downstream analyses. (Figure 7) as these individuals are likely hybrids or the result of a technical artifact of sequencing.

## Contemporary temporal Ne calculation

*Methods*

In order to standardize the number of gene copies contributing to the estimation of Ne, we subsampled to 50 individuals from each birth year. Years with fewer than 35 individuals were removed from subsequent analyses. We then called genotypes in the selected individuals using allele frequency as priors in ANGSD. SNPs meeting the following criteria were accepted: posterior probability greater than 0.85 (-postCutoff 0.85), a SNP p-value greater than 1e-6 (-SNP\_pval 1e-6), found in greater than 50% of individuals (-minInd 1472), minimum mapping quality of 20 of greater (-minMap 20), minimum base quality of 20 or greater (-minQ 20), and a minimum minor allele frequency of at least 0.05 (-minMaf 0.05), and genotypes were written as numbers (-doGeno 2) in a geno file. The geno file was read into R for further filtration using the snpR package45. Within snpR we filtered SNPs that violated Hardy-Weinberg Equilibrium (HWE=0.99) and did not have read coverage in at least 75% of individuals in each year (min\_ind=0.75).

We made one-generation and all-by-all temporal method estimates of using three different estimators: Nei & Tajima46, Pollak47, and Jorde & Ryman48. Temporal estimations give the harmonic mean between two different time points ( and ). For our first estimations we sought to set a specific time point by using years that were separated by one-generation. Thus, one-generation temporal method estimates of were made between time points and in order to find the effective population size of generation . To do this we exported the snpR genotype data as a genepop file and imported it into NeEstimator49 to make estimates in sequential years with sequencing data. Next, to get a broad understanding of how contemporary is changing through time, we performed all-by-all generations temporal method estimates. In these estimates and , where increases to represent every other year in the dataset until estimates have been made for all possible combination pairs of generations.

*Results*

Birth years 1993, 2000, 2007 were all removed from subsequent analyses due to having an insufficient number of individuals (Table 6). A total of 27,809 loci were read into R for snpR filtration. After Hardy-Weinberg and minimum yearly individual purging a total of 911 loci remained.

One-generation birth year estimates were made from 1995 to 1999 and from 2008 to 2019 due to the availability of samples. All temporal-method estimations from 1995 to 1997, 1999, and 2008 to 2010 show an above 5,000 or infinite. For birth year 1998, the Nei & Tajima, and Pollak estimators show below 1,000, however, the Jorde & Ryman method estimates to be above 5,000. More recent estimates show consistently lower with estimations from 2011 to 2017, and 2019 having an of 4,000 or less for all temporal-method estimators. Additionally, most estimates within these years are below 1,500. Birth year 2018 shows the Nei and Tajima estimation of to be above 5,000 but both the Jorde & Ryman, and Pollak estimators infer an below 2,500 (Figure 8).

All-by-all generation estimates were made, and data was plotted as a point located at the mean generation between and (Figure 9). Similar to the one-generation experiment, estimates made further back in time are more consistently infinite or above 10,000. The more recent estimations, notably from 2013 to 2020, are both decreasing and below 2,500.

## Long-term genetic diversity calculation

*Methods*

Since the value of is directly related to genetic diversity through the equation ,where (theta) is a measure of genetic diversity and is the organism’s mutation rate, we wanted to see if the size of translated to genetic diversity loss. To quantify genetic diversity through time we used two different estimates of ––the normalized number of segregating sites (Watterson, )50 and average pairwise nucleotide differences (Pi, )51.

To make estimates of genetic diversity ( estimates), we obtained a global estimate of the folded site frequency spectrum. First, we acquired site allele frequency likelihoods using the reference genome ANGSD (angsd -GL 2 -doSaf 1 -minMapQ 10 -minQ 20). Next, we estimated the maximum likelihood of the site frequency spectrum (SFS) in realSFS (realSFS -maxIter 100 -fold 1). Finally, we calculated for each site by using the realSFS (realSFS saf2theta) and converted those data into logscale per-site with thetaStat (thetaStat print).

*Results*

Genetic diversity measures significantly decline from 1995 to 2020 (p = 1.82e-4 and 2.95e-3 for and , respectively). Per-base estimates of range from 2.55e-4 to 7.12e-5 and estimates of range from 1.50e-4 to 6.47e-5 (Figure 10).

## Ne Discussion

Two previous studies estimated contemporary in the wild population of delta smelt using 12-15 markers. Fisch et al. 20116 found the to be decreasing during the 2003 to 2009 study period6. In contrast, Finger et al. 20175 found the could not be accurately estimated from 2011 to 2014 due to infinite confidence intervals52. Multiple factors may have contributed to this discrepancy: 1) different versions of NeEstimator49,53 were used between the two studies; 2) differences in the number of informative loci analyzed; or 3) the number of generations factored into the analysis.

In this study we removed non-neutral loci in order to make unbiased temporal estimates. Our results show a broad decline of delta smelt effective population size taking place between 1995 and 2020. In general, estimates made with birth year cohorts from the 1990’s and early 2000’s were more varied and more frequently infinite, negative (indicating is too large to accurately be estimated), or greater than 5,000. More recent estimates starting around birth year 2013 show less variation, narrower confidence intervals and lower estimates of (Figure 8 and 9).

Our estimates corroborated our temporal estimates. We quantified genetic diversity through two statistics––average pairwise nucleotide difference (pi) and the normalized number of segregating sites. We found both parameters to be declining with pi to be slightly more stable. This is expected as low frequency alleles are more rapidly lost as populations decline. Our diversity estimates are consistent with our estimates that show an overall decline over the past 25 years. There is a lot more here to talk about.

# Task 3 Domestication Selection

* Background
* <insert methods>
* <insert methods>
* Discussion

## Background

tktktktkt

## Methods 1

tktktktkt

## Methods 2

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## Domestication Discussion

tktktktkt

# Task 4: Sex Marker

* Background
* DNA sampling & sequencing
* Genome wide association study
* Depth Analysis
* K-mer analysis
* Discussion

## Background

Sex determination in fish is a highly variable trait54 and understanding its mechanisms is crucial for understanding both the biology of a species and for gaining insight into the evolution of sex chromosomes and genetic mechanisms underlying sex determination55. Fish represent the most diverse group of vertebrates on earth with over 30,000 described species56. With this diversity and constant exposure to variable environments comes a vast array of morphological, physiological, behavioral, developmental and sexual mechanisms57–60. In teleost fishes, sex determination can be genetic or environmental and varies even between closely related species54,61–63. 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 determination62. 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)64. 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 survival65.

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, providing more coverage of the genome than Sbf1. RAD sequencing libraries were prepared at the GVL according to Ali et al (2016)42 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 in the program ANGSD43 using males as controls (0) and females as cases (1). The goal for this GWAS was to find alleles associated with a particular 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 11). The two SNPs most associated with sex in delta smelt had LRT scores of 37.854854 and 35.802804 which correspond to p-values of 7.621e-10 and 2.183e-9, respectively. Despite being highly associated with sex the genotypes at these loci were not diagnostic of sex (Table 7).

## Depth analysis

*Methods*

If sex determination in delta smelt is caused by chromosomal differences, it would be expected that the heterogametic sex (e.g. XY) would have roughly half the sequencing depth (the number of sequences that cover a given locus) of the homogametic sex (e.g. 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 aforementioned 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 genomic 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. Finally, we totaled the difference of coverage between male and females and sorted the output to look 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 therefore did not identify any sex specific loci in this analysis.

## K-mer analysis

*Methods*

In our k-mer (where k is equal to a specified sequence length) 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 used the software sourmash66 to creat 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 . 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 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 12). 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 13). We found 44 contigs with k-mer mean abundance in the male sequencing data that had zero abundance in the female sequencing data (Figure 14).

We mapped the putative Y data back to the male reference genome and found the reads mapped to multiple regions within the genome (Table 8). However, we did not find a significant difference in male versus female read depth at locations across the putative Y regions.

## Sex Marker Discussion

Our study thoroughly probed the genome and two RAD-sequencing data sets in multiple ways to identify sex-specific markers, but we did not find an obvious sex-determining region. This means that it is unlikely that delta smelt have straightforward chromosomal sex-determination, though we cannot yet rule it out. While we did not find diagnostic SNPs, we did find paths forward for further analysis that may result in an understanding delta smelt sex determination. For example, we found candidate loci via GWAS using RADseq data and also via k-mer analysis using the linked-read sequencing data generated for the genome assembly. The GWAS found two markers on Chromosome 5 that were significantly associated with sex but did not have alleles that diagnostic of sex. Also, k-mer analysis detected sequencing data only found within the male individual’s linked-read sequencing data which could contain a sex determining region or SNPs diagnostic of sex. The post k-mer analysis depth analysis showed that the observed increase in male specific k-mers at 50% abundance of the normally distributed peak of the female k-mer abundance is consistent with the male sequence data potentially having heterogametic (male sex-specific) regions in its genome (such as the 50% ratio between Y chromosomes when paired with X chromosomes in human males). However, we could not identify or confirm sex-specific markers with the RADseq data generated for this project. Interestingly, many contigs containing male-specific k-mers were located on Chromosome 9. While there is a clear increase in associated SNPs on Chromosome 9, none met the significance threshold or were found to be diagnostic of sex. An additional important observation is that the k-mer analysis revealed male-specific linked-read sequencing data from an individual male aligned to multiple regions throughout the genome. This may indicate that sex determination in delta smelt is polygenic (determined by several genes or locations scattered throughout the genome rather than a single region as in the human Y chromosome) but further sequencing and analysis is needed to test this hypothesis.

Our work shows a need for further investigation using high-coverage whole-genome resequencing (WGS) data from a cohort of male and female fish to more evenly survey the genome in hopes of identifying sex-specific markers. While RAD sequencing data provides an adequate distribution of discrete locations throughout the genome of individuals, high-coverage WGS data more comprehensively surveys the entire genome of individuals, rather than just a fraction.

# Conclusion

As the refuge population of delta smelt become increasingly important for the preservation of the species it is essential that genetic resources and sequencing data are available to inform population management and improve reintroduction planning. In this study, we utilized third and next-generation sequencing technologies to generate a chromosomal-level genome assembly. We combined this assembly with multi-generational RADseq data to make demographic inferences of effective population size, observe genetic diversity through time, observe the genetic effects of domestication selection and interrogate the genome for sex specific markers

Mandi, I know Shawn mentioned wanting something written for how our findings should and shouldn’t be used for management… I’m not well versed in what to say re: management of the wild or captive populations and thought you might want to add in your thoughts/

# Glossary & Acronyms

**BUSCO score** – the percent of highly conserved universal single copy orthologs found within an assembly. Percentage derived from assigned lineage (e.g. Actinopterygii)

**CABA** – Center for Aquatic Biology and Aquaculture

**contig** – a continuous stretch of DNA sequence created from a consensus of reads

**coverage** – (or depth) the number of sequences that cover a given locus

**depth** – (or coverage) the number of sequences that cover a given locus

**DNA** – deoxyribonucleic acid

**FCCL** – Fish Conservation & Culture Laboratory

**genetic** **marker** – a location in the genome with a SNP that can be used for analysis

**HMW** – high molecular weight

**k-mer** – a sequence of length k

**movie** – the time specified for collecting sequencing data from a SMRT Cell

**PBS** – phosphate-buffered saline

**QC** – quality control

**RAD-sequencing** – restriction site associated DNA sequencing that produces genetic markers spread throughout the genome. Markers are located at each location that contains the cut site of the specific restriction enzyme used to shear DNA for sequencing.

**scaffold** – a string of DNA sequences with potential gaps created from chaining contigs together. Scaffolds are created by using sequence data, and relative position and orientation data. They may contain gaps, denoted by the letter N.

**SNP** – single nucleotide polymorphism

**UC Davis Sequencing Center** – UC Davis DNA Technologies & Expression Analysis Core

# Software Versions

ANGSD=0.934

BUSCO=4.0.6

samtools=1.10

bwa=0.7.17-r1188

bedtools=2.29.2

chromonomer=1.13

htslib=1.10.2

SALSA2=2.0

scaff10x=4.2

kat=2.4.2

ccs=3.3.0

picard=2.23.3

IPA=1.3.1

Genomescope2=2.0

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