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Population Structure and Connectivity of a Groundwater Crustacean Across Northwestern Montana Aquifers

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POPULATION STRUCTURE AND CONNECTIVITY OF A GROUNDWATER CRUSTACEAN ACROSS NORTHWESTERN MONTANA AQUIFERS

by

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A Thesis

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Abstract

Floodplain aquifer systems are diverse and heterogenous ecosystems that serve many ecological functions, including habitat provision to a range of groundwater species. The Flathead River system of northwestern Montana is home to many floodplain aquifers of great ecological importance to the region. The basic biology, population structure, and dispersal patterns of obligate groundwater organisms that reside within the aquifers of the Flathead are still largely unknown. In this study, we investigate the population structure of one such taxon, an undescribed species of the amphipod genus *Stygobromus*. For our low-coverage RADseq dataset, we tested the suitability of three different analysis pipelines: Stacks, GATK, and ANGSD. We found three distinct genetic groups that corresponded to different floodplains of the Flathead River. These results suggest that geographic separation and possible hydrologic barriers are leading to differentiation of these populations. We discuss the possible role of glacial refugia in promoting genetic diversity and the implications for aquifer research and groundwater conservation.

Introduction

River floodplains serve numerous ecological functions, including essential roles in the hydrologic cycle, nutrient transport (Stanford & Ward 1988; Tockner*, et al* 2010), physical alteration of surrounding land areas through flooding and continuous erosion (Tockner*, et al* 2010), and habitat provision for a wide range of species (Lundberg*, et al* 2000; Dudgeon*, et al* 2006; Meyer*, et al* 2007; Hauer*, et al* 2016). Gravel-bed river floodplains are additionally benefited by their associated aquifers which, through hydrologic and nutrient exchange, support a diverse community of microbes, crustaceans, and aquatic insects, many of which are not found in surface waters (Stanford & Ward 1988; David M. Pepin & F. Richard Hauer 2002). These vast and unique floodplain ecosystems are imperiled by threats that include groundwater depletion, pollution, and habitat alteration (Tockner & Stanford 2002; Danielopol*, et al* 2003; Dudgeon*, et al* 2006; Sabater & Tockner 2009; Hauer*, et al* 2016). To better understand and protect river and aquifer ecosystems, aquatic scientists need to focus research efforts on understanding their connectivity, ecological processes, and biodiversity.

Organisms living in aquifers face numerous challenges, including variable interstitial pore size, limited oxygen and other nutrient availability, low water temperatures, and lack of sunlight. These complex and heterogeneous systems would pose a challenge to any species not well adapted to their extremes. These organisms also face anthropogenic challenges. In the face of global warming,

groundwater depletion, and human activities like mining, aquifers are likely to experience drastic changes in a variety of characteristics, including temperature, sediment load, groundwater depth, and recharge rate (Allen 2011; Bouchaou*, et al* 2011; Kløve*, et al* 2014; Kurylyk, MacQuarrie & Voss 2014). While many characteristics of aquifers can vary, water temperature remains relatively constant within an aquifer compared to the main river channel (Case 1995), and aquifers are reliant on steady recharge rates from surface flows for water and nutrients (Stanford, Lorang & Hauer 2005; Helton*, et al* 2012). With little known about the environmental tolerances of groundwater organisms, there is an urgency to aquifer research.

Floodplains of alluvial, gravel-bed river systems have been characterized as shifting habitat mosaics, with the distribution of habitat patches changing over time (Stanford*, et al* 2005). The heavily braided Flathead River is one such gravel-bed river system, and is strongly influenced by spring snowmelt and flooding regimes. This system is also part of the Crown of the Continent Ecosystem, whose headwaters streams contribute to three great North American rivers (Columbia, Missouri, and Saskatchewan), draining to three different oceans. Research on the Flathead River system includes studies spanning nearly half a century of the Nyack, one of the best understood temperate floodplains in the world.

The floodplain groundwaters of the Flathead River are closely coupled to surface flows; as discharge in the main channel rises and falls, so too does the

connected subsurface flow (Stanford & Ward 1988). Likewise, the Flathead's aquatic invertebrate communities are tightly linked between surface and aquifer habitats. Many species of invertebrates live, grow, and reproduce within the variable interstitial spaces of the porous aquifer (Stanford & Ward 1993; Case 1995; Canton & Chadwick 2000). Both obligate and facultative groundwater organisms (stygobionts and amphibionts respectively) can be found up to five km from the main river channels, and up to ten meters below ground (Stanford & Gaufin, 1974; Stanford & Ward, 1988). Connections between the groundwater and surface flows allow amphibiotic species, such as stoneflies, to travel from the aquifer to the main channel where they emerge as adults (Stanford & Ward 1993). While biodiversity varies across different groundwater systems, members of subphylum Crustacea (*e.g.*, isopods and amphipods) are typically among the most abundant taxa (Stoch 1995; Deharveng*, et al* 2009; Gibert & Culver 2009).

One such crustacean group in the Flathead aquifers is *Stygobromus*, a genus of stygobiotic amphipods. (Holsinger 1978). There are roughly 130 described species of *Stygobromus* globally (Kosnicki 2019), with the majority from the eastern United States, and fewer than ten known from Eurasia (Holsinger 1987; Sidorov, Holsinger & Takhteev 2010) and Canada (Holsinger 1980; Bousfield & Holsinger 1981). This is likely due to a lack of research in the western U.S. and elsewhere, and is thus not an accurate reflection of species distribution. There are currently only

five described *Stygobromus* species in Montana, none of which have been reported in the Flathead River (Holsinger 1974; Wang & Holsinger 2001).

While worldwide research on *Stygobromus* has included investigations of cryptic speciation and phylogeography (Lefébure*, et al* 2006; Finston*, et al* 2007; Lefébure*, et al* 2007; Ethridge, Gibson & Nice 2013), morphological differentiation (Culver*, et al* 2010; Kosnicki, Julius & Gibson 2019), and behavior (Gilbert, Keany & Culver 2018; Kosnicki 2019), there are still significant gaps in our ecological understanding of them. North American *Stygobromus* habitats include streams and lakes within caves, water pockets in epikarst (the bedrock layer overlying the aquifer where water storage and transport occur), seeps, and hyporheic zones beneath and along rivers or streams where the groundwater and surface water mix (Holsinger 1974; Ward & Holsinger 1981). Distribution of *Stygobromus* species tends to be restricted, with many species endemic to a specific river or river system and, in extreme cases, to a single cave (Holsinger 1978; Holsinger 1974).

Recent genetic studies have offered important insights into the spatial scale of population connectivity of groundwater amphipods, which varies according to local geographic factors. Lucas *et al*. (2016) used genotyping-by-sequencing to obtain a single nucleotide polymorphism (SNP) dataset of various endangered taxa, including *Stygobromus*, in Comal Springs, Texas, USA, finding high overall migration rates for amphipods. This high migration rate and apparent lack of spatial and reproductive barriers could be explained by the study's limited geographic scope, a

1.5 km range along Comal Springs, a single drainage. However, the authors did note that stygobiont populations had lower migration rates than their surface-water counterparts, most likely due to their isolated habitats. Additionally, gene flow between populations was unconstrained by directionality of water flow within this spring system, diverging from expectations of passive downstream drift. A more expansive mtDNA study across multiple drainage basins in Western Australia, separated by several hundred kilometers, reported no current gene flow between basins (Finston*, et al* 2007). Tributary boundaries and other hydrological barriers led to isolated and highly divergent populations. It is not clear if populations present in larger river floodplains would have higher levels of structure or experience more gene flow between floodplains. However, a small study of *Stygobromus* populations on the Flathead has shown little population structure between floodplains approximately 70 km apart (Jordan*, et al* 2019).

In recent years, the growth, development, and plunging cost of next-generation sequencing technologies has led to a dramatic increase in the number of studies using next-gen data to answer a broad range of population genomic questions (e.g., Buermans & Den Dunnen 2014; Hendricks*, et al* 2018). To address the influx of data, there have been several programs created to process raw sequencing data and to identify loci and SNPs, such as Stacks, GATK, and ANGSD. Stacks is capable of reference-based or *de novo* locus building, and is usually used with medium to high-coverage data (Catchen, *et al* 2013). The Stacks pipeline begins

by demultiplexing and cleaning the raw reads, building loci from a subset of individuals, generating a catalog of these loci, and then matching the remaining reads to this catalog. These matches are used to generate contigs, call genotypes, and then a maximum-likelihood statistical model is used to distinguish SNPs from possible sequencing errors. Other pipelines, such as GATK and ANGSD, have been created to utilize genotype likelihoods to account for statistical uncertainty in low-coverage data. GATK first calls haplotypes for each sample, and then uses a joint-probability method to call genotypes and SNPs (McKenna*, et al* 2010). The ANGSD approach also uses the GATK model to calculate genotype likelihoods, but utilizes these likelihoods for downstream analyses as well, instead of calling genotypes (Korneliussen, Albrechtsen & Nielsen 2014). Working in this probabilistic framework allows the program to incorporate statistical uncertainty surrounding sequencing and mapping into the analyses.

These programs also differ in practical components such as program run time, ease of use, filtering capabilities, and compatibility with other analysis and filtering tools. While several studies have begun the important work of comparing these and other pipelines (Pan*, et al* 2015; Shafer*, et al* 2017; Wang*, et al* 2017; Rochette, Rivera-Colón & Catchen 2019), more work is needed to determine their suitability and practical application to varied data sets, especially those with low coverage. Based on the large genome size of other Amphipoda taxa (Rees, *et al* 2007;

Jeffery, Yampolsky & Gregory 2016), we suspected our study would need to utilize a pipeline suited for analysis of low-coverage data.

In this study, we use genomic sequencing to better understand how groundwater invertebrate populations are structured and connected along three floodplains of the Flathead River system. We expand upon the initial findings of Jordan *et al.* (2019), with larger sample sizes and additional study sites, to have a better view of *Stygobromus* populations and gain a deeper understanding of aquifer and groundwater connections. In particular, we investigate (i) the suitability of three different pipelines for analyzing low-coverage RAD-seq data; (ii) the overall structure of *Stygobromus* populations along the Flathead River; and (iii) potential gene flow between floodplains.

Methods

Study Area

The Flathead River of northwestern Montana is a major tributary of the Clark Fork River, and is fed by three, fifth-order tributaries, the North, Middle, and South Forks, all of which have segments designated as US National Wild and Scenic Rivers. Sites within the current study occur along the Middle and North Forks, as well as the main stem of the river below the confluence of the three forks (Figure 1a-b). The North Fork floodplain is contained within Flathead National Forest, the Middle Fork sites along the Nyack floodplain are on private agricultural land, and the main stem sites are outside the city limits of Kalispell, adjacent to or on private agricultural land. Sampling wells are on floodplains of varying sizes and differ in elevation and proximity to the river (Table 1). Water temperature taken from the wells at time of sampling was variable, but ANOVA and post hoc Tukey tests showed the only significant difference between wells occurred for one of the main stem wells, ST5R. The water temperature in this well was higher than all other wells besides the other main stem well, GRBD ($F(5,67)= 6.39$, $p=6.54e-5$; ST5R-Wurtz, $p=0.00027$; ST5R-HA15, $p = 0.0094$; ST5R-HA19, $p =0.00015$, ST5R-HA9, $p=0.017$; Table 1). All other comparisons were found to not significantly differ (Figure 2).

Field Collection

We collected *Stygobromus* samples from May to August of 2017 and 2018. Each study site consisted of a well fitted with slotted PVC pipe, 5.08 to 7.62 cm (2.0-3.0 inches) in diameter. New wells were drilled using a GeoProbe®, a truck-mounted hydraulic drill, along the North and Middle Forks, as well as the main stem (Figure 1). At each site, we inserted a hose attached to a gasoline-powered diaphragm pump into the well, filtering water through a 250-μm-mesh net for approximately ten minutes. While pumping, we raised and lowered the hose through the length of the well in order to sample groundwater at all available levels. The pumping rate varied depending on the depth and amount of water present in the aquifer, ranging from 15 lpm to >100 lpm. For samples that included a large amount of sediment, we first elutriated the sample several times using water collected from the well to separate organisms from sediment. We immediately picked macroinvertebrates and stored them in 95% ethanol.

Wet Lab Work

We sorted a total of 285 *Stygobromus* individuals into morphospecies and compared them using North American keys and species descriptions from Holsinger (1974; 1978). Using light and scanning electron microscopy, we determined that our samples do not morphologically align with any of the five described *Stygobromus* species in Montana, four of which have only been found in areas over 200 km south

of Kalispell. The fifth species, *Stygobromus glacialis*, is a high-elevation cave species (Wang and Holsinger 2001). Notable differences included: gnathopod shape, antenna I length, U3 ramus length, spine count on uropod 1, and presence of spines on posterior plates (Figures A1 and A2). Damaged individuals that lacked key appendages used for morphospecies separation (*e.g.*, telsons, antennae, gnathopods) were removed from genomic analysis to prevent inaccurate morphospecies assignment. We found one morphospecies to be the most common at our sites (n=235; Table 1) and refer to it as *Stygobromus* sp. 1 throughout this paper. In order to better study landscape-level genomic variation and focus on population-specific variation, we chose this morphospecies for genomic analysis. Additionally, based on a cursory visual inspection and known occurrence in sampled wells, we believe this species corresponds to that studied by Jordan *et al*. (2019).

We processed *Stygobromus* sp. 1 individuals prior to DNA extraction by rinsing them in distilled water and removing the abdomen to avoid genetic contamination from foreign material in the GI tract. Abdomens were individually stored in 95% ethanol. We then lysed sample tissues in individual wells of a 96-well plate using a homemade tissue homogenization buffer. We cleaned the lysed tissues with a mix of homemade Hyb buffer and SeraMag beads (GE Healthcare), and then washed this with a hybridization buffer solution to extract clean DNA from the samples. Extracted DNA was then quantified from representative samples across the

plate using a NanoDrop (Thermo Scientific) to approximate the quantity of DNA extracted.

For genomic library preparation, we followed the BestRAD protocol (Ali, *et al* 2016). We quantified DNA concentrations using standard Picogreen and Invitrogen Qubit 2.0 (Thermo Scientific) assays. We then standardized the samples to 10 ng/µl and brought them to a total volume of 15 µl using an ep*Motion* robot 5075 TMX (Eppendorf). We followed this with digestion using the restriction enzyme SbfI, an 8-bp cutter, as we expected a large genome size (Jeffery, Yampolsky & Gregory 2016). We ligated BestRAD adaptors to the sticky ends of fragmented DNA using the adaptor ligation master mix (New England Biolabs) and then added 2 µl of 50nM BestRAD adaptors, which include unique 8-bp barcodes, to every well.

Individuals from each 96-well extraction plate were then combined into three pooled samples. We concentrated these samples to 50 µl and sheared them via sonication using an E220 Focused-ultrasonicator (Covaris). We used streptavidin beads to recover DNA fragments with properly ligated BestRAD adaptors. Using the NEBNext Ultra™ DNA Library Prep Kit for Illumina, we ligated the Illumina-compatible adaptors to prepare fragments for sequencing and then size selected for 300-400 bp inserts. We performed a 10-cycle PCR on the library and sent it to Novogene (Chula Vista, CA) for sequencing on an Illumina HiSeq X sequencer. The final sequenced dataset for morphospecies 1, analyzed here, consisted of 235 individuals with paired-end reads that were each 150 bp in length.

Parameter Optimization

As a quality control check, we ran FastQC v0.11.7 (Andrews 2010) on data from each plate, both the forward and reverse reads. We then preliminarily processed sequences using the software Stacks 2.2 (Catchen, *et al* 2011). Stacks is a widely-used package for organizing and assembling restriction-enzyme-digest-based sequence data from raw reads (Rochette & Catchen 2017). Through the Stacks *process_radtags* step, we demultiplexed the raw reads and removed those of low quality with the default flag filters in Stacks of -c and -q (Phred quality score < 10 over 15% of read length). We then ran FastQC followed by MultiQC 1.7 (Ewels*, et al* 2016) to compare the quality of the processed, demultiplexed, and filtered samples to the original plates. The average number of forward and reverse reads was 2.20 million per sample. Based on this, we removed nine individuals which had fewer than 0.5 million reads sequenced, resulting in a total of 226 individuals with an average number of reads of 2.28 million.

There are several parameter values that can be adjusted within Stacks, the most important for stack creation being M and n, which respectively set the number of differences allowed between stacks and between loci (Rochette & Catchen 2017). To optimize parameter choices in Stacks, we followed the steps of Rochette and Catchen (2017) using a subset of the data which consisted of five high-coverage individuals from each of the six sampling sites in the initial dataset (n=30, Table A1). After running the Stacks pipeline with increasing values of M and n, we found the

values that would lead to the highest number of loci were M=7 and n=7 across our test samples (Figures A2 and A3). In the *ustacks* coverage report, our samples were found to have an average coverage of 25.78. However, following PCR duplication removal in *gstacks*, this dropped to 5.75, indicating high rates of PCR duplication and low-overall coverage in our data (Table A1).

Pipeline Comparison

In order to compare the efficacy of the most-commonly used RAD analysis pipelines on low-coverage data, we carried out preliminary population structure analyses on results generated from Stacks, GATK, and ANGSD (Figure 3). In Stacks, we followed the *de novo* pipeline methodology to generate SNP data from raw reads. ANGSD and GATK are reference-based approaches, and use a joint -genotyping method to generate genotype likelihoods. We used GATK to test how utilizing these likelihoods for genotype and SNP calling would impact population structure analyses. We used ANGSD to address problems associated with potentially low-coverage depth (Korneliussen*, et al* 2014). In this approach, we did not call SNPs or genotypes, but worked directly with genotype likelihoods which allows for the inclusion of statistical uncertainty in analyses (Nielsen*, et al* 2012; Fumagalli*, et al* 2013; Korneliussen*, et al* 2014). In each analysis, we used parameter values that were as similar as possible given the idiosyncrasies of each pipeline.

Pseudo-reference genome assembly

We first generated a pseudo-reference genome sequence as two of the methods require reference-aligned reads. For this, we used the Stacks subset data of *de novo* contigs which we filtered to only include sequences longer than 300 bp. We then created a super scaffold that would serve as the pseudo-reference genome from these contigs, using the python tool ScaffoldStitcher (available at https://github.com/ameliahaj/ScaffoldStitcher). To prepare the raw reads for analysis and remove possible PCR duplicates, we ran *clone_filter* within Stacks. We used Trimmomatic 0.38 (Bolger, Lohse & Usadel 2014) to remove any remaining adaptor sequences and trim reads with average quality scores across a sliding window of four bases of 15 or below. To reassess the quality of the reads following trimming, we used FastQC and MultiQC analyses.

We then created an index in BWA v.0.7.17-r1198-dirty and aligned the trimmed reads to the reference genome using BWA-MEM (Li 2013). To convert the generated SAM files into indexed binary alignment map (BAM) files we used SAMTools v1.3.1 (Li, *et al* 2009). We determined the number of aligned reads in each BAM file using SAMTools, to confirm consistent values across samples.

Stacks

We used the catalog generated from the subsampled, parameter-optimized data to rerun Stacks with all individuals. We continued using M=7 in *ustacks* and

n=7 in *cstacks*. Other parameter values varied through the pipeline, including, in *ustacks*: the lower and upper bounds of the error rate (--bound_low 0.001, --bound_high 0.05); in *gstacks*: PCR duplicate removal (--rm_pcr_duplicates); in *populations*: the minimum minor allele frequency (--min_maf 0.05), the max observed heterozygosity (--max_obs_het 0.6), the number of populations (-p 6), the proportion of individuals in each population (-r .6), and analyses were limited to one random SNP per locus (--write_random_snp). We left all other parameters in the pipeline at their default values.

GATK

We began the GATK analysis by first creating a sequence dictionary for the reference file using *CreateSequenceDictionary* in Picard 2.20.4, and then ran *HaplotypeCaller* in GATK 3.7. To produce genotype likelihoods, we combined the generated vcf files in groups of 10, using *CombineGVCFs* and then ran *GenotypeGVCFs*. We removed indels, and then hard filtered within GATK on quality by depth (QD < 2.0), strand bias (FS > 60.0), mapping quality (MQ <40.0 and MQRankSum < -12.5), and read position rank sum test (ReadPosRankSum <-8.0). We then used vcftools v.0.1.16 to filter based on minor allele count (--mac 3), maximum alleles (--max-alleles 2), and minor allele frequency (--maf 0.05).

ANGSD

To account for loci that deviated from Hardy Weinberg Equilibrium (HWE), we first ran the HWE test in ANGSD (-doHWE 1) separately for each population using the BAM files (Meisner & Albrechtsen 2019). We used a conservative p-value cutoff of 0.001, and removed all loci from subsequent analyses that were found to deviate in two or more populations using a custom R script. All ANGSD analyses were performed with the following filters: minimum mapping quality score of 10, minimum base quality score of 20, minimum minor allele frequency of 0.05, sites with a p-value less than 1e-6, and the GATK genotype likelihood model. We generated a Beagle file of the genotype likelihoods in ANGSD, with additional parameters that tasked the program with inferring the major and minor alleles from the genotype likelihoods (-doMajorMinor), calculating the allele frequencies (-doMaf 1), and requiring sites to be present in 50% of included individuals (-minInd 113).

Population Structure

Following the generation of SNPs or allele frequency data from each of the programs (Stacks, GATK, and ANGSD), we ran principal component analyses (PCAs) for each. For both Stacks and GATK, we used the variant call format (vcf) file generated from the program post filtering to generate PCAs. We first used *glPcaFast* in adegenet (Jombart & Ahmed 2011) to perform the PCA and then ggplot2 v3.2.1 (Wickham 2016) in R to plot them. To compare the ANGSD pipeline, we estimated

the covariance matrix from the ANGSD-generated genotype likelihoods in PCAngsd (Meisner & Albrechtsen 2018). We used a custom R script (I. Saglam and M. Fumagalli, pers. comm.) and ggplot2 to visualize the PCA. Regardless of parameter choice and number of loci/sites retained, the patterns within the data were consistent across all programs (details below). For most downstream population statistics, we therefore proceeded with ANGSD which has been shown to be advantageous for dealing with challenges associated with low-coverage data (Korneliussen*, et al* 2014).

To estimate pairwise F_{ST} values between sample sites, we used ANGSD to first calculate unfolded site allele frequency likelihoods (SAF) for each site. We then used realSFS to generate the folded site-frequency spectrum (SFS), because we lacked an ancestral state to compare, and to calculate a 2d site-frequency spectrum for each pair of sites. The unfolded SAF and folded SFS were then used to calculate pairwise F_{ST} values. Importantly, we did not use any filters in ANGSD (i.e. -minInd, -minMAF, or -SNP_pval) that would distort allele frequency distribution, and thus, the SFS. We created a visual matrix of the pairwise F_{ST} values in R using corrplot 0.84.

We also used the genotype likelihoods to infer individuals' ancestry based on admixture using NGSadmix v 32 (Skotte, Korneliussen & Albrechtsen 2013). We tested varying numbers of genetic clusters (K) from 1-10, each with 10 independent replicates. To determine the optimal value of K, we followed the Evanno (2005)

method using the CLUMPAK program (Kopelman*, et al* 2015). We visualized the best K values using R (R Core Team 2019). We tested for isolation by distance using Mantel's test (Mantel 1967). For genetic distance, we normalized the previously calculated F_{ST} values (Slatkin 1995). For geographic distance we used point-to-point measures between wells on the same floodplain, and river distances were calculated between wells on separate floodplains. We calculated river distances using network analysis in ArcGIS. Individual wells were snapped (moved) to the nearest surface water channel and the river distances were calculated from these locations (Table A2). We used VEGAN v. 2.5-6 (Oksanen*, et al* 2019) in R to run the Mantel test.

Between Floodplain Migration

To investigate relative migration, we used the function *divMigrate* in the R package diveRsity v1.9.90 (Sundqvist*, et al* 2016) with 1000 bootstraps. Because *divMigrate* requires genepop files, we used the Stacks-generated genepop file created with the same parameters used for PCA generation, but consolidated sites within each floodplain to one population (total n=3). To account for possible assumptions of certain statistics (e.g. no migration), we calculated several differentiation statistics including Jost's D and Nei's G_{ST} and compared them (Verity & Nichols 2014). While originally created for analysis of microsatellite data, this program has been used effectively for SNP based datasets (Pazmiño*, et al* 2017; Woodings*, et al* 2018; Dang*, et al* 2019).

Results

Pipeline Comparison

The preliminary run of Stacks to generate contigs for the pseudo-reference (used for GATK and ANGSD) resulted in 20,065 contigs. We removed contigs less than 300 bp in length, and retained 20,053 contigs. The average number of alignments for each sample to the pseudo-reference was 2,036,440.

The pipelines of Stacks, GATK, and ANGSD vary drastically in their modeling, assumptions and filtering capabilities. We accounted for this by performing basic tests of population structuring, PCAs, and used limited filtering for each analysis. The number of SNPs or sites (Stacks: 1,431 SNPs, GATK: 84,207 SNPs, and ANGSD: 287,358 sites), filtering level, and variance explained by the data (Figure 4) all differed drastically between the three analyses; however, we found the general patterns of results to be consistent across methods. In all three PCAs, three groups emerged that paralleled the three floodplains of the river, with individuals from the same floodplain grouping together. For the subsequent analyses we used the ANGSD pipeline, which previous research has shown to be more suitable for low-coverage data based on its use of genotype likelihoods in downstream analyses (Korneliussen*, et al* 2014; Rochette*, et al* 2019).

Population Structure

To investigate the overall structure of *Stygobromus* sp. 1 populations, we calculated pairwise F_{ST} values between them (Figure 5). These results were consistent with the output from PCAngsd (Figure 4). Sites on the same floodplain had a lower average pairwise F_{ST} value (global mean of 0.0076) than sites on separate floodplains (global mean of 0.045), indicative of more isolation between floodplains. Comparisons between the North Fork site and the other populations, had similar pairwise F_{ST} values, while comparisons between the Middle Fork (Nyack floodplain) sites had highest pairwise F_{ST} values when paired with the main stem sites.

Through the admixture analysis and Evanno (2005) method, we identified a best K value of two (Figure 6), which again highlighted geographic structuring of these groups (Figure 7). There was a clear distinction between the main stem and Middle Fork sites, with the North Fork individuals demonstrating a relatively even split between the other two clusters. A plot based on a K value of three further revealed population structure based on river fork. With three genetic clusters, the North Fork samples formed a new genetic cluster. Further review of higher K values (e.g. K=4) did not reveal any further structuring.

The Mantel test revealed a significant, positive correlation between genetic and geographic distance (r= 0.531, p=0.0111). With increasing distance between sites, there is a moderate increase in genetic divergence.

Between Floodplain Migration

To further investigate population differentiation, we calculated and plotted migration networks using G_{ST} and D using *divMigrate*. G_{ST} and D statistics led to slightly variable relative migration plots, but when the significance values of these pairwise comparisons were calculated (and non-significant values replaced with zero) these two statistics produced identical plots (Figures 8 and A4). The networks showed migration into both the North Fork and Flathead main stem populations. Consistent with the F_{ST} values, there was limited migration into the Middle Fork population despite its closer proximity to the main stem population.

Discussion

Pipeline Comparison

Initial population structure results were consistent across different pipelines, including Stacks, GATK, and ANGSD, as evidenced by similar patterns in their PCAs. This suggests that for analyses of population structure, the Stacks and GATK pipelines may perform as well as ANGSD on low-coverage data. Further research utilizing both real and simulated data could reveal that for some population-genetic questions and analyses, pipeline choice is less critical than previously thought. Researchers should confirm their findings using multiple pipelines and be cautious of pipeline choice biasing their results.

Population Structure

The overall genetic structure of *Stygobromous* sp. 1 populations was strongly influenced by geography. As expected, individuals sampled in wells from the same floodplain were genetically similar, with pairwise F_{ST} values approaching 0 (Figure 5), suggesting that they belong to a single population. We did not find strong evidence for multiple populations within any of the sampled floodplains, meaning that across contiguous suitable habitat, *Stygobromus* sp. 1 individuals showed no genetic structure. On the Middle Fork, this could be explained by the close proximity of sites to each other (< 2 km). However, given the relatively large distance between the Kalispell sites (8.76 km), the low pairwise F_{ST} value is more intriguing.

There are several possible explanations for this finding. First, there could be unimpeded movement of individuals within the Kalispell floodplain, indicative of limited barriers to dispersal (high connectivity) within the alluvial aquifer. Second, the increase in flow for both surface and subsurface water following spring snowmelt could facilitate *Stygobromus* dispersal over large distances. Third, large population size is preventing local differentiation of subpopulations due to drift, even across the span of several kilometers. Lastly, relatively recent, post-Glacial, population expansion has yet to yield many genetic differences between these subpopulations.

Based on the positive correlation between genetic and geographic distance found by the Mantel test, we expected the most northern site (Wurtz) would be the most genetically distinct. However, the Middle Fork population was the most genetically differentiated from the others, as evidenced by the PCA, higher pairwise F_{ST} values, and relative migration networks. It is possible that there are geological or hydrological barriers to dispersal, preventing movement to and from the Middle Fork sites. However, evidence of gene flow, based on the *divMigrate* analysis, from the Middle Fork to the North Fork population challenges the assumption of purely downstream migration.

Our results are somewhat at odds with recent findings of more genetically homogenous *Stygobromus* sp. 1 populations along the Flathead (Jordan, *et al* 2019). These researchers found low levels of genetic differentiation between floodplains

for both the obligate groundwater organism, *Stygobromus,* and the amphibiotic stonefly, *Paraperla frontalis*. Using the same restriction enzyme and sampling from some of the same wells, we found *Stygobromus* sp. 1 populations to be more geographically structured. With larger sample sizes and additional study sites, including the North Fork population, ours is a more holistic view of the structuring of these populations along the Flathead River system.

Our study also noticeably differs from the findings of Lucas *et al*. (2016), who did not find a relationship between geographic distance and differentiation in their analysis of *Stygobromus* subpopulations in a single drainage basin in Comal Springs. While our pairwise F_{ST} values were similar, there was an order-of-magnitude difference in the geographic sampling of our studies. Unlike our study sites, separated by over 50 km, the Comal Springs sites were separated by only a few kilometers. These inconsistencies suggest that population structure of *Stygobromus* is idiosyncratic, and dependent on geological history, or factors such as flow rate or connectivity of the aquifers being sampled.

Biogeography

At the last glacial maximum (LGM), approximately 15,000 years ago (Alt 2001), much of northwestern Montana, including the Flathead Basin, lay beneath continental ice sheets and the waters of Lake Missoula (Smith, *et al* 2018). Failure of the ice dam, combined with rapid melting of the Cordilleran ice sheet and its

northward retreat, led to massive flooding down the Clark Fork and, eventually, the Columbia River. While the geologic effects of this have been well studied, in terms of river and landscape morphology (Pardee 1910; Pardee 1942; Smith, Blood & LaFave 2000; Smith 2006; Smith*, et al* 2018), less understood is the effect it had on groundwater systems and the organisms that reside there. For species whose dispersal capabilities and phylogenetic history are unknown, such as current *Stygobromus* sp. 1 populations, there are many questions remaining about their recolonization of the Flathead river system. For example, did the current populations move into newly available terrain from a central refugium following glacial retreat, or did they move independently from multiple refugia?

This question has been answered for several sky-island taxa from this region. Research on alpine stoneflies found varying evolutionary responses to glaciation with current populations originating from refugia (Hotaling*, et al* 2018; Hotaling*, et al* 2019). Knowles (2001) found that the existence of multiple Pleistocene refugia promoted divergence of montane grasshopper populations. Evidence, including the fact that current geographic locations do not align with genetic similarity, suggests divergence occurred while those populations were in separate refugia during the glacial period.

Similar pressure could have led to divergence of the *Stygobromus* sp. 1 populations along the Flathead River, as here too we see a mismatch in spatial proximity and genetic similarity. *Stygobromus* distribution north of the southern

Pleistocene glacial boundary is limited (Holsinger 1980; Bousfield & Holsinger 1981; Holsinger & Shaw 1987; Smith 1985). However, the researchers who have discovered them have hypothesized that these species survived the glacial period by finding refugia in caves or deep groundwater in ice-free regions. Support for this hypothesis has been found for other groundwater organisms in Iceland (Kristjánsson & Svavarsson 2007; Kornobis*, et al* 2010) and the Alps (Lefébure*, et al* 2007). If current populations in the Flathead aquifers existed in multiple refugia during the Pleistocene, and then recolonized the Flathead Forks separately, it could explain the patterns of genetic differentiation we observed. It is also possible that the current North Fork and main stem populations originated from a single refugium, separate from that of the Middle Fork population.

Significance & Future Research

With the geographic structuring of these populations, they may continue towards reproductive isolation and speciation. As populations diverge and new species boundaries emerge, there will be a need to protect genetic diversity within the aquifer. With diverging populations along the Flathead, some may harbor individuals better adapted to changing environmental conditions. This is especially critical as aquifer systems face threats including groundwater depletion and global warming (Rood*, et al* 2008; Hay, Markstrom & Ward-Garrison 2011; Hauer*, et al* 2016). Our data hint at possible complications in line with this reasoning including an increase in water temperature (Table 1 and Figure 2) in the more southern sites

that could be reflective of future trends for sites further north. Additionally, while sampling, we did note fewer *Stygobromus* sp. 1 individuals in the Kalispell floodplain than the other two floodplains. Given that the environmental tolerances of *Stygobromus* and other groundwater species are still unknown, there is concern for the future habitability of these systems. These is a need for more research on *Stygobromus* sp. 1, and the aquifer in general, as the risk of biodiversity loss increases.

There are still many questions remaining for these groundwater populations. Future studies should aim to include more sites along the Flathead River system, including sites along the South Fork. Additionally, including the other morphospecies discovered along the Flathead could allow researchers to better understand the timeline and phylogenetic history of *Stygobromus* species of the Flathead, especially considering the short time frame of introduction since the Pleistocene glaciation. A future diffusion approximation for demographic inference (dadi) analysis could help reveal the history of these populations.

Beyond increasing the overall species diversity of the aquatic system, groundwater invertebrates also contribute to the functioning and overall health of the aquifer (Boulton*, et al* 2008). The importance of aquifers is highlighted by their connections with the surrounding ecosystems, and contributions to landscape diversity (Hauer*, et al* 2016). Aquifers have also been known to serve as refugia for species during disturbances such as flooding or droughts (Sedell*, et al* 1990). These

connections and roles emphasize the need to protect the Flathead River aquifers and their inhabitants, especially given that the Flathead occurs near ecologically valuable and protected areas, such as Glacier National Park.

References

Ali, O.A., O'Rourke S.M., Amish S.J., Meek M.H., Luikart G., Jeffres C. and Miller M.R. (2016) RAD capture (Rapture): flexible and efficient sequence-based genotyping. *Genetics*, **202**, pp.389-400.

Allen D. (2011) Climate change impacts on valley-bottom aquifers in mountain regions: case studies from British Columbia, Canada. In: *Climate change effects on groundwater resources: a global synthesis of findings and recommendations* (Eds H. Treidel, J.L. Martin-Bordes & J.J. Gurdak), pp. 249-264. CRC Press, Boca Raton, Florida.

Alt D.D. (2001) *Glacial Lake Missoula: and its humongous floods*. Mountain Press Publishing, Missoula, Montana.

Andrews S. (2010) FASTQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bolger A.M., Lohse M. & Usadel B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* **30,** 2114-2120.

Bouchaou L., Tagma T., Boutaleb S., Hssaisoune M. & El Morjani, Z.E.A. (2011) Climate change and its impacts on groundwater resources in Morocco: the case of the Souss-Massa basin. In: *Climate change effects on groundwater resources: a global synthesis of findings and recommendations* (Eds J.J. Gurdak, H. Treidel & J.L. Martin-Bordes). IAH - International Contributions to Hydrogeology. CRC Press, Boca Raton, Florida.

Boulton A.J., Fenwick G.D., Hancock P.J. & Harvey M.S. (2008) Biodiversity, functional roles and ecosystem services of groundwater invertebrates. *Invertebrate Systematics,* **22,** 103-116.

Bousfield E.L. & Holsinger J.R. (1981) A second new subterranean amphipod crustacean of the genus *Stygobromus* (Crangonyctidae) from Alberta, Canada. *Canadian Journal of Zoology,* **59,** 1827-1830.

Buermans H. & Den Dunnen J.T. (2014) Next generation sequencing technology: advances and applications. *Biochimica et Biophysica acta (BBA)-Molecular Basis of Disease,* **1842,** 1932-1941.

Canton S.P. & Chadwick J.W. (2000) Distribution of the subterranean amphipod *Stygobromus* in central Colorado streams, with notes on the interstitial community. *Western North American Naturalist,* **60,** 130-138.

Case G.L. (1995) *Distribution and abundance of zoobenthos in channel, springbrook, and hyporheic habitats of an alluvial floodplain.* Thesis or Dissertation, ProQuest Dissertations Publishing.

Catchen J.M., Amores A., Hohenlohe P., Cresko W. & Postlethwait J.H. (2011) Stacks: building and genotyping loci *de novo* from short-read sequences. *G3: Genes, Genomes, Genetics,* **1,** 171-182.

Catchen J., Hohenlohe P.A., Bassham S., Amores A. & Cresko W.A. (2013) Stacks: an analysis tool set for population genomics. *Molecular Ecology,* **22,** 3124-3140.

Culver D.C., Holsinger J.R., Christman M.C. & Pipan T. (2010) Morphological differences among eyeless amphipods in the genus *Stygobromus* dwelling in different subterranean habitats. *Journal of Crustacean Biology,* **30,** 68-74.

Dang B.T., Rahman M.A., Tran S.Q. & Glenner H. (2019) Genome-wide SNP analyses reveal population structure of *Portunus pelagicus* along Vietnam coastline. *PloS One,* **14,** e0224473.

Danielopol D.L., Griebler C., Gunatilaka A. & Notenboom J. (2003) Present state and future prospects for groundwater ecosystems. *Environmental Conservation,* **30,** 104-130.

Deharveng L., Stoch F., Gibert J., Bedos A., Galassi D., Zagmajster M., Brancelj A., Camacho A., Fiers F. & Martin P. (2009) Groundwater biodiversity in Europe. *Freshwater Biology,* **54,** 709-726.

Dudgeon D., Arthington A.H., Gessner M.O., Kawabata Z., Knowler D.J., Lévêque C., Naiman R.J., Prieur-Richard A., Soto D., Stiassny M.L.J. & Sullivan C.A. (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews,* **81,** 163-182.

Ethridge J.Z., Gibson J.R. & Nice C.C. (2013) Cryptic diversity within and amongst spring-associated *Stygobromus* amphipods (Amphipoda: Crangonyctidae). *Zoological Journal of the Linnean Society,* **167,** 227-242.

Evanno G., Regnaut S. & Goudet J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology,* **14,** 2611-2620.

Ewels P., Magnusson M., Lundin S. & Käller M. (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics,* **32,** 3047-3048.

Finston T.L., Johnson M.S., Humphreys W.F., Eberhard S.M. & Halse S.A. (2007) Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape. *Molecular Ecology,* **16,** 355-365.

Fumagalli M., Vieira F.G., Korneliussen T.S., Linderoth T., Huerta-Sánchez E., Albrechtsen A. & Nielsen R. (2013) Quantifying population genetic differentiation from next-generation sequencing data. *Genetics,* **195,** 979-992.

Gilbert H., Keany J. & Culver D.C. (2018) Response of shallow subterranean freshwater amphipods to habitat drying. *Subterranean Biology,* **28,** 15.

Gibert J. & Culver D.C. (2009) Assessing and conserving groundwater biodiversity: an introduction. *Freshwater Biology,* **54,** 639-648.

Hauer F.R., Locke H., Dreitz V.J., Hebblewhite M., Lowe W.H., Muhlfeld C.C., Nelson C.R., Proctor M.F. & Rood S.B. (2016) Gravel-bed river floodplains are the ecological nexus of glaciated mountain landscapes. *Science Advances,* **2,** e1600026.

Hay L.E., Markstrom S.L. & Ward-Garrison C. (2011) Watershed-scale response to climate change through the twenty-first century for selected basins across the United States. *Earth Interactions,* **15,** 1-37.

Helton A.M., Poole G.C., Payn R.A., Izurieta C. & Stanford J.A. (2012) Scaling flow path processes to fluvial landscapes: An integrated field and model assessment of temperature and dissolved oxygen dynamics in a river-floodplain-aquifer system. *Journal of Geophysical Research: Biogeosciences,* **117**.

Hendricks S., Anderson E.C., Antao T., Bernatchez L., Forester B.R., Garner B., Hand B.K., Hohenlohe P.A., Kardos M. & Koop B. (2018) Recent advances in conservation and population genomics data analysis. *Evolutionary Applications,* **11,** 1197-1211.

Holsinger J.R. (1974) Systematics of the subterranean amphipod genus *Stygobromus* (Gammaridae), part I: species of the western United States. *Smithsonian Contributions to Zoology,* **160,** 1-63.

Holsinger J.R. (1978) Systematics of the subterranean amphipod genus *Stygobromus* (Crangonyctidae): Part II. Species of the eastern United States. *Smithsonian Contributions to Zoology,* **266**, 1-142.

Holsinger J.R. (1980) *Stygobromus canadensis,* a new subterranean amphipod crustacean (Crangonyctidae) from Canada, with remarks on Wisconsin refugia. *Canadian Journal of Zoology,* **58,** 290-297.

Holsinger, J. R. (1987). Redescription of the stygobiont amphipod crustacean *Stygobromus pusillus* (Crangonyctidae) from the Soviet Union, with comments on taxonomic and zoogeographic relationships. *Journal of Crustacean Biology*, ***7***, 249-257.

Holsinger J.R. & Shaw D.P. (1987) *Stygobromus quatsinensis*, a new amphipod crustacean (Crangonyctidae) from caves on Vancouver Island, British Columbia, with remarks on zoogeographic relationships. *Canadian Journal of Zoology,* **65,** 2202-2209.

Hotaling S., Giersch J.J., Finn D.S., Tronstad L.M., Jordan S., Serpa L.E., Call R.G., Muhlfeld C.C. & Weisrock D.W. (2019) Congruent population genetic structure but differing depths of divergence for three alpine stoneflies with similar ecology and geographic distributions. *Freshwater Biology,* **64,** 335-347.

Hotaling S., Muhlfeld C.C., Giersch J.J., Ali O.A., Jordan S., Miller M.R., Luikart G. & Weisrock D.W. (2018) Demographic modelling reveals a history of divergence with gene flow for a glacially tied stonefly in a changing post-Pleistocene landscape. *Journal of Biogeography,* **45,** 304-317.

Jeffery N.W., Yampolsky L. & Gregory T.R. (2016) Nuclear DNA content correlates with depth, body size, and diversification rate in amphipod crustaceans from ancient Lake Baikal, Russia. *Genome*, **60**, 303-309.

Jombart T. & Ahmed I. (2011) adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics,* **27,** 3070-3071.

Jordan S., Hand B.K., Hotaling S., DelVecchia A., Malison R., Nissley C., Luikart G. & Stanford J. (2019) Genomic data reveal similar genetic differentiation in aquifer species with different dispersal capabilities and life histories. *Biological Journal of the Linnean Society*, https://doi.org/10.1093/biolinnean/blz173.

Kløve B., Ala-Aho P., Bertrand G., Gurdak J.J., Kupfersberger H., Kværner J., Muotka T., Mykrä H., Preda E. & Rossi P. (2014) Climate change impacts on groundwater and dependent ecosystems. *Journal of Hydrology,* **518,** 250-266.

Knowles L.L. (2001) Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshopprers. *Molecular Ecology,* **10,** 691-701.

Kopelman N.M., Mayzel J., Jakobsson M., Rosenberg N.A. & Mayrose I. (2015) Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources,* **15,** 1179-1191.

Korneliussen T.S., Albrechtsen A. & Nielsen R. (2014) ANGSD: analysis of next generation sequencing data. *BMC Bioinformatics,* **15,** 356.

Kornobis E., Pálsson S., Kristjansson B.K. & Svavarsson J. (2010) Molecular evidence of the survival of subterranean amphipods (Arthropoda) during Ice Age underneath glaciers in Iceland. *Molecular Ecology,* **19,** 2516-2530.

Kosnicki E. (2019) Life-history aspects of *Stygobromus pecki*. Bio-West, Inc., San Marcos, Texas.

Kosnicki E., Julius E.P. & Gibson J.R. (2019) Variation in the number of lateral telson spines of *Stygobromus flagellatus* (Benedict, 1896) (Amphipoda: Crangonyctidae), a subterranean species from Texas, USA. *Journal of Crustacean Biology,* **39,** 186-188.

Kristjánsson B.K. & Svavarsson J. (2007) Subglacial refugia in Iceland enabled groundwater amphipods to survive glaciations. *The American Naturalist,* **170,** 292-296.

Kurylyk B.L., MacQuarrie K.T.B. & Voss C.I. (2014) Climate change impacts on the temperature and magnitude of groundwater discharge from shallow, unconfined aquifers. *Water Resources Research,* **50,** 3253-3274.

Lefébure T., Douady C.J., Gouy M., Trontelj P., Briolay J. & Gibert J. (2006) Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. *Molecular Ecology,* **15,** 1797-1806.

Lefébure T., Douady C.J., Malard F. & Gibert J. (2007) Testing dispersal and cryptic diversity in a widely distributed groundwater amphipod (*Niphargus rhenorhodanensis*). *Molecular Phylogenetics and Evolution,* **42,** 676-686.

Li H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv preprint arXiv:1303.3997*.

Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G. & Durbin R. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics,* **25,** 2078-2079.

Lucas L.K., Gompert Z., Gibson J.R., Bell K.L., Buerkle C.A. & Nice C.C. (2016) Pervasive gene flow across critical habitat for four narrowly endemic, sympatric taxa. *Freshwater Biology,* **61,** 933-946.

Lundberg J.G., Kottelat M., Smith G.R., Stiassny M.L.J. & Gill A.C. (2000) So many fishes, so little time: An overview of recent ichthyological discovery in continental waters. *Annals of the Missouri Botanical Garden,* **87,** 26-62.

Mantel N. (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research,* **27,** 209-220.

McKenna A., Hanna M., Banks E., Sivachenko A., Cibulskis K., Kernytsky A., Garimella K., Altshuler D., Gabriel S. & Daly M. (2010) The henome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research,* **20,** 1297-1303.

Meisner J. & Albrechtsen A. (2018) Inferring population structure and admixture proportions in low-depth NGS data. *Genetics,* **210,** 719-731.

Meisner J. & Albrechtsen A. (2019) Testing for hardy-weinberg equilibrium in structured populations using genotype or low-depth NGS data. *Molecular Ecology Resources*, **19,** 1144-1152.

Meyer J.L., Strayer D.L., Wallace J.B., Eggert S.L., Helfman G.S. & Leonard N.E. (2007) The contribution of headwater streams to biodiversity in river networks. *Journal of the American Water Resources Association,* **43,** 86-103.

Nielsen R., Korneliussen T., Albrechtsen A., Li Y. & Wang J. (2012) SNP calling, genotype calling, and sample allele frequency estimation from new-generation sequencing data. *PloS One,* **7,** e37558.

Oksanen J., Blanchet F.G., Friendly M., Kindt R., Legendre P., McGlinn D., O'Hara R.B., Simpson G.L., Solymos P., H. Stevens M.H., Szoecs E. & Wagner H. (2019) vegan: Community ecology package. R package version 2.5-6.

Pan J., Wang B., Pei Z., Zhao W., Gao J., Mao J. & Wang X. (2015) Optimization of the genotyping-by-sequencing strategy for population genomic analysis in conifers. *Molecular Ecology Resources,* **15,** 711-722.

Pardee J.T. (1910) The glacial lake Missoula. *The Journal of Geology,* **18,** 376-386.

Pardee J.T. (1942) Unusual currents in glacial lake Missoula, Montana. *Bulletin of the Geological Society of America,* **53,** 1569-1600.

Pazmiño D.A., Maes G.E., Simpfendorfer C.A., Salinas-de-León P. & van Herwerden L. (2017) Genome-wide SNPs reveal low effective population size within confined management units of the highly vagile Galapagos shark (*Carcharhinus galapagensis*). *Conservation Genetics,* **18,** 1151-1163.

Pepin D.M. & Hauer R.F. (2002) Benthic responses to groundwater–surface water exchange in 2 alluvial rivers in northwestern Montana. *Journal of the North American Benthological Society,* **21,** 370-383.

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rees D.J., Dufresne F., Glemet H. & Belzile C. (2007) Amphipod genome sizes: first estimates for Arctic species reveal genomic giants. *Genome*, **50**, 151-158.

Reid B.L. (2007) *Energy flow in a floodplain aquifer ecosystem.* Dissertation, University of Montana.

Rochette N.C. & Catchen J.M. (2017) Deriving genotypes from RAD-seq short-read data using Stacks. *Nature Protocols,* **12,** 2640-2659.

Rochette N.C., Rivera-Colón A.G. & Catchen J.M. (2019) Stacks 2: Analytical methods for paired-end sequencing improve RADseq-based population genomics. *Molecular Ecology,* **28,** 4737-4754.

Rood S.B., Pan J., Gill K.M., Franks C.G., Samuelson G.M. & Shepherd A. (2008) Declining summer flows of Rocky Mountain rivers: changing seasonal hydrology and probable impacts on floodplain forests. *Journal of Hydrology,* **349,** 397-410.

Sabater S. & Tockner K. (2009) Effects of hydrologic alterations on the ecological quality of river ecosystems. In: *Water Scarcity in the Mediterranean*, pp. 15-39. Springer, Berlin, Heidelberg.

Sedell J.R., Reeves G.H., Hauer F.R., Stanford J.A. & Hawkins C.P. (1990) Role of refugia in recovery from disturbances: modern fragmented and disconnected river systems. *Environmental Management,* **14,** 711-724.

Shafer A.B.A., Peart C.R., Tusso S., Maayan I., Brelsford A., Wheat C.W. & Wolf J.B.W. (2017) Bioinformatic processing of RAD-seq data dramatically impacts downstream population genetic inference. *Methods in Ecology and Evolution,* **8,** 907-917.

Sidorov D.A., Holsinger J.R. & Takhteev V.V. (2010) Two new species of the subterranean amphipod genus *Stygobromus* (Amphipoda: Crangonyctidae) from Siberia, with new data on *Stygobromus pusillus* (Martynov) and remarks on morphology and biogeographic relationships. *Zootaxa,* **2478,** 41-58.

Skotte L., Korneliussen T.S. & Albrechtsen A. (2013) Estimating individual admixture proportions from next generation sequencing data. *Genetics,* **195,** 693-702.

Slatkin M. (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics,* **139,** 457-462.

Smith D.G. (1985) The occurrence of the troglobitic amphipod, *Stygobromus tenuis tenuis* (Smith)(Crangonyctidae) in the Taconic Mountains of southwestern Massachusetts (USA): A case for the existence of a subterranean refugium in a glaciated region. *International Journal of Speleology,* **14,** 31-37.

Smith L.N. (2006) Stratigraphic evidence for multiple drainings of glacial Lake Missoula along the Clark Fork River, Montana, USA. *Quaternary Research,* **66,** 311-322.

Smith L.N., Blood L. & LaFave J.I. (2000) Quaternary geology, geomorphology, and hydrogeology of the upper Flathead River valley area, Flathead County, Montana. *Geologic field trips, western Montana and adjacent areas: Rocky*

Mountain Section of the Geological Society of America, University of Montana, 41-63.

Smith L.N., Sohbati R., Buylaert J., Lian O.B., Murray A. & Jain M. (2018) Timing of lake-level changes for a deep last-glacial Lake Missoula: optical dating of the Garden Gulch area, Montana, USA. *Quaternary Science Reviews,* **183,** 23-35.

Stanford J.A. & Gaufin A.R. (1974) Hyporheic communities of two Montana rivers. *Science,* **185,** 700-702.

Stanford J.A., Lorang M.S. & Hauer F.R. (2005) The shifting habitat mosaic of river ecosystems. *Internationale Vereinigung für theoretische und angewandte Limnologie: Verhandlungen,* **29,** 123-136.

Stanford J.A. & Ward J.V. (1993) An ecosystem perspective of alluvial Rivers: connectivity and the hyporheic corridor. *Journal of the North American Benthological Society,* **12,** 48-60.

Stanford J.A. & Ward J.V. (1988) The hyporheic habitat of river ecosystems. *Nature,* **335,** 64-66.

Stoch F. (1995) The ecological and historical determinants of crustacean diversity in groundwaters, or: Why are there so many species? *Mémoires de Biospéologie,* **22,** 139-160.

Sundqvist L., Keenan K., Zackrisson M., Prodöhl P. & Kleinhans D. (2016) Directional genetic differentiation and relative migration. *Ecology and Evolution,* **6,** 3461-3475.

Tockner K., Pusch M., Borchardt D. & Lorang M.S. (2010) Multiple stressors in coupled river-floodplain ecosystems. *Freshwater Biology,* **55,** 135-151.

Tockner K. & Stanford J.A. (2002) Riverine flood plains: present state and future trends. *Environmental Conservation,* **29,** 308-330.

Trontelj P., Douady C.J., Fiser C., Gibert J., Goricki Š, Lefébure T., Sket B. & Zaksek V. (2009) A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? *Freshwater Biology,* **54,** 727-744.

Valett H., Hauer F. & Stanford J. (2014) Landscape influences on ecosystem function: local and routing control of oxygen dynamics in a floodplain aquifer. *Ecosystems,* **17,** 195-211.

Verity R. & Nichols R.A. (2014) What is genetic differentiation, and how should we measure it—GST, D, neither or both? *Molecular Ecology,* **23,** 4216-4225.

Wang, D. & Holsinger J.R. (2001) Systematics of the subterranean amphipod genus *Stygobromus* (Crangonyctidae) in western North America, with emphasis on the *hubbsi* group. *Amphipacifica*, **3**, 39–147.

Wang X., Ye X., Zhao L., Li D., Guo Z. & Zhuang H. (2017) Genome-wide RAD sequencing data provide unprecedented resolution of the phylogeny of temperate bamboos (Poaceae: Bambusoideae). *Scientific Reports,* **7,** 11546.

Wickham H. (2016) *ggplot2: elegant graphics for data analysis*. Springer-Verlag, New York.

Woodings L.N., Murphy N.P., Doyle S.R., Hall N.E., Robinson A.J., Liggins G.W., Green B.S., Cooke I.R., Bell J.J. & Strugnell J.M. (2018) Outlier SNPs detect weak regional structure against a background of genetic homogeneity in the Eastern Rock Lobster, *Sagmariasus verreauxi*. *Marine Biology,* **165,** 185.

Table 1. Study site characteristics. Average temperature was measured during sampling periods (May-Oct) of 2017-2019.

River	Floodplain	Well	Latitude	Longitude	Elevation (m)	Distance from river (m)	Average Temp. (°C)	Sample size (n)
Main Stem	Kalispell	GRBD	48.3167	-114.20841	905	45	9.73	37
		ST5R	48.239856	-114.23505	912	31	10.4	38
North Fork	Wurtz	WUG4	48.902669	-114.37888	1145	30	7.73	49
Middle Fork	Nyack	HA15	48.462634	-113.81941	1012	230	9.13	33
		HA19	48.478264	-113.8284	1009	110	8.26	39
		HA9	48.476222	-113.83679	1010	650	9.37	39

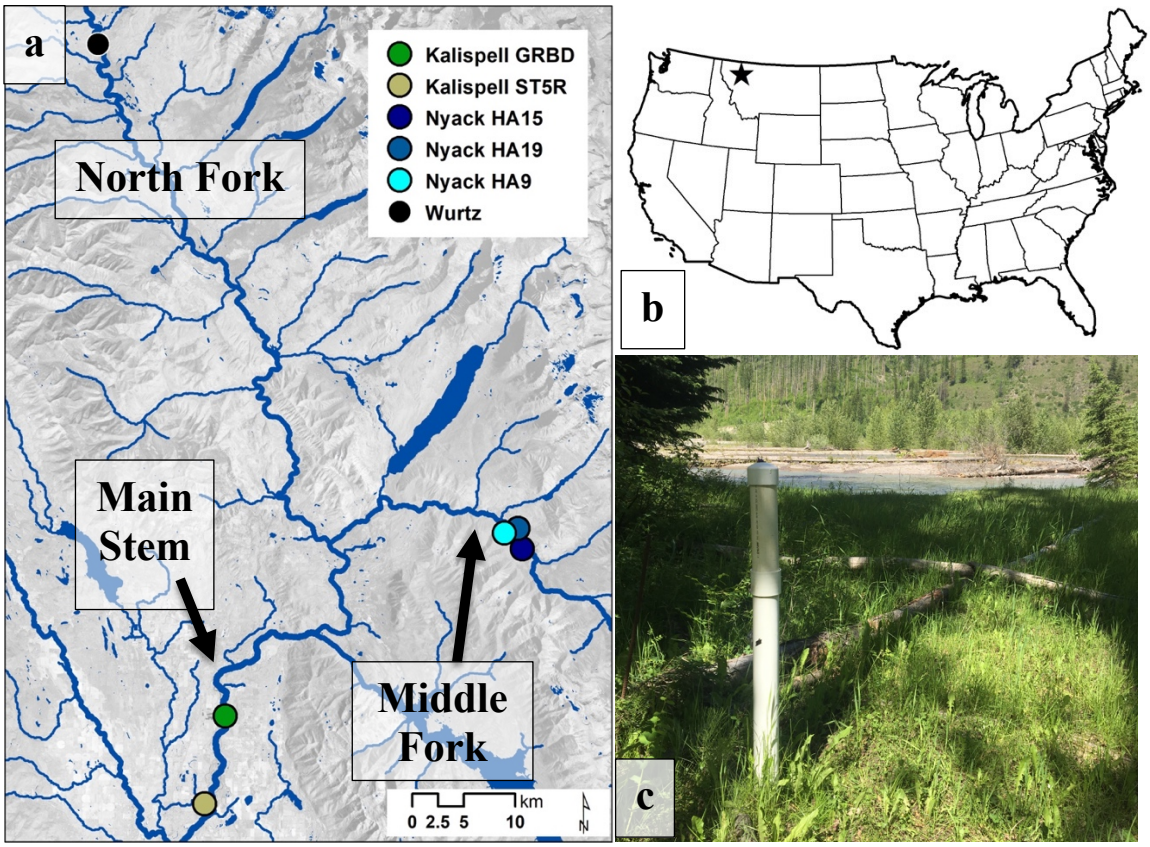


Figure 1. Study sites along the Flathead River, including a) the North Fork, Middle Fork, and main stem, b) Study region within the USA, and c) a well on the Middle Fork of the river.

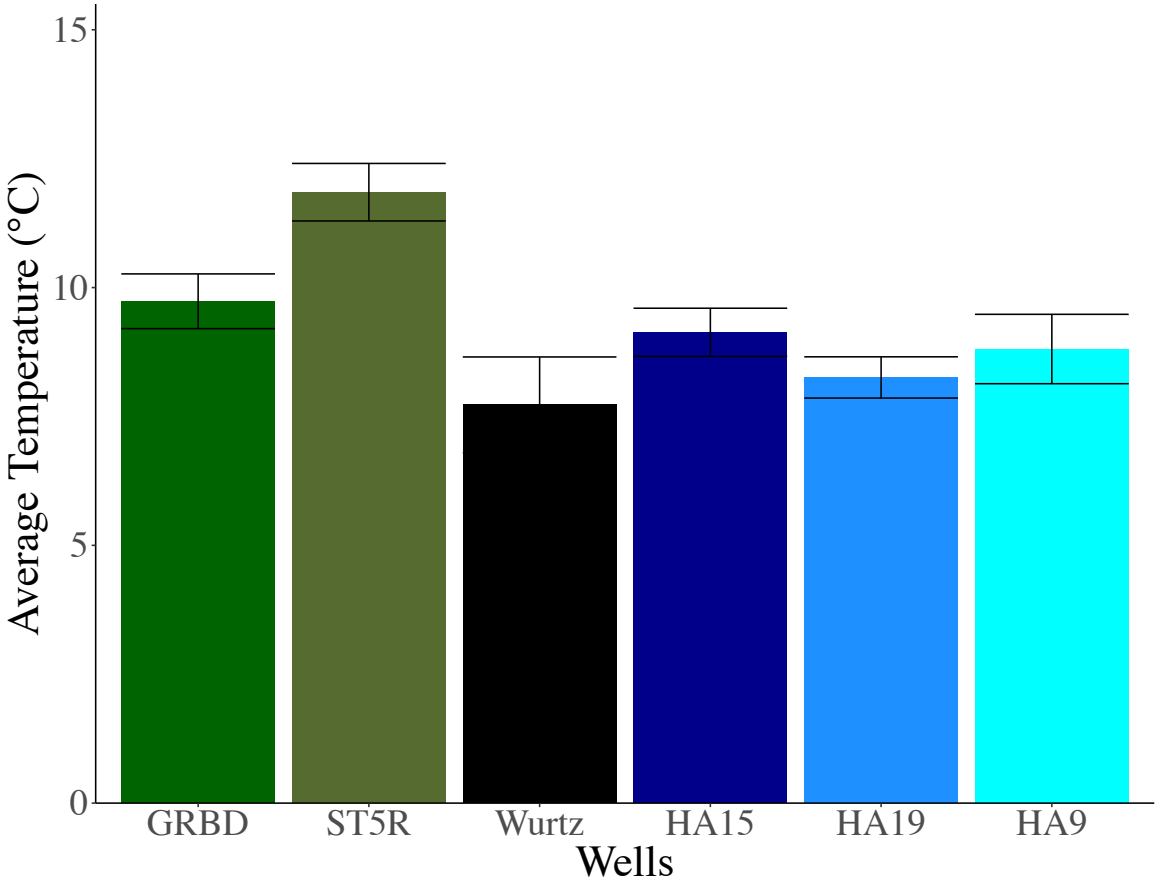


Figure 2. Average water temperature in each well. Error bars represent standard error of the mean.

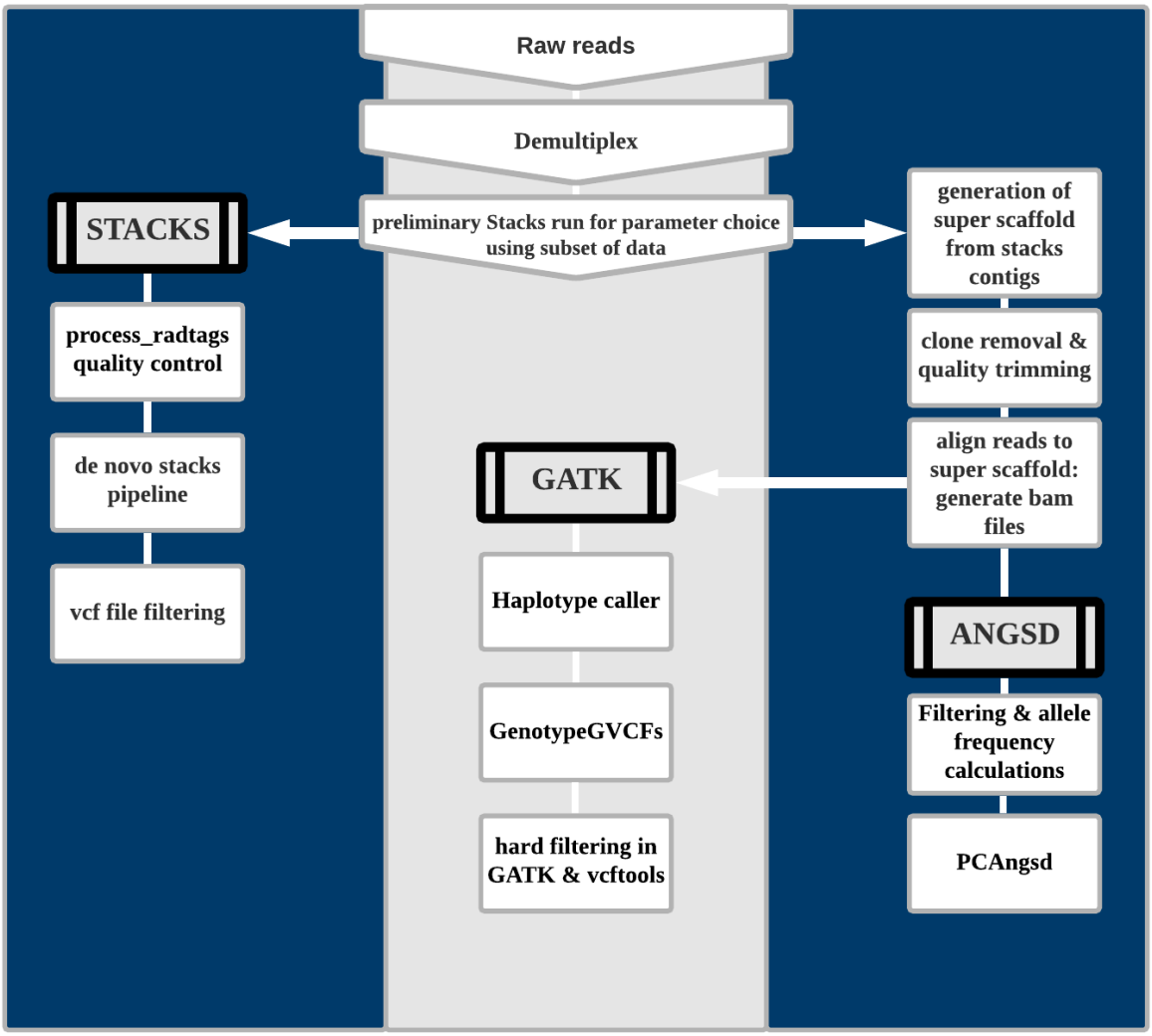


Figure 3. Visualization of PCA workflow for each pipeline: Stacks, GATK, and ANGSD.

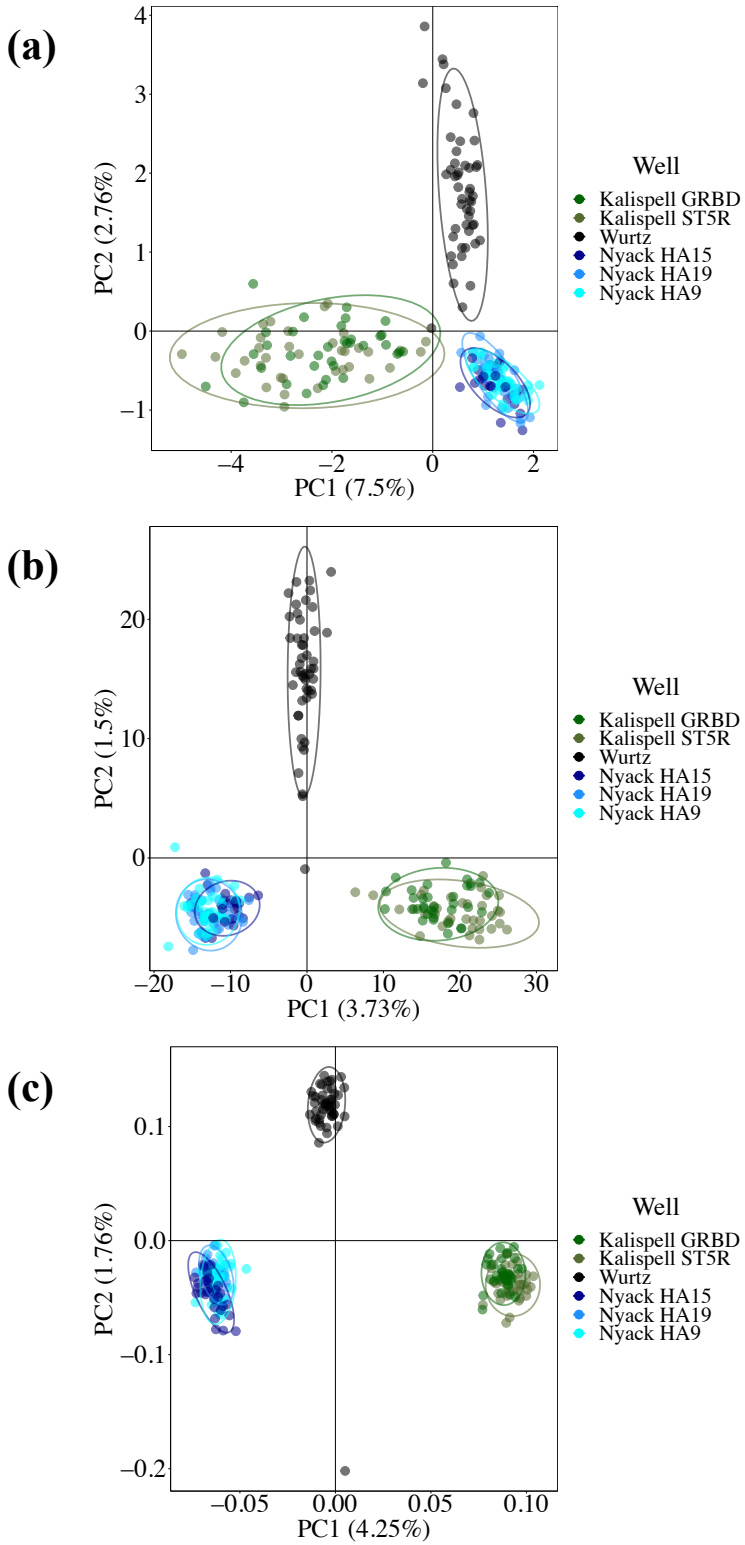


Figure 4. Principal component analyses of 226 individuals generated from a) Stacks, b) GATK, and c) ANGSD.

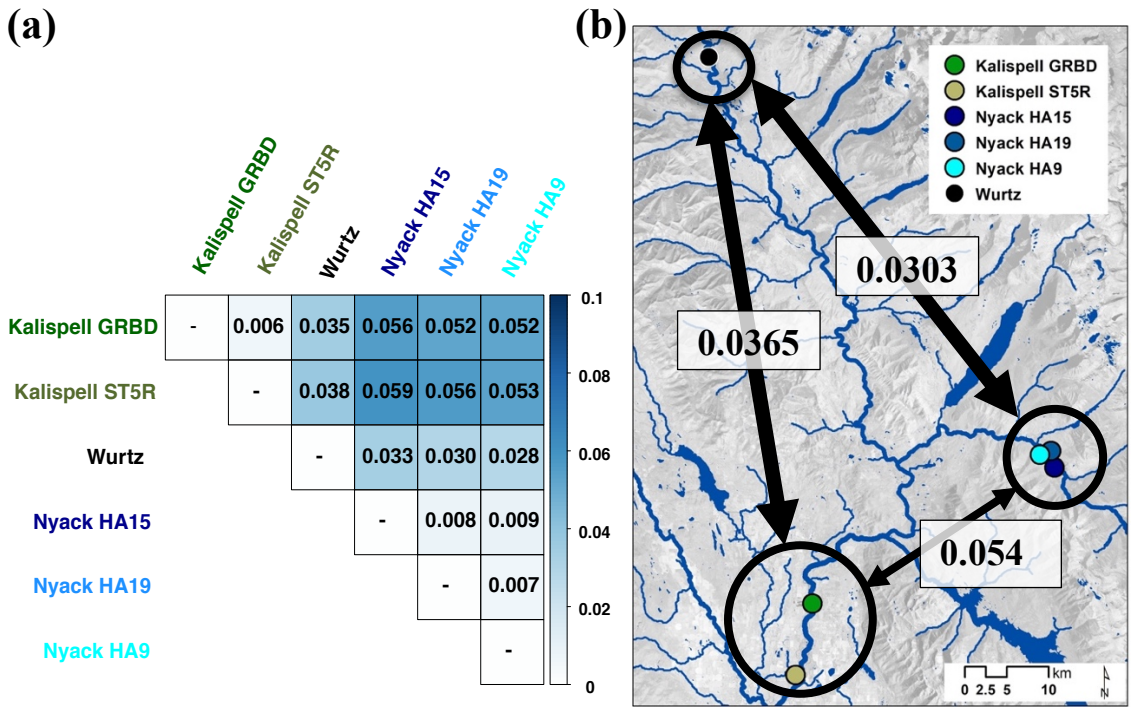


Figure 5. Pairwise F_{ST} values generated from ANGSD. a) Well to well comparisons, b) Average pairwise F_{ST} values generated between floodplains.

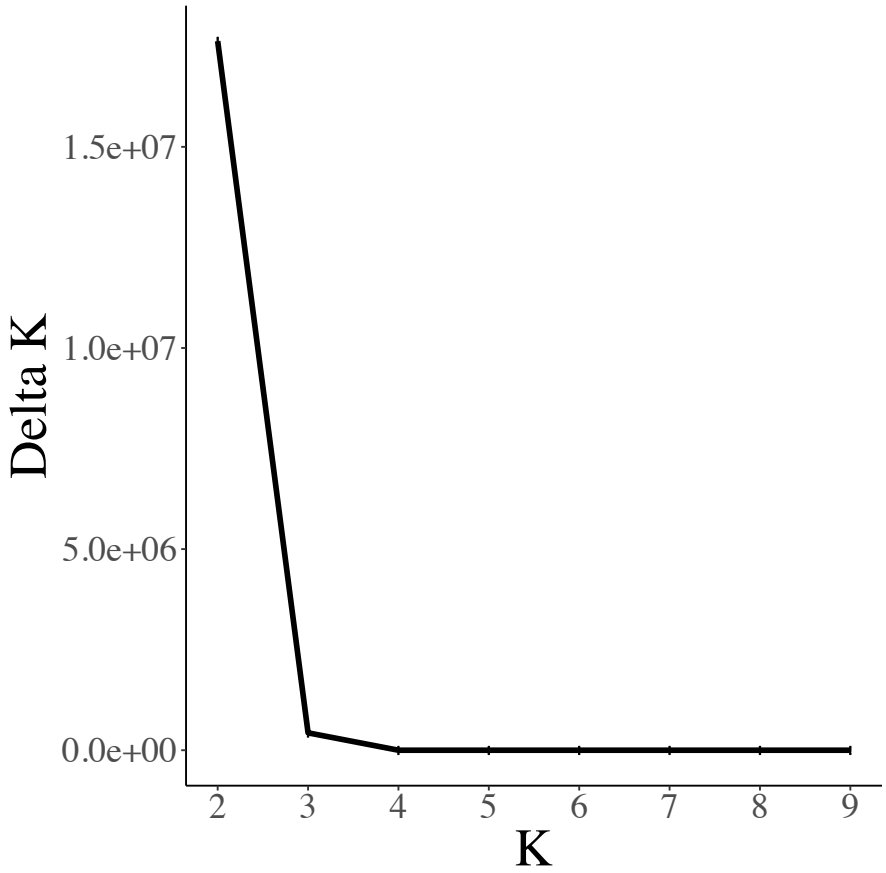


Figure 6. Determination of the best value of K based on ΔK.

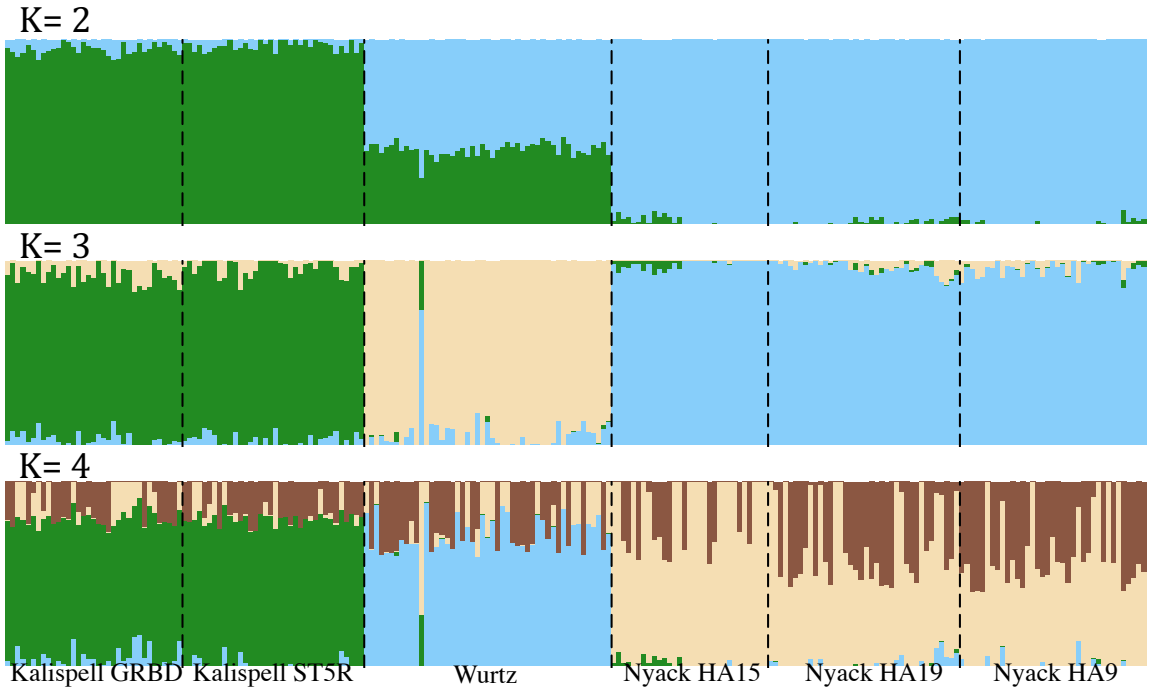


Figure 7. Admixture plots using K=2, 3, and 4. Each line on the x axis represents an individual, and the y axis represents the admixture proportions

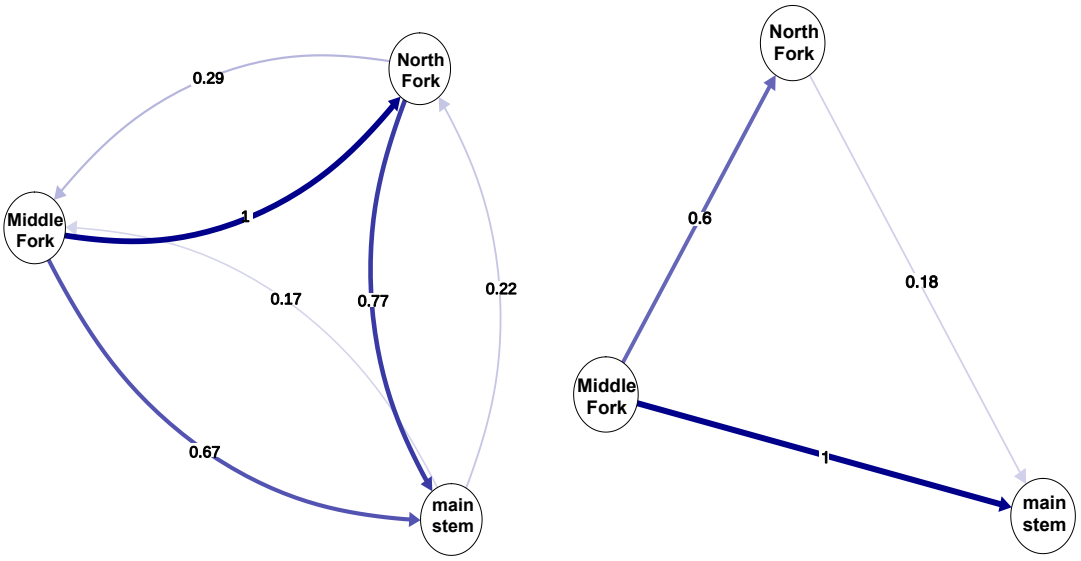


Figure 8. Relative (left) and significant (right) migration networks created from G_{ST} values, noted on the connecting arrows. Circles denote populations.

Appendix

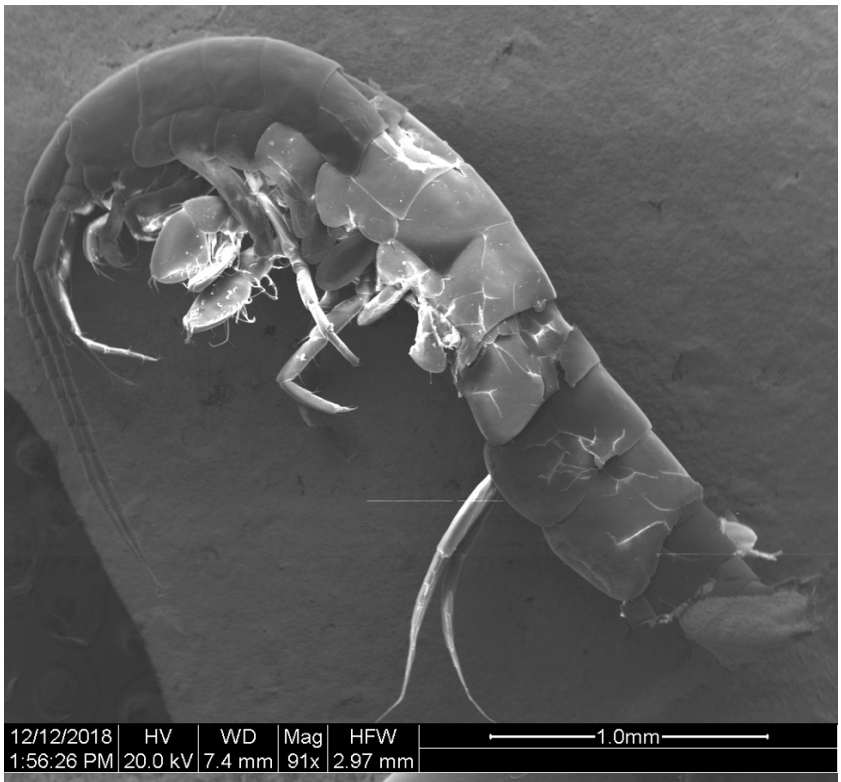
Table A1. Coverage reports prior to and following PCR duplicate removal in Stacks, for the subset of samples with the highest reported coverage (ustacks coverage and mean coverage, respectively).

Population	Well	Sample	ustacks coverage	n loci	Mean Coverage	PCR duplication rate
Main Stem	Kalispell GRBD	SS_212	20.21	41639	5.75	0.692
		SS_226	19.78	40891	5.398	0.699
		SS_228	19.78	42212	5.375	0.702
		SS_242	24.83	42448	6.999	0.697
		SS_247	19.16	41275	5.367	0.697
	Kalispell ST5R	SS_183	30.84	46017	6.712	0.77
		SS_184	29.25	44492	6.309	0.769
		SS_189	33.12	47121	7.113	0.774
		SS_197	30.41	44737	6.388	0.775
		SS_200	31.57	44358	6.658	0.774
North Fork	Wurtz	SS_131	28.83	43257	6.262	0.767
		SS_164	25.47	42217	5.626	0.76
		SS_165	31.99	44843	6.645	0.776
		SS_168	29.18	41899	6.138	0.772
		SS_172	25.33	41183	5.504	0.766
Middle Fork	Nyack HA15	SS_103	15.84	31897	3.4	0.746
		SS_104	19.47	36612	4.135	0.762
		SS_110	15.81	34644	3.623	0.741
		SS_112	15.94	36566	3.715	0.742
		SS_117	18.66	37718	4.421	0.74
	Nyack HA19	SS_15	23.68	41442	4.91	0.777
		SS_18	22.7	41136	4.763	0.773
		SS_26	22.9	41208	4.731	0.776
		SS_32	22.13	43946	4.873	0.766
		SS_37	26.48	42921	5.588	0.775
	Nyack HA9	SS_55	42.32	46349	9.103	0.775
		SS_56	26.99	47856	5.885	0.778
		SS_74	28.49	43799	5.867	0.784
		SS_76	34.71	47712	7.273	0.794
		SS_87	37.42	48113	7.984	0.786
Average			25.78	42350	5.751	0.757

Table A2. Corrected pairwise Fst comparisons and distances used to perform mantel test.

Site 1	Site 2	Fst	Corrected Fst	Distance (km)
Kalispell GRBD	Kalispell ST5R	0.0061	0.0061	8.77
Kalispell GRBD	Wurtz	0.035	0.0363	107.1
Kalispell GRBD	Nyack HA15	0.0558	0.0591	58.9
Kalispell GRBD	Nyack HA19	0.0522	0.0551	55.9
Kalispell GRBD	Nyack HA9	0.052	0.0549	56.8
Kalispell ST5R	Wurtz	0.0379	0.0394	117.3
Kalispell ST5R	Nyack HA15	0.0592	0.0629	69.1
Kalispell ST5R	Nyack HA19	0.0562	0.0595	66.1
Kalispell ST5R	Nyack HA9	0.0532	0.0562	67.1
Wurtz	Nyack HA15	0.0334	0.0346	102.0
Wurtz	Nyack HA19	0.0295	0.0304	99.0
Wurtz	Nyack HA9	0.0282	0.0290	99.9
Nyack HA15	Nyack HA19	0.0083	0.0084	1.86
Nyack HA15	Nyack HA9	0.0092	0.0093	1.98
Nyack HA19	Nyack HA9	0.0068	0.0068	0.66

(a)



(b)

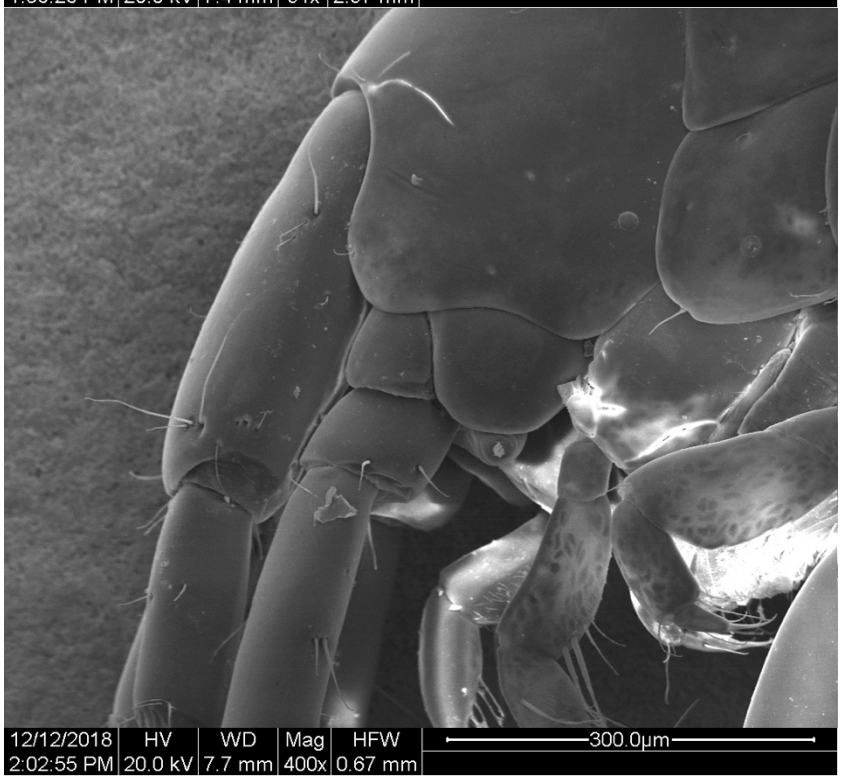


Figure A1. Scanning electron microscope images of one *Stygobromus* sp. 1 individual featuring a) overall body shape, and b) zoomed in view of mouthparts and head.

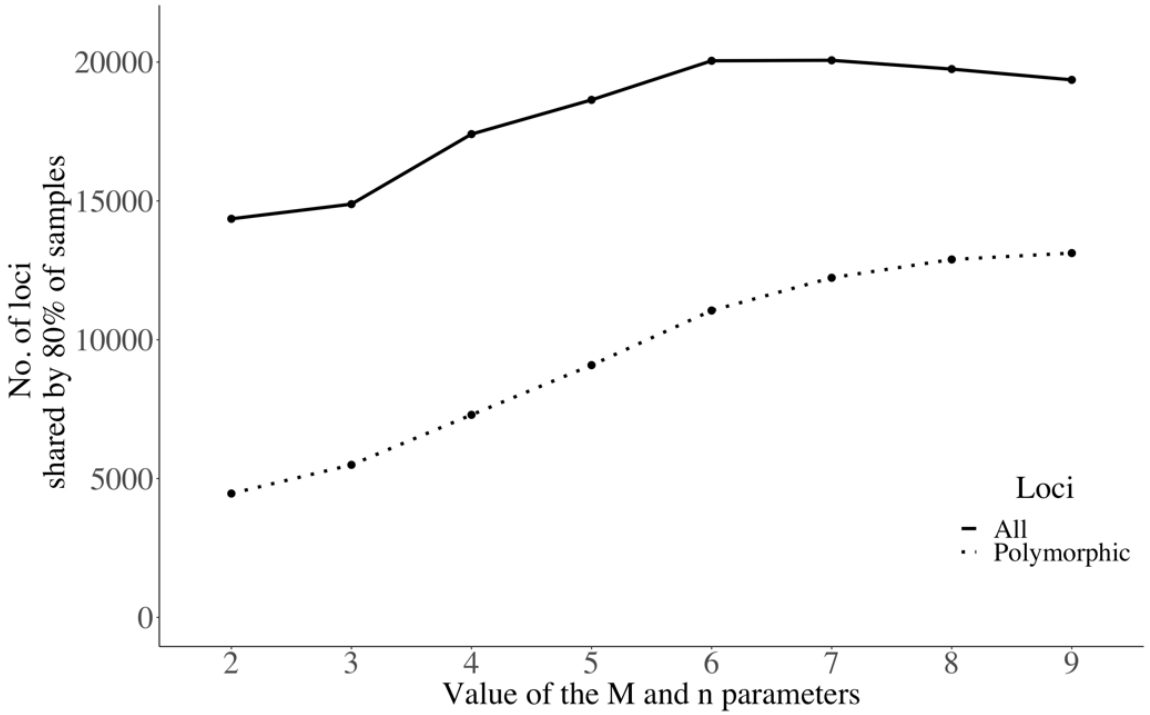


Figure A2. Number of loci and SNPs shared by 80% of samples with catalog assemblies of different M and n parameters.

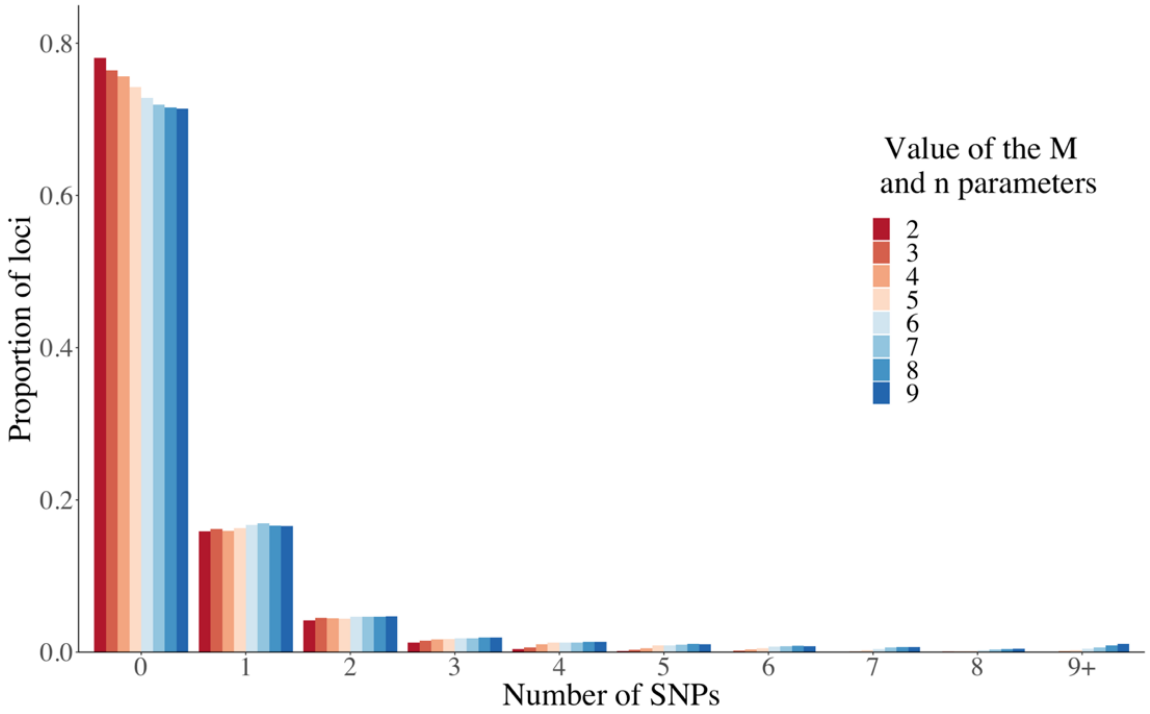


Figure A3. Proportion of loci with specific SNP counts from catalog assemblies of different M and n parameters.

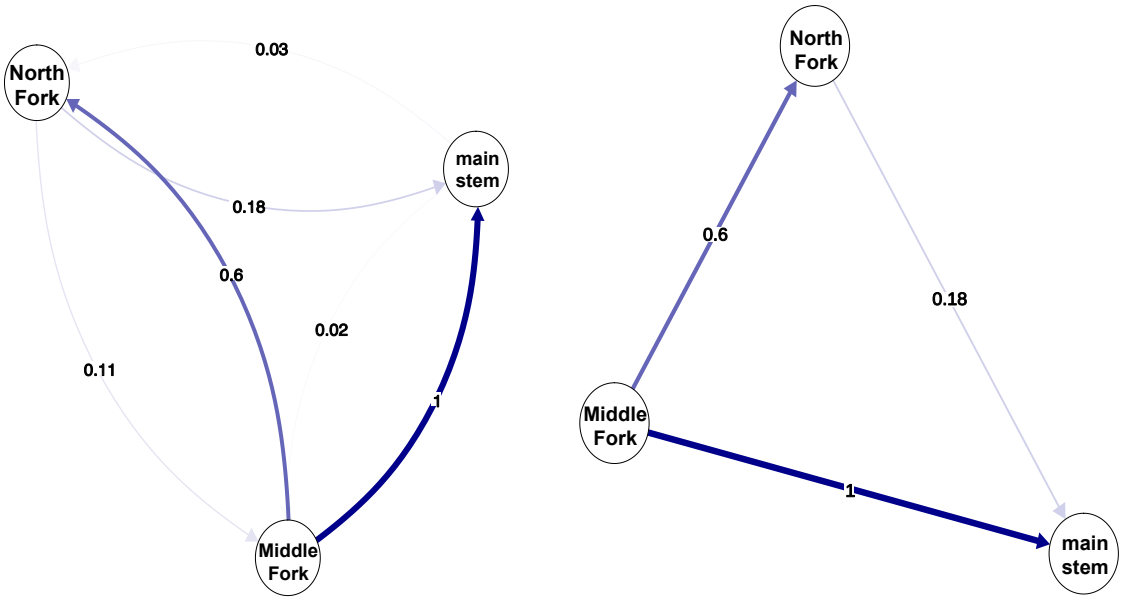


Figure A4. Relative (left) and significant (right) migration networks created from D values, noted on the connecting arrows. Circles denote populations.