# Population genetic structure of the freshwater mussel *Potamilus alatus* in the state of Minnesota

A Thesis

SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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May 2020

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## Acknowledgements

I would like to thank my adviser Dr. Andrew Simons, for taking me on as a student and being a patient and thoughtful resource and mentor as I grew as a scientist. I would also like to thank my additional committee members Sharon Jansa and Loren Miller for providing sound advice and feedback as I turned my research ideas in to a project. I must additionally thank Loren Miller for allowing me to use his lab for genetic work, and letting me pick his brain over various technical and statistical problems I encountered while doing this research.

I am deeply indebted to Bernard Sietman of the Minnesota Department of Natural Resources, who brought me in to the world of malacology and helped me learn to identify and find freshwater mussels in the field. In addition, I thank Mark Hove for lending me field work equipment and teaching me and my field crew how to safely and effectively dive in Minnesotan rivers.

Field work takes a tremendous amount of effort and resources, and mine would not have been possible without the help of my excellent field crew including Paul Leingang and Nolan Iwinski, and my wonderful labmates Sean Keogh, Peter Hundt and Joshua Egan. I'd also like to thank two fellow graduate students Ami Thomspon and Yana Eglit, who encouraged me through my frustrations and helped me find solutions.

Finally, I'd like to thank my family, who encouraged my interest in the natural world from a young age, and whose support and love have made me who I am today.

## Abstract

Freshwater mussels (family Unionidae) have been declining across North America at an alarming rate. Numerous conservation strategies have been invoked to slow or reverse this decline, with hatcheries and transport of individuals from healthy populations to ailing ones being a common technique. However, moving individuals from one population to another can have unintended consequences if the individuals from the source population are genetically distinct, so it is imperative that conservationists understand the underlying patterns of genetic diversity of the species they are trying to save. This study examines populations of the mussel *Potamilus alatus* across the state of Minnesota in order to determine the extent of genetic connectivity among different geographic populations are. Analyses of seven different microsatellite loci found that populations within the Mississippi River watershed are genetically similar; however, a population along the Red Lake River is isolated and unique. Any future conservation efforts with *Potamilus alatus* should take these differences in to account when taking broodstock for hatcheries or transporting individuals between populations, in order to avoid outbreeding depression, or transplanting individuals into environments for which not be genetically suited.

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## Introduction

Freshwater ecosystem biodiversity is arguably the most threatened in the world (Dudgeon et al. 2005), with extinction rates estimated to be as high or higher than in any terrestrial habitat (Ricciardi and Rasmussen 2001). Within those ecosystems, mollusks are among the most threatened taxa (Strayer et al. 2004): especially those from the family Unionidae, over half of which are extinct or in danger of extinction in the near future in North America (Bogan 1993).

Mussels from the family Unionidae, often referred to as unionids, have a unique life history that can impact their conservation. As an adaptation to living in riparian environments, these mussels have a specialized larval stage called glochidia, which attach to a fish's fins or gills. While on their fish host, the glochidia transform to fully functional juvenile mussels, and drop off at a new location (Strayer et al. 2004). Each unionid species has a different subset of fish species they can use as hosts, some species are generalist and can use almost any fish as host, while others can use only one species (Haag 2012).

There are numerous reasons for the decline of Unionidae in the United States. Early threats to came from the button trade, where hooks and rakes were used to harvest large numbers of mussels from rivers, mainly in the Midwest (Anthony and Downing 2001). The causes of more recent declines are harder to identify but are likely due to a combination of altered river flow and fragmented population by impoundments such as dams, and water quality degradation from farming and industrial practices (Haag 2012). In some cases, impoundments have kept host fish from migrating upstream, preventing mussels upstream of the dam from reproducing (Kelner and Sietman 2000). Invasive quagga and zebra mussels constitute a further issue, competing with unionids for space

and possibly food, attaching themselves to Unionids and preventing successful burial in the substrate (Nichols and Amberg 1999).

Conservation efforts to counteract the decline of unionid populations taken several strategies. Many federal and state owned hatcheries have successfully reared and released mussels, re-establishing or augmenting populations (Jones et al. 2006). Others have transferred adult mussels from healthy populations to threatened ones (Sheehan et al. 1989). Mussels are sometimes moved from habitats compromised by human activities such as bridge construction, and relocated to new areas. This translocation can be across great distances (Tiemann et al. 2016, Cope and Waller 1995).

However, efforts to transfer or augment populations may unintentionally cause harm. Populations may be susceptible to genetic threats from: inbreeding or outbreeding. Inbreeding depression occurs when related individuals mate and produce genetically unfit offspring that threaten that population's survival. In the long-term, small population size and inbreeding may result in insufficient allelic diversity to adapt to the environment (Frankham et al. 2002). These inbreeding threats may be caused by isolation of previously connected populations, for example following dam construction or habitat destruction (Fuller 2017). Inbreeding depression may also occur when hatchery programs take broodstock from a single, genetically homogeneous source. Outbreeding depression occurs when previously-isolated, locally adapted populations breed with outside populations, producing offspring less fit to their specific environment (Frankham et al. 2002). This can be caused by man-made infrastructure such as canals connecting different watersheds (Hoffman et al. 2018), by conservation efforts moving adult mussels between watersheds (Haag and Williams 2013), or hatcheries sourcing non-local individuals for their broodstock (Jones et al. 2006). These issues have led to a

widespread acceptance that population genetics should be taken in to account in mussel conservation efforts (Haag 2012).

Our understanding of unionid population genetics remains poor compared to that of vertebrate taxa. This is partially due to the difficulties in sampling mussels—many species are endangered, thus complicating conventional tissue sampling approaches which usually involve taking a section of mantle tissue with a razor blade (Inoue et al. 2015). Alternative methods such as cell or eDNA collection are subject to issues such as PCR (Polymerase Chain Reaction) inhibition by tannins and humic acids, and difficulties with separation of proteins from DNA (Henley et al. 2006). In addition, there are fewer genetic resources available for developing population level studies, such as published genetic markers.

The majority of genetic studies of mussels have been done on endangered species (Zanatta and Murphy 2008; Curole et al. 2004; Galbraith et al. 2015; Kelly and Rhymer 2005; Menon 2019), focusing on current conservation issues. However, this means most of our knowledge of unionid genetics is based on species that have faced high human impact, and are thus likely to inhabit a much smaller portion of their original range, exist as more fragmented populations, and may experience more inbreeding than wide ranging, more abundant unionid species (Berg et al.2007).

In contrast, some unionid species are not experiencing population decline and might further understanding of unimpacted unionid genetics. One of these species is *Potamilus alatus*, a mussel found in the Mississippi River watershed from Ontario to Arkansas (Hua 2005). It has a single fish host, the freshwater drum *Aplodinotus grunniens* (Sietman et al. 2018) and is widespread and abundant in most of its range (Funk 1957). However, previous declines in unionid populations have happened rapidly, and may someday

occur for *Potamilus alatus*, so it is important to collect population genetic data while it is available.

The goal of this study is to determine whether *Potamilus alatus* in Minnesota exists as one continuous interbreeding population, or multiple genetically distinct populations. Genetically distinct populations are hypothesized to exist in the Red River watershed due to its isolation from the rest of the state's drainages, and in the Chippewa River, due to its distance from the large continuous mussel beds found on the Mississippi and St. Croix Rivers.

## Methods

### *Sample Locations and Collection*

Mussel collection sites were chosen in consultation with Bernard Sietman at the Minnesota Department of Natural Resources (MDNR), using locations of previous MDNR surveys. Sites were chosen to maximize the number of drainages included in the study. Drainages included were the Mississippi, Minnesota, St. Croix and Red rivers. Collecting was ideally done from the main river stem of each drainage, but when the main river could not be accessed for logistical or safety reasons, specimens were collected from tributaries instead. Individuals were collected via SCUBA in major rivers and snorkeling in tributaries. There were five sample sites: Red Lake River (RLR) a tributary to the Red River of the North, Chippewa River (CHR) tributary to the Minnesota River, Mississippi (MSP) on the main stem, and the St. Croix River at Interstate Park (SCR – I) and Hudson (SCR – H) (See Table 1 and Figure 1). Mussels were collected on MNDNR special permit #19473.

Samples were taken using Epicenter oral buccal swabs (catalog # QEC0925). Mussels were gently opened until swabs could easily be inserted, and swabs rubbed gently against the mantle tissue as described in Henley et al. (2006). Mussels were then

returned to the river. Swabs were then placed in 95% ethanol and stored in a -30°C freezer until used for DNA extraction.

### *DNA Extraction and Amplification*

DNA was extracted using a Qiagen DNeasy kit (catalog #69506), except for SCR – H samples which were extracted with a FastDNA spin kit (MP biomedicals, catalog #116540000). Extractions followed kit protocol for DNA extraction from tissue, with swabs physically removed with sterile forceps after the incubation or cell lysis step depending on the DNA extraction method used. After extraction, samples from the MNR and SCR – I sites were run through a Zymo Research One Step PCR Inhibitor Removal Kit (catalog # D6030). Final DNA extraction volumes were 200 µL. Samples were checked with a spectrometer to insure the products of the extraction contained DNA, but were not diluted to any standard concentration.

Microsatellite markers developed for closely related species were tested for amplification via polymerase chain reaction (PCR) in *Potamilus alatus,* using tissue samples from *Potamilus alatus* in the Bell Museum's invertebrate collection. The markers were originally developed for *Leptodea leptodon* (O'Bryhim et al. 2012) and *Potamilus capax* (Diaz-Ferguson 2011). Nine microsatellite markers were successfully amplified in tissue samples, and displayed enough variation for use in fragment length analysis in mucus samples collected from the wild (Table 2). An M-13 tail with fluorescently labeled dye on the 5' end of the forward primer was used for amplification for fragment length analysis, following techniques developed by Boutin-Ganache et al. (2001). PCR reactions were performed in 15 µL volumes containing 3.85 µL template DNA, 3.25 µL deionized water, 6.75 µL GoTaq master mix (catalog # M7123), 0.15 µL M-13 tailed forward primer, 0.5 µL reverse primer , and 0.5 µL M-13 primer labeled with either VIC, PET, NED or FAM fluorescent dyes purchased from MP Biomedical. Samples from the SCR-I, SCR-H and

CHR sites were difficult to amplify, most likely due to PCR inhibition from tannic and humic acids from the sample site; thus, bovine serum albumin was added to the water in the PCR reaction to a final concentration of 1 mg/mL (from New England Biolabs, catalog # B9001S) solution, following recommendations in Kreader (1996). All primers were kept at a 10 µM concentration.

Each PCR experiment included a reaction with template replaced by deionized water as negative control. Microsatellite markers were amplified using one of three amplification regimes: Group #1 (2 markers, Lele45, Lele47): 5 minute denaturation at 95° C, 40 cycles of 30 seconds annealing at 95° C, 30 seconds at 65° C, 30 seconds at 72° C, and a 72° C extension at the end for 5 minutes followed by 4° C hold; Group #2 (Lele3, Lele24, Lele33, Lele6): 95 °C for five minutes, 20 cycle of 95° C for 30 seconds, 65° C for 30 seconds (decreasing temperature by 0.2° C each cycle, i.e. touchdown PCR), 75° C for 30 seconds; then 20 cycles of 95° C for 30 seconds, 55° C for seconds, and 75° C for 30 seconds, ending with a 75° C extension phase for 5 minutes and a hold at 4° C; Group #3 (PCD 110, PCD 122, PCD 123): 94° C for 10 minutes, 33 cycles of 94° C for 30 seconds, 56° C for 30 seconds (decreasing temperature by 0.2° C each cycle), 74° C for 1 minute, followed by a 5 minute extension phase at 72° C and a 4° C hold. Material was amplified using an Applied Biosystems Veriti thermal cycler.

To confirm whether the PCR amplification was successful, amplified product was run on a polyacrylamide gel for 1 hour and then stained with ethidium bromide and visualized using UV light. PCR products were then loaded onto a 96-well plate for fragment length analysis. Markers with different dye tags were combined into the same well. Fragment length analysis was done by the University of Minnesota Genomics Center, using an ABI 3730xl capillary electrophoresis platform and Liz-500 as a ladder. Output data were saved as ABI files and exported into the fragment length analysis add-on in Geneious

version 6.1.6 (http://www.geneious.com, Kearse et al. 2012). There, the dataset was fit to the Liz-500 ladder, and each individual in the dataset was scored to remove false peaks. Once scored, individuals were binned into allele size categories based on repeat length and this data exported in a .csv table for statistical analysis. Individuals missing more than 3 markers were excluded from analyses.

| Abbreviation | Description | County | Main Stem River | Watershed | Coordinates | Date |
|---|---|---|---|---|---|---|
| SCR - I | Main stem of the St. Croix river at Interstate park, between the boat launch and Folsom Island. | Chisago | St. Croix River | Upper Mississippi | N 45.393557, W -92.665169 | 16 May 2015 |
| SCR - H | St. Croix River near Hudson, just south of Can Island. | Washington | St. Croix River | Upper Mississippi | N 44.967398, W -92.767879 | 29 June 2016 |
| CHR | Chippewa River at highway 70 bridge crossing, about ¼ mile up and downstream of bridge. | Chippewa | Minnesota River | Upper Mississippi | N 44.940727, W -95.730761 | 21 July 2015 |
| RLR | Red Lake River near High Landing, ¼ mile west of 30$^{th}$ Ave NE. | Pennington | Red River of the North | Souris-Red-Rainy | N 48.039096, W -95.820019 | 6 August 2015 |
| MSP | Mississippi River, about a mile south of Coon Rapids Dam. | Hennepin | Mississippi River | Upper Mississippi | N 45.062374, W -93.285229 | 10 September 2015 |

**Table 1.** Collection locations for five populations of *Potamilus alatus* in Minnesota in 2015 and 2016. Watersheds are named and defined by the United State Geological Survey (USGS).

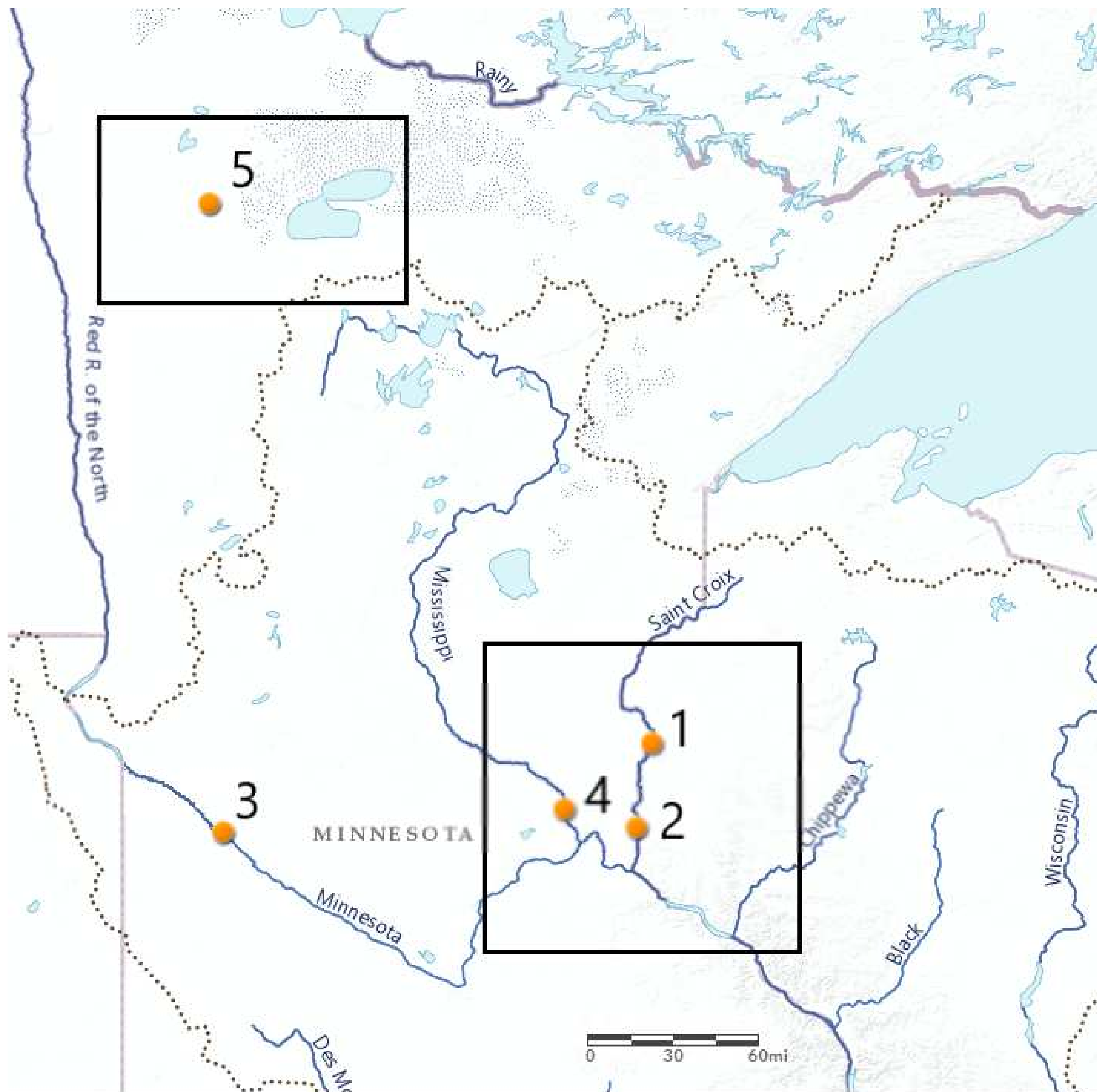


**Figure 1.a**. Sampling site locations for collection of *Potamilus alatus*, overview of all sites.1 – SCR-I, 2 – SCR-H, 3 – CHR, 4 – MSP, 5 – RLR. Map created using the Esri Hydro Reference Overlay in ArcGIS. Dashed lines show watershed boundaries.

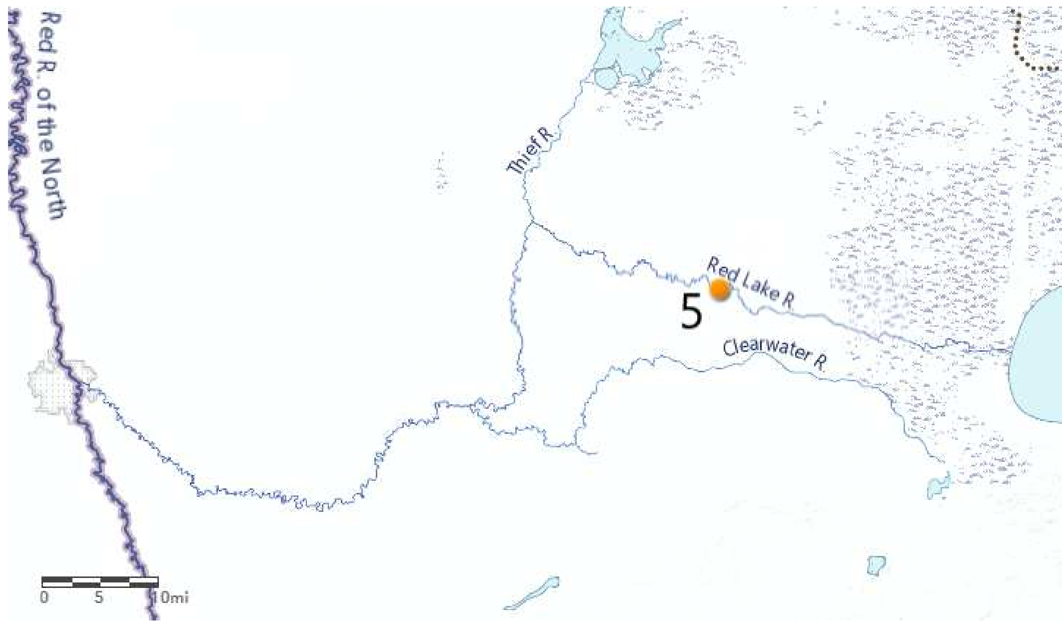


**Figure 1.b.** Sampling site locations for collection of *Potamilus alatus*. 5 – RLR. Map created using the Esri Hydro Reference Overlay in ArcGIS. Dashed lines show watershed boundaries

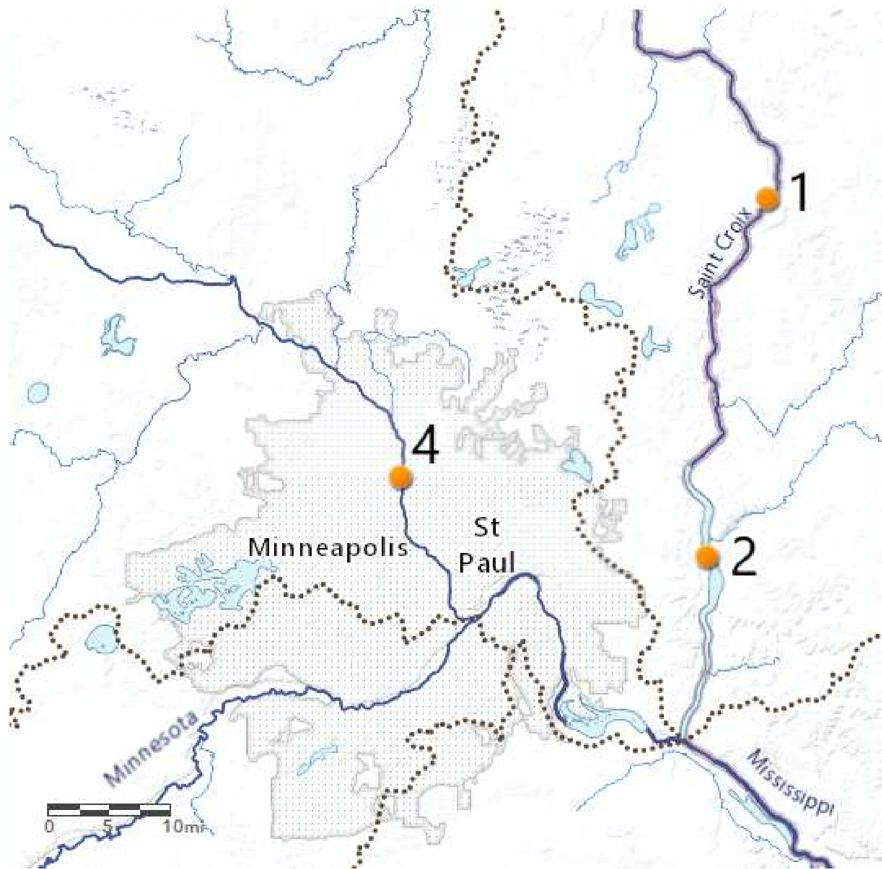


**Figure 1c.** Sampling site locations for collection of *Potamilus alatus* in the Mississippi River drainage. 1 – SCR-I, 2 – SCR-H, 4 – MSP. Map created using the Esri Hydro Reference Overlay in ArcGIS. Dashed lines show watershed boundaries.

| Marker Name | Group | Primer Sequence | Repeat Motif | Genbank #/ Citation |
|---|---|---|---|---|
| Lele45 | 1 | F: GCTAAGCTAAAGCTACAAATCACGC<br>R: AGTACACCTGAAATGACAATATGGC | ATCT | O'Bryhim et al. 2012 |
| Lele47 | 1 | F: TTCTTTGAGTGTCATTAGACGTGG<br>R: TGCCAGGCTGGATTTATAGG | AATG | O'Bryhim et al. 2012 |
| Lele3 | 2 | F: GAACAGTTATAATAGACCCTTAGTGTTACC<br>R: TTTGCAGTAAATAACTGAACCCG | AC | O'Bryhim et al. 2012 |
| Lele6 | 2 | F: GCTGGCTAACGTATCTGTAATGG<br>R: TTTGCAGTAAATAACTGAACCCG | ATAC | O'Bryhim et al. 2012 |
| Lele24 | 2 | F: CCATCAAAGACGTCGGAGC<br>R: CTCGGGTGCAATGTAGAGAGC | AAAC | O'Bryhim et al. 2012 |
| Lele33 | 2 | F: GGGTTAAAGTAGAACGGCGG<br>R: AACAGACCCTTAAATGGTGTCC | AAAG | O'Bryhim et al. 2012 |
| PCD110 | 3 | F: TGCAGTTTACAGTGGCAGTA<br>R: GCAGACGGAGTTGAGATG | TAGA | HM991153 |
| PCD122 | 3 | F: ATCGGATCAATTTTAGCAGTAG<br>R: TCCCCGTATTGTGCATAGATAC | TATC | HM991157 |
| PCD123 | 3 | F: ACAGAAGGACAGCCACACATAC<br>R: GTCCGACACCAATTTTATCTGA | AGAT | HM991158 |

**Table 2.** Microsatellite markers and associated primers used in analysis of population genetics of *Potamilus alatus*.

### *Data Analysis*

Scored alleles were analyzed with the program Microchecker (Oosterhout 2004) to look for evidence of genetic linkage, Hardy-Weinberg equilibrium, null alleles and allelic stuttering, and alleles with consistently high null frequencies were removed. The remaining alleles were analyzed in GeneAlEx version 6.503 (Peakall and Smouse 2006, 2012) to estimate divergence between populations using $F_{st}$ values. A hierarchical AMOVA analysis was run using the region feature in GeneAlEx, designating each watershed as a region (Red Lake River in the Souris-Red-Rainy watershed, all others in

the Upper Mississippi watershed), in order to compare variation among watersheds with variation among populations. HPrare (Kalinowski 200) used to estimate allelic richness with a rarefaction approach that standardizes to a common sample size. NeEstimator (Do et al. 2014) was used to estimate the effective population size using the molecular co-ancestry technique developed by Nomura (2008).

The number of genetically distinct populations was determined via the program Structure (Pritchard et al. 2000), which estimates the number of distinct genetic clusters, i.e., populations, and estimates the ancestry contributed by each population to individuals. The program was run with both locprior (which takes in to account the geographic information from each individual) and without, all other settings were default. There was a burnin length of 10,000 followed by another 50,000 repetitions. Output for different populations was compared using the ΔK method of Evanno et al. (2005) to determine the optimal number of genetically distinct. Poptree (Takezaki et al. 2014) was used to infer a phylogenetic tree of populations using neighbor joining methods. Bottleneck (Piry et al. 1999) was used with default settings to detect recent genetic bottlenecks in populations based on excess heterozygotes at each locus relative to expectations at mutation-drift equilibrium

## Results

Null alleles were found in six loci (PCD 123, PCD 110, Lele 3, Lele 122, Lele 45 and Lele 6); however, they were inconsistently detected and in low frequencies (well below 20 percent) in all populations except for Lele 45 and Lele 6. Lele 6 and Lele 45 were dropped from the dataset and all other analyses were run with the remaining 7 loci. PCD 123 also had a high frequency of null alleles near the cutoff amount (17%) so analyses were run with and without PCD 123 in the dataset: dropping PCD 123 did not change

any broad trends among the data, but did increase $F_{st}$ along with standard error. There was no evidence of genetic linkage disequilibrium in any of populations or loci pairs. With this reduced dataset, individuals with more than three missing markers were removed from analysis (Table 3).

Rarefication analysis found a range of 4.59 alleles (RLR) to 8.31 (CHP) (Table 4). Lele 24 had the lowest number of alleles per population (3-5) while PCD 122 had the highest (6-20). Private alleles (alleles found in one population and no others) occurred in every population. The population of St. Croix River at Hudson had the highest number of private alleles—found at almost every locus. The Red Lake River had the lower number of private alleles, found only in two loci already characterized by high allele diversity.

| Name | Number of Individuals |
| --- | --- |
| St. Croix River – Interstate Park (SCR – I) | 31 |
| St. Croix River – Hudson (SCR-H) | 14 |
| Chippewa River (CHR) | 40 |
| Mississippi River (MSP) | 27 |
| Red Lake River (RLR) | 37 |

**Table 3**. Number of *Potamilus alatus* individuals used in analyses from each location, after individuals with more than three missing markers were removed.

Expected and observed heterozygosity was similar for all populations, except for the Chippewa River population which had a lower observed heterozygosity compared to other populations (table 4). See appendix 2 for a list of all expected and observed heterozygosity in each loci and population.

| Population | Allelic Richness | $F_{IS}$ | $H_o$ | $H_e$ | Estimated $N_e$ |
|---|---|---|---|---|---|
| St. Croix River – Interstate Park (SCR – I) | 7.41 | 0.061 | 0.712 | 0.777 | Infinite |
| St. Croix River – Hudson (SCR-H) | 7.72 | 0.091 | 0.781 | 0.773 | 999 |
| Chippewa River (CHR) | 7.36 | 0.097 | 0.669 | 0.749 | 1945 |
| Mississippi River (MSP) | 8.31 | 0.015 | 0.787 | 0.798 | 1904 |
| Red Lake River (RLR) | 4.59 | 0.007 | 0.622 | 0.643 | 434 |

**Table 4.** Allelic richness, $F_{IS}$, and expected and observed heterozygosity values and estimated Ne for five populations of *Potamilus alatus*. $N_e$ was calculated using the software NE estimator using the linkage disequilibrium method, infinite Ne results imply the real $N_e$ was too large for the sample size to distinguish the effects of disequilibrium from sampling error.

All population pairwise $F_{st}$ values were found to be significantly different than zero (Table 5; including data from PCD 123). $F_{st}$ values were the highest between the Red Lake River population and all others, ( 0.071 - 0.089), while only 0.013-0.039 between all other populations. AMOVA results found most diversity explained within and among individuals ($p <0.001$). However 8% of diversity was explained with variation between watersheds (between the Red Lake River population and all others), and only 3% explained by variation between the four populations within the Mississippi watershed.

| | SCR-I | SCR-H | CHR | RLR | MSP |
|---|---|---|---|---|---|
| St. Croix River – Interstate Park (SCR – I) | 0 | - | - | - | - |
| St. Croix River – Hudson (SCR-H) | 0.025 | 0 | - | - | - |
| Chippewa River (CHR) | 0.013 | 0.027 | 0 | - | - |
| Red Lake River (RLR) | 0.081 | 0.089 | 0.089 | 0 | - |
| Mississippi River (MSP) | 0.025 | 0.039 | 0.033 | 0.071 | 0 |

**Table 5.** $F_{st}$ values between populations on *Potamilus alatus* in Minnesota rivers. $P < 0.001$ in all pairings.

The ΔK method found strong support for K=2 populations within Structure (Figure 2). There was no substantial difference in results between runs with locprior and without, results are shown with locprior. Red Lake River samples were consistently grouped in the same population, whereas all other populations were assigned to another ancestral group (Figure 3). The Evanno method is known to often detect the highest level of structure among populations (Evanno et al. 2005), so higher K values were examined to assess possible finer sub-structure. At K = 3 and 5 (the number of sample sites), the Red sample consistently formed one group while no other presumed ancestry was associated with a sampling site, indicating a lack of further sub-structure. Program Poptree supported a grouping of all Mississippi watershed populations together, with the Red Lake River as an outgroup (Figure 3). Bottleneck (Piry et al. 1999) found no evidence of past genetic bottlenecks in any population.

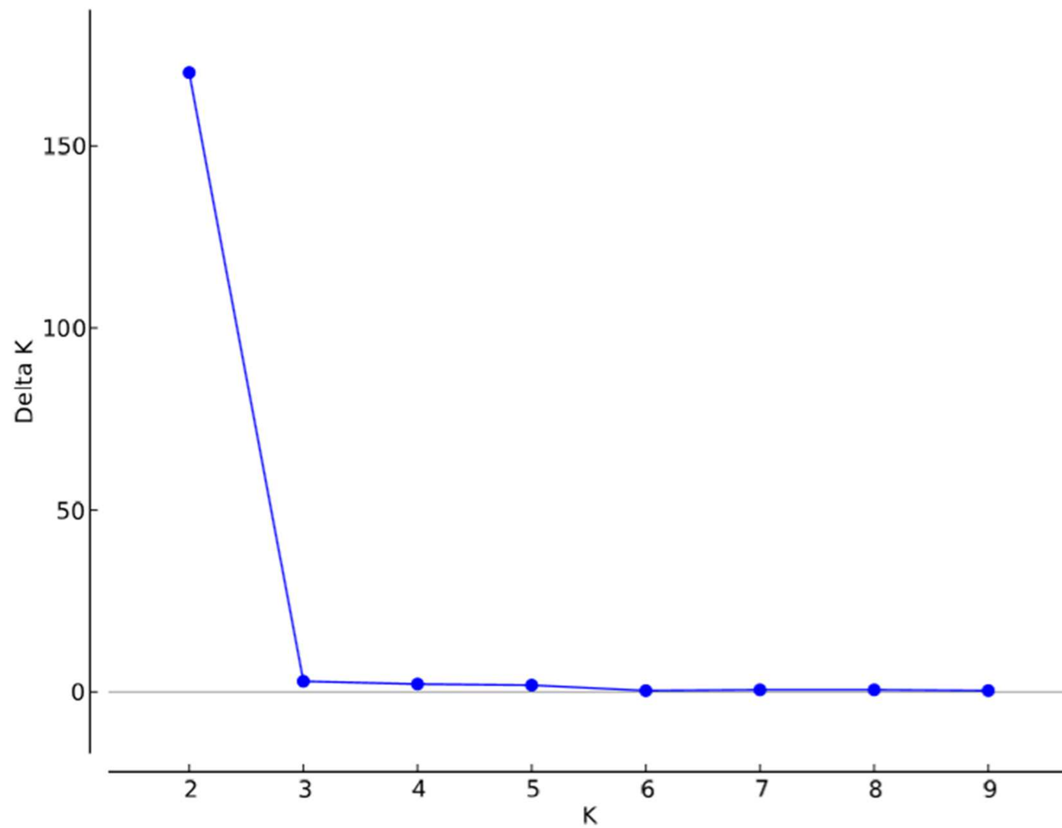


**Figure 2.** Delta K values for *Potamilus alatus* populations evaluating assumed numbers of distinct genetic clusters, i.e., populations (K) = 1-10 in Structure, each K value run with 20 replicates. The largest value of delta K indicates the number of distinct populations best supported by the data (i.e., K=2). Output analyzed using Structure Harvester (Dent and vonHoldt 2012).

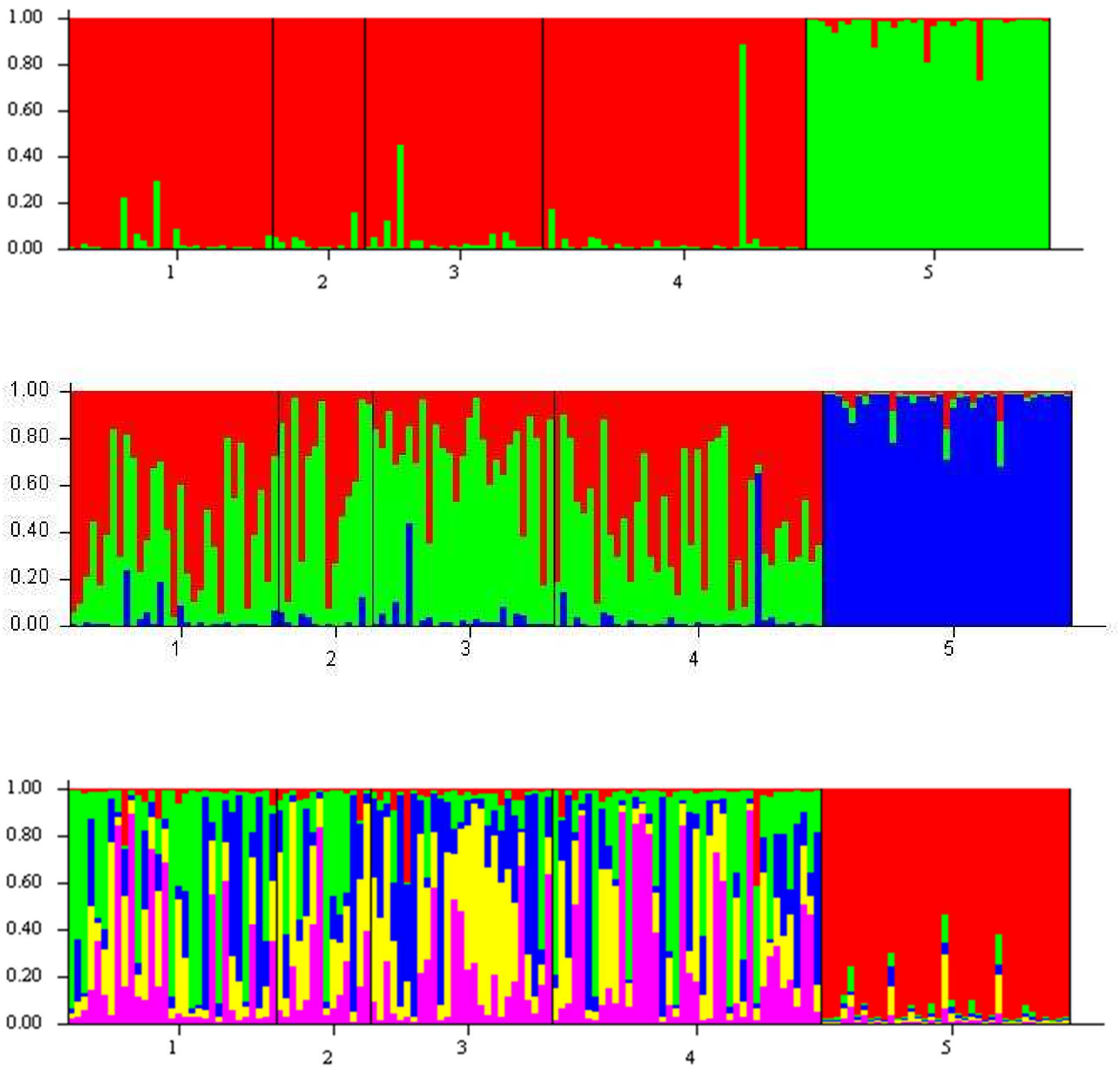


**Figure 3.** Bar graph results for Structure analysis of *Potamilus alatus* for various assumed population (K). Top: K =2, middle: K=3, bottom: K=5 Numbers below bar graphs correspond to geographic populations. 1 = SCR-I, 2= SCR-H, 3 = CHR, 4 = MSP, 5 = RLR. Vertical lines represent individuals, colors represent proportion of assigned ancestry.

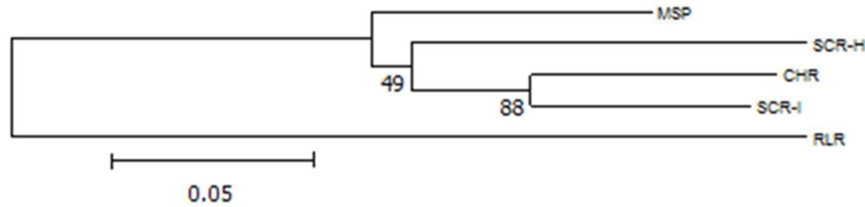


**Figure 4**. Neighbor-Joining tree using microsatellite data made using program PoptreeW (Takezaki et al. 2014) for five populations of *Potamilus alatus*. Numbers at branches indicate bootstrap values out of 100 replicates.

## Discussion

Effective conservation of species benefits from a good understanding of the genetic diversity and connectivity between their populations (Haag 2012). This may be difficult for small populations with fragmented distributions. Studies of wide-ranging, abundant species can provide useful information for conservation managers should these species later decline (Berg et al. 2007).

This study examined the population genetics of *Potamilus alatus*, a wide-ranging species of freshwater mussel. Results suggest the existence of weak structure between the four populations found in the Mississippi River watershed (SCR-I, SCR-H, MSP and CHR). In contrast, the Red Lake River population was consistently grouped as a separate population by Structure, had relatively high $F_{st}$ values, and was found to be the most distantly related population by Poptree.

The Red Lake River population has the highest $F_{st}$ values between it and other populations. It also exhibits the lowest genetic diversity within population, in terms of number of both overall and unique alleles. Though there was no evidence of a genetic bottleneck in this population, the extremely low number of unique alleles in this population suggests that it was possibly founded by a small set of migrants from the Mississippi watershed, which would bring along a subset of alleles found in the more

genetically diverse Mississippi River. This is congruent with patterns observed in Freshwater Drum, which show decreased genetic diversity in the Red River drainage system compared to others, and appear to have relatively recently colonized the Red River from the Mississippi River system (Jarvinen 2011).

The Red River was historically connected to the Minnesota and Mississippi rivers via the glacial river Warren, which drained Lake Agassiz (the remnant of which is now Lake Winnipeg) south to the Gulf of Mexico about 11,700 – 9,500 years ago (Wright 1990). Lake Winnipeg now drains north to Hudson Bay via Lake Nelson, and the Red River flows north into Lake Winnipeg (Wright 1990). The remnants of glacial river Warren now make up the Minnesota River, whose headwaters are separated from the headwaters of the Red River by a roughly 1.6 km long strip of land called the Traverse Gap. This area floods regularly, allowing the gap to close and the Red and Minnesota drainages to be briefly connected (Spading 1999). The building of a dike and culvert boxes on Lake Traverse in 1941 and 1943 has reduced the chances of interbasin flow, but it’s estimated that the two drainages still connect roughly once every ten years (Spading 2001). Presumably, the gap flooded even more frequently before the dike was installed, and there are historic accounts of this flooding and possible interbasin fauna transfer before the construction of modern day dikes (Featherstonhaugh 1847). There are other places where the two drainages come close, and tagged Walleye *Sander vitreus* have been documented crossing the continental divide; thus, there appear to be many opportunities for faunal transfer between the two watersheds (Ludwig and Leitch 1995).

Previous studies examining genetic differentiation in fish and mussels between the Red River of the North and other river systems have found mixed results. Using mitochondrial DNA, studies have found no difference between populations in the upper Mississippi and Red River basins in the Common Mudpuppy *Necturus maculosus maculosus* (Stedman

2016) or the freshwater mussel Plain Pocketbook *Lampsilis cardium* (Monroe 2008). A microsatellite study on Walleye (Stepien et al. 2009) did find significant differentiation between Red River populations and the others, including nearby Mississippi river populations. Another microsatellite study on Pugnose Shiners *Notropis anogenus* (McCuster et al. 2014) found different populations on tributaries of the Red River to be genetically closer to nearby populations in other watersheds than to each other, and suggest this may be because Pugnose Shiners may be used as a bait fish and individuals could have been transferred across watersheds by humans.

Overall, studies using microsatellite markers, as opposed to mitochondrial DNA, appear to show more pronounced differences between Red River populations and Mississippi populations. The ecology of the study organism might also affect whether there is significant gene flow between basins. Pugnose Shiners and Common Mudpuppies are more likely to inhabit small headwaters than Walleye and Freshwater Drum (the host for *Potamilus alatus*). Since the Red River basin and Mississippi basins connect when shallow headwater regions flood, organisms that live in deeper waters might be less susceptible to transfer between basins. There are numerous dams separating each of the sampled populations in different rivers, ranging from ten to 93 feet in height (National Inventory of Dams – USACE), which do not appear to be influencing population structure. Though there have been numerous studies showing that dams influence the distribution and abundance of unionids (Watters 1996, McRae et al. 2004, Tiemann et al. 2007), studies that looked at the genetic effects of impoundments suggest that the relatively long lifespan of unionid species helps insulate them from fragmentation (Fuller 2017). In addition, *Potamilus alatus* uses only Freshwater Drum as its fish host, a species which is highly mobile and has been documented to move hundreds of kilometers in a year. (Funk 2011). They are also known to at least occasionally pass

across the tallest dams in Minnesota, presumably via locks (Stiras 2014). The mobility of fish hosts appears to play a large role in freshwater mussel dispersal abilities, as mussels with less mobile fish hosts tend to have more differentiation between populations than species with highly mobile ones (Mock et al. 2013). *Potamilus alatus* are also long term brooders - glochidia attach to their host in the fall, and do not drop off until spring (Sietman et al. 2018), giving them a higher chance to move away from their parental population.

Although the Mississippi watershed populations appear to exhibit high genetic similarity, there is still evidence for small but significant differentiation between them. This could lead to issues if any of these populations are used as sources to establish new populations in the future, especially if two or more of these are combined as a source, as this could lead to outbreeding depression (Houde et al. 2015). In particular, the Mississippi River population was the only one collected on the main stem of a large river, and may have unique genetic adaptations for large river living not found in the other populations sampled. The Red Lake River appears to be more isolated, and an effort should be made to not move mussels between the Red River and Mississippi River watersheds, as this population may have accumulated unique alleles in a different watershed. In addition, the Red Lake River population had much lower allelic richness than other populations, and contained the lowest number of private alleles, and should not be used as a single source population for transplants as any transplanted population might not have the necessary genetic diversity to adapt to a new environment (Houde et al. 2015) Further studies of the Red River may show whether populations in this watershed show high connectivity within the watershed, or whether they are more distinct from each other than from the populations of the Mississippi watershed.

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## Appendix 1 – Allele frequencies for all seven loci in all populations

| **Allele Frequencies and Sample Size by Populations** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Locus** | **Allele/n** | | **SCR-I** | **SCR-H** | **CHR** | **RLR** | **MSP** |
| **Lele 24** | **N** | | 31 | 14 | 40 | 37 | 27 |
| | | **180** | 0.000 | 0.000 | 0.013 | 0.000 | 0.000 |
| | | **188** | 0.177 | 0.143 | 0.238 | 0.338 | 0.259 |
| | | **192** | 0.065 | 0.000 | 0.038 | 0.000 | 0.019 |
| | | **196** | 0.000 | 0.143 | 0.000 | 0.000 | 0.056 |
| | | **200** | 0.710 | 0.607 | 0.675 | 0.527 | 0.611 |
| | | **204** | 0.048 | 0.107 | 0.038 | 0.135 | 0.056 |
| **Lele 3** | **N** | | 22 | 14 | 35 | 34 | 20 |
| | | **378** | 0.000 | 0.000 | 0.000 | 0.000 | 0.050 |
| | | **380** | 0.023 | 0.000 | 0.014 | 0.000 | 0.025 |
| | | **382** | 0.000 | 0.000 | 0.000 | 0.000 | 0.025 |
| | | **384** | 0.205 | 0.214 | 0.143 | 0.000 | 0.175 |
| | | **386** | 0.159 | 0.179 | 0.200 | 0.000 | 0.125 |
| | | **388** | 0.068 | 0.143 | 0.029 | 0.441 | 0.025 |
| | | **390** | 0.114 | 0.036 | 0.057 | 0.029 | 0.075 |
| | | **392** | 0.023 | 0.000 | 0.057 | 0.000 | 0.000 |
| | | **394** | 0.068 | 0.071 | 0.171 | 0.000 | 0.100 |
| | | **396** | 0.205 | 0.107 | 0.114 | 0.235 | 0.100 |
| | | **398** | 0.068 | 0.107 | 0.100 | 0.015 | 0.100 |
| | | **400** | 0.068 | 0.036 | 0.029 | 0.000 | 0.100 |
| | | **402** | 0.000 | 0.000 | 0.029 | 0.044 | 0.000 |
| | | **404** | 0.000 | 0.000 | 0.000 | 0.044 | 0.025 |
| | | **406** | 0.000 | 0.071 | 0.000 | 0.088 | 0.000 |
| | | **408** | 0.000 | 0.000 | 0.000 | 0.103 | 0.000 |
| | | **410** | 0.000 | 0.036 | 0.043 | 0.000 | 0.050 |
| | | **412** | 0.000 | 0.000 | 0.014 | 0.000 | 0.025 |
| **Lele 33** | **N** | | 28 | 14 | 40 | 34 | 20 |
| | | **128** | 0.125 | 0.214 | 0.150 | 0.088 | 0.175 |
| | | **132** | 0.000 | 0.179 | 0.000 | 0.000 | 0.025 |
| | | **136** | 0.214 | 0.179 | 0.263 | 0.191 | 0.275 |
| | | **140** | 0.161 | 0.179 | 0.013 | 0.088 | 0.100 |
| | | **172** | 0.411 | 0.250 | 0.438 | 0.632 | 0.400 |
| | | **176** | 0.089 | 0.000 | 0.088 | 0.000 | 0.025 |
| | | **180** | 0.000 | 0.000 | 0.050 | 0.000 | 0.000 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **PCD 123** | **N** | | 29 | 13 | 35 | 37 | 25 |
| | | **155** | 0.000 | 0.000 | 0.043 | 0.000 | 0.000 |
| | | **158** | 0.000 | 0.154 | 0.000 | 0.000 | 0.000 |
| | | **159** | 0.052 | 0.000 | 0.171 | 0.203 | 0.140 |
| | | **162** | 0.000 | 0.154 | 0.000 | 0.000 | 0.000 |
| | | **163** | 0.034 | 0.000 | 0.129 | 0.135 | 0.140 |
| | | **167** | 0.121 | 0.000 | 0.057 | 0.000 | 0.020 |
| | | **170** | 0.000 | 0.077 | 0.000 | 0.000 | 0.000 |
| | | **171** | 0.069 | 0.000 | 0.086 | 0.257 | 0.100 |
| | | **174** | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | | **175** | 0.190 | 0.000 | 0.086 | 0.027 | 0.040 |
| | | **178** | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | | **179** | 0.121 | 0.000 | 0.071 | 0.000 | 0.100 |
| | | **182** | 0.000 | 0.269 | 0.000 | 0.000 | 0.000 |
| | | **183** | 0.017 | 0.000 | 0.057 | 0.000 | 0.060 |
| | | **186** | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | | **187** | 0.103 | 0.000 | 0.129 | 0.000 | 0.120 |
| | | **190** | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | | **191** | 0.034 | 0.000 | 0.043 | 0.000 | 0.020 |
| | | **194** | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | | **195** | 0.052 | 0.000 | 0.000 | 0.000 | 0.100 |
| | | **199** | 0.034 | 0.000 | 0.057 | 0.297 | 0.040 |
| | | **202** | 0.000 | 0.115 | 0.000 | 0.000 | 0.000 |
| | | **203** | 0.052 | 0.000 | 0.014 | 0.000 | 0.040 |
| | | **206** | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 |
| | | **207** | 0.086 | 0.000 | 0.014 | 0.000 | 0.000 |
| | | **211** | 0.000 | 0.000 | 0.029 | 0.000 | 0.060 |
| | | **215** | 0.000 | 0.000 | 0.000 | 0.000 | 0.020 |
| | | **219** | 0.034 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | **227** | 0.000 | 0.000 | 0.014 | 0.027 | 0.000 |
| | | **235** | 0.000 | 0.000 | 0.000 | 0.014 | 0.000 |
| | | **239** | 0.000 | 0.000 | 0.000 | 0.041 | 0.000 |
| **PCD 110** | **N** | | 31 | 13 | 40 | 37 | 25 |
| | | **264** | 0.000 | 0.000 | 0.000 | 0.000 | 0.100 |
| | | **267** | 0.177 | 0.000 | 0.075 | 0.000 | 0.040 |
| | | **268** | 0.000 | 0.000 | 0.000 | 0.068 | 0.400 |
| | | **271** | 0.419 | 0.577 | 0.650 | 0.054 | 0.100 |
| | | **275** | 0.016 | 0.000 | 0.063 | 0.000 | 0.020 |
| | | **279** | 0.032 | 0.038 | 0.013 | 0.257 | 0.020 |
| | | **283** | 0.032 | 0.038 | 0.000 | 0.014 | 0.020 |
| | | **287** | 0.016 | 0.038 | 0.088 | 0.000 | 0.040 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | **291** | 0.032 | 0.077 | 0.013 | 0.000 | 0.040 |
| | | **295** | 0.081 | 0.115 | 0.075 | 0.000 | 0.100 |
| | | **299** | 0.048 | 0.077 | 0.000 | 0.000 | 0.020 |
| | | **303** | 0.048 | 0.000 | 0.025 | 0.000 | 0.000 |
| | | **305** | 0.000 | 0.000 | 0.000 | 0.311 | 0.000 |
| | | **307** | 0.048 | 0.000 | 0.000 | 0.243 | 0.000 |
| | | **311** | 0.000 | 0.038 | 0.000 | 0.000 | 0.040 |
| | | **315** | 0.048 | 0.000 | 0.000 | 0.000 | 0.040 |
| | | **319** | 0.000 | 0.000 | 0.000 | 0.054 | 0.020 |
| **Lele 47** | **N** | | 31 | 10 | 31 | 18 | 19 |
| | | **175** | 0.000 | 0.050 | 0.000 | 0.000 | 0.000 |
| | | **179** | 0.161 | 0.000 | 0.129 | 0.000 | 0.053 |
| | | **183** | 0.048 | 0.100 | 0.129 | 0.000 | 0.105 |
| | | **187** | 0.274 | 0.250 | 0.323 | 0.139 | 0.132 |
| | | **191** | 0.210 | 0.350 | 0.226 | 0.611 | 0.211 |
| | | **195** | 0.145 | 0.150 | 0.065 | 0.083 | 0.368 |
| | | **199** | 0.048 | 0.000 | 0.032 | 0.139 | 0.079 |
| | | **203** | 0.097 | 0.050 | 0.000 | 0.000 | 0.026 |
| | | **207** | 0.000 | 0.000 | 0.016 | 0.000 | 0.000 |
| | | **211** | 0.016 | 0.000 | 0.016 | 0.028 | 0.000 |
| | | **215** | 0.000 | 0.000 | 0.048 | 0.000 | 0.026 |
| | | **219** | 0.000 | 0.000 | 0.016 | 0.000 | 0.000 |
| | | **227** | 0.000 | 0.050 | 0.000 | 0.000 | 0.000 |
| **PCD 122** | **N** | | 28 | 12 | 37 | 32 | 25 |
| | | **220** | 0.000 | 0.000 | 0.014 | 0.000 | 0.040 |
| | | **224** | 0.000 | 0.000 | 0.014 | 0.000 | 0.000 |
| | | **260** | 0.036 | 0.000 | 0.041 | 0.000 | 0.020 |
| | | **268** | 0.018 | 0.042 | 0.000 | 0.000 | 0.000 |
| | | **272** | 0.000 | 0.000 | 0.014 | 0.000 | 0.000 |
| | | **276** | 0.000 | 0.000 | 0.014 | 0.000 | 0.040 |
| | | **284** | 0.000 | 0.000 | 0.041 | 0.000 | 0.040 |
| | | **288** | 0.000 | 0.000 | 0.068 | 0.000 | 0.000 |
| | | **292** | 0.089 | 0.000 | 0.081 | 0.000 | 0.020 |
| | | **296** | 0.018 | 0.042 | 0.027 | 0.000 | 0.000 |
| | | **300** | 0.000 | 0.083 | 0.068 | 0.000 | 0.080 |
| | | **304** | 0.107 | 0.042 | 0.189 | 0.000 | 0.080 |
| | | **308** | 0.196 | 0.167 | 0.095 | 0.016 | 0.080 |
| | | **312** | 0.036 | 0.083 | 0.068 | 0.656 | 0.120 |
| | | **316** | 0.125 | 0.125 | 0.081 | 0.031 | 0.120 |
| | | **320** | 0.107 | 0.125 | 0.041 | 0.000 | 0.100 |
| | | **324** | 0.089 | 0.083 | 0.027 | 0.016 | 0.040 |

| | | | | | |
|---|---|---|---|---|---|
| **328** | 0.018 | 0.000 | 0.027 | 0.000 | 0.060 |
| **332** | 0.018 | 0.042 | 0.068 | 0.031 | 0.040 |
| **336** | 0.000 | 0.042 | 0.000 | 0.000 | 0.020 |
| **340** | 0.036 | 0.042 | 0.000 | 0.000 | 0.040 |
| **344** | 0.071 | 0.042 | 0.014 | 0.000 | 0.000 |
| **348** | 0.000 | 0.000 | 0.014 | 0.250 | 0.020 |
| **352** | 0.018 | 0.000 | 0.000 | 0.000 | 0.000 |
| **360** | 0.000 | 0.000 | 0.000 | 0.000 | 0.020 |
| **364** | 0.018 | 0.042 | 0.000 | 0.000 | 0.000 |
| **368** | 0.000 | 0.000 | 0.000 | 0.000 | 0.020 |

## Appendix 2 – He and Ho for all seven loci in all populations

| Pop | Locus | N | Ho | He | Probability | Significance |
|---|---|---|---|---|---|---|
| **SCR-I** | **Lele 24** | 31 | 0.516 | 0.458 | 0.908 | ns |
| | **Lele 3** | 22 | 0.682 | 0.858 | 0.476 | ns |
| | **Lele 33** | 28 | 0.929 | 0.736 | 0.026 | * |
| | **PCD 123** | 29 | 0.483 | 0.899 | 0 | *** |
| | **PCD 110** | 31 | 0.645 | 0.773 | 0.059 | ns |
| | **Lele 47** | 31 | 0.871 | 0.819 | 0.665 | ns |
| | **PCD 122** | 28 | 0.857 | 0.896 | 0.797 | ns |
| | **Lele 45** | 26 | 0.269 | 0.835 | 0 | *** |
| **SCR-H** | **Lele 24** | 14 | 0.714 | 0.579 | 0.687 | ns |
| | **Lele 3** | 14 | 0.929 | 0.865 | 0.675 | ns |
| | **Lele 33** | 14 | 1 | 0.796 | 0.259 | ns |
| | **PCD 123** | 13 | 0.692 | 0.852 | 0.721 | ns |
| | **PCD 110** | 13 | 0.615 | 0.636 | 0.017 | * |
| | **Lele 47** | 10 | 0.6 | 0.775 | 0.096 | ns |
| | **PCD 122** | 12 | 0.833 | 0.92 | 0.214 | ns |
| | **Lele 45** | 10 | 0.1 | 0.805 | 0 | *** |
| **CHP** | **Lele 24** | 40 | 0.4 | 0.485 | 0.025 | * |
| | **Lele 3** | 35 | 0.857 | 0.876 | 0 | *** |
| | **Lele 33** | 40 | 0.85 | 0.707 | 0.002 | ** |
| | **PCD 123** | 35 | 0.571 | 0.903 | 0.001 | ** |
| | **PCD 110** | 40 | 0.525 | 0.554 | 0.936 | ns |
| | **Lele 47** | 31 | 0.774 | 0.803 | 0.563 | ns |
| | **PCD 122** | 37 | 0.703 | 0.916 | 0.002 | ** |
| | **Lele 45** | 38 | 0.421 | 0.885 | 0 | *** |
| **MSP** | **Lele 24** | 27 | 0.407 | 0.553 | 0.476 | ns |
| | **Lele 3** | 20 | 0.85 | 0.9 | 0.151 | ns |
| | **Lele 33** | 20 | 1 | 0.723 | 0.332 | ns |
| | **PCD 123** | 25 | 0.68 | 0.903 | 0 | *** |
| | **PCD 110** | 25 | 0.68 | 0.8 | 0.01 | ** |
| | **Lele 47** | 19 | 0.895 | 0.781 | 0.855 | ns |
| | **PCD 122** | 25 | 1 | 0.926 | 0.421 | ns |
| | **Lele 45** | 22 | 0.455 | 0.854 | 0.015 | * |
| **RR** | **Lele 24** | 37 | 0.595 | 0.59 | 0.205 | ns |
| | **Lele 3** | 34 | 0.794 | 0.727 | 0 | *** |
| | **Lele 33** | 34 | 0.706 | 0.548 | 0.251 | ns |

| | | | | | |
|---|---|---|---|---|---|
| **PCD 123** | 37 | 0.595 | 0.783 | 0.158 | ns |
| **PCD 110** | 37 | 0.541 | 0.768 | 0.022 | * |
| **Lele 47** | 18 | 0.5 | 0.58 | 0.125 | ns |
| **PCD 122** | 32 | 0.625 | 0.504 | 0.991 | ns |
| **Lele 45** | 16 | 0 | 0 | na | na |

**Key: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001**