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DIFFERENTIATING BETWEEN INVASIVE AND NATIVE POPULATIONS OF BIGHEAD AND SILVER CARP USING MS-AFLP

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By

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A Thesis

Submitted in Partial Fulfillment of the Requirements for the

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When a species is introduced outside their native range, the genetic diversity of the introduced population is generally decreased due to the founder effect, and the fitness of individuals in the introduced population may decrease due to inbreeding depression. Invasive species are a paradox to this paradigm because while the initial population size of an invasive species may be small in their non-native range, the individuals are able to survive, eat, and reproduce so successfully, that they have deleterious effects on native species. One mechanism that invasive species use to overcome a lack of genetic diversity and adapt to their new environment is CpG methylation, a heritable and environmentally influenced epigenetic modification that regulates the expression of certain genes to alter phenotypes without altering an organism's DNA sequence.

Bighead and silver carps, two species of bigheaded carp native to eastern Asia, are believed to have been introduced to the United States in the 1970s. Since that time, populations of both bighead and silver carp have surged, particularly in the Mississippi River drainage, where they compete with native planktivores for food, injure boaters, and threaten the multi-million dollar fisheries industry in the Great Lakes.

In this study, methylation-sensitive amplified fragment length polymorphisms (MS-AFLPs) were used to analyze the genetic and epigenetic diversity of bighead and silver carp

from the Gan, Pearl, and Yangtze rivers in their native China and from the Illinois River in the United States. While the heterozygosity of silver carp in Illinois was not found to be significantly lower than that of silver carp in China, the silver carp in Illinois did show a significantly higher level of methylation compared to Chinese silver carp. There is evidence that CpG methylation may play a significant role in allowing silver carp to adapt and thrive in an introduced environment.

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CHAPTER 1

INTRODUCTION

Epigenetics

Epigenetics is the study of changes in gene expression that are not explained by alterations in DNA sequence (Holliday 1994; Holliday 2006; Richards 2006). Epigenetic mechanisms such as DNA methylation, histone modification, and chromatin structure are responsible for these changes in gene expression (Holliday 1994; Holliday 2006). This allows for phenotypic plasticity, expression of multiple phenotypes from the same genotype (Pigliucci 2005). Epigenetic states may be heritable (Richards 2006; Richards 2011). Epigenetic changes can be influenced by environmental factors such as temperature, food availability, and chemical pollutants, and may allow organisms to quickly adapt their phenotype in response to new environments and changing climate conditions (Richards et al. 2010). Epigenetic changes can occur within a much smaller time frame than strictly genetic evolution, and may be responsible for the rapid phenotypic changes associated with invasive species (Bossdorf et al. 2008).

There are several molecular processes by which epigenetic mechanisms can alter gene expression by activating, reducing, or disabling the activity of specific genes (Bossdorf et al. 2008). The best-studied epigenetic mechanism is DNA methylation (Bender 2004), which occurs when a methyl group is added to a cytosine, most frequently when the cytosine is followed by a guanine in the DNA sequence. These methylation sites, known as CpG sites, tend to be clustered in the regulatory region of genes (Bossdorf et al. 2008). However, there is a complicated relationship between CpG methylation and variation in gene expression (Nätt et al. 2012).

DNA methylation can be screened using a methylation-sensitive Amplified Fragment

Length Polymorphism (MS-AFLP), a modification of the traditional AFLP protocol (Cervera et al. 2002; Vos et al. 1995). In this technique, genomic DNA is digested using restriction endonucleases, typically *Eco*RI and *Msp*I/*Hpa*II. The methylation state of the DNA will differentially affect the ability of *Msp*I and *Hpa*II to cleave at the recognition sequence CpCpGpG. *Msp*I is not sensitive to methylation and cleaves both methylated and non-methylated sites, while *Hpa*II is methylation sensitive, and will only cleave the site if it is not methylated. In AFLP, fragments generated by the restriction digest are ligated to adapters that allow them to be selectively amplified using polymerase chain reaction (PCR). The PCR products can then be separated and visualized using polyacrylamide gel or capillary electrophoresis (Reyna-López 1997). This technique is relatively inexpensive and straight-forward to perform, which makes it a good, first choice for screening for epigenetic diversity.

In invasive species, epigenetics may play a role in the adaptation of invaders in their introduced habitat through phenotypic plasticity (Richards et al. 2012; Pigliucci 2001; Schrey et al. 2012; Blanchet 2012). Researchers in the budding field of ecological epigenetics study the mechanisms by which epigenetic variation produces phenotypes that allow organisms to fine tune gene expression in a particular environment (Schrey et al. 2013; Kilvitis et al. 2014; Bossdorf and Zhang 2011). Ecological epigenetics is particularly helpful in understanding how exotic and invasive species are able to adapt and thrive in their introduced habitats (Schrey et al. 2014; Richards et al. 2008; Richards et al. 2012).

In recent years, there have been several studies that have examined the relationship between epigenetics and the successful invasion of non-native organisms in novel environments. The house sparrow (*Passer domesticus*) is a songbird species that has been introduced throughout much of the world. Introduced populations typically have low genetic diversity due

to a small initial population size, but tend to be phenotypically variable. Liebl et al. (2013) analyzed DNA methylation patterns of a population of house sparrows that was introduced to Kenya in the 1950s. They found that despite the low levels of genetic diversity within the introduced population, the Kenyan sparrows had high epigenetic variation that was negatively correlated with genetic variation and positively correlated with inbreeding. This suggests that methylation is the mechanism responsible for the high levels of phenotypic plasticity observed in introduced populations with low genetic diversity.

Richards et al. (2008, 2012) investigated the epigenetic variation present in Japanese knotweed (*Fallopia japonica*), a plant species that has been introduced in Europe and the United States. They grew samples of Japanese knotweed from three different habitat types in a common garden and subjected the plants to controlled salt treatments. They found that when knotweed cultivars from all three habitat types were grown under the same conditions, there was significant phenotypic variation. The plants exhibited almost no genetic diversity between habitat types, but there were high levels of epigenetic diversity, which shows that epigenetic variation could allow invasive species like the Japanese knotweed to compensate for low genetic variation and successfully colonize new environments.

There have also been several studies that have linked phenotypically plastic traits to epigenetic variation in wild populations of fishes. Male Atlantic salmon (*Salmo salar*) parr can achieve early maturation faster in some rivers than in others. Morán and Pérez-Figueroa (2011) used AFLP to investigate genetic variation between Atlantic salmon males in different rivers and at different maturation stages. They also used methylation-sensitive AFLP to show the epigenetic differentiation of male salmon from different rivers and at different maturation stages. While no significant genetic differentiation was found for both comparisons, there were

significant levels of epigenetic variation between rivers and between different maturity states. This shows that epigenetic modifications may allow for variation in certain phenotypic traits that are not mediated by genetic variation.

In another study, Shao et al. (2014) investigated the role of DNA methylation in environmental sex reversal in the half-smooth tongue sole (*Cynoglossus semilaevis*), a marine fish found in the coastal waters of China. While the sex of most organisms is determined at fertilization by genetic factors, in some species like the half-smooth tongue sole, a variety of environmental factors can cause the primary sex of the organism to be reversed during development via a process called environmental sex reversal. Shao et al. found that sex reversal in the half-smooth tongue sole may be due to methylation of genes in the sex determination pathways, and that these methylation modifications were inherited in the genetically female offspring of pseudomale fish (i.e. a genetically female fish that has undergone sex reversal and displays male sex characteristics). Thus, epigenetic modifications such as DNA methylation on specific genes can cause heritable changes in the regulation and expression of those genes.

Invasive Bigheaded Carp

Silver carp (*Hypophthalmichthys molitrix*; Valenciennes 1844) and bighead carp (*Hypophthalmichthys nobilis*; Richardson 1845) are two species of bigheaded carp invasive to the waters of the Mississippi River basin in North America. Native to freshwater streams and rivers in eastern Asia (Kamilov and Komrakova 1999; Jennings 1988), bighead and silver carps were first introduced in the United States in the early 1970s as a result of escapement from aquaculture facilities in Arkansas (Freeze and Henderson 1982; Williamson and Garvey 2005). Populations of these invasive species flourished, and both species quickly became established in many freshwater rivers throughout the central United States.

Bighead and silver carps are large and deep-bodied generalist planktivores (Spataru and Gophen 1985). They have large heads with eyes situated near the ventral side of the head. The diet of both species consists primarily of zooplankton, but they can also consume phytoplankton and detritus when zooplankton is less abundant (Williamson and Garvey 2005; Sampson et al. 2009). Both bighead and silver carps display plasticity in their diets, and have been shown to alter the length of their digestive tracts to adapt to different food types (Ke et al. 2008). Both bighead and silver carps have been introduced to lakes, reservoirs, and ponds in more than 30 countries in Europe, Asia, North America, and South America (Jennings 1988).

Silver carp are native to the large rivers of southeastern Asia, with the Amur River in eastern Russia forming the northern boundary of their range (Kamilov and Komrakova 1999; Konradt 1965). Silver carp have small scales that are silver in color (Kolar et al. 2005). The gill rakers of silver carp are fused into a sponge-like structure with small pores that allow the fish to filter plankton as small as 8 μm from the water (Sampson et al. 2009; Kolar et al. 2005).

Bighead carp are endemic to the Yellow, Yangtze, and Pearl rivers of eastern China, the Tumannaya and Razdolnaya rivers south of the Amur River in eastern Siberia, and in North Korea (Kolar et al. 2005). Bighead carp have larger heads than silver carp and have black and grey mottling along the sides of the body (Jennings 1988). Bighead carp have very fine gill rakers that act as a sieve, straining food particles as small as 17 μm from the water (Sampson et al. 2009; Kolar et al. 2005). As with many invasive species, nonnative bigheaded carps can cause significant damage to the ecosystems in which they have been introduced. Bigheaded carps are voracious eaters and are extremely fecund (Kolar et al. 2005; Schrank and Guy 2002; Williamson and Garvey 2005), making them a significant threat to many native species throughout their introduced range.

Bighead and silver carps have multiple negative effects on local fauna in their introduced range. Both species compete with native planktivores for food and habitat resources, and have been known to increase turbidity (Wu et al. 1997; Laws and Weisburd 1990), lower water quality (Kolar et al. 2005), and decrease dissolved oxygen in the water (Vybornov 1989), which can also be detrimental to native species. Most notably, gizzard shad, bigmouth buffalo, paddlefish, and the emerald shiner have experienced population declines as a result of competition with bighead and silver carps for zooplankton and phytoplankton (Sampson et al. 2009; Schrank et al. 2003; Williamson and Garvey 2005; Hayer et al. 2014; Kolar et al. 2005). The invasive carp species can also introduce new diseases and parasites to which native fishes have not acquired resistance (Kolar et al. 2005).

Notably, silver carp display a jumping behavior when startled by boat motors or other disturbances. They can leap several meters out of the water, and can cause expensive damage to boats and other watercraft, and potentially life-threatening injuries to boaters and people enjoying recreational water sports (Kolar et al. 2005). This unique behavior of invasive silver carp in the Mississippi and Illinois rivers has garnered much public attention, and has become an issue of particular concern for people who use these waterways.

Both bighead and silver carps are already established in the Middle Mississippi and Illinois rivers, but there is currently no evidence of established populations of bigheaded carps in the Laurentian Great Lakes. Lake Michigan faces an especially high risk of invasion by bigheaded carps because shipping channels in Chicago connect Lake Michigan to the Illinois River (DeGrandchamp et al. 2008), where populations of bighead and silver carps are robust. Lake Michigan currently supports multi-million dollar commercial trout and salmon fisheries. The establishment of bigheaded carps into Lake Michigan would be devastating to these

fisheries, and likely would have a significant economic impact in the surrounding region. The introduction of other exotic fish species into the Great Lakes, such as the sea lamprey (*Petromyzon marinus*), alewife (*Alosa pseudoharangus*), and white perch (*Morone americana*), resulted in significant population declines in native fishes, and caused millions of dollars in damage to commercial fisheries (Mills et al. 1994; Lavis et al. 2003; Benjamin and Bence 2003).

Genetic surveillance using environmental DNA (eDNA) is currently being used as an early detection tool to screen for bigheaded carps in the Great Lakes (Jerde et al. 2011; Jerde et al. 2013). In order to prevent bigheaded carps from becoming established in Lake Michigan, several barriers have been put into place, including an electric barrier along the upper reach of the Illinois River (Moy 2005). While these barriers appear to be effective so far at preventing the further dispersal of bigheaded carps, they are not infallible, and more research on bigheaded carps is necessary to ensure that carp from the Mississippi and Illinois rivers are not permitted entry into the Great Lakes basin.

Bigheaded carps in the Mississippi River basin display considerable phenotypic variation. They grow larger and more rapidly than the species in their native range (Williamson and Garvey 2005), and have different spawning habitat preferences than their conspecifics in Asia (DeGrandchamp et al. 2008). Bighead and silver carps in North America also have extended spawning periods and earlier maturation times relative to native bigheaded carp populations (Williamson and Garvey 2005). Understanding if epigenetic mechanisms may contribute to such phenotypic plasticity will help predict future invasions of bighead and silver carps, and will aid in the creation of management plans to ease propagule pressure.

Several studies have examined the genetic diversity of native and introduced bighead and silver carps. Li et al. (2010) used the mitochondrial 16S ribosomal RNA gene and D-loop

regions to analyze the genetic diversity and variation in bighead carp from the Yangtze, Pearl, and Amur rivers in China, the Danube River in Hungary, and the Mississippi River basin in the United States. Li et al. found that introduced populations of bighead carp from the Mississippi River basin had significantly lower haplotype diversity than native populations of bighead carp in China. The low haplotype diversity in the Mississippi River basin is consistent with the low levels of genetic diversity that is expected in populations of organisms introduced outside their native range. Despite the low haplotype diversity exhibited by introduced carp in the United States, the population in the Mississippi River basin exhibited moderate nucleotide diversity. The discrepancy between haplotype and nucleotide diversity in carp from the Mississippi River basin may be explained by vast population expansion throughout the introduced range, a high degree of adaptation in the introduced range, or from multiple introductions of bighead carp in the Mississippi River basin.

In a similar study, Li et al. (2011) examined silver carp from the same locations as Li et al. 2010 (Yangtze, Pearl, and Amur rivers in China, the Danube River in Hungary, and the Mississippi River basin in the United States). Silver carp from the Mississippi River basin exhibited significantly lower haplotype and nucleotide diversity than native silver carp populations in China. These results suggest that silver carp in the Mississippi River basin may have low genetic diversity due to the founder effect, in which an introduced population may have reduced genetic diversity due to a low initial population size. In another more recent study, Farrington et al. (2017) examined bighead and silver carp from throughout their North American range and found that genetic diversity of both species was higher than expected with relatively little population structure. The genetic diversity of both bighead and silver carp from North America was still 75-90% lower than that of bighead and silver carp individuals from Asia.

While there have been many studies on the biology, geographic range, population characteristics, and genetics of invasive bigheaded carps, there have been no studies to date on the epigenetic mechanisms behind the successful invasion and establishment of bighead and silver carps in North America. Further research is necessary to determine the genetic and epigenetic factors that make bigheaded carps such successful invaders in the freshwaters of the United States. A greater understanding of the underlying genetics and epigenetics of these species will help in future management efforts and will help prevent further invasion into susceptible habitats like the Great Lakes.

In this study, both the genetic and epigenetic variation in introduced bighead and silver carps in the Illinois River were assessed and compared to the genetic and epigenetic variation in native bighead and silver carps from the Yangtze River in China. The goal of this study was to compare the genetic and epigenetic variation in native populations of bighead and silver carps to that of introduced populations in the Illinois River using AFLPs. I used methylation-sensitive AFLPs (MS-AFLPs) to look for DNA methylation in both native and invasive populations (Yamamoto and Yamamoto 2004; Schrey et al. 2013). I compared the methylation patterns between the invasive and native populations to determine if significant epigenetic differentiation has taken place.

I hypothesize that epigenetic differentiation has allowed bighead and silver carps in North American rivers to overcome decreased genetic diversity and increased inbreeding due to the small size of the initial invasive population. If epigenetic differentiation is the mechanism behind the successful invasion and establishment of bighead and silver carps in the Mississippi River basin, I would expect to see epigenetic diversity exceeding genetic diversity in invasive silver carp. I would also expect epigenetic diversity in Illinois silver carp to exceed the

epigenetic diversity of native silver carp from China.

HYPOTHESES

H_0: No significant genetic differentiation will be observed between Chinese and Illinois samples of bighead and silver carp.

H_1: Significant genetic differentiation will be observed between Chinese and Illinois samples of bighead and silver carp.

H_0: No significant epigenetic differentiation will be observed between Chinese and Illinois samples of bighead and silver carp.

H_2: Significant epigenetic differentiation will be observed between Chinese and Illinois samples of bighead and silver carp.

H_0: No differences in magnitude of genetic and epigenetic diversity and differentiation will be observed between bighead and silver carp populations in China and Illinois.

H_3: Significant differences in magnitude of genetic and epigenetic diversity and differentiation will be observed between bighead and silver carp populations in China and Illinois.

CHAPTER 2

METHODS

Sample Collection

Carp fin clips (n = 100) were collected in October 2014 from Starved Rock State Park on the Illinois River. Collection was done by commercial fishermen using gill nets. The carp were placed in individual pens, and were weighed and measured. Fin clips were taken from the pectoral fin of each individual and were preserved in 95% ethanol. Carp samples (n = 101) were obtained during the summer 2015 from the Yangtze, Pearl, and Gan rivers in China (samples provided by Dr. Jingou Tong at the Institute of Hydrobiology, Chinese Academy of Sciences). Fin clips were taken and preserved in 95% ethanol.

DNA Isolation

DNA was extracted from each sample using the Qiagen DNeasy kit following a modified version of the Animal Tissue Extraction protocol. To increase DNA concentrations, each sample was eluted with only 100 μL of AE elution buffer and the elution step was repeated using the resulting flow-through of the first elution. This yielded a sufficient volume of DNA to perform MS-AFLP assays. The concentration of DNA in the isolates was quantified using a NanoDrop 2000 spectrophotometer. Isolates were standardized at 50 ng/ μL prior to performing MS-AFLP analysis.

SNP Analysis

To confirm the genetic identity of the specimens, DNA isolates were submitted to the lab of Dr. James Lamer at Western Illinois University. The Lamer lab used 44 single nucleotide polymorphism (SNP) loci to identify samples as bighead carp, silver carp, or hybrids (Lamer et al. 2015). Any hybrids or first or second generation backcrosses were excluded from further

analyses. Ultimately, 30 Illinois bighead carp samples, 69 Illinois silver carp samples, and 89 Chinese silver carp samples, including 29 silver carp samples from Gan River, 24 silver carp samples from Pearl River, and 36 silver carp samples from Yangtze River were analyzed.

Genetic Analysis

MS-AFLPs were performed following the Wendel Lab AFLP protocol (http://www.eeob.iastate.edu/faculty/WendelJ/aflp.htm 08 August 2016). Genomic DNA was digested using the restriction endonucleases *Msp*I and *EcoR*I. *EcoR*I cleaves dsDNA at the recognition site G/AATTC and generates restriction fragments containing a 4 bp overhang of AATT on the 5' end. *Msp*I cleaves dsDNA at the recognition site CCGG, and will cleave DNA regardless of whether the internal cytosine residue contains a 5-methyl group. Restriction digests had a final volume of 20 µL and included approximately 200 ng genomic DNA, 2.0 µL 10X NEB buffer, 10 units *EcoR*I, 10 units *Msp*I, and 13.8 µL H_2O. Restriction digests were then incubated at 37°C for 3 hours. 50 µLM adapter pairs were prepared for both *EcoR*I and *Msp*I. Forward and reverse adapters for each restriction enzyme were combined and denatured at 95°C for 5 minutes, then allowed to re-anneal by slowly lowering the temperature to 20° over 30 minutes in a thermal cycler.

Adapter pairs were then ligated to the DNA restriction fragments. Total ligation volume was 40.0 µL and will contain 4.0 µL 10X ligase buffer, 75 pmol of *EcoR*I adapter, 75 pmol of *Msp*I adapter, 20 units of T4 DNA ligase, 12.99 µL H_2O, and the 20 µL digested DNA. Ligations were allowed to incubate at 16°C overnight. After ligation, the samples were diluted with 160 µL H_2O.

Polymerase chain reaction (PCR) was performed using preselective primers *Eco*RI+1 and *Hpa*II/*Msp*I+0 to amplify a subset of the restriction fragments. A list of primers used in

preselective PCR is compiled in Table 2.1. Total PCR volume was 50 µL and contained 25 µL 2X PCR Master Mix (Fisher Scientific, Lenaxa KS); final concentration = 0.625 units *Taq* DNA polymerase, 75 mM Tris-HCL, 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 0.01% Tween 20, 200 µM each dNTP), 0.8 µM of *Eco*RI+1 primer, 0.8 µM of *Hpa*II/*Msp*I+1 primer, 7 µL H_2O, and 10 µL of diluted ligations. Reactions were carried out with an initial denaturing at 75°C for 2 minutes (min), 20 cycles of 94°C for 30 seconds (s), 56°C for 30 s, and 72°C for 2 min, and a final extension at 60°C for 30 min. Preselective PCR products were diluted with 720 µL H_2O.

Selective amplification of preselective PCR products using +3 primers was performed in a selective PCR reaction. A list of primers used in selective PCR is compiled in Table 2.2. Total PCR volume was 20 µL and included 10 µL PCR Master Mix (same as for preselective PCRs), 0.25 µM of 6FAM labeled *Eco*RI+3 primer, 0.25 µM of HEX labeled *Eco*RI+3 primer, 1.25 µM of unlabeled *Msp*I+3 primer, 1.5 µL H_2O, and 5.0 µL of diluted preselective PCR product. Reactions were carried out with an initial denaturing at 94° C for 2 min, followed by 9 cycles of 94° C for 30 s, 65° C for 30 s, dropping the annealing temperature 1 degree each cycle (e.g., 65, 64, 63, 62, 61, 60, 59, 58, 57), 31 cycles of 94° C for 30 s, 56° C for 30 s, 72° C for 2 min, and a final extension at 60° C for 5 min.

The resulting amplicons from the selective PCR were resolved using a 3130*xl* 16 capillary genetic analyzer (Applied Biosystems). The protocol for AFLP analysis using the 3130*xl* genetic analyzer was as follows: 9.1 µL highly deionized formamide, 0.2 µL 400 ROX dye internal-lane size standard (Gel Company, San Francisco CA), and 0.7 µL selective PCR product for a final volume of 10 µL . The samples were denatured at 95° C for 3 min prior to the 3130*xl* run. Results were analyzed using GENEMAPPER 4.0 (Applied Biosystems, Beverly, MA).

Epigenetic Analysis

A second MS-AFLP restriction digest was performed using the methylation-sensitive restriction endonuclease *Hpa*II in place of *Msp*I. Genomic DNA was digested using the restriction endonucleases *EcoR*I and *Hpa*II. *Hpa*II is an isoschizomer of *Msp*I and has the same CCGG recognition sequence as *Msp*I, but cleavage is prevented when the internal cytosine residue is methylated. *Hpa*II restriction digests had a final volume of 20 µL and included approximately 200 ng genomic DNA, 2.0 µL 10X NEB buffer, 10 units *EcoR*I, 10 units *Hpa*II, and 13.8 µL H_2O. Restriction digests were incubated at 37°C for 3 hours.

100 µM adapter pairs were prepared for *EcoR*I and *Hpa*II. Adapters were annealed and ligated following the same protocol that was used for *Msp*I fragments. Preselective PCR was performed using +1 primers to amplify a subset of the restriction fragments following the same protocol that was used for *Msp*I fragments. A list of primers used in preselective PCR is compiled in Table 2.1. Selective amplification using +3 labeled primers and +4 unlabeled primers was performed in a selective PCR reaction following the same protocol that was used for AFLP. A list of primers used in selective PCR is compiled in Table 2.2. The resulting amplicons from the selective PCR were resolved using a 3130*xl* 16 capillary genetic analyzer (Applied Biosystems) following the same protocol that was used to visualize *Msp*I fragments. 1166 loci were scored for each individual.

Genetic Statistical Analyses

Heterozygosities of Illinois bighead carp samples, Illinois silver carp, and Chinese silver carp were calculated using GENALEX version 6.1 (Peakall and Smouse 2006). The heterozygosity of each population was calculated using the equation for the unbiased expected heterozygosity (uHe), which adjusts for sample size. $uHe = \frac{2N}{2N-1} * 2pq$ where

$N = sample\ size,\ q = 1 - band\ frequency$, and $p = 1 - q$.

Haplotype diversity was calculated using the results of the *Msp*I digest for silver carp samples from both Illinois and China using GENALEX. A pairwise AMOVA was conducted to calculate the amount of variation between haplotypes of silver carp samples from Illinois and haplotypes of silver carp samples from China. All pairwise and hierarchical AMOVA were run using GENALEX. The test statistic derived from each AMOVA was ϕ_{PT}, which is a measure of variation among populations. The significance of ϕ_{PT} was determined via permutation test in GENALEX using 999 permutations with the p-value indicating the rank (highest to lowest) of the observed ϕ_{PT} among the 1000 ϕ_{PT} values. A two-tailed homoscedastic *t* test was also run to test whether the haplotypes differed significantly between Illinois and Chinese silver carp samples.

To estimate genetic differentiation between populations of invasive bighead carp in Illinois and invasive silver carp in Illinois, a pairwise AMOVA that compared variation in haplotypes between Illinois bighead carp population and Illinois silver carp was conducted.

A pairwise AMOVA was conducted to estimate genetic variation among haplotypes of Illinois silver carp, Gan River silver carp, Pearl River silver carp, and Yangtze River silver carp.

A hierarchical AMOVA was conducted to partition genetic variation of silver carp between regions (Illinois vs. China), among populations within regions (Gan River, Pearl River, and Yangtze River in China), and within populations (Gan River, Pearl River, Yangtze River, and Illinois River).

Pairwise ϕ_{PT} was calculated among all pairs of samples and a neighbor-joining tree was constructed with Illinois bighead carp as an outgroup using MEGA version 7.0 (Kumar et al. 2016).

Individual assignment testing was performed using two STRUCTURE analyses, which assigns individuals to populations based on patterns in the genotypes of the individuals without a priori sampling information (Pritchard et al. 2000). The first STRUCTURE analysis included bighead carp and silver carp from both Illinois and China. The second STRUCTURE analysis was run to determine the number of populations among only silver carp samples, including individuals from both Illinois and China. The STRUCTURE analyses were run with a 30,000 burn-in period, 100,000 repetitions, and using K values 1-6. Each data set was run 6 times to check for variation in population assignments. The results of the STRUCTURE analyses were uploaded to STRUCTURE Harvester, an online program that collates data and uses the Evanno method to determine the number of populations (K) that best fit the data (Earl and vonHoldt 2012; Evanno et al. 2005). The STRUCTURE Harvester program produces graphs for ΔK and Ln(K). Ln(K) graph plots the mean estimated probability of the data for each value of K. The maximum value of ΔK denotes the number of populations that best fit the data provided that Ln(K) is not greatest for K=1. The slope of the Ln(K) graph tends to asymptote off such that as the value of K increases, the increase in Ln(K) gets increasingly small. As the value of K increases, the variance in Ln(K) also increases. The ΔK graph finds the value of K where there is a large increase in Ln(K) and/or an increase in the variance of Ln(K) as K increases from K to K+1.

Epigenetic Statistical Analyses

I followed the approach of Blouin et al. (2010) to evaluate whether there were differences in the amount of methylation between silver carp from Illinois and China. Each MS-AFLP locus was designated as 1-1 if it a band was present in both the *Msp*I and *Hpa*II digests, 1-0 if a band was present in *Hpa*II but not in *Msp*I, 0-1 if a band was present in *Msp*I but not *Hpa*II, and 0-0 if

no bands were present in both *Msp*I and *Hpa*II digests. Four two-tailed homoscedastic *t* tests were performed to test the following: (*i*) whether the average number of 1-0 bands (bands revealed by *Hpa*II, but not *Msp*I) differed significantly between Illinois silver carp and silver carp from China (Gan, Pearl, and Yangtze River sites) (*ii*) whether the average number of 1-1 bands (bands present in both *Msp*I and *Hpa*II digests) differed significantly between Illinois and Chinese silver carp populations (*iii*) whether the average number of 0-1 bands plus 1-0 bands (total number of methylated bands identified) differed significantly between the invasive and native silver carp populations (*iv*) whether the percentage of all methylated bands locus by locus among the total number of bands (0-1 +1-0)/(0-1 + 1-0 + 1-1) detected differed significantly between Illinois and Chinese silver carp.

Discrete populations of fish may be differentially methylated at different sites, but this variation could be missed if only testing average levels of methylation between populations. While *t* tests are useful for looking for overall differences in methylation, they often miss locus-by-locus variation in methylation. To test whether the band frequency differed between Illinois and Chinese silver carp, two-tailed Fisher's exact tests were run on each individual locus using a 5% criterion (Blouin et al. 2010).

Epihaplotypes were created by assigning both Type I (1-1) and Type IV (0-0) as a 0 and assigning Type II (1-0) and Type III (0-1) methylation as a 1. Epihaplotype diversity was calculated for Illinois bighead carp and silver carp samples from both Illinois and China using GENALEX. A pairwise AMOVA was run to estimate the proportion of variation shared between Illinois silver and bighead carp epihaplotypes. Another pairwise AMOVA was run to calculate the amount of variation between epihaplotypes of silver carp from Illinois and China. The percentages of methylation per locus and per individual were also calculated for all silver

carp samples from Illinois and China. A two-tailed homoscedastic *t* test was run to test whether the epihaplotype diversity differed significantly between Illinois and Chinese silver carp samples.

To test whether epihaplotype diversity significantly exceeded haplotype diversity, two two-tailed homoscedastic *t* tests were run: the first testing the difference between haplotype diversity and epihaplotype diversity in silver carp samples from Illinois, and the second testing the difference between haplotype diversity and epihaplotype diversity in silver carp samples from China.

A pairwise AMOVA was run to estimate the proportion of variation shared between Illinois bighead carp haplotypes and epihaplotypes. A second AMOVA was run to calculate the variation between haplotypes and epihaplotypes of Illinois silver carp. Final pairwise AMOVA was run to partition variation between the haplotypes of silver carp from China with the epihaplotypes of the same Chinese silver carp individuals.

Table 2.1. A list of primers used to perform Preselective PCR.

Preselective Primer Name	**Sequence**
EcoRI+1	TACTGCGTACCAATTCA
HpaII/MspI+0	ATCATGAGTCCTGCTCGG

Table 2.2. A list of primers used to perform Selective PCR (selective portion of each primer is underlined).

Selective Primer Name	**Sequence**
EcoRI+AGC	6FAM-TACTGCGTACCAATTCAGC
EcoRI+ACG	HEX-TACTGCGTACCAATTCACG
EcoRI+AAC	HEX-TACTGCGTACCAATTCAAC
EcoRI+ACA	6FAM-TACTGCGTACCAATTCACA
HpaII/MspI+TCAT	ATCATGAGTCCTGCTCGGTCAT
HpaII/MspI+TCAC	ATCATGAGTCCTGCTCGGTCAC

CHAPTER 3

RESULTS

SNP Analysis

The results of the SNP analysis identified 28 of the Illinois River samples as being pure bighead carp (Table 3.1). One Illinois River sample was identified as a fourth generation bighead carp backcross with 41 diagnostic bighead carp alleles and 3 heterozygote alleles. For the purposes of this analysis, this backcrossed individual was considered to be a bighead carp. No bighead carp were identified among any of the samples from China.

36 of the Illinois River fish were identified as pure silver carp. 10 individuals from the Illinois River were identified as third generation silver carp backcrosses, and 23 individuals from the Illinois River were identified as fourth generation silver carp backcrosses. All third and fourth generation silver carp backcrosses were considered to be Illinois silver carp in further analyses. One Illinois River fish was identified to be a late generation silver carp backcross x late generation silver carp backcross. This individual was eliminated from further analyses.

32 samples from China were identified as pure silver carp. Six of these pure silver carp samples originated in the Gan River, 1 from the Pearl River, and the remaining 25 silver carp samples came from the Yangtze River. 2 Pearl River individuals were identified as third generation silver carp backcrosses. In addition, 55 individuals from China were identified as fourth generation silver carp backcrosses. 23 of these fourth generation silver carp backcrossed individuals came from the Gan River, 21 of these samples came from the Pearl River, and 11 samples came from the Yangtze River. All third and fourth generation silver carp backcrosses were considered to be silver carp for the purposes of this project. 3 samples from the Gan River and 7 samples from the Pearl River were identified as late generation silver carp backcross x late

generation silver carp backcross. These 10 individuals were eliminated from subsequent analyses. The genetic identities of 1 sample from the Pearl River and 1 sample from the Yangtze River were not able to be resolved through SNP analysis, and were eliminated from the analysis.

Genetic Analysis

The unbiased expected heterozygosity of the Illinois bighead carp population was calculated to be 0.154. The calculated heterozygosity of the Illinois silver carp population was 0.182. The Chinese silver carp population (all three rivers) had a heterozygosity of 0.175. The Chinese silver carp were also broken down into the three sampling localities. Silver carp from the Gan River in China had an unbiased expected heterozygosity of 0.202. Silver carp from the Pearl River locality had a heterozygosity of 0.170. Yangtze River silver carp samples had a heterozygosity of 0.114 (Table 3.2).

The mean haplotype of bighead carp samples from Illinois was 0.145 (Table 3.3). The mean haplotype diversity of silver carp samples from Illinois was 0.176. The mean haplotype diversity of all silver carp samples from China was 0.157. The Gan River silver carp samples had a haplotype diversity of 0.193. The Pearl River silver carp samples had a mean haplotype diversity of 0.167, and the Yangtze River silver carp had a mean haplotype diversity of 0.110.

The output of the pairwise AMOVA assessing variation in haplotypes between Illinois bighead carp and Illinois silver carp produced a ϕ_{PT} value of 0.501 with p = 0.001 (Table 3.4).

The output of the pairwise AMOVA assessing variation in haplotypes between silver carp from Illinois and silver carp from China produced a ϕ_{PT} value of 0.132 with p = 0.001 (Table 3.5). The result of the two-tailed *t* test analyzing the difference between the haplotype diversities of invasive and native silver carp samples was P = 0.00177.

The pairwise AMOVA assessing variation in haplotypes among silver carp from the

Illinois River, Gan River, Pearl River, and Yangtze River produced a ϕ_{PT} value of 0.163 with $p < 0.001$ (Table 3.6).

The hierarchical AMOVA partitioning variation in haplotypes of silver carp between regions (Illinois and China), among populations within regions (Illinois River in Illinois; Gan River, Pearl River, and Yangtze River in China), and within populations (Illinois River, Gan River, Pearl River, and Yangtze River) produced a ϕ_{RT} of 0.048, which is the test statistic that calculates the variation between silver carp in the Illinois River in Illinois and the Gan, Pearl, and Yangtze Rivers in China (Table 3.7). The hierarchical AMOVA also produced a ϕ_{PR} of 0.133, which calculates the variation between silver carp in Illinois and China, and a ϕ_{PT} of 0.175, which calculates the variation across the full data set. $p < 0.001$ for all test statistics derived from the hierarchical AMOVA.

A ϕ_{PT} matrix was created using GENALEX, from which a neighbor-joining tree for all silver carp samples was constructed using Illinois bighead carp as an outgroup (Figure 3.1).

The first STRUCTURE analysis included both bighead and silver carps, and identified 3 discrete populations. The slope of the Ln(K) graph for the first STRUCTURE analysis began to level off at K=3 (Figure 3.2). The ΔK graph from the STRUCTURE Harvester program produced a maximum value of ΔK at K=3 (Figure 3.3.). This analysis placed the bighead carp into one group, the Illinois River and Gan River silver carp into another group, and the Pearl River and Yangtze River silver carp into a third group at K=3, with some individuals from the Gan River assigning with the Pearl and Yangtze River fish (Figure 3.4).

A second STRUCTURE run included only silver carp (individuals from Illinois and China). This analysis identified two discrete populations. The slope of the Ln(K) began to level off at K=2 (Figure 3.5). The ΔK graph from the STRUCTURE Harvester program indicated a

maximum value of ΔK at K=2 (Figure 3.6). This analysis placed the Yangtze River and Pearl River silver carp into one group, and the Illinois River and Gan River silver carp into another group at K=2 with some individuals from the Gan River assigning with the Pearl and Yangtze River fish (Figure 3.7).

Epigenetic Analysis

The epihaplotype diversity of bighead carp from Illinois was 0.213 (Table 3.8). The epihaplotype diversity of silver carp samples from Illinois was 0.239. The epihaplotype diversity of silver carp from China was 0.204. The epihaplotype diversity of silver carp from the Gan River was 0.235. The Pearl River silver carp had an epihaplotype diversity of 0.210, and the Yangtze River silver carp samples had an epihaplotype diversity of 0.166.

The result of the two-tailed *t* test analyzing the difference between the epihaplotype diversities of silver carp from Illinois and China was $P = 5.173 \times 10^{-8}$. The average percentage of methylation per locus for silver carp samples from Illinois was 25.9%, and the average percentage of methylation per locus for silver carp samples from China was 26.2%. The average percentage of methylation per individual silver carp from Illinois was 25.9%. The average percentage of methylation per individual silver carp from China was 20.3% (Table 3.9).

The result of the *t* test analyzing the difference between haplotype and epihaplotype diversities in Illinois silver carp was $P = 6.082 \times 10^{-17}$. The result of the *t* test analyzing the difference between haplotype and epihaplotype diversities in Chinese silver carp was $P = 2.607 \times 10^{-26}$.

The pairwise AMOVA estimating variation between Illinois bighead and silver carp epihaplotypes produced a ϕ_{PT} of 0.497 with p = 0.001 (Table 3.10).

The pairwise AMOVA estimating variation between Illinois and Chinese silver carp

epihaplotypes produced a ϕ_{PT} of 0.116 with p = 0.001 (Table 3.11).

A pairwise AMOVA assessing variation between Illinois bighead carp haplotypes and epihaplotypes produced a ϕ_{PT} value of 0.281 with $p < 0.001$ (Table 3.12).

A pairwise AMOVA assessing variation between Illinois silver carp haplotypes and epihaplotypes produced a ϕ_{PT} value of 0.408 with $p < 0.001$ (Table 3.13).

A final pairwise AMOVA estimating variation between Chinese silver carp haplotypes and epihaplotypes produced a ϕ_{PT} value of 0.334 with $p < 0.001$ (Table 3.14).

Of the four *t* tests looking at differences in the average number of 1-0 bands, 1-1 bands, 1-0 + 0-1 bands, and the percentage of all methylated bands per the total number of bands, only one test had significant results (Table 3.15). The 1-0 *t* test results indicated a significant difference (p = 0.0345) between the number of methylated sites identified by the methylation-sensitive enzyme *Hpa*II in invasive silver carp collected from the Illinois River versus silver carp collected from China. Approximately 63% of the bands in all the silver carp samples were methylated sites (0-1 or 1-0) (Table 3.15).

Of the 1166 loci tested using two-tailed Fisher's exact tests, 343 loci (29.42%) differed significantly in frequency between Illinois and Chinese silver carp ($\alpha = 0.05$). Of those 343 loci that were found to be significantly different, 231 (67.35%) of the sites were hypermethylated in invasive Illinois silver carp relative to the silver carp from China.

Table 3.1. Results of SNP analysis on all carp samples.

Silver carp diagnostic	Heterozygote	Bighead carp diagnostic	Individual ID	Sampling Locality	Genotype
0	0	44	CRP-012	Illinois River	BHCP
0	0	44	CRP-013	Illinois River	BHCP
0	0	44	CRP-014	Illinois River	BHCP
0	0	44	CRP-015	Illinois River	BHCP
0	0	44	CRP-016	Illinois River	BHCP
0	0	44	CRP-018	Illinois River	BHCP
0	0	44	CRP-019	Illinois River	BHCP
0	0	44	CRP-027	Illinois River	BHCP
0	0	44	CRP-028	Illinois River	BHCP
0	0	43	CRP-029	Illinois River	BHCP
0	0	44	CRP-032	Illinois River	BHCP
0	0	44	CRP-035	Illinois River	BHCP
0	0	44	CRP-036	Illinois River	BHCP
0	0	44	CRP-037	Illinois River	BHCP
0	0	44	CRP-040	Illinois River	BHCP
0	0	44	CRP-041	Illinois River	BHCP
0	0	44	CRP-042	Illinois River	BHCP
0	0	44	CRP-044	Illinois River	BHCP
0	0	44	CRP-045	Illinois River	BHCP
0	0	44	CRP-046	Illinois River	BHCP
0	0	44	CRP-047	Illinois River	BHCP
0	0	44	CRP-059	Illinois River	BHCP
0	0	44	CRP-064	Illinois River	BHCP
0	0	44	CRP-071	Illinois River	BHCP
0	0	44	CRP-072	Illinois River	BHCP
0	0	44	CRP-074	Illinois River	BHCP
0	0	44	CRP-079	Illinois River	BHCP
0	0	44	CRP-099	Illinois River	BHCP
0	14	30	CRP-007	Illinois River	BxBH2
0	3	41	CRP-043	Illinois River	BxBH4
39	4	0	CRP-001	Illinois River	BxSV3
37	6	0	CRP-005	Illinois River	BxSV3
38	6	0	CRP-009	Illinois River	BxSV3
37	7	0	CRP-023	Illinois River	BxSV3
40	4	0	CRP-025	Illinois River	BxSV3
38	6	0	CRP-051	Illinois River	BxSV3

Table 3.1. Continued.

Silver carp diagnostic	Heterozygote	Bighead carp diagnostic	Individual ID	Sampling Locality	Genotype
40	4	0	CRP-052	Illinois River	BxSV3
39	4	0	CRP-080	Illinois River	BxSV3
40	4	0	CRP-088	Illinois River	BxSV3
36	8	0	CRP-093	Illinois River	BxSV3
40	4	0	PEA-010	Pearl River	BxSV3
20	2	0	PEA-021	Pearl River	BxSV3
41	3	0	CRP-004	Illinois River	BxSV4
43	1	0	CRP-008	Illinois River	BxSV4
42	2	0	CRP-011	Illinois River	BxSV4
41	2	0	CRP-020	Illinois River	BxSV4
41	3	0	CRP-021	Illinois River	BxSV4
43	1	0	CRP-024	Illinois River	BxSV4
43	1	0	CRP-026	Illinois River	BxSV4
43	1	0	CRP-030	Illinois River	BxSV4
40	3	0	CRP-033	Illinois River	BxSV4
42	1	0	CRP-034	Illinois River	BxSV4
40	3	0	CRP-039	Illinois River	BxSV4
43	1	0	CRP-049	Illinois River	BxSV4
43	1	0	CRP-054	Illinois River	BxSV4
42	2	0	CRP-069	Illinois River	BxSV4
43	1	0	CRP-075	Illinois River	BxSV4
43	1	0	CRP-076	Illinois River	BxSV4
42	2	0	CRP-081	Illinois River	BxSV4
43	1	0	CRP-082	Illinois River	BxSV4
41	2	0	CRP-083	Illinois River	BxSV4
43	1	0	CRP-087	Illinois River	BxSV4
41	3	0	CRP-095	Illinois River	BxSV4
42	2	0	CRP-096	Illinois River	BxSV4
43	1	0	CRP-097	Illinois River	BxSV4
42	2	0	GAN-002	Gan River	BxSV4
43	1	0	GAN-003	Gan River	BxSV4
43	1	0	GAN-004	Gan River	BxSV4
41	3	0	GAN-007	Gan River	BxSV4
43	1	0	GAN-009	Gan River	BxSV4
42	1	0	GAN-010	Gan River	BxSV4
43	1	0	GAN-011	Gan River	BxSV4

Table 3.1. Continued.

Silver carp diagnostic	Heterozygote	Bighead carp diagnostic	Individual ID	Sampling Locality	Genotype
43	1	0	GAN-012	Gan River	BxSV4
43	1	0	GAN-013	Gan River	BxSV4
43	1	0	GAN-014	Gan River	BxSV4
42	2	0	GAN-016	Gan River	BxSV4
41	3	0	GAN-018	Gan River	BxSV4
42	2	0	GAN-019	Gan River	BxSV4
43	1	0	GAN-020	Gan River	BxSV4
42	1	0	GAN-021	Gan River	BxSV4
41	3	0	GAN-022	Gan River	BxSV4
43	1	0	GAN-023	Gan River	BxSV4
43	1	0	GAN-024	Gan River	BxSV4
42	2	0	GAN-028	Gan River	BxSV4
42	2	0	GAN-029	Gan River	BxSV4
43	1	0	GAN-030	Gan River	BxSV4
43	1	0	GAN-031	Gan River	BxSV4
42	2	0	GAN-032	Gan River	BxSV4
43	1	0	PEA-001	Pearl River	BxSV4
43	1	0	PEA-003	Pearl River	BxSV4
39	2	0	PEA-004	Pearl River	BxSV4
43	1	0	PEA-006	Pearl River	BxSV4
42	1	0	PEA-007	Pearl River	BxSV4
40	3	0	PEA-008	Pearl River	BxSV4
43	1	0	PEA-013	Pearl River	BxSV4
43	1	0	PEA-015	Pearl River	BxSV4
40	3	0	PEA-016	Pearl River	BxSV4
41	3	0	PEA-017	Pearl River	BxSV4
42	2	0	PEA-020	Pearl River	BxSV4
42	2	0	PEA-022	Pearl River	BxSV4
41	3	0	PEA-024	Pearl River	BxSV4
42	2	0	PEA-025	Pearl River	BxSV4
43	1	0	PEA-026	Pearl River	BxSV4
41	3	0	PEA-027	Pearl River	BxSV4
43	1	0	PEA-028	Pearl River	BxSV4
42	2	0	PEA-029	Pearl River	BxSV4
43	1	0	PEA-030	Pearl River	BxSV4
42	2	0	PEA-031	Pearl River	BxSV4

Table 3.1. Continued.

Silver carp diagnostic	Heterozygote	Bighead carp diagnostic	Individual ID	Sampling Locality	Genotype
43	1	0	PEA-032	Pearl River	BxSV4
43	1	0	YAN-006	Yangtze River	BxSV4
42	2	0	YAN-008	Yangtze River	BxSV4
41	2	0	YAN-010	Yangtze River	BxSV4
43	1	0	YAN-011	Yangtze River	BxSV4
43	1	0	YAN-013	Yangtze River	BxSV4
43	1	0	YAN-016	Yangtze River	BxSV4
43	1	0	YAN-018	Yangtze River	BxSV4
43	1	0	YAN-020	Yangtze River	BxSV4
43	1	0	YAN-029	Yangtze River	BxSV4
43	1	0	YAN-034	Yangtze River	BxSV4
43	1	0	YAN-036	Yangtze River	BxSV4
39	4	1	CRP-090	Illinois River	FxSV
41	2	1	GAN-001	Gan River	FxSV
43	0	1	GAN-015	Gan River	FxSV
42	1	1	GAN-026	Gan River	FxSV
42	1	1	PEA-002	Pearl River	FxSV
39	1	2	PEA-011	Pearl River	FxSV
41	2	1	PEA-012	Pearl River	FxSV
42	0	2	PEA-014	Pearl River	FxSV
42	1	1	PEA-018	Pearl River	FxSV
41	2	1	PEA-019	Pearl River	FxSV
42	1	1	PEA-023	Pearl River	FxSV
1	0	1	PEA-005	Pearl River	Inconclusive
0	0	0	YAN-021	Yangtze River	Inconclusive
43	0	0	CRP-002	Illinois River	SVCP
43	0	0	CRP-003	Illinois River	SVCP
44	0	0	CRP-006	Illinois River	SVCP
44	0	0	CRP-010	Illinois River	SVCP
44	0	0	CRP-017	Illinois River	SVCP
44	0	0	CRP-022	Illinois River	SVCP
44	0	0	CRP-031	Illinois River	SVCP
44	0	0	CRP-038	Illinois River	SVCP
44	0	0	CRP-048	Illinois River	SVCP
44	0	0	CRP-050	Illinois River	SVCP
44	0	0	CRP-053	Illinois River	SVCP

Table 3.1. Continued.

Silver carp diagnostic	Heterozygote	Bighead carp diagnostic	Individual ID	Sampling Locality	Genotype
44	0	0	CRP-055	Illinois River	SVCP
44	0	0	CRP-056	Illinois River	SVCP
44	0	0	CRP-057	Illinois River	SVCP
44	0	0	CRP-058	Illinois River	SVCP
44	0	0	CRP-060	Illinois River	SVCP
44	0	0	CRP-061	Illinois River	SVCP
44	0	0	CRP-062	Illinois River	SVCP
44	0	0	CRP-063	Illinois River	SVCP
44	0	0	CRP-065	Illinois River	SVCP
43	0	0	CRP-066	Illinois River	SVCP
44	0	0	CRP-067	Illinois River	SVCP
44	0	0	CRP-068	Illinois River	SVCP
43	0	0	CRP-070	Illinois River	SVCP
44	0	0	CRP-073	Illinois River	SVCP
44	0	0	CRP-077	Illinois River	SVCP
43	0	0	CRP-078	Illinois River	SVCP
44	0	0	CRP-084	Illinois River	SVCP
43	0	0	CRP-085	Illinois River	SVCP
44	0	0	CRP-086	Illinois River	SVCP
42	0	0	CRP-089	Illinois River	SVCP
43	0	0	CRP-091	Illinois River	SVCP
44	0	0	CRP-092	Illinois River	SVCP
44	0	0	CRP-094	Illinois River	SVCP
44	0	0	CRP-098	Illinois River	SVCP
44	0	0	CRP-100	Illinois River	SVCP
44	0	0	GAN-005	Gan River	SVCP
44	0	0	GAN-006	Gan River	SVCP
43	0	0	GAN-008	Gan River	SVCP
44	0	0	GAN-017	Gan River	SVCP
44	0	0	GAN-025	Gan River	SVCP
44	0	0	GAN-027	Gan River	SVCP
44	0	0	PEA-009	Pearl River	SVCP
44	0	0	YAN-001	Yangtze River	SVCP
44	0	0	YAN-002	Yangtze River	SVCP
44	0	0	YAN-003	Yangtze River	SVCP
44	0	0	YAN-004	Yangtze River	SVCP

Table 3.1. Continued.

Silver carp diagnostic	Heterozygote	Bighead carp diagnostic	Individual ID	Sampling Locality	Genotype
44	0	0	YAN-005	Yangtze River	SVCP
44	0	0	YAN-007	Yangtze River	SVCP
44	0	0	YAN-009	Yangtze River	SVCP
44	0	0	YAN-012	Yangtze River	SVCP
44	0	0	YAN-014	Yangtze River	SVCP
44	0	0	YAN-015	Yangtze River	SVCP
44	0	0	YAN-017	Yangtze River	SVCP
44	0	0	YAN-019	Yangtze River	SVCP
44	0	0	YAN-022	Yangtze River	SVCP
44	0	0	YAN-023	Yangtze River	SVCP
44	0	0	YAN-024	Yangtze River	SVCP
44	0	0	YAN-025	Yangtze River	SVCP
44	0	0	YAN-026	Yangtze River	SVCP
44	0	0	YAN-027	Yangtze River	SVCP
44	0	0	YAN-028	Yangtze River	SVCP
44	0	0	YAN-030	Yangtze River	SVCP
44	0	0	YAN-031	Yangtze River	SVCP
44	0	0	YAN-032	Yangtze River	SVCP
44	0	0	YAN-033	Yangtze River	SVCP
22	0	0	YAN-035	Yangtze River	SVCP
44	0	0	YAN-037	Yangtze River	SVCP

Table 3.2. Unbiased expected heterozygosity of bighead carp from Illinois, silver carp from Illinois, and silver carp from China (all three rivers).

Population	Heterozygosity (H_e)	Standard Error
Illinois Bighead	0.154	0.005
Illinois Silver	0.182	0.005
China Silver	0.175	0.005
Gan River Silver	0.202	0.006
Pearl River Silver	0.170	0.006
Yangtze River Silver	0.114	0.005

Table 3.3. Mean haplotype diversity of bighead carp from Illinois, silver carp from Illinois, and silver carp from China (all three rivers).

Population	Haplotype Diversity (h)	Standard Error
Illinois Bighead	0.145	0.005
Illinois Silver	0.176	0.005
China Silver	0.157	0.003
Gan River Silver	0.193	0.005
Pearl River Silver	0.167	0.005
Yangtze River Silver	0.110	0.005

Table 3.4. Pairwise AMOVA assessing variation in haplotypes between Illinois bighead carp and Illinois silver carp.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	4581.574	4581.574	107.012	50%
Within Pops	97	10331.314	106.508	106.508	50%
Total	98	14912.889		213.521	100%

$\phi_{PT} = 0.501$, $p = 0.001$

Table 3.5. Pairwise AMOVA assessing variation in haplotypes between silver carp from Illinois and silver carp from China.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	1546.418	1546.418	18.347	13%
Within Pops	156	18753.044	120.212	120.212	87%
Total	157	20299.462		138.559	100%

$\phi_{PT} = 0.132$, $p = 0.001$

Table 3.6. Pairwise AMOVA assessing variation in haplotypes among silver carp from the Illinois, Gan, Pearl, and Yangtze rivers.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Among Pops	3	2798.244	932.748	22.199	16%
Within Pops	154	17501.219	113.644	113.644	84%
Total	157	20299.462		135.843	100%

$\phi_{PT} = 0.163$, $p = 0.001$

Table 3.7. Hierarchical AMOVA partitioning variation in haplotypes between regions (Illinois and China), among populations within regions (Illinois River in Illinois; Gan, Pearl, and Yangtze rivers in China), and within populations (Illinois, Gan, Pearl, and Yangtze rivers).

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Regions	1	1546.418	1546.418	6.679	5%
Among Pops	2	1251.826	625.913	17.508	13%
Within Pops	154	17501.219	113.644	113.644	82%
Total	157	20299.462		137.832	100%

$\Phi_{RT} = 0.048$, $p = 0.001$
$\Phi_{PR} = 0.133$, $p = 0.001$
$\Phi_{PT} = 0.175$, $p = 0.001$

Table 3.8. Mean epihaplotype diversity of bighead carp from Illinois, silver carp from Illinois, and silver carp from China (all three rivers).

Population	Epihaplotype Diversity (epih)	Standard Error
Illinois bighead	0.213	0.006
Illinois silver	0.239	0.005
China silver	0.204	0.003
Gan River silver	0.235	0.006
Pearl River silver	0.210	0.006
Yangtze River silver	0.166	0.005

Table 3.9. Average percentage of loci methylated per locus and per individual for Illinois silver carp and silver carp from China.

Population	Average % Methylated per Locus	Average % Methylated per Individual
Illinois bighead	26.4	26.4
Illinois silver	25.9	25.9
China silver	26.2	20.3

Table 3.10. Pairwise AMOVA estimating variation between Illinois and Chinese silver carp epihaplotypes.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	5210.616	5210.616	121.657	50%
Within Pops	97	11942.778	123.121	123.121	50%
Total	98	17153.394		244.779	100%

$\phi_{PT} = 0.497$, $p = 0.001$

Table 3.11. Pairwise AMOVA estimating variation between Illinois and Chinese silver carp epihaplotypes.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	1538.019	1538.019	18.024	12%
Within Pops	156	21367.734	136.973	136.973	88%
Total	157	22905.753		154.996	100%

$\phi_{PT} = 0.116$, $p = 0.001$

Table 3.12. Pairwise AMOVA assessing variation between Illinois bighead carp haplotypes and epihaplotypes.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	3132.533	3132.533	100.786	48%
Within Pops	58	6318.600	108.941	108.941	52%
Total	59	9451.133		2209.728	100%

$\phi_{PT} = 0.281$, $p = 0.001$

Table 3.13. Pairwise AMOVA assessing variation between Illinois silver carp haplotypes and epihaplotypes.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	6201.268	6201.268	88.025	41%
Within Pops	136	17343.464	127.525	127.525	59%
Total	137	23544.732		215.551	100%

$\phi_{PT} = 0.408$, $p = 0.001$

Table 3.14. Pairwise AMOVA estimating variation between Chinese silver carp haplotypes and epihaplotypes.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percentage of Molecular Variance
Between Pops	1	5902.893	5902.893	64.871	33%
Within Pops	176	22777.315	129.417	129.417	67%
Total	177	28680		194.287	100%

$\phi_{PT} = 0.334$, $p = 0.001$

Table 3.15. Mean numbers of 1-1 bands (no methylated sites), 1-0 bands (produced by *Hpa*II only), 1-0 + 0-1 (total number of methylated sites), and % 1-0 + 0-1 (percentage of methylated sites detected across all bands).

Dependent Variable	Illinois Silver Carp Mean	Chinese Silver Carp Mean	P
1-1	13.73	14.65	0.3349
1-0	4.16	5.09	0.0345
1-0 + 0-1	17.82	18.04	0.7836
% 1-0 + 0-1	64.53	62.09	0.1356

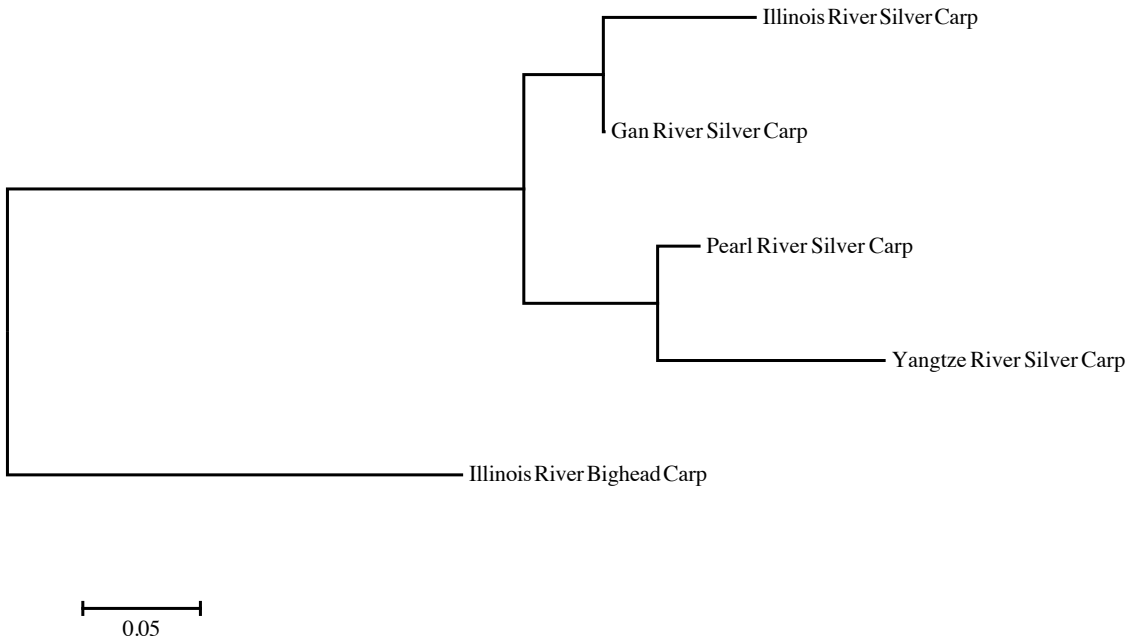

Figure 3.1. Neighbor-joining tree of pairwise ϕ_{PT} among Illinois bighead and silver carp, and silver carp from the Gan, Pearl, and Yangtze rivers in China.

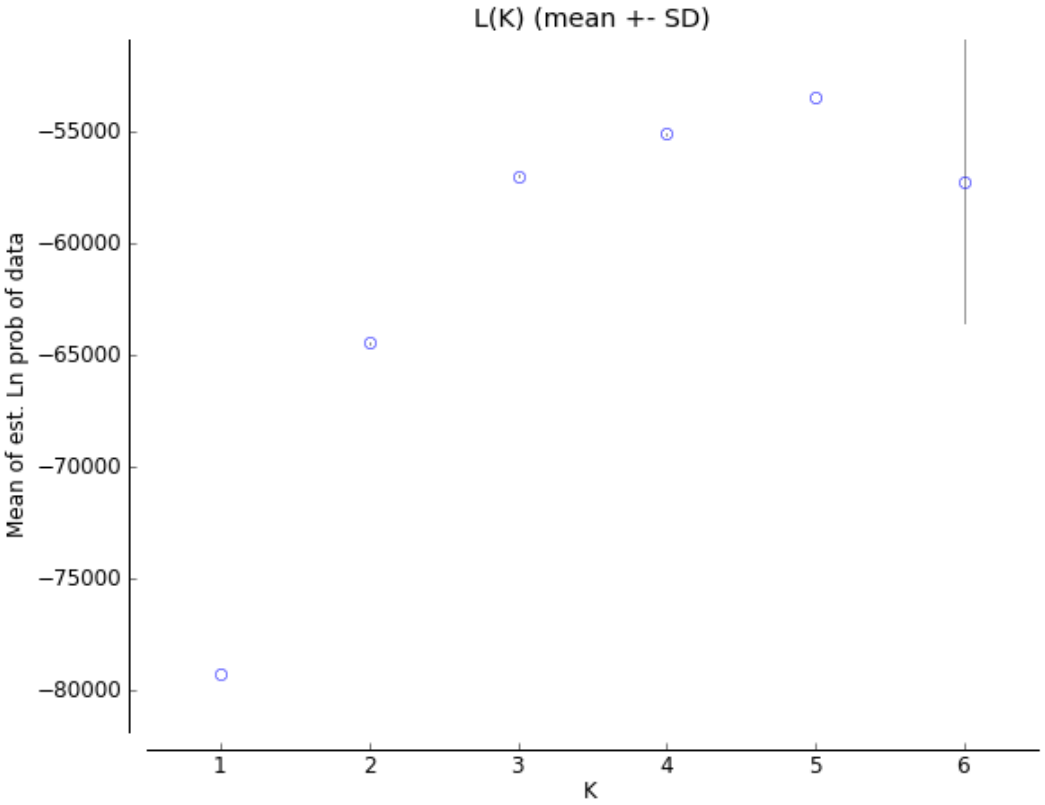

Figure 3.2. Ln(K) graph generated using STRUCTURE Harvester showing the probability of the data for each value of K for both bighead carp and silver carp.

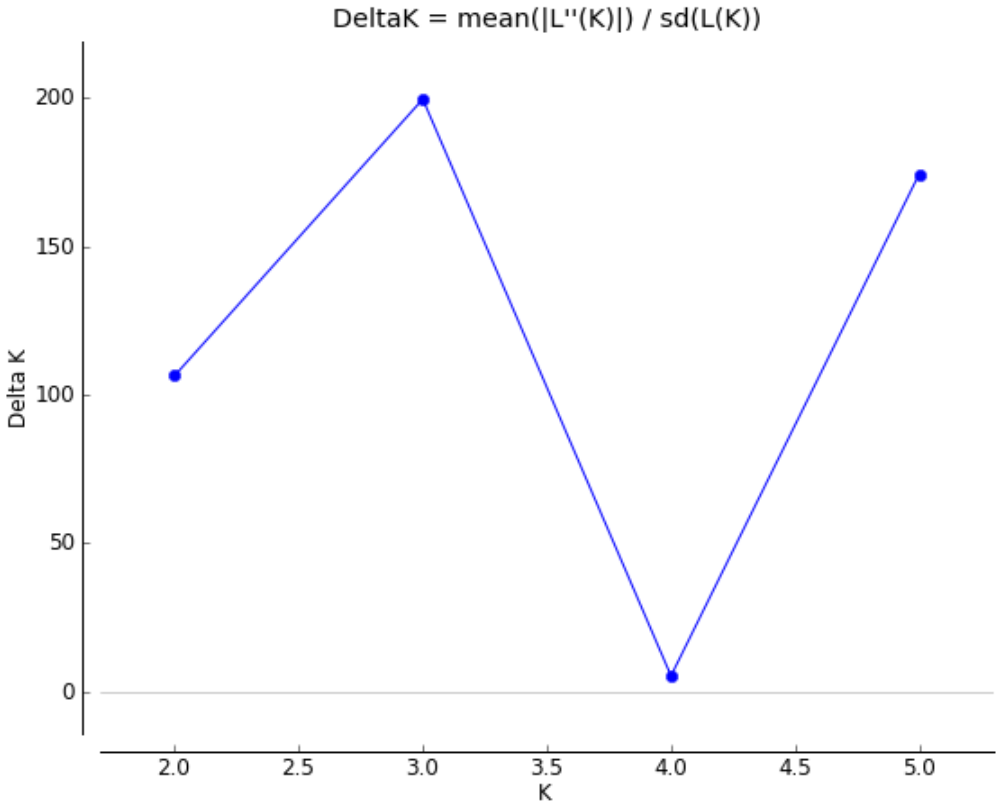

Figure 3.3. ΔK graph generated using STRUCTURE Harvester to show the number of purported populations (K) that best fit the data for both bighead carp and silver carp.

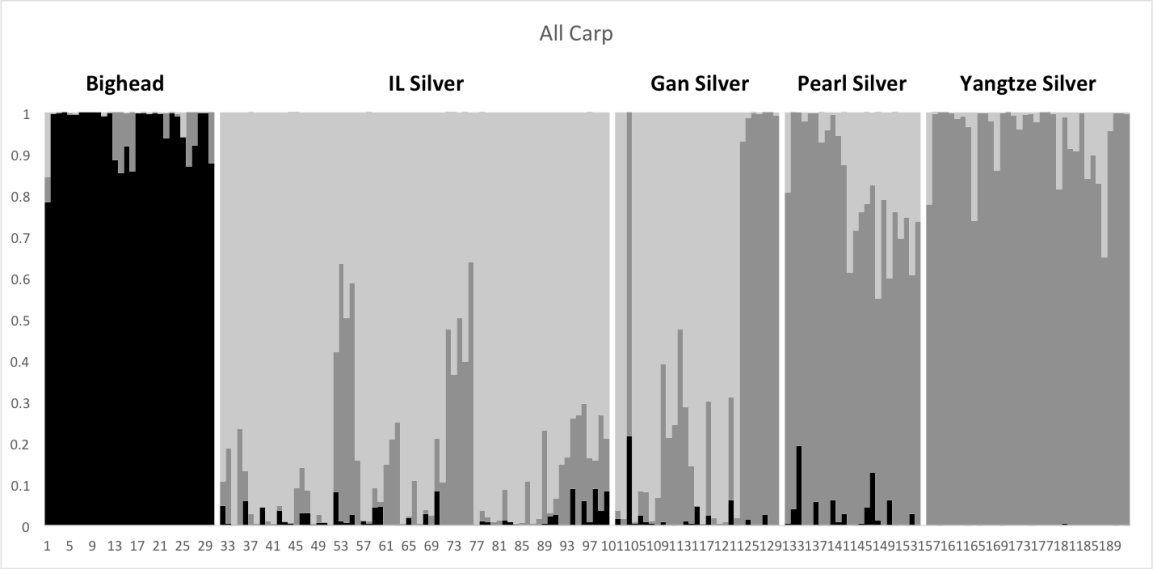

Figure 3.4. Bighead carp and silver carp STRUCTURE analysis for K=3.

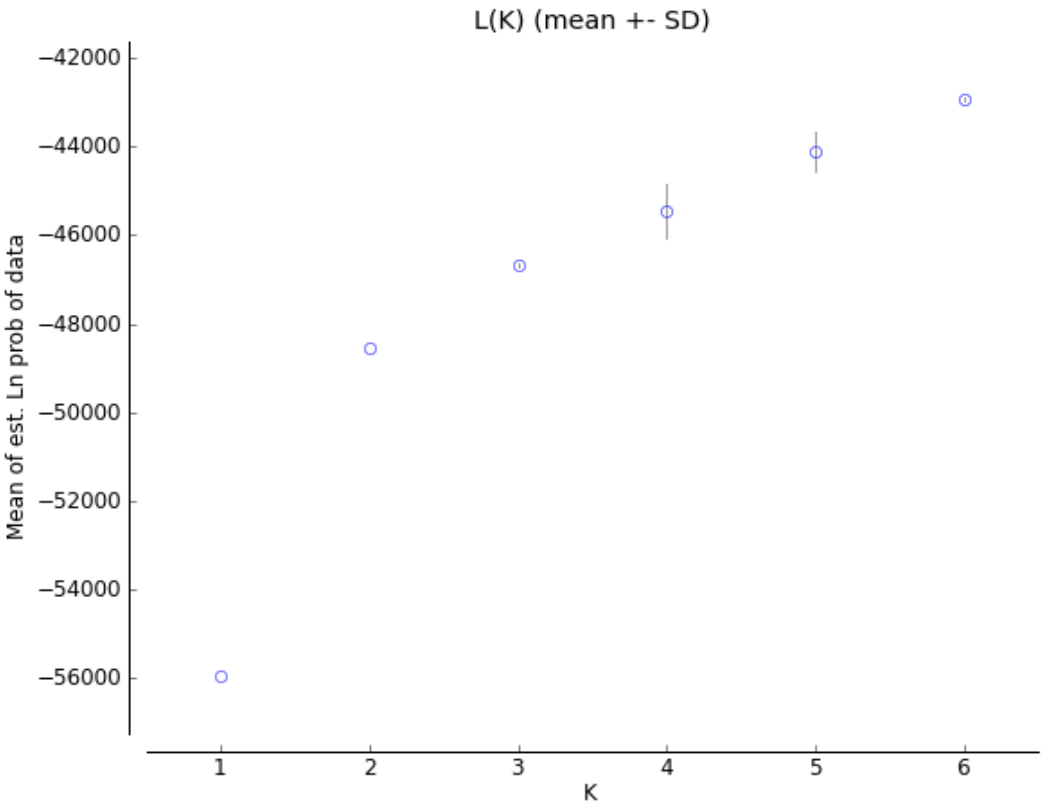

Figure 3.5. Ln(K) graph generated using STRUCTURE Harvester showing the probability of the data for each value of K for only silver carp from both Illinois and China.

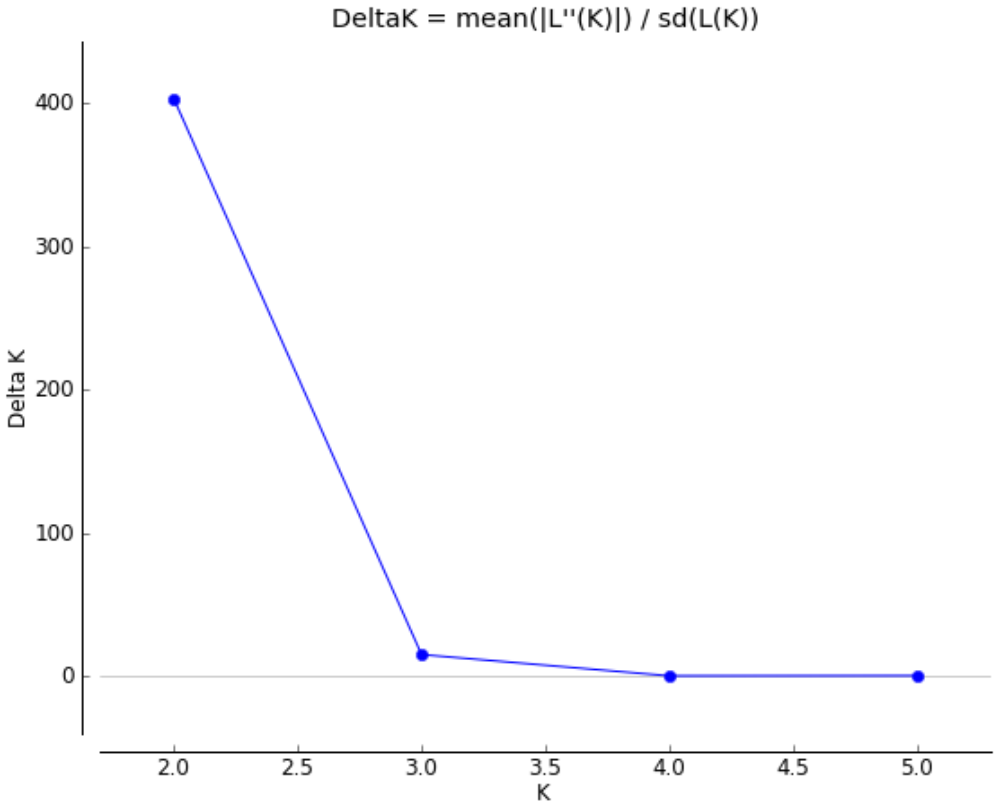

Figure 3.6. ΔK graph generated using STRUCTURE Harvester to show the number of purported populations (K) that best fit the data for only silver carp from both Illinois and China.

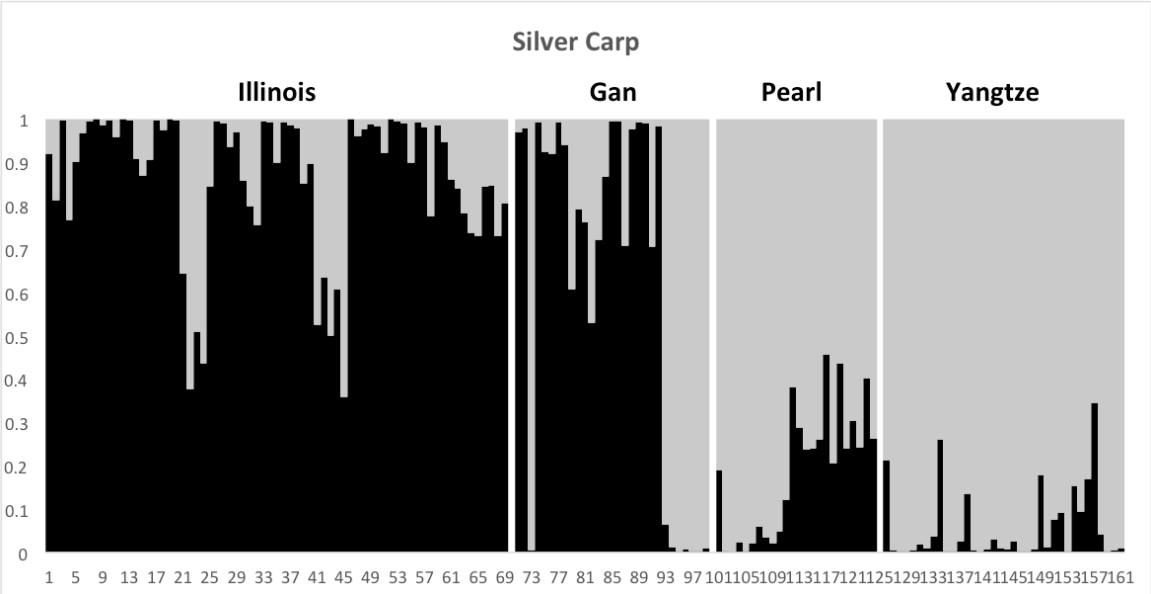

Figure 3.7. Silver carp STRUCTURE analysis for K=2.

CHAPTER 4

DISCUSSION

Heterozygosity was highest in the Gan River population of silver carp. The Illinois River silver carp had the second highest heterozygosity, followed by the Pearl River carp. The Yangtze River silver carp had the lowest heterozygosity. Analysis of haplotype diversity showed similar results with haplotype diversity of silver carp samples from Illinois exceeding haplotype diversity of silver carp samples from China. These results are surprising considering that invasive populations often have genetic variation that is much lower than native populations of the same species. We would expect to see a lower heterozygosity in Illinois silver carp than in the Chinese silver carp due to the founder effect, which reduces genetic diversity when a new population is established by a small number of individuals. There are several factors that may explain this deviation from the expected outcome. First, overfishing has decimated populations of bighead and silver carps in China (Chen et al. 2004). In 2003, the Three Gorges Dam was constructed in the Yangtze River. This dam, as well as other dams, has significantly altered hydrological regimes and seasonal water levels below the dam, interfering with natural carp reproduction and recruitment (Fu et al. 2003; Duan et al. 2009). We also know that bighead and silver carps are stocked in China (Jingou Tong, personal communication), and fish stocking can result in a loss of genetic variation (Araki and Schmid 2010). It is likely that some of my samples came from stocked carp. The combination of overfishing, loss of habitat, and stocking may have reduced genetic variation in silver carp in China. It is also believed that invasive silver carp populations in the United States may be a result of multiple introductions, perhaps from multiple distinct populations, which would ultimately increase the initial size of the founding population and therefore increase genetic variation in the invasive population. Other factors such

as propagule pressure, rapid population expansion, and migration can also increase the genetic diversity of introduced populations. If these factors are taken into account, it seems more plausible that the heterozygosity of the Illinois silver carp would not be lower than that of the Chinese silver carp population. These results seem to agree with a recently published study by Farrington et al. (2017), which found high levels of genetic diversity in both bighead and silver carps in the United States.

The pairwise AMOVA comparing Illinois bighead carp haplotypes to Illinois silver carp haplotypes showed that equal amounts of variation were distributed between and within species. The pairwise AMOVA that partitioned variation between Illinois and Chinese silver carp haplotypes, indicated that the Illinois silver carp shared more variation with the Chinese silver carp than with the Illinois bighead carp. This result is unsurprising, as we would expect two discrete populations of the same species to share more variation than two different species.

The hierarchical AMOVA calculating variation between Illinois and China, among rivers within Illinois and China, and within populations, had a ϕ_{RT}, which quantifies the variation of silver carp between Illinois and China, that was not significant. Thus, variation among the Gan, Pearl, and Yangtze rivers in China is greater than the variation between Illinois and China. This can be explained by the low pairwise distance between silver carp from Illinois and the Gan River ($\Phi_{PT} = 0.065$), which increases the variation among Chinese rivers and decreases the variation between regions.

Likewise, the neighbor-joining tree (Figure 1) clustered the Gan River silver carp population with the Illinois River silver carp population. It is possible that the silver carp originally introduced in the Illinois River had origins in the Gan River. However, there is no documentation detailing the locality from which the invasive silver carp were obtained. The

genetic distance between native and invasive silver carp may be further delineated with a larger sample size of individuals from both China and the United States. As expected, the bighead carp outgroup fell out as being much more distantly related to the silver carp as the populations of silver carp were to each other. This result is supported by the STRUCTURE analysis, which identified three distinct populations: Illinois bighead carp, Illinois + Gan River silver carp, and Pearl + Yangtze River silver carp. I can therefore reject my first null hypothesis, which states that "[n]o significant genetic differentiation between bighead and silver carp populations in China and Illinois." There were significant differences in haplotype frequencies between Illinois and the combined samples from the three Chinese rivers, although there were smaller differences between the Illinois and Gan rivers than between the Pearl and Yangtze.

The STRUCTURE analysis (Figure 4) that included both bighead and silver carps identified three discrete groups. The results at K=3 showed a clear delineation between bighead carp and silver carp from both Illinois and China, and divided the silver carp into one group from the Illinois and Gan rivers and another group from the Pearl and Yangtze rivers. Another STRUCTURE analysis (Figure 7) included only silver carp, and identified two discrete populations. STRUCTURE assigned silver carp from the Pearl and Yangtze rivers mostly to one population and assigned Illinois River and Gan River silver carp mostly to another, although some of the Gan River silver carp were more similar to Pearl and Yangtze river carp than to Illinois carp. These results were also consistent with the results of the neighbor-joining tree (Figure 1).

The epihaplotype diversity of the silver carp samples from Illinois was significantly greater than the epihaplotype diversity of the silver carp samples from China. The AMOVA analyzing the difference between haplotypes indicated there was a moderately low degree of

epigenetic differentiation (12%) between Illinois and Chinese silver carp. Thus, I can reject my second null hypothesis which states "[n]o significant epigenetic differentiation will be observed between Chinese and Illinois samples of bighead and silver carp."

Did silver carp from Illinois and China differ in the amount of methylation? In this analysis, significantly less Type II (1-0) methylation was observed in Illinois silver carp than native Chinese silver carp (Table 13, $p = 0.0345$), but there was no significant overall difference (Type II and Type III) in methylation between Illinois and Chinese silver carp. The Fisher's exact tests identified 29.42% of the individual loci as differing significantly between silver carp in China and Illinois. These results indicate that while the total number of methylation events did not differ significantly between native Chinese silver carp and invasive silver carp in Illinois, the individual bands did differ in frequency between populations. 67.34% of these sites were found to be hypermethylated in Illinois carp relative to the Chinese population. Also, the results of the three AMOVAs that compared variation between haplotypes and epihaplotypes for Illinois bighead, Illinois silver, and Chinese silver carp showed that Illinois bighead and Illinois silver carp haplotypes and epihaplotypes were more different than Chinese silver carp haplotypes and epihaplotypes. Therefore, I can reject my third null hypothesis, which states that "[s]ignificant differences in magnitude of genetic and epigenetic diversity and differentiation will be observed between bighead and silver carp populations in China and Illinois." These data indicate that Illinois carp may indeed utilize differential CpG methylation as a means of adapting to novel environments.

CHAPTER 5

CONCLUSION

Bighead and silver carps are already established throughout the Mississippi River basin, and while there is no evidence to date that invasive bighead and silver carps are established in Lake Michigan or other Laurentian Great Lakes, there continues to be increasing pressure on the barriers that prevent these invasive carp from gaining access to Lake Michigan. The invasion of bighead and silver carps poses a significant threat to the native fishes and invertebrates in the Mississippi Basin, and could wreak havoc on the multi-million dollar fishing industry in the great lakes. Invasive species continue to be a genetic paradox to researchers. They seem to be unaffected by the founder effect, which states that a new population founded by only a few individuals should have reduced genetic diversity and therefore a decrease in overall fitness. The founder effect should limit the ability of a species to successfully establish populations outside of the species' native range. However, invasive species are able to overcome the challenge posed by the founder effect, and despite decreased genetic diversity are able to produce populations of individuals that thrive in their introduced locality. This study, in addition to several other ecological epigenetics studies examining epigenetics in invasive species, shows that epigenetic mechanisms such as DNA methylation may be involved in invasive species' success.

DNA methylation mediates gene activity, allowing certain genes to be activated or repressed. Methylation changes can be passed from parent to offspring, and can be influenced by environmental factors such as food availability and temperature. This study demonstrated that invasive silver carp in the Illinois River are more methylated than native silver carp from three rivers in China. While these results indicate a significant difference in methylation between the two populations of silver carp, further study with a larger sample size and a broader

geographic scope (particularly through the inclusion of invasive bigheaded carp throughout the Mississippi River basin) would be beneficial to definitively determine whether invasive silver carp utilize increased epigenetic modifications like DNA methylation to overcome low levels of genetic diversity.

Understanding the driving factors behind the success of invasive species may help us mitigate the spread of current invasive species and predict and prevent future invasions of alien species. Future studies should focus on DNA methylation and other epigenetic mechanisms as a way for invasive species to compensate for the decreased genetic variation associated with a small initial size of the introduced population.

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