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POPULATION GENETIC STRUCTURE AND EFFECTIVE POPULATION SIZE OF THE THREERIDGE MUSSEL (*AMBLEMA PLICATA*) IN SOUTHEAST OKLAHOMA ASSESSED USING MICROSATELLITES.

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POPULATION GENETIC STRUCTURE AND EFFECTIVE POPULATION SIZE OF THE THREERIDGE MUSSEL (*AMBLEMA PLICATA*) IN SOUTHEAST OKLAHOMA ASSESSED USING MICROSATELLITES.

A THESIS APPROVED FOR THE
DEPARTMENT OF BIOLOGY

BY

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I dedicate this thesis to my mother, Kylene Olson, who instilled in me an interest in biology at an early age. I also dedicate this thesis to my loving and supportive family and friends.

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Abstract

A myriad of anthropogenic factors have led to substantial declines in North America's freshwater mussel populations over the last century. A greater understanding of mussel dispersal abilities, genetic structure, and effective population sizes is imperative to improve conservation strategies. This study used nine microsatellite loci to investigate the genetic structure among mussel beds and estimate effective population sizes of *Amblema plicata* in beds in the Little River in southeast Oklahoma. I genotyped a total of 270 individuals from nine mussel beds distributed throughout a large extent (156 km) of the Little River. Currently, low flow conditions associated with drought, rather than impoundments, is likely the major driver of genetic structuring in the Little River. Genetic structuring was present within the Little River, with three upstream sites forming a distinct genetic cluster from six sites downstream. Years with low flow due to drought likely decreases, or blocks, gene flow between mussels beds in the upper Little River and beds in the lower Little River. Gene flow among beds in the lower Little River may not be impacted due to the higher flows in the lower part of the river. Gene flow in *A. plicata* can occur through the movement of host fish, sperm dispersal, larval thread dispersal, and/or juvenile drift. Estimates of effective population sizes (N_e) of large mussel beds were low compared to the total abundance (N) of *A. plicata* in each large bed with an average N_e/N ratio of 6.4%. Information on the genetic structure and estimates of effective population sizes of mussel beds can provide useful information on the spatial scale at which conservation plans should focus and the population sizes that should be sustained through relocation and restocking programs.

Introduction

Human activities are driving an ongoing mass extinction where we are losing species much more quickly than new species are arising (Ceballos et al. 2015). Conservation genetics aims to prevent further loss of biodiversity through the use of genetic techniques. Populations of many species have been reduced to small numbers and isolated by habitat fragmentation. Conservation genetics examines the negative, genetic consequences of small, isolated populations such as inbreeding, loss of genetic diversity, the accumulation of deleterious alleles, and reduced gene flow. An understanding of the negative impacts small population sizes and fragmentation of populations of a species can help aid conservation of that species (Frankham et al. 2010).

Globally, aquatic faunas are experiencing extinction rates that mirror extinction rates in tropical forests (Ricciardi and Rasmussen 1999; Dudgeon et al. 2006). Habitat deterioration due to pollution and sedimentation, habitat fragmentation and altered flow regimes due to impoundments, stream channelization and dredging, and the introduction of invasive species have led to significant declines and extinction in multiple freshwater taxa in North America (Strayer and Dudgeon 2010). The mean estimate of recent extinction rates (percent loss per decade) across major North American freshwater faunal groups (amphibians, crayfishes, fishes, gastropods, and mussels) is 0.5%, with estimates ranging from 0.1% in crayfishes to 1.2% in mussels (Ricciardi and Rasmussen 1999). Ricciardi and Rasmussen (1999) also estimated future extinction rates using an exponential decay model and found that the mean extinction rate estimate was 3.7%, with estimates ranging from 2.4% in fishes to 6.4% in mussels. Thus, freshwater

biodiversity in North America is highly threatened and requires effective conservation strategies to slow down population declines and extinction rates.

Freshwater mussels (Bivalvia: Unionoida, hereafter "mussels") are a highly diverse and imperiled group of animals. North America has the highest diversity of mussels with approximately 300 species. Of these 300 species, roughly 70% are considered threatened, vulnerable, endangered, or extinct (Lydeard et al. 2004). Even species that are not of conservation concern have shown decreases in abundance over time (Anthony and Downing 2001, Vaughn et al. 2015). Declines in mussel populations can be attributed to a variety of factors, such as habitat destruction and fragmentation, introduction of invasive species, pollution and commercial exploitation of shells for the pearl and pearl button industries (Lydeard et al. 2004; Strayer et al. 2004). Conservation of the remaining mussel fauna is a priority, but without an understanding of basic population biology of mussels, developing successful conservation plans may not be possible. One emerging conservation tool is the propagation and restocking of mussels (FMCS 2016). To do this successfully, we need to understand the spatial scale of genetic structuring and effective population sizes of mussel beds.

Mussels often occur in dense aggregations called mussel beds (hereafter "beds") that are separated by stretches of river with no or very few mussels (Strayer 2004). Mussel larvae (glochidia) are obligate ectoparasites on fish, but adults are sedentary (Barnhart et al. 2008). Thus, gene flow among and within beds occurs through four means of dispersal: movement of glochidia attached to host fish, movement of glochidia before they are attached to fish, as conglutinates or larval threads, movement of sperm, and juvenile drift (Strayer et al. 2004; Schwalb et al. 2011a, 2011b; Zanatta and Wilson

2011; Ferguson et al. 2013; Irmscher 2014; Galbraith et al. 2015; Irmscher and Vaughn 2015; Schwalb et al. 2015). Mussel beds that are connected through gene flow are essentially one large metapopulation that contains more individuals than a single, isolated mussel bed (Vaughn 2012). Generally, small populations have a higher frequency of inbreeding than large populations, and reduced genetic diversity due to genetic drift (Frankham et al. 2010). Inbreeding can result in the buildup of deleterious, recessive alleles in small populations. Populations with low genetic diversity may have a reduced ability to respond to stochastic environmental changes. Both inbreeding and low genetic diversity can increase the risk of extinction of populations (Chen 1993; Frankham 1995b; Newman and Pilson 1997; Frankham et al. 2010).

Genetic structure in mussels is the sum of connectivity among beds due to gene flow through the dispersal of host fish, sperm, glochidia in larval threads or conglutinates, and juvenile drift; and the isolation of populations due to dispersal barriers such as impoundments or stretches of unsuitable habitat (Galbraith et al. 2015). The genetic structure of mussel beds can indicate which beds are connected through gene flow, and which beds are distinct, isolated populations. Genetic structure coupled with the locations of related individuals (in this case parent-offspring relationships) can indicate the spatial scale at which gene flow is occurring. Many studies have used genetic data to estimate relatedness among individuals across a range of taxa including fish (Suk et al. 2010; Perrier et al. 2014) and freshwater mussels (Roe 2010; Ferguson et al. 2013). Habitat disturbances and fragmentation can influence the genetic structure of mussel beds by blocking gene flow among beds (Watters 1996; Strayer et al. 2004; Newton et al. 2008; Schwalb et al. 2011; Galbraith et al. 2015). A number of studies

have evaluated genetic structure in unionid mussels (Berg et al. 1998; Kelly and Rhymer 2005; Berg et al. 2007; Elderkin et al. 2006; Zanatta and Wilson 2011; Galbraith et al. 2015); but, to our knowledge, only one other study has determined the locations of parent-offspring relationships and estimated effective populations sizes of mussel beds (Ferguson et al. 2013).

The goals of this study were to gain an understanding of connectivity between mussel beds and the effective population sizes of mussel beds. I used microsatellites to evaluate population genetic structure and effective population size in a common, widespread mussel species, the threeridge mussel (*Amblema plicata*), in a medium-sized south-central U.S. river known for its diverse and relatively healthy mussel and fish populations (Vaughn and Taylor 1999; Vaughn 2003; Matthews et al. 2005). The Little River is fragmented by both large and low head dams (Vaughn and Taylor 1999; Allen et al. 2013), which might restrict gene flow and result in distinct genetic clusters of individuals upstream and downstream of dams (i.e. genetic structuring). My objectives were to (1) assess the population genetic structure of *Amblema plicata* in the Little River, (2) determine the number of close family relationships (parent-offspring or sibling relationships) among and within mussel beds, and (3) estimate the effective population size of each sampled mussel bed.

Methods

Study Species

Amblema plicata is a wide-ranging mussel species found throughout central and eastern North America, and it's one of the most abundant species in the Little River. *Amblema plicata* is a host generalist, but typically uses fish in the sunfish (Centrarchidae) and perch (Percidae) families (Division of Molluscs, The Ohio State University, Mussel Host Database). Male *A. plicata*, like all unionids, broadcast their sperm into the water column to fertilize females downstream (Haag 2012). Female *A. plicata* release larval threads, which are mucus threads with attached glochidia, into the water column to infect host fish (Haag 2012). Gene flow within and among *A. plicata* subpopulations can occur through the dispersal of fish hosts, sperm, glochidia in larval threads, and juvenile drift.

Study Area

During the summers of 2015 and 2016, I collected *A. plicata* tissue samples from eight mussel beds in the Little River and one mussel bed in the Glover River, a tributary of the Little River, in southeast Oklahoma (Figure 1). The Little River is influenced by two large impoundments and two small low head dams. Pine Creek Dam (constructed in 1969) impounds the main stem of the Little River, and the Mountain Fork River, a major tributary of the Little River, is impounded by the Broken Bow Dam (constructed in 1968), which is a hypolimnetic release dam (Vaughn and Taylor 1999; Matthews et al. 2005). Cold water from Broken Bow Dam has eliminated most mussels in the lower Mountain Fork River and the lower Little River below the confluence of these two rivers. The Mountain Fork River joins the Little River approximately 65 km

downstream of Pine Creek Dam. The Glover River is unimpounded and enters the Little River approximately 30 km downstream of Pine Creek Dam. The two low head dams are located on the main stem of the Little River, one between the outflow of Pine Creek Reservoir and the confluence of the Glover River, and the other between the Glover and Mountain Fork River confluences with the Little River (Figure 1).

Sampling Strategy

Tissue samples from 30 individual *A. plicata* were collected from each site for a total of 270 samples across all sites. Five of the nine sites (sites: 1, 5, 6, 7, and 8) were large mussel beds (>50 m long). These sites were quantitatively sampled with quadrats (Vaughn et al. 1997). Twenty, 0.25 m^2 quadrats were placed randomly along transects throughout the mussel bed and excavated to a depth of 15 cm. The length and width of large mussel beds were measured in meters in order to approximate the area of each large bed. The density (mussels/m^2) of *A. plicata* was calculated using the quadrat data, and the total abundance of *A. plicata* in each large mussel bed was estimated by multiplying the density by the area of each bed. Semi-quantitative time searches (Vaughn et al. 1997) were conducted for an hour at four small mussel beds (<50 m long; sites: 2, 3, 4, and 9). Mussels were located tactilely or visually while snorkeling or scuba diving over the mussel bed. I collected approximately 20 mg of mantle tissue from each mussel and stored it in 95% ethanol. I also measured the shell length of every mussel sampled.

DNA Extraction and Genotyping

DNA was extracted using the methods of the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Microsatellites were used to assess population genetic

structure and effective population size. I amplified nine microsatellite loci using primers developed for *Amblema neislerii*: Anec101, Anec114, Anec122, Anec126, Aned103, Aned104, Aned108, Aned126, and Aned140 (Díaz-Ferguson et al. 2011). I amplified these loci using a variety of different PCR conditions provided by James Cureton (personal communication) and Galbraith et al. (2015) (Table A1). I used the ILS600 red size standard (Promega, Madison, Wisconsin, U.S.) and analyzed the PCR products on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, California, U.S.). Alleles were binned and scored in GeneMapper V3.7 (Applied Biosystems).

Data Analysis

I used GenAlEx 6.502 to calculate expected (H_e) and observed (H_o) heterozygosities and to check for deviations from Hardy-Weinberg equilibrium (HWE) (Peakall and Smouse 2006, 2012). I checked for linkage disequilibrium within and among mussel beds with GENEPOP V4.6 (Raymond and Rousset 1995; Rousset 2008). I estimated null allele frequencies with MICRO-CHECKER (van Oosterhout et al. 2004). Subpopulation pairwise F_{st}'s were calculated with GENEPOP V4.6 (Raymond and Rousset; Rousset 2008). I ran exact G tests to check for significant allelic (genic) differentiation and genotypic differentiation (F_{st} values) in GENEPOP V4.6. Additionally, I used the excluding null alleles (ENA) method to estimate subpopulation pairwise F_{st}'s in the presence of null alleles with FreeNA (Chapuis and Estoup 2007). The ENA method corrects for the positive bias null alleles have on the estimation of F_{st} by excluding null alleles and only using visible alleles to calculate F_{st}. I ran paired Mantel tests with 9999 permutations in GenAlEx 6.502 (Peakall and Smouse 2006, 2012) using the uncorrected pairwise F_{st}'s and ENA adjusted pairwise F_{st}'s and

geographic distances (river kilometers between sites measured with the path function in Google Earth Pro) to analyze genetic isolation-by-distance (IBD) across all sites.

I used STRUCTURE (Version 2.3.4; Pritchard et al. 2000), which uses a Bayesian clustering method to assign individuals to beds and infer genetic structure, to evaluate population genetic structure. Across all runs, I assumed independent allele frequencies and allowed for individuals to be admixed among subpopulations. In order to assist clustering, I used the sampling location of each individual as prior information. Each run had an initial burn-in period of 50,000 and was followed by an additional 100,000 MCMC replicates. I ran 10 iterations for each value of K (genetic clusters). Values of K ranged from 1-9 and were based on the number of mussel beds sampled. STRUCTURE HARVESTER (V0.6.94; Earl and vonHoldt 2012) was used to determine the number of genetic clusters (K) that fit the data best. The value of K that corresponds to the greatest $P(X \mid K)$ value was identified as the number of genetic clusters in the study area, which according to Evanno et al. (2005), is a good predictor of the real number of genetic clusters. I used the FullSearch algorithm in CLUMPP (Version 1.1.2; Jakobsson and Rosenberg 2007) to find the optimal alignment of 10 replicate cluster analyses from STRUCTURE with K = 2, and distruct (Version 1.1; Rosenberg 2004) was used to graphically represent the individual assignment scores of all 270 individuals across the 9 mussel beds. Beds that belonged to the same genetic cluster were grouped together and checked for evidence of recent population declines using a Wilcoxon test in BOTTLENECK (Version 1.2.02; Cornuet and Luikart 1996) under the infinite allele and two-phase models with 1000 iterations.

Microsatellite genotypes were used to estimate the number of close family relationships (parent-offspring or sibling relationships) among and within mussel beds using ML-Relate (Kalinowski et al. 2006), which is a program that calculates maximum likelihood estimates of relatedness and accommodates null alleles. I used NeEstimator (Version 2.01; Do et al. 2014, Waples and Do 2008) to calculate the effective population size (N_e) of each mussel bed using the linkage disequilibrium (LD) method. I calculated the proportion of reproductively active *A. plicata* individuals (N_e/N) in each of the five large mussel beds by dividing the effective population size by the total abundance.

Results

I genotyped 30 *A. plicata* individuals from each bed for a total of 270 individuals. There are missing data for the Anec126 locus for one individual and the Aned103 locus for 12 individuals. The number of alleles ranged from 4 to 31 across loci and beds (Table 1). Anec122 had the lowest number of alleles across beds with 4 alleles, while Anec126 had the highest number of alleles across beds with 31 alleles (Table 1). The mean numbers of alleles across loci were lower at upstream sites (8.33-10.22) than downstream sites (10.89-12.89; Table 1). Mean observed heterozygosities (H_o) ranged from 0.50 to 0.63, and mean expected heterozygosities (H_e) ranged from 0.66 to 0.79 among sites (Table 1). MICRO-CHECKER calculated null allele frequencies ranging from -0.22 to 0.41. Null alleles were present at three loci (Aned103, Aned104, and Aned108) across all nine sites and at one locus (Anec101) at eight of the nine sites (Table 2). Null alleles were also present at two loci (Anec126 and Aned140) at two sites per locus. A negative null allele frequency indicates a heterozygote excess, which occurred at 22 locus-bed combinations. Deviations from HWE due to heterozygote deficiencies occurred across all sites for two loci (Anec101 and Aned104), while Aned103 and Aned108 deviated from HWE at eight and six sites, respectively (Table 1). The remaining five loci deviated from HWE at three or fewer sites (Anec114 – 0, Anec122 – 1, Anec126 – 3, Aned126 – 3, and Aned140 – 3; Table 1). There was no evidence of linkage disequilibrium between loci across all subpopulations. Linkage disequilibrium between two or fewer loci was detected within six sites.

Uncorrected pairwise F_{st} values ranged from -0.0007 (between sites 6 and 8) and 0.0537 (between sites 1 and 3; Table 3). There was significant ($P<0.05$) allelic and

genotypic differentiation (F_{st}) at 33 and 21 of the 36 subpopulation pairs, respectively (Table 3). The paired Mantel test found a significant positive relationship between genetic (F_{st}) and geographic (river km) distance within the Little River ($P < 0.01$, $R = 0.75$). Pairwise F_{st}ENA (Excluding Null Alleles) values ranged from -0.0002 (between sites 5 and 7) and 0.0497 (between sites 1 and 3; Table 4). Pairwise geographic distances ranged from 0.39 km to 155.79 km (Table 5; Figure 1). The paired Mantel test found a significant positive relationships between the adjusted genetic distance (F_{st}ENA) and geographic distance (river km) within the Little River ($P < 0.001$, $R = 0.73$; Figure 2). Analysis of population genetic structure revealed two genetic clusters among *A. plicata* subpopulations (Figure 3). Sites 1-3 form one cluster and sites 4-9 form the second cluster ($F_{st} = 0.0112$, $P < 0.001$). Figure 3 shows a gradient where individuals downstream of the confluence of the Glover and Little Rivers have a higher proportion of cluster two (orange) in their assignment scores than individuals upstream of the confluence that have a higher proportion of cluster one (blue) in their assignment scores, which suggests that there is significant isolation-by-distance in the *A. plicata* population in the Little River. There was no evidence of recent population bottlenecks in the two genetic clusters.

ML-Relate found a total of 84 close family relationships (likely parent-offspring or sibling relationships) out of 36,315 pairwise relationships, of which 19 involved individuals within the same bed and 65 involved individuals found in different beds. Familial relationships within beds were recovered from six sites (1, 2, 4, 5, 6, and 9; Figure 1). Distances between close relatives ranged from meters (within beds) up to ~135 km (sites 1 and 8).

Large mussel beds had *A. plicata* densities ranging from 3.5 to 9.4 individuals per square meter, and small beds had catch per unit efforts (CPUE) ranging from 34 to 82 individuals per hour (Table 6). The abundance of *A. plicata* in large beds ranged from 1,572 individuals in a 449 m^2 bed to 61,776 individuals in a 7,020 m^2 bed. The effective population sizes of the five large beds were relatively low ranging from 251 (95% CI: 90-Infinite) at site 8 to 1,492 (95% CI: 138-Infinite) at site 7; and the effective population sizes of small beds ranged from 556 (95% CI: 109-Infinite) to infinity (Table 6). The mean proportion of individuals breeding (N_e/N) among the five large mussel beds was 0.064.

Discussion

My results indicate that there is genetic structure among subpopulations of *Amblema plicata* in the Little River. Three sites upstream of the confluence of the Glover and Little Rivers formed a genetic cluster and six sites downstream of the confluence formed another cluster. Although I found no evidence of a recent population bottleneck at the upstream sites, the low mean numbers of alleles across loci at upstream sites suggests that these sites have decreased population sizes and that there is lower genetic diversity at these upstream sites. A likely mechanism underlying these differences could be restricted gene flow during periods of drought, which is common and cyclical in the southcentral U.S. and has been shown to lead to decreases in mussel population sizes in rivers in this region (Galbraith et al. 2010; Atkinson et al. 2014; Vaughn et al. 2015). The Little River above the confluence of the Glover River is smaller and higher gradient than more downstream reaches, and during droughts riffle areas can become dry or very shallow (Vaughn 2003; Matthews et al. 2005). Gene flow among mussel beds requires sufficient flow for the movement of fish hosts, sperm, and glochidia. Irmscher and Vaughn (2015) found that the movement of fish hosts in the Little River was restricted during drought periods. Thus, low water conditions during droughts may restrict gene flow between sites above and below the confluence of the Glover River, leading to the genetic structuring that I observed. I did not observe genetic structuring among sites in the lower river (below the confluence of the Glover River), and this is likely because there is sufficient gene flow among these sites. Other studies have also found no genetic structuring of mussel populations where stream

flows are consistent and unfragmented (Szumowski et al. 2012; Ferguson et al. 2013; Galbraith et al. 2015).

Although the Little River is fragmented by both large and small low head dams, this fragmentation does not appear to be affecting gene flow among *A. plicata* subpopulations in the river. Pine Creek Dam (constructed in 1969) impounds the river itself; and thus should impede host fish migration and prevent gene flow between beds up and downstream of that dam. However, site 1 is upstream of Pine Creek Dam and is part of the same genetic cluster as sites 2 and 3, which are downstream of the dam. Broken Bow Dam (constructed in 1968) is a hypolimnetic release dam on a major tributary of the Little River, the Mountain Fork River. Cold water constantly flowing into the Little River via the Mountain Fork has caused significant declines in Little River mussel beds downstream from the release (Vaughn and Taylor 1999); this could also hinder gene flow. Finally, small low head dams on the Little River main stem might also slow down gene flow. However, I did not find distinct genetic clusters upstream and downstream of any of the dams; rather, I found that sites upstream and downstream of Pine Creek Dam (sites 1-3) formed a single genetic cluster. Other studies have also shown that dams can have little effect on genetic structure in mussels (Kelly and Rhymer 2005; Szumowksi et al. 2012). In the Little River, the lack of apparent fragmentation effects by dams is likely due to two factors. First, these dams were constructed relatively recently (late 1960s) but mussels, including *A. plicata*, are long-lived organisms with long generation times (Haag and Rypel 2011). In a study of growth and longevity of mussels in southeast Oklahoma using dendrochronological techniques, maximum ages of adult *A. plicata* from three rivers ranged from 53-79

years old (Sansom et al. 2016). Thus, there have likely not been enough mussel generations to cause genetic isolation up and downstream of the large dams. Second, the Little River experiences frequent high flows (Matthews et al. 2005) and fish hosts should be able to freely migrate over low head dams during these flood events.

This study is one of the first to examine the locations of familial relationships in mussels to gain an understanding of the scale at which gene flow is occurring. These relationships could be parent-offspring or sibling relationships, but are an indicator of close relatedness. The locations of familial relationships indicated that gene flow in *A. plicata* might be occurring at large geographic distances. In two cases, closely related mussels were separated by ~135 river kilometers (sites 1 and 8), which likely happened before the construction of Pine Creek Dam. Ferguson et al. (2013) found large distances between parents and offspring in *Lampsilis cardium* populations in Ohio; 18 of 29 parent-offspring relationships involved individuals that were >3.8 km apart. The genetic structure of *A. plicata* populations is the result of gene flow among and within beds, which occurs through host fish movement, sperm dispersal, larval thread dispersal, and juvenile drift. The primary host fish for *A. plicata*, bass and sunfish (Centrarchidae), are highly mobile and known to travel long distances. In addition, *A. plicata* is a host generalist that can use many species of fish as hosts; so even if the migration of one host species is impeded, *A. plicata* may still be able to use other fish hosts for dispersal. Finally, the long-distance transport of sperm broadcasted by males, larval threads broadcasted by females, and juveniles by stream flow may connect distant, as well as adjacent, beds via gene flow. Any of these four modes of gene flow could influence the locations of offspring relative to their parents or of sibling mussels.

This study, to my knowledge, is the first to compare the effective population sizes of mussel beds to the total subpopulation size (N_e/N). Mean estimates of N_e/N ranged from 0.10-0.11 from 192 published estimates across 102 species (Frankham 1995a). I found that estimates of effective population sizes of *A. plicata* estimated from microsatellite genotypes were small relative to the total subpopulation sizes measured by quantitatively sampling mussels. The mean proportion of breeding mussels in the five large beds where total subpopulation size was available was 0.064. While this proportion is lower than Frankham's mean estimate of 0.10-0.11, it's not substantially lower. Other broadcast spawning species have widely variable N_e/N ratios. Hedgecock et al. (1992) estimated the N_e/N ratio for Pacific oysters (*Crassostrea gigas*) and found that $N_e/N < 10^{-6}$. Another broadcast spawning species, sea bass (*Atractoscion nobilis*), had N_e/N ratios ranging from 0.27 to 0.40 (Bartley et al. 1992). My results are corroborated by other studies that have also found effective population sizes that seem low but in different mussel species (Kelly and Rhymer 2005; Roe 2010; Ferguson et al. 2013). Kelly and Rhymer (2005) found that estimates of effective population sizes in *Lampsilis cariosa* ranged from 150 to 1,850 individuals across nine sites in three river drainages in Maine while Ferguson et al. (2013) found that estimates of effective population sizes in *Lampsilis cardium* ranged from 1.5 to 205.8 individuals across eight sites in Ohio. Roe (2010) estimated the effective population size of *Quadrula fragosa* in the St. Croix River as 149.2 individuals. Estimates of effective population sizes in other mussel species seem low, but this needs to be confirmed by comparing to the total population sizes. Estimating the effective population size of mussel beds is difficult because gene flow can increase the estimate of effective population size. Regardless,

restocking programs should ensure that population sizes are large enough to support effective population sizes that can sustain the mussel population.

This study provides a deeper understanding of the population genetics of a common mussel species, but there are limitations to the results. First, four of the nine loci consistently deviated from HWE due to heterozygote deficiencies; and estimated null allele frequencies were high at these four loci (0.08-0.41), indicating that many alleles did not amplify during PCR. Secondly, I could only get nine of 11 loci to successfully amplify with PCR. Additional loci would provide more resolution when trying to estimate relatedness between individuals and effective population sizes. Furthermore, all studies of *A. plicata* genetic structure to date have used microsatellite primers developed for *A. neislerii* (Díaz-Ferguson et al. 2011). Primers developed specifically for *A. plicata* may amplify more successfully. Finally, single nucleotide polymorphisms (SNPs) may provide better resolution, as there is no limitation on the number of loci.

This study provides important information on the genetic structure and effective population size of a common mussel species. Such information can be used to help manage and conserve not only common species, but also rare mussel species. Galbraith et al. (2015) found that mussel genetic structure did not vary as a function of rarity, and suggested that common species could be used as surrogates for rare species. Rare species (*Epioblasma triquetra* and *Ptychobranchus fasciolaris*) exhibited the same geographic patterns of genetic structuring as common species (*Amblema plicata* and *Lasmigona costata*)(Galbraith et al. 2015). Sampling for common species is less time intensive and thus less expensive than sampling for rare species.

I found significant genetic structuring of *A. plicata* subpopulations within a large extent (156 km) of the Little River. However, other studies have found that genetic structuring of mussel populations is typically not present within rivers; but, rather, across watersheds (Szumowski et al. 2012; Ferguson et al. 2013; Galbraith et al. 2015). Within the Little River, genetic structuring among beds appears to be influenced by flow conditions. Gene flow among beds in the upper reaches may potentially be impacted more by low water levels during drought than gene flow among beds in the lower parts of the river due to river size, which may account for the distinct genetic clusters found in the upper and lower parts of the river. In stretches of river with genetically similar beds, individuals could be translocated from healthy beds to beds that are declining (Galbraith et al. 2015). Additionally, management could use individuals from stable beds to propagate mussels to be restocked into beds that are suffering from declines. Obviously, in order for relocation and restocking programs to be successful, there must be high quality, unfragmented habitat. My study, unlike most other studies on genetic structuring in mussels, found genetic structuring among mussel beds within a single watershed, which emphasizes the importance of understanding mussel population genetics before implementing conservation strategies such as propagation and relocation programs. Information on the genetic structure and effective population size of mussel populations can provide useful information on the spatial scale at which conservation plans should focus on and the population size that should be sustained through relocation and restocking programs.

Table 1. Genetic diversity metrics per locus and site of *Amblema plicata* in the Little River. Figure 1 shows the locations of each site. n = number of individuals genotyped per locus. H_o = observed heterozygosity. H_e = expected heterozygosity. Bold font indicates departures from Hardy-Weinberg equilibrium.

Locus	Site / Metric	1	2	3	4	5	6	7	8	9	All
Anec101	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	15	6	11	7	8	16	13	10	11	27
	H_o	**0.80**	**0.33**	**0.50**	**0.20**	**0.40**	**0.53**	**0.43**	**0.33**	**0.37**	0.43
	H_e	0.85	0.70	0.80	0.77	0.78	0.88	0.85	0.79	0.81	0.80
Anec114	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	6	9	10	13	12	11	12	9	11	16
	H_o	0.80	0.90	0.87	0.93	0.87	0.90	0.77	0.90	0.83	0.86
	H_e	0.78	0.84	0.82	0.87	0.89	0.85	0.88	0.85	0.86	0.85
Anec122	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	2	2	2	2	2	3	3	2	3	4
	H_o	0.50	0.27	0.10	0.47	0.23	**0.30**	0.30	0.30	0.27	0.30
	H_e	0.47	0.28	0.10	0.44	0.34	0.35	0.26	0.30	0.24	0.31
Anec126	n	30	30	30	30	30	30	30	29	30	269
	# of alleles	12	20	20	20	24	19	24	19	22	31
	H_o	0.90	1.00	**0.83**	0.90	0.87	**0.80**	**0.90**	0.83	0.97	0.89
	H_e	0.38	0.92	0.91	0.93	0.94	0.93	0.94	0.93	0.95	0.92
Aned103	n	28	30	30	30	30	29	28	26	27	258
	# of alleles	4	7	7	8	7	7	6	5	5	12
	H_o	**0.21**	0.30	**0.37**	**0.37**	**0.10**	**0.28**	**0.32**	**0.19**	**0.22**	0.26
	H_e	0.60	0.55	0.68	0.70	0.76	0.73	0.65	0.73	0.59	0.66

Table 1. Continued

Locus	Site / Metric	1	2	3	4	5	6	7	8	9	All
Aned104	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	8	12	8	12	14	13	15	12	11	19
	H_o	**0.57**	**0.33**	**0.37**	**0.43**	**0.57**	**0.50**	**0.37**	**0.53**	**0.27**	0.44
	H_e	0.78	0.87	0.82	0.87	0.89	0.90	0.92	0.83	0.88	0.86
Aned108	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	13	16	18	15	20	20	16	26	25	29
	H_o	**0.57**	**0.57**	**0.63**	**0.63**	0.67	**0.60**	**0.47**	0.80	0.80	0.64
	H_e	0.80	0.89	0.89	0.86	0.90	0.94	0.90	0.94	0.94	0.90
Aned126	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	8	11	6	11	11	13	14	14	13	18
	H_o	0.43	0.70	0.23	0.53	0.63	**0.67**	**0.70**	**0.63**	0.77	0.59
	H_e	0.38	0.60	0.22	0.59	0.60	0.68	0.68	0.68	0.75	0.57
Aned140	n	30	30	30	30	30	30	30	30	30	270
	# of alleles	7	9	7	10	11	14	12	10	13	16
	H_o	0.87	**0.70**	0.63	**0.60**	0.83	0.90	**0.70**	0.80	0.73	0.75
	H_e	0.71	0.76	0.73	0.82	0.83	0.87	0.84	0.85	0.80	0.80
Mean	n	29.78	30.00	30.00	30.00	30.00	29.89	29.78	29.44	29.67	269
	# of alleles	8.33	10.22	9.89	10.89	12.11	12.89	12.78	11.89	12.67	19.11
	H_o	0.63	0.57	0.50	0.56	0.57	0.61	0.55	0.59	0.58	0.57
	H_e	0.70	0.71	0.66	0.76	0.77	0.79	0.77	0.77	0.76	0.74

Table 2. Null allele frequencies per locus across the nine sites. Negative null allele frequencies indicate a heterozygote excess at a given locus and site. Bold font indicates the presence of null alleles at a given locus and site.

Site	1	2	3	4	5	6	7	8	9
Locus									
Anec101	0.03	**0.26**	**0.19**	**0.35**	**0.24**	**0.19**	**0.25**	**0.27**	**0.27**
Anec114	-0.01	-0.04	-0.03	-0.04	0.02	-0.03	0.06	-0.04	0.02
Anec122	-0.03	0.02	-0.05	-0.03	0.13	0.06	-0.16	0.00	-0.14
Anec126	-0.01	-0.05	0.04	0.02	0.04	**0.07**	0.02	**0.06**	-0.01
Aned103	**0.29**	**0.20**	**0.22**	**0.23**	**0.41**	**0.29**	**0.23**	**0.34**	**0.28**
Aned104	**0.14**	**0.30**	**0.28**	**0.25**	**0.18**	**0.22**	**0.30**	**0.18**	**0.34**
Aned108	**0.14**	**0.18**	**0.14**	**0.13**	**0.13**	**0.18**	**0.24**	**0.08**	**0.08**
Aned126	-0.22	-0.14	-0.12	0.07	-0.03	0.00	-0.02	0.04	-0.03
Aned140	-0.12	0.04	0.06	**0.14**	0.00	-0.03	**0.08**	0.02	0.03

Table 3. Pairwise F_{st} values. Bold font indicates significant allelic differentiation between subpopulations ($P < 0.05$). Asterisks indicate significant genotypic differentiation between subpopulations ($P < 0.05$). See Figure 1 for site locations.

Site	1	2	3	4	5	6	7	8	9
1	0.0000								
2	**0.0309***	0.0000							
3	**0.0537***	**0.0225***	0.0000						
4	**0.0298***	**0.0151***	**0.0343***	0.0000					
5	**0.0275***	**0.0087**	**0.0218***	**0.0014**	0.0000				
6	**0.0291***	**0.0115**	**0.0221***	**0.0056**	-0.0053	0.0000			
7	**0.0421***	**0.0038**	**0.0192***	**0.0120***	**-0.0033**	**-0.0047**	0.0000		
8	**0.0294***	**0.0157***	**0.0249***	**0.0164***	-0.0041	**-0.0007**	**0.0023**	0.0000	
9	**0.0489***	**0.0164***	**0.0281***	**0.0144***	**-0.0040**	**-0.0026**	-0.0028	**0.0010**	0.0000

Table 4. Pairwise F_{st}ENA (Excluding Null Alleles) values. See Figure 1 for site locations.

Site	1	2	3	4	5	6	7	8	9
1	0.0000								
2	0.0283	0.0000							
3	0.0497	0.0231	0.0000						
4	0.0257	0.0155	0.0350	0.0000					
5	0.0239	0.0103	0.0233	0.0012	0.0000				
6	0.0268	0.0096	0.0238	0.0030	-0.0039	0.0000			
7	0.0413	0.0048	0.0209	0.0109	-0.0002	-0.0030	0.0000		
8	0.0280	0.0144	0.0249	0.0144	-0.0006	-0.0006	0.0016	0.0000	
9	0.0459	0.0147	0.0273	0.0124	0.0004	-0.0006	-0.0033	0.0028	0.0000

Table 5. Pairwise geographic distances (river km) between sites. See Figure 1 for site locations.

Site	1	2	3	4	5	6	7	8	9
1	0.00								
2	74.56	0.00							
3	102.66	28.10	0.00						
4	86.96	12.40	15.94	0.00					
5	101.96	27.40	30.94	15.00	0.00				
6	131.31	56.75	60.29	44.35	29.35	0.00			
7	134.31	59.75	63.29	47.35	32.35	3.00	0.00		
8	134.70	60.14	63.68	47.74	32.74	3.39	0.39	0.00	
9	155.79	81.23	84.77	68.83	53.83	24.48	21.48	21.09	0.00

Table 6. Demographic metrics for *A. plicata* at each site. Area (m^2); density (mussels/m^2); total number of *A. plicata* individuals; effective population size; and proportion of individuals breeding were estimated for large beds because quantitative sampling using quadrats was only completed at large beds. CPUE = catch per unit effort. N = total number of individuals. N_e = effective population size. N_e/N = proportion of individuals breeding. Small beds are indicated with an asterisk by the site number. Negative N_e values can be explained by sampling error and interpreted as an infinite N_e since there was no evidence in the genetic characteristic that there was a finite number of parents (NeEstimator V2.01 Help File).

Site	Area (m^2)	Density (mussels/m^2)/ CPUE (mussels/hr)	N	N_e (LDN_e)	N_e/N
1	449	3.5	1572	295	0.188
2*	-	75	-	-192	-
3*	-	41	-	-258	-
4*	-	82	-	556	-
5	2598	3.6	9353	567	0.061
6	4949	4.0	19796	742	0.037
7	4900	9.4	46060	1492	0.032
8	7020	8.8	61776	251	0.004
9*	-	34	-	-793	-

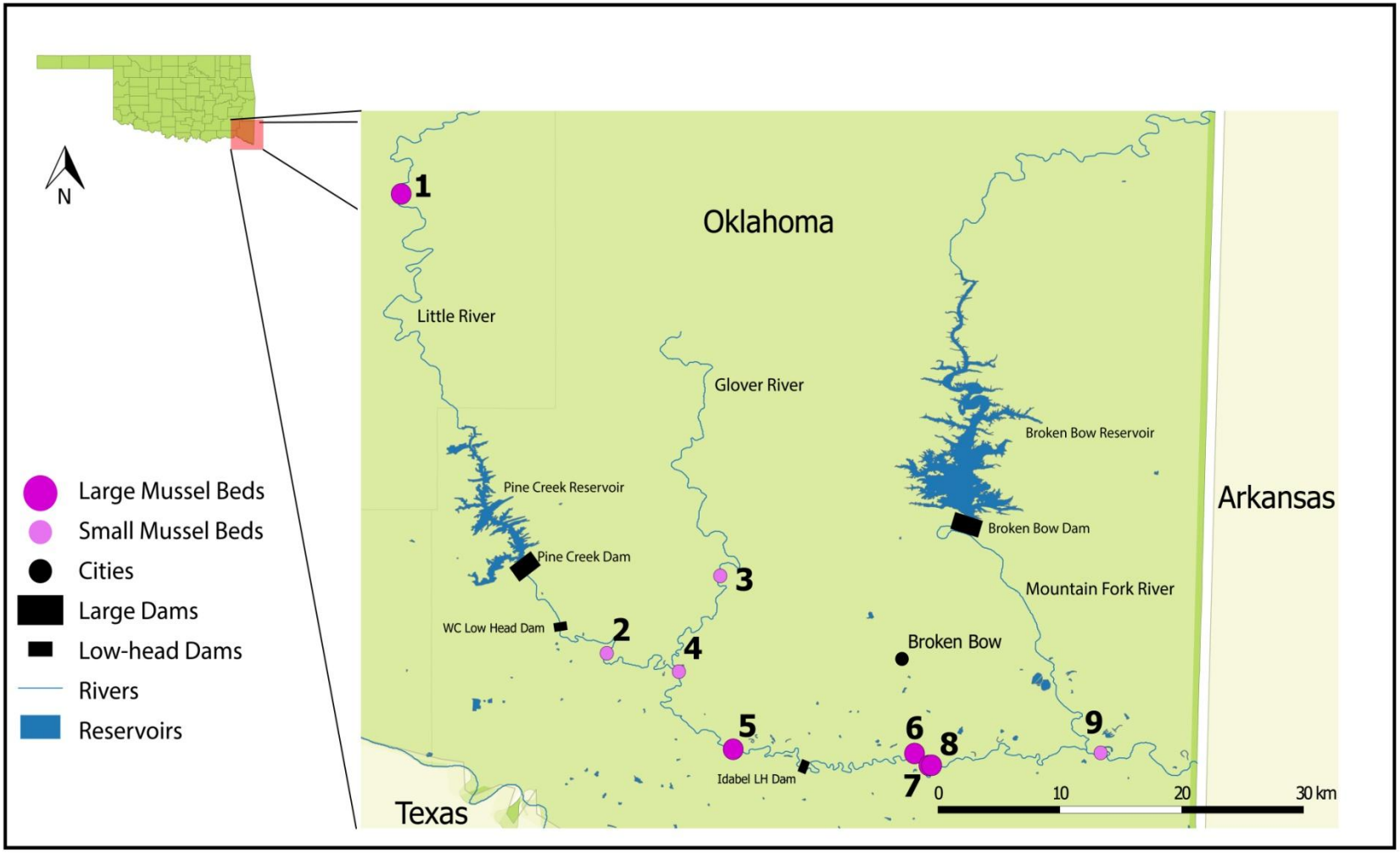

Figure 1. Sampling sites in the Little River.

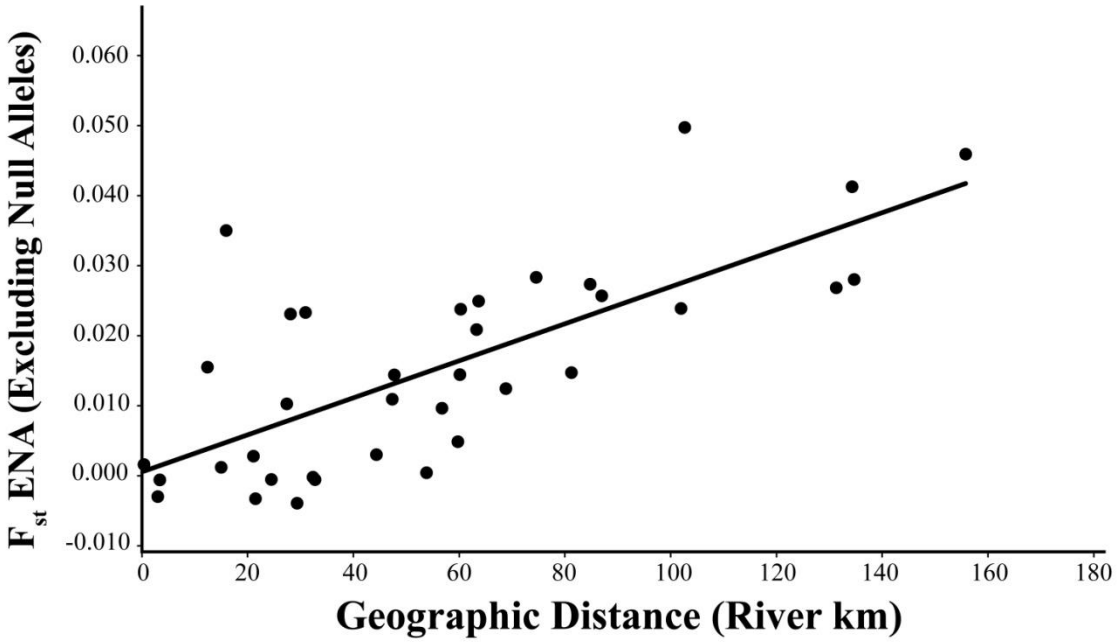

Figure 2. Relationship between geographic distance (river km) and genetic distance (F_{st}ENA). There was a significant positive relationship between geographic and genetic distance among nine *A. plicata* subpopulations in the Little River ($P < 0.001$, $R = 0.73$).

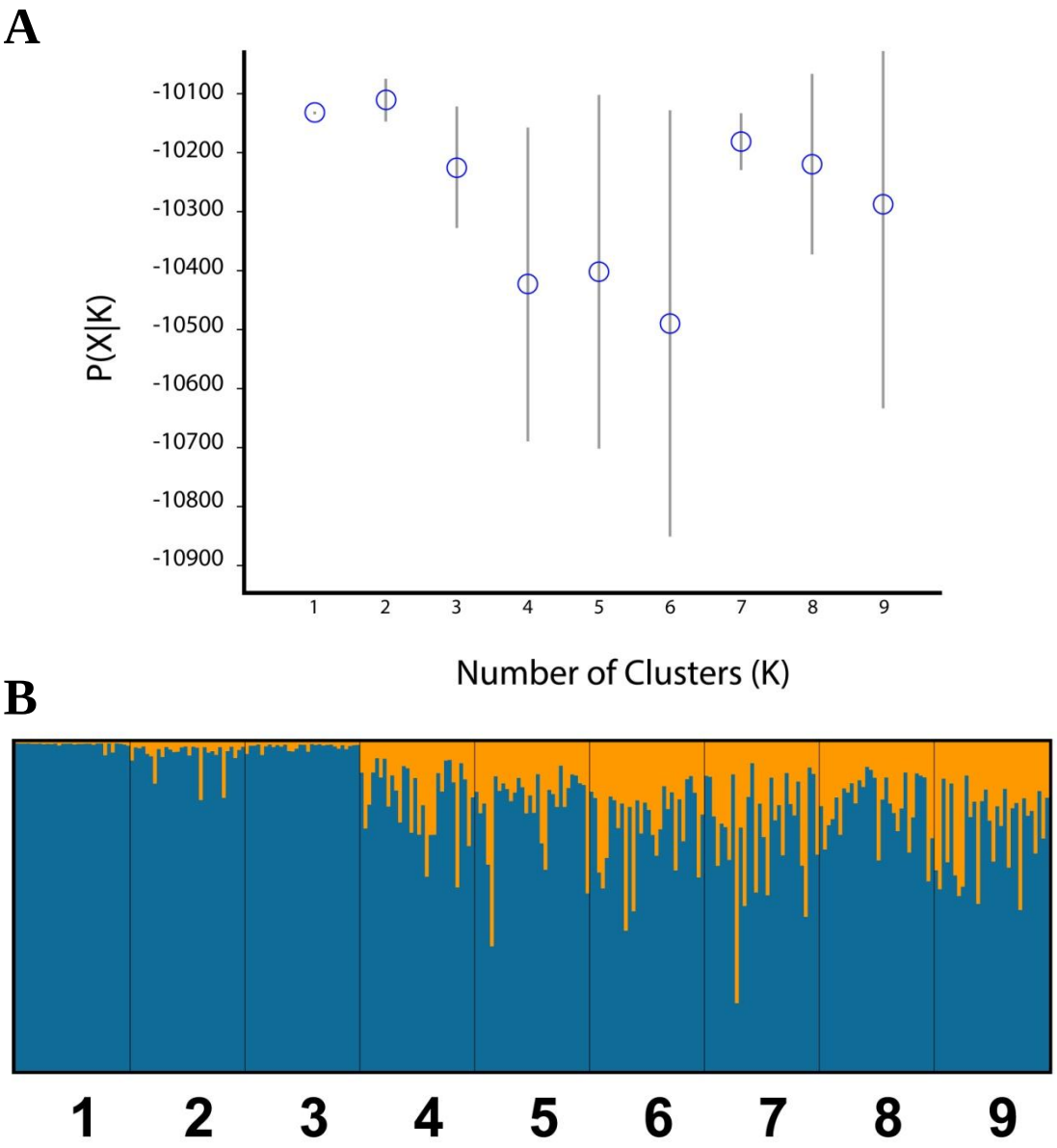

Figure 3. Bayesian clustering analysis of *Amblema plicata* genetic structure among nine mussel beds in the Little River. **A.** Mean (±SD) of P(X|K) of 10 iterations across different values of K (number of genetic clusters). **B.** Individual assignment scores with K = 2 reveal that there are two genetic clusters among the nine subpopulations within the Little River. Sites 1-3 form one cluster and sites 4-9 form the second cluster.

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Appendix A – PCR Reaction Mixes and Conditions

Table A1. PCR reaction mixes and conditions for all nine loci. Anec101, Anec122, Aned104, Aned126, and Aned140 used the same PCR conditions from James Cureton([a]). Annealing temperatures for Aned103, Aned108, Anec114, and Anec126 varied and are from Galbraith et al. 2015([b]).

Locus	PCR Reaction Mix Per Sample	PCR Conditions
Anec101[a]	6.25 µl GoTaq® Green Master Mix (Promega) 1.50 µl Labeled Primer (10 µM, Forward) 1.50 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.50 µl DNA	[a]30 s at 94°C 4 cycles of: [10 s at 94°C, 20 s at 58°C, 90 s at 72°C] 4 cycles of: [10 s at 94°C, 20 s at 56°C, 90 s at 72°C] 4 cycles of: [10 s at 94°C, 20 s at 54°C, 90 s at 72°C] 15 cycles of: [10 s at 94°C, 20 s at 52°C, 90 s at 72°C] 15 cycles of: [10 s at 94°C, 20 s at 50°C, 90 s at 72°C] 10 min at 72°C
Anec122[a]	6.25 µl GoTaq® Green Master Mix (Promega) 1.00 µl Labeled Primer (10 µM, Forward) 1.00 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.50 µl DNA	
Aned104[a]	6.25 µl GoTaq® Green Master Mix (Promega) 0.75 µl Labeled Primer (10 µM, Forward) 0.75 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.50 µl DNA	
Aned126[a]	6.25 µl GoTaq® Green Master Mix (Promega) 0.40 µl Labeled Primer (10 µM, Forward) 0.40 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.00 µl DNA	
Aned140[a]	6.25 µl GoTaq® Green Master Mix (Promega) 1.00 µl Labeled Primer (10 µM, Forward) 1.00 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.50 µl DNA	
Aned103[b]	6.25 µl GoTaq® Green Master Mix (Promega) 0.75 µl Labeled Primer (10 µM, Forward) 0.75 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.50 µl DNA	[b]10 min at 94°C 35 cycles of: [45 s at 94°C, 60 s at 60°C, 60 s at 72°C] 7 min at 72°C

Aned108[b]	6.25 µl GoTaq® Green Master Mix (Promega) 1.50 µl Labeled Primer (10 µM, Forward) 1.50 µl Unlabeled Primer (10 µM, Reverse) 1.75 µl ddH_2O 1.50 µl DNA	[b]10 min at 94°C 35 cycles of: [45 s at 94°C, 60 s at 54°C, 60 s at 72°C] 7 min at 72°C
Anec114[b]	0.25 µl TopTaq™ DNA Polymerase (Qiagen) 1.00 µl 10X Buffer 0.80 µl 800 µM dNTPs 0.33 µl Labeled Primer (1 µM, Forward) 0.33 µl Unlabeled Primer (1 µM, Reverse) 5.29 µl ddH_2O 2.00 µl DNA	[b]10 min at 94°C 35 cycles of: [45 s at 94°C, 60 s at 51°C, 60 s at 72°C] 7 min at 72°C
Anec126[b]	0.25 µl TopTaq™ DNA Polymerase (Qiagen) 1.00 µl 10X Buffer 0.80 µl 800 µM dNTPs 0.33 µl Labeled Primer (1 µM, Forward) 0.33 µl Unlabeled Primer (1 µM, Reverse) 5.29 µl ddH_2O 2.00 µl DNA	[b]10 min at 94°C 35 cycles of: [45 s at 94°C, 60 s at 48°C, 60 s at 72°C] 7 min at 72°C

Appendix B – Microsatellite Genotypes

Table B1. Microsatellite genotypes (i.e., the first three and last three digits represent distinct alleles as the number of base pairs) for all 270 individuals across nine mussel beds. Thirty individuals were sampled from each bed. # = individual identification number ranging from 1-30 for each bed. See Figure 1 for site locations.

Site	#	Anec101	Anec114	Anec122	Anec126	Aned103	Aned104	Aned108	Aned126	Aned140
1	1	222252	152152	204204	232268	266274	270270	256264	206206	250262
1	2	238282	160164	196204	224228	266274	250270	248248	206206	270274
1	3	238282	152152	204204	232268	274274	262262	248296	194206	270274
1	4	222252	148164	196196	244268	266266	258262	244248	206206	270274
1	5	200238	152152	204204	244272	266266	266274	252252	206206	270270
1	6	202252	156160	196204	208240	274274	262262	248248	206206	262274
1	7	222238	152164	204204	224228	274274	250266	248280	206206	266270
1	8	200252	156160	204204	208208	266270	262274	252276	206206	266270
1	9	222222	140160	196204	232232	274274	266274	256292	206206	262270
1	10	252252	152164	204204	232236	270270	262262	248300	206226	266270
1	11	238284	160160	196204	228232	274274	262270	248252	206206	266270
1	12	200296	152156	196204	272272	266266	262274	248300	206250	266270
1	13	176208	160160	196204	228244	266266	270270	248296	206206	270270
1	14	176288	156164	196204	228252	266266	266266	204276	206206	262270
1	15	222222	152160	196204	228232	266274	262266	204204	206226	270274
1	16	252252	148160	204204	244272	274274	254254	248248	206206	258270
1	17	238284	160164	196204	208232	266266	270270	244248	206262	262270
1	18	252252	156164	196204	244268	258258	262274	276276	206206	242262
1	19	200252	140164	196204	232240	258266	254262	248248	206206	262270
1	20	252292	152156	196204	228252	266266	258262	248256	206206	262270
1	21	200252	140152	196204	232260	270270	262262	244300	206206	270270
1	22	222266	152156	196196	232252	266266	266278	296300	206258	262266
1	23	252252	152164	204204	208268	266266	262262	248248	206254	262266
1	24	252296	148152	204204	208252	266266	258274	240240	206262	242262
1	25	222266	160164	196196	232244	274274	262262	248248	206254	262274
1	26	200296	160164	196196	208248	266274	250254	292292	206214	262270
1	27	222252	140164	196204	208272	266266	266266	248248	206214	266270
1	28	200246	152152	204204	248252	266266	266274	276276	194206	266270
1	29	252316	160164	204204	228268	000000	262262	248276	206250	262270

1	30	200246	160164	196204	244252	000000	250262	280308	206206	270270
2	1	200296	148160	196204	232276	266270	278278	244244	206226	262266
2	2	176176	148160	204204	240296	266266	270274	244248	206258	266266
2	3	252252	152160	204204	200228	266266	258258	256256	206250	266270
2	4	176176	152160	196204	224252	266266	266270	248248	206210	242242
2	5	200200	164164	204204	232240	266266	262262	272272	206206	266266
2	6	252252	140148	204204	244272	266266	290290	256296	206246	266266
2	7	176222	148148	204204	232252	270270	238238	264264	206206	258270
2	8	176176	148156	204204	252272	266270	274274	244244	206254	262270
2	9	252252	156160	204204	232268	270270	262270	248252	194206	246266
2	10	252252	152152	196204	236248	274274	258258	264300	206206	274274
2	11	222252	144160	204204	252272	270270	262262	296296	206206	262274
2	12	252252	152172	204204	216228	266266	270278	284284	206206	254266
2	13	252252	152160	204204	204244	266266	270278	252284	206234	250266
2	14	176176	148156	204204	232240	266266	278278	244312	206206	266270
2	15	252252	152156	204204	232244	262266	262262	244256	206226	262270
2	16	200252	148152	196204	204244	266266	254254	220296	226242	262270
2	17	222252	156160	204204	244300	266266	262262	248296	194206	270274
2	18	252252	148160	204204	232260	266266	266266	264268	194206	266266
2	19	176176	152160	204204	228244	270270	294294	292296	194206	254258
2	20	176176	148164	196204	232268	266266	266266	256268	194206	250270
2	21	176176	140152	204204	236288	266270	250250	248248	206250	270274
2	22	176176	160168	204204	204248	266278	262262	228280	194206	266270
2	23	176252	148160	196204	228244	266270	262262	268268	206254	246270
2	24	200252	148156	196196	228272	266266	262278	248248	206206	266266
2	25	200296	152168	204204	256304	250274	258274	276276	206238	266270
2	26	200200	156172	204204	244248	266266	266270	248296	226250	270274
2	27	252252	168172	204204	228268	266274	254254	244248	206206	266266
2	28	222252	140152	204204	236240	274274	226226	256296	194206	270270
2	29	200246	160168	196204	224228	258266	262270	312312	206258	266270
2	30	176176	140144	196204	232272	266266	266270	248296	206206	266270
3	1	252252	152168	204204	296300	246246	266266	264284	206206	274278
3	2	222222	144160	204204	248296	270270	258270	256256	206206	270270
3	3	222266	156172	204204	296296	278278	254254	256304	206206	266270
3	4	252252	152152	204204	252284	266266	262262	284284	206206	270278

3	5	252252	140144	204204	284284	262266	258266	288288	206250	262270
3	6	252270	140168	204204	232272	262266	270278	248256	206254	242266
3	7	222252	160160	204204	236296	262274	262262	260260	206206	266270
3	8	216222	160180	196204	240276	262274	274278	296296	206206	266266
3	9	252252	160172	204204	232236	270270	258266	256296	206206	270270
3	10	204252	152160	204204	236264	266266	262262	284320	206218	270270
3	11	252266	144152	204204	296296	266266	258262	256312	206206	266270
3	12	176176	156156	204204	292300	266266	274274	304304	206234	270278
3	13	176176	156160	196204	292296	266270	266266	220236	206218	274278
3	14	222266	156160	204204	232296	262262	246266	304320	206206	262270
3	15	222252	152160	196204	228304	266266	254254	252256	206206	270278
3	16	252252	140160	204204	224248	266266	266270	296320	206210	270278
3	17	218218	152160	204204	236268	270270	266274	220312	206206	250270
3	18	222252	152160	204204	296296	266266	262262	256300	206206	262262
3	19	200246	156160	204204	224268	266266	254254	256276	206206	266278
3	20	176176	156160	204204	236236	266266	262262	256300	206206	266270
3	21	192200	140156	204204	272296	266274	274274	256296	206206	266266
3	22	176246	160168	204204	248252	266270	254254	244256	206206	270270
3	23	200252	160164	204204	244248	266266	254254	232232	206206	270270
3	24	200246	156156	204204	208256	266266	266266	300304	206206	242262
3	25	176176	156168	204204	248284	290290	262262	256320	206206	278278
3	26	252252	168172	204204	244300	270274	254254	256256	206206	262262
3	27	176176	152156	204204	288292	270270	258258	260264	206206	266270
3	28	176176	148152	204204	272284	266270	258270	232232	206210	266270
3	29	222266	160164	204204	232300	266270	262262	268268	206206	270274
3	30	176176	148160	204204	232296	266270	266274	260260	206206	270270
4	1	288288	148160	204204	228228	266266	258258	252256	206214	270278
4	2	252252	168192	196204	252292	270270	266266	264300	206206	246254
4	3	176252	148152	204204	260284	258258	274274	260260	246250	262278
4	4	176176	148168	204204	252268	270270	266266	248296	206258	270270
4	5	252252	152160	204204	236244	266266	254254	248248	206206	274278
4	6	222222	152160	196204	196256	270270	266266	232248	206206	242242
4	7	176176	148160	204204	228268	266266	262266	248248	206226	270270
4	8	222222	156160	204204	244260	250274	266282	220244	226254	274274
4	9	176176	156160	196204	212240	270270	262262	276276	206206	266266

4	10	222222	160172	204204	232236	254270	278278	244260	206206	274282
4	11	252252	132152	196196	232256	270278	246262	248296	230258	250274
4	12	252252	160184	196204	232300	266270	266266	248256	206206	266274
4	13	222222	148152	196204	248252	270270	270274	248296	206206	250262
4	14	200252	148160	196196	256276	270274	246246	248248	206206	270282
4	15	176176	140160	204204	240244	270274	258274	292296	206250	266266
4	16	296296	140148	204204	244252	262270	278278	248260	206206	254254
4	17	222252	156160	196204	252292	270270	226226	248272	206206	270270
4	18	296296	152168	196204	212244	270270	234234	220256	206258	262270
4	19	252252	152176	196204	264292	258270	238254	288296	206238	266278
4	20	200222	168192	196204	276276	266266	246254	272272	206250	266270
4	21	252252	144160	204204	236272	258258	258262	244276	206250	266266
4	22	252252	168176	204204	232256	266266	274274	232232	242250	274274
4	23	176176	148152	196204	228264	266266	266266	248256	206206	262270
4	24	252252	152152	196196	228244	266266	262262	252252	206210	242270
4	25	252252	148164	196204	256268	274278	246262	248248	206214	270274
4	26	296296	152152	204204	252284	270270	258274	236292	250250	246266
4	27	176176	164176	196204	232272	262266	258274	256260	206206	270270
4	28	176184	144152	196204	232232	270270	254258	296296	206206	270274
4	29	200296	156168	196204	220240	266270	254266	248292	230250	262270
4	30	296296	140144	204204	244304	266266	274274	248248	206246	270270
5	1	222222	144148	204204	268272	250274	254254	244256	206206	254270
5	2	252252	140152	196204	268272	266266	242270	244284	230270	254266
5	3	200200	152168	204204	208272	274274	246262	252252	206206	250274
5	4	200200	148168	196204	220288	270270	262262	252296	206238	262278
5	5	200200	160160	204204	264288	270270	226230	252256	238246	246246
5	6	252252	160172	204204	240244	270270	262266	240248	206206	270274
5	7	176222	132164	204204	252268	270270	258258	244248	206206	270290
5	8	176176	156160	204204	264264	266266	254278	248248	206230	270270
5	9	222222	140156	204204	232232	270270	254266	252256	206230	270270
5	10	252252	148152	196204	208232	254254	258262	260260	206250	270278
5	11	200252	152152	204204	236320	266266	254266	248288	206210	246262
5	12	200200	156164	204204	228260	266266	258258	248272	206206	270274
5	13	200252	140172	196196	256264	266266	250266	292304	206206	262270
5	14	228252	164172	196204	212252	258258	238266	252288	194206	270274

5	15	200200	144156	196204	236248	262262	274278	244256	206246	266270
5	16	176176	152152	204204	216256	270270	250250	248268	230254	266266
5	17	176252	160176	204204	200252	266266	274278	244244	194206	262274
5	18	176252	140148	204204	228232	266266	226226	292292	194206	254258
5	19	200200	148156	196196	264296	270270	258262	236252	206222	258274
5	20	252252	136144	196204	208236	258258	262262	252260	206254	250270
5	21	222222	140148	196204	236236	270270	266278	304304	206254	270278
5	22	200200	140144	204204	232244	266274	266266	248296	230250	250266
5	23	222252	160160	196196	228292	266266	258266	228284	206206	270270
5	24	222316	144168	204204	252268	274274	246286	244244	206206	242274
5	25	222266	156160	204204	248260	250274	250250	248280	194206	250266
5	26	176176	148168	204204	240240	262262	254270	224248	206206	266270
5	27	176252	140164	204204	204240	262262	262262	220220	206206	266270
5	28	176176	156164	204204	212264	266266	278278	248248	206234	262270
5	29	222316	152156	204204	208276	266266	266266	264280	206206	242254
5	30	200296	160176	204204	252304	270270	242242	300300	194206	246270
6	1	176176	148152	204204	244272	266266	254258	232288	206258	258262
6	2	252252	148164	204204	244272	274274	262266	308308	206206	254266
6	3	252270	152152	204204	236236	274274	234234	256256	218250	246254
6	4	270292	140144	196204	208284	270270	262262	224292	206246	258266
6	5	176176	140160	228228	232236	270274	266274	264304	230230	270294
6	6	200296	152160	196204	236240	266266	250274	272296	206254	242270
6	7	200246	136152	196204	252252	266266	262270	300300	206246	250270
6	8	222266	140144	196204	268268	266270	270282	248284	206234	270290
6	9	208222	148152	196204	232256	266282	266270	252296	206250	266270
6	10	200200	156172	204204	272280	250250	282282	240272	206230	270278
6	11	222222	160164	204204	232248	266266	246246	276276	206226	278278
6	12	226226	148160	204204	300300	254254	246246	260260	206206	258270
6	13	216216	156168	196204	228240	258266	262262	232232	206250	266270
6	14	200200	160164	204204	252268	250250	274278	296296	242258	262262
6	15	200200	148160	204204	240272	270270	262262	260268	206206	266270
6	16	222230	152160	204204	244248	258258	254254	268268	206238	242270
6	17	176176	140168	196204	232248	270270	262274	224252	206246	254274
6	18	222266	144160	204204	268300	270270	258258	260284	206230	246250
6	19	222222	148164	196196	256256	258282	226266	248252	206206	246266

6	20	200222	160160	204204	232252	266266	254266	232248	242250	270274
6	21	176176	160168	204204	252284	000000	258258	240240	206206	262286
6	22	222266	144160	196204	224280	266274	270274	248248	206206	270282
6	23	200200	148164	204204	252272	270270	258266	244272	234238	242266
6	24	196196	156164	204204	196228	266270	270270	244248	206206	242270
6	25	246252	140160	204204	264292	266266	234238	220252	206206	254270
6	26	222252	160160	204204	236244	266266	254254	260260	206226	274286
6	27	222266	152160	196204	240268	266266	270270	220268	206258	266274
6	28	238252	152156	204204	232256	270270	274274	220264	206206	266270
6	29	200296	156160	204204	268268	266270	246262	288288	206230	262282
6	30	176188	128140	204204	204232	266266	250250	244248	194222	270270
7	1	252252	160180	204204	228232	266270	250250	244244	194206	262270
7	2	252252	160160	204204	232268	266278	258286	244300	194206	274274
7	3	252252	144164	204204	208236	278278	254254	264264	206230	258270
7	4	176176	160164	196204	244292	270274	234234	300300	194206	246282
7	5	200200	160168	204204	204264	266270	250254	244244	206250	270270
7	6	252252	144144	204204	232236	262266	242254	304304	202206	274290
7	7	270274	136156	196204	232276	266270	270270	264272	206234	258270
7	8	252252	144160	196204	244256	000000	258262	256256	194206	262270
7	9	200216	148148	204204	212292	270270	282282	292292	230238	254278
7	10	252252	148184	204204	268284	270270	226226	256276	206206	266266
7	11	184230	156160	204204	204236	000000	246246	304316	206254	266266
7	12	222222	140172	204204	216312	270270	262278	260268	206206	266270
7	13	222222	152152	204204	240244	266266	258258	248300	206206	270270
7	14	200296	140148	196204	244280	246246	246266	300304	206206	266294
7	15	176184	144164	204204	200296	266278	250250	304304	206206	266266
7	16	176176	140144	204204	276332	270270	274274	244244	206222	270278
7	17	230230	140148	204204	248256	266266	282282	296296	206250	246270
7	18	176176	164164	204204	236236	266266	262262	296296	194226	274294
7	19	252252	140144	196204	264272	266270	226254	244244	206222	250266
7	20	200246	148160	204204	232264	266266	254254	300300	234258	270274
7	21	176176	152156	204224	232240	266266	234234	240252	206206	258258
7	22	176176	140152	196204	252252	266266	254266	256256	206206	254278
7	23	200200	152152	204204	240240	266266	222222	260260	206254	266270
7	24	222266	148168	204204	248292	266266	274274	244252	206250	254278

7	25	222252	140160	204204	272280	266266	270270	288300	206206	266274
7	26	200296	148168	196204	204300	266266	262262	240248	242246	258270
7	27	200296	144160	204204	256280	266270	226258	244316	234250	270274
7	28	222266	152160	204204	228232	274274	250270	276300	206210	262266
7	29	200216	152164	196204	244248	274274	262278	244248	206206	274274
7	30	222316	156156	204204	244252	270270	246246	256256	210250	270270
8	1	200222	140160	204204	252252	274274	254262	252252	222254	270282
8	2	200246	156156	204204	248268	000000	250266	280308	206250	246254
8	3	222252	144164	196204	252264	266266	250262	252256	238238	246262
8	4	216216	144160	204204	228236	000000	262262	268276	222234	270270
8	5	200252	160160	204204	244272	270270	270274	244312	246258	262262
8	6	200252	160172	204204	248292	270270	262286	232244	210254	254266
8	7	200200	156168	204204	252252	270270	262262	276276	206206	262270
8	8	252252	144160	204204	272296	270270	226262	220248	206206	242262
8	9	252252	148164	196196	232268	274274	258262	264272	206218	258270
8	10	252252	156160	196204	244248	266270	290290	236236	206234	262262
8	11	238238	164168	204204	240268	262266	262262	248248	234250	266270
8	12	200200	152160	196204	260276	266278	266266	236284	206206	266270
8	13	222252	144156	204204	208232	266266	262266	256304	206206	270270
8	14	222222	160160	204204	248272	266266	258258	292320	206206	270274
8	15	200200	144160	204204	236244	266266	266274	244276	206206	270278
8	16	252252	148160	204204	264280	270270	226226	252256	206226	254266
8	17	222222	164168	196204	232280	266266	254270	304316	194206	258270
8	18	222266	148164	196204	204260	262262	258266	256260	206206	266278
8	19	176176	148152	196204	252288	000000	262262	224248	206230	278278
8	20	200200	148164	204204	288292	278278	250250	248296	194206	242266
8	21	200200	156164	204204	260260	270270	262262	240300	206206	254274
8	22	252252	152160	204204	000000	270270	226262	236276	206206	266282
8	23	176176	164172	204204	204304	266266	262278	260264	206206	266270
8	24	230230	144164	196204	204244	266270	258278	220220	206234	270278
8	25	200200	152164	204204	248288	000000	250278	256256	206234	254262
8	26	222222	152164	204204	252252	266266	262262	296300	206254	266282
8	27	222252	164172	204204	252276	262262	266266	264280	206218	242270
8	28	252252	140164	196204	228228	262262	258258	256288	206242	242270
8	29	200296	148164	196204	204240	274274	246246	288296	222234	266270

8	30	200296	152160	204204	248260	266274	250254	228292	206226	246246
9	1	200200	144148	204204	204228	270270	266266	288308	206242	270274
9	2	200200	148156	204204	204296	270270	246246	252288	206206	266270
9	3	222222	152172	204204	268272	266274	226266	232256	206206	270274
9	4	200200	144148	196204	228240	242242	262262	244280	250250	262270
9	5	222222	160160	204204	248296	270270	270270	300300	194206	270270
9	6	208208	144160	204204	208276	270270	226262	236300	206234	266270
9	7	200252	156164	204204	236256	266270	234234	240248	230262	270282
9	8	200246	144148	204204	236248	266270	246278	284316	206250	274298
9	9	222222	148156	196204	224268	266266	254254	244284	206210	270274
9	10	252252	156160	204204	204264	266270	254254	232248	206230	270290
9	11	200200	136152	196204	196252	266266	266266	248268	210214	270274
9	12	252252	156156	204204	240292	270270	246254	216308	234246	254270
9	13	200200	156160	204204	224236	270270	270270	264300	206206	270270
9	14	176176	144152	204204	248252	270270	262262	260264	194206	270270
9	15	218222	152156	204204	240268	266266	258258	288288	206206	254254
9	16	200252	144152	204204	236240	266270	226226	240276	206254	254274
9	17	252252	144144	196204	252252	270270	270270	204256	206234	270270
9	18	200222	136152	204204	208228	270270	266266	212252	206250	270274
9	19	176176	136172	196204	232240	266266	278278	248300	206246	270274
9	20	252252	148176	204204	200264	000000	262262	292292	246246	266270
9	21	222252	140156	196204	236276	270270	238262	248304	194206	262278
9	22	176176	148152	204204	264268	000000	266270	244244	206238	242262
9	23	176184	160168	204204	196232	246246	262262	220220	238250	234234
9	24	176288	152152	204204	196272	266266	262262	220244	206226	246274
9	25	200228	160160	204204	256300	266266	258258	256264	206234	242242
9	26	208208	136140	196204	232300	266266	258258	248256	206246	262266
9	27	222266	160164	204204	212300	266270	258258	244264	230242	258274
9	28	252252	152160	204204	256288	266266	250250	228228	194206	266270
9	29	200200	160164	204224	268280	266266	246258	276292	206206	266266
9	30	200246	148160	204204	272292	000000	266278	260296	194206	262270