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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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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 | - |

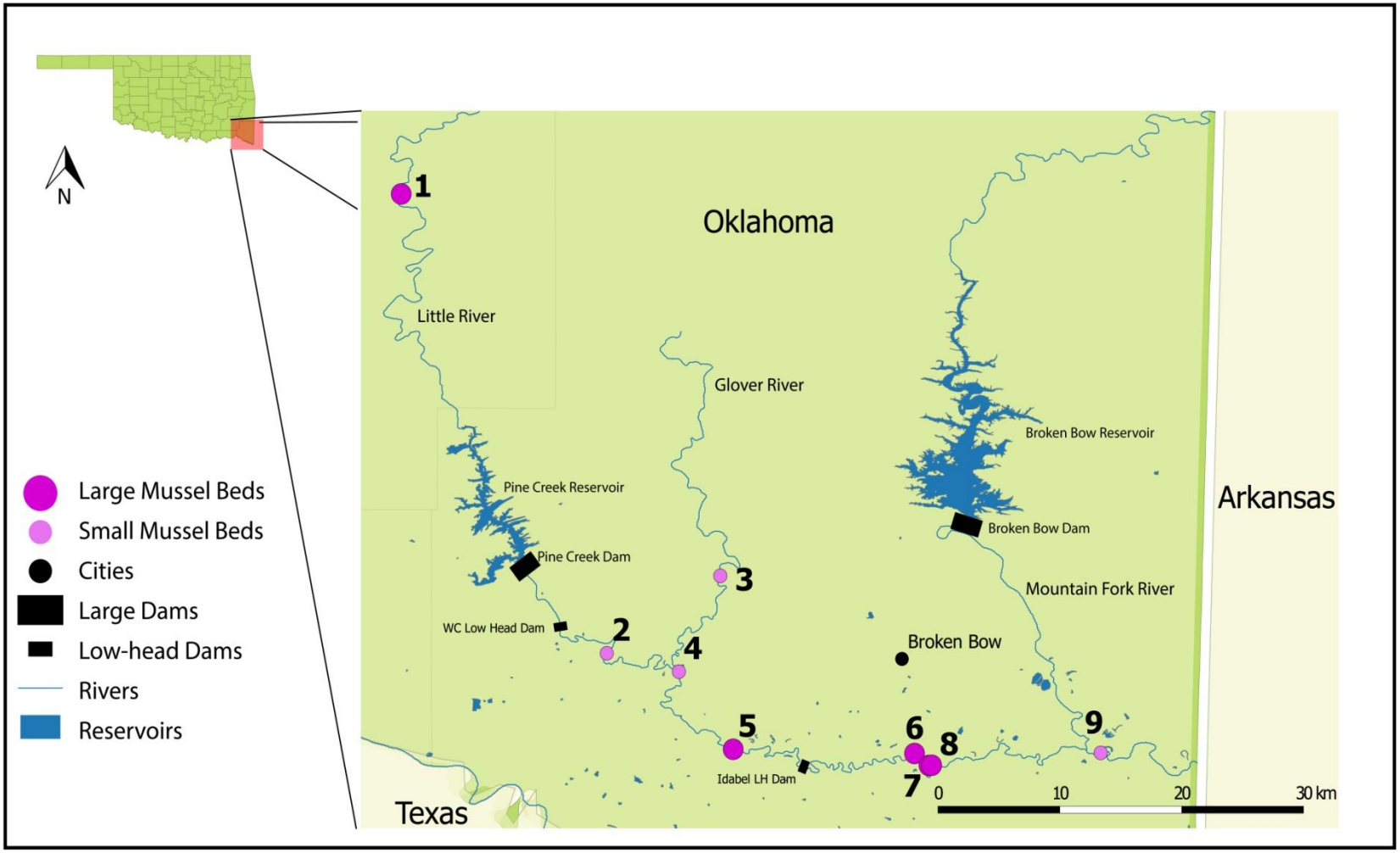



**Figure 1.** Sampling sites in the Little River.

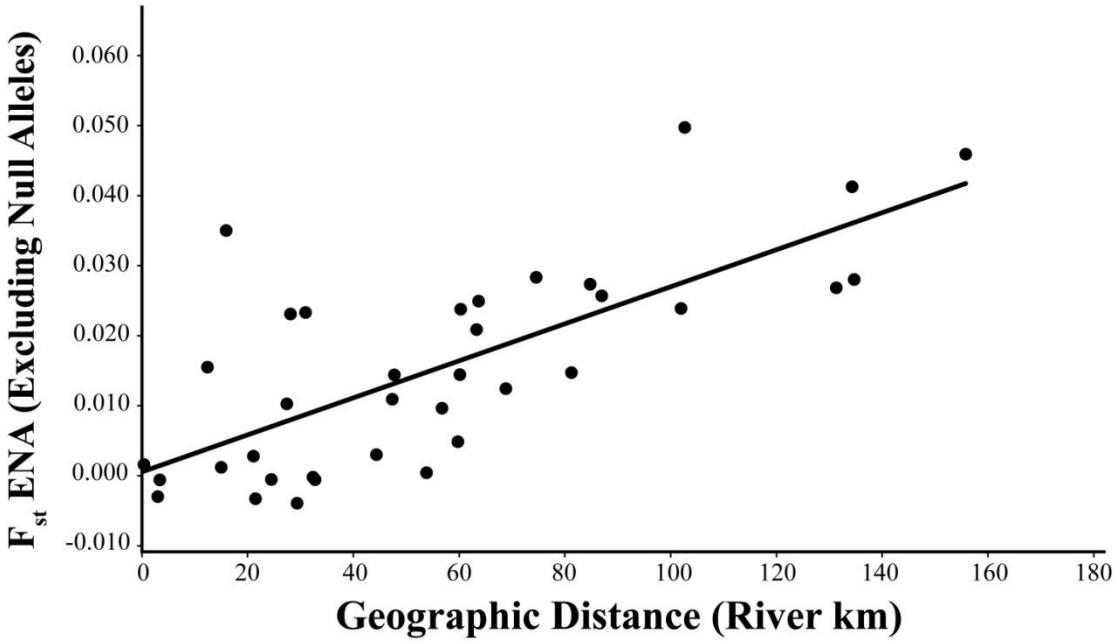



**Figure 2.** Relationship between geographic distance (river km) and genetic distance ($F_{st}$ENA). There was a significant positive relationship between geographic and genetic distance among nine *A. plicata* subpopulations in the Little River ($P < 0.001$, $R = 0.73$).

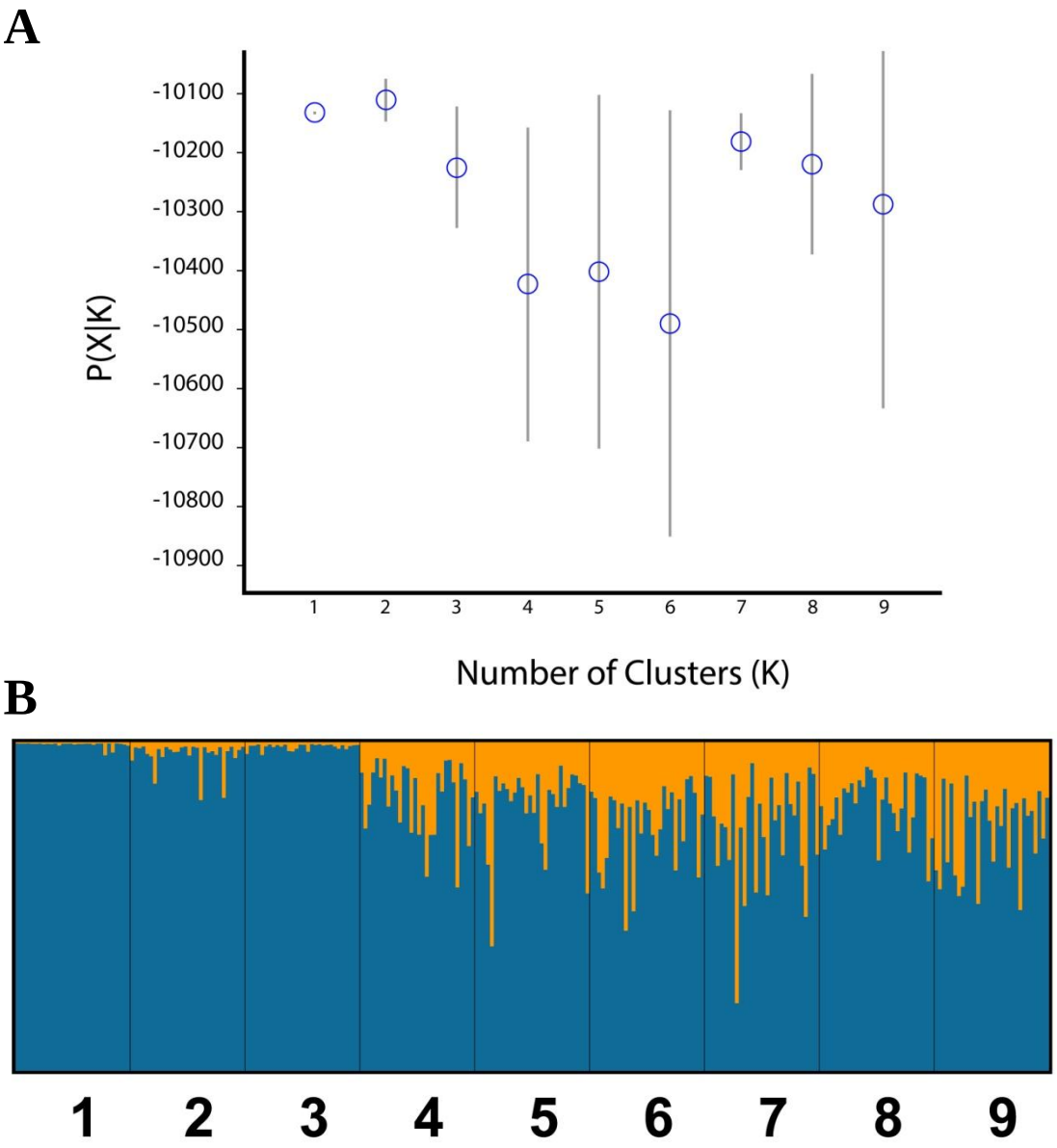



**Figure 3.** Bayesian clustering analysis of *Amblema plicata* genetic structure among nine mussel beds in the Little River. **A.** Mean (±SD) of P(X|K) of 10 iterations across different values of K (number of genetic clusters). **B.** Individual assignment scores with K = 2 reveal that there are two 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)<br>1.50 µl Labeled Primer (10 µM, Forward)<br>1.50 µl Unlabeled Primer (10 µM, Reverse)<br>1.75 µl $ddH_2O$<br>1.50 µl DNA | [a]30 s at 94°C<br>4 cycles of:<br>[10 s at 94°C,<br>20 s at 58°C,<br>90 s at 72°C]<br>4 cycles of:<br>[10 s at 94°C,<br>20 s at 56°C,<br>90 s at 72°C]<br>4 cycles of:<br>[10 s at 94°C,<br>20 s at 54°C,<br>90 s at 72°C]<br>15 cycles of:<br>[10 s at 94°C,<br>20 s at 52°C,<br>90 s at 72°C]<br>15 cycles of:<br>[10 s at 94°C,<br>20 s at 50°C,<br>90 s at 72°C]<br>10 min at 72°C |
| Anec122[a] | 6.25 µl GoTaq® Green Master Mix (Promega)<br>1.00 µl Labeled Primer (10 µM, Forward)<br>1.00 µl Unlabeled Primer (10 µM, Reverse)<br>1.75 µl $ddH_2O$<br>1.50 µl DNA | |
| Aned104[a] | 6.25 µl GoTaq® Green Master Mix (Promega)<br>0.75 µl Labeled Primer (10 µM, Forward)<br>0.75 µl Unlabeled Primer (10 µM, Reverse)<br>1.75 µl $ddH_2O$<br>1.50 µl DNA | |
| Aned126[a] | 6.25 µl GoTaq® Green Master Mix (Promega)<br>0.40 µl Labeled Primer (10 µM, Forward)<br>0.40 µl Unlabeled Primer (10 µM, Reverse)<br>1.75 µl $ddH_2O$<br>1.00 µl DNA | |
| Aned140[a] | 6.25 µl GoTaq® Green Master Mix (Promega)<br>1.00 µl Labeled Primer (10 µM, Forward)<br>1.00 µl Unlabeled Primer (10 µM, Reverse)<br>1.75 µl $ddH_2O$<br>1.50 µl DNA | |
| Aned103[b] | 6.25 µl GoTaq® Green Master Mix (Promega)<br>0.75 µl Labeled Primer (10 µM, Forward)<br>0.75 µl Unlabeled Primer (10 µM, Reverse)<br>1.75 µl $ddH_2O$<br>1.50 µl DNA | [b]10 min at 94°C<br>35 cycles of:<br>[45 s at 94°C,<br>60 s at 60°C,<br>60 s at 72°C]<br>7 min at 72°C |

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

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|---|---|---|---|---|---|---|---|---|---|---|
| 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 |

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|---|---|---|---|---|---|---|---|---|---|---|
| 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 |
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