Genetic variability and geographic structure of *Lampsilis higginsii* mussels in the upper Mississippi River and tributaries

FINAL REPORT

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ABSTRACT

Freshwater mussels are one of the most imperiled faunas of animals in the world. Genetic studies can be used to help conserve these animals. *Lampsilis higginsii* (Higgins' Eye) is a federally endangered mussel in the Upper Mississippi River basin. *L. higginsii* currently occurs in the Mississippi, St. Croix and Wisconsin rivers and has suffered a 50% reduction in its range since 1965. Populations of *L. higginsii* in the Mississippi River and lower St. Croix River are threatened with extirpation due to the alien zebra mussel. As a result, refuges in rivers that are zebra-mussel free have been proposed to preserve this species. The goal of this study was to characterize the genetic structure and diversity of *L. higginsii* as a basis for conservation and management actions. In a previous study of a small number of individuals (16) from three populations, using a portion of the mitochondrial gene *cytochrome-b*, we found that there was no evidence for genetic differences among the populations. Surprisingly, there was a high level of genetic variation within the populations. In the current study, we sampled 20-30 individuals from each of four localities, plus 15 others from three additional localities. We characterized 130 individuals for 1027 nucleotides from segments of three mitochondrial DNA genes: *cytochrome-b*, *cytochrome c oxidase I,* and *16S rRNA*. We detected 24 genetic forms (haplotypes), which clustered into four groups (clades). Again, we did not find evidence of differentiation among populations, but we found a high level of genetic diversity within populations. These findings suggest that if relocations occur, a large number of individuals should be used in order to preserve as much genetic variation as possible. Based on the estimates of frequencies of the mtDNA clades we detected, we recommend that more than 100 females should be used in the propagation program. As in the previous study, we found that a small number of individuals sampled contained the mitochondrial DNA (mtDNA) form typically found in *Lampsilis siliquoidea*, a common and widespread congener that occurs in the Midwest. Similar observations have been made in other animal groups. Additional research is required to determine the cause of this finding and the implications it has for conservation of *L. higginsii*.

INTRODUCTION

The Higgins' Eye Pearlymussel, *Lampsilis higginsii*, is a Federally endangered species, which is in jeopardy of extinction throughout its historic range, the Upper Mississippi River (UMR) and several associated tributaries (U.S. Fish and Wildlife Service, 2003). The primary threat is infestation by the alien zebra mussel (*Dreissena polymorpha*), which has colonized and spread throughout much of the UMR. Plans for recovery of this species include a Relocation Plan submitted by the St. Paul District (MVP), Army Corps of Engineers (COE), to augment and/or create *L. higginsii* populations throughout its range (U.S. Army Corps of Engineers, 2002). The Relocation Plan includes relocation of adult individuals and propagation of larvae and juveniles. For propagation of larvae and juveniles, fertilized gravid females are collected from the wild and the larvae (glochidia) are harvested. Glochidia are then allowed to attach on host fish. The fish are either held in cages in a free-flowing river until the larval mussels drop off, or free-swimming fish with their attached glochidia are released into a river. Each year a different group of females is used as glochidia donors, then those females are returned to the wild so that they can contribute their genes to the free-living mussel population in the future.

Information on the genetic characteristics of *L. higginsii* populations is needed to make scientifically sound decisions regarding the numbers, localities, and logistical concerns of proposed relocations. If genetic variability is high, then a large number of individuals should be relocated to adequately preserve the genetic variation of the species. Likewise, if genetic variability if high, then a large number of females should be used as sources for propagation projects. If populations differ from one another genetically, then animals should be relocated to areas that contain similar genetic profiles.

Prior genetic research on *L. higginsii* has demonstrated that this species contains a high level of mitochondrial DNA genetic variation within populations and that there is little evidence for genetic differences among the populations (Bowen and Richardson, 2000). The earlier research was based on samples of 5-6 individuals from each of three populations and used a segment of the mitochondrial gene *cytochrome-b* to characterize the genetic structure of the species. The surprising finding of a high level of mitochondrial DNA genetic variation within the populations indicated that more individuals need to be sampled to get a precise estimate of genetic variability.

Little is know about mitochondrial DNA genetic variability within populations of unionacean mussels, one of the most imperiled groups of animals in North America. Most research using mussel mitochondrial DNA has examined differences among populations and among species (Mulvey et al. 1997; Roe and Lydeard, 1998; King et al, 1999; Turner et al., 2000; Serb et al., 2003). Most research on variation within populations has used nuclear DNA markers, such as allozymes (Mulvey et al., 1997; Berg et al., 1998). The research presented in this report, on mitochondrial DNA variation within and among populations of *L. higginsii,* represents novel information on a taxon of important conservation priority.

Two research objectives have been identified: (1) Assess the genetic variability and geographic structure within and among populations of *L. higginsii* in the Upper Mississippi, St. Croix, and Wisconsin Rivers. (2) Make management recommendations regarding relocations of individuals among populations, specifically with regard to (a) numbers of animals to be relocated and (b) appropriate geographical sites for relocations.

To achieve these objectives, a Research Work Order (RWO) was established that provided funding to Iowa State University, Dr. Bonnie Bowen, Principal Investigator, through the Iowa Cooperative Fish and Wildlife Research Unit. The source of these funds was an MIPR Interagency Agreement from the U.S. Army Corps of Engineers, St. Paul District, to the U.S. Geological Survey, Reston, VA. RWO funds, in the amount of $36,046, were used (1) to hire a laboratory assistant, Ms. Kim Jones Glenn, who worked from November 2001 through August, 2002, (2) to purchase expendable laboratory supplies, (3) to pay for DNA sequencing services at Iowa State University, (4) to pay for travel costs for Dr. Bowen to present findings and meet with colleagues at the meeting of the Freshwater Mollusk Conservation Society meeting in March 2003, and (5) to support 12% of Dr. Bowen's salary between September 2001 and April 2003.

METHODS

Tissue samples from mantle (Berg et al., 1995) of 130 *L. higginsii* were obtained from the U.S. Army Corps of Engineers, St. Paul District and other cooperating collectors. Tissue samples were collected at the following sites: 1) Hudson, St. Croix River; 2) Lansing, Upper Mississippi River (UMR), River Mile (RM) 661; 3) East Channel-Prairie du Chien, UMR, RM635; 4) McMillan Island, UMR, RM618; 5) Cassville, UMR, RM606; 6) Cordova, UMR, RM504 and 7) Orion, Wisconsin River. Tissue was preserved in 95-100% ethanol. DNA was

extracted from 10 to 20 milligrams (mg) of tissue using the Puregene DNA Extraction Kit (Gentra Systems). Following extraction, DNA was stored at -20C.

We amplified portions of three mitochondrial genes, *cytochrome-b (cyt-b)*, *cytochrome c oxidase I (COI)* and *16S rRNA* (*16S*), using the polymerase chain reaction (PCR) (see Table 1 for primers and references). Double stranded PCR amplifications were conducted in 50 µl reactions containing 3.0 mM Promega $MgCl_2$, 1.14X Promega Buffer, 0.23 mM of each dNTP, 0.3 µM of each primer, 1 unit Promega Taq, and at least 50 ng DNA. The thermal cycler program varied, depending on the gene being amplified (Table 2). Following successful amplification, PCR products were purified and concentrated with Microcon-100 concentrators (Milipore), quanitifed with a fluorometer, and sequenced at the Iowa State University DNA Sequencing Facility on an ABI 377 (Applied Biosystems) automated sequencer.

Table 1. Primer sequences used to amplify mitochondrial DNA genes using the Polymerase Chain Reaction in *L. higginsii*.

mtDNA gene	**Forward primer (5'—3')**	**Reverse primer (5'—3')**	**Source**
Cyt-b	LS151F: AAGAAGTATCATTGCGGTTG	LS270R: TGTGGGGCGACTGGTATTACTAA	Bowen (modified from Merritt et al., 1998)
COI	CO1FINTA: GGTCAACAAATCATAAAGATATTGG	CO1RINTA: TAAACTTCAGGGTGACCAAAAAATCA	Bowen (modified from Folmer et al., 1994)
16s RNA	16Sint3L: TGAGCGTVCTAAGGTAGC	16Sint4H: AKCCAACATCGAGGTCGCAA	Turner et al., 2000.

Table 2. Thermocycler programs used to amplify mitochondrial DNA genes in *L. higginsii*.

STEP	***Cyt-b***	***COI***	***16S***
1	94C, 60 sec	94C, 40 sec	94C, 30 sec
2	40C, 60 sec	55C, 60 sec	50C, 30 sec
3	72C, 60 sec	72C, 90 sec	72C, 60 sec
4	29 cycles to step 1	31 cycles to step 1	29 cycles to step 1
5	4C indefinitely	72C, 5 min	4C indefinitely
		4C indefinitely	

Sequences were aligned by hand with Se-Al (http://evolve.zoo.ox.ac.uk/software.html ?id=seal) and/or with CLUSTAL-W (Thompson et al., 1994; http://www.ebi.ac.uk/clustalw/). Each gene segment was aligned individually and all haplotypes were compared to determine the total number of unique haplotypes at each gene. Representatives of each haplotype were then chosen (usually the individual with the longest sequence) for phylogenetic analyses. Analyses of phylogeny and sequence divergence among haplotypes are based on these representative individuals. Representative haplotypes of each of the three genes were aligned and trimmed, then the trimmed sequences were combined for analyses of the three genes together.

We estimated several measures of genetic variability within populations and species. Most analyses were performed on the 115 individuals from the four sampling locations with >20 individuals (Hudson-St. Croix, Lansing, Cassville, and Cordova). Some tables and figures include the small samples from the other three locations (East Channel-Prairie du Chien, McMillan Island, and Wisconsin River). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al., 2001; http://www.megasoftware.net/). We estimated the fraction of the total mtDNA variation that was distributed within and among populations by conducting an Analysis of Molecular Variance (AMOVA) using GENO (software written by Rodney Dyer, rodney@iastate.edu). To determine the pattern of relationships among the populations and haplotypes, phylogenetic analyses were conducted

using PAUP* 4.0 (Swofford, 2002; http://paup.csit.fsu.edu/) and Mr. Bayes (Hulsenbeck and Ronquist, 2001; http://morphbank.ebc.uu.se/mrbayes/).

We estimated the number of genetically contributing females that would be needed to have a 95% probability of including a rare variant (frequency 3% in the population) using binomial probabilities. We calculated the probability of obtaining a rare variant for different numbers of females using the following formula: Prob = $1 - [(1-f)^N]$, where f = frequency of the rare allele or clade and N = number of females contributing gametes.

For f = 0.03 and N = 5, Prob = $1 - [0.97^5] = 1-0.86 = 0.14$;

For f = 0.03 and N = 100, Prob = $1 - [0.97^{100}] = 1-0.05 = 0.95$.

RESULTS AND DISCUSSION

ASSESSMENT OF GENETIC VARIATION IN Lampsilis higginsii: *mtDNA HAPLOTYPE NUMBER AND ORGANIZATION*

In *L. higginsii*, we found 24 haplotypes at the combined sequence (all three genes) of 1027 bp (Table 3). These sequences differed from one another by 1 to 75 base pairs, or 0.1 to 6.9% sequence divergence. Since many of the haplotypes were very similar, yet some more divergent, we grouped them into clades using phylogenetic methods. We used these clades to look for patterns within and among the four main localities.

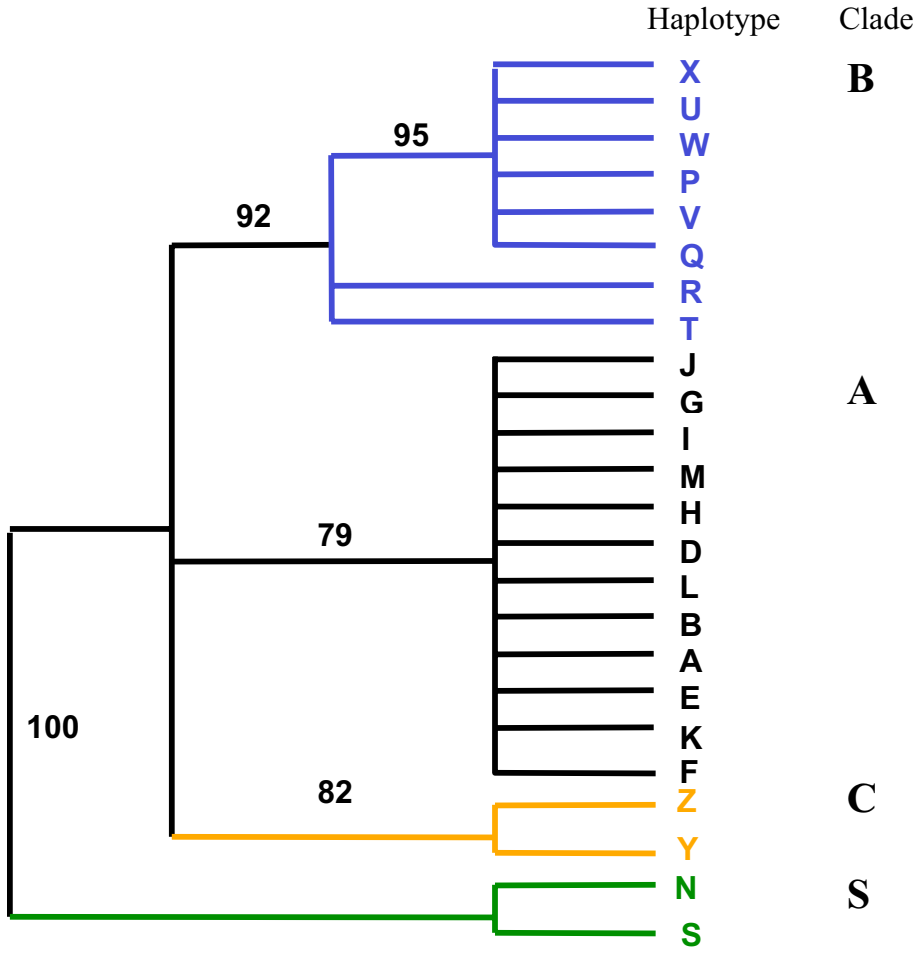
Table 3. Number of sites, number of variable sites (with and without Clade S), number of haplotypes and number of clades for three mtDNA gene segments individually and combined.

	Cyt-b	COI	16S	3 genes combined
No. sites	359	353	335	1047
No. variable sites with Clade S	44	28	29	101
No. variable sites without Clade S	19	10	10	39
No. haplotypes	15	11	13	24

Phylogenetic analysis showed that the 24 haplotypes clustered into four clades, which were well supported by statistical tests (bootstrap analysis) (see Fig. 1). Analysis of genetic variation within and among clades showed that Clades A, B, and C were separated by 1.2 to

1.4% sequence divergence, whereas Clade S was separated from the others by 6.9% sequence divergence (Table 4). Clade S contains two haplotypes that were the same or similar to those found in the congener *L. siliquoidea*. We found that some *L. higginsii* contained the mtDNA characteristic of *L. siliquoidea* in our previous survey of mtDNA variation at the *cyt-b* locus in *L. higginsii* (Bowen and Richardson, 2000). Surprisingly, representatives of all four clades identified in the current study were found in the initial survey of 16 individuals that was reported by Bowen and Richardson (2000). The current study provides large enough sample sizes to estimate the frequencies of the haplotypes and clades.

Figure 1. Phylogenetic tree showing relationships among the 24 haplotypes of *L. higginsii* mtDNA, based on 1027 bp of segments of *cyt-b*, *COI*, and *16S*. Strict consensus tree is based on maximum parsimony analysis, 1000 bootstrap analyses. Four clades were identified.



Analysis of each of the three genes individually also showed many haplotypes among the 130 individuals sampled (Table 3). Clade S was clearly different from the other clades at all three genes. The *Cyt-b* gene had the most variable sites in the analysis with Clade S, the most variable sites without Clade S, and the most haplotypes. It also had the highest sequence divergence in pair-wise comparisons among clades (Table 4). *Cyt-b* was the gene studied in the initial survey of 16 samples of *L. higginsii* (Bowen and Richardson, 2000) and it remains the most variable and most informative locus of those tested for population-level studies. In her study of *Cyprogenia aberti,* Serb found a high level of haplotype diversity in the mtDNA gene ND-2 (Serb, pers. comm.), but that gene was not examined in *L. higginsii*. The *cyt-b* primers that were used in the earlier study of *L. higginsii* and in this one were developed by Bowen. They have not been used by many other unionid researchers, so comparative data from other species are lacking. We recommend that this locus be used in future studies of freshwater mussels when the goal is to identify as many variable sites as possible.

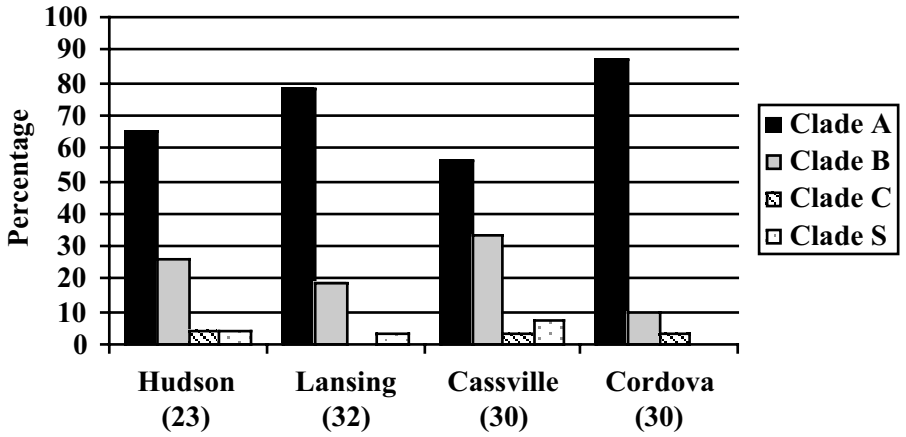
Table 4. Percent sequence divergence within and among clades, based on 24 haplotypes for 1047 bp using MEGA.

% sequence divergence (p-dist)	Cyt b Mean (SE)	COI Mean (SE)	16S Mean (SE)	3 genes combined
Clade A vs B	1.9 (0.63)	1.4 (0.53)	1.4 (0.50)	1.4 (0.33)
Clade A vs C	1.5 (0.53)	1.2 (0.47)	1.2 (0.53)	1.2 (0.30)
Clade A vs S	7.8 (1.41)	n/a	6.3 (1.25)	6.9 (0.77)
Clade B vs C	2.3 (0.63)	1.3 (0.53)	0.8 (0.38)	1.4 (0.33)

GEOGRAPHIC DISTRIBUTION OF HAPLOTYPES

We explored the potential for genetic differentiation among populations by examining the distribution of the haplotype clades among the four primary sampling localities. If populations were genetically differentiated, we would expect clades and haplotypes to be found in a limited number of localities and each locality would contain only a sample of the clades and haplotypes. This is not what we found. We found that most clades were present in all four localities and that most localities contained three or four clades (Fig. 2, Table 5).

Figure 2. Percentages of haplotype clades at four localities from the St. Croix and Mississippi Rivers sampled in 2001, based on 1027 bp from three mtDNA genes. (N) is sample size for each locality.



The Cassville population contained the most haplotypes, 13. Cordova, the southernmost site, contained only eight, even though we sampled 30 individuals. The Hudson-St. Croix sample, the northernmost sampling locality, contained seven haplotypes, the fewest number of any locality. Fewer individuals (23) were sampled at this site, but it is noteworthy that Hudson-St. Croix contained none of the variants of Clade A (haplotypes B-M), which occurred at the other sites. Most of these haplotypes were single base substitutions that occurred in <5 individuals. The lack of these haplotypes at Hudson-St. Croix indicates that this population may have been established more recently and there may have been less time for mutations to accumulate. This result would be consistent with the establishment of this population since the last glacial maximum.

Table 5. Frequencies of all 24 haplotypes for three genes combined for all sampling localities. Sample IDs of reference individuals for each haplotype are given, as well as clade designations.

SAMPLE ID	Haplotype	Clade	Hudson-St. Croix	Lansing	Cassville	Cordova	EastChannel	McMillan Island	Wisconsin River
Mc17	A	A	15	20	14	20	3	6	3
Mc13	B	A	-	2	-	-	-	1	-
CV43	D	A	-	-	1	-	-	-	-
WR134	E	A	-	1	-	-	-	-	-
WR162	F	A	-	1	-	-	-	-	-
CDV21	G	A	-	-	-	1	-	-	-
CV39	H	A	-	-	1	-	-	-	-
CDV26	I	A	-	-	-	3	-	-	-
C65	J	A	-	-	-	1	-	-	-
WR153	K	A	-	1	-	-	-	-	-
CV45	L	A	-	-	1	-	-	-	-
CDV29	M	A	-	-	-	1	-	-	-
HU4	P	B	1	-	2	-	-	-	-
WR163	Q	B	1	1	-	1	-	-	-
CV31	R	B	1	1	3	-	-	-	1
CV42	T	B	-	-	1	-	-	-	-
CV40	U	B	-	-	1	-	-	-	-
HU23	V	B	3	3	2	-	1	-	-
P22	W	B	-	-	1	-	-	-	-
C60	X	B	-	1	-	2	-	-	-
HU5	Y	C	1	-	-	-	-	-	-
CDV20	Z	C	-	-	1	1	-	-	-
HU24	S	S	1	1	1	-	-	-	-
CV44	N	S	-	-	1	-	-	-	-
Total # haplotypes	24	4	7	10	13	8	2	2	2
Total # individuals			23	32	30	30	4	7	4

AMOVA showed that most of the variation was within populations and little was among populations (Table 6). This result is consistent with the finding of the earlier study, but not typical for other species of unionids that have been examined (see Turner et al., 2000). It is especially unusual to find different mtDNA haplotypes and clades that are as divergent as the ones found in *L. higginsii* to be present in the same population.

Table 6. Analysis of Molecular Variance (AMOVA) for 115 *Lampsilis higginsii* from the St. Croix and Mississippi Rivers, 2001-2002.

Source of Variation	Percentage of Variation
Among Populations	3.5%
Within Populations	96.5%

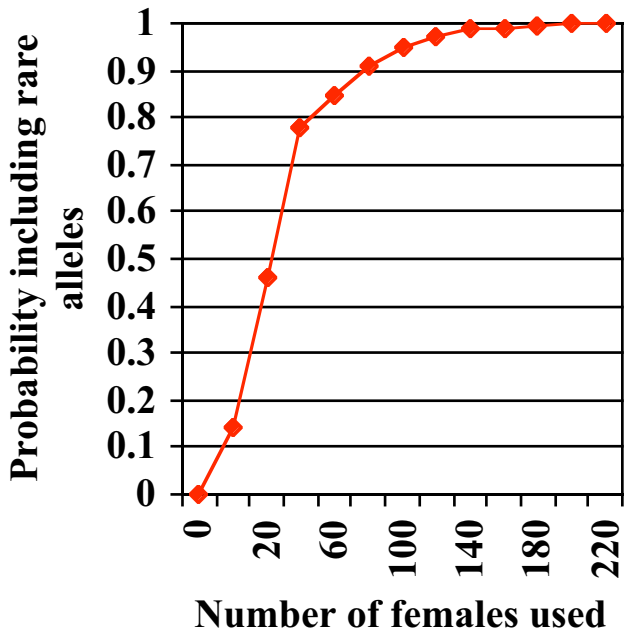
***PRESERVATION OF GENETIC DIVERSITY OF* L. higginsii**

The genetic markers that were examined in this study are assumed to be "neutral" markers that reflect the genetic variation and the history of population processes in the species. We assume that these markers are not important in adaptations of the animals to their environments. Nevertheless, the preservation of this variation is an appropriate goal for the conservation and management of this endangered species. There is likely to be genetic variation present in the species that functions in the current environment and there is also likely to be variation could be necessary for future adaptive evolution. Hence we recommend that relocation and propagation programs involve enough individuals to assure that the rare genetic variants that we detected will be preserved in future generations.

We estimated that ~100 females would need to contribute offspring in a new population to have a 95% probability of including a rare variant (frequency 3% in the population) (Fig. 3). We chose 3% as the frequency of the rare variant, because this was th efrequency observed for rare mtDNA clades in *L. higginsii* (see Fig. 2 and Table 5). The current propagation plan involves using 20-60 females per year, with females sampled and juveniles produced for at least 5 years (see Appendix). Thus, if all females used in the propagation program contributed offspring to the new population, then the total number of females used is adequate to preserve the rare alleles in at least one population. It is likely, however, that the genetic contributions of the donor females will not be equal, and the glochidia of some females may not persist at all. There are many unknown factors, such as the success rates of juveniles from individual females and specific years that may reduce the amount of genetic variation that is preserved. At the present time we do not have a quantitative estimate of the success rates of females that contribute offspring that have been raised in propagation facilities. In fact, we do not know the relative contributions of different females in wild populations, either. If 50% of donor females contribute

offspring to a new population, then 200 females would need to be used in the propagation program to have a 95% chance of including a rare allele. If 25% of donor females contribute offspring, then 400 females would need to be used. Our observation that there are many mtDNA haplotypes present in the wild argues that many females have made genetic contributions to the extant populations we sampled. The most effective way to assure that a substantial sample of the genetic variation will be preserved in the future is to establish new populations with representatives from many females.

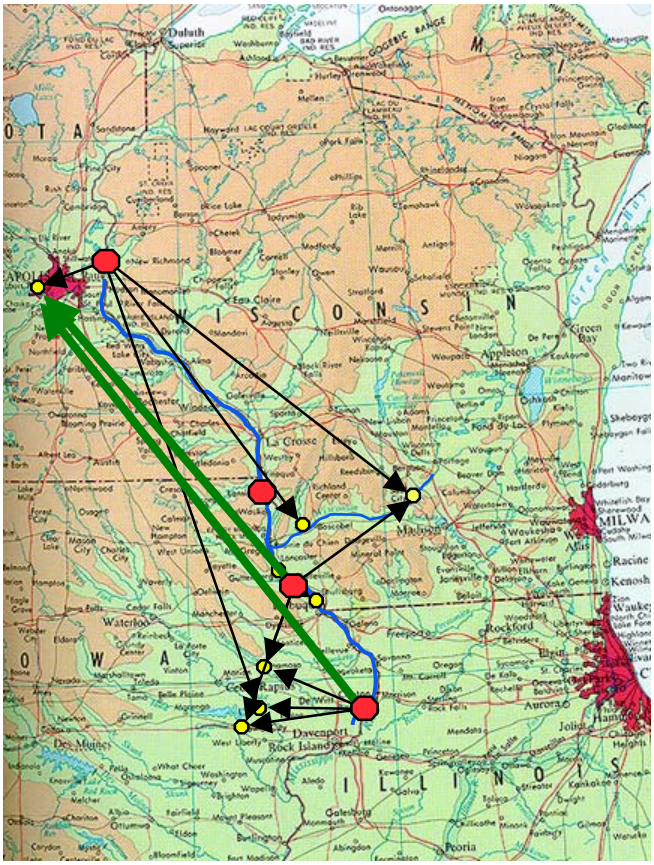
Figure 3. Probability of including a rare allele (frequency of 3% in the population) as a function of the number of females used in the propagation program.



RELOCATION SITES: MOVEMENTS OF INDIVIDUALS AMONG POPULATIONS AND ESTABLISHMENT OF NEW POPULATIONS.

In recent years, the relocation plan has included moving individuals from existing populations (Cassville and Cordova) to a central site (Pool 2 near Minneapolis) and founding new populations in the Cedar, Iowa, Mississippi, Rock, Wapsipinicon, and Wisconsin Rivers. There have been a few introductions of juveniles from the propagation facility into existing populations, but these activities are less common. Based on data provided by the Mussell Coordination Team (Appendix), I have constructed a map (Fig. 4) that shows the sources and destinations of individuals moved during the relocation and propagation programs.

Figure 4. Map of relocations of adults (bold green line from Cassville and Cordova to Pool 2), and larvae/juveniles from 2000-2004. Sources of females were Hudson-St. Croix (northern red dot), Cassville (third red dot from top) and Cordova (southern red dot). List of destination sites is in Appendix 1.



SUMMARY OF OBJECTIVE ACCOMPLISHMENTS:

Objective 1: Assess the genetic variability and geographic structure within and among populations of L. higginsii *in the Upper Mississippi, St. Croix, and Wisconsin Rivers.*

Genetic variability within populations: We found a high level of genetic variation in the mitochondrial DNA of *L. higginsii* individuals sampled in 2001. This is consistent with the findings based on a small number of individuals sampled in 1998 (Bowen and Richardson, 2000). We found three groups (clades) of mtDNA forms (haplotypes) that differed from each other by ~1.3% of their genetic sequences (based on 1027 base pairs). When this level of genetic difference is observed within a species, it is usually found in geographically isolated populations (Avise, 2000). We also found one clade (Clade S) of mtDNA haplotypes that differed from the other three by ~7% sequence divergence. This level of genetic difference is characteristic of the degree of difference among species.

A noteworthy finding is that all four of the clades found in these 130 individuals were recovered in the initial sample of 16 individuals in 1998 (Bowen and Richardson, 2000). This "good luck" reflects a high level of genetic diversity in the populations sampled in 1998, especially Prairie du Chien and Campbell's Island. Prairie du Chien, the most important critical habitat for *L. higginsii* prior to the zebra mussel infestation of the 1990's, contained the highest density of *L. higginsii* (U.S. Fish and Wildlife Service, 2003) and highest genetic diversity (Bowen and Richardson, 2000). Today that population of *L. higginsii* has been severely reduced. During two years of sampling in 1999 and 2000, only one individual was collected (U.S. Fish and Wildlife Service, 2003).

We found some individuals with Clade S, which contains the mtDNA haplotypes commonly found in *L. siliquoidea*, as we had found in 1998. The frequency of this clade was lower in the individuals sampled in 2001, occurring in 3 of 130 individuals, compared to 3 of 16 in 1998. The current study does not allow us to determine whether the occurrence of the siliquoidea haplotypes in higginsii is due to hybridization or whether a common ancestor of *L. higginsii* and *L. siliquoidea* shared this haplotype. Ongoing studies of nuclear DNA using microsatellites may help resolve this question.

In the current study, we sampled >20 individuals from each population, which has allowed us to estimate the frequency of the clades in the various populations. We found that Clade A is

the most common clade, occurring in 57% (Cassville) to 87% (Cordova) of individuals. This clade contained the highest number of unique haplotypes (12). Clade B, which was found in all populations, occurred in 10% (Cordova) to 33% (Cassville) of individuals. This clade contained eight haplotypes. Clade C was clearly distinct from Clades A and B, yet it was found in only three individuals, once each in Hudson, Cassville, and Cordova. Other clades may exist in *L. higginsii*, but were not detected in the sample of 20-30 from each population.

A high level of mtDNA diversity within populations has been found in *Cyprogenia aberti* (Jeanne Serb, pers. comm.). The clades found in this species reflect morphological variation and may indicate cryptic species. Morphological differences have not been noted in *L. higginsii* clades and cannot be tested for the individuals sampled in this study because voucher shells were not collected. Future studies of *L. higginsii* should attempt to determine whether there are morphological differences among individuals with different mtDNA haplotypes.

The genetic forms we detected using mtDNA markers are not a direct measure of adaptive genetic variation in this species. Nevertheless, the fact that we found such a high level of variation indicates that this species has the potential for having adaptive traits and has genetic variation that could be the basis for evolutionary change in the future. We recommend that the relocation and propagation plans include measures that will assure that this high level of genetic variation will be preserved for future generations of *L. higginsii*. The management recommendations presented below assume that it is desirable to maintain this variation.

Genetic variability among populations: L. higginsii has an unusual geographic distribution of the mtDNA clades we observed. Rather than finding these divergent clades distributed in different populations, we found them mixed in the same population. It is likely that this pattern reflects historical isolation during previous glacial maxima, with dispersal, mixing, and gene flow in the Mississippi River following the glacial retreat. We observed mixing of the different clades in the previous study, as well (Bowen and Richardson, 2000). The mitochondrial genome does not indicate that these populations represent genetically distinct entities, such as Evolutionarily Significant Units or Management Units, at this time. It is possible that nuclear genes could show genetic differences among the populations. Studies of nuclear microsatellite DNA are currently underway in this laboratory. Initial results indicate a high level of genetic variation, as we found with mtDNA. Statistical tests are being conducted to determine whether populations differ in their nuclear DNA.

Objective2: Make management recommendations regarding relocations of individuals among populations, specifically with regard to (a) number of animals to be relocated and (b) appropriate geographical sites for relocations.

Although we did not find evidence of genetic stocks, the exceptionally high level of genetic variation we found suggests that careful consideration needs to be given to management of the genetic resources of *Lampsilis higginsii*. Many of the management recommendations that were made in 2000 (Bowen and Richardson, 2000) still apply following this study. We recommend that as many individuals as possible be used for relocation and propagation efforts in order to preserve the genetic diversity found in *L. higginsii*. Based on the population genetic analysis of the current study, we are able to estimate the frequencies of the various haplotypes and clades, which allows us to make more specific recommendations regarding the number of individuals that should be relocated. Recommendations from the previous study (Bowen and Richardson, 2000) were presented in a hierarchical fashion, depending on whether "extirpation is imminent" or whether "extirpation is not imminent and drastic management actions were 5 to 10 years away." Since that report was written, "drastic management actions" have been undertaken with the movement and propagation of millions of larval *L. higginsii*. Therefore, this report will focus on recommendations for the ongoing relocation and propagation efforts.

It is necessary to consider two aspects of relocation: (1) the establishment of new populations where *L. higginsii* currently does not occur, and (2) the augmentation of existing populations to increase their size and viability. In both of these situations, populations can be manipulated (a) with individuals produced in propagation facilities and (b) with individuals from existing populations. Each of these situations requires somewhat different considerations to ensure the genetic health and viability of the population.

1a. New populations are established with propagated individuals. In the case of the establishment of new populations of *L. higginsii*, it is important to include enough founding members so that a large proportion of the genetic variation is introduced into the new population. Based on our estimation of the frequencies of the rare clades and haplotypes in *L. higginsii*, we calculated the probability of retaining rare forms (occur in ~3% of individuals) for various numbers of breeding females (Fig. 3). Based on this simple calculation using binomial probabilities, we calculated that ~100 females are needed to

have 95% confidence that the rare forms would be included in genetic material of the breeding females. The current propagation plan includes using 50 to 80 females each year for ~5 years. Thus, an adequate number of females will be used to preserve the genetic diversity detected in this study if all females successfully contribute offspring to the new population. Because we expect that not all females used in the propagation program will successfully contribute offspring, we consider this to be a minimum number of females. If the number of females collected for the propagation project (50-80/year) is not too large a fraction of the reproducing population, this plan should be continued as long as artificial propagation occurs. Although the recommendation to use 100 females makes it likely that most genetic diversity will be preserved, it does not assure that each population will contain the full complement of genetic diversity that exists in the species. As the propagation and relocation effort continues, it is important to continue to add genetic material to each population from as many females as possible.

Reintroduction of progeny from same female in multiple years could lead to a reduction in genetic variation in the population, by reducing the effective population size (N_e) and promoting genetic drift. The current propagation plan includes collecting glochidia from a cohort of females and releasing host fish that are carrying glochidia in 2004, then releasing cage-reared juveniles from this same female in 2006. This practice is occurring because the Mussel Relocation Team has determined that releasing cage-reared juveniles at some sites is more likely to be successful than releasing fish carrying glochidia parasites. The progeny released in 2004 and 2006 are from the same age cohort, yet they represent multiple attempts to introduce progeny produced by a cohort of females. This practice could result in a large genetic contribution from fewer females. This practice will occur in 2004/2006 (year of collection of glochidia and release of fish carrying glochidia/year of release of 2-year-old cage-reared juveniles) and 2005/2007. We caution against using genetic material from the same females in multiple years unless new females are used also.

We recommend that the geographic locations of the source females that are used for production of glochidia and juveniles should be similar to the newly established populations. As we have noted above, we have not found evidence for genetic differences

among populations with mtDNA. This indicates that the populations we sampled have not been genetically isolated from one another for an extended period of evolutionary time (tens of thousands to millions of years). The lack of mitochondrial DNA divergence does not rule out the possibility that adaptations exist among the populations we sampled. We are especially concerned that there may be differences along the north-south gradient that reflect adaptations to differences in water temperature and other effects of seasonality. The current relocation activities (see Fig. 4 and Appendix) have included mixing individuals from different populations, especially relocating progeny from the Hudson-St. Croix populations to populations in the southern part of the range. We recommend that this movement of individuals among populations be minimized in the future. This recommendation regarding the geographic source and destination of relocated individuals applies to all four scenarios discussed here (1a, 1b, 2a, and 2b).

1b. New populations are established with adults and juveniles are moved from existing populations to new sites. As discussed above, we recommend that more than 100 females be used as genetic donors to new populations. For long-term population viability, conservation geneticists recommend local populations of ≥500 individuals. If a population was initiated with at least 100 females and an equal number of males, and reproduction occurs, then a population size of ≥500 could be achieved. For many endangered species involved in captive propagation programs, even for many species of unionid mussels currently being propagated, it is not possible to use >100 females. Local populations may contain only a few individuals, not all are reproductively active, and some individuals need to be left to breed in the wild. We are in an enviable position with *L. higginsii*, in that we have enough individuals available to conduct a propagation program that will preserve the high level of genetic diversity present in this species.

2a. Existing populations are augmented with larvae and juveniles produced in a propagation facility. When existing populations are augmented with propagated individuals (supportive breeding) there are two concerns: the number of introduced individuals compared to the number of individuals already present in the population, and the geographic location of source population of the introduced individuals. When

considering the relationship between the number of introduced and established individuals, it is important to recognize the potential for the loss of genetic variation due to a reduction in effective population size (the "Ryman-Laikre effect"; Ryman and Laikre, 1991). This can happen if there is unequal or selective reproduction with the result that the cultured offspring genotypes "swamp" the native genotypes, resulting in loss of genetic variation. Thus, it is necessary to monitor the genotypes of individuals produced in the propagation facility as those in the wild and to see if there is a change over time in genotypes in the wild population. When augmenting existing populations, it is also important to know the geographic locations of the source populations to assure that the source and destination populations are in close geographic proximity and to avoid mixing individuals from different populations, as discussed in (1a) above.

2b. Existing populations are augmented with adults and juveniles are moved from existing populations to new sites. When animals are moved among populations, the most important parameter to consider is the geographic location of the source animals and the destination population. In some endangered species, there has been a specific plan to mix individuals among populations to reduce inbreeding, with the goal of a "genetic rescue" on an extremely small population. That is not the case with *L. higginsii*; no populations are known to be extremely small or in danger of extinction due to inbreeding effects. Hence, there is no reason to deliberately mix individuals among the populations. Adult *L. higginsii* individuals were moved from Cassville and Cordova to Pool 2 (see Fig. 4) to prevent extirpation of Cassville and Cordova populations when they were heavily infested with zebra mussels in 2001. At that time there were no *L. higginsii* in Pool 2, but the effect of the movement was to increase the chances for mixing Cassville and Cordova genotypes when natural reproduction occurred subsequent to the move. The current relocation plan does not emphasize the movements of adults to existing populations, but if that activity is considered in the future, we recommend that exchanges occur among populations that are geographically close to each other.

NEEDS FOR FURTHER RESEARCH

As the Relocation Plan for *L. higginsii* has progressed during the past four years, the need for genetic information has become more evident. A number of questions have arisen that can be addressed with further genetic and ecological studies. As the propagation and relocation efforts continue, we feel that the genetic consequences of these activities should be evaluated in an adaptive management framework. The *L. higginsii* relocation program is the largest, most comprehensive program of propagation of a species of freshwater mussel. The scope and long-term nature of the program offer the opportunity to gather important information that can help guide future programs on species that have more limited ranges and more fragile population sizes.

In this report we have shown that to preserve the high level of genetic variation that we found in *L. higginsii*, ≥100 females contribute genetic material to the new populations. These calculations assume that the progeny of all of those females will live to reproduce in the future. It is unknown, however, what proportion of females and their progeny will be successful. If the offspring of a few females are highly successful, and most fail, then the number of females used for propagation should be much larger than 100. Data are needed to assess the proportion of successful females. These data are extremely difficult to obtain, but genetic techniques such as microsatellites have the potential to address this question. We are currently conducting an assessment of the suitability of microsatellites in *L. higginsii*. In addition to expanded genetic studies, we recommend that additional mathematical models be used to evaluate the number of individuals that should be used in the propagation program. The simple model used here does not include any estimates of variance or confidence intervals.

We have recommended that individuals produced in the propagation facility be moved to populations that are geographically near to the source populations. In the recent past, there has been considerable mixing of individuals from different source populations (see Fig. 4). We suggest that a study be initiated to determine the success of individuals from different source populations in those populations where mixing has occurred. It is appropriate to evaluate whether single-source or mix-source populations are more successful. Such information could be used to inform future propagation and relocation programs for endangered freshwater mussels.

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REFERENCES

Avise, J. C. 2000. Phylogeography: The history and formation of species. Harvard University Press. Cambridge, MA.

Berg, D.J, W. R. Haag, S. I. Guttman, and J. B. Sickel. 1995. Mantle biopsy: a technique for nondestructive tissue-sampling of freshwater mussels. J. N. Am. Benthol. Soc. 14:577-581.

Berg, D.J., E.G. Cantonwine, W.R. Hoeh, and S.L. Guttman. 1998. Genetic structure of *Quadrula quadrula* (Bivalvia: Unionidae): Little variation across large distances. J. Shellfish Research 17:1365-1373.

Bowen, B.S., and W. Richardson. 2000. Genetic characterization of *Lampsilis higginsii*. Final Report to U.S. Fish and Wildlife Service, Bloomington, MN.

Folmer, O., W. R. Hoeh, M. B. Black & R. L. Vrijenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from metazoan invertebrates. Molecular Marine Biology and Biotechnology 3:294-299.

Huelsenbeck, J. P and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

King, T. L., M. S. Eackles, B. Gjetvaj, and W. R. Hoeh. 1999. Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Unionidae): conservation implications of range discontinuity. Molecular Ecology 9:S65-S78.

Kumar, S., K. Tamura, I.B. Jakobsen, and M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17:1244-1245.

Merritt, T. J. S., L. Shi, M.C. Chase, M. A. Rex, R. J. Etter, and J. M. Quattro. 1998. Universal cytochrome b primers facilitate intraspecific studies in molluscan taxa. Molecular Marine Biology and Biotechnology. 7:7-11.

Mulvey, M., C. Lydeard, D.L. Pyer, K.M. Hicks, J. Brim-Box, J.D. Williams, and R.S. Butler. 1997. Conservation genetics of North American freshwater mussels *Amblema* and *Megalonaias*. Conservation Biology 11:868-878.

Roe, K.J., and C. Lydeard. 1998. Molecular systematics of the freshwqter mussel genus *Potamilus* (Bivalvia: Unionidae). Malacologia 39:195-205.

Ryman, N., and L. Laikre. 1991. Effects of supportive breeding on the genetically effective population size. Conservation Biology 5:325-329.

Serb, J.M., J.E. Buhay, and C. Lydeard. 2003. Molecular systematics of the North American freshwater bivalve genus *Quadrula* (Unionidae: Ambleminae) based on mitochondrial ND1 sequecnes. Mol. Phyl. Evol. 28:1-11.

Swofford, D. L. 1998. PAUP* 4.0: Phylogenetic Analysis Using Parsimony. Sinauer Associates, Inc.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680.

Turner, T.F., J.C. Trexler, J.L. Harris, and J.L. Haynes. 2000. Nested cladistic analysis indicates population fragmentation shapes genetic diversity in a freshwater mussel. Genetics 154:777-785.

U.S. Army Corps of Engineers. 2002. Definite project report and environmental assessment for relocation plan for the endangered Higgins' eye pearlymussel (Lampsilis higginsii), Upper Mississippi River and tributaries, Minnesota, Wisconsin, Iowa, and Illinois. July 2002. U.S. Army Corps of Engineers, St. Paul District, MN. 56pp + Appendices.

U.S. Fish and Wildlife Service. 2003. Higgins' Eye Pearlymussel (*Lampsilis higginsii*) draft recovery plan: first revision. Ft. Snelling, MN.

Appendix

Sources of Females and Destinations of Larvae/Juveniles from Propagation Program

Table provided by Dennis Anderson, August 18, 2004, dennis.d.anderson@mvp02.usace.army.mil

Table 1. Number of donor females used (including translocated adult females) for relocation sites among years for *L. higginsii* propagation efforts.

		Strain and Year																														
		Cassville									Cordova								St. Croix									Wisconsin			Total by	Total by
	Method	2000*	2001	2002	2003	2004	2005	2006	2007	Total	2001*	2002	2003	2004	2005	2006	2007	Total	2000	2001	2002	2003	2004	2005	2006	2007	Total	2003	2004	Total	metapopulation	Relocation site
Cedar River	free release fish										10		35	43	50	50		188		20	19						39				227	227
Iowa River - Iowa City	free release fish			8						8	10		35	43	50	50		188													196	196
Wisconsin River - Lower	free release fish																		5								5				5	
Wisconsin River - Prairie du Sac	free release fish				21		10	10	10	51									5	20	19						44	2	3	3	98	
Wisconsin River - Orion EHA	open bottom cages				21	19	10	10	10	70										20							20		3	3	93	
	sub-adults					19	10	10	10	49																						117
Wapsipinicon River - Anamosa	free release fish			8			10	10		38	10	15	35	43	50	50		203													241	
Wapsipinicon River - Central City	free release fish			8			10	10		38	10		35	43	50	50		188													226	241
Rock River	sub adults				21	19	10	10	10	70				43	50	50	20	163													233	233
Pool 17	sub adults				21	19	10	10	10	70				43	50	50	20	163													233	233
Pool 2 - Hidden Falls	adults	41								41	118							118													159	
Pool 2 - Lower Pool 2	sub-adults																				19	18	5	10	10		62				62	221
Pool 3 - upper pool	adults	41								41																					41	
Pool 3 - Sturgeon Lake Pool 3	sub adults																			20	19	18	5	10	10		82				82	123
Wisconsin Channel Pool 4	sub adults																			20	19	18	5	10	10		82				82	82
Site to be determined	sub adults				21	19	10	10	10	70				43	50	50	20	163													233	233
Total No. females used		82		8	21	19	10	10	10	**160**	128	15	35	43	50	50	20	**341**	5	20	19	18	5	10	10		**87**	2	3	**3**	**591**	**591**

*Note - A total of 99 and 100 Cassville adult females and males were translocated to pool 2 and upper pool 3 in 2000. A total of 271 Cordova adult females and males were translocated to pool 2 in 2001.