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# Genetic characterization of *Lampsilis higginsii*

## 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. *Lampsilis 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. However, the genetic variation of populations among and within rivers where *L. higginsii* currently resides is unknown. The goal of this study was to characterize the genetic structure and diversity of *Lampsilis higginsii* as a basis for conservation and management actions. We sampled individuals from three populations and used the mitochondrial gene *cytochrome-b* to characterize the genetic structure of the species. 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, suggesting that *L. higginsii* may have had much larger populations in the past or that it may have occurred in isolated refugia during times of glacial advances. These findings suggest that if translocations occur, a large number of individuals should be collected in order to preserve as much genetic variation as possible. We also found that three of the 16 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 *Lampsilis higginsii*.


## INTRODUCTION

Unionacean mussels are the most imperiled group of freshwater invertebrates in North America, with 72% of all taxa identified as endangered, threatened or of special concern (Williams et al. 1993). The fauna as a whole is a priority concern of the U. S. Fish and Wildlife Service (USFWS), National Park Service, and most state agencies in the eastern U.S. because of the severe and immediate threat from zebra mussels, as well as rapid and widespread loss of

habitat. Efforts to conserve unionacean mussels require an understanding of the genetic diversity in natural populations and the recognition of evolutionarily significant units. Management actions for species with high gene flow and little among population variation will differ substantially from that of species with restricted gene flow and large among population variation. Whereas, preservation of only several populations that have high gene flow will conserve most of its diversity, protection of numerous populations within a geographic region will be necessary for taxa that have low gene flow. Endangered species recovery plans often identify characterization of the genetic structure as a high priority task for recovery of the species; yet, management tasks are usually determined without *a priori* knowledge of the genetic stocks being managed.

*Lampsilis higginsii* (Higgins' Eye) is a federally endangered mussel in the Upper Mississippi River basin. *Lampsilis higginsii* currently occurs in the Mississippi, St. Croix and Wisconsin rivers and has suffered a 50% reduction in its range since 1965 (Havlik 1981). Populations of *L. higginsii* in the Mississippi River and lower St. Croix River are threatened by extirpation from the zebra mussel. As a result, refuges in rivers that are zebra-mussel free have been proposed to preserve this species. However, the genetic variation of populations among and within rivers where *L. higginsii* currently resides is unknown. The goal of this study was to characterize the genetic structure and diversity of *Lampsilis higginsii* as a basis for conservation and management actions. Our specific objectives were to: (1) Determine the genetic structure of the species within and between major rivers, (2) Identify genetically distinct populations, and (3) Develop management recommendations based on characterization of genetic stocks.

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 Upper Midwest Environmental Sciences Center, Biological Resources Division, U.S. Geological Survey, Dr. William Richardson Project Director. The source of some of these funds ($10,000) was an Interagency Agreement from the Fish and Wildlife Service to the Upper Midwest Environmental Sciences Center. RWO funds, in the amount of $15,000, were used (1) to hire a laboratory assistant, Ms. Tonia Schwartz, who worked 1/2 time from October, 1998 through May, 1999, (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 and Ms. Schwartz to present preliminary findings and meet with colleagues at the meeting of the Freshwater Mollusk

Conservation Society meeting in March 1999, and (5) to support 10% of Dr. Bowen's salary from October, 1998 through April, 1999.

## METHODS

### *SAMPLING*

We obtained samples of *L. higginsii* during the summer of 1998 from three localities: (1) St. Croix River, near Lakeland, Minnesota, River Mile (RM) 16, (2) Mississippi River near Prairie du Chien, Wisconsin, RM 635, and (3) Mississippi River near Campbell Island, Moline, Illinois, RM 490. Sampling localities were chosen to maximize the potential for finding genetically different populations. We chose populations at the northern and southern limits of the range (St. Croix and Campbell Island) and one intermediate between them (Prairie du Chien). Limited funding prevented us from collecting additional samples. Individual mussels were collected by divers and personnel experienced in identification of *L. higginsii*. Mussels were identified in the field by Dr. Andrew Miller, U.S. Corps of Engineers, Waterways Experiment Station, Vicksburg, MS, and Dr. Diane Waller, Western Wisconsin Technical College, La Crosse, WI. We obtained mantle and foot samples (5-15 mg; Berg et al. 1995) from 5-7 individuals at each location. We chose this number of animals because there is usually little variation in mitochondrial DNA sequences within populations, and many current studies rely on similar sample sizes (Barrowclough et al. 1999, King et al. 1999). Animals were marked, photographed, and returned to the river. No voucher shells were collected, as specified in the Endangered Species collecting permit. Tissue samples were also collected from two individuals of *Amblema plicata* to use as comparative samples (outgroups in phylogenetic analyses). After receipt of samples from collectors, the study director assigned a sample code to each specimen and recorded all collection information in the study logbook. When samples were sent to Iowa State University (ISU) for analysis, they were identified only by their sample code. Collecting location was not revealed to the ISU investigators until DNA sequence analyses had been completed.

Samples of *Lampsilis siliquoidea* were collected from lakes in northern Minnesota during 1996 as part of a study on genetic variation in this species (Tuxbury 1997). Tissues and DNA have been stored at –80°C or –20°C since 1996 and were available for use in this study. We used

DNA that had been extracted from these samples to develop appropriate techniques for *L. higginsii*. Once we had established an appropriate genetic marker in *L. siliquoidea* (see below), we obtained DNA sequence data from five individuals from each of three lakes, Leech, Loon, and Bluewater, all in the Mississippi River drainage. This sampling design for *L. siliquoidea* was similar to that used for *L. higginsii* and allowed us to compare the patterns of genetic variation within and among populations for the two species. DNA sequence data was also collected from one individual *Lampsilis siliquoidea* from Whitefish Lake, in the Hudson Bay drainage.

### *DNA EXTRACTION AND SEQUENCING TECHNIQUES FROM SMALL SAMPLES*

DNA from *Lampsilis siliquoidea* was extracted from frozen tissue using a C-TAB/phenol/chloroform technique standardized in the ISU laboratory in 1996 (Tuxbury 1997). Because *L. higginsii* samples had been preserved in ethanol in 1998, we tested to see whether tissue preserved in ethanol would yield DNA suitable for PCR and mtDNA sequencing. We placed small quantities of *L. siliquoidea* tissue in ethanol, then used the standard procedure to extract DNA. We compared the results of ethanol-preserved DNA with DNA extracted from frozen tissue and found no difference in our ability to amplify and sequence mtDNA genes.

The quantity of tissue obtained from the tissue biopsy procedure was very small (2-15 mg). We were concerned that the standard C-TAB/phenol/chloroform extraction technique, which required many rinses in which the DNA pellet might accidentally be lost, was unsuitable for such small samples. We consulted with colleagues who were also using DNA techniques to study freshwater mussels, and we learned of a commercially-available DNA extraction kit that would be suitable for our work with small samples from an Endangered Species. We purchased the kit (Puregene DNA Extraction Kit from Gentra Systems) and tested it thoroughly with ethanol-preserved material from *L. siliquoidea.* After confirming that the kit produced high quality DNA for PCR amplification and DNA sequencing, we extracted DNA from all *L. higginsii.* DNA was stored at –20°C after extraction.

### *DEVELOPMENT OF mtDNA GENE USEFUL FOR INTRASPECIFIC STUDIES*

We used mitochondrial DNA sequence variation to characterize the genetic structure of the target mussel species. We chose to assay mitochondrial DNA because of its strength for revealing relationships among closely related species and among populations of the same

species. Mitochondrial DNA is especially appropriate for the study of Endangered Species because the mitochondrial genome of populations becomes differentiated more rapidly than does the nuclear genome (Moritz 1994). Furthermore, it is possible to detect differences among populations using mitochondrial DNA with fewer individuals than are required when using nuclear DNA. As proposed by Moritz (1994), one of the criteria for distinguishing Evolutionarily Significant Units (ESUs) is the occurrence of different forms of mitochondrial DNA in different populations, with no forms shared between populations. ESUs also show significant divergence of genetic material in the nuclear genome. Populations that are different, but not as highly divergent as ESUs may warrant conservation status as Management Units (MUs). MUs are recognized as populations with significant divergence of genetic material in the mitochondrial or nuclear genome, even if there is not complete divergence (Moritz 1994). Our investigation of mitochondrial DNA in *Lampsilis higginsii* provided the opportunity to identify MUs, if significant differences among populations were found in the mitochondrial DNA. If we found complete differentiation among populations in the mitochondrial DNA, then this study would have been an initial step in identifying ESUs. Analysis of the nuclear genome would then be called for to further define ESUs.

Only a few studies have been conducted using mitochondrial DNA analysis within a species of freshwater mussel (Lydeard et al. 1996, Mulvey et al. 1997, King et al. 1999), but it holds great promise for identifying Management Units (Mulvey et al. 1998). Sequence variation at the *16s ribosomal RNA* (*16s rRNA*) and the *cytochrome oxidase I* (*COI*) genes have been documented within populations of freshwater mussels.

Initially, we explored DNA amplification using PCR and DNA sequencing methods for the *16s rRNA* and *COI* genes from mitochondrial DNA in *Lampsilis siliquoidea*, a closely related congener to *Lampsilis higginsii*, that is common and readily available. Primer sets used for PCR amplification are shown in Table 1. We successfully amplified the *COI* gene and obtained DNA sequence data from individuals from three Minnesota lakes. We found no variation in the 350 base pair fragment. We were unsuccessful in our attempts to amplify the *16s rRNA* gene. Thus, neither of these genes was suitable for measuring intraspecific genetic variation in *Lampsilis*. Rather than continue to pursue these two genes, we decided to turn our attention to a third mitochondrial gene, *cytochrome-b*, which is often more variable than *COI* and *16s rRNA*. Late in 1998, at about the time we had obtained uniform sequences for *COI* and were looking for a

more productive genetic marker, a paper was published with primers for *cytochrome-b* from a number of mollusc species (Merritt et al. 1998).

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

| MtDNA gene | Forward primer (5'—3') | Reverse primer (5'—3') |
|---|---|---|
| 16s RNA | CGACTGTTTAACAAAAACAT | CCGTTCTGAACTCAGCTCATGT |
| COI | GGTCAACAAATCATAAAGATATTGG | TAAACTTCAGGGTGACCAAAAAATCA |
| Cyt-b | AAGAAGTATCATTGCGGTTG | TGTGGGGCGACTGGTATTACTAA |

We developed the methodology to allow us to determine the DNA sequence for the *cytochrome-b* gene, which had never previously been assayed in a unionacean mussel. *Cytochrome-b* had been amplified and sequenced a several species of molluscs, primarily marine bivalves and terrestrial and freshwater gastropods. We used these published degenerate primers and published PCR conditions to amplify *cytochrome-b* in *L. siliquoidea*. Following successful amplification, PCR products were purified and concentrated with Microcon-100 concentrators and sequenced at the Iowa State University DNA Sequencing Facility on an ABI 377 (Perkin-Elmer) automated sequencer. Based on this sequence, forward and reverse primers were designed specifically for *Lampsilis siliquoidea* (Table 1). Using these primers, a 420 base pair region of *cytochrome-b* was amplified for 14 *Lampsilis siliquoidea*, four or five individuals from each of three lakes. 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 mg DNA. The thermal cycler program had 30 cycles at 94°C for 1 min, 40°C for 1 min, 72°C for 1 min, and an indefinite hold at 4°C. PCR products were purified and concentrated as described above and sequenced at the ISU DNA Sequencing facility. We found five different *cytochrome-b* sequences in these 14 *Lampsilis siliquoidea*. Individuals differed from one another by only one base pair, but this level of difference was typical within a species and was encouraging enough to warrant further development. Next, we verified that we could amplify *cytochrome-b* in *L. higginsii*. We then amplified *cytochrome-b* in 16 *Lampsilis higginsii* and two *Amblema plicata*. We reported the

development of the *cytochrome-b* gene and preliminary data on variation in *L. siliquoidea* at the meeting of the Freshwater Mollusk Conservation Society meeting in March 1999.

***STATISTICAL ANALYSES:***

Sequences were aligned by hand with Se-Al (http://evolve.zoo.ox.ac.uk/Se-Al/Se-Al.html) and/or with CLUSTAL-W (Higgins and Sharp 1989). We estimated several measures of genetic variability within populations and species, including numbers of haplotypes observed, Nei's (1987) gene diversity (which is comparable to heterozygosity) and its variance, and Tajima's (1983) index of nucleotide diversity ($\pi$) and its variance. We estimated gene and nucleotide diversities for each population (lake or river sampling location). In addition, we pooled all samples for each species so that we could compare the overall level of diversity between species. Gene diversity and nucleotide diversity were calculated using ARLEQUIN (http://anthropologie.unige.ch/arlequin/). We calculated the percent sequence difference among haplotypes by counting pair-wise differences by hand. We report the range of pair-wise differences within each population. We estimated the fraction of the total mtDNA variation that was distributed within and among populations by conducting an Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992), using ARLEQUIN. To determine the pattern of relationships among the populations, we conducted phylogenetic analysis using standard techniques (Swofford et al. 1996), including maximum parsimony analyses (PAUP*4.0, Swofford 1998).

## RESULTS

### *ASSESSMENT OF GENETIC VARIATION IN* Lampsilis siliquoidea *AND* Lampsilis higginsii

We obtained the mitochondrial DNA sequence of *cytochrome-b* from 14 *L. siliquoidea*, four or five from each of three lakes in Northern Minnesota, and 16 *L. higginsii*, five or six from each of the target populations in the St. Croix and Mississippi Rivers. We sequenced at least 360 base pairs of the *cytochrome-b* gene for all individuals.

In *L. siliquoidea,* we found five different forms (or haplotypes) of the *cytochrome-b* gene (Table 2, Appendix 1). Ten individuals shared a single haplotype, which we have designated the S1 (Siliquoidea-1) haplotype. The remaining four individuals each had a unique haplotype (S2-S5) that differed from S1 by one base substitution, or 0.3% of the 360 base pair sequence. This

is a small amount of difference, and is likely the result of random mutations at the *cytochrome-b* gene. In the largest lake, Leech Lake, we found four haplotypes in the five individuals we sampled. Bluewater Lake contained two haplotypes, S1 in four mussels and a variant (S2) in one. In Loon Lake, all four mussels sampled had the S1 haplotype. Gene diversity ($\pi$), which is comparable to heterozygosity and measures the variation in terms of haplotypes, ranged from 0 in Loon Lake to 0.9 in Leech Lake. The overall gene diversity for *L. siliquoidea,* with all samples pooled, was 0.28. Nucleotide diversity, which measures the variation in terms of DNA nucleotide substitutions, ranged from 0 to 0.003 in the lakes, with an overall level of 0.001.

In *L. higginsii*, we found eight haplotypes, which differed from one another by 1-34 base pairs (Table 2, Appendix 1). Two of the haplotypes found in *L. higginsii*, S1 and S6, were the same or very similar to those found in *L. siliquoidea*. This is an unusual finding, though not unknown in animals (Lehman et al. 1991). We have calculated gene diversity and nucleotide diversity (and their variances) in two ways (Table 2): (1) including all samples identified as *L. higginsii*, and (2) excluding the samples that contained mtDNA similar to *L. siliquoidea* (S1 and S6 haplotypes). Considering all eight haplotypes found in *Lampsilis higginsii,* the haplotypes represent 0.3-9.4% sequence divergence. When the S1 and S6 haplotypes were not included in the analysis, the remaining six haplotypes (H1 to H6) found in *Lampsilis higginsii* represent 0.3-2.5% sequence divergence (1 to 9 of 360 base pairs). This is a high level of variation, especially in an endangered species. For comparison, in *Lampsilis siliquoidea*, we found 0.3% sequence divergence among all haplotypes within the species.

The measures of gene diversity (heterozygosity) and nucleotide diversity were also higher in *Lampsilis higginsii* than in *Lampsilis siliquoidea*. Gene diversity for all *Lampsilis higginsii* samples ranged from 0.7 to 0.9 in the three populations, with an overall level of 0.85. Nucleotide diversity ranged from 0.008-0.055 in the three populations, with an overall level of 0.034. When we excluded the S1 and S6 haplotypes from the analysis of *Lampsilis higginsii*, we found levels of gene diversity and nucleotide diversity that were lower, but not as low as those found in *Lampsilis siliquoidea*. Gene diversity ranged from 0.7-1.0, with an overall level of 0.6 and nucleotide diversity ranged from 0.008 to 0.015, with an overall level of 0.011. Thus, our measures of genetic variability indicate that *Lampsilis higginsii* has a surprisingly high level of variation for an endangered species, especially when compared with its congener, *Lampsilis siliquoidea.*

Table 2. Measures of genetic variation in *Lampsilis higginsii* from the Mississippi and St. Croix Rivers and in *Lampsilis siliquoidea* from three lakes in Minnesota.

| Species | Population | No. individuals | No. haplotypes | % sequence divergence | Gene diversity | Variance | Nucleotide diversity | Variance |
|---|---|---|---|---|---|---|---|---|
| *L. higginsii* all samples | Overall | 16 | 8 | 0.28-9.2 | 0.85 | 0.075 | 0.033 | 0.018 |
| | St. Croix | 5 | 3 | 0.28-2.2 | 0.70 | 0.218 | 0.008 | 0.006 |
| | Prairie du Chien | 5 | 4 | 0.56-9.2 | 0.90 | 0.161 | 0.055 | 0.035 |
| | Campbell Island | 6 | 4 | 1.9-9.2 | 0.80 | 0.172 | 0.035 | 0.021 |
| *L. higginsii* without S haplotypes | | 13 | 6 | 0.28-2.5 | 0.78 | 0.104 | 0.011 | 0.007 |
| | St. Croix | 5 | 3 | 0.28-2.2 | 0.70 | 0.218 | 0.008 | 0.006 |
| | Prairie du Chien | 3 | 3 | 0.56-2.5 | 1.00 | 0.272 | 0.015 | 0.013 |
| | Campbell Island | 5 | 3 | 1.4-2.5 | 0.70 | 0.218 | 0.012 | 0.008 |
| *L. siliquoidea* | Overall | 15 | 5 | 0.28-0.56 | 0.48 | 0.154 | 0.001 | 0.001 |
| | BlueWater | 5 | 2 | 0.28 | 0.40 | 0.237 | 0.001 | 0.001 |
| | Leech | 5 | 4 | 0.28-0.56 | 0.90 | 0.161 | 0.003 | 0.003 |
| | Loon | 5 | 1 | 0 | 0 | 0 | 0 | 0 |

The haplotypes found in *L. higginsii* were not restricted to individual populations (Table 3), as would be expected if there were Management Units (MUs) or genetic stocks. H1 was found in all populations, H3 was found in St. Croix and Campbell Island, and H6 was found in

Table 3. Localities and occurrence of *cytochrome-b* haplotypes in *Lampsilis higginsii* from the Mississippi and St. Croix Rivers, 1998.

| Mantle Sample Code | Locality | Haplotype |
|---|---|---|
| 1 | St. Croix River, WI | H3 |
| 11 | St. Croix River, WI | H1 |
| 36 | St. Croix River, WI | H1 |
| 38 | St. Croix River, WI | H2 |
| 39 | St. Croix River, WI | H1 |
| 10 | Prairie du Chien, WI | S1 |
| 16 | Prairie du Chien, WI | H6 |
| 27 | Prairie du Chien, WI | S1 |
| 29 | Prairie du Chien, WI | H5 |
| 32 | Prairie du Chien, WI | H4 |
| 12 | Campbell Island, IL | S6 |
| 17 | Campbell Island, IL | H3 |
| 19 | Campbell Island, IL | H6 |
| 33 | Campbell Island, IL | H1 |
| 35 | Campbell Island, IL | H1 |
| 37 | Campbell Island, IL | H1 |
| 15 | Campbell Island, IL | H1 |

Prairie du Chien and Campbell Island. S1 was found in Prairie du Chien and S6 was found in Campbell Island. The St. Croix sample, the northernmost population, contained three haplotypes, two of which it shared with Campbell Island, the southernmost population. The Prairie du Chien and Campbell Island populations each contained four haplotypes, one of which they shared with each other. AMOVA showed that most of the variation was within populations and little was among populations (Table 4). When all *Lampsilis higginsii* samples were included in the analysis, there was more variation among populations than there was when the S1 and S6 haplotypes were excluded. However, neither analysis showed statistically significant differentiation among populations. Likewise, no differentiation was found among populations of *Lampsilis siliquoidea*.

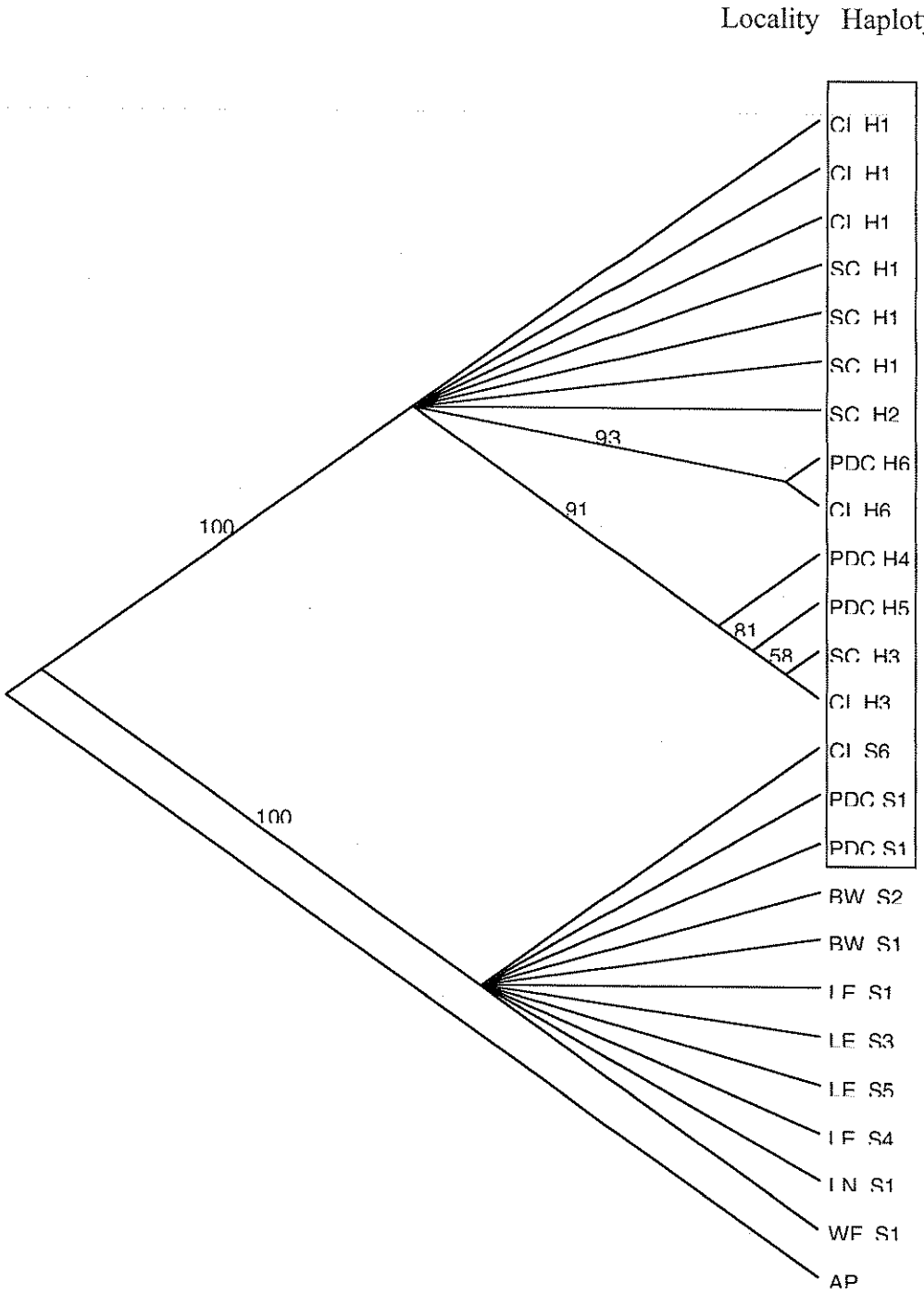
Table 4. Analysis of Molecular Variance (AMOVA) for *Lampsilis higginsii* from the St. Croix and Mississippi Rivers, 1998.

| Samples used in analysis | Source of Variation | Percentage of Variation |
|---|---|---|
| All *Lampsilis higginsii* | | |
| | Among Populations | 1.34 |
| | Within Populations | 98.66 |
| *Lampsilis higginsii* | | |
| without S1 and S6 | Among Populations | 0.00 |
| | Within Populations | 100.00 |
| All *Lampsilis siliquoidea* | | |
| | Among Populations | 0.00 |
| | Within Populations | 100.00 |

Phylogenetic analysis of the *cytochrome-b* mitochondrial gene shows two three clusters of genetic types of *Lampsilis higginsii*, one containing haplotypes H1, H2, and H6, a second containing H3, H4, and H5, and a third containing S1 and S6 (Figure 1). Each of these forms was found in two or more populations. Thus, there is not a strong correspondence between genetic types and geographic location. The Prairie du Chien population contained more unique haplotypes, and hence more genetic variation, than did the St. Croix population. Phylogenetic analysis showed that some of the Prairie du Chien haplotypes (e.g. H4) are closely related to haplotypes (e.g. H3) in the St. Croix River, to the north, and Campbell Island, to the south.

The most surprising finding of our study was that some of the *L. higginsii* had the same (S1) or a similar (S6) mtDNA haplotype at the *cytochrome-b* gene as did most of the *L. siliquoidea.* These "siliquoidea-like" *L. higginsii* were collected at Campbell Island (one mussel—S6 type) and Prairie du Chien (two mussels—S1 type). The siliquoidea (S1 and S6) *cytochrome-b* haplotypes differed from the higginsii (H1 through H6) haplotypes by 7.5-9.4 % of the sequence (27-34 of 360 base pairs). The amount of difference between the *cytochrome-b* S and H sequences is substantial and typical of the degree of difference found between two species.

Figure 1. Phylogenetic tree, using maximum parsimony, showing relationships among haplotypes for *Lampsilis higginsii* samples (enclosed in box) from St. Croix River (SC), Prairie du Chien (PD) and Campbell Island (CI), and *Lampsilis siliquoidea* samples from Bluewater Lake (BW), Leech Lake (LE), Loon Lake (LN), and Whitefish Lake (WL). *Amblema plicata* (AP) was used as an outgroup. See text and Table 2 for explanation of haplotypes. Numbers indicate boostrap branch support.



## DISCUSSION

We found that the 16 *Lampsilis higginsii* we sampled contained a surprisingly high number of forms (haplotypes) of *cytochrome-b* mitochondrial DNA. Further, the amount of difference among these haplotypes was large compared to differences typically seen within a species. Differences as large as we found among haplotypes are typically found in animals that have large population sizes (tens of thousands or more) or that have discrete populations that have been isolated from one other for many (thousands of) generations, during which time random genetic changes have accumulated. It is possible that the various haplotypes evolved during periods of glacial advance, when *Lampsilis higginsii* was distributed in several isolated populations. As the glaciers receded, *L. higginsii* became more widely distributed and the populations mixed. The pattern of genetic variation we see today may be a reflection of the isolation in the past and the current mixing of populations. An alternative explanation is that *Lampsilis higginsii* has or had in the past a very large population size that would have supported a high amount of variation.

The mitochondrial genome does not indicate that these populations represent genetically distinct entities at this time. It is possible that nuclear genes could show genetic differences among the populations, although we do not consider this likely.

There are several possible explanations for the finding that three L. higginsii showed the cytochrome-b sequence of L. siliquoidea. First, the specimens could have been misidentified. The collectors were experienced field biologists who are unlikely to have made such an error. Photographs of the animals we sampled were examined by two unionid experts, Diane Waller (examined photographs from both populations with animals that contained siliquoidea-type DNA) and Kevin Cummings (examined photographs from one population with animals that contained siliquoidea-type DNA). Both observers stated that all photographs appeared to be *Lampsilis higginsii*. Further, Mr. Cummings commented that the habitats of the two species do not overlap, hence we were not likely to have collected *Lampsilis siliquoidea* from our sampling sites. Unfortunately we do not have voucher shells to examine. A second possible explanation for the finding that three *L. higginsii* showed the *cytochrome-b* sequence of *L. siliquoidea* is that samples were mixed up in the laboratory, either during the DNA extraction from tissue or during

DNA amplification. Mislabeling during DNA extraction is highly unlikely because *L. siliquoidea* DNA was extracted in 1996 and *L. higginsii* DNA was extracted in 1999. It is not possible for us to repeat the extraction procedure because we have no extra tissue from *Lampsilis higginsii*. Mislabeling during DNA amplification is also unlikely because amplification of DNA from *L. siliquoidea* and *L. higginsii* occurred on different days. To eliminate the possibility that amplification products had become contaminated, we re-amplified DNA of the two individuals with the S1 haplotype and had the new PCR products sequenced. In both cases, the individuals were again determined to have the S1 haplotype. A third possible explanation for the finding of *L. siliquoidea* mtDNA in *L. higginsii* is that there has been hybridization between the species. If a female *L. siliquoidea* had been fertilized by sperm from *L. higginsii*, then the progeny would have had mtDNA like their mother, *L. siliquoidea*. Because the mtDNA in somatic tissues, such as mantle and foot, comes only from the mother, and because all somatic cells in an individual contain the same form of mtDNA, hybrid individuals appear like the mother; they do not have an intermediate form of mtDNA. Hybridization could have taken place recently or even hundreds of generations ago. A fourth possible explanation for the finding of the *L. siliquoidea* mtDNA in *L. higginsii* is that the siliquoidea-like mtDNA haplotype was present in a common ancestor of these two species and it was passed to both species. Eventually we expect species to lose these shared haplotypes as evolutionary time progresses, but it is possible that not enough time has elapsed in the evolution of *L. siliquoidea* and *L. higginsii*. It is possible to distinguish between these possibilities with data from the nuclear genome, as discussed in the section on Needs for Further Research.

## *SUMMARY OF OBJECTIVE ACCOMPLISHMENTS:*

*Objective 1: Determine the genetic structure of the species within and between major rivers.*

We determined that *Lampsilis higginsii* contains a high level of genetic variation, higher than its congener, *Lampsilis siliquoidea*. We identified six distinct forms (haplotypes) of the mitochondrial DNA *cytochrome-b* gene that occurred only in *Lampsilis higginsii* (H1-H6) and five forms of *cytochrome-b* in *Lampsilis siliquoidea* (S1-S5). In addition, we found three *Lampsilis higginsii* individuals that contained a form of *cytochrome-b* the same or similar to that typically found in *Lampsilis siliquoidea* (S1, S6). Further research is needed to determine

whether this finding is due to hybridization or whether a common ancestor of *Lampsilis higginsii* and *Lampsilis siliquoidea* shared this *cytochrome-b* haplotype. It is likely that a more thorough survey of *Lampsilis higginsii*, both within and among populations, would reveal additional genetic variation.

Most of the forms of *cytochrome-b* we identified were not restricted to one river system. Some forms were found in all three systems we sampled. A statistical analysis of genetic variation did not find differences among the rivers.

*Objective 2: Identify genetically distinct populations.*

We did not find evidence for genetically distinct populations of *Lampsilis higginsii*. The statistical analysis mentioned above found that most of the genetic variation was within populations, not among populations.

*Objective 3: Develop management recommendations for based on characterization of genetic stocks.*

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*.

First, we are in an enviable position with this species -- we have documented a great deal of genetic variation in an Endangered Species. This is not often the case. We have an obligation to make management decisions that acknowledge this unusual situation and call for management practices that will perpetuate this high level of diversity in *Lampsilis higginsii*.

Second, to manage such great genetic variation, without losing a significant portion of the gene pool, population sizes must not be allowed to get too small. At this time we can not estimate what "too small" is, but there is clearly a need to keep the populations stable. To make a quantitative estimate of minimum population size, we need to obtain a more accurate estimate of the level of genetic variation in the species. This will require samples of more animals from more populations.

Third, if animals are to be transferred to refugia, we can only support such transfers if (1) they involve large numbers of individuals and (2) the movements are within the Upper Mississippi River Basin (UMRB) proper. Until we have a larger genetic data set, with greater

spatial coverage, we are unwilling to recommend movements of *L. higginsii* into other basins or from other basins into the UMRB.

Fourth, because of the unexpected nature of these findings it is difficult to make specific management recommendations regarding relocation of *L. higginsii*. We can suggest several scenarios based on the urgency of potential management actions.

I. If extirpation is imminent within the next several years (e.g., zebra mussel invasion is causing severe and widespread mortality of unionids in the Upper Mississippi Basin) we would suggest, based on the genetic evidence, that movement of individuals among any of these three sites would be acceptable. We found a mixture of genetic forms among the three sites we sampled, not distinct site-specific genetic stocks. This finding suggests that if an emergency translocation were necessary we would likely not be mixing individuals among Evolutionarily Significant Units. To ensure the preservation of as much genetic variation as possible, many individuals should be collected from each population that is to be translocated. Because of the high amount of genetic variation at these sampled sites, we could be introducing new genetic material into the receiving sites, with unknown consequences.

II. If extirpation is not imminent and drastic management actions were 5 to 10 years away, we recommend an attempt be made to resolve the question of exactly how much variation exists in these and other populations. We found many haplotypes (8 different haplotypes in 16 animals) that are fairly different from each other (1-2% sequence divergence) and distributed throughout the sampled range of *Lampsilis higginsii*. It is likely that there were other haplotypes we did not sample. We do not know, for example, whether the St. Croix population actually contained some siliquoidea-type forms (S1-S6) that we did not detect. Likewise, the Prairie de Chien population may contain H1, which was found in both St. Croix and Campbell Island samples. The unusual pattern of mitochondrial DNA variation in *Lampsilis higginsii*--high variability with many haplotypes shared among populations--means that we need to use the frequencies of the different haplotypes (forms) as our basis of describing the genetic differences among the populations. The AMOVA analysis indicates that we have no evidence that the haplotype frequencies are different among the populations. At the present time we really do not have a good estimate of haplotype frequencies. To accurately describe the genetic composition of each population using frequencies of the various haplotypes, we need more individuals from each population and we need to evaluate the nuclear genome. In addition, we need to complete

our examination of genetic variation of *Lampsilis higginsii* in the UMRB by sampling other populations of the species, especially those in the Wisconsin River. Only after these issues are resolved will we be entirely certain of the best strategy for movement of individuals among sites on the Mississippi River or into other river systems in the Basin.

## *NEEDS FOR FURTHER RESEARCH*

Our findings indicate that the genetic variation we detected at the mitochondrial *cytochrome-b* gene is not distributed among distinct populations. But our findings also indicate that we have much to learn about the patterns of genetic variation within and among *L. higginsii* populations. The variation we have found is likely just a portion of that which exists in this species. To fully understand the nature of the genetic populations, we must study additional individuals, including additional populations and larger samples from each population. It is especially important to obtain samples from populations farther south and in any tributaries to the Mississippi River that have extant populations of *L. higginsii.*

Studies of additional individuals and populations could be accomplished by collecting additional individuals from the field, although such a request may not be granted for this Endangered Species. If museums hold any ethanol-preserved samples of *Lampsilis higginsii*, these samples could be a valuable resource to determine the genetic characteristics of populations of this species. Extensive museum samples might make it possible to determine a more complete range of the variation in the species, and whether the various haplotypes are associated with populations from tributaries that may have served as refugia during periods of glacial advance. If museum samples are found that have the S1 or S6 haplotype, characteristic of *L. siliquoidea,* it would be possible to examine the shells to confirm their morphological identity.

In addition to studying more individuals and populations, our understanding of the genetics of *L. higginsii* would be enhanced by the study of markers from the nuclear genome. Nuclear DNA could provide evidence for genetic differences among populations, although at the present time we do not think there is a strong likelihood that it will. Nuclear DNA does, however, provide a powerful method to determine whether the *L. higginsii* individuals with the S1 and S6 haplotypes are the progeny of recent hybridization between the species. A colleague at

the Leetown Science Center, USGS, is currently using our DNA to develop a nuclear technique (Amplified Fragment Length Polymorphisms; AFLPs) for measuring genetic variation in *L. siliquoidea*. It is possible that he may find diagnostic markers that distinguish *L. higginsii* and *L. siliquoidea*. If he does, then we could use those markers to determine whether the S1 and S3 *L. higginsii* carry nuclear genes characteristic of *L. siliquoidea*. Such information would tell whether these individuals are truly hybrids.

## ACKNOWLEDGEMENTS

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Appendix 1. DNA sequences of *cytochrome-b* haplotypes from *Lampsilis siliquoidea* and *Lampsilis higginsii*. Haplotypes S1-S5 were found in *L. siliquoidea*, H1-H6, S1 and S6 were found in *L. higginsii*. Asterisks under the columns indicate positions that do not differ among the 12 haplotypes. Spaces indicate one or more differences among haplotypes. Alignment was performed with CLUSTAL W(1.5).

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Haplotype          DNA Sequence

S1                 --CGACTGTTATTANTAATCTTTTATCAGCAATCCCCTATATTGGAAAAACCTTAGTATA
S2                 -----------------------------ATCCCCTATATTGGAAAAACCTTAGTATA
S3                 -GCGACTGTTATTACTAATCTTTTATCAGCAATCCCCTATATTGGAAAAACCTTAGTATA
S4                 -GCGACTGTTATTACTAATCTTTTATCAGCAATCCCCTATATTGGAAAAACCTTAGTATA
S5                 GGCGACTGTTATTACTAATCTTTTATCAGCAATCCCCTATATTGGAAAAACCTTAGTATA
S6                 -GCGACTGTTATTACTAATCTTTTATCAGCAATCCCCTATATTGGAAAAACCTTAGTATA
H1                 -GCGACTGTTATTACTAACCTCTTATCAGCAATCCCATATGTTGGGAAAACCTTAGTATA
H2                 -GCGACTGTTATTACTAACCTCTTATCAGCAATCCCATATGTTGGGAAAACCTTAGTATA
H3                 ----ACTGTTATTANTAACCTCTTATCAGCAATCCCATATGTCGGGAAAACCTTAGTATA
H4                 --CGACTGTTATTACTAACCTCTTATCAGCAATCCCATATGTCGGGAAAACCTTAGTATA
H5                 --CGACTGTTATTACTAACCTCTTATCAGCAATCCCATATGTCGGGAAAACCTTAGTATA
H6                 -GCGACTGTTATTACTAACCTCTTATCAGCAATCCCATATGTTGGGAAAACCTTAGTATA
                                           ***** *** * ** ***************

S1                 TTGGTTATGAGGGGGATTCTCAGTATCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
S2                 TTGGTTATGAGGGGGATTCTCAGTATCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
S3                 TTGGTTATGAGGGGGATTCTCAGTATCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
S4                 TTGGTTATGAGGGGGATTCTCAGTATCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
S5                 TTGGTTATGAGGGGGATTCTCAGTATCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
S6                 TTGGTTATGAGGGGGATTCTCAGTATCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
H1                 TTGGTTGTGGGGAGGATTCTCAGTGTCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
H2                 TTGGTTGTGGGGAGAATTCTCAGTGTCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
H3                 TTGGTTGTGGGGAGGATTCTCAGTGTCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
H4                 TTGGTTGTGGGGAGGATTCTCAGTGTCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
H5                 TTGGTTGTGGGGAGGATTCTCAGTGTCTAACGCAACCTTAAACCGATTTTTTGTTCTTCA
H6                 TTGGTTGTGGGGAGGATTCTCAGTGTCTAACGCAACCTTAAATCGATTTTTTGTTCTTCA
                   ****** ** ** * ********* ****************** ******************

S1                 CTTCGTTCTTCCCTTTGCTATTGCAGCTTTCGCCGCAGTACATCTCCTATTTCTCCATGA
S2                 CTTCGTTCTTCCCTTTGCTATTGCAGCTTTCGCCGCAGTACATCTCCTATTCCTCCATGA
S3                 CTTCGTTCTTCCCTTTGCTATTGCAGCTTTCGCCGCAGTACATCTCCTATTTCTCCATGA
S4                 CTTCGTTCTTCCCTTTGCTATTGCAGCTTTCGCCGCAGTACATCTCCTATTTCTCCATGA
S5                 CTTCGTTCTTCCCTTTGCTATTGCAGCTTTCGCCGCAGTACATCTCCTATTTCTCCATGA
S6                 CTTCGTTCTTCCCTTTGCTATTGCAGCTTTCGCCGCAGTACATCTCCTATTTCTCCATGA
H1                 CTTTGTTCTTCCCTTTGCTATTGCAGCTTTTGCCGCAGTACATCTCCTATTTCTTCATGA
H2                 CTTTGTTCTTCCCTTTGCTATTGCAGCTTTTGCCGCAGTACATCTCCTATTTCTTCATGA
H3                 CTTTGTTCTTCCATTTGCTATTGCAGCTTTTGCCGCAGTACATCTCCTATTTCTTCATGA
H4                 CTTTGTTCTTCCATTTGCTATTGCAGCTTTTGCCGCAGTACATCTCCTATTTCTTCATGA
H5                 CTTTGTTCTTCCATTTGCTATTGCAGCTTTTGCCGCAGTACATCTCCTATTTCTTCATGA
H6                 CTTTGTTCTTCCATTTGCTATTGCAGCTTTTGCCGCAGTACATCTCCTATTTCTTCATGA
                   *** ******** ***************** ******************** ** *****
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| Haplotype | DNA Sequence |
|---|---|
| S1 | AATAGGTTCTAACAACCCTTTAGGTATCTCCTCAAACACTAACCTTATCCCATTTCACAT |
| S2 | AATAGGTTCTAACAACCCTTTAGGTATCTCCTCAAACACTAACCTTATCCCATTTCACAT |
| S3 | AATAGGTTCTAACAACCCTTTAGGTATCTCCTCAAACACTAACCTTATCCCATTTCACAT |
| S4 | AATAGGTTCTAACAACCCTTTAGGTATCTCCTCAAACACTAACCTTATCCCATTTCACAT |
| S5 | AATAGGTTCTAACAACCCTTTAGGTATCTCCTCAAACACTAACCTTATCCCATTTCACAT |
| S6 | AATAGGTTCTAACAACCCCTTAGGTATCTCTTCAAACACTAACCTTATCCCATTTCACAT |
| H1 | AACAGGCTCTAACAACCCATTAGGTATCTCCTCAAACACTAACCTTATTCCATTCCACAT |
| H2 | AACAGGCTCTAACAACCCATTAGGTATCTCCTCAAACACTAACCTTATTCCATTCCACAT |
| H3 | AACAGGCTCTAACAACCCATTAGGTATCTCCTCAAACACTAACCTTATTCCATTCCACAT |
| H4 | AACAGGCTCTAACAACCCATTAGGTATCTCCTCAAACACTAACCTTATTCCATTCCACAT |
| H5 | AACAGGCTCTAACAACCCATTAGGTATCTCCTCAAACACTAACCTTATTCCATTCCACAT |
| H6 | GACAGGCTCTAACAACCCATTAGGTATCTCCTCAAACACTAATCTTATTCCATTCCACAT |
| | * *** ********** ********** ********** ***** ***** ***** |
| S1 | TTTTTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTTTAGGAGCATTATCTTTTAT |
| S2 | TTTTTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTTTAGGAGCATTATCTTTTAT |
| S3 | TTTTTACACAATAAAAGACGTTGCTGGGTTTATTGTTCTTTTAGGAGCATTATCTTTTAT |
| S4 | TTTTTACACAACAAAAGACGTTGTTGGGTTTATTGTTCTTTTAGGAGCATTATCTTTTAT |
| S5 | TTTTTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTTTAGGAGCATTATCTTTTAT |
| S6 | TTTTTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTTTAGGAGCATTATCTTTTAT |
| H1 | TTTCTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTCTAGGAGTATTAACTTCCAT |
| H2 | TTTCTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTCTAGGAGTATTAACTTCCAT |
| H3 | TTTCTACACAACAAAAGACGCTGCTGGGTTTATTGTTCTTCTAGGAGTATTAACTTCCAT |
| H4 | TTTCTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTCTAGGAGTATTAACTTCCAT |
| H5 | TTTCTACACAACAAAAGACGCTGCTGGGTTTATTGTTCTTCTAGGAGTATTAACTTCCAT |
| H6 | TTTCTACACAACAAAAGACGTTGCTGGGTTTATTGTTCTTCTAGGAGTATTAACTTTCAT |
| | *** ******* ******** ** ********** ****** **** *** ** |
| S1 | CTGCTTATTTTTTCCTAACCTCCTAACCGATCCAGAAAACTTTATCCCAGCAAATCCCTT |
| S2 | CTGCTTATTTTTTCCTAACCTCCTAACCGATCCAGAAAACTTTATCCCAGCAAATCCCTT |
| S3 | CTGCTTATTTTTTCCTAACCTCCTAACCGATCCAGAAAACTTTATCCCAGCAAATCCCTT |
| S4 | CTGCTTATTTTTTCCTAACCTCCTAACCGATCCAGAAAACTTTATCCCAGCAAATCCCTT |
| S5 | CTGCTTATTTTTTCCTAACCTCCTAACCGATCCAGAAAACTTTATCCCAGCAAATCCCTT |
| S6 | CTGCTTATTTTTTCCTAACCTCCTAACCGATCCAGAAAACTTTATCCCAGCAAATCCCTT |
| H1 | CTGTTTGTTCTTTCCTAACCTCCTAACCGACCCAGAAAACTTTATTCCAGCAAATCCCCT |
| H2 | CTGTTTGTTCTTTCCTAACCTCCTAACCGACCCAGAAAACTTTATTCCAGCAAATCCCCT |
| H3 | CTGTTTATTCTTTCCTAACCTCCTAACCGACCCAGAAAACTTTATTCCAGCAAACCCCCT |
| H4 | CTGTTTGTTCTTTCCTAACCTCCTAACCGACCCAGAAAACTTTATTCCAGCAAACCCCCT |
| H5 | CTGTTTGTTCTTTCCTAACCTCCTAACCGACCCAGAAAACTTTATTCCAGCAAACCCCCT |
| H6 | CTGTTTGTTCTTTCCTAACCTCCTAACCGACCCAGAAAACTTTATTCCAGCAAATCCCCT |
| | *** ** ** ******************** ************** ******** *** * |
| S1 | AAGAACCCCAGTGCATATTCAACCCGAATGATACT- |
| S2 | AAGAACCCCAGTGCATATTCAACCCGAATGATACT- |
| S3 | AAGAACCCCAGTGCATATTCAACCCGAATGATACT- |
| S4 | AAGAACCCCAGTGCATATTCAACCCGAATGATACTT |
| S5 | AAAAACCCCAGTGCATATTCAACCCGAATGATACT- |
| S6 | AAGAACCCCAGTGCATATTCAACCCGAATGATACTT |
| H1 | AAGAACCCCAGTGCATATTCAACCCGAATGATACT- |
| H2 | AAGAACCCCAGTGCATATTCAACCCGAATGATACT- |
| H3 | AAGAACCCCAGTACACATTCAACCCGAATGAT---- |
| H4 | AAGAACCCCAGTGCACATTCAACCCGAATGATAC-- |
| H5 | AAGAACCCCAGTACACATTCAACCCGAATGATACT- |
| H6 | AAGAACCCCAGTGCATATTCAACCCGAATGATACT- |
| | ** ********* ** ********** |

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June 20, 2002

Richard Neves
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Dear Dick,

Teresa Newton and Bill Richardson passed along to me your request for the report on genetics of *Lampsilis higginsii*, which I have enclosed. I haven't published the data yet in a peer-reviewed publication because I am currently analyzing 130 additional samples of *L. higginsii* . Once the new project is completed, I will submit the entire study for publication.

Thanks for your interest in this work. Please get in touch if you have any questions.

Sincerely,

Bonnie

Bonnie Bowen
Affiliate Assistant Professor
bsbowen@iastate.edu

Encl.