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DNA Sequence Analysis of the Freshwater Mussel *Lampsilis hydiana* (Bivalvia: Unionidae) in Select Ozark and Ouachita Mountain Streams of Arkansas

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Nearly 300 species of freshwater mussels are found in North America; this represents the greatest diversity of freshwater mussels in the world (Williams et al., 1993; Lydeard and Mayden, 1995). Although it is not clearly understood which environmental and historical factors have fostered such diversity in North America, there is scientific evidence of anthropogenic factors causing the rapid decline of many species of freshwater mussels (Neves et al., 1997). The unionid mussels have recently become one of the most endangered freshwater organisms in North America (Williams et al., 1993; Stein and Flack, 1997; Master et al., 1998). The primary threats to these mussels include pollution, increased sedimentation from stream alterations, loss of fish species that host the parasitic unionid larvae (glochidia), and the introduction of the exotic zebra mussel (*Dreissena polymorpha*) (Williams et al., 1993; King et al., 1999). To date, there has been very little research done on the genetic diversity of unionid mussels (Liu et al., 1996; Roe and Lydeard, 1998). The traditional method of classifying taxa based on physical characteristics is challenging with mussels because they do not have many distinguishing features (Lydeard and Roe, 1998). Furthermore, knowing the exact geographical range of an endangered species (or sub-species) can provide insight as to which anthropogenic factors may be the most detrimental to the organism's survival (Roe et al., 2001). Finally, conservational laws and methods cannot be implemented until the endangered organism(s) is properly classified and its geographical range is known (Lydeard and Roe, 1998).

The objective of this study was to examine a ~300 bp region of mitochondrial DNA in the 16s rRNA gene of *Lampsilis hydiana* and compare our results with those of Turner et al. (2000). Previous to this study, the only genetics research available for this species was done by Turner et al. (2000) who found five haplotypes using the 16S rRNA gene.

Since *L. hydiana* can be found in many different streams in Arkansas, we hypothesized that the *Lampsilis* populations would exhibit a population structure that reflects isolation-by-distance. For example, populations that are further apart geographically have greater genetic variation than populations that are closer together which could potentially interbreed.

Mussel Samples.--*Lampsilis hydiana* mussels were collected from the North and Middle forks of the Illinois Bayou and the South Fourche La Fave River of Arkansas (Table 1.) Live specimens were brought back to the Department of Biology, Arkansas Tech Univ. in Russellville, AR and were killed by immersion in a jar of 95% ethanol and stored at -20° C.

DNA Extraction, Sequencing and Sequencing Analysis.--DNA was extracted from a small piece of muscular foot tissue (<1 cm^3) using the following phenol/chloroform extraction protocol. Nine hundred µL of Lifton Buffer (0.2M sucrose, 0.05M EDTA, 0.1M Tris, 0.5% SDS) was added to a 50 mL plastic tube with foot tissue. Forty-five µL of Proteinase K (Promega, Madison, WI) were added and the tissue was homogenized with a glass stirring rod and placed on ice for one hour. One hunded µL of 8M potassium acetate was added and the samples were vortexed briefly and again chilled on ice for one hour. The solution was transferred to a new microtube and centrifuged at 14000 RPM for fifteen minutes. Supernatant was transferred to a new microtube, 2 µL of RNAse (Promega), were added to the new tube, and it was incubated at room temperature for twenty minutes. One hundred µL chloroform/isoamyl alcohol (CIA) and 300 µL of equilibrated phenol was added, samples were vortexed, incubated at room temp for five minutes, and centrifuged at 14000 RPM for five min. The aqueous phase was pipetted to a new microtube, 400 µL CIA were added, and the sample was vortexed and centrifuged at 14000 RPM for five min. The aqueous phase was pipetted to a new microtube, then 100 µL of 3M sodium acetate were added. Three hunded µL of isopropanol was added, and sample was stored at –20°C for three days. The samples were then centrifuged at 14000 RPM for 30 minutes, then 1 mL of chilled 70% ETOH was added to the DNA pellet. The samples were spun at 14000 RPM for five minutes, supernatant removed and the DNA pellet was rewashed with 1 mL chilled 70% ETOH and was centrifuged for five min. Supernatant was removed and the DNA pellet was allowed to air dry for at least fifteen minutes. Finally, 200 µL of distilled H_2O were added, DNA was resuspended for two hours at room temperature and then stored at 4°C.

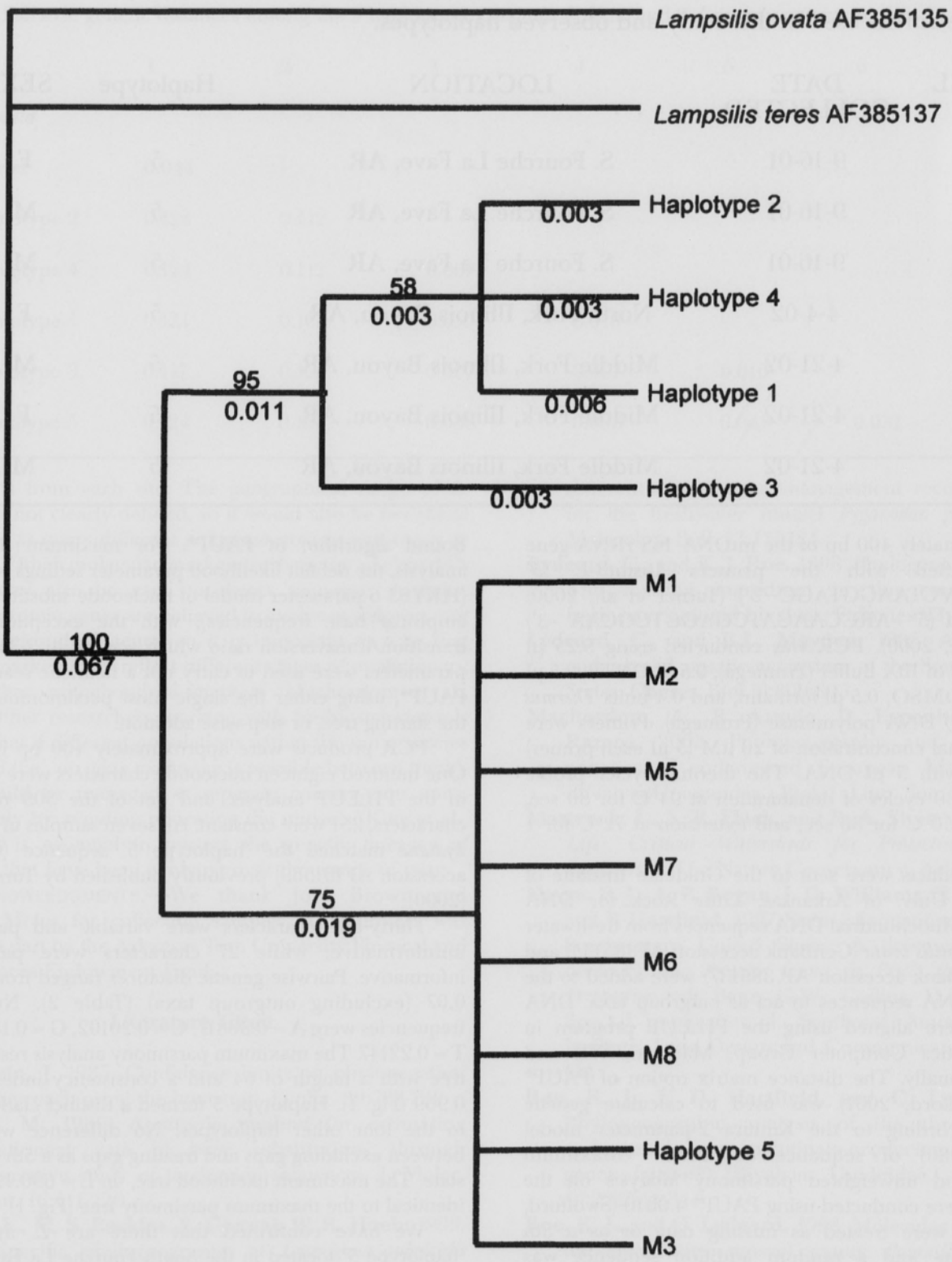
Lampsilis ovata AF385135
Lampsilis teres AF385137
Haplotype 2
0.003
58
0.003
Haplotype 4
0.003
95
0.011
Haplotype 1
0.006
Haplotype 3
0.003
M1
100
0.067
M2
M5
M7
75
0.019
M6
M8
Haplotype 5
M3

Table 1. Specimens used in this study and observed haplotypes.

MUSSEL ID	DATE COLLECTED	LOCATION	Haplotype	SEX
M1	9-16-01	S. Fourche La Fave, AR	5	F
M2	9-16-01	S. Fourche La Fave, AR	5	M
M3	9-16-01	S. Fourche La Fave, AR	5	M
M5	4-4-02	North Fork, Illinois Bayou, AR	5	F
M6	4-21-02	Middle Fork, Illinois Bayou, AR	5	M
M7	4-21-02	Middle Fork, Illinois Bayou, AR	5	F
M8	4-21-02	Middle Fork, Illinois Bayou, AR	5	M

Approximately 400 bp of the mtDNA 16S rRNA gene were amplified with the primers 16sint3-L (5' –TGAGCGTVCTAAGGTAGC- 3') (Turner et al., 2000) and 16sint4-H (5' –AKCCAACATCGAGGTCGCAA –3') (Turner et al., 2000). PCR was conducted using 9.25 µl DH_2O, 3.0 µl of 10X Buffer (Promega), 0.5 µl DNTP, 0.5 µl BSA, 0.5 µl DMSO, 0.5 µl formalin, and 0.4 units *Thermus aquaticus (Taq)* DNA polymerase (Promega). Primers were added at a final concentration of 20 µM (5 µl each primer) and mixed with 5 µl DNA. The thermal cycler profile consisted of 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min.

PCR products were sent to the Graduate Institute of Technology, Univ. of Arkansas, Little Rock for DNA sequencing. Mitochondrial DNA sequences from freshwater mussels *Lampsilis ovata* (GenBank accession AF385135) and *L. teres* (GenBank accession AF 385137) were added to the *L. hydiana* DNA sequences to act as outgroup taxa. DNA sequences were aligned using the PILEUP program in GCG (Genetics Computer Group, Madison, WI) and adjusted manually. The distance matrix option of PAUP* 4.0b10 (Swofford, 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura, 1980) of sequence evolution. Maximum likelihood and unweighted parsimony analysis on the alignments were conducted using PAUP* 4.0b10 (Swofford, 2001). Gaps were treated as missing data or as a 5th character state and a random addition sequence was employed. The reliability of trees was tested with a bootstrap test (Felsenstein, 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, the default likelihood parameter settings were used (HKY85 6-parameter model of nucleotide substitution, and empirical base frequencies), with the exception of the transition/transversion ratio which was set to 2.345. These parameters were used to carry out a heuristic search using PAUP*, using either the single most parsimonious tree as the starting tree, or step-wise addition.

PCR products were approximately 400 bp in length. One hundred eighteen nucleotide characters were excluded in the PILEUP analysis, and out of the 309 remaining characters, 251 were constant. All seven samples of *Lampsilis hydiana* matched the 'haplotype 5' sequence (GenBank accession AF191569) previously published by Turner et al., (2000).

Thirty-one characters were variable and parsimony-uninformative, while 27 characters were parsimony-informative. Pairwise genetic distances ranged from 0.00 to 0.07 (excluding outgroup taxa) (Table 2). Nucleotide frequencies were A = 0.38817, C = 0.20102, G = 0.18934 and T = 0.22147. The maximum parsimony analysis resulted in a tree with a length of 64 and a consistency index (CI) of 0.969 (Fig. 1). Haplotype 5 formed a distinct clade relative to the four other haplotypes. No difference was found between excluding gaps and treating gaps as a 5th character state. The maximum likelihood tree, -ln L = 690.49790, was identical to the maximum parsimony tree (Fig. 1).

We have confirmed that there are *L. hydiana* of 'haplotype 5' located in the South Fourche La Fave River, and this same haplotype is found in the North and Middle forks of the Illinois Bayou. Since sample sizes were small, future research should be conducted including more

Table 2. Pairwise genetic distances among the 5 haplotypes *Lampsilis hydiana* and 2 *Lampsilis* outgroup taxa.

		1	2	3	4	5	6	7
1	*L. ovata*	-						
2	*L. teres*	0.044	-					
3	Haplotype 2	0.124	0.112	-				
4	Haplotype 4	0.124	0.112	0.007	-			
5	Haplotype 1	0.121	0.108	0.010	0.010	-		
6	Haplotype 3	0.117	0.104	0.007	0.007	0.010	-	
7	Haplotype 5	0.124	0.107	0.038	0.038	0.041	0.031	-

specimens from each site. The geographical range of *L. hydiana* is not clearly defined, so it would also be beneficial to sample as many different Arkansas streams and rivers as possible. Other molecular markers such as the nuclear ITS region may also be used to detect sequence diversity. Nuclear protein genes are believed to evolve at a slower rate than mitochondrial genes, so it is important to note that nuclear markers may reflect different stages of evolutionary history than mitochondrial markers (Machordom et al., 2003). Other researchers working with freshwater mussels suggest that if different populations within the same species are found (i.e., no gene exchange is possible between them), they should be managed as separate conservation units, particularly by avoiding relocating the mussels (King et al., 1999). It is essential to protect the genetic lineages of unionids that have been evolving over thousands of years.

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