Articles

Endangered Rough Pigtoe Pearlymussel: Assessment of Phylogenetic Status and Genetic Differentiation of Two Disjunct Populations

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Abstract

We conducted a genetic characterization of two extant populations of an endangered freshwater mussel, the rough pigtoe *Pleurobema plenum*, in the Clinch River, Tennessee of the Tennessee River basin, and the Green River, Kentucky, of the Ohio River basin for purposes of conservation recovery planning. First, phylogenetic status of this species within the *Pleurobema cordatum* species complex (*P. cordatum*, *plenum, rubrum*, *sintoxia*) was assessed using mitochondrial *ND1* deoxyribonucleic acid (DNA) sequences, which showed that all investigated species of *Pleurobema* are genetically distinct. Our results indicated that *P. cordatum*, *P. plenum*, *P. rubrum*, and *P. sintoxia* each represent monophyletic clades; however, the latter two species were closely related, separated by only one to three nucleotide differences. Further, DNA sequence haplotypes from both populations of *P. plenum* grouped together into one monophyletic clade and did not support characterizing the populations as separate species. Thus, our phylogenetic analysis confirms that populations of *P. plenum* in the Clinch and Green rivers are the same species. Second, we assessed genetic differentiation between *P. plenum* populations in each river by analyzing variation at eight nuclear DNA microsatellite loci, where F_{ST} (=0.023) and Jost's D (=0.175) indicated genetically differentiated populations. Because these populations are geographically and demographically independent, and differentiated at nuclear microsatellite loci, recognition as management units is recommended. Additional studies are needed to determine whether there are differences at adaptive or life-history traits, such as fish host usage, shell morphology, and soft anatomy between populations, and to further investigate the phylogenetic relationship of *P. rubrum* and *P. sintoxia*.

Keywords: Rough pigtoe; *Pleurobema plenum*; endangered species; freshwater mussel; mitochondrial DNA; nuclear DNA microsatellites

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Introduction

Freshwater mussels are considered one of the most imperiled faunal groups in the United States, with 213 (72%) of the 297 species that are known from U.S. waters listed as endangered, threatened, or of special concern at the state or federal level (Williams et al. 1993). At least 29 species in the United States are already considered extinct (Neves et al. 1997; Haag and Williams 2013). Because mussels live in sediments of streams and lakes, they are vulnerable to natural and anthropogenic impacts, including severe floods and droughts, habitat alteration from dams, various types of pollution entering streams, substrate destabilization, sedimentation from agriculture and urban environments, and many other factors (Neves et al. 1997). To help conserve and protect mussels, natural resource management agencies have initiated monitoring and research programs to better understand their status and life-history requirements (Strayer et al. 2004). Increasingly, research also is focusing on resolving numerous taxonomic and population genetic issues to assist managers with conservation planning (Jones et al. 2006a; Berg et al. 2007).

The rough pigtoe *Pleurobema plenum* was listed as an endangered species in 1976 (U.S. Fish and Wildlife Service 1976) under the U.S. Endangered Species Act (ESA 1973, as amended). It was once widespread throughout the Ohio River basin, but has declined by $>$ 90% throughout its range in the 20th century (U.S. Fish and Wildlife Service 1984; Parmalee and Bogan 1998). Historically, the species occurred in the Ohio River and many of its main tributaries, including the Muskingum, Scioto, Green, Wabash, Tennessee, Cumberland, and other rivers (Watters et al. 2009). *P. plenum* are known to occur in only three locations in the United States: 1) the Green River and its tributary the Barren River, Kentucky, 2) in the Tennessee River in Alabama and Tennessee, and 3) in the Clinch River, Tennessee. All three locations are separated by hundreds of river miles and isolated by multiple dams. Life-history data for *P. plenum* are limited, but like other species in the genus, it is thought to produce and release glochidia within conglutinates in the summer (a short-term summer brooder) and to utilize cyprinids as its primary fish hosts (Parmalee and Bogan 1998; Watters et al. 2009).

The species belongs to a complex of four morphologically similar-looking species, including Ohio pigtoe (*Pleurobema cordatum*), *P. plenum*, pyramid pigtoe (*Pleurobema rubrum*), and round pigtoe (*Pleurobema sintoxia*; Figure 1). On the basis of examining the distribution, shell morphology, and soft anatomy, A.E. Ortmann concluded that the complex was comprised of four subspecies or forms, and not four separate species (Ortmann 1909, 1910, 1911). However, nearly 50 y later, D.H. Stansbery at The Ohio State University reexamined the data and stated:

> The extreme "big-river" form is the one found furthest upstream in the Clinch River system where it is accompanied by the "small stream" form without a trace of intermediates. At several localities, two forms of the complex exist side by side maintaining their identity without perceptible intergradation and in the Green River at Munfordville, Kentucky, four forms of this complex are found in numbers on the same riffle without intermediate specimens. The indication is that this complex is composed either of four very closely related, very similar yet distinct, species or is a single species exhibiting a gene-linkage or other genetic phenomenon as yet unrecognized as occurring in nature (Stansbery 1967).

Stansbery's reference to the "big-river" and "small-stream" forms in the Clinch River likely refers to *P. rubrum* and *P. plenum*, respectively. He went further and described each of the four phenotypic forms as a separate species, provided a set of shell characters, and developed a key to identify each respective species in the complex. Although malacologists have long recognized these species or phenotypic forms on the basis of subtle morphological differences among shells (Ortmann 1918; Stansbery 1967; Watters et al. 2009), considerable taxonomic uncertainty has existed for the group and only recently were several members of the group tested for phylogenetic distinctiveness using molecular markers (Campbell and Lydeard 2012). Further, the Endangered Species Act listing (U.S. Fish and Wildlife Service 1976) for *P. plenum* was based on morphological data; molecular genetic data were lacking at the time of listing for this species, and now are needed to supplement these morphologically based distinctions (U.S. Fish and Wildlife Service 1984).

Hence, a genetic study of the two largest remaining populations of *P. plenum* in the Green and Clinch rivers was initiated to answer two main questions: 1) within the *P. cordatum* species complex, are these conchologically similar species phylogenetically distinct, and 2) are the two largest populations of *P. plenum* genetically differentiated? Answering the first question is central to resolving the conservation status of *P. plenum*; for example, if one or more members of the *P. cordatum* species complex are found to be genetically indistinguishable from *P. plenum*, conservation status may not be warranted. However, if *P. plenum* is a valid species, determining whether the two largest populations are genetically differentiated is important for recovery planning. Implementation of certain recovery strategies for *P. plenum*, such as release of propagated juvenile mussels and translocation of adult mussels to protected habitats, is contingent upon achieving a thorough understanding of the species' life history and population genetics (U.S. Fish and Wildlife Service 1984).

Methods

Tissue collection and preparation

Mantle tissue from live mussels was sampled in 2004 from various river locations: 1) *P. plenum*, Clinch River, Hancock County, Tennessee, and Green River, near Munfordville, Hart County, Kentucky; samples of *P. plenum* from the Tennessee River were not included in

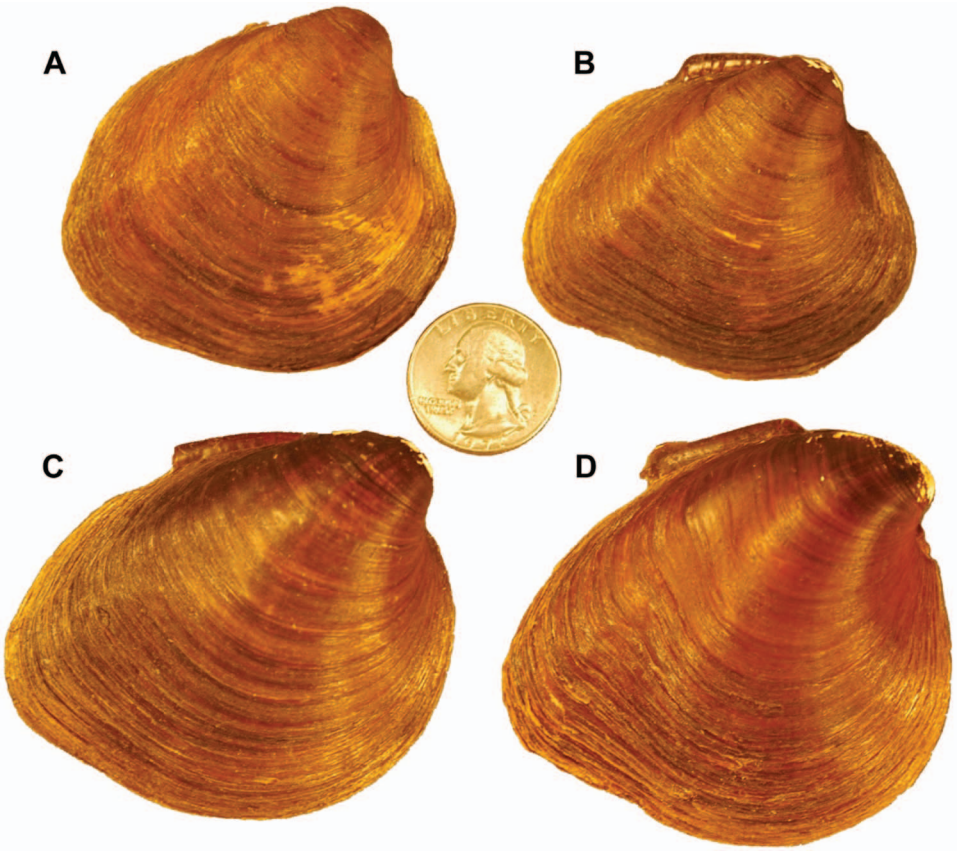


Figure 1. Shells (right valve shown) of the four species belonging to the *Pleurobema cordatum* complex: (**A**) *Pleurobema plenum* collected from Green River, Munfordville, Hart County, Kentucky; (**B**) *Pleurobema cordatum* collected from Green River, Munfordville, Hart County, Kentucky; (**C**) *Pleurobema sintoxia* collected from Ohio River, 5 miles southeast of New Richmond, Campbell County, Kentucky; and (**D**) *Pleurobema rubrum* collected from Clinch River, Anderson County, Tennessee. Shells and locality data are at The Ohio State University Museum and were photographed by J.W.J. in 2006.

the study because this population is very small and occurs at extremely low density at known sites of occurrence; 2) *P. cordatum*, Green River, Munfordville, Hart County, Kentucky, and Tennessee River, Hardin County, Tennessee; 3) *P. rubrum*, Clinch River, Hancock County, Tennessee; and 4) *P. sintoxia*, Green River, Munfordville, Hart County, Kentucky (Figure 2). Sample sizes for each species collected for this study are reported in Table 1. A small piece of mantle tissue (20–30 mg) was collected nonlethally from live mussels from each population (Naimo et al. 1998). Tissues were preserved in 95% ethanol and stored at −20°C before

Table 1. Sampling locations and sample sizes for mitochondrial (mt) deoxyribonucleic acid (DNA) sequences and DNA microsatellite loci for investigated species of *Pleurobema*, where mantle tissues were sampled from live mussels in 2004 at various river locations, including: 1) *P. plenum*, Clinch River, Hancock County, Tennessee, and Green River, near Munfordville, Hart County, Kentucky; 2) *P. cordatum*, Green River, Munfordville, Hart County, Kentucky, and Tennessee River, Hardin County, Tennessee; 3) *P. rubrum*, Clinch River, Hancock County, Tennessee; and 4) *P. sintoxia*, Green River, Munfordville, Hart County, Kentucky. Sample size does not include DNA sequences obtained from GenBank.

Species	Sampling location	Total sample size	mtDNA	Microsatellite DNA							
				Microsatellite loci							
			ND1	*D119*	*D3*	*C121*	*D104*	*D9*	*B11*	*D115*	*D129*
P. plenum	Clinch River	15	15	15	15	15	15	15	15	13	13
P. plenum	Green River	19	15	19	19	17	18	19	19	15	17
P. cordatum	Green River	16	16	15	15	15	15	14	14	12	14
P. cordatum	Tennessee River	2	2	—	—	—	—	—	—	—	—
P. rubrum	Clinch River	4	4	—	—	—	—	—	—	—	—
P. sintoxia	Green River	4	4	—	—	—	—	—	—	—	—

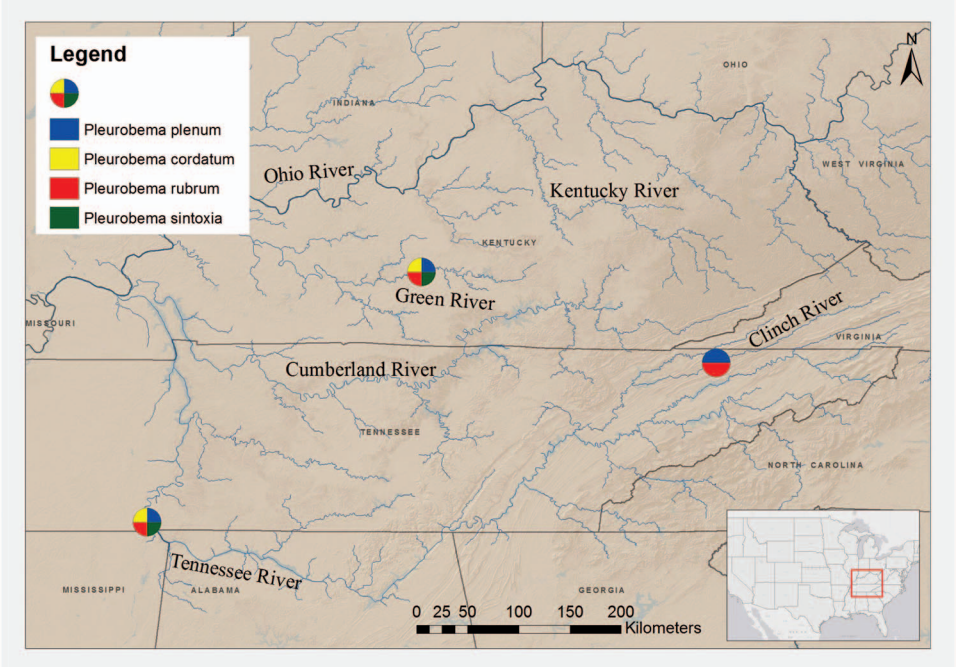


Figure 2. Sampling locations for *Pleurobema* species collected in 2004 from the Clinch and Tennessee rivers, Tennessee, and Green River, Kentucky. The pie-chart colored circles indicate approximate locations of each site sampled during the study. The occurrence and sympatry of each species per site is shown by color in each circle.

deoxyribonucleic acid (DNA) extraction. Total genomic DNA was isolated from ~20 mg of fresh mantle tissue using the Purgene DNA extraction kit (Gentra Systems) following the manufacturer's instructions. The concentration of DNA was determined using a Hoefer TKO 1000 fluorometer to provide a standardized quantity for use in the polymerase chain reaction (PCR).

DNA sequences

Mitochondrial DNA (mtDNA) sequences from the first subunit of NADH dehydrogenase (*ND1*) were amplified by PCR in a PTC-200 thermal cycler (MJ Research) using primers and conditions reported in Serb et al. (2003). The PCR amplification solutions for *ND1* consisted of 100 ng of genomic DNA, 1× PCR buffer, 4.0 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 1.0 μM each primer, and 1.5 U Ampli*Taq* DNA polymerase in a total volume of 20 μL. The PCR thermal cycling conditions were 95°C for 7 min, followed by 35 cycles of 94°C for 40 s, 47°C for 60 s, and 72°C for 90 s; a final extension at 72°C for 2 min; and a final hold at 4°C. All PCR products were sequenced with a Big Dye terminator cycle sequencing kit with Ampli*Taq* DNA polymerase (Applied Biosystems, Inc. [ABI]). Cycle sequence reactions were purified using a Qiagen DNA purification kit before electrophoresis and sequencing using an ABI 3100 automated DNA sequencer at the Virginia Bioinformatics Institute.

The DNA sequences were aligned and edited using the alignment algorithm in the program SEQUENCHER (version 3.0, Gene Codes Corporation). In addition, *ND1* haplotypes of *P. rubrum* (AY655117.1, sampled from Duck River, Venable Spring, Marshall County, Tennessee), *P. sintoxia* (AY613815.1, sample from Cumberland River, Big

Table 2. Deoxyribonucleic acid (DNA) primer sequences used to amplify the eight microsatellite loci in this study from 2004 to 2006 for *Pleurobema plenum* and *Pleurobema cordatum*. Primers were developed in 2003–2004 by the U.S. Geological Survey Leetown Science Center, Kearneysville, West Virginia from DNA of *Pleurobema clava* (*Pcl*) The locus name, forward and reverse primer sequences, and base-pair size range of observed microsatellite alleles for each locus are shown.

Locus name	Primer sequences	Base-pair range
PclD119	F: ACATCAGCTCATGGCAAATAAC	
	R: CGGTTGTCCTTAATATAAACGTG	204–292
PclD3	F: ATGGCGTGATGGTTCTATT	
	R: AAGGGAGGATGGACTTATTAAC	275–419
PclC121	F: TCAAAAGACCCTCTTAGCATAG	
	R: AGATTGGGAGCCTATCACA	148–276
PclD104	F: TATCAACCCCAGACATTACCAG	
	R: GTTTCTGATGACAAATCCCTTC	114–274
PclD9	F: AATTCCTTAACGTCAAGTTCCTC	
	R: GCAATATAAGCAACACGATACG	195–361
PclD106	F: TGCTGTAATAAACATTTGTCACCTAC	
	R: CAAACATTGTGTGCAGTTAGGAC	174–308
PclB11	F: GGCGTAGTTTGAACCATTC	
	R: TTGAAATCTGCCCCATAAC	211–247
PclD115	F: TGAAGTAAGCAATCTCAACAGG	
	R: GCGACTGCATTAACATAAGATG	176–244
PclD129	F: TGCAAAGAGCGATAGACATAA	
	R: CAATCGTAAACAACGAATTACC	145–181

Table 3. Haplotypes and variable sites of mitochondrial deoxyribonucleic acid (DNA) sequences of *Pleurobema plenum*, Clinch River (*Pp*CR); *P. plenum*, Green River (*Pp*GR); *Pleurobema cordatum*, Green River (*Pc*GR); *P. cordatum*, Tennessee River (*Pc*TR); *Pleurobema sintoxia*, Green River (*Ps*GR); and *Pleurobema rubrum*, Clinch River (*Pr*CR) amplified in this study from 2004 to 2006. * indicates shared haplotypes between the Clinch and Green river populations.

Haplotype	*N*	Base pairs
PpClinch01	1	ACCTACACTCTCCAGATTAACGTTTTGGAACGTTCTTTTTACTCCATTTCACGTCTTGGCGCCAAATCA
PpClinch02	2	...T.....C...T....ATT.....T..G.....T....C.T.T.....A.....T.
PpClinch03	1	...T.....TC...T....ATT.....G.....T....C.T.T.....T.
PpClinch04	3	...T...T...C...T....ATT.....G.....CT....C.T.T....C.....
PpClinch05	1	...T.....C...T....ATT.....G.....T....C.T.T.....
PpClinch06	1	...T.....C...T....ATT.....G.....T....C.T.TT.....
PpClinch07 *	4	...T.....C...T....ATT.....G.....T....C.T.T.....
PpClinch08	1	...T.....C...T....ATT.....G.....CT....C.T.T.....
PpClinch09	2	...T.....C...T....ATT.....G.....T....C.T.T.....
PpClinch10	1	...T.....C...T....ATT.....G.....CT....C.T.T.....C..
PpClinch11 *	5	...T.....C...T....ATT.....G.....T....C.T.T.....T.
PpGreen12	3	...T....C...C...T....ATT.....G.....T....C.T.T.....
PpGreen13	2	...T.....C...T....ATT.....AG.....T....C.T.T.....
PpGreen14	1	...T.....C...T....ATT.....G.....T....C.T.T.....T.
PpGreen15	1	...T.....C...T....ATT.....G.....T....C.T.T.....A.....
PpGreen16	1	...T.....C...T....ATT.....G.....T....C.T.T....C.....
PcGreen01	1	.TTC.T...T.T...CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T..TGG.CT.
PcGreen02	1	.TTC.T...T.T.A.CC....ACC...A.G.A.CT..C.C..CTT.A.C...A.T....T..TGG.CT.
PcGreen03	1	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....TC..GG.CTG
PcGreen04	1	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CTG
PcGreen05	1	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TTGA.C...A.T....T...GG.CTG
PcGreen06	3	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CTG
PcGreen07	1	TTTC.T-..T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CTG
PcGreen08	1	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T..TGG.CT.
PcGreen09	2	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CT.
PcGreen10	2	.TTC.T...T.T...CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CTG
PcGreen11	1	.TTC.T.T.T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CTG
PcGreen12	1	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CTC..C.C...TT.A.C...A.T....T...GG.CT.
PcGreen13	1	TTTC.T-..T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C.G.A.T....T...GG.CTG
PcTenn14	1	.TTC.T...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.T....T...GG.CTG
PcTenn15	1	.TTCGT...T.T.A.CC.G.ACC...A.G.A.CT..C.C...TT.A.C...A.....T...GG.CTG
PsGreen01	2	...C....C.C.TG.GC.GTT.CCC.A....AC...C..C.T..T.CCC..T....CA..AT...GCT.
PsGreen02	2	...C....C.C.TG.GC.GTT.CCC.A....AC...C..C.T..T.CCC..T....CA..AT...GCT.
AY613815.1	1	...C....C.C.TG.GT.GTTACCC.A....AC.....CGT..T.CCC..T...CCA..AT...GCT.
PrClinch01	1	...C....C.C.TG.GC.GTTACCC.A....AC.....CGT..T.CCC..T...T.CA..AT...GCT.
PrClinch02	1	...C....C.C.TG.GC.GTTACCCCA....AC.....CGT..T.CCC..T...T.CA..AT...GCT.
PrClinch03	2	...C....C.C.TG.GC.GTTACCC.A....AC.....CGT..T.CCC..T...CCA..AT...GCT.
AY655117.1	1	..TC..G.C.C.TG.GC.GTTACCC.A....AC.....CGT..T.CCC..T...CCA..AT...GCT.

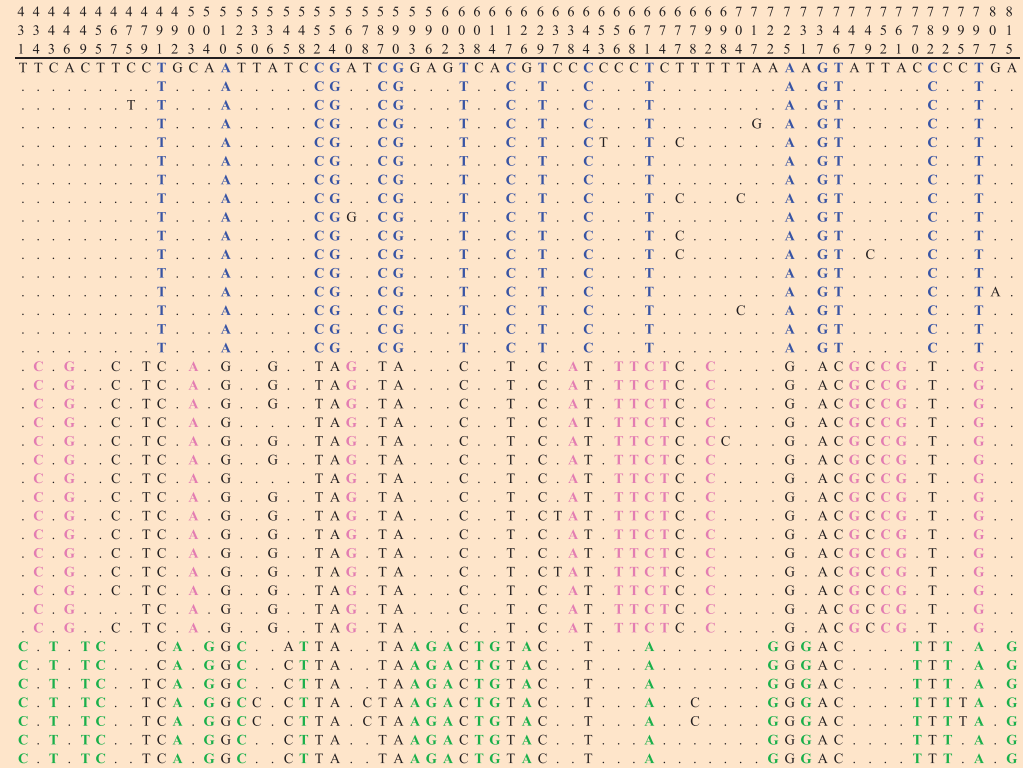
South Fork, Station Camp Creek, Scott County, Tennessee), and two lampsiline mussel species, the plain pocketbook *Lampsilis cardium* (EF446096) and the Higgin's eye pearlymussel *Lampsilis higginsii* (EF213061), were obtained from GenBank and aligned within the DNA sequence data set; the latter two sequences were used to represent outgroup taxa. Phylogenetic analysis was conducted primarily to assess genetic distinctiveness of DNA sequence haplotypes of *P. plenum* relative to those of *P. cordatum*, *P. rubrum*, and *P. sintoxia*. Analysis of variable nucleotide sites was used to infer ancestral genealogical relationships among haplotypes and to provide statistical support for any inferred taxonomic groups. Per the phylogenetic species concept (Cracraft 1983), taxa forming a monophyletic clade were considered a single species. The model of sequence evolution used for the analysis was determined by the program MODELTEST 3.6 (Posada and Crandall 1998); searches were conducted using the Kimura two-parameter model (Hasegawa et al. 1985). Pair-wise genetic distances among haplotypes were estimated using uncorrected *P*-distance in MEGA 3.1 (Kumar et al. 2004). A phylogenetic reconstruction was estimated using Bayesian inference in MrBayes version 3.2.1 (Huelsenbeck and Ronquist 2001). MrBayes was run for 1 million generations and eight chains, sampling trees every 500 generations, and posterior probabilities were calculated using the tree topologies that remained after the burn-in trees from 20,000 generations were excluded (i.e., after the tree score likelihood values had stabilized). Stabilization of likelihood scores was confirmed visually by plotting scores in Microsoft Excel to determine where scores stabilized asymptotically.

DNA microsatellites

Primers for nine nuclear DNA microsatellite loci were developed and characterized using DNA from a closely related species (clubshell *Pleurobema clava*) by Dr. Tim King and colleagues at the U.S. Geological Survey–Biological Resources Division, Kearneysville, West Virginia (Table 2). The PCR amplification solutions (Eackles and King 2002) consisted of 100 ng of genomic DNA, 1 × PCR buffer, 2 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 0.5 μM each primer, and 1.0 U Ampli*Taq* DNA polymerase (ABI) in a total volume of 20 μL. The PCR thermal cycling conditions were those of Eackles and King (2002): 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 1 min, followed by a final extension at 72°C for 1 min and a hold at 4°C.

Amplification products containing microsatellite loci were examined for size polymorphism using an ABI 3100 automated sequencer and GENOTYPER (ABI) software to

Table 3. Extended.



determine allele size. Results were stored as GENESCAN files; GENEMAPPER software (ABI) was used to visualize allele size and score the results. All alleles were scored initially by N.J. during the project. However, if difficult-to-interpret allele chromatograms were observed, they were re-examined and scored a second time by J.W.J. Loci that proved too difficult to score were amplified again and alleles rescored. Microsatellite data sets were tested for genotyping errors caused by null alleles, stuttering, and short-allele dominance using a Monte Carlo simulation of expected allele size differences using MICROCHECKER (Van Oosterhout et al. 2004). Populations were screened for linkage disequilibrium between all pairs of loci and for deviations from Hardy–Weinberg equilibrium (HWE) at each locus using the program ARLEQUIN, version 3.0 (Excoffier et al. 2005). Significance of linkage disequilibrium pair-wise tests was determined using the likelihood ratio test with 10,000 permutations (Slatkin and Excoffier 1996), and HWE using the exact test with a Markov chain of 1,000,000 steps and 100,000 dememorization steps (Guo and Thompson 1992). We used a sequential Bonferroni correction to account for type I errors associated with all multiple pair-wise comparisons (Rice 1989).

Genetic variability across microsatellite loci for populations of *P. plenum* and *P. cordatum* was quantified in terms of percentage of polymorphic loci, observed heterozygosity, expected heterozygosity, number of alleles per locus, total number of alleles, number of private alleles, and allele frequencies per locus. Evidence for a bottleneck in each population and locus was tested for using the Garza–Williamson index (*M*-ratio) as implemented in ARLEQUIN, which is the ratio of the number of alleles to range in allele size; values below 0.7 suggest the occurrence of a bottleneck (Garza and Williamson 2001). Evidence for inbreeding within each population was tested for using F_{IS}, which measures the extent of departure from HWE due to inbreeding within a subpopulation(s) and can range from −1.0 (all heterozygotes) to +1.0 (all homozygotes; Wright 1965). Genic differentiation for each population pair at each locus was tested for significant differences using an exact *G* test of allele frequency heterogeneity following the algorithm of Raymond and Rousset (1995). A Markov chain Monte Carlo randomization method was used to calculate an unbiased estimate of the exact probability for each single locus comparison. The randomization test was conducted in Genepop on the Web version 4.2 (available at: http://genepop.curtin.edu.au/genepop_op3.html), where we ran 100 batches with 10,000 iterations per batch and 10,000 dememorization steps; for each population pair comparison, a multilocus *P*-value was computed using Fisher's

Table 4. Summary of intraspecific mitochondrial deoxyribonucleic acid (mtDNA) sequence variation at the *ND1* gene obtained in this study from 2004 to 2006 among investigated populations and species of *Pleurobema* in the Clinch and Tennessee rivers, Tennessee, and Green River, Kentucky, including: 1) sample size (*n*); 2) number of haplotypes; 3) mean number of base-pair (bp) differences (*k*) and range of bp differences among haplotypes within samples for each species; 4) pair-wise genetic distance among haplotypes; (5) haplotype diversity (*h*), i.e., the probability that two randomly chosen mtDNA sequences were different in a sample; and 6) nucleotide diversity (π), i.e., the probability that two randomly chosen homologous nucleotides were different in the sample.

Species	Sampling Location	*n*	Number of haplotypes	Mean number of bp differences *k* (range)	Pair-wise divergence*	Haplotype diversity (*h*)	Nucleotide diversity (π)
P. plenum	Clinch River, Tennessee	15	11	4.2 (0–7)	0.000–0.008	0.95238	0.00500
P. plenum	Green River, Kentucky	15	7	2.7 (0–5)	0.000–0.006	0.87912	0.00324
P. cordatum	Green River, Kentucky	16	13	2.9 (0–6)	0.000–0.007	0.96667	0.00343
P. cordatum	Tennessee River, Tennessee	2	2	3.0 (0)	0.000–0.004	1.00000	0.00361
P. sintoxia	Green River, Kentucky	4	2	0.7 (0–1)	0.000–0.001	0.66667	0.00080
P. rubrum	Clinch River, Tennessee	4	3	4.5 (0–7)	0.000–0.008	0.83333	0.00542

* Estimated using uncorrected *P*-distance.

method for combining probabilities across loci. Population differentiation was quantified using the F_{ST} (Wright 1978) metric in ARLEQUIN, which ranges in value from 0 (no differentiation) to 1 (complete differentiation; Wright 1978; Balloux and Lugon-Moulin 2002). However, because F_{ST} will approach zero when gene diversity is high, *D*—an estimator of actual differentiation developed by Jost (2008)—was estimated using the online software SMOGD at http://www.ngcrawford.com/django/jost/ (Crawford 2010). This metric also ranges from 0 to 1, but may provide a more accurate estimate of differentiation due to genetic drift and gene flow at highly polymorphic loci.

Results

Phylogenetic analysis of mtDNA sequences

The matrix of aligned *ND1* mtDNA sequences contained 831 base pairs (bp), of which 133 were variable among species (Table 3). Of these variable sites, 26 (shown in blue) were seemingly fixed (contained only one nucleotide) for all individuals within *P. plenum*, 30 (in pink) were fixed within *P. cordatum*, and 38 (in green) were fixed within both *P. sintoxia* and *P. rubrum*. In addition, one site (in red) at bp position 200 was fixed only in *P. sintoxia*, and one site (also in red) at bp position 227 was fixed only in *P. rubrum*. Excluding DNA sequences for outgroups obtained from GenBank, observed nucleotide site variation defined 36 haplotypes within and among the investigated species of *Pleurobema*; 11 in *P. plenum* from Clinch River, 7 in *P. plenum* from Green River, 13 in *P. cordatum* from Green River, 2 in *P. cordatum* from Tennessee River, 2 in *P. sintoxia* from Green River, and 3 in *P. rubrum* from Clinch River (Tables 3 and 4). Haplotype names were abbreviated to include the species, river location, and DNA haplotype number (e.g., PpClinch01 refers to *P. plenum* sampled from the Clinch River and is designated the first haplotype for the species in our study). Haplotypes PpClinch07 ($N = 2$ in Clinch River and $N = 2$ in Green River) and PpClinch11 ($N = 3$ in Clinch River and $N = 2$ in Green River) were shared between the Clinch and Green river populations (Table 3; Figure 3). Overall, the investigated populations exhibited high intraspecific haplotypic diversity. However, within populations, haplotypes were characterized by low divergence ($< 0.05\%$ as measured by uncorrected *P*-distance) and nucleotide diversity, typically exhibiting only a few nucleotide differences among haplotypes.

Phylogenetic analysis of mtDNA sequence haplotypes showed *P. plenum* to be a genetically distinct, monophyletic lineage (Figure 3). More important, sampled individuals from the Clinch and Green river populations of *P. plenum* grouped together into one distinct clade. Only minor differences, typically one to two bp, were observed among haplotypes between the Clinch and Green river populations, and further, no fixed nucleotide differences were observed between populations. Similarly, sampled individuals of *P. cordatum*, *P. rubrum*/*P. sintoxia* also grouped together into their own respective clades; all recovered clades were well supported statistically by high posterior probability values (Figure 3). The tree topology placed *P. plenum* and *P. cordatum* together as sister taxa, and *P. rubrum* and *P. sintoxia* together as sister taxa. However, these latter two species were closely related and separated by only four fixed nucleotides (two of which were diagnostic when compared with *P. plenum* and *P. cordatum*). Two of these haplotypes, PrClinch03 (*P. rubrum*) from this study and AY613815.1 (*P. sintoxia*), collected from the Cumberland River drainage, Big South Fork at Station Camp Creek, Scott County, Tennessee (Campbell et al. 2005), were nearly identical and differed by only one nucleotide, and hence grouped together (Table 3; Figure 3).

Population genetic analysis of DNA microsatellites

Allele frequencies at each microsatellite locus for each population are reported in Table S1, Supplemental Material. Summary statistics for variation across eight of the microsatellite loci are reported in Table 5. Locus *D106* was consistently out of HWE across all populations and was too difficult to score because of stuttering. Hence, it was removed from our analyses. Significant deviations from HWE ($\alpha = 0.05$) were observed in *P. plenum* (Clinch River) at loci *D119*, *D3*, *C121*, and *D104*; in *P. plenum* (Green) at loci *D104* and *D115*; and in *P. cordatum* (Green)

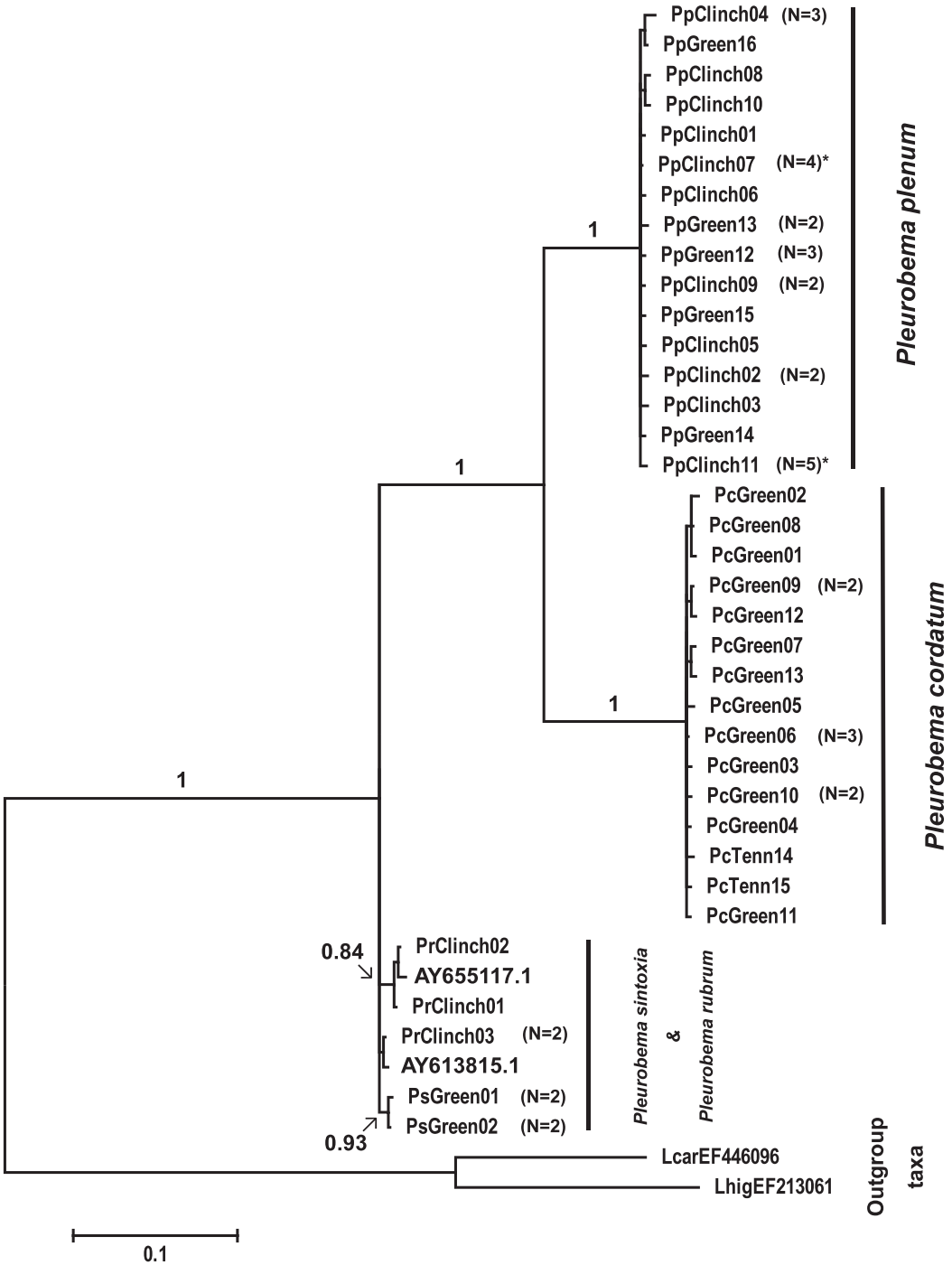


Figure 3. Phylogenetic relationships among the investigated *Pleurobema* species inferred from mitochondrial deoxyribonucleic acid (mtDNA) *ND1* region (831 base pairs) using a Kimura two-parameter model of nucleotide substitution and Bayesian consensus trees. Analyses were conducted from 2005 to 2006. Numbers on branches are calculated posterior probabilities for nodes. Final average standard deviation of split frequencies was 0.009104, with the most likely tree possessing a −ln likelihood of −2,108.592, with a burn-in set to 20,000 and the mean −ln likelihood of −2,115.105. Numbers in parentheses at the end of each haplotype name represent the number of observed haplotypes, where Pp = *P. plenum*, Pc = *P. cordatum*, Pr = *P. rubrum*, and Ps = *P. sintoxia*, and AY655117.1, AY613815.1, EF446096, and EF213061 are Genbank accession numbers for *ND1* DNA sequences of *P. rubrum*, *P. sintoxia*, *Lampsilis cardium*, and *L. higginsi*, respectively. *N* = 1 for haplotype names without numbers in parentheses. * indicates shared haplotypes between the Clinch and Green rivers.

Table 5. Summary of genetic variation among eight microsatellite deoxyribonucleic acid (DNA) loci examined in this study from 2004 to 2006 for *Pleurobema plenum* in the Clinch River, Tennessee, and Green River, Kentucky, and *Pleurobema cordatum* in the Green River, Kentucky, where N = number of individuals genotyped per locus, H_o = observed heterozygosity, H_e = expected heterozygosity, A = number of observed alleles per locus, range = number of nucleotide base-pair differences between the shortest and longest microsatellite alleles observed per locus, M-ratio = approximate ratio of A and range, and F_{IS} = inbreeding coefficient. Values in bold are significantly different from each other ($P < 0.05$).

Species	Locus	*N*	H_o	H_e	*A*	Range	*M*-ratio	F_{IS}
P. plenum (CR)	*PclD119*	15	**0.47**	**0.91**	12	60	0.19	0.50
	PclD3	15	**0.73**	**0.88**	10	68	0.14	0.17
	PclC121	15	**0.93**	**0.96**	16	144	0.11	0.03
	PclD104	15	**0.67**	**0.92**	10	66	0.15	0.28
	PclD9	15	0.93	0.92	13	54	0.24	−0.01
	PclB11	15	0.87	0.89	13	50	0.25	0.03
	PclD115	13	0.93	0.90	9	50	0.18	−0.02
	PclD129	13	1.0	0.89	9	44	0.20	−0.12
	Mean	14.5	0.82	0.91	11.5	67	0.18	0.11
	SD	0.93	0.18	0.03	2.5	32.2	0.05	0.20
P. plenum (GR)	*PclD119*	19	0.89	0.96	22	164	0.13	0.07
	PclD3	19	0.79	0.91	15	68	0.22	0.14
	PclC121	17	1.0	0.97	26	142	0.18	−0.03
	PclD104	18	**0.61**	**0.92**	16	104	0.15	0.35
	PclD9	19	0.79	0.90	14	72	0.19	0.12
	PclB11	19	0.79	0.93	17	46	0.36	0.15
	PclD115	15	**0.60**	**0.89**	10	50	0.20	0.33
	PclD129	34	0.82	0.89	12	48	0.24	0.08
	Mean	20	0.79	0.92	16.5	87	0.21	0.15
	SD	5.8	0.13	0.03	5.2	45.4	0.07	0.13
P. cordatum (GR)	*PclD119*	17	1.0	0.93	13	104	0.12	−0.08
	PclD3	17	0.94	0.92	13	74	0.17	−0.02
	PclC121	17	**0.76**	**0.96**	19	162	0.12	0.17
	PclD104	17	0.82	0.90	10	34	0.29	0.04
	PclD9	16	1.0	0.97	20	182	0.11	−0.03
	PclB11	16	0.37	0.49	9	34	0.26	0.20
	PclD115	28	**0.29**	**0.87**	9	50	0.18	0.74
	PclD129	16	1.0	0.86	8	32	0.24	−0.16
	Mean	18	0.77	0.86	12.6	84	0.19	0.11
	SD	4.1	0.29	0.16	4.6	60	0.07	0.28

at loci *C121* and *D115*. Deviations from HWE and linkage equilibrium at some of the loci used in our study could be the result of loss of genetic diversity due to recent population bottlenecks, or high levels of inbreeding, the latter perhaps due at least in part to the facultative hermaphroditism occasionally observed in freshwater mussels (van der Schalie 1970). Significant linkage disequilibrium ($\alpha = 0.05$) was observed for eight pairs of loci in *P. plenum* (Clinch River); zero pairs in *P. plenum* (Green River); and zero pairs in *P. cordatum* (Green River). The MICROCHECKER analyses indicated no evidence for stuttering or short-allele dominance in the eight loci included in the analysis. However, the possible presence of null alleles was detected in *P. plenum* (Clinch River) at locus *D119*, in *P. plenum* (Green River) at loci *D104* and *D115*, and in *P. cordatum* (Green River) at loci *C121* and *D115*.

Observed and expected heterozygosities were high and at similar levels in *P. plenum* (Clinch River) and *P. plenum* (Green River), followed by *P. cordatum* (Green River; Table 5). However, mean numbers of alleles per locus, total numbers of alleles observed, and numbers of private alleles were greatest in the latter two populations. Many private alleles were observed in all three populations (1–13) and at every locus (4–33; see Table S1). On average, 45% of the alleles observed at a locus were private alleles to a population. Observed M-ratio values for all three populations and all loci were below 0.5, and mean F_{IS} values were > 0.1 (Table 5).

The exact G test was highly significantly different ($P < 0.0001$) across loci for all three population pair comparisons; results per locus are available in Table S2, Supplemental Material. The level of allele frequency divergence among populations was low to moderate on the basis of F_{ST} estimates (Table 6). Pair-wise F_{ST}

Table 6. Pair-wise F_{ST} (below diagonal) and D (above diagonal) estimates among populations of *Pleurobema plenum* and *Pleurobema cordatum* using data from eight microsatellite loci in this study from 2004 to 2006. All pair-wise comparisons for F_{ST} are significant $pP < 0.05$).

Species	*P. plenum* (Clinch River)	*P. plenum* (Green River)	*P. cordatum* (Green River)
P. plenum (Clinch River)	—	0.175	0.415
P. plenum (Green River)	0.023	—	0.236
P. cordatum (Green River)	0.087	0.065	—

comparisons ranged from 0.023 to 0.087, with the lowest differentiation between *P. plenum* (Clinch River) and *P. plenum* (Green River), and the greatest between *P. plenum* (Clinch River) and *P. cordatum* (Green River) on the basis of microsatellite data. In contrast, although patterns of population differentiation were similar, the level of allele frequency differentiation among populations was higher on the basis of *D* estimates, which ranged from 0.175 to 0.415, with the most closely related taxa again being the two populations of *P. plenum* and the most distantly related being comparisons between *P. plenum* and *P. cordatum*.

Discussion

Phylogenetic analysis of *ND1* mtDNA sequences revealed that populations of *P. plenum* in the Clinch and Green Rivers are closely related and grouped together into a single monophyletic clade. More important, haplotypes from these two rivers shared 26 diagnostic nucleotides, clearly separating *P. plenum* from the other three species belonging to the *P. cordatum* complex. Further, both populations of *P. plenum* contained high levels of within-population genetic variation at the *ND1* marker, but the variation was typically in the form of single nucleotide polymorphisms at particular sites. Although these polymorphic nucleotides defined many unique haplotypes, the observed genetic variation did not separate the two populations of *P. plenum* into different monophyletic clades. Thus, observed mtDNA variation between these two populations is indicative of low intraspecific genetic divergence, but not of two evolutionarily diverged monophyletic lineages typically observed between species.

A key result of the *ND1* mtDNA sequence analysis is that all morphologically defined members of the *P. cordatum* species complex were phylogenetically distinct. The close phylogenetic relationship between *P. rubrum* and *P. sintoxia* warrants further investigation, as these two species were separated from each other by only two diagnostically fixed nucleotides at the *ND1* gene. However, sample sizes ($N = 5$) for each species were low, and only one mtDNA gene was examined in our study. Therefore, we consider this finding preliminary until more individuals and nucleotides are investigated.

Our findings corroborate those of Campbell et al. (2005) and Campbell and Lydeard (2012) who also showed that several of the species in the *P. cordatum* complex were phylogenetically distinct on the basis of analysis of mtDNA haplotypes. These two earlier studies, however, did not include DNA sequences for *P. plenum*, analyzing only *P. cordatum*, *P. rubrum*, and *P. sintoxia*. We specifically focused on *P. plenum*'s phylogenetic standing within the group, utilizing larger sample sizes from each population and those of three other *Pleurobema* species in the complex. Even though the shell characteristics of these species are quite similar in appearance, they are generally distinguishable from each other by trained biologists; our results showed that sampled individuals exhibited species-specific mtDNA haplotypes, which, when considered together, qualifies them as species under the phylogenetic species concept (Cracraft 1983). Furthermore, these species are sympatric through significant portions of their ranges; thus, they appear to be reproductively isolated (i.e., individuals of each species occur together in space and time but do not exchange gametes to produce progeny) from each other and hence also qualify as species under the biological species concept (Mayr and Ashlock 1991). For example, sympatric populations of *P. plenum* and *P. cordatum* in the Green River were diverged at both mtDNA sequences and nuclear DNA microsatellites, again supporting the inference that these species are reproductively isolated from each other. The presence of private alleles and high genetic differentiation indicates that gene flow between these two species is infrequent or absent.

Results of our phylogenetic analysis support recognition of *P. plenum* as a valid species, and those of our population genetic analysis showed considerable differentiation at nuclear DNA microsatellites between populations in the Clinch and Green rivers. These populations are separated by hundreds of river miles, and numerous dams that block host fish migration between them; hence, natural gene flow is no longer possible. As defined by Moritz (2002), management units are demographically independent populations that exhibit significant divergence of either mtDNA or nuclear DNA, which are managed to ensure viability of a larger evolutionary significant unit or species. The Clinch and Green river populations of *P. plenum* are geographically and demographically independent, and as shown in this study, diverged at microsatellite loci, suggesting that they meet Moritz's definition of management unit. Depending on conservation goals, management options may range from keeping the Clinch and Green river populations on separate demographic paths to restoring gene flow by removing barriers or through assisted migration. Managers should carefully consider balancing the relative risks of inbreeding and outbreeding depression on long-term viability of remaining populations or those that may become established to recover the species (Edmands 2007; Frankham et al. 2011).

Additional work will be required to assess whether adaptively significant differences distinguish these populations. Hence, we recommend that studies be

conducted on these populations to assess possible differences in biology or life-history traits, such as glochidia dimensions, soft anatomy, gravidity period, and fish host usage (Jones et al. 2006b). For example, if fish host usage between the two populations differed greatly, important adaptive differentiation may exist. Results of such studies would help identify management actions appropriate for recovery of the species. Finally, the hypothesis presented by Stansbery (1967) that *P. cordatum, P. plenum, P. rubrum*, and *P. sintoxia* represent separate species generally has been confirmed by this study and those of Campbell et al. (2005) and Campbell and Lydeard (2011). However, the taxonomic relationship between *P. rubrum* and *P. sintoxia* will require further investigation, with potential implications for understanding the distribution and abundance of these taxa.

Supplemental Material

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Table S1. Allele frequencies of DNA microsatellites examined for *Pleurobema plenum* and *Pleurobema cordatum* for samples collected in 2004 from the Clinch River, Tennessee and Green River, Kentucky. Allele sizes are given in number of base pairs, including the primer flanking regions. Private alleles to a population are shown in bold font.

Found at DOI: http://dx.doi.org/10/3996/052013-JFWM-036.S1 (14 KB DOCX).

Table S2. Genic differentiation exact *G* test *P*-values are reported for each locus and population pair, where GPC is *Pleurobema cordatum* Green River, Kentucky, GPP is *Pleurobema plenum* Green River, Kentucky, and CPP is *P. plenum* Clinch River, Tennessee.

Found at DOI: http://dx.doi.org/10/3996/052013-JFWM-036.S2 (12 KB DOCX).

Reference S1. U.S. Fish and Wildlife Service. 1984. Rough pigtoe pearly mussel recovery plan. U.S. Fish and Wildlife Service, Atlanta, Georgia.

Available at: http://ecos.fws.gov/speciesProfile/profile/speciesProfile.action?spcode = F00P.

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